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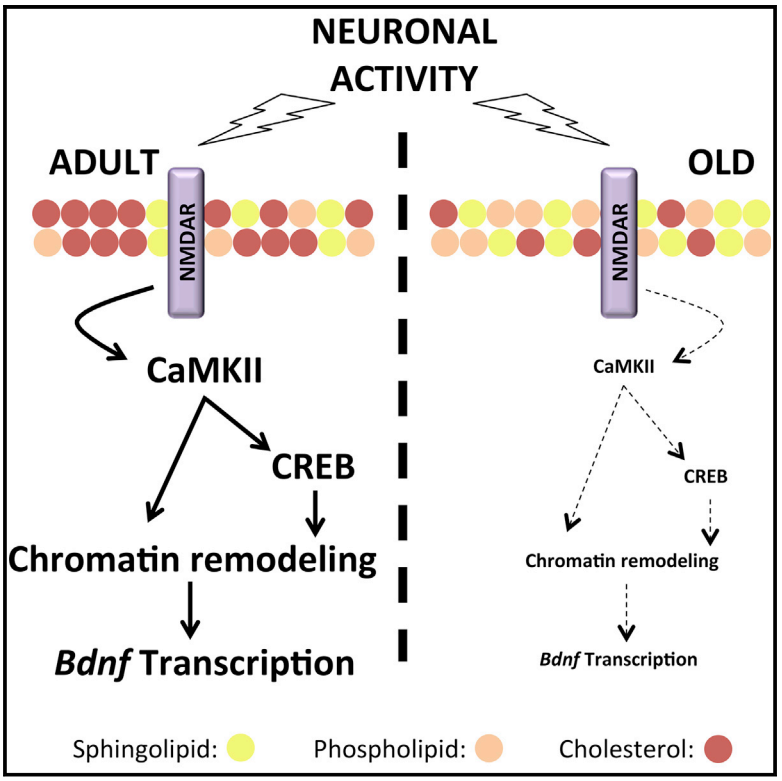
Aging Triggers a Repressive Chromatin State at *Bdnf* Promoters in Hippocampal Neurons

Graphical Abstract



Highlights

- Aging impairs the transcriptional induction of *Bdnf* by synaptic stimulation
- Age-linked membrane cholesterol reduction underlies weak synaptic signaling
- Weak synaptic signaling determines the repressive chromatin state at *Bdnf* promoters
- Preventing cholesterol loss in old mice rescues *Bdnf* transcription and improves cognition

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In Brief

Palomer et al. find that, unlike what occurs in adult animals, expression of the neurotrophic factor *Bdnf* does not respond to synaptic activity in the hippocampus of old mice. In addition, they show that age-associated cholesterol loss weakens the signaling pathway required for chromatin remodeling and transcription of this gene.

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Aging Triggers a Repressive Chromatin State at *Bdnf* Promoters in Hippocampal Neurons

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SUMMARY

Cognitive capacities decline with age, an event accompanied by the altered transcription of synaptic plasticity genes. Here, we show that the transcriptional induction of *Bdnf* by a mnemonic stimulus is impaired in aged hippocampal neurons. Mechanistically, this defect is due to reduced NMDA receptor (NMDAR)-mediated activation of CaMKII. Decreased NMDAR signaling prevents changes associated with activation at specific *Bdnf* promoters, including displacement of histone deacetylase 4, recruitment of the histone acetyltransferase CBP, increased H3K27 acetylation, and reduced H3K27 trimethylation. The decrease in NMDA-CaMKII signaling arises from constitutive reduction of synaptic cholesterol that occurs with normal aging. Increasing the levels of neuronal cholesterol in aged neurons in vitro, ex vivo, and in vivo restored NMDA-induced *Bdnf* expression and chromatin remodeling. Furthermore, pharmacological prevention of age-associated cholesterol reduction rescued signaling and cognitive deficits of aged mice. Thus, reducing hippocampal cholesterol loss may represent a therapeutic approach to reverse cognitive decline during aging.

INTRODUCTION

Aging is characterized by a progressive decline in cognitive capacities. It is unlikely that the decline of a complex process like cognition arises from the altered expression or activity of a single factor. However, changes in global regulators of gene expression, such as epigenetic mechanisms, may contribute to the aged brain phenotype. In support of this hypothesis, epigenetic regulatory mechanisms have been shown to be deregulated in the aged brain and in many age-related neurodegenerative disorders (Kosik et al., 2012; Peleg et al., 2010; Penner et al., 2010; Sanchez-Mut and Gräff, 2015). Studies in diverse systems have shown that reduced gene transcription in aged tissues is associated with increased levels of repressive chromatin marks (H3K9Me2-Me3 or H3K27Me3), decreased levels of activating chromatin marks (H3K9Ac, H3K14Ac, H4K12Ac, or H4K20Me1), and/or decreased levels of chromatin marks linked to transcriptional elongation (H3K36Me3; Peleg et al., 2010; Tang et al., 2011; Walker et al., 2013; Wang et al., 2010). In addition, the levels of histone deacetylases (HDACs) undergo age-associated changes (Baltan, 2012; Chouliaras et al., 2013). Thus, deregulation of chromatin-modifying enzymes occurs in the aging brain.

Epigenetic deregulation in the aging brain could arise from defects in the signal transduction of neuronal stimuli. For example, changes in subunit composition, localization, and/or assembly of neurotransmitter receptors may alter their activity and downstream signaling. The expression levels and lipidic surroundings of receptors change with age and affect their internalization and lateral diffusion (Ledesma et al., 2012; Martin et al., 2014a). One of the lipid changes that has the most profound effect on membrane structure is the reduction in cholesterol content, which was observed in the synaptic fraction of the hippocampus of aged mice (Martin et al., 2008; Sodero et al., 2011b) and in the hippocampus and cortex of mouse models of Alzheimer's disease (Fabelo et al., 2012; Xie et al., 2003). Reduced cholesterol content in the aged brain was also reported in humans, in both non-Alzheimer's and Alzheimer's brain samples (Mason et al., 1992; Molander-Melin et al., 2005; Mulder et al., 1998; Roher et al., 2002; Söderberg et al., 1990; Svennerholm et al., 1991, 1994, 1997; Thelen et al., 2006). In support of a direct relationship between age-associated cholesterol reduction and cognitive deficits, recent work demonstrated that restoring cholesterol levels in the hippocampus of old mice is sufficient to rescue biochemical, electrophysiological, and behavioral (cognitive) deficits (Martin et al., 2014a). Furthermore, restoring brain cholesterol levels rescues synaptic and cognitive dysfunction in a mouse model of Huntington's disease (Valenza et al., 2015). All these data suggest that cholesterol deficiency in the old may play a role in the epigenetic deregulation of genes involved in learning and memory and consequently in the cognitive deficits typical of this stage of life.

