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ΔFosB Regulates Gene Expression and Cognitive Dysfunction in a Mouse Model of Alzheimer's Disease.

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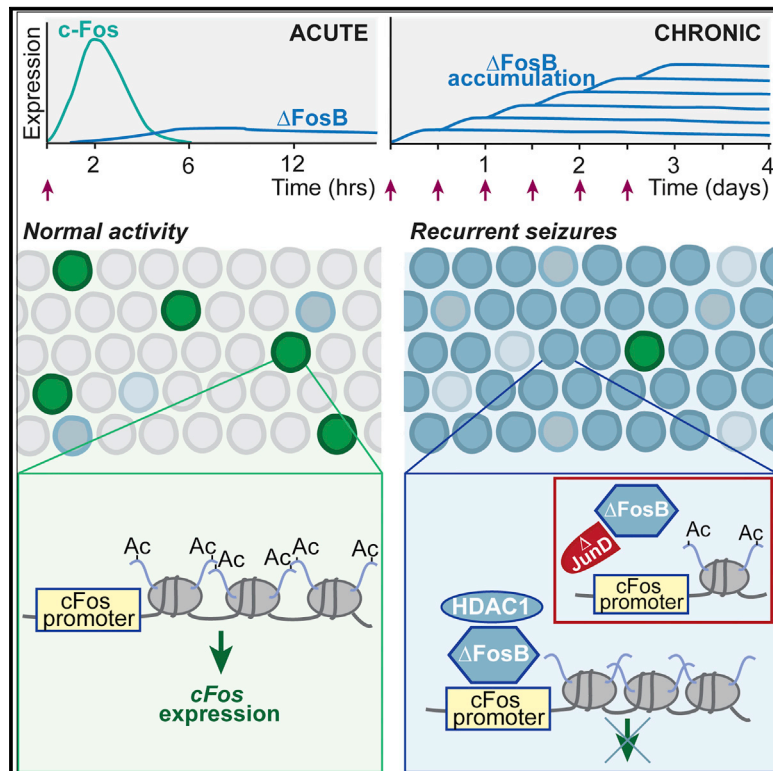
Authors

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Δ FosB Regulates Gene Expression and Cognitive Dysfunction in a Mouse Model of Alzheimer's Disease

Graphical Abstract



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In Brief

Corbett et al. report that, in mouse models of Alzheimer's disease, spontaneous seizures induce expression of Δ FosB in the dentate gyrus, where it triggers histone modifications and suppresses gene expression important for memory. Due to its long half-life, Δ FosB may persistently affect gene expression and memory, even when seizures are infrequent.

Highlights

- Spontaneous seizures in APP mice increase Δ FosB in the dentate gyrus
- Δ FosB binds and recruits HDAC to suppress expression of genes such as c-fos
- Blocking Δ FosB signaling restores gene expression and normalizes memory in APP mice
- Δ FosB's long half-life enables persistent regulation of gene expression



Δ FosB Regulates Gene Expression and Cognitive Dysfunction in a Mouse Model of Alzheimer's Disease

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SUMMARY

Alzheimer's disease (AD) is characterized by cognitive decline and 5- to 10-fold increased seizure incidence. How seizures contribute to cognitive decline in AD or other disorders is unclear. We show that spontaneous seizures increase expression of Δ FosB, a highly stable Fos-family transcription factor, in the hippocampus of an AD mouse model. Δ FosB suppressed expression of the immediate early gene *c-Fos*, which is critical for plasticity and cognition, by binding its promoter and triggering histone deacetylation. Acute histone deacetylase (HDAC) inhibition or inhibition of Δ FosB activity restored *c-Fos* induction and improved cognition in AD mice. Administration of seizure-inducing agents to nontransgenic mice also resulted in Δ FosB-mediated suppression of *c-Fos*, suggesting that this mechanism is not confined to AD mice. These results explain observations that *c-Fos* expression increases after acute neuronal activity but decreases with chronic activity. Moreover, these results indicate a general mechanism by which seizures contribute to persistent cognitive deficits, even during seizure-free periods.

INTRODUCTION

Alzheimer's disease (AD) is the most prevalent type of dementia among the elderly and is associated with prominent impairments in hippocampal memory (Burgess et al., 2002; Selkoe, 2002; Tulving and Markowitsch, 1998). Many current treatments are aimed at preventing disease progression in at-risk individuals or early in disease. A central component of these therapeutic efforts is the prevention of cognitive decline. Therefore, comprehensive understanding of the neuronal and network alterations associated with cognitive dysfunction in AD is critical to enable development of effective therapeutics.

It is becoming increasingly clear that network dysfunction, which can manifest as seizures and epileptiform activity, plays a contributing role in AD-related cognitive deficits. AD is associated with a 5- to 10-fold increased risk in seizures (Amatniek et al., 2006; Larner, 2010; Lozsadi and Larner, 2006). This rate may be underestimated due to the incidence of subclinical epileptiform activity and non-convulsive seizures, which in a majority of cases precede or coincide with the diagnosis of amnesic mild cognitive impairment or AD (Vossel et al., 2013). A recent prospective study found that 42.4% of AD patients with no clinical history of seizures exhibited subclinical epileptiform activity, and the presence of such activity was associated with faster rates of cognitive decline (Vossel et al., 2016). Furthermore, multiple mouse models of AD exhibit epileptiform activity (Chin and Scharfman, 2013; Kam et al., 2016; Minkeviciene et al., 2009; Palop et al., 2007; Palop and Mucke, 2009). Because patients with intractable epilepsy and mouse models of epilepsy display hippocampal memory deficits (Hermann et al., 2006; Hoppe et al., 2007; Stafstrom et al., 1993), the epileptiform activity associated with AD may contribute to cognitive deficits as well. Indeed, antiepileptic drug treatment reduces network dysfunction and improves hippocampus-dependent cognition in prodromal AD patients and mouse models of AD (Bakker et al., 2012; Sanchez et al., 2012). However, the mechanisms by which seizures induce persistent cognitive deficits in AD and epilepsy are poorly understood, particularly because seizure frequency is often not high.

Neuronal activity alters expression of many genes in the hippocampus, with important consequences for learning and memory. One of the most widely studied is *Fos*, a gene whose protein product, *c-Fos*, is critical for synaptic plasticity and hippocampal memory (Fleischmann et al., 2003; He et al., 2002; Tischmeyer and Grimm, 1999). Recent studies demonstrated that neuronal activity induces formation of DNA double-strand breaks (Madabhushi et al., 2015), including within the promoters of early-response genes, including *Fos*, which facilitates their expression (Madabhushi et al., 2015). Therefore, it might be expected that *c-Fos* expression should be high in conditions of chronic network hyperactivity. However, hippocampal *c-Fos* expression is biphasic, increasing after acute neuronal activity but decreasing in chronically active neurons (Calais et al.,

2013; Renthal et al., 2008; Sheng and Greenberg, 1990; Tsankova et al., 2004). The mechanisms governing such biphasic regulation of c-Fos in the hippocampus are not clear. Decreased hippocampal c-Fos expression in mouse models of AD and epilepsy has been regarded as a potential contributor to hippocampus-dependent cognitive deficits (Calais et al., 2013; Palop et al., 2003, 2005; Chin et al., 2005; España et al., 2010). Therefore, it is critical to define the molecular mechanisms responsible for reducing c-Fos expression.

Δ FosB is an activity-dependent transcription factor in the immediate early gene family and is an alternatively spliced product of the *FosB* gene. However, unlike the products of other immediate early genes, such as c-Fos, Δ FosB has an unusually long half-life that allows it to accumulate and remain in chronically active cells for weeks (McClung et al., 2004; Nestler et al., 2001). Studies of Δ FosB function in the nucleus accumbens after chronic exposure to drugs of abuse revealed that Δ FosB epigenetically regulates a number of gene targets critical in addiction, including c-Fos, via histone modifications (Renthal et al., 2008). The term “epigenetic” is used here to indicate histone modifications that alter gene expression.

We therefore hypothesized that high levels of neuronal activity induced by seizures may similarly lead to accumulation of Δ FosB in the hippocampus, where it would alter the expression of target genes, such as c-Fos, causing long-term consequences for hippocampal function. Indeed, in the hippocampus, overexpression of Δ FosB can exert effects on learning and memory (Eagle et al., 2015). In the present study, we examined expression of Δ FosB in a transgenic mouse model of AD neuropathology that exhibits spontaneous seizures, and in a pharmacological model of epilepsy. We demonstrate that seizure-induced Δ FosB expression in the hippocampus is present and epigenetically suppresses c-Fos expression in both models. Moreover, blocking either histone deacetylation or Δ FosB activity ameliorated effects on both c-Fos expression and memory impairment. Our results reveal a molecular mechanism that explains why hippocampal c-Fos expression is chronically reduced in conditions associated with aberrant neuronal hyperactivity, and highlight potential therapeutic avenues to improve cognitive function in both AD and epilepsy.

