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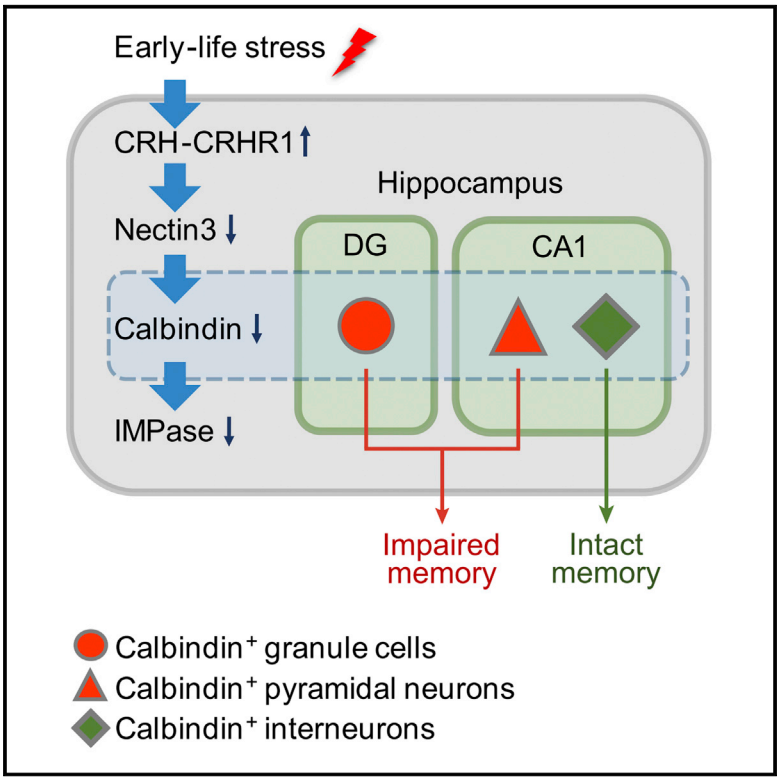
Suppressed Calbindin Levels in Hippocampal Excitatory Neurons Mediate Stress-Induced Memory Loss

Graphical Abstract



Highlights

- Early-life stress suppresses calbindin levels in the adult mouse hippocampus
- Calbindin knockdown in CA1 or DG excitatory neurons impairs spatial memory
- Calbindin knockdown in CA1 interneurons preserves long-term spatial memory
- Stress downregulates calbindin levels via a corticotropin-releasing hormone receptor

Authors

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In Brief

Li et al. demonstrate that early-life stress suppresses hippocampal calbindin levels through the CRHR1-nectin3 system. Reduced calbindin levels in hippocampal excitatory, but not inhibitory, neurons mediate stress-induced spatial memory impairment.

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Report

Suppressed Calbindin Levels in Hippocampal Excitatory Neurons Mediate Stress-Induced Memory Loss

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SUMMARY

Calbindin modulates intracellular Ca^{2+} dynamics and synaptic plasticity. Reduction of hippocampal calbindin levels has been implicated in early-life stress-related cognitive disorders, but it remains unclear how calbindin in distinct populations of hippocampal neurons contributes to stress-induced memory loss. Here we report that early-life stress suppressed calbindin levels in CA1 and dentate gyrus (DG) neurons, and calbindin knockdown in adult CA1 or DG excitatory neurons mimicked early-life stress-induced memory loss. In contrast, calbindin knockdown in CA1 interneurons preserved long-term memory even after an acute stress challenge. These results indicate that the dysregulation of calbindin in hippocampal excitatory, but not inhibitory, neurons conveys susceptibility to stress-induced memory deficits. Moreover, calbindin levels were downregulated by early-life stress through the corticotropin-releasing hormone receptor 1-nectin3 pathway, which in turn reduced inositol monophosphatase levels. Our findings highlight calbindin as a molecular target of early-life stress and an essential substrate for memory.

INTRODUCTION

Calbindin D-28K (abbreviated as calbindin) binds Ca^{2+} with fast kinetics and intermediate affinity (Faas et al., 2011; Kojetin et al., 2006), and it acts as a buffer, sensor, and transporter of intracellular Ca^{2+} (Schmidt, 2012). In the hippocampus, calbindin shows a cell subtype-specific expression pattern: it is present in glutamatergic neurons, including mature granule cells in the dentate gyrus (DG) (Celio, 1990), superficial CA1 pyramidal neurons (Kohara et al., 2014), and CA3 granule cells (Szabadics et al., 2010), as well as in a subpopulation of GABAergic interneurons in the CA1 (Jinno and Kosaka, 2006).

Calbindin localizes to both axonal boutons and dendritic spines and dynamically modulates synaptic plasticity. Presynaptic calbindin facilitates vesicular release and paired pulse facilitation (PPF) (Blatow et al., 2003; Pan and Ryan, 2012; Westerink et al., 2012), while postsynaptic calbindin is necessary for the maintenance of long-term potentiation (LTP) in DG and CA1 excitatory neurons (Molinari et al., 1996; Westerink et al., 2012). Calbindin also influences learning and memory. In transgenic calbindin-knockout mice, spatial reference memory and cued fear memory are compromised (Harris et al., 2016; Molinari et al., 1996). Moreover, reduced DG calbindin levels correlate with impaired hippocampus-dependent memory in animal models of Alzheimer's disease (Palop et al., 2003; Sun et al., 2008). Nonetheless, overexpression of calbindin in DG neurons disrupts PPF, LTP, and spatial memory (Dumas et al., 2004), indicative of its homeostatic role in synaptic plasticity and memory.

Dysregulation of calbindin is implicated in stress-related psychiatric disorders. In patients with depression or schizophrenia, calbindin-expressing interneurons are decreased in number in the neocortex (Beasley et al., 2002; Maciag et al., 2010; Rajkowska et al., 2007). Notably, calbindin expression levels are developmentally regulated and peak during the early postnatal period in the rodent neocortex (Alcántara et al., 1993), when the developing brain is highly sensitive to stress exposure (Liao et al., 2014; Liu et al., 2016; Yang et al., 2015). Severely stressful experiences around this critical period, which increase the risk for psychiatric disorders later in life (Humphreys and Zeanah, 2015; Nemeroff, 2016), could alter calbindin levels and the density of calbindin-positive neurons in the hippocampus (Giachino et al., 2007; Seidel et al., 2008; Xu et al., 2011) and other stress-related brain regions (Gos et al., 2014; Helmeke et al., 2008).