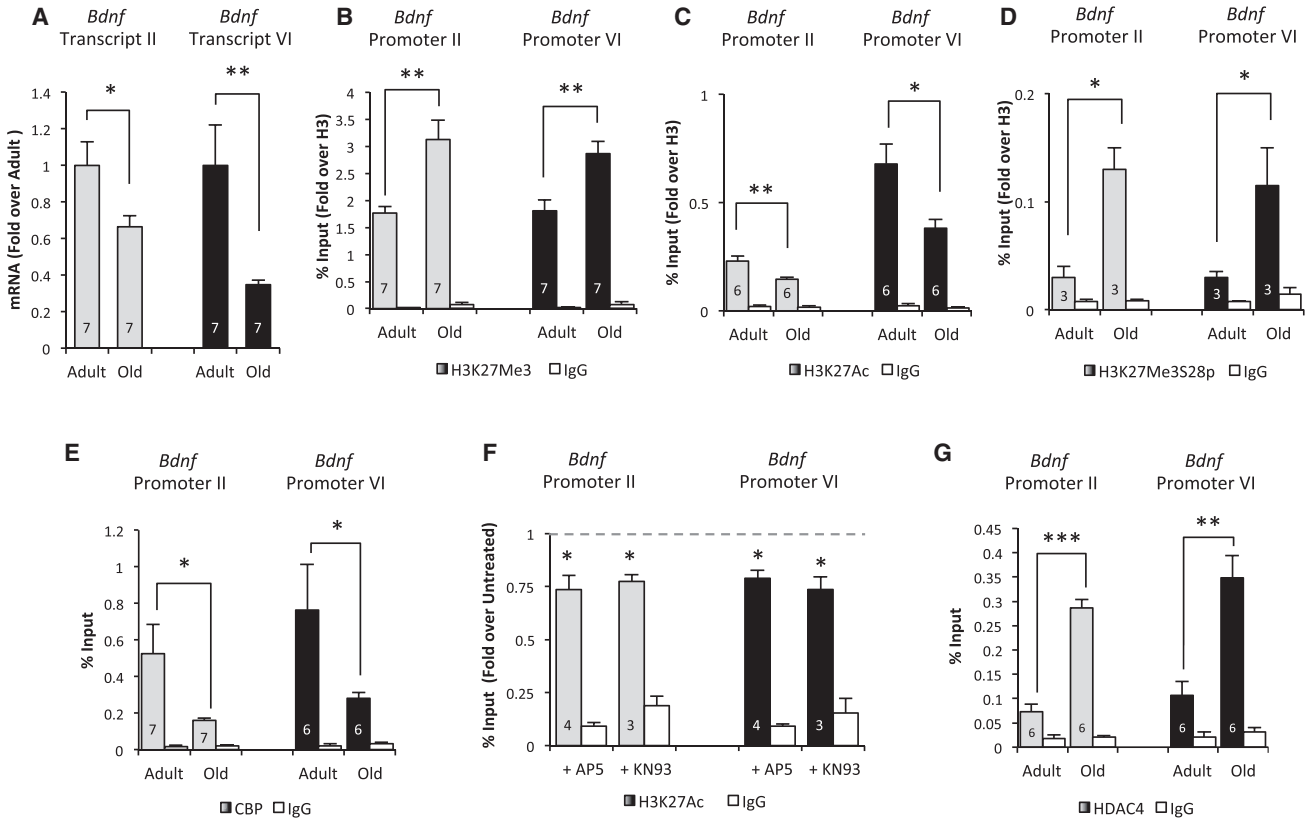

Figure 1. Age-Associated Chromatin Alterations at *Bdnf* Promoters

(A) qRT-PCR analysis showing the levels of *Bdnf* mRNAs transcribed from promoters II and VI in hippocampus from adult (8 months old) and old (20 months old) mice. (B–G) ChIP results showing the levels of histone and regulatory proteins bound at *Bdnf* promoters in hippocampus from adult and old mice: (B) H3K27Me3, (C) H3K27Ac, (D) H3K27Me3S28p, (E) CBP, and (G) HDAC4. (F) ChIP results showing the levels of H3K27Ac found at the *Bdnf* promoters after treatment of neuronal cultures with the NMDAR inhibitor AP5 or with the CaMKII inhibitor KN93. The control values (untreated) taken as 1 are indicated by a dashed line.

Data are represented as mean ± SEM. Statistical analysis was performed by Student's t test or Mann-Whitney (A–E and G) and one-sample t test (F). The value inside the bars indicates the number of animals or independent experiments; asterisks indicate the p values (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) obtained in the comparisons of adult versus old (A–E and G) or treatments versus control conditions (F). For specific statistical analysis and data, see Experimental Procedures and Supplemental Information.

RESULTS

Epigenetic Repression of *Bdnf* Promoters in the Hippocampus of Old Mice

BDNF is vital for learning and memory (Lu et al., 2014). The *Bdnf* gene contains multiple promoters that are specifically regulated by different stimuli (Aid et al., 2007). The rodent *Bdnf* gene possesses structural complexity that results in multiple splicing variants. It has nine exons, eight of which have their own promoter and are directly spliced to exon IX, the exon containing the coding sequence (Aid et al., 2007; Liu et al., 2006). Transcripts containing exons II and VI are rapidly synthesized after determined neuronal stimuli and transported to distal dendrites, where they play an important role in architectural and functional plasticity (Baj et al., 2011; Palomer et al., 2016). We found that the levels of mRNAs containing *Bdnf* exons II or VI are significantly reduced in the hippocampus of 20-month-old mice compared to adult (8 months old) mice (Figure 1A).

To investigate why these transcripts are reduced in older mice, we analyzed the epigenetic marks present at their respective promoters in the hippocampus of adult and old mice. Chromatin immunoprecipitation (ChIP) revealed that activating (H3K27Ac) and repressive (H3K27Me3) marks coexist at these promoters. Both promoters were enriched (1.6-fold) for H3K27Me3 and depleted (0.4-fold) of H3K27Ac in the hippocampus of old mice compared to adult animals (Figures 1B and 1C). The repressive H3K27Me3 histone mark is recognized by the polycomb repressor complex 1/2 (PRC1/2), leading to chromatin compaction and transcriptional inhibition (Margueron and Reinberg, 2011). It has been reported that phosphorylation of H3K27Me3 at Serine 28 (H3K27Me3S28p) displaces PRC2 from chromatin, facilitating transcriptional activation (Gehani et al., 2010). By ChIP experiments, we observed that the increase in H3K27Me3 at *Bdnf* promoters of old hippocampus is accompanied by a higher phosphorylation of at Serine 28 (Figure 1D), suggesting that the reduced basal transcription of *Bdnf* promoters II and VI in old neurons is not solely

explained by enhanced H3K27Me3-mediated transcriptional repression.

It was recently shown that H3K27 acetylation at *Bdnf* promoters II and VI in hippocampal neurons is mediated by active CREB, which recruits the histone acetyl transferase CBP to these promoters after NMDA receptor (NMDAR) activation (Palomer et al., 2016). Consistent with a defect in H3K27 acetylation in aged neurons, our ChIP experiments revealed reduced (0.7-fold) levels of CBP at these two promoters in aged hippocampus (Figure 1E). Furthermore, it has been shown that HDAC2 regulates memory formation and is enriched at the promoters of genes involved in synaptic remodeling and plasticity, including the *Bdnf* promoter (Guan et al., 2009). Consistently, our ChIP results show that hippocampal aging is accompanied by the accumulation of HDAC2 at *Bdnf* promoters II and VI (Figure S1A). Altogether, our data indicate that decreased H3K27Ac and expression of *Bdnf* promoters II and VI in aged hippocampus reflect both reduced histone acetylation and enhanced histone deacetylation.

Reduced NMDAR Signaling Promotes Histone Deacetylation at *Bdnf* Promoters

Different signaling pathways may underlie the decreased levels of CBP bound to *Bdnf* promoters II and VI in the hippocampus of aged mice (described earlier). A strong candidate to mediate these age-associated defects is the NMDAR-Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)-CREB pathway. It has been reported that long-term depression (LTD, one of the processes underlying memory formation) requires CaMKII activity (Mayford et al., 1995; Pi et al., 2010) and that CREB activation, required for CBP recruitment to *Bdnf* promoters, is dependent on CaMKII (Palomer et al., 2016). Thus, defects in the NMDAR-CaMKII pathway in the aged brain could lead to impaired CBP recruitment at *Bdnf* promoters and consequently lower levels of H3K27 acetylation. To test this hypothesis, we first analyzed whether NMDAR activity regulates the basal epigenetic modifications at *Bdnf* promoters. Inhibition of NMDARs with the antagonist AP5 (Davies and Watkins, 1982), followed by ChIP experiments, revealed that inhibiting NMDAR reduced the level of H3K27Ac at *Bdnf* promoters II and VI in basal conditions (Figure 1F). As expected, CaMKII phosphorylation was also reduced when NMDARs were inhibited with AP5 (Figure S2A). Thus, the NMDA-CaMKII pathway is required for H3K27Ac at *Bdnf* promoters II and VI.