RESULTS

Seizure-Induced Δ FosB Correlates with Impairments in Hippocampal c-Fos Expression and Cognition in APP Mice

Experimentally induced seizures can increase Δ FosB expression in the hippocampi of nontransgenic (NTG) wild-type mice (Chen et al., 1997; McClung et al., 2004), but the consequences of such increases on downstream gene expression are not clear. To investigate a link between seizures, Δ FosB, and c-Fos in AD mice, we first evaluated the relationship between seizures and hippocampal Δ FosB expression in a commonly used transgenic mouse model that expresses mutant human amyloid precursor protein (APP) (line J20; Mucke et al., 2000). We performed electroencephalogram (EEG) recordings in APP mice at 4–6 months of age and found that the higher the frequency of epileptiform activity or seizures in APP mice, the greater the increase in Δ FosB

expression levels in the dentate gyrus (DG) (Figures 1A and 1B). Overall Δ FosB expression was higher in APP mice relative to NTG controls, both by immunohistochemistry (Figure 1C) and by western blot analysis (Figures S1A and S1B). To confirm that Δ FosB expression in APP mice is driven by spontaneous epileptiform activity, we treated NTG and APP mice with the antiepileptic drug levetiracetam (75 mg/kg) for 14 days. Levetiracetam reduced epileptiform activity (Figure S2) consistent with previous studies (Sanchez et al., 2012) and also reduced Δ FosB expression in APP mice to levels that were no longer significantly different from NTG mice (Figure 1D).

To determine whether such increased Δ FosB expression in the DG is related to c-Fos expression, we confirmed our previous findings of reduced c-Fos expression in the DG of APP mice (Chin et al., 2005; Palop et al., 2005; Figures 1E and 1F). In addition, we observed that APP mice with the highest Δ FosB expression had the lowest numbers of c-Fos-immunoreactive (IR) cells in the DG and that the magnitude of Δ FosB and c-Fos expression was inversely correlated in APP mice on both a mouse-by-mouse and a cell-by-cell basis (Figures 1G and 1H). Because c-Fos expression is tightly linked to spatial memory (Fleischmann et al., 2003; He et al., 2002; Tischmeyer and Grimm, 1999), we examined whether Δ FosB expression levels in APP mice were related to performance in the Morris water maze. Indeed, the magnitude of Δ FosB expression correlated with the distance to find the hidden platform in the water maze (Figure 1I). We also observed some variability in the water maze performance of NTG mice (Figure 1I), which can sometimes be randomly affected even in control conditions. There was no obvious systematic relationship with Δ FosB levels, which did not vary greatly in NTG mice.

The increase in DG Δ FosB expression is not restricted to the particular line of APP mice used in our studies (line J20), as Tg2576 and presenilin/amyloid precursor protein (PSAPP) mice also exhibited increased Δ FosB-IR in the DG (Figures 1J, 1K, S1E, and S1F). All of these lines of mice express human APP with mutations linked to autosomal dominant AD, produce high levels of A β (Chin, 2011), and exhibit epileptiform activity (Chin and Scharfman, 2013; Corbett et al., 2013; Kam et al., 2016; Minkeviciene et al., 2009; Palop et al., 2007). Notably, similarly aged I5A mice, which overexpress wild-type human APP and do not produce high levels of A β (Mucke et al., 2000), do not exhibit increased Δ FosB (Figures 1L and S1G).

Epigenetic Mechanism of c-Fos Suppression in APP Mice

Δ FosB suppresses c-Fos expression in nucleus accumbens by directly binding to the c-Fos promoter and recruiting HDAC1, thereby promoting deacetylation of histone H4 (Renthal et al., 2008). We hypothesized that this mechanism could also be responsible for the decreased c-Fos expression observed in DG of APP mice. Indeed, chromatin immunoprecipitation (ChIP) experiments demonstrated increased binding of Δ FosB to the c-Fos promoter in the hippocampus of APP mice (Figure 2A). Interactions between Δ FosB and HDAC1 have been characterized in nucleus accumbens (Renthal et al., 2008), and co-immunoprecipitation experiments showed Δ FosB also binds HDAC1 in the hippocampus (Figure 2B). Consistent with this,

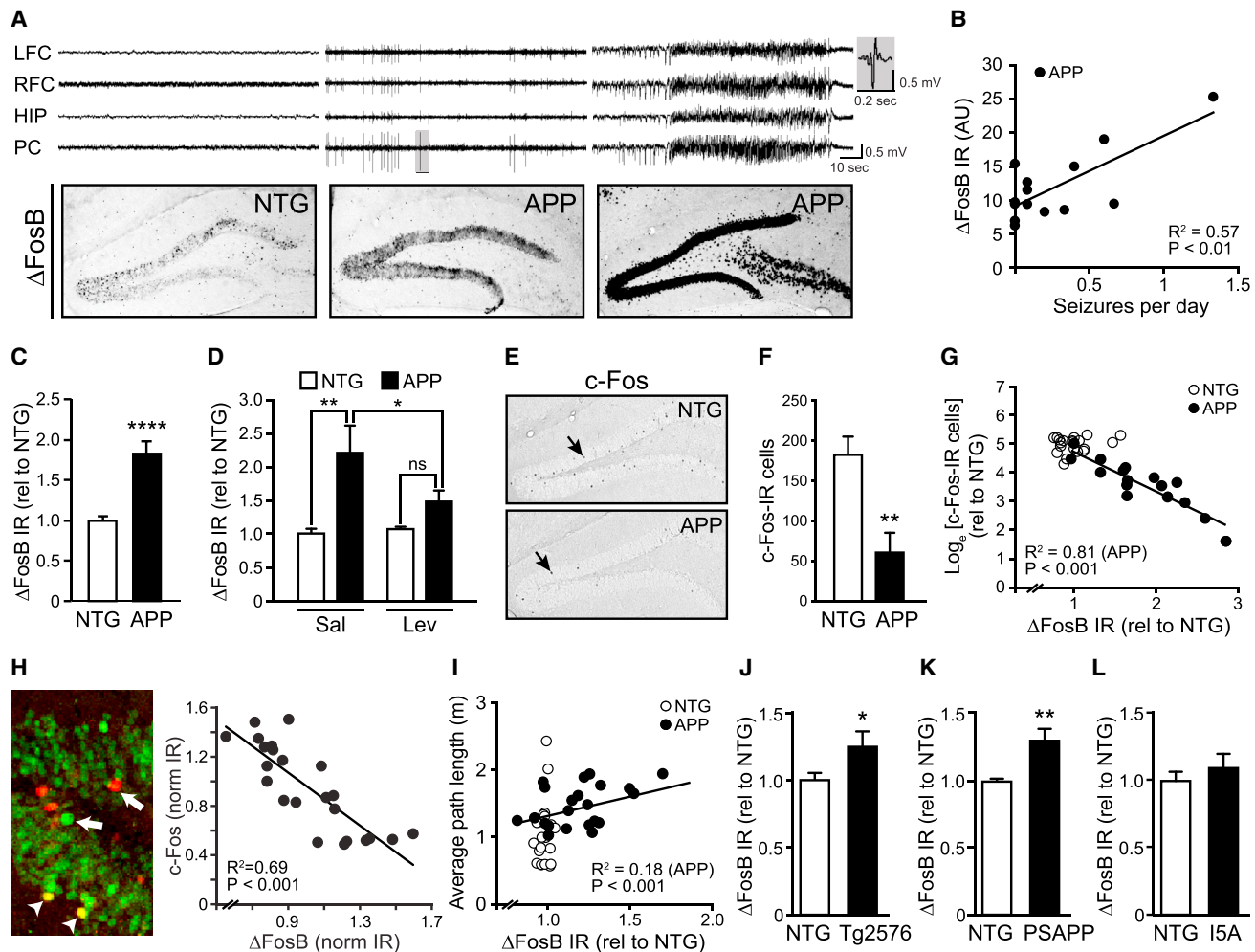


Figure 1. Epileptiform Activity and Seizures in APP Mice Were Associated with Increased Δ FosB and Decreased c-Fos in the Hippocampus

(A) EEG (upper panels) and corresponding immunohistochemical staining for Δ FosB (lower panels) in 4- to 6-month-old APP mice. A representative epileptiform discharge in the shaded region in the middle set of traces is magnified on the right side of the panel. Electrodes were in left and right frontal cortex (L/RFC), hippocampus (HIP), and parietal cortex (PC).