Although accumulating evidence suggests the involvement of calbindin in early-life stress-induced synaptic and cognitive deficits, two major questions remain unsolved. First, how do early-life stressful experiences influence calbindin expression in heterogeneous hippocampal neurons with distinct cellular

properties and circuit connectivity? Second, how do altered calbindin levels in each neuronal population influence hippocampus-dependent memory under basal or stressful conditions? In this study, we aimed to investigate the molecular mechanisms underlying early-life stress-induced hippocampal calbindin alterations and dissect the contribution of calbindin in each neuronal population to spatial memory. Our findings reveal that early-life stress suppresses hippocampal calbindin levels through the corticotropin-releasing hormone receptor 1 (CRHR1)-nectin3 system, and they demonstrate that reduced calbindin levels in hippocampal excitatory, but not inhibitory, neurons modulate stress-induced spatial memory impairments.

RESULTS

Correlated Hippocampal Calbindin Reductions and Spatial Memory Impairments in Early-Life-Stressed Adult Mice

In the adult mouse hippocampus, calbindin expression shows a cell-type-specific pattern (Figure 1A). During postnatal development, hippocampal calbindin levels peaked on postnatal day 9 (P9) (Figure 1B), and this transient increase coincided with a period sensitive to stress (Liu et al., 2016). We found that stress exposure during this period inhibited calbindin protein expression in all subregions of the adult hippocampus (Figures 1C and S1A). The stress effect on calbindin expression was also evident on P9 when the stress procedure ended, but not in adolescence (Figure S1B). Immunostaining further revealed that, in postnatally stressed adult mice, calbindin immunoreactivity in CA1 pyramidal neurons and dentate granule cells (Figure 1D), as well as the number of calbindin-positive interneurons in the CA1 (Figure 1E), was significantly decreased, indicating that early-life stress downregulates hippocampal calbindin levels in both excitatory and inhibitory neurons. In addition, the levels of calretinin, another Ca^{2+}-binding protein that is highly homologous to calbindin (Rogers, 1987), and the number of neuronal nuclei antigen-positive neurons remained unchanged between groups (Figures S1C and S1D).

In the hippocampus-dependent object location task, postnatally stressed adult mice failed to discriminate the displaced object from the non-displaced one, and they performed significantly worse than the controls (Figure 1F), indicative of spatial memory deficits. The total time it took to explore the objects during the acquisition phase of this task and the anxiety level as evaluated by the open field test were comparable between groups (Figures S1E and S1F). Furthermore, cognitive performance strongly correlated with calbindin immunoreactivity in the CA1 or DG, but not with the number of calbindin-positive interneurons in the CA1 (Figures 1G and S1G). This indicates that calbindin levels in CA1 and DG excitatory neurons, but not CA1 interneurons, are important for spatial memory.

Calbindin Knockdown in Both CA1 and DG Neurons Mimicked Early-Life Stress-Induced Spatial Memory Loss

To investigate the role of hippocampal calbindin in spatial memory, we first injected AAV-shCalb1 into the DG and CA1 of adult C57BL/6N mice to knock down calbindin levels in both regions ($Calb1^{DGCA1\text{-}KD}$; Figures 2A, S2A, and S2B). Compared to the controls, $Calb1^{DGCA1\text{-}KD}$ mice exhibited significant reductions of calbindin immunoreactivity in the CA1 and DG and the number of calbindin-positive interneurons in the CA1 (Figures 2B–2D). No off-target effect of AAV-shCalb1 on calretinin levels was noticed (Figure S2C).

In the object location task, $Calb1^{DGCA1\text{-}KD}$ mice failed to show object discrimination and performed worse than the controls (Figures 2E and S2D), mimicking the effects of early-life stress on long-term spatial memory. In the Y-maze spontaneous alternation task, $Calb1^{DGCA1\text{-}KD}$ mice had significantly reduced spontaneous alternation rates (Figure 2F) and an increased number of same arm returns (Figure S2E) compared to the controls, indicative of impaired short-term spatial memory. Anxiety-related behavior remained unchanged in $Calb1^{DGCA1\text{-}KD}$ mice (Figure S2F). Moreover, in control and $Calb1^{DGCA1\text{-}KD}$ mice, both the discrimination index in the object location task and spontaneous alternation rates in the Y-maze task significantly correlated with calbindin immunoreactivity in the CA1 or DG, but not with the number of calbindin-positive interneurons in the CA1 (Figures 2G and S2G). These data suggest that the reduction of calbindin levels in both DG and CA1 neurons reproduces the cognitive effects of early-life stress and that hippocampal calbindin is necessary for spatial memory.

Because all DG and CA1 neuron subtypes were affected in $Calb1^{DGCA1\text{-}KD}$ mice, we next dissected the contribution of calbindin in each neuronal population to spatial memory.

Calbindin Knockdown in DG Excitatory Neurons Impaired Spatial Memory

To selectively reduce calbindin levels in DG excitatory granule cells ($Calb1^{DGGlu\text{-}KD}$), we injected AAV-shCalb1 into the DG region of adult C57BL/6N mice (Figures 3A, S3A, and S3B). In $Calb1^{DGGlu\text{-}KD}$ mice, calbindin immunoreactivity in the DG was markedly reduced, while calbindin levels in CA1 neurons remained unchanged (Figures 3B–3D and S3C). $Calb1^{DGGlu\text{-}KD}$ mice showed deficits in both long-term spatial recognition memory (Figures 3E and S3D) and short-term spatial working memory (Figures 3F and S3E), with comparable anxiety levels to control mice (Figure S3F).

Calbindin Knockdown in CA1 Excitatory, but Not Inhibitory, Neurons Impaired Spatial Memory

To selectively knock down calbindin in CA1 excitatory pyramidal neurons ($Calb1^{CA1Glu\text{-}KD}$), we infused AAV-LSL-MIRshCalb1 into the CA1 region of adult Camk2α-Cre mice (T29-1 line; Figures 4A and S4A). In $Calb1^{CA1Glu\text{-}KD}$ mice, calbindin immunoreactivity in the stratum pyramidale of CA1 was selectively reduced (Figures 4B and 4C), whereas calbindin levels in the DG and the number of calbindin-positive interneurons in the CA1 were unaffected (Figures 4D and S4B). Similar to the cognitive deficits of $Calb1^{DGCA1\text{-}KD}$ and $Calb1^{DGGlu\text{-}KD}$ mice, $Calb1^{CA1Glu\text{-}KD}$ mice exhibited impairments in spatial recognition memory (Figures 4E and S4C) and spatial working memory (Figures 4F and S4D), but they had normal anxiety-related behavior (Figure S4E).