HDAC4 plays an important role in the transcriptional control of synaptic plasticity and memory genes. The association of HDAC4 with chromatin is negatively regulated by CaMKII, in turn a target of NMDA activity (Backs et al., 2006; McKinsey et al., 2000; Sando et al., 2012). Thus, we tested whether HDAC4 is enriched at *Bdnf* promoters of the old hippocampus, where NMDA-CaMKII activity is low. We observed a substantial increase (3.3-fold) in HDAC4 at *Bdnf* promoters II and VI in hippocampal nuclei obtained from old mice by ChIP (Figure 1G). To further establish that the NMDAR-CaMKII pathway controls H3K27 acetylation at *Bdnf* promoters, we treated hippocampal neuronal cultures with KN93, a CaMKII-specific inhibitor (Sumi et al., 1991). KN93 treatment led to reduced basal levels of H3K27Ac at *Bdnf* promoters, determined by ChIP (Figure 1F). In contrast, treatment with KN92, a structural analog lacking the ability to inhibit CaMKII, did not show any effect (Figure S2B; Knafo et al., 2012). Altogether, our findings suggest that impaired NMDAR-CaMKII activity contributes to the altered epigenetic regulation of *Bdnf* promoters in aged neurons.

Bdnf Induction by NMDA Is Impaired in the Hippocampus of Old Mice

We next analyzed whether the response of *Bdnf* promoters II and VI to NMDAR stimulation is affected by their repressive epigenetic state in old neurons (Figure 1). Hippocampal slices obtained from adult and old mice were stimulated with a low dose of NMDA (20 μM), a concentration widely used to induce NMDAR-LTD (Ehlers, 2000; Fernández-Monreal et al., 2012; Lee et al., 1998; Lin et al., 2000), and the level of the specific transcript was determined by qPCR. We found that NMDA triggered an increase in mRNAs containing exons II and VI 10 min after stimulation in slices from 8-month-old mice but not in those from 20-month-old mice (Figure 2A). NMDA treatment also led to increased levels of activating signals (H3K27Ac and CBP) at promoters II and VI in samples from the 8-month-old mice but not from old mice, observed by ChIP (Figures 2B and 2C). Furthermore, the ChIP experiments highlighted that NMDA-LTD leads to the depletion of HDAC4 from these promoters in the adult but not in the old animals (Figure 2D). Consistent with our findings that epigenetic deregulation is associated with age-associated decreases in NMDA signaling, we found that the NMDA-LTD stimulus triggered the activation of CREB (Figures 2E and 2F) and CaMKII (Figures 2G and 2H) in hippocampal slices obtained from adult but not old mice. We observed that NMDAR stimulation did trigger HDAC2 depletion from *Bdnf* promoters in old hippocampal slices (Figure S1B). Therefore, the impaired induction of *Bdnf* promoters II and VI by synaptic activity in the aged hippocampus correlates specifically with the impaired induction of CaMKII-CREB-CBP signaling and HDAC4 displacement.

Age-Associated Cholesterol Reduction Leads to Weak NMDAR Signaling

To test whether the naturally occurring age-associated cholesterol reduction (see Introduction; Figure 3A) plays a role in the weak NMDAR-CaMKII signaling of old neurons, we incubated hippocampal slices from old mice with a cholesterol-rich solution (MβCD-Ch), using a protocol known to restore membrane cholesterol levels to the adult mouse levels (Figure 3B; Martin et al., 2014a). We performed ChIP and found that cholesterol addition to hippocampal slices from old mice is sufficient to induce increased levels of H3K27Ac and reduced levels of HDAC4 at *Bdnf* promoters II and VI in basal, unstimulated neurons (Figures 3C and 3D), resembling the epigenetic state found in adult animals. Cholesterol-treated slices also displayed significantly increased basal CaMKII activity (Figure 3E) and restored levels of the histone acetyltransferase CBP at these *Bdnf* promoters (Figure 3F). Moreover, the cholesterol treatment enhanced basal CREB activity, indicated by increased phosphorylation at Serine 133 (Figure 3G). Therefore, adding cholesterol to the hippocampus of old mice is sufficient to promote epigenetic changes at *Bdnf* promoters II and VI associated with activation.

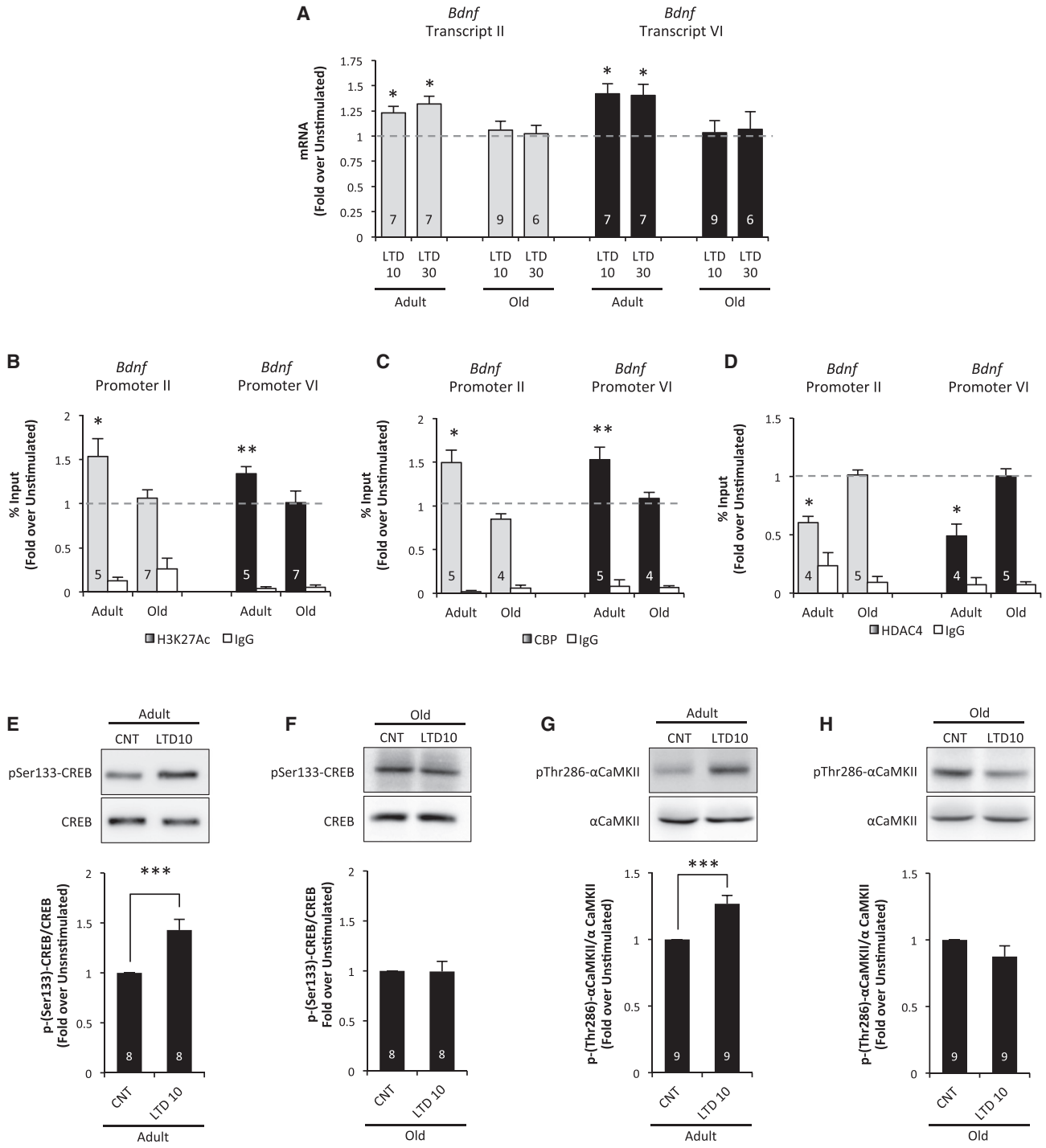

Figure 2. Impaired *Bdnf* Transcription after LTD in Hippocampal Slices from Old Mice

(A) qRT-PCR analysis in hippocampal slices showing that the levels of *Bdnf* mRNAs transcribed from promoters II and VI increase in adult (8 months old) but not in old (20 months old) mice at 10 and 30 min after NMDA stimulation.