(B) Increased hippocampal Δ FosB immunoreactivity (IR) is associated with epileptiform activity and frequency of seizures (APP; $n = 14$).

(C) Increased Δ FosB IR in the DG of APP versus NTG mice ($n = 12$ /genotype).

(D) 14-day treatment of APP mice with levetiracetam reduces Δ FosB IR ($n = 4$ or 5 per treatment/genotype; two-way repeated measures (RM) ANOVA with Bonferroni post hoc tests).

(E) Images of c-Fos-IR in NTG and APP mice.

(F) Reduced number of c-Fos-IR cells in the DG of APP versus NTG mice ($n = 16$ – 18 /genotype).

(G) Expression of Δ FosB and c-Fos is inversely related in the DG of APP mice ($n = 19$ mice).

(H) Expression of Δ FosB and c-Fos is also inversely related in individual cells within the DG ($n = 23$ cells). Arrows indicate cells expressing primarily Δ FosB (green) or c-Fos (red); arrowheads indicate cells expressing both Δ FosB and c-Fos (yellow).

(I) Average path length to find the hidden platform in the Morris water maze is associated with hippocampal Δ FosB expression ($n = 21$ or 22/genotype).

(J–L) Increased Δ FosB IR was detected in the DG of Tg2576 ($n = 9$ or 10/genotype) (J) and PSAPP mice ($n = 5$ /genotype) (K), but not in I5A mice ($n = 7$ – 9 /genotype) (L).

AU, arbitrary unit. Data were normalized to NTG control values (rel to NTG). * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$. Error bars indicate SEM. See also [Figures S1 and S2](#) and [Tables S3 and S4](#).

a target of HDAC1, histone H4, was hypoacetylated at the c-Fos promoter in the hippocampus of APP mice (Figure 2C). No significant differences in histone H3 acetylation at the c-Fos promoter were observed between NTG and APP mice (Figures 2D and 2E), indicating relative specificity. Because histone H4 acetylation is

important for gene transcription (Grunstein, 1997; Lee et al., 1993; Struhl, 1998; Tsankova et al., 2004), we measured c-Fos mRNA in the hippocampus of NTG and APP mice and confirmed that APP mice exhibited decreased levels of hippocampal c-Fos mRNA compared to NTG controls (Figure 2F). Together, these

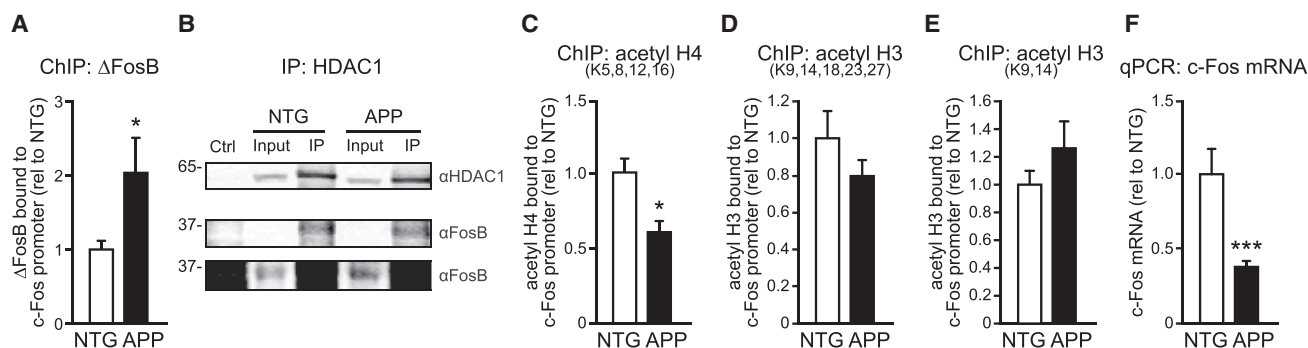


Figure 2. Epigenetic Suppression of the c-Fos Gene in the Hippocampus of APP Mice

(A) Increased Δ FosB binding to the c-Fos promoter in the hippocampus APP versus NTG mice (n = 8/genotype).
 (B) Δ FosB co-immunoprecipitates (IP) with HDAC1 in the hippocampus of NTG and APP mice. Two exposures of the same Δ FosB blot are shown.
 (C) c-Fos promoter is hypoacetylated on histone H4 in APP versus NTG mice (n = 7/genotype).
 (D and E) No changes in tetra-acetyl histone H3 (n = 8/genotype) (D) or di-acetyl histone H3 (n = 8/genotype) (E) were detected in APP versus NTG mice.
 (F) Reduced c-Fos mRNA expression in APP versus NTG mice (n = 12/genotype).
 Data were normalized to NTG control values (rel to NTG). *p < 0.05; ***p < 0.001. Error bars indicate SEM. See also Tables S3 and S4.

data support the hypothesis that increased hippocampal Δ FosB suppresses c-Fos expression in APP mice.

Pharmacologically Induced Seizures Are Sufficient to Increase Δ FosB and Suppress c-Fos in NTG Mice within Days

We next investigated whether seizures alone, outside of the context of other AD-related factors, were sufficient to drive Δ FosB-mediated c-Fos suppression and hippocampal memory deficits in NTG mice. We assessed Δ FosB and c-Fos IR in the DG of NTG mice given a single convulsant injection of kainic acid (KA) or saline control and examined mice either 2 hr later as seizures were occurring or 3 days later when seizures were over. Mice injected with KA exhibited dose-dependent behavioral seizures that were observed in the first 2 hr post-injection (Figure S3A). For mice that were sacrificed 2 hr after KA injection, high doses (15 and 25 mg/kg) of KA caused severe behavioral seizures and increased levels of both Δ FosB and c-Fos compared to saline-treated mice (Figures 3A, 3B, S1A, and S1C), whereas no changes were observed in mice receiving a low dose (5 mg/kg) of KA that did not elicit behavioral seizures.

Three days after KA injection, Δ FosB expression remained increased in mice that received high doses of KA, consistent with its long half-life, and was unchanged in mice that received low doses of KA (Figures 3C, S1A, and S1D). Δ FosB expression persisted for at least 4 weeks after KA injection (Figure S3B). At 3 days post-injection, low doses of KA increased c-Fos expression, whereas high doses of KA decreased c-Fos expression (Figure 3D). The dose-dependent relationship between Δ FosB and c-Fos expression was positively correlated 2 hr after KA (Figure 3E) but inversely correlated 3 days after KA (Figure 3F). These results suggest that at 2 hr post-KA, Δ FosB had not yet suppressed c-Fos expression; however, by 3 days post-KA, it had. Therefore, induction of seizures in NTG mice appears to be sufficient, after 3 days, to simulate the increased Δ FosB/decreased c-Fos signature observed in the DG of APP mice. A summary of the cellular changes following different seizure severities is provided in Figure 3G.

To determine whether the decreased c-Fos levels observed in the DG of mice exposed to high doses of KA were also due to Δ FosB-mediated epigenetic suppression, we performed ChIP experiments. Three days after mice were administered 15 mg/kg KA, there was increased binding of Δ FosB to the Fos promoter compared to saline-treated mice (Figure 3H) and also decreased hippocampal Fos mRNA expression (Figure 3I).

We investigated whether the increased Δ FosB expression in KA-treated mice was also associated with hippocampal memory deficits. We used the object location memory task, a hippocampus-dependent spatial memory task (Faust et al., 2013; Scharfman and Binder, 2013). Mice received weekly injections of either saline or KA (15 mg/kg) for 3 weeks to ensure maximal Δ FosB expression and persistent decreases in c-Fos prior to behavioral testing. Mice that received saline injections displayed intact memory, but mice that received KA injections exhibited impaired memory (Figure 3J). Together, these data support the hypothesis that seizures are sufficient to drive Δ FosB-mediated suppression of c-Fos and contribute to hippocampal memory deficits.