Next, we specifically reduced calbindin levels in CA1 GABAergic interneurons ($Calb1^{CA1GABA\text{-}KD}$) by expressing

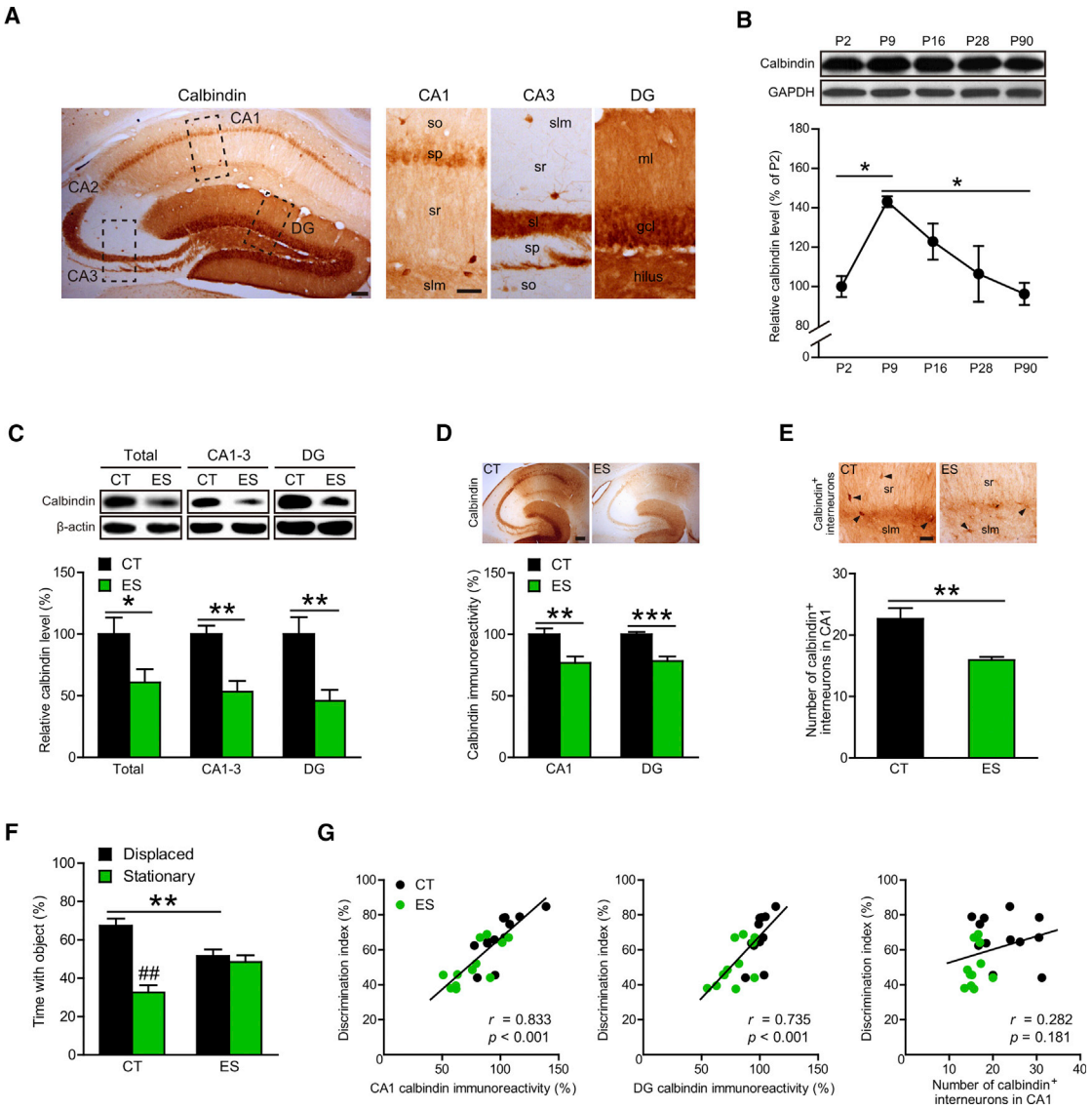

Figure 1. Correlated Hippocampal Calbindin Reductions and Spatial Memory Deficits in Adult Mice with Early-Life Stress Exposure

(A) In the adult hippocampus, calbindin localized in both excitatory neurons (including superficial CA1 pyramidal neurons and mature dentate granule cells) and a subpopulation of interneurons in the CA1 and CA3. Scale bar, 100 μm.

(B) Hippocampal calbindin protein levels were developmentally regulated and peaked on P9.

(C) Early-life stress suppressed calbindin protein expression in the adult hippocampus, involving all hippocampal subregions.

(D) Immunostaining revealed that, in postnatally stressed adult mice, calbindin levels in CA1 pyramidal neurons and dentate granule cells were decreased. Scale bar, 200 μm.

(E) The number of calbindin-positive interneurons in the CA1 (arrowheads) was decreased in stressed mice. Scale bar, 50 μm.

(F) In the object location task, control mice distinguished the displaced object from the non-displaced one, whereas stressed mice failed to discriminate the objects and performed worse than the controls.

(G) The discrimination index in the object location task correlated with calbindin immunoreactivity in CA1 and DG excitatory neurons, but not with the number of CA1 calbindin-positive interneurons.

CT, control; DG, dentate gyrus; ES, early-life stress; gcl, granule cell layer; ml, molecular layer; P, postnatal day; sl, stratum lucidum; slm, stratum lacunosum-moleculare; so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $^{\#\#}p < 0.01$. In this and subsequent figures, data are presented as mean ± SEM.