(B–D) ChIP analysis showing the levels of H3K27Ac (B), CBP (C), and HDAC4 (D) bound at *Bdnf* promoters 10 min after LTD induction in hippocampal slices from adult and old mice. The data represent relative values with respect to non-stimulated controls (dashed line).

(E and F) Western blot and its quantification, showing that the levels of phospho-CREB increase in adult hippocampal slices (E) but not in old hippocampal slices (F) 10 min after NMDA stimulation.

(G and H) Western blot and its quantification, showing that the levels of phospho-CaMKII increase in adult hippocampal slices (G) but not in old hippocampal slices (H) 10 min after NMDA stimulation.

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To determine whether the effect of cholesterol was due to the potentiation of the signaling through NMDARs, we pre-incubated the cholesterol-treated, old hippocampal slices with the NMDAR antagonist AP5. The treatment of old slices with cholesterol and AP5 led to reduced H3K27Ac and increased HDAC4 levels, although the changes were not significant (Figure 3). Thus, although cholesterol treatment restored the biochemical pathways and epigenetic marks associated with gene activation in a NMDAR activity-dependent manner (Figures 3E–3G), it did not increase the levels of *Bdnf* mRNAs II and VI in the steady state (Figure S1C), suggesting that other cholesterol-independent regulatory mechanisms also participate in the repressive state of *Bdnf* expression in the non-stimulated context.

To further substantiate that reduced levels of cholesterol affect NMDAR signaling and the epigenome in the aged hippocampus, we decreased the cholesterol content in hippocampal slices from 8-month-old mice. Slices were treated with cholesterol oxidase (COase) for 30 min, a protocol previously used in our laboratory to induce a mild cholesterol reduction without affecting cell survival (Figure S2C; Brachet et al., 2015). Lowering cholesterol levels in slices from these younger mice resulted in enrichment of HDAC4 and depletion of CBP and H3K27Ac at *Bdnf* promoters II and VI (Figures S2D–S2F). In agreement, lowering cholesterol in hippocampal neurons in culture triggered CaMKII de-phosphorylation (Figure S2A). Altogether, this suggests that the reduction in hippocampal cholesterol could contribute to reduced NMDAR signaling, which is in turn responsible for the low basal CaMKII activity and the repressive chromatin status, characterized by lower H3K27Ac levels at *Bdnf* promoters II and VI.

Cholesterol Rescues LTD-Induced *Bdnf* Transcription in Old Neurons

Given the observation that cholesterol treatment of old neurons in basal, non-stimulated conditions restores an epigenetic state at *Bdnf* promoters II and VI so that it is similar to that of younger, adult hippocampus, we next tested whether exogenous cholesterol would enhance *Bdnf* transcription after NMDA stimulation. By ChIP, we found that cholesterol enrichment of hippocampal slices from old mice was required for NMDA-induced recruitment of CBP, H3K27 acetylation, and HDAC4 depletion at *Bdnf* promoters II and VI (Figures 4A–4C). Furthermore, cholesterol treatment of these slices led to NMDA-induced activation of CaMKII and CREB (Figures 4D and 4E). More importantly, and consistent with a true functional effect, the addition of cholesterol rescued the impaired NMDA-induced transcription of *Bdnf* promoters II and VI in old hippocampal slices, mimicking the effect of NMDA on hippocampal slices from younger mice (Figure 4F).

To further test that decreased cholesterol and the consequent reduction in the NMDA-CaMKII pathway contribute to the weak NMDA-induced expression of *Bdnf* transcripts II and VI in old mice, we reduced cholesterol in hippocampal slices from adult 8-month-old mice by treating with COase before LTD induction. COase treatment of the slices from adult mice impaired the activation of CaMKII and CREB 10 min after NMDA stimulation (Figures S3A and S3B). Moreover, COase treatment of slices from adult mice impaired LTD-induced chromatin remodeling and *Bdnf* transcription (Figures S3C–S3F).

H3K27Me3 Demethylation at *Bdnf* Promoters Is Cholesterol Dependent

We performed ChIP to investigate whether cholesterol affects levels of the repressive modification H3K27Me3 at the *Bdnf* promoter. We found that cholesterol treatment of old slices resulted in decreased basal levels of the repressive mark H3K27Me3, which required NMDAR signaling, because it was prevented by the NMDA antagonist AP5 (Figure 5A). Furthermore, COase-mediated cholesterol depletion of slices obtained from 8-month-old mice resulted in higher levels of H3K27Me3 at *Bdnf* promoters II and VI (Figure 5B). We also found that the reduction in H3K27Me3 levels triggered by LTD is impaired in old neurons (Figure 5C). The chemical LTD-induced reduction in H3K27Me3 is enhanced by cholesterol treatment of slices from old mice and prevented by COase-mediated cholesterol reduction in slices from adult mice (Figure 5C). Thus, membrane cholesterol controls the levels of H3K27Me3 at *Bdnf* promoters through the NMDAR-CaMKII pathway, probably via the H3K27Me3 demethylase JMJD3, which we have shown to be recruited to the *Bdnf* promoters by the CREB/CBP complex (see Palomer et al., 2016).

Prevention of Age-Associated Cholesterol Loss Rescues Hippocampal *Bdnf* Transcription and Enhances Cognition in Old Mice

Based on these results, we hypothesized that preventing the hippocampal cholesterol loss that occurs during aging may rescue the membrane signaling and epigenetic changes at *Bdnf* promoters and may ameliorate some of the cognitive deficits typical of this stage of life. The age-associated reduction in hippocampal cholesterol arises partly from increased expression of the *Cyp46A1* gene, which encodes the major cholesterol catabolic enzyme in the brain (Cyp46; Lund et al., 1999). High levels of Cyp46 were observed in the hippocampus of elderly humans and in patients with certain pathological, neurodegenerative conditions (reviewed in Martín et al., 2014b). To test this hypothesis, we inhibited Cyp46 activity in old mice by treating them with the antifungal azolic derivative voriconazole. Voriconazole inhibits the enzyme sterol-14a-demethylase (Cyp51) in fungi, which is involved in the ergosterol synthesis, but targets Cyp46 in mammalian cells (Shafaati et al., 2010). Previous work established that intraperitoneal voriconazole crosses the blood-brain barrier, effectively inhibiting Cyp46 activity (Shafaati et al., 2010). Voriconazole was administrated to 19-month-old mice in their drinking water for 45 days. We observed a significant decrease in the age-associated loss of cholesterol in the

CNT, unstimulated control; LTD10, NMDA treated. Data are represented as mean ± SEM. Statistical analysis by ANOVA or Kruskal-Wallis (A) and one-sample t test or Mann-Whitney (B–H). The value inside the bars indicates the number of independent experiments; the asterisks indicate the p values (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) obtained in the comparisons to controls indicated as a dashed line (A–D) or labeled as CNT (E–H). For specific statistical analysis and data, see Supplemental Information.

