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Neuron-specific menin deletion leads to synaptic dysfunction and cognitive impairment by modulating p35 expression

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Summary

Menin (*MEN1*) is a critical modulator of tissue development and maintenance. As such, *MEN1* mutations are associated with multiple endocrine neoplasia type 1 (MEN1) syndrome. Although menin is abundantly expressed in the nervous system, little is known with regards to its function in the adult brain. Here, we demonstrate that neuron-specific deletion of *Men1* (CcKO) impacts dendritic branching and spine formation, resulting in defects in synaptic function, learning and memory. Further, we find that menin binds to the *p35* promoter region to facilitate *p35* transcription. As a primary Cdk5 activator, p35 is mainly expressed in neurons and is critical for brain development and synaptic plasticity. Restoration of p35 expression in the hippocampus and cortex of *Men1* CcKO mice rescues synaptic and cognitive deficits associated with *Men1* deletion. These results reveal a critical role for menin in synaptic and cognitive function by modulating the p35-Cdk5 pathway.

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Author Contributions

K.Z., H.X., and J.Z. conceptualized the study. K.Z, H.C., L.L., H.Y., S.M., and D.W. prepared and maintained the mice. K.Z. designed and performed morphological analysis and biochemical assays. K.Z., H.C., G.Y., and C.G. performed behavior tests. G.Y. performed electrophysiology experiments and Golgi staining. H.S. supervised the electrophysiology experiment. H.Z., and Z.J. performed the microarray assays and Luciferase assays. C.G and H.L performed the primary neuronal culture. K.Z, H.X., and J.Z. wrote the manuscript. M.X., Y.Z., T.Y.H., Y.Z., and G.B. discussed and edited the manuscript. J.Z. supervised the project.

Declaration of Interests

The authors declare no competing interests

Data and Software Availability

The accession number for the micro-array data reported in this paper is GEO: GSE115344

Keywords

Menin; Synaptic function; Cognition; p35; Cdk5

Introduction

Menin is a scaffold protein encoded by the multiple endocrine neoplasia type 1 (*MEN1*) gene in humans (*Men1* in mice). Although menin is ubiquitously expressed (Stewart et al., 1998), its biological function appears to be tissue-specific (Matkar et al., 2013). Loss of function *MEN1* gene mutations are causal to *MEN1* syndrome, which is a dominantly inherited disease characterized by tumor formation in endocrine organs including the pituitary gland, parathyroid gland, and pancreatic islets (Chandrasekharappa et al., 1997; Lemmens et al., 1997). Menin is a major contributor to tissue development and maintenance (Guru et al., 1999; Stewart et al., 1998). Homozygous *Men1* deletion in mice has been reported to produce developmental defects in multiple organs, including defective neural tube closure, with embryonic lethality between embryonic days (E)11.5–13.5 (Bertolino et al., 2003; Engleka et al., 2007; Scacheri et al., 2001). Furthermore, Menin can promote postsynaptic clustering of neurotransmitter receptors and synapse formation in Lymnaea neurons (Getz et al., 2016; van Kesteren et al., 2001). Clinical studies indicated that *MEN1* patients also present psychosis or polyneuropathy (de Paiva et al., 2012; Kito et al., 2005). These reports indicate that menin may be involved in neuronal development and function. Menin is capable of interacting with diverse proteins to regulate a variety of cellular functions by controlling gene transcription in a variety of organs (Balogh et al., 2006). Menin interacts with transcriptional activators include c-Myb (Jin et al., 2010) and histone modifiers such as mixed lineage leukemia proteins (MLL)-1 and −2 and histone H3 lysine 4 (H3K4) methyltransferases (Hughes et al., 2004; Yokoyama et al., 2004) to promote gene expression. Menin also interacts with the transcription factor JunD (Agarwal et al., 1999) and histone deacetylase (HDAC)1/2 (Gao et al., 2009) to suppress gene expression. However, the physiological function of menin in neuronal development and cognition remains unknown.