Persistent Δ FosB Expression Leads to Desensitization of c-Fos Expression with Repetitive Seizures

c-Fos mRNA induction in the cortex is attenuated after repeated neuronal stimulation by either brain injury or seizures (Calais et al., 2013; Ivkovic et al., 1994; Renthal et al., 2008; Winston et al., 1990). We hypothesized that, similar to the effect of chronic exposure to drugs of abuse (Renthal et al., 2008), repeated seizures may lead to desensitization of c-Fos expression due to the upregulation and persistent presence of Δ FosB in the hippocampus. We therefore examined whether increased Δ FosB expression coincided with desensitization of c-Fos mRNA induction in the hippocampus following multiple KA injections. We first verified the expression time course of c-Fos mRNA by injecting NTG mice with a single dose of 15 mg/kg of KA and then assessing levels of hippocampal c-Fos mRNA at various time points. As expected, c-Fos mRNA was robustly increased 1 hr post-KA and returned to baseline levels 4.5 hr post-KA (Figure 4A). We then injected NTG mice with KA (15 mg/kg) either one, two, three,

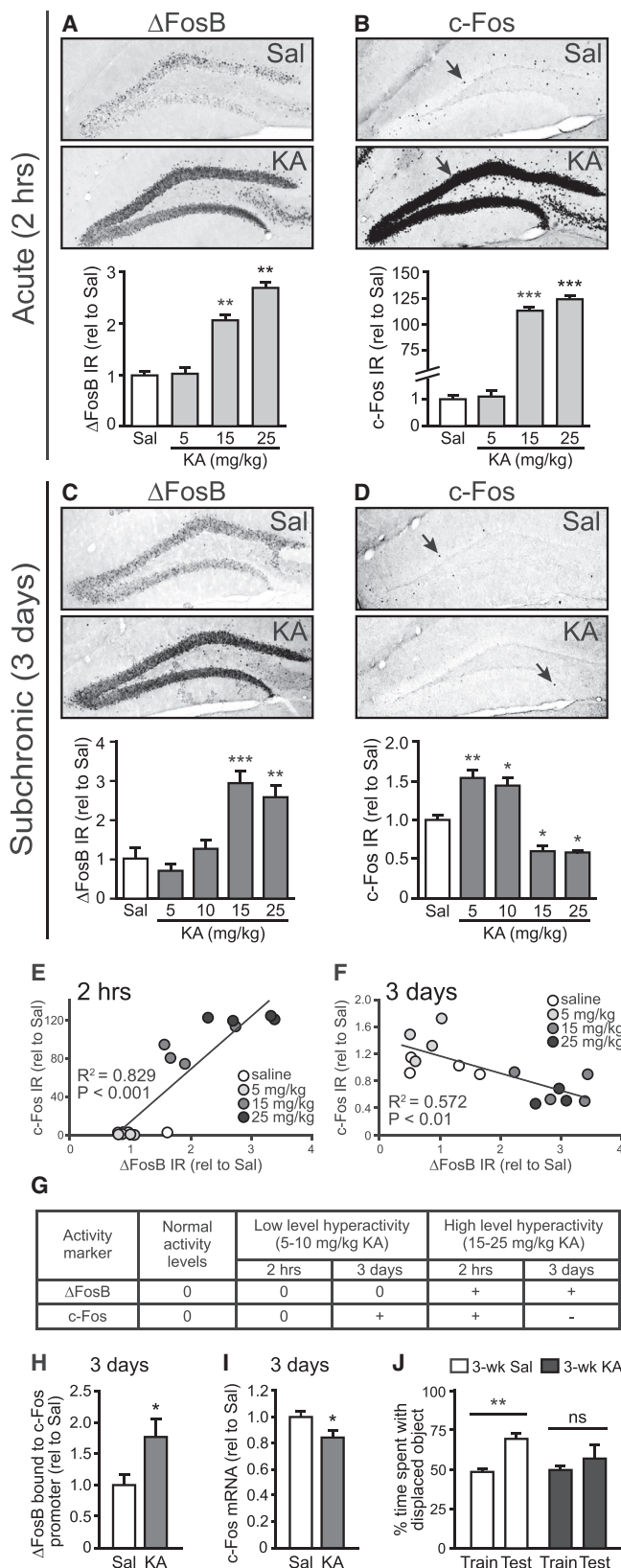


Figure 3. Pharmacologically Induced Seizures Are Sufficient to Increase Δ FosB and Suppress c-Fos in NTG Mice within Days

(A and B) At 2 hr after single administration of 15 or 25 mg/kg KA, there were increases in Δ FosB IR (n = 4/group) (A) and c-Fos IR (n = 4/group) (B).

(C) Δ FosB IR is increased 3 days after single administration 15 or 25 mg/kg KA (n = 4/group).

(D) c-Fos IR is increased 3 days after single administration of 5 or 10 mg/kg KA but decreased 3 days after 15 or 25 mg/kg KA (n = 4/group).

(E) Δ FosB and c-Fos expression are positively correlated with increasing doses of KA when assessed 2 hr after KA injection.

(F) Δ FosB and c-Fos expression are inversely correlated with increasing doses of KA when assessed 3 days after KA injection.

(G) Summary of results from (A)–(D). “0” indicates no change from control, whereas “+” and “-” indicate increase and decrease from control, respectively.

(H) Increased Δ FosB binding to the c-Fos promoter in mice 3 days after 15 mg/kg KA (n = 5/group).

(I) Decreased Fos mRNA expression in mice 3 days after 15 mg/kg KA (n = 8 or 9/group).

(J) NTG mice treated with three weekly doses of saline, but not 15 mg/kg KA, show preference for the displaced object in the test phase of the object location memory task (n = 6/group).

Data were normalized to saline-treated control values (rel to Sal). * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$. ns, non-significant. Error bars indicate SEM. See also Figures S1 and S3 and Tables S3 and S4.

four, or five times, with injections spaced 4.5 hr apart for the first four injections and 12 hr apart for the fourth and fifth injections. Mice were sacrificed 1 hr following their final injection, and hippocampal Δ FosB protein and c-Fos mRNA expression was assessed. Increased expression of Δ FosB protein was first detected after the second injection, and remained high throughout the remainder of the protocol (Figure 4B), and coincided with desensitization of c-Fos mRNA expression (Figure 4C). These results support the hypothesis that persistent Δ FosB upregulation can desensitize c-Fos expression to repeated stimulation. The mean maximum seizure score in mice injected multiple times with KA was lower than in mice injected only once (Figure S4A), so other factors, such as modification of glutamate receptors (Borbély et al., 2009; Világi et al., 2009), may also be involved in attenuation of c-Fos induction and seizures scores. The multiple KA injections did not result in any obvious cell death (Figure S4B).

HDAC1 Contributes to the Suppression of Physiological c-Fos Induction in the Hippocampus of APP Mice

Given that Δ FosB suppresses c-Fos expression during seizures, we hypothesized that prolonged levels of Δ FosB might serve as a persistent brake that limits c-Fos expression during physiological neuronal activity. Indeed, APP mice exhibit impaired induction of c-Fos expression after exploration of a novel environment (NE) (Palop et al., 2003), but the mechanisms were not clear. Because Δ FosB binds HDAC1 in the hippocampus, and suppression of c-Fos in the nucleus accumbens is dependent on Δ FosB-mediated recruitment of HDAC1 to the c-Fos promoter (Kennedy et al., 2013; Renthal et al., 2008), we predicted that acute inhibition of HDAC1 would improve physiological c-Fos induction.

To test this hypothesis, we treated mice with the class I histone deacetylase (HDAC) inhibitor 4-phenylbutyric acid (4-PBA) 1 hr prior to exposure to NE. After exploring the NE for 2 hr, mice

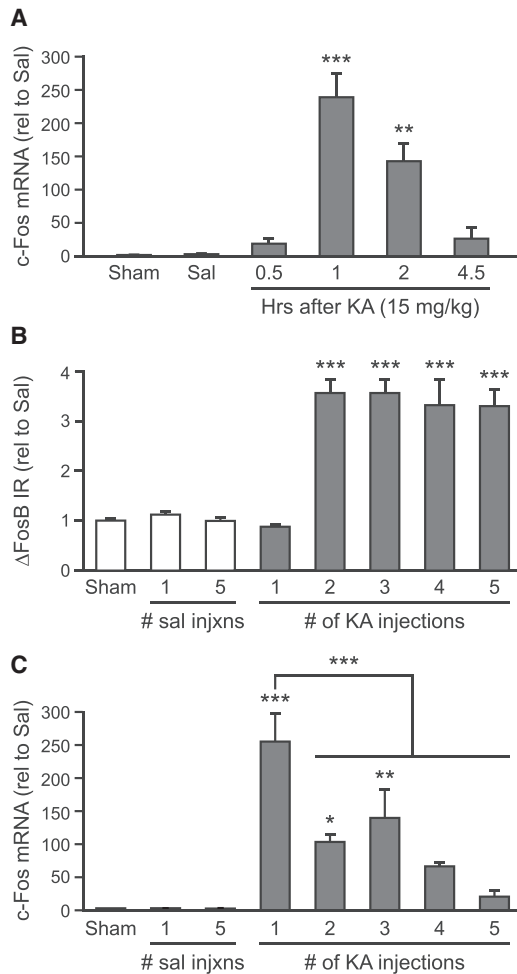


Figure 4. Persistent Δ FosB Expression Leads to Desensitization of c-Fos Expression with Repetitive Seizures

(A) Hippocampal c-Fos mRNA expression in NTG mice is increased 1 or 2 hr after single administration of 15 mg/kg KA but returns to baseline at 4.5 hr ($n = 3$ or 4/group).