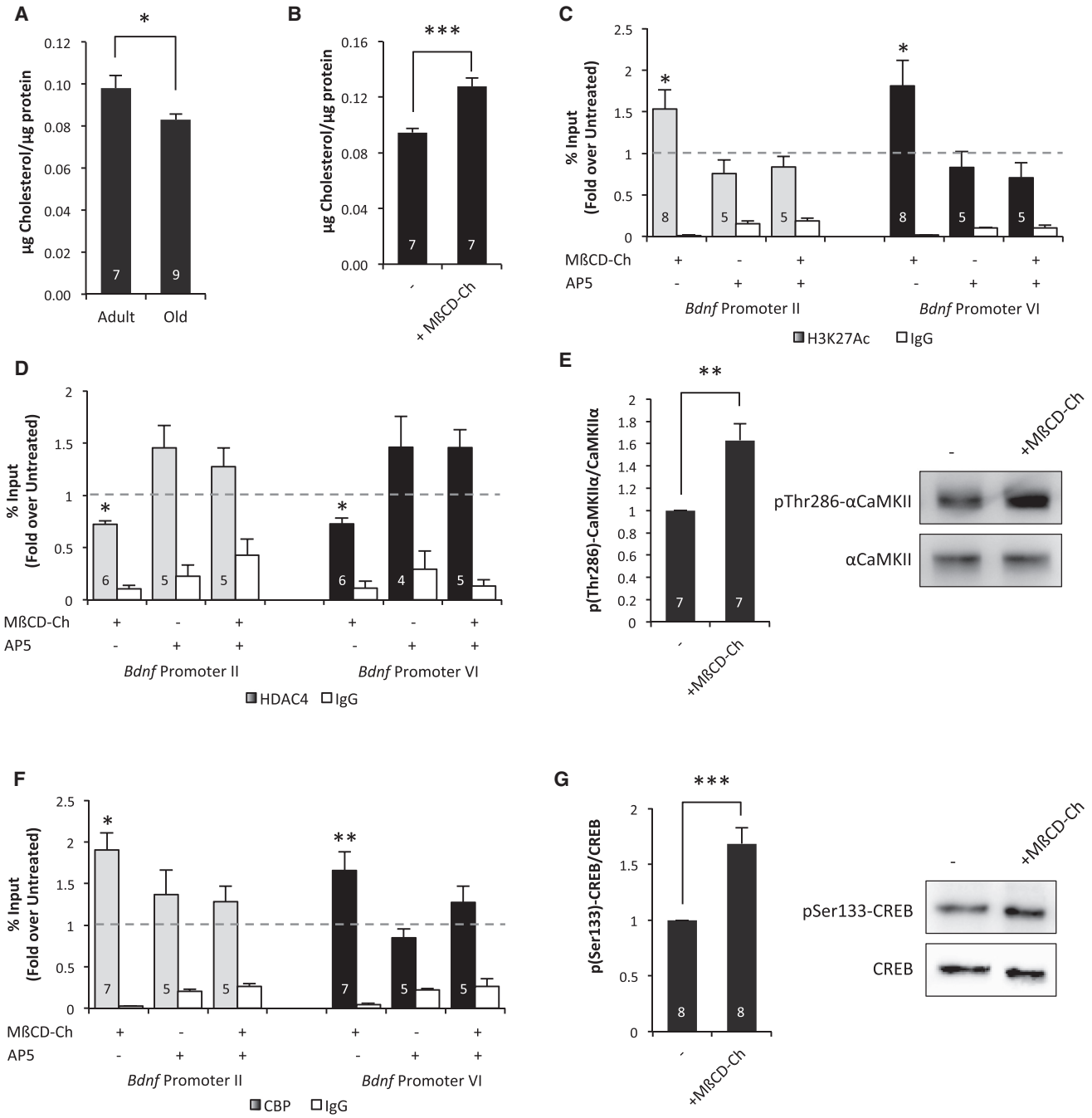

Figure 3. Cholesterol Rescues the *Bdnf* Epigenetic State through NMDAR Activation in Old Hippocampal Slices

(A) Cholesterol quantification of adult and old hippocampus.

(B) Cholesterol quantification of control and MßCD-Ch-treated old hippocampal slices.

(C and D) ChIP analysis showing the levels of H3K27Ac (C) and HDAC4 (D) bound at *Bdnf* promoters in hippocampal slices from old mice treated with MßCD-Ch and/or AP5. The data are expressed as relative values with respect to untreated controls (dashed line).

(E) Western blot and its quantification, showing the levels of active and total αCaMKII in control or MßCD-Ch-treated old hippocampal slices.

(F) ChIP analysis showing the levels of CBP bound at *Bdnf* promoters in hippocampal slices from old mice treated with MßCD-Ch and/or AP5.

(G) Western blot and its quantification, showing the levels of phosphorylated and total CREB in control or MßCD-Ch-treated old hippocampal slices.

Data are represented as mean ± SEM. Statistical analysis by Student's t test or one-sample t test (A, B, E, and G) and ANOVA or Kruskal-Wallis (C, D, and F). The value inside the bars indicates the number of animals or independent experiments; asterisks indicate *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared to adult samples (A) or untreated samples, indicated as a minus sign (B, E, and G) or dashed line (C, D, and F). For specific statistical analysis, see Supplemental Information.