Early postnatal brain development involves proper coordination of dendritic branching and synapse formation in neurons. Regulation of neuronal density and morphology is critical in facilitating higher brain function such as learning and memory (Ho et al., 2011). Neuronal Cyclin-dependent kinase 5 (Cdk5) maintains a variety of established roles in brain function to include neuronal migration (Cheung et al., 2006), spine formation (Lai et al., 2012), synaptic plasticity, cognitive function and memory formation (Guan et al., 2011; Hawasli et al., 2007; Ohshima et al., 2005). Cdk5 activation is dependent on its association with two neuronal activators, p35 (Cyclin Dependent Kinase 5 Regulatory Subunit 1, Cdk5r1) and p39 (Cyclin Dependent Kinase 5 Regulatory Subunit 2, Cdk5r2) (Hisanaga and Saito, 2003; Saito et al., 2003). p35 appears to be the predominant Cdk5 activator in the brain as a 90% reduction in Cdk5 kinase activity is observed in $p35^{-/-}$ adult mouse brain (Ohshima et al., 2001). Although genetic *Cdk5* mutations in human patients have not yet been reported, reductions in p35 have been reported in postmortem brain samples from patients with schizophrenia (Engmann et al., 2011; Ohshima et al., 2005; Sudhof, 2012). p35 is a short-

lived protein with a half-life of 20 to 30 minutes (Patrick et al., 1998); suggesting that regulation of p35 expression may be fundamental to the modulation of p35/Cdk5 activity (Dhavan and Tsai, 2001). Currently, little is known with respect to the transcriptional regulation of p35 in brain development and homeostasis.

Here we demonstrate a role for menin in the regulation of synaptic function. We find that menin binds to the *p35* promoter to promote *p35* transcription. As such, *Men1* deficiency leads to reduced *p35* expression which impairs synaptic function by abolishing *Cdk5* activity. Neuron-specific deletion of *Men1* in mice results in synaptic dysfunction and cognitive deficits. Further, overexpressing *p35* in a conditional *Men1* knockout mouse model reverses impairments in dendritic branching, spine density and synaptic plasticity, as well as in hippocampus-dependent memory. Therefore, menin is crucial for maintaining *p35* transcription and is required for normal synaptic activity and memory formation.

Results

Generation of neuron-specific *Men1* knockout mice

The *MEN1* gene shows a high degree of conservation between mouse and human homologues. *Men1* gene activity can be detected as early as gestational day 7, and is expressed in almost all organs in the adult mouse (Stewart et al., 1998). We verified menin brain expression in mice at postnatal stages and in adult. Menin was detected in all postnatal mouse tissues analyzed, with the exception of kidney and was expressed at high levels in cortex, hippocampus, brainstem and cerebellum (Figure 1a). In brain, menin protein was detected mainly in the nuclei of developing or adult mouse hippocampus and cortex (Figure S1a-b). Menin was also detectable in cultured neurons co-labeled with Tuj1, indicating that menin is expressed in differentiated neurons (Figure S1c). We then examined progressive menin expression during development and observed increased menin expression during postnatal stages in the hippocampus (Figure 1b). *In situ* hybridization results from Allen Brain Atlas similarly demonstrate high *Men1* mRNA expression in the cortex, hippocampus and cerebellum, and robust expression in the hippocampus (Figure 1c). These results indicate that menin may be involved in hippocampus-dependent function.