(B) DG Δ FosB IR was increased in mice that received multiple administrations of 15 mg/kg KA.

(C) The increase in c-Fos mRNA expression after each KA injection is attenuated with multiple injections, which is first evident when Δ FosB expression increases as shown in (B).

Data were normalized to saline-treated control values (rel to Sal). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Error bars indicate SEM. See also [Figure S4](#) and [Tables S3](#) and [S4](#).

were sacrificed and their brains processed to assess c-Fos-IR cells in the DG. Mice treated with saline and/or left in their home cages were used as controls. As expected, saline-treated NTG, but not APP, mice exhibited a greater number of total c-Fos-IR cells when exposed to the NE than when left in their home cages ([Figure 5A](#)). Surprisingly, neither NTG nor APP mice treated with 4-PBA 1 hr prior to placement in the NE exhibited increased total numbers of c-Fos-IR cells compared to their home cage controls ([Figure 5B](#)). We initially thought that the lack of effect might be due to non-specific HDAC blockade, because 4-PBA inhibits all class I HDACs (HDACs 1–3 and 8). To

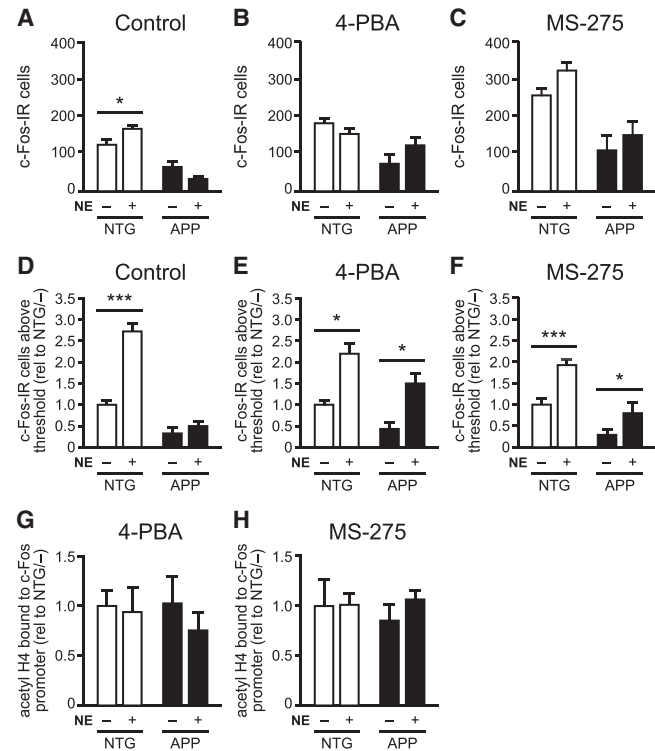


Figure 5. Acute HDAC Inhibition Partially Restores c-Fos Induction in APP Mice

(A) NTG, but not APP, mice exhibit increased total c-Fos IR in the hippocampus after exploration of a novel environment for 2 hr (NE+) relative to home cage controls (NE–; $n = 9$ or 10/group).

(B and C) Neither NTG nor APP mice exhibit significant increases in the total number of c-Fos IR cells when exposed to NE 1 hr after 4-PBA treatment ($n = 6$ /group) (B) or 2 hr after MS-275 treatment ($n = 8$ –10/group) (C).

(D) In control conditions, NTG, but not APP, mice exhibit increased numbers of c-Fos-IR cells above optical threshold when exposed to NE versus home cage ($n = 9$ or 10/group).

(E and F) Both NTG and APP mice exhibit increased number of c-Fos-IR cells above optical threshold when exposed to NE 1 hr after 4-PBA treatment ($n = 6$ /group) (E) or 2 hr after MS-275 treatment ($n = 8$ –10/group) (F).

(G and H) Histone H4 acetylation on the Fos promoter in APP mice is normalized after treatment with 4-PBA ($n = 6$ /group) (G) or MS-275 ($n = 6$ /group) (H).

Data in (D)–(F) were normalized to NTG home cage control values (rel to NTG/–). * $p < 0.05$; *** $p < 0.001$. Error bars indicate SEM. See also [Figures S5](#) and [S6](#) and [Tables S3](#) and [S4](#).

inhibit HDAC1 more specifically, we treated NTG and APP mice with MS-275, an inhibitor of HDAC1–3 that has a median inhibition concentration (IC_{50}) that is 8- to 30-fold lower for HDAC1 than for HDACs 2 and 3 ([Bahari-Javan et al., 2012](#); [Hu et al., 2003](#); [Khan et al., 2008](#)). The brain penetration of MS-275 has also been characterized ([Hooker et al., 2010](#); [Simonini et al., 2006](#)), allowing us to administer a dose predicted to be near the IC_{50} for HDAC1. However, similar to 4-PBA treatment, treatment with MS-275 2 hr before placement in the NE did not increase the total number of c-Fos-IR cells of NTG or APP mice compared to home cage controls ([Figure 5C](#)).

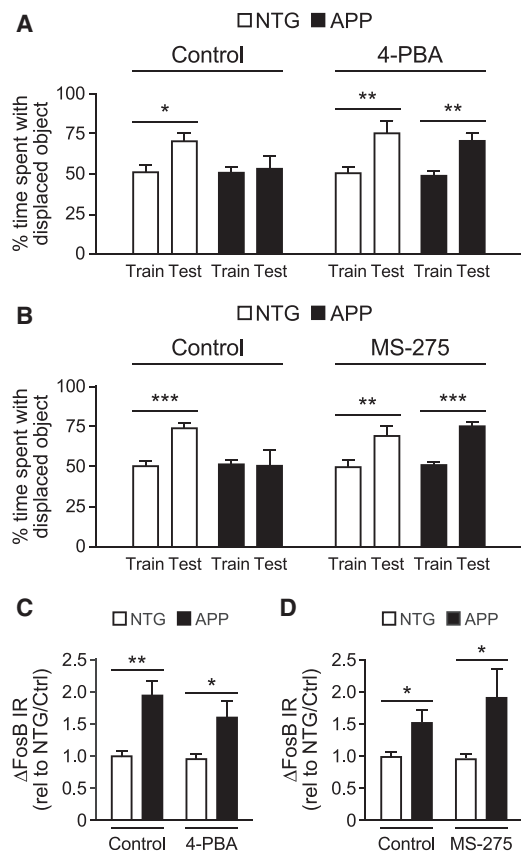


Figure 6. Acute HDAC Inhibition Improves Hippocampus-Dependent Learning and Memory in APP Mice

(A and B) APP mice exhibited increased time interacting with the displaced object during the test phase of the task, when, prior to training, they were treated with either 4-PBA (n = 5 or 6/group) (A) or MS-275 (n = 6/group) (B) relative to treatment with vehicle (control).

(C and D) Increased Δ FosB expression in APP versus NTG mice is unaffected by 4-PBA (n = 5 or 6/group) (C) or MS-275 (n = 7/group) (D).

Data in (C) and (D) were normalized to vehicle-treated NTG values (rel to NTG/Ctrl). *p < 0.05; **p < 0.01; ***p < 0.001. Error bars indicate SEM. See also Tables S3 and S4.