AAV-LSL-MIRshCalb1 in the CA1 region of GAD2-Cre mice (Figures 4G and S4F). Compared to the controls, the density of calbindin-positive CA1 interneurons was significantly reduced in *Calb1*$^{CA1GABA\text{-}KD}$ mice, with calbindin immunoreactivity in CA1 pyramidal neurons and dentate granule cells unchanged (Figures 4H–4J and S4G). In contrast to mice with calbindin levels reduced in either all neurons or excitatory neurons in the CA1/DG, calbindin knockdown in CA1 interneurons

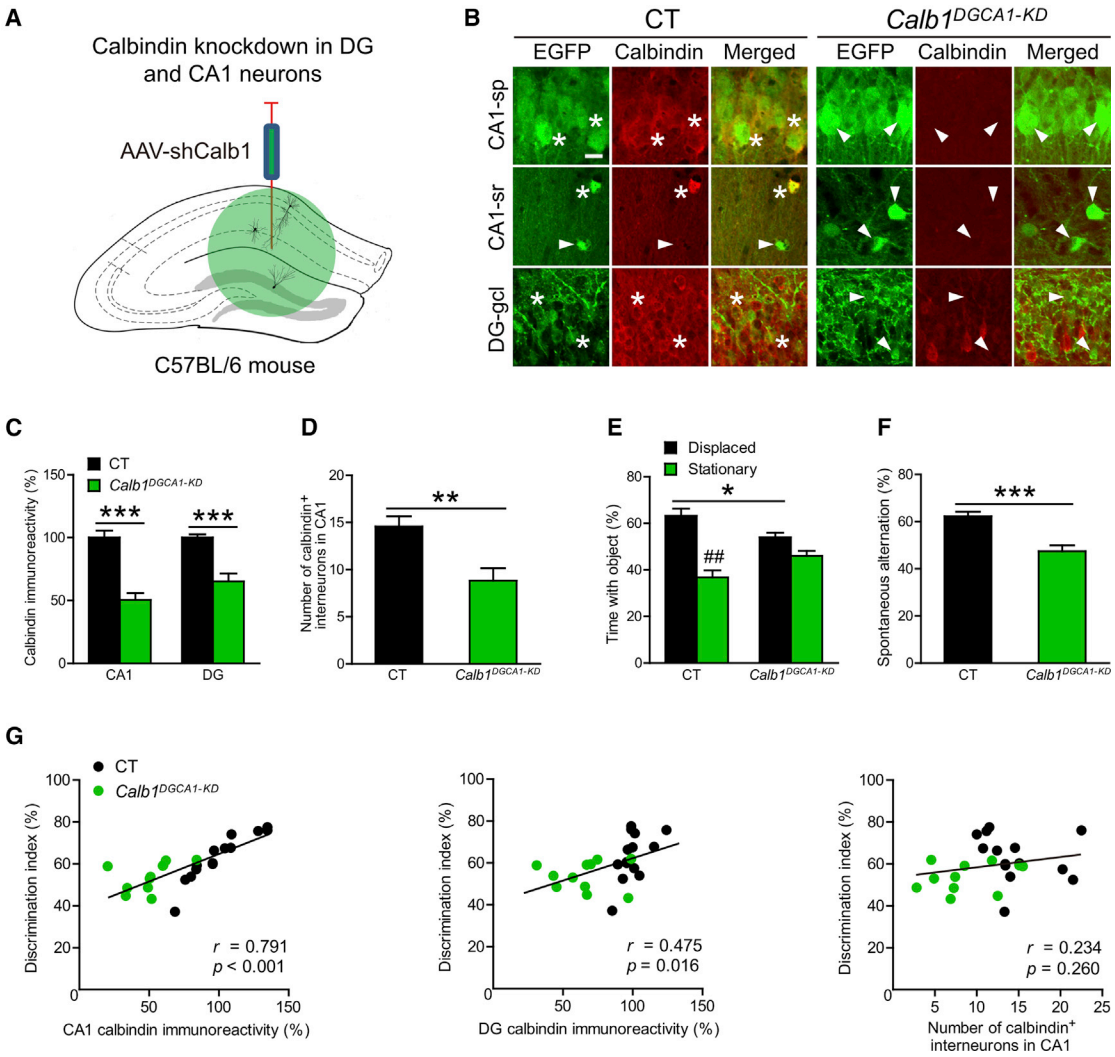

Figure 2. Calbindin Knockdown in Both DG and CA1 (*Calb1*$^{DGCA1\text{-}KD}$) Impaired Spatial Memory

(A) Schematic showing the microinjection of knockdown virus (AAV-shCalb1) into the DG and CA1 of adult C57BL/6N mice.

(B) Representative images showing the expression of EGFP and calbindin in the hippocampus of control and *Calb1*$^{DGCA1\text{-}KD}$ mice. Asterisks indicate neurons that co-express EGFP and calbindin, while arrowheads indicate EGFP-expressing cells without detectable calbindin expression. Scale bar, 20 μm.

(C and D) In *Calb1*$^{DGCA1\text{-}KD}$ mice, (C) calbindin immunoreactivity in the CA1 and DG and (D) the number of calbindin-positive CA1 interneurons were markedly reduced.

(E) Compared to control mice that successfully distinguished the displaced object from the stationary one, *Calb1*$^{DGCA1\text{-}KD}$ mice showed impaired spatial recognition memory.

(F) In the Y-maze spontaneous alternation task, *Calb1*$^{DGCA1\text{-}KD}$ mice showed impaired spatial working memory.

(G) In control and *Calb1*$^{DGCA1\text{-}KD}$ mice, the discrimination index in the object location task correlated with calbindin immunoreactivity in CA1 and DG excitatory neurons, but not with the number of CA1 calbindin-positive interneurons.

*p < 0.05; **p < 0.01; ***p < 0.001; $^{\#\#}$p < 0.01.

did not disrupt spatial recognition memory under basal conditions (Figure 4K). Furthermore, at 4 hr after an acute severe stress challenge (6 min of forced swim), *Calb1*$^{CA1GABA\text{-}KD}$ mice still showed intact object discrimination, whereas the performance of control mice was impaired (Figures 4L and S4H). We further performed the Y-maze delayed non-match-to-sample test that is more demanding than the spontaneous alternation task, and we found that short-term spatial working memory of *Calb1*$^{CA1GABA\text{-}KD}$ mice was generally comparable to the controls, with only transient and subtle impairments on days 2 and 7 (Figures S4I and S4J). Object exploration and anxiety level were not altered in *Calb1*$^{CA1GABA\text{-}KD}$ mice (Figures S4K and S4L).

Together, these results indicate that the reduction of calbindin levels in CA1 pyramidal neurons impairs spatial memory, whereas the downregulation of calbindin in CA1 interneurons buffers the negative consequences of acute stress on long-term spatial memory.