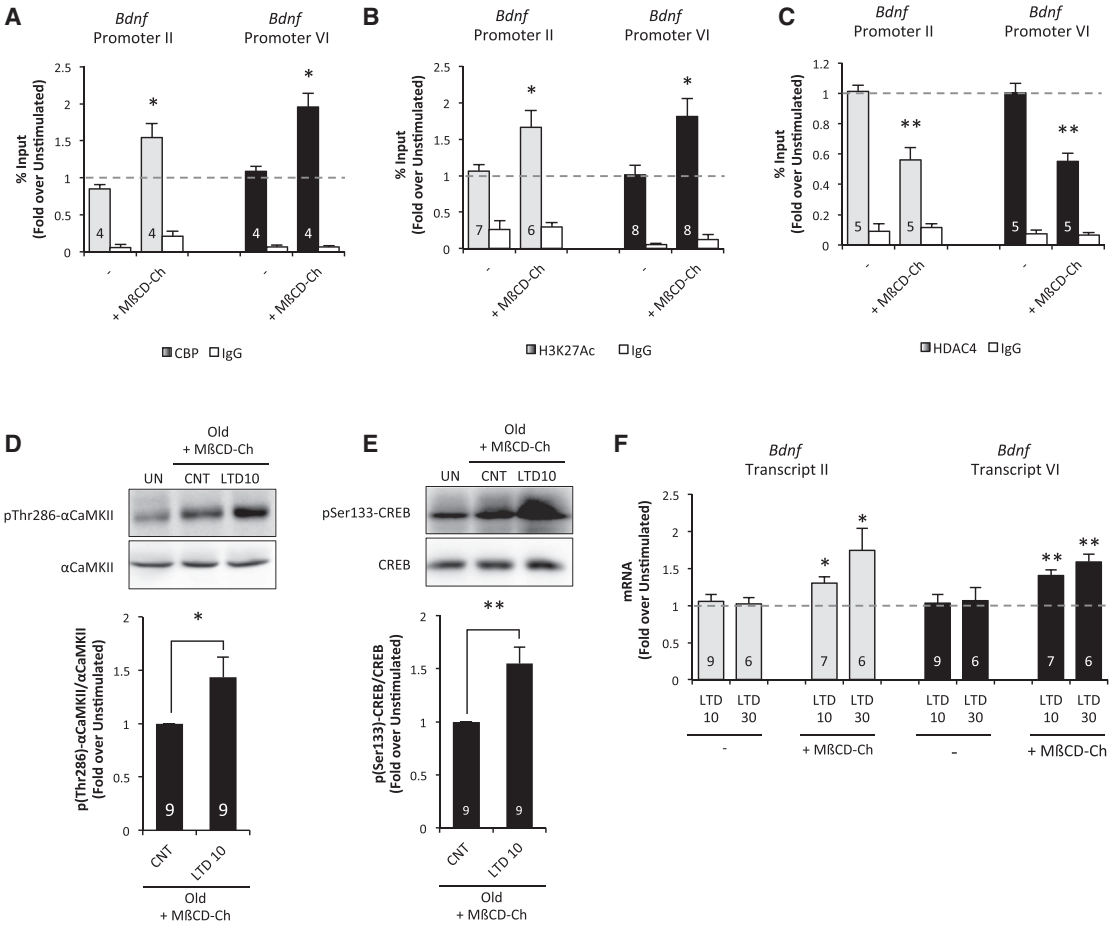

Figure 4. Cholesterol Rescues *Bdnf* Induction by NMDA-LTD in Old Hippocampus

(A–C) The ChIP analysis shows that NMDA stimulation triggers the recruitment of CBP and H3K27 acetylation (A and B) and the decrease of HDAC4 (C) at *Bdnf* promoters II and VI in old hippocampal slices pretreated with MßCD-Ch to restore the normal levels of this lipid. These changes are not observed in cholesterol-untreated old samples (−). The asterisks indicate the p values obtained by comparison of NMDA-stimulated samples with their own unstimulated controls, taken as reference (value = 1, dashed line).

(D and E) Western blot and its quantification, showing the levels of active and total αCaMKII and CREB in cholesterol-untreated slices (UN), MßCD-Ch-treated slices, unstimulated controls (CNT), and MßCD-Ch-treated, NMDA-stimulated (LTD10), old hippocampal slices. Reactivation of the NMDAR-CaMKII pathway by cholesterol leads to CREB and CaMKII phosphorylation in basal conditions. NMDA stimulation leads to further activation of these proteins.

(F) qRT-PCR analysis showing that MßCD-Ch treatment in old hippocampal slices rescues the induction of *Bdnf* promoters II and VI by NMDA. Cholesterol-treated (MßCD-Ch) or cholesterol-untreated (−) slices were stimulated with NMDA, samples were taken at 10 and 30 min after stimulation and compared to each unstimulated control (dashed line).

Data are represented as mean ± SEM. Statistical analysis was performed by one-sample t test (A–E) and ANOVA or Kruskal-Wallis (F). The value inside the bars indicates the number of animals or independent experiments; asterisks indicate *p < 0.05; **p < 0.01; for specific statistical analysis, see Supplemental Information.

hippocampus of voriconazole-treated mice compared to vehicle-treated animals (Figure 6A). Furthermore, we observed increased levels of *Bdnf* transcripts II and VI 10 and 30 min after LTD induction of hippocampal slices from voriconazole-treated mice (Figure 6B). In contrast, low transcriptional induction was observed after LTD induction of slices from the vehicle-treated group. Voriconazole treatment also resulted in increased levels of H3K27Ac and decreased levels of H3K27Me3 at *Bdnf* promoters II and VI after LTD (Figures 6C and 6D). Thus, the partial rescue of cholesterol levels in mice treated with voriconazole was paralleled by epigenetic changes at *Bdnf* promoters and by improved LTD-induced response.

To determine whether voriconazole treatment improves the cognitive abilities of old mice, voriconazole- or vehicle-treated mice were subjected to three behavioral tests: novel object location, Morris water maze, and contextual fear conditioning. In the novel location task, the exploration phase was similar in voriconazole- or vehicle-treated animals; however, during the retention session, animals chronically treated with voriconazole spent more time exploring the object that was placed in a novel position, indicating better spatial memory than vehicle-treated mice (Figure 6E).

In the hidden-platform version of the Morris water maze test, which evaluates hippocampal function, both groups showed

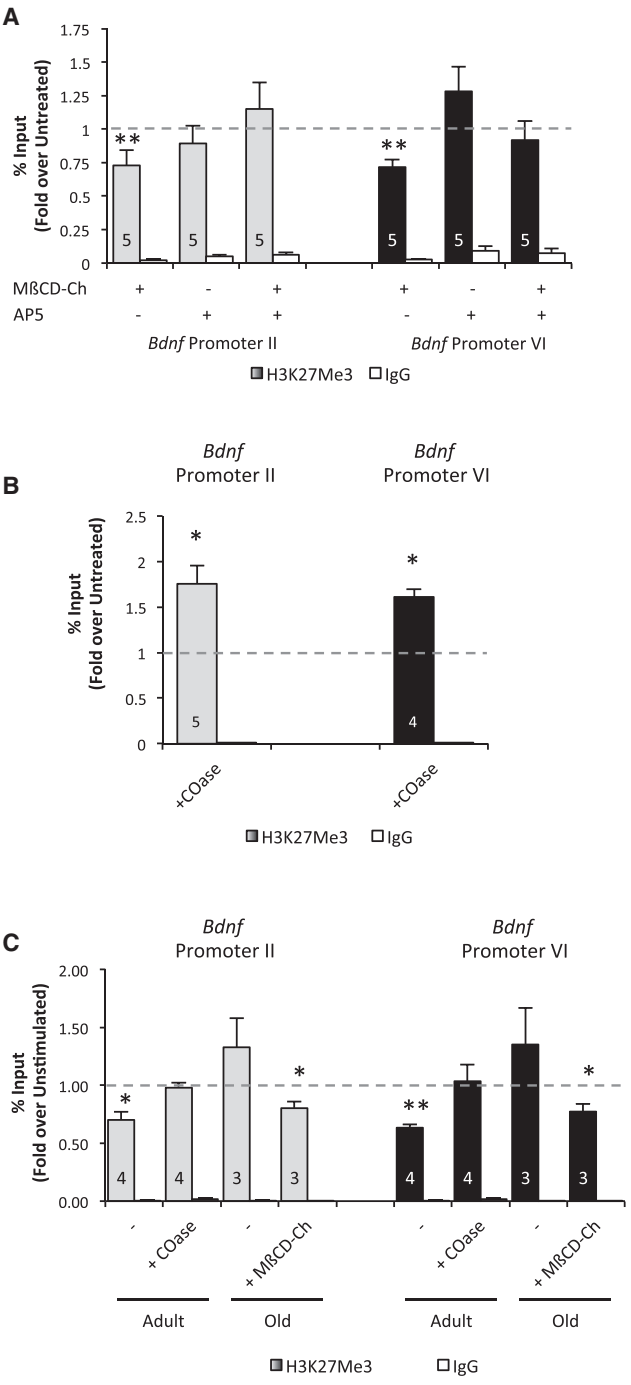

Figure 5. H3K27Me3 Demethylation at *Bdnf* Promoters Requires Cholesterol and NMDAR Activity in Old Neurons

(A) ChIP analysis showing the levels of H3K27Me3 bound at *Bdnf* promoters in hippocampal slices from old mice treated with MßCD-Ch and/or AP5. H3K27Me3 demethylation is observed when cholesterol is replenished in old hippocampal slices but not when cholesterol is added in the presence of AP5. No significant differences were observed in neurons treated with AP5.

(B) ChIP analysis showing that cholesterol depletion (COase) results in increased H3K27Me3 bound to *Bdnf* promoters compared to untreated controls.

(C) ChIP results showing that H3K27Me3 demethylation is triggered by LTD in adult but not in old neurons. Cholesterol depletion (COase) in adults impairs demethylation, whereas it is rescued by cholesterol replenishment in old slices (MßCD-Ch). The values show the levels of H3K27Me3 10 min after NMDA stimulation with respect to unstimulated controls.

Data are represented as mean ± SEM. Statistical analysis by Kruskal-Wallis (A) or one-sample t test (B and C). The value inside the bars indicates the number of animals or independent experiments; asterisks indicate *$p < 0.05$; **$p < 0.01$ compared untreated or control samples, indicated as a dashed line. For specific statistical analysis, see Supplemental Information.

improved performance during all successive trials throughout the 4 days of spatial training. Path length, swimming speed, and time to reach the platform were unchanged by the treatment, indicating that controls and voriconazole-treated animals learned the location of the platform at an equivalent rate (Figures S4A–S4E). However, in the memory probe test, voriconazole-treated animals spent significantly more time in the target quadrant compared with the controls, indicating that voriconazole improved spatial reference memory (Figure 6F).