Because c-Fos is only expressed in a sparse population of DG cells at baseline, we hypothesized that increases in physiologically induced c-Fos following HDAC inhibition might be detectable only in select granule cells that were highly active. Furthermore, because HDAC inhibition appears to increase the baseline number of DG c-Fos-IR cells in home cage controls (Figures 5A–5C), it was possible that the specific population of c-Fos-IR cells induced during NE exploration was difficult to detect by total cell counts. Therefore, we investigated whether acute HDAC inhibitor treatment increased c-Fos IR in a specific population of DG cells capable of inducing c-Fos at high levels. To do this, we set an optical threshold during quantification so that only DG cells with high c-Fos-IR were counted (Figure S5). Using this quantification method, we found that saline-treated NTG mice exposed to the NE exhibited higher numbers of c-Fos-IR cells above threshold compared to home cage controls whereas saline-treated APP mice did not demonstrate this effect (Figure 5D).

However, when treated with either 4-PBA or MS-275 prior to placement in the NE, both NTG and APP mice that explored the NE exhibited increased numbers of c-Fos-IR cells above threshold compared to home cage controls (Figures 5E and 5F). To verify that treatment with 4-PBA and MS-275 inhibited HDAC1, we confirmed that there were no significant differences in hippocampal histone H4 acetylation at the c-Fos promoter among any groups treated with 4-PBA or MS-275 (Figures 5G and 5H). Moreover, no differences in the number of object interactions during NE exploration were observed between NTG and APP mice, regardless of treatment (Figures S6A–S6C), indicating that the induction of c-Fos was not due to behavioral alterations. We also confirmed that APP mice exhibited increased Δ FosB-IR in the DG compared to NTG controls, regardless of treatment or cage condition (Figures S6D–S6F). Δ FosB-IR was inversely related to the number of c-Fos-IR cells above threshold in saline and MS-275-treated APP mice and trended toward an inverse relation in 4-PBA-treated APP mice (Figures S6G–S6I). Moreover, acute HDAC inhibitor treatment did not affect the number of spikes observed by EEG recording, indicating that the alterations in c-Fos expression were not due to gross changes in EEG activity (Figure S6J). Together, these results demonstrate that HDAC1 contributes to the suppression of physiological c-Fos induction in the DG of APP mice.

Acute HDAC1 Inhibition Improves Hippocampus-Dependent Memory Deficits in APP Mice

Physiological c-Fos induction is critical for hippocampal memory (Fleischmann et al., 2003; He et al., 2002). Therefore, we hypothesized that acute HDAC inhibition would also improve hippocampal memory in APP mice if administered prior to training. We assessed hippocampal memory using the object location memory task. We treated mice with saline, 4-PBA, or MS-275 prior to training in the object location memory task. Saline-treated NTG mice, but not APP mice, spent an increased percentage of time interacting with the displaced object during testing compared to training. When treated with 4-PBA or MS-275, both NTG and APP mice spent an increased percentage of time interacting with the displaced object during the testing phase (Figures 6A and 6B). We hypothesize that Δ FosB recruits HDAC1 to deacetylate its gene targets, and thus, HDAC1 functions downstream of Δ FosB. Therefore, inhibition of HDACs with either 4-PBA or MS-275 should not affect Δ FosB levels, especially with the acute time frame of treatment, given that the half-life of Δ FosB is 8 days. Consistent with our hypothesis, APP mice displayed increased levels of Δ FosB in the DG compared to NTG controls, regardless of treatment (Figures 6C and 6D). Moreover, acute treatment with HDAC inhibitors did not non-specifically alter spiking frequency in APP mice (Figure S6J). These results support the hypothesis that HDAC1 actively and acutely suppresses the expression of genes important for hippocampal memory and contributes to cognitive deficits in APP mice.

Direct Inhibition of Δ FosB Improves c-Fos Induction and Hippocampus-Dependent Memory in APP Mice

Our results suggest that direct inhibition of Δ FosB should also improve DG c-Fos induction and spatial memory deficits in

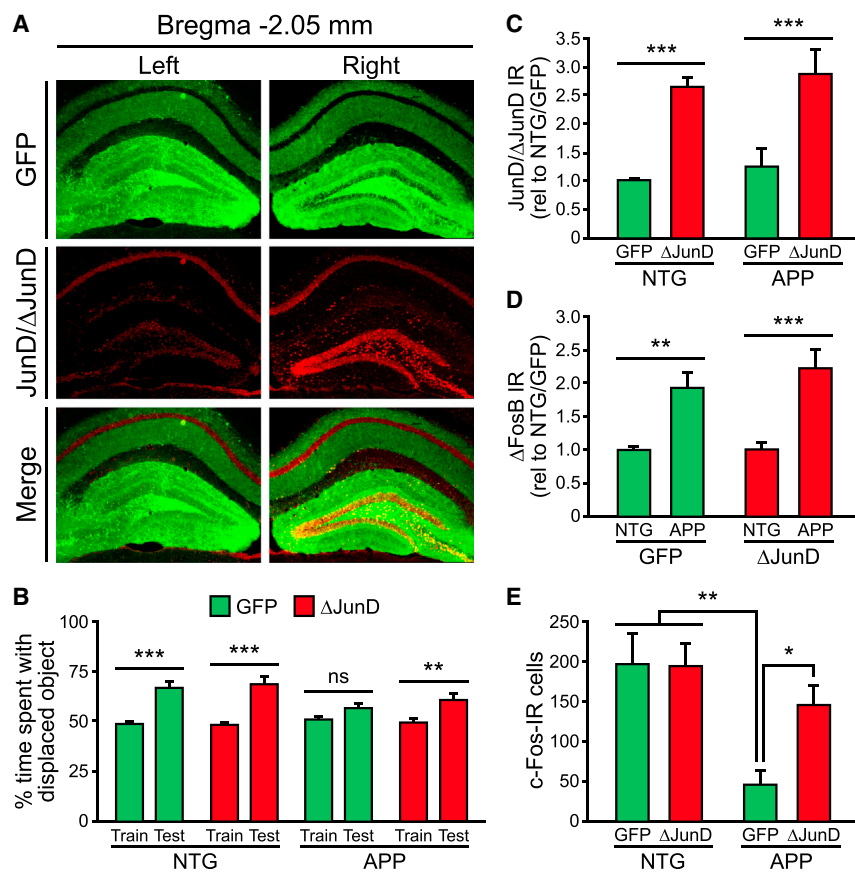


Figure 7. Direct Inhibition of Δ FosB Signaling Improves c-Fos Expression and Hippocampus-Dependent Memory in APP Mice

(A) Unilateral AAV-GFP/ Δ JunD infusion into the right DG achieves robust Δ JunD overexpression in comparison with AAV-GFP infused into the contralateral side.

(B) Bilateral AAV- Δ JunD infusion into the DG of APP mice improves performance in the object location memory task.

(C) Bilateral AAV-GFP/ Δ JunD (Δ JunD) infusion into the DG of NTG and APP mice achieves robust Δ JunD overexpression versus AAV-GFP (GFP) control.

(D) Δ JunD overexpression does not impact Δ FosB expression in APP mice.

(E) Δ JunD overexpression restores c-Fos expression in APP mice.

JunD/ Δ JunD and Δ FosB quantification data were normalized to AAV-GFP-treated NTG values (rel to NTG/GFP). $n = 5-8$ /group for (B)–(E). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Error bars indicate SEM. See also Figure S7 and Tables S3 and S4.

APP mice. To test whether this is true, we used Δ JunD, an N-terminally truncated mutant of JunD, the normal binding partner of Δ FosB. When expressed in neurons, Δ JunD serves as a dominant-negative inhibitor of Δ FosB (Berton et al., 2007; Vialou et al., 2010).

To achieve Δ JunD overexpression in the DG, we utilized an adeno-associated virus serotype 2 (AAV2) delivery system (Vialou et al., 2010). We confirmed Δ JunD overexpression in the DG by stereotactically infusing AAV2 carrying GFP alone (AAV-GFP) into the left DG and AAV2 carrying both GFP and Δ JunD (AAV-GFP/ Δ JunD) into the right DG of NTG mice and assessed Δ JunD expression at 28 days. Using an antibody that recognizes both Δ JunD and endogenous JunD, we verified that AAV-GFP/ Δ JunD overexpressed Δ JunD by nearly 3-fold above endogenous JunD levels (Figures 7A and S7).

To test whether Δ JunD overexpression improves hippocampal memory and increases c-Fos expression in the DG of APP mice, we infused either AAV-GFP or AAV-GFP/ Δ JunD bilaterally into the DG of NTG and APP mice and tested them in the object location memory task 28 days later. NTG mice spent more time interacting with the displaced object in the testing phase of the task, regardless of treatment (Figure 7B). APP mice that received AAV-GFP spent similar amounts of time with the displaced object in both training and testing phases, demonstrating impaired hippocampal memory (Figure 7B). However, APP mice that received AAV-GFP/ Δ JunD spent an increased percentage of

time interacting with the displaced object in the testing phase (Figure 7B), indicating that Δ JunD overexpression improves hippocampal memory in APP mice.