Given that menin is a major contributor to normal tissue development and maintenance (Guru et al., 1999; Stewart et al., 1998), it is not surprising that homozygous whole-body *Men1* deletion results in embryonic lethality at E12 (Bertolino et al., 2003). The physiological function of menin in brain, however, remains largely unknown. To specifically disrupt the *Men1* gene in the nervous system, we generated *Nestin-Cre*;$Men1^{F/F}$ conditional knockout mice (NcKO) by crossing *Nestin-Cre* animals (Tronche et al., 1999) with mice carrying floxed *Men1* alleles ($Men1^{F/F}$) (Scacheri et al., 2004) (Figure 1c). We found that NcKO mice were viable from birth, but were found to be inviable with lethality apparent in early postnatal stages where most NcKO animals died at 4 weeks (Figure 1d). Given that the *Nestin-Cre* transgene induced 90% recombination in brain tissues from E12 (Trumpp et al., 1999), we observed that *Nestin-Cre* mediated *Men1* deletion resulted in early postnatal lethality. Early lethality, therefore limited our ability to investigate the effects of menin on synaptic and cognitive function using this system. To abrogate menin expression specifically in neurons at later stages during development, we crossed $Men1^{F/F}$ mice (Ctrls) with

CamkIIα-Cre mice to generate *CamkIIα-Cre*;*Men1*$^{F/F}$ conditional knockout mice (CcKO) to facilitate Cre-dependent deletion after birth (Akbarian et al., 2002) (Figure 1c). CcKO animals exhibit longer longevity than NcKO mice, and lethality in CcKO mice was delayed until 4 months of age (Figure 1d). Body weight is slightly decreased at postnatal stages, where no differences were observed in CcKO and littermate controls at P60 (Figure 1e). Menin deletion in CcKO mice was confirmed by western blotting and immunostaining (Figure 1f-1g). No appreciable differences in cortical or hippocampal size and cortical thickness were observed between CcKO and Ctrl mice by Nissl staining (Figure 1h).

Impaired learning and memory in *Men1* CcKO mice

As mentioned above, menin is ubiquitously expressed and its expression increases during postnatal development in the hippocampus. Since the hippocampus is fundamental to learning and memory, we assessed potential cognitive deficits in CcKO mice using behavioral tests.

First, we performed rotarod assays to test for locomotor activity and found no differences between CcKO and Ctrl mice (Figure 2a). In Morris water maze tests, we found that CcKO mice showed impaired learning with improved escape latency compared to controls (Ctrl) during the four-day training phase (Figure 2b). No differences were observed in total distance traveled between CcKO mice and Ctrls, indicating that neuronal menin deletion had little or no effect on locomotor activity (Figure 2c). Moreover, during the probe trial test where the hidden platform was removed on day 5, CcKO mice spent significantly less time in the target quadrant (Figure 2d); CcKO mice also demonstrated fewer entries into the platform location and required a longer time period to travel from the entry point to the target zone compared to Ctrls (Figure 2e-2f). We next evaluated contextual and cued learning response in these mice using fear conditioning tests. Freezing response was found to be significantly reduced in response to contextual testing, but not in cued testing in CcKO mice (Figure 2g-2h), indicating that *Men1* deletion results in defective hippocampal-associated, but not amygdala-dependent memory associated with fear. Together, these behavioral tests demonstrate that neuronal menin regulates cognition-related behavior, and its loss severely impairs hippocampus dependent learning and memory in mice.

Impaired synaptic function and neuronal morphology in CcKO mice

Synaptic dysfunction can lead to alterations in learning and memory. Thus, we performed an electrophysiological characterization of synaptic function in the hippocampal CA1 region in Ctrl and CcKO mice. We observed a substantial reduction in high frequency stimulation (HFS)-induced long-term potentiation (LTP) in CcKO mice (Figure 3a-3b). Input-output response analysis indicates that CcKO mice showed decreased excitatory synaptic transmission compared to Ctrl mice (Figure 3c). Paired-pulse facilitation (PPF) ratios remained unchanged between CcKO with Ctrl animals (Figure 3d). Given that these synaptic defects are postsynaptic, we examined levels of the AMPA receptor GluR1, as well as synaptophysin and PSD95 in the hippocampus and in cultured primary neurons. Notably, all components were reduced in menin-deficient hippocampus or primary neurons compared to Ctrls (Figure 3e). Brain development during early postnatal stages involves dendritic spine formation and dendritic branching, both of which also contribute to LTP formation. Golgi