In the cued fear-conditioning training test, both groups displayed similar levels of freezing before, during, and after tone-shock pairings (Figure S4F). However, in the contextual fear-conditioning test, which is primarily contingent on hippocampal function, voriconazole-treated animals exhibited improved memory recall compared to controls (Figure 6G; Figure S4G). During the auditory-cued fear memory test, which depends on amygdala but not hippocampal function, both animal groups displayed similar freezing behavior (Figure S4H) and similar sensitivity to the electric shock. These findings suggest that voriconazole increases contextual fear conditioning through an improvement of hippocampal function.

In addition to the beneficial effects of long-term treatment with voriconazole, we found that short-term voriconazole treatment restores synaptic function. We treated hippocampal slices obtained from old mice with 10 nM voriconazole for 2 hr and observed rescue of NMDA-LTD (Figures S5A and S5B). Finally, to link the effects of voriconazole with NMDA signaling, we analyzed the levels of PSD95 and the NMDAR subunit NR1 in control and in voriconazole-treated old mice. We found that the total levels of PSD95 do not change with voriconazole treatment, neither in total hippocampal extract nor in hippocampal synaptosomal fraction (Figures S6A and S6B). However, NR1 levels increase in the synaptic fraction of voriconazole-treated mice, without a change in total levels of NR1 (Figures S6C and S6D). These findings suggest the beneficial effects of voriconazole may be mediated partly by the more efficient recruitment of NMDAR to synapses. This possibility is in line with the work of Korinek et al. (2015), who showed that NMDAR activity is impaired in low-cholesterol contexts, without changes in total surface NMDAR.

DISCUSSION

The existence of a functional link between changes in the brain cholesterol content and cognitive deficits is anticipated: cholesterol is an essential constituent of eukaryotic membranes, and as such, any alteration in this sterol will have an impact on the function of brain cells. Intuitively, a change in cholesterol content

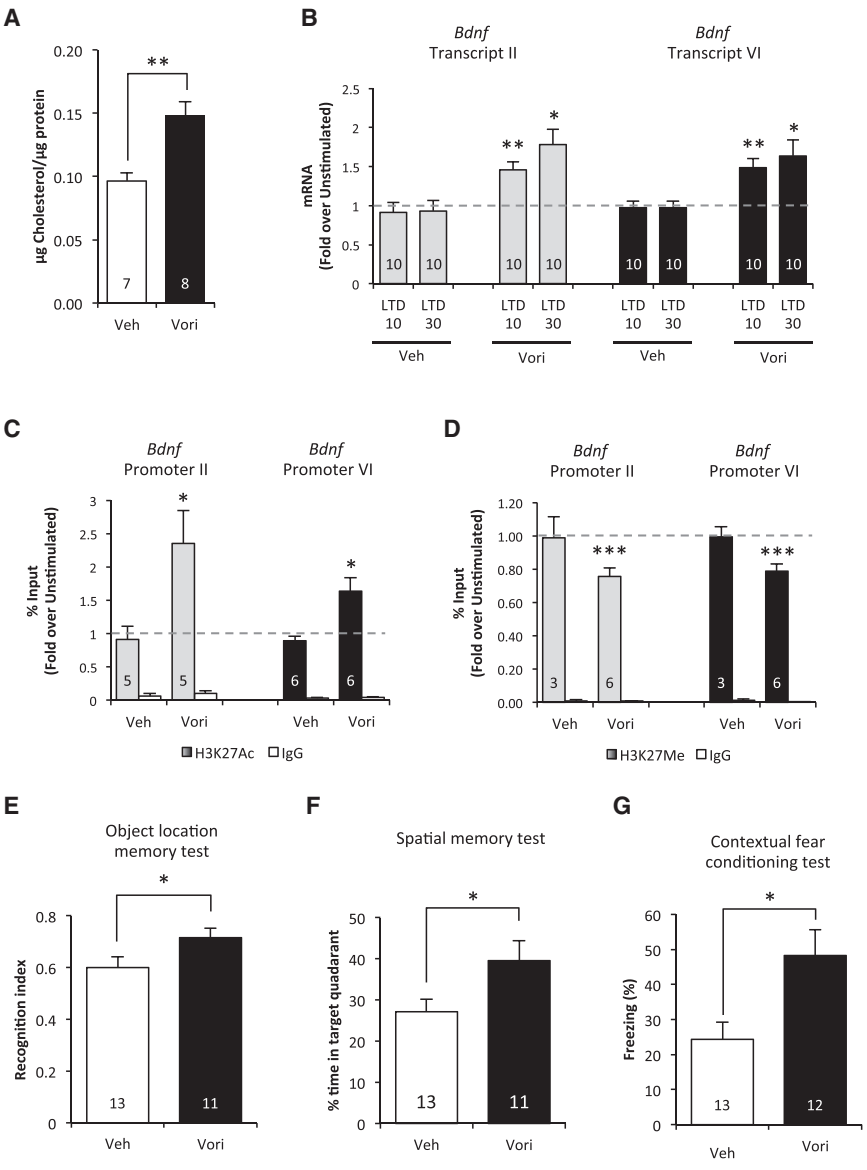

Figure 6. Oral Administration of Voriconazole to Old Mice Rescues *Bdnf* Epigenetic Regulation and Transcription after LTD and Improves Behavior

(A) Cholesterol quantification in hippocampus of voriconazole- and vehicle-treated old mice.

(B) Levels of mRNAs transcribed from promoters II and VI after LTD in hippocampal slices of voriconazole- or vehicle-treated mice.

(C and D) ChIP analysis showing the levels of H3K27Ac and H3K27Me3 at these Bdnf promoters 10 min after NMDA stimulation in hippocampal slices of voriconazole- or vehicle-treated mice.

(E–G) Behavioral tests of old mice treated for 45 days with voriconazole or vehicle. (E) Object location memory test, (F) Morris water maze spatial memory test, and (G) contextual fear-conditioning test.

Veh, vehicle; Vori, voriconazole. Data are represented as mean ± SEM. Statistical analysis by Student's t test (A and E–G) or one-sample t test (C and D) and ANOVA (B). The value inside the bars indicates the number of animals or independent experiments; asterisks indicate *p < 0.05; **p < 0.01; ***p < 0.001 compared to vehicle (A and E–G) or to control, indicated as a dashed line (B–D). For specific statistical analysis, see Supplemental Information.

could lead to cognitive alterations by both short- and long-range actions. Synaptic transmission is particularly sensitive to a disturbance in cholesterol levels, probably because synaptic vesicle release at the presynaptic terminal and the response to signals through neurotransmitter receptors on the postsynaptic side rely entirely on membranous compartments and membrane-bound signaling pathways, many of which depend on the integrity of cholesterol-rich microdomains (reviewed in Hering et al., 2003; Ledesma et al., 2012; Renner et al., 2009). The long-range action of cholesterol would be via the regulatory role that membrane cholesterol plays on membrane receptors, likely influencing the activity of downstream kinases, e.g., p38MAPK or MSK1/2, which can influence gene expression by phosphorylating chromatin proteins (Gehani et al., 2010; Palomer et al., 2016). We demonstrate that the loss of cholesterol in the hippocampus of old mice contributes to the impaired induction of two key *Bdnf* promoters in response to a cognitive stimulus. We discovered that old hippocampal neurons have a reduced capacity to activate the biochemical signals normally arising by NMDAR stimulation; as a consequence, the epigenetic remodeling that leads to transcriptional stimulation of *Bdnf* promoters II and VI by LTD does not occur. All these events were rescued by directly increasing cholesterol levels in the hippocampus of old mice or by preventing age-associated brain cholesterol loss.