After behavioral testing, we verified that JunD-IR was increased in NTG and APP mice receiving AAV-GFP/ Δ JunD infusion (Figure 7C) and that Δ FosB expression was increased in APP mice regardless of

the type of virus infused (Figure 7D). We then quantified the total number of c-Fos-IR cells in the DG of NTG and APP mice receiving AAV infusions. Δ JunD overexpression did not affect c-Fos expression in the DG of NTG mice. APP mice that received AAV-GFP/ Δ JunD exhibited increased numbers of c-Fos-IR cells in the DG compared to APP mice that received AAV-GFP (Figure 7E). This result indicates that Δ FosB directly mediates the suppression of c-Fos expression in the DG of APP mice.

DISCUSSION

A growing body of evidence suggests that network dysfunction in the form of epileptiform activity contributes to cognitive deficits in AD, even in early stages of the disease (Bakker et al., 2012; Vossel et al., 2013, 2016). Understanding how specific neuronal alterations contribute to cognitive deficits may therefore highlight additional therapeutic avenues to improve the quality of life for AD patients. Moreover, because long-term treatment with antiepileptic drugs is associated with many adverse effects, targeting more specific mechanisms may enable development of treatments with fewer side effects.

Our studies reveal a molecular mechanism by which epileptiform activity might contribute to cognitive deficits in AD and epilepsy. This mechanism highlights seizure-induced Δ FosB as a transcription factor that critically regulates hippocampal memory, although other effects of seizures and/or APP/ $A\beta$ may also

play a role. Due to the unusually long half-life of Δ FosB, even infrequent epileptiform activity would be sufficient to maintain persistent high levels of Δ FosB. Therefore, Δ FosB is well poised to modulate gene expression over extended periods of time, which may be part of why cognitive deficits persist even during seizure-free periods in AD and epilepsy patients, as well as in relevant mouse models.

A critical implication of Δ FosB-mediated c-Fos suppression is that it also explains the biphasic expression curve of c-Fos expression after sustained high levels of neuronal activity. Hippocampal c-Fos expression is increased by acute neuronal activity but decreased by chronic activity (McClung et al., 2004; Renthal et al., 2008; Sheng and Greenberg, 1990; Tsankova et al., 2004). The gradual but persistent nature of activity-induced Δ FosB expression directly accounts for the biphasic expression profile of c-Fos. Acute neuronal activity induces c-Fos expression prior to the accumulation of Δ FosB to high enough levels in the nucleus to regulate gene expression. Following chronic neuronal activity, persistent increases in Δ FosB allow it to reach a threshold that enables regulation of genes, such as c-Fos. Therefore, although c-Fos expression is frequently used as a marker of neuronal activity, it may only accurately reflect activity under acute conditions. However, the simultaneous use of both Δ FosB and c-Fos as activity markers provides a more accurate readout of neuronal activity.

We focused on Δ FosB-mediated suppression of hippocampal c-Fos expression as a mechanism for hippocampal memory deficits. However, the extent to which the normalization of other Δ FosB-targeted genes contribute to cognitive improvement was not investigated. We chose to investigate Δ FosB-mediated c-Fos suppression because the role of c-Fos in synaptic plasticity and hippocampal memory is well established (Fleischmann et al., 2003; He et al., 2002; Tischmeyer and Grimm, 1999). Furthermore, Δ FosB may not be the sole mechanism underlying hippocampal c-Fos suppression in APP mice. Other factors, such as altered regulation of CREB-regulated transcription co-activator-1 (España et al., 2010; Saura, 2012; Yiu et al., 2011) and increased levels of miR-181 (Rodriguez-Ortiz et al., 2014), have also been implicated in reduced c-Fos expression exhibited by mouse models of AD.

We hypothesized that Δ FosB actively suppresses the expression of genes important for cognition in the hippocampus and that even a temporary relief of this suppression would improve hippocampal memory. Indeed, acute HDAC inhibition improved hippocampal c-Fos induction and hippocampal memory in APP mice. Although acute HDAC inhibition may have improved memory in APP mice by altering the expression of many genes important for cognition in addition to c-Fos, the increase in c-Fos is likely to have contributed strongly to the efficacy of acute HDAC inhibition. Chronic treatment with class 1 HDAC inhibitors, including 4-PBA, improved learning and memory in APP mice (Kilgore et al., 2010; Ricobaraza et al., 2009), but our results demonstrate that even a temporary relief of gene suppression can improve cognition when administered prior to training in a memory paradigm. For AD patients, acute treatment options may minimize side effects by allowing for an infrequent but well-timed schedule of administration (e.g., during family visitation), thus improving overall quality of life.

Acute HDAC inhibition did not increase the total number of c-Fos-IR cells in the DG of APP mice following exploration in the NE paradigm; however, it did increase c-Fos expression in a certain population of neurons. As such, it is possible that acute HDAC inhibition is unable to affect more severe and/or chronic epigenetic mechanisms that may have occurred, such as histone or DNA methylation. Overexpression of Δ JunD, a more chronic treatment that might alter multiple modes of epigenetic modification, was able to improve the total number of c-Fos-IR neurons in the DG of APP mice, lending support for this hypothesis.

Understanding how network dysfunction induces neuronal alterations that cause cognitive deficits emphasizes the importance of the role that seizures play in AD. Furthermore, many diseases, including schizophrenia (Hyde and Weinberger, 1997), autism (Viscidi et al., 2013), Down syndrome (Lott et al., 2012), fragile X syndrome (Hecht, 1991), Rett syndrome (Jian et al., 2006), and Angelman syndrome (Pelc et al., 2008), are all accompanied by both seizures and cognitive deficits. Therefore, it is possible that seizures and network dysfunction may be common denominators contributing to cognitive deficits in many brain disorders. In addition, seizure-induced increases in Δ FosB may contribute to deficits in cognition or other behaviors via alteration of downstream gene targets that are already vulnerable due to compromised genetic backgrounds.

EXPERIMENTAL PROCEDURES

Mice

4- to 6-month-old heterozygous transgenic and NTG mice from lines hAPP-J20, Tg2576, and PSAPP were used in these experiments. Male and female mice were used unless otherwise specified, and sex was balanced within a cohort. All mice referred to as APP mice were of the line hAPP-J20, which express a human APP (hAPP) gene carrying Swedish (K670N and M671L) and Indiana (V717F) familial AD mutations under control of the platelet-derived growth factor β -chain promoter, on a C57BL/6 background (Mucke et al., 2000). Tg2576 mice express hAPP carrying the Swedish familial autosomal dominant (FAD) mutation (Hsiao et al., 1996). PSAPP mice express hAPP carrying the Swedish mutation and presenilin-1 with FAD mutation (M146L; Holcomb et al., 1998). For brain harvesting, mice were flush-perfused transcardially with diethyl pyrocarbonate (DEPC)-treated saline. Hemibrains were frozen on dry ice for biochemistry or postfixed in 4% paraformaldehyde for immunohistochemistry. All procedures were approved by the Thomas Jefferson University and Baylor College of Medicine Institutional Animal Care and Use Committees.

Drug Treatment

Levetiracetam was dissolved in saline at 15 mg/mL and administered intraperitoneally (i.p.) at a dose of 75 mg/kg every 8 hr for 14 days. KA (Sigma) was dissolved in saline at 2 mg/mL and administered i.p. at doses described in Results. 4-PBA (Aldrich) was dissolved with equimolar NaOH in ddH₂O to 0.125 M and injected i.p. at a dose of 200 mg/kg 1 hr prior to NE or object placement paradigms. A stock solution of 50 mg/mL MS-275 in DMSO was dissolved into a working solution of 1.5 mg/mL MS-275 in saline containing 20% DMSO, which was administered at 6 mg/kg via subcutaneous injection 2 hr prior to NE or object placement paradigms.