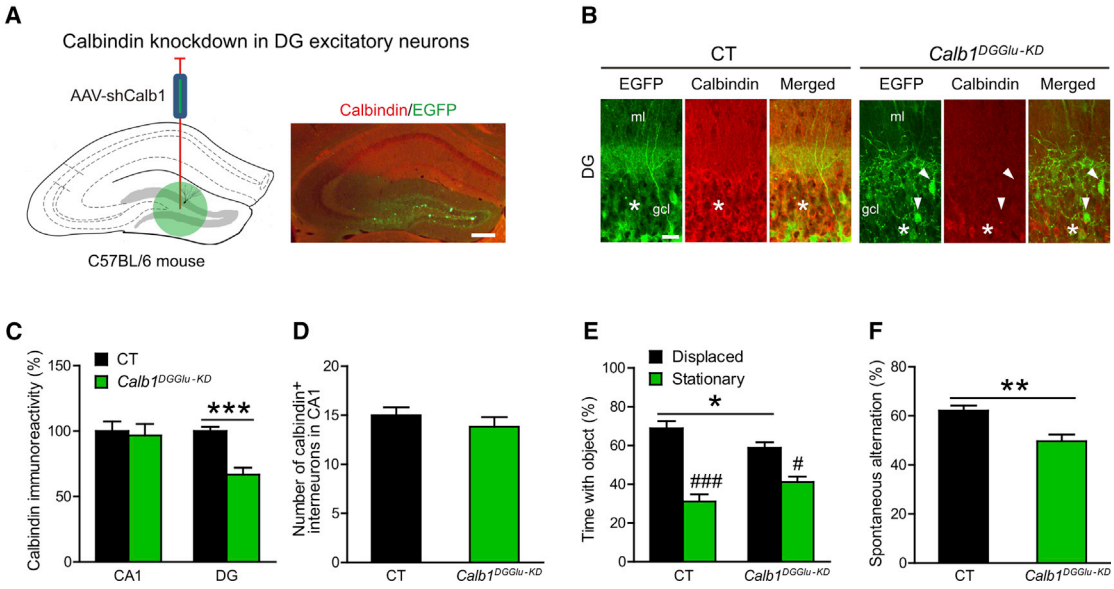

Figure 3. Calbindin Knockdown in DG Glutamatergic Granule Cells (*Calb1*$^{DGGlu-KD}$) Induced Spatial Memory Deficits

(A) Left: schematic showing the microinjection of AAV-shCalb1 into the DG region of adult C57BL/6N mice. Right: region-specific expression of EGFP in the DG is shown. Scale bar, 200 μm.

(B) Magnified images showing EGFP- and calbindin-expressing neurons in the DG. Asterisks indicate DG neurons that co-express EGFP and calbindin, while arrowheads indicate EGFP-expressing neurons without detectable calbindin expression. Scale bar, 20 μm.

(C and D) In *Calb1*$^{DGGlu-KD}$ mice, (C) calbindin immunoreactivity in the DG, but not the CA1, was selectively reduced. (D) The number of calbindin-positive CA1 interneurons remained unchanged between groups.

(E) Although both control and *Calb1*$^{DGGlu-KD}$ mice showed object preference, control mice performed better than *Calb1*$^{DGGlu-KD}$ mice.

(F) Spatial working memory was also impaired in *Calb1*$^{DGGlu-KD}$ mice.

*p < 0.05; **p < 0.01; ***p < 0.001; $^{\#}$p < 0.05; $^{\#\#\#}$p < 0.001.

The CRHR1-Nectin3 Pathway Mediates the Downregulation of Hippocampal Calbindin Levels by Early-Life Stress

Having revealed that hippocampal calbindin could modulate early-life stress-induced spatial memory deficits, we then dissected the molecular pathway responsible for the regulation of calbindin levels by early-life stress. In neonatally stressed adult mice, systemic blockade of CRHR1 by antalarmin during stress exposure normalized the protein levels of nectin3 (Figure 5A), a cell adhesion molecule linking CRHR1 to stress-induced memory loss (Wang et al., 2013) and restored spatial memory performance (Figures S5A–S5E). Most importantly, CRHR1 blockade attenuated the effects of early-life stress on hippocampal calbindin levels in both neonatal (Figure S5F) and adult (Figure 5B) mice, indicating that stress reduces calbindin levels via the CRHR1-nectin3 pathway.

We further investigated how nectin3 might interact with calbindin, and we observed that nectin3 and calbindin partially colocalized in adult hippocampal neurons, including CA1 pyramidal neurons, CA1 interneurons, and DG granule cells (Figure 5C). A similar colocalization pattern was observed on P9 (Figure S5G). Moreover, hippocampal calbindin levels were reduced in *Nectin3*$^{DGCA1-KD}$ mice (Figures 5D and S5H) whose nectin3 expression in the DG and CA1 was suppressed (Figure S5I). However, in *Calb1*$^{DGCA1-KD}$ mice, hippocampal nectin3 levels remained unchanged (Figure S5J), suggesting that calbindin serves as a downstream molecule of the CRHR1-nectin3 pathway.

Calbindin can activate inositol monophosphatase (IMPase), a key enzyme in the phosphatidylinositol signaling pathway that modulates memory (Berggard et al., 2002; Figueiredo et al., 2016; Levi et al., 2013). We observed that early-life stress reduced IMPase levels in the adult hippocampus, especially in CA1-3 subregions (Figure S5K), which can be reversed by CRHR1 antagonism (Figure S5L). Knockdown of either nectin3 (Figure 5E) or calbindin (Figure 5F) significantly reduced hippocampal IMPase levels, mimicking the effects of early-life stress. Taken together, IMPase may be a common endpoint of the CRHR1-nectin3 pathway and calbindin in modulating the cognitive impact of early-life stress.

DISCUSSION

Our results demonstrate that reduced calbindin levels in CA1 and DG excitatory neurons mediate early-life stress-induced memory loss, whereas reduced calbindin levels in CA1 interneurons may potentially increase resilience to acute stress-induced long-term memory impairments. Moreover, CRHR1 and nectin3 are required for the regulation of calbindin levels by early-life stress, thus identifying a molecular mechanism linking stress-elevated CRHR1 signaling and disrupted synaptic adhesion to cognitive dysfunction.