staining indicated that neurons from cortex and hippocampus in CcKO mice exhibited significant reductions in dendritic branching and spine density (Figure 3f-3h). Similarly, we also observed deficits in dendritic branching and synapse formation in NcKO mice at P20 (Figure S2a-d). *Men1*-deficient primary cortical NcKO neurons from E16.5 embryos were also seen to feature defects in dendritic branching and synapse formation compared to wild-type neurons (Figure S2e-h). These results indicate that impaired LTP is most likely due to a reduction in functional synapses and defects in postsynaptic function in brain-specific *Men1* deletion mice.

Loss of menin reduces p35 expression and Cdk5 activity

To explore molecular mechanisms associated with the phenotypes observed upon neuronal *Men1* deletion, we profiled transcriptomes from Neuro2a (N2a) cells transfected *Men1 siRNA* using Agilent Mouse 4 X 44K microarrays. Altogether, 1040 genes showed altered expression in 2/2 samples (>2-fold difference to controls, FDR-corrected $p < 0.05$; Figure S3a). Within a pool of 1040 genes, approximate 67% of the genes analyzed were down-regulated. Gene Ontology (GO) biological process analysis indicates that developmental pathways associated with the central nervous system were significantly represented in the dataset (FDR corrected $p<0.05$; Figure S3b, labeled in red).

Among identified genes related to neuronal development that were modulated with *Men1* downregulation, we identified a reduction in the Cdk5 activator p35 (Figure 4a). As the fundamental activator of Cdk5, p35 associates with Cdk5 to maintain physiological synaptic formation/function (Su et al., 2011); synaptic plasticity (Fischer et al.,2005; Li et al., 2001); and cognitive function/memory formation (Guan et al., 2011; Hawasli et al., 2007; Ohshima et al., 2005). Given that synaptic defects and impaired cognition in Men1 CcKO mice phenocopies defects reported in Cdk5 conditional knockout mice, p35 is a likely candidate in mediating physiological menin function in neuronal development and cognition.

Using *Men1* siRNAs, we verified reductions in both *p35* protein and mRNA levels in N2a cells (Figure 4b). In contrast, overexpression of menin resulted in increased *p35* protein and mRNA expression in N2a cells (Figure 4c). We then measured *p35* levels in CcKO mice, and found that p35 but not Cdk5 expression was significantly decreased in brains from CcKO mice and NcKO mice compared to aged-matched Ctrl mice respectively (Figure 4d-e). Further, we also observed decreased p35 expression in *Men1* deficient neurons in culture (Figure 4f). p35 expression was also decreased in *Men1 shRNA* transfected neurons, while p35 staining intensity was dramatically increased with GFP-*Men1* transfection, with little or no change in Cdk5 expression/staining (Figure S4a-c, indicated by arrows). Moreover, menin overexpression markedly rescued p35 expression in MEN1 knockout neurons (Figure 4g). As p35 is markedly decreased in CcKO mice, we also observed that Cdk5 kinase activity is similarly significantly deceased in CcKO mouse brain (Figure 4h).

Menin binds the *p35* promoter locus and promotes *p35* transcription

Menin is involved in regulatory aspects of epigenomic modulation of gene expression, such as its association with *H3K4me3* modification (Matkar et al., 2013). Peak *H3K4me3* levels were reported in the *p35* promoter in the cortex of mouse brain (Figure 5a)

(www.encodeproject.org), which indicates that menin may modulate *p35* transcription through epigenomic *H3K4me3* modification. Through use of *H3K4me3* ChIP assays using three distinct primer pairs targeting the *p35* promoter locus (Figure 5b-c), we observed robust *H3K4me3* in the *p35* promoter in WT neurons; while conversely, H3K4me3 occupancy at the p35 promoter locus dramatically decreased in *Men1* knockout neurons (