KA Injections and Seizure Severity Scoring

NTG mice were injected with KA IP at specified doses and monitored for 2 hr. Seizure severity and elapsed times since injection were recorded using a modified Racine scale (Racine, 1972; Roberson et al., 2007): 0, normal behavior; 1, immobility; 2, generalized spasm, tremble, or twitch; 3, tail extension; 4, forelimb clonus; 5, generalized clonic activity with loss of posture; 6, bouncing or running seizures; 7, full tonic extension; and 8, death. Acute KA

groups were sacrificed 2 hr following a single KA injection (15 mg/kg). Sub-chronic KA groups were sacrificed 3 days following a single KA injection (15 mg/kg). For mice receiving multiple KA injections, all injections were separated by 4.5 hr except for the fourth and fifth injections, which were separated by 12 hr. Unless otherwise specified, mice were sacrificed 0.5 hr following their final injection.

Video-EEG Monitoring

Electrodes were implanted as described in [Supplemental Experimental Procedures](#). EEG recordings were performed on at least 2 different days for a minimum of 4 hr each using a Stellate Harmonie acquisition system. EEG signal processing was performed using native Stellate software and Labchart Pro (AD Instruments). The frequency and total number of spikes for each mouse was analyzed.

AAV-Mediated Gene Transfer

AAV2 carrying CMV- Δ JunD-IRES2-eGFP (AAV-GFP/ Δ JunD) or CMV-eGFP (AAV-GFP) was previously described ([Berton et al., 2007](#); [Vialou et al., 2010](#)), demonstrating that these constructs are neurotropic and achieve stable neuronal gene expression within 18–22 days of infusion into the brain. One microliter of virus solution was stereotaxically infused unilaterally or bilaterally into the hippocampus at rostral (–1.7 mm anterior/posterior [A/P], 1.2 mm medial/lateral [M/L], and 2 mm dorsal/ventral [D/V] from bregma) and caudal (–2.7 mm A/P, 2 mm M/L, and 2.1 mm D/V from bregma) coordinates. Approximately 2×10^9 infectious particles were infused into each hippocampus. Mice were allowed to recover for 28 days post-surgery before behavior testing/sacrifice.

Morris Water Maze

Female NTG and APP mice (4–6 months old; 20–24/genotype) were tested as described in detail in the [Supplemental Experimental Procedures](#). Mice were first tested in cued platform sessions, two trials per day for 2 days. Hidden platform training involved six trials/day over 7 days. After the last training trial, the platform was removed and mice were given a 60-s probe trial test. Performance was video tracked and analyzed using TopScan (CleverSys). The experimenter was blinded to genotypes during testing.

Object Location Memory Task

The object location memory task was based on previously described protocols ([Scharfman and Binder, 2013](#)) and is described in detail in the [Supplemental Experimental Procedures](#). Briefly, mice were trained in three trials to recognize set locations of two identical objects in an empty mouse cage, and the time spent exploring each object during each of the three trials was recorded. After a 2-hr delay, the mouse was placed back in the test cage, in which one of the objects had been displaced. The time spent exploring the displaced versus the non-displaced object was recorded. For HDAC inhibitor treatments, training began 1 hr following i.p. injection with saline or 4-PBA or 2 hr following subcutaneous injection with 20% DMSO in saline or MS-275. For KA experiments, mice were injected with saline or 15 mg/kg KA once a week for 3 weeks and trained/tested 3 days following the third injection.

Novel Environment

Female NTG and APP mice were placed in a NE either without treatment, 1 hr following 4-PBA treatment, or 2 hr following MS-275 treatment. Home cage control mice for each group received the same treatment in close temporal proximity. The home cages of mice contained only bedding, a water bottle, and a food hopper. The NE cage included a novel bedding material, a banana slice, the grid of a pipette tip rack, a 50-mL conical vial cap, and a repeat pipetter tip wrapped in red tape. The top of the cage lid was removed for observation. Mice explored the NE for 2 hr while being video monitored and were then sacrificed. The number of object interactions was quantified for each mouse in 10-min bins at 1, 50, and 110 min into the recording.

Immunohistochemistry

Tissue preparation and immunohistochemistry were performed as described ([Chin et al., 2005](#); [Corbett et al., 2013](#)). Brains were sectioned at 30 μ m. Antibodies are listed in [Table S1](#). For staining with 3,3'-diaminobenzidine (DAB),

further amplification was accomplished using Avidin-Biotin Complex (Vectastain). DAB (Sigma) was used as a chromagen. Δ FosB-IR quantification was performed by assessing the mean pixel intensity using ImageJ software. Quantification of c-Fos was performed by total cell count or by using the threshold function provided by Metamorph. A pixel intensity threshold was set such that ~40–50 (~30% of total) cells were above threshold in the DG of NTG mice from the homecage group. This threshold was used for all mice, and the number of c-Fos-IR cells above threshold was recorded. For immunofluorescence, Metamorph software was used to quantify optical density throughout the rostral-caudal extent of the DG.

Western Blot

Western blot analysis was performed as described ([Corbett et al., 2013](#)). The hippocampus from one hemisphere of each mouse was isolated and homogenized with a Polytron tissue homogenizer in ice-cold radioimmunoprecipitation assay (RIPA) buffer. Equal amounts of protein were resolved by SDS-PAGE on 4%–12% gels, transferred to nitrocellulose, and probed with primary antibodies listed in [Table S1](#). IR-dye-conjugated secondary antibodies were used for detection and quantification using a LI-COR Odyssey infrared imaging system.

Co-immunoprecipitation

Hippocampi were lysed in immunoprecipitation buffer (320 mM sucrose, 10 mM Tris-HCl [pH 7.4], 10 mM EDTA, and 1% sodium deoxycholate) and diluted 1:5 in binding buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 0.5 mM EDTA, and 0.5% Triton-X). 4 μ g of rabbit anti-HDAC1 (Abcam) was added to immunoprecipitate HDAC1 prior to western blot analysis. HDAC1 and FosB were immunoblotted.

RNA Extraction

RNA extraction was adapted from instructions from the QIAGEN RNeasy kit. Briefly, hippocampi were homogenized in RLT/ β -mercaptoethanol buffer by passing the lysate through an 18G needle followed by a 25G needle. After centrifugation, RNA was extracted and eluted with nuclease-free water. Final RNA concentration was measured using a NanoDrop 2000 spectrophotometer.

qRT-PCR

Reverse transcription was performed using the Taqman Gold RT-PCR kit (ABI). qPCR was performed with an ABI 7500 PCR machine using SYBR Green as a fluorophore. Primers used to amplify cDNA are listed in [Table S2](#).

Chromatin Immunoprecipitation

ChIP was performed similarly to described methods ([Renthal et al., 2008](#); [Tsankova et al., 2004](#)). Hippocampi were subdissected and fixed in 1% formaldehyde. Samples were sonicated to generate genomic fragments 200–1,000 bp in length and pre-cleared with Protein A beads (Millipore) prior to incubation with antibodies listed in [Table S1](#) at 4°C overnight. The antibody-chromatin complex was immunoprecipitated with Protein A beads, washed with a series of buffers (Millipore), and then chromatin was eluted and reverse cross-linking performed with Proteinase K. DNA was purified via phenol-chloroform extraction. Final DNA concentration was measured using the NanoDrop 2000. qPCR was performed with an ABI 7500 PCR machine using SYBR green as a fluorophore. Primers used to amplify the *c-fos* promoter and *β -actin* promoter are listed in [Table S2](#).

Statistical Analysis

Statistical analyses were performed using Prism 5. Raw values for data presented as “relative to NTG controls” are listed in [Table S3](#). Differences between means were assessed using a two-tailed, unpaired Student's *t* test unless otherwise indicated. Differences among three or more means were assessed using one-way ANOVA and Tukey post hoc tests. Two-way ANOVA using Bonferroni or Student-Newman-Keuls post hoc tests were used to assess differences in analyses with two variables. Repeated-measures two-way ANOVA with Bonferroni post hoc tests were used for analyzing object location memory data. Regression analysis was used to detect correlations. To assess the relationship between c-Fos-expressing cells and Δ FosB expression, c-Fos cell counts were log transformed in order to perform a linear regression and

test for statistical significance. Detailed results of all statistical analyses are listed in Table S4.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and four tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2017.06.040>.

AUTHOR CONTRIBUTIONS

B.F.C. and J.C. conceived the project. B.F.C., X.Z., E.J.N., and J.C. designed the experiments. B.F.C., X.Z., M.S.P., U.T., J.C.Y., D.M.I., I.P., A.H., C.-H.F., G.S.S., A.A.A., S.A., L.Z., and J.C. performed the experiments and analyzed the data. All authors discussed the results, and B.F.C., J.C.Y., and J.C. wrote the manuscript.

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