Calbindin is expressed early from the embryonic stage (Morante-Oria et al., 2003), and it changes in levels during postnatal development (Alcántara et al., 1993). We observed that

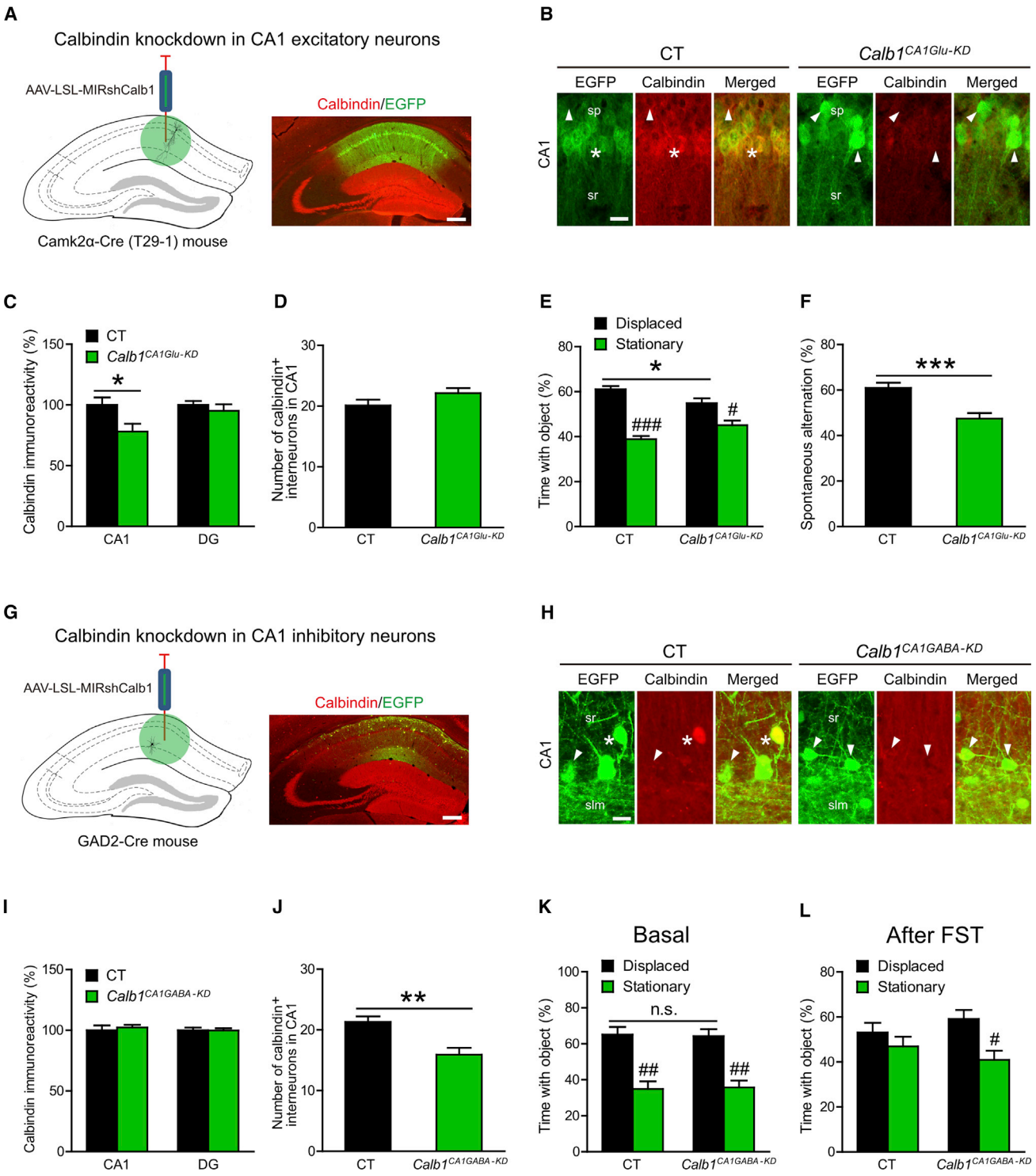

Figure 4. Calbindin Knockdown in CA1 Glutamatergic Neurons (*Calb1*$^{CA1Glu-KD}$), but Not GABAergic Neurons (*Calb1*$^{CA1GABA-KD}$), Impaired Spatial Memory

(A) Left: schematic showing the microinjection of a Cre-dependent knockdown virus (AAV-LSL-MIRshCalb1) into the CA1 region of adult Camk2α-Cre mice. Right: region-specific expression of EGFP in CA1 pyramidal neurons is shown. Scale bar, 200 μm.

(B) Magnified images showing EGFP- and/or calbindin-expressing neurons in the CA1. Asterisks indicate CA1 pyramidal neurons that co-express EGFP and calbindin, while arrowheads indicate CA1 neurons that express either EGFP or calbindin. Scale bar, 20 μm.

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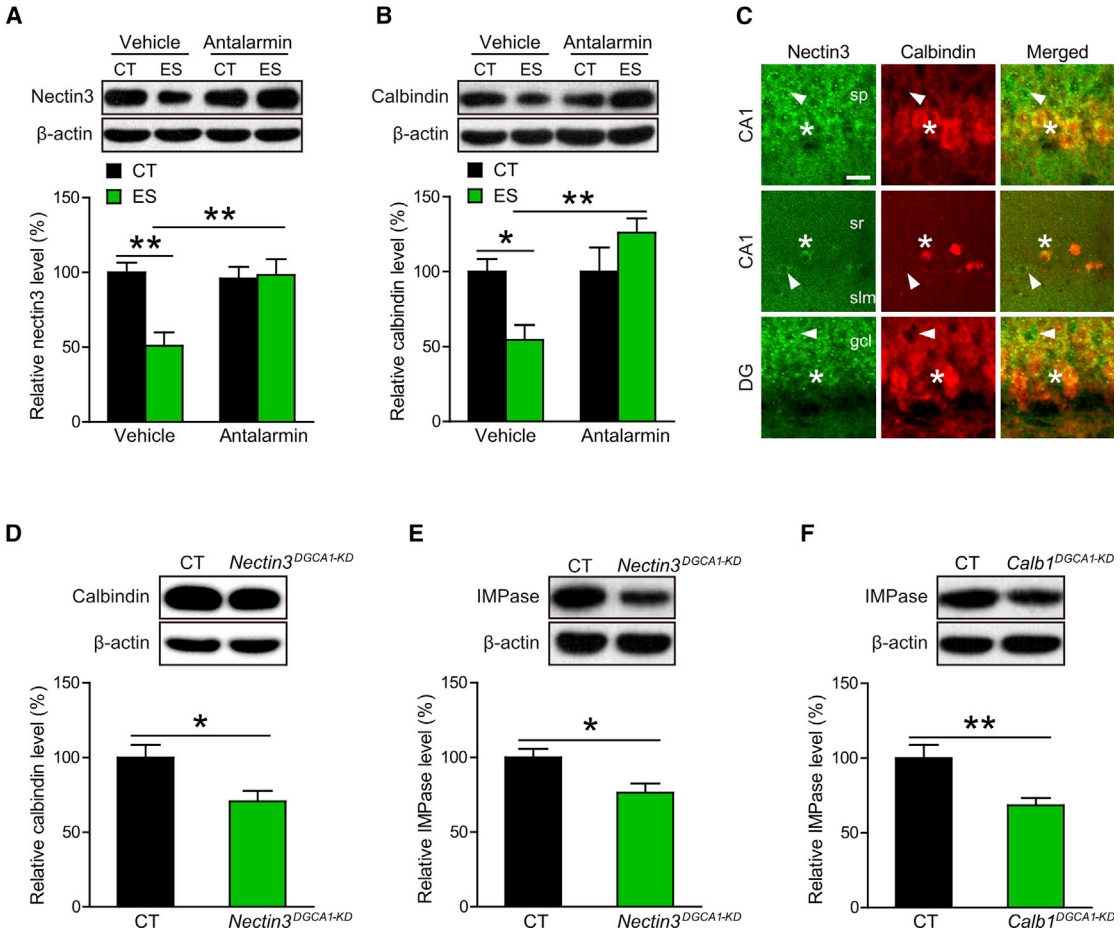