Although a reduced cholesterol environment promotes the repressive epigenetic state at *Bdnf* promoters, it does not seem to be the only factor responsible for the low basal expression of *Bdnf* promoters II and IV found in elderly mice. Cholesterol treatment of old, unstimulated neurons was not sufficient to increase the basal levels of these transcripts, despite enhanced CaMKII activity and altered features at the *Bdnf* promoters associated with active transcription, including reduced binding of HDAC4, depletion of H3K27Me3, enrichment of H3K27A, and increased binding of CBP. However, the cholesterol replenishment rescued the ability of these promoters to be transcriptionally induced by NMDA. From these results, one could argue that cholesterol-dependent and cholesterol-independent mechanisms coexist to restrict the transcription of *Bdnf* promoters in the basal conditions and that all these restrictions are removed after neuronal stimulation with NMDA in the presence of normal levels of cholesterol.

One strong candidate to exert repression in the basal condition is HDAC2, which accumulates at the *Bdnf* promoters and

seems to respond to a different, cholesterol-independent signaling pathway in the old (Figure S1B). Another candidate is the repressor protein CDYL, which is present at *Bdnf* promoter II and can be degraded by NMDA or KCl stimulation (Palomer et al., 2016; Qi et al., 2014). In this case, the reactivation of the NMDAR-CaMKII pathway by cholesterol in the basal state would not be sufficient to trigger CDYL degradation, though a stronger signal induced by treatment with NMDA and cholesterol could lead to CDYL degradation and promoter induction. Another possibility is that cholesterol replenishment per se is not sufficient to restore *Bdnf* expression in unstimulated old neurons due to an age-associated reduction of a putative transcriptional activator. Along these lines, one could speculate that an age-related decay of SIRT1 may promote the inhibition of CREB synthesis by microRNA (miRNA) 134 (Gao et al., 2010), resulting in low levels of CREB with age. However, we did not observe a decrease in the total levels of SIRT1 or CREB in old hippocampi compared with adult ones (Figures S1D and S1E). Furthermore, the rescue of *Bdnf* expression after LTD in cholesterol-treated old hippocampi was not accompanied by a change in the total levels of SIRT1 or CREB (Figures S1F and S1G). Future work is needed to dissect the full mechanisms repressing *Bdnf* transcription in old mice.

However, the reduction of hippocampal cholesterol with age seems to play an important role in the age-associated impairment of NMDA-induced *Bdnf* transcription. Treatment of old neurons with cholesterol improved NMDAR signaling toward CaMKII. Cholesterol treatment of old neurons was also required for NMDA-induced changes at the *Bdnf* promoter, including depletion of phosphorylated HDAC4, CREB activation, CREB-p/CBP recruitment, and increased H3K27Ac levels, and for NMDA-LTD-induced expression from *Bdnf* promoters II and VI. Pre-treatment of old neurons with the NMDAR antagonist AP5 abrogated the effects of cholesterol, indicating the attenuated NMDAR signaling arising from reduced cholesterol levels underlie the epigenetic changes at the *Bdnf* promoter. Low cholesterol levels may perturb the organization or dynamics of receptors in the plasma membrane. It was recently shown that reduced levels of cholesterol in the plasma membrane result in defective AMPA receptor recycling in hippocampal neurons from old mice (Martin et al., 2014a).

Cholesterol reduction in the aged hippocampus may have several causes. Among these, increased transcription of *Cyp46A1* has been proposed to explain cholesterol reduction in a number of circumstances (reviewed in Martín et al., 2014b). Cyp46 hyper-activation has been observed in cells subjected to stress in vitro (Lund et al., 1999; Sodero et al., 2011a, 2011b) and in Alzheimer's brains and in other brain pathologies with abundant stress (Bogdanovic et al., 2001; Cartagena et al., 2008; Teunissen et al., 2007). Our current results, based on the use of the Cyp46 inhibitor voriconazole, also fit with this hypothesis: oral administration of voriconazole in aged mice reduced the typical cholesterol loss associated with aging, favored a transcriptionally poised epigenetic state at *Bdnf* promoters, and improved cognitive abilities. In light of these observations, we can conclude that, as proposed by Murphy and Partridge (2008), *Cyp46A1* may be one of the upregulated genes responsible for the aging phenotype of the mammalian brain. Furthermore, its inhibition appears a likely target to prevent or mitigate, at least to a certain extent, the cognitive deficits of the aged. Knockout mice constitutively lacking *Cyp46A1* expression exhibit severe deficiencies in spatial, associative, and motor learning associated to developmental defects (Kotti et al., 2006). Therefore, in light of our findings, one can envision the following scenario: when at basal levels of expression, as occurs during development and in adulthood, Cyp46's activity may be essential for optimal cognition; when activity is abnormally high, for instance, during aging or chronic stress conditions (Alzheimer's), Cyp46's activity may be deleterious. Tissue-specific inducible knockout of *Cyp46A1* may help to address this question.

We have previously described the immediate consequences of age-associated cholesterol loss in neurons and how these may contribute to the cognitive deficits of the old (Martin et al., 2014a). Here, we show that decreased hippocampal cholesterol levels in old mice disrupt NMDAR activation and, in turn, epigenetic regulation of *Bdnf* and likely other immediate early-memory genes (Figures S6E and S6F). Pharmacological inhibition of Cyp46, the enzyme responsible for the major brain cholesterol catabolic pathway (Lund et al., 2003), rescued the behavioral deficits and associated poor induction of *Bdnf* by cognitive stimuli in aged mice, suggesting that the cognitive decline associated with aging may be reversible.

EXPERIMENTAL PROCEDURES

Animal Handling

All experiments were performed in accordance with European Union guidelines (2010/63/UE) regarding the use of laboratory animals.

Hippocampal Slices

Hippocampal slices were prepared from C57BL/J adult (8 ± 2 months old) mice and old (20 ± 1 month old) mice. Hippocampi were extracted in dissection solution (10 mM D-glucose, 4 mM KCl, 26 mM NaHCO3, 233.7 mM sucrose, 5 mM MgCl2, 1:1,000 phenol red), oxygen saturated with carbogen (95% O_2/5% CO_2), and sliced in an automatic tissue chopper (McIlwain Tissue Chopper, Standard Table, 220 V, Ted Pella) to obtain 400 μm hippocampal slices. Then, slices were kept for 1 hr in artificial cerebrospinal fluid (ACSF; 119 mM NaCl, 2.5 mM KCl, 1 mM NaH_2PO_4, 11 mM glucose, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$). (Osmolarity was adjusted to 290 Osm, and ACSF was oxygen saturated with carbogen.) Finally, the experiments were performed in ACSF.

Statistical Analysis

All values are presented as mean + SEM. Data normality and variances were tested by the Shapiro-Wilk test and Levene test, respectively. For non-parametric values, comparisons between two groups of data were analyzed by Mann-Whitney U test, comparisons between more than two groups were analyzed by Kruskal-Wallis. The Friedman test was used to analyze behavioral studies. For normal distributed data, parametric tests were used. Comparisons between two groups were analyzed by Student's t test. If no equal variances were found in one of the groups, the statistical test applied was one-sample t test. For comparisons of more than two groups of data, ANOVA was used; post hoc was performed assuming or not assuming equal variances. In the figures, asterisks indicate p values as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and twelve tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.08.028.

AUTHOR CONTRIBUTIONS

E.P. contributed to the conception, design, analysis, and interpretation of the data; performed the experiments; and wrote the article. A.M.-S. contributed the biochemistry of ex vivo experiments. S.B. and C.V. performed the behavior experiments. T.A. and D.B. performed the electrophysiology experiments. M.G.M. and C.G.D. conceived the project; contributed with the experimental design and interpretation of the data; and wrote the article.

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