Figure 5. The Interplay among Calbindin, Stress Mediators, and Related Molecules

(A and B) Early-life stress reduced hippocampal (A) nectin3 and (B) calbindin levels in vehicle-injected mice, while repeated CRHR1 blockade reversed the stress effects.

(C) Calbindin and a cell adhesion molecule nectin3 partially colocalized in superficial CA1 pyramidal neurons, CA1 interneurons, and DG granule cells. Asterisks indicate neurons co-expressing nectin3 and calbindin, while arrowheads indicate nectin3-immunoreactive neurons without detectable calbindin expression.

(D) Nectin3 knockdown in both DG and CA1 (*Nectin3*$^{DGCA1\text{-}KD}$) reduced hippocampal calbindin protein levels.

(E and F) Hippocampal IMPase levels were decreased by the knockdown of (E) nectin3 or (F) calbindin.

*p < 0.05; **p < 0.01.

hippocampal calbindin levels increased after birth and peaked around P9. This transient increase falls in a critical developmental period of the hippocampus (Liao et al., 2014; Liu et al., 2016). Similar to previous findings (Seidel et al., 2008; Xu et al., 2011), we found that stress exposure during this critical period reduced calbindin levels in subtypes of CA1 and DG neurons in

(C and D) In *Calb1*$^{CA1Glu\text{-}KD}$ mice, (C) calbindin immunoreactivity in the stratum pyramidale of CA1 was selectively reduced. (C) Calbindin immunoreactivity in the DG and (D) the number of calbindin-positive CA1 interneurons were comparable between groups.

(E) Although both control and *Calb1*$^{CA1Glu\text{-}KD}$ mice showed object preference, control mice performed better than *Calb1*$^{CA1Glu\text{-}KD}$ mice.

(F) *Calb1*$^{CA1Glu\text{-}KD}$ mice had spatial working memory deficits.

(G) Left: schematic showing the microinjection of AAV-LSL-MIRshCalb1 into the CA1 region of adult GAD2-Cre mice. Right: region-specific expression of EGFP in CA1 interneurons is shown. Scale bar, 200 μm.

(H) Magnified images showing EGFP- and/or calbindin-expressing neurons in the CA1. Asterisks indicate CA1 interneurons that co-express EGFP and calbindin, while arrowheads indicate CA1 interneurons that express either EGFP or calbindin. Scale bar, 20 μm.

(I and J) In *Calb1*$^{CA1GABA\text{-}KD}$ mice, (J) the number of calbindin-positive interneurons in the CA1 was significantly reduced. (I) Calbindin immunoreactivity in CA1 and DG excitatory neurons was comparable between groups.

(K) Control and *Calb1*$^{CA1GABA\text{-}KD}$ mice showed object preference and performed similarly under stress-free conditions.

(L) At 4 hr after a 6-min forced swim test (FST), control mice showed impaired performance in the object location task, whereas *Calb1*$^{CA1GABA\text{-}KD}$ mice still distinguished the displaced object from the stationary one.

n.s., not significant. *p < 0.05; **p < 0.01; ***p < 0.001; $^{\#}$p < 0.05; $^{\#\#}$p < 0.01; $^{\#\#\#}$p < 0.001.

adulthood. Although inconsistent results have been reported (Giachino et al., 2007), these and our data are not mutually exclusive considering the differences in experimental animals and stress paradigms. Moreover, our study pinpoints the importance of classifying hippocampal calbindin-expressing neurons into structurally and functionally distinct subtypes to dissect their differential roles in memory.

Our major finding is the differential contribution of calbindin expressed by distinct hippocampal neurons to stress-induced memory deficits. We found that calbindin protein levels in CA1 or DG excitatory neurons, but not CA1 interneurons, strongly correlated with spatial memory performance. Accordingly, calbindin knockdown in CA1 and/or DG excitatory neurons reproduced the effects of early-life stress and disrupted both short- and long-term spatial memory, consistent with studies using calbindin-knockout mice (Harris et al., 2016; Molinari et al., 1996). In contrast, calbindin knockdown in CA1 interneurons preserved long-term spatial memory under basal conditions. This indicates that early-life stress mainly targets calbindin-containing excitatory neurons to disrupt memory. In the dorsal CA1, calbindin-expressing pyramidal neurons show complex dendritic morphology and receive circuit-specific inputs to modulate learning (Li et al., 2017), while, in the DG, calbindin marks functionally mature granule cells. Although we did not monitor the electrophysiological properties of CA1 and DG excitatory neurons with reduced levels of calbindin, considering its role in the immediate and lasting modifications of synaptic activities (Blatow et al., 2003; Molinari et al., 1996; Pan and Ryan, 2012; Westerink et al., 2012), the observed cognitive deficits are likely ascribed to abnormal activity of corresponding neuron ensembles and neural circuits.

Interneurons are highly diverse in structural, neurochemical, and functional properties (Kepecs and Fishell, 2014). In the CA1 region, interneurons immunoreactive for calbindin account for ~20% of caudal ganglionic eminence-derived interneurons (Wierenga et al., 2010). CA1 calbindin-expressing interneurons receive excitatory (~70%) and inhibitory (~30%) innervations (Gulyás et al., 1999) as well as neuromodulatory inputs (Freund et al., 1990). These interneurons are heterogeneous in morphology and connectivity, with some targeting the dendrites of local pyramidal neurons and others sending long-range projections to the septum (Gulyás and Freund, 1996; Gulyás et al., 1999). We found that calbindin knockdown in CA1 interneurons preserved spatial recognition memory both before and following an acute adult stress challenge. This raises the possibility that reduced calbindin levels in CA1 interneurons by early-life stress might increase the resistance to a future stress challenge, which merits further investigations. Moreover, in mice with calbindin knockdown in CA1 interneurons, short-term spatial working memory was transiently and subtly impaired under basal conditions. This is reminiscent of mice with AMPA or NMDA receptors selectively deleted in hippocampal parvalbumin-expressing interneurons (Fuchs et al., 2007; Korotkova et al., 2010), indicating that excitatory and inhibitory neurons in the CA1 region differentially modulate specific components of spatial memory.

Another key finding is the molecular substrates modulating the effects of early-life stress on hippocampal calbindin expression. Repeated, but not single, stress exposure downregulates nectin3 levels in hippocampal neurons (van der Kooij et al., 2014), which is dependent on CRHR1 (Wang et al., 2013). Here we found a similar suppression of hippocampal calbindin levels by early-life stress via CRHR1. Remarkably, the cell adhesion molecule nectin3 colocalizes and interacts with calbindin in hippocampal neurons, indicative of nectin3 as an upstream molecule of calbindin. Moreover, calbindin has been shown to interact with and activate IMPase (Berggard et al., 2002; Levi et al., 2013), a key enzyme in the phosphatidylinositol-signaling pathway. We found that early-life stress lastingly lowered hippocampal IMPase levels, which can be reproduced by knockdown of either nectin3 or calbindin and prevented by CRHR1 blockade. Together with previous evidence on the involvement of CRHR1 and nectin3 in stress-induced memory loss, the current findings suggest that the molecular components of the CRHR1-nectin3 pathway are critical modulators of the cognitive impact of early-life stress. Interventions that block CRHR1 or normalize the levels of nectin3, calbindin, or IMPase in hippocampal neurons are promising therapeutic strategies for early-life stress-related disorders, which can be implemented around the time window of stress exposure or during young adulthood (Regev and Baram, 2014).

Beyond these findings, several questions remain to be answered. The most important one is how various stressors dynamically influence the activity of calbindin-expressing hippocampal neurons and the expression of calbindin in distinct neuron subtypes. Moreover, studies using sophisticated approaches, including calbindin-Cre mouse lines and viral tools with neuron subtype-specific promoters, are required to reveal the impact of hippocampal calbindin knockdown at both cellular and circuit levels. Finally, more evidence is needed on whether reducing calbindin levels in CA1 interneurons indeed conveys resilience to the negative stress effects on spatial memory.

In summary, our study reveals that reduced calbindin levels in hippocampal excitatory neurons by early-life stress lead to cognitive deficits, and it identifies a molecular pathway that mediates such effects.

EXPERIMENTAL PROCEDURES

Animals

Adult male C57BL/6N mice were purchased from Vital River Laboratories (Beijing, China). The B6.Cg-Tg(Camk2α-Cre)T29-1Stl/J mice (stock number 005359, Jackson Laboratory) have Cre recombinase preferentially expressed by excitatory CA1 pyramidal neurons. The *Gad2*$^{tm2(cre)Zjh}$/J mice (stock number 010802, Jackson Laboratory) have Cre recombinase expressed in GABAergic interneurons. All experiments were approved by the Peking University Committee on Animal Care and Use and the Animal Advisory Committee at Zhejiang University, and they were performed in compliance with the NIH's Guide for the Use and Care of Laboratory Animals.

Early-Life Stress Procedure

The limited nesting and bedding material paradigm was performed as previously described (Yang et al., 2015). For details, see the Supplemental Experimental Procedures.

Stereotaxic Surgery and Viral Microinjection

We used adeno-associated virus (AAV) 2/8 vectors to suppress calbindin or nectin3 protein levels. To achieve calbindin or nectin3 knockdown in CA1 and/or DG cells, AAV-shCalb1, AAV-shNectin3, and the control virus were used. To selectively knock down calbindin protein levels in CA1 excitatory

or inhibitory neurons, a Cre recombinase-responsive AAV (AAV-LSL-MIRshCalb1) and a control virus (AAV-LSL-MIRshScr) were used. For details, see the Supplemental Experimental Procedures.

Behavioral Testing

Anxiety-related behavior and spatial memory were assessed as previously described (Wang et al., 2012, 2013; Yang et al., 2015). For details, see the Supplemental Experimental Procedures.

Immunostaining and Image Analysis

Serial coronal or horizontal sections (30 μm thick) were prepared through the dorsal hippocampus using a cryostat (Leica, Wetzlar, Germany). For details about immunostaining and image analysis, see the Supplemental Experimental Procedures.

Western Blot

Hippocampal samples containing 30 μg protein were resolved by 10% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA), which were then labeled with primary antibodies at 4°C (overnight). After incubation with horseradish peroxidase-conjugated secondary antibodies at room temperature (3 hr), bands were visualized and quantified by densitometry. See the Supplemental Experimental Procedures for details.

Statistical Analysis

SPSS 16.0 (Chicago, IL, USA) and GraphPad Prism 5 (San Diego, CA, USA) were used to perform statistical analyses. Data were first checked for normality using Kolmogorov-Smirnov test. For between-group comparisons, Student's t test and Mann-Whitney U test were applied for normally and non-normally distributed data, respectively. For multiple group comparisons, data were analyzed by ANOVA followed by Bonferroni post hoc test when appropriate. Correlations were assessed by Pearson correlation coefficient. Statistical outliers with values that fell beyond two SDs from the mean were excluded from analysis. The sample size and statistical results for main figures are summarized in Table S1. Data are reported as mean ± SEM. Statistical significance was defined at $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.10.006.

AUTHOR CONTRIBUTIONS

The project was conceived by X.-D.W. and supervised by X.-D.W. and T.-M.S. J.-T.L., X.-M.X., J.-Y.Y., Y.-X.S., X.-M.L., and X.-X.W. performed the experiments. J.-T.L., X.-M.X., J.-Y.Y., and Y.-A.S. analyzed the data and drafted the results. X.-D.W., T.-M.S., J.-T.L., M.V.S., and Y.-J.L. wrote the manuscript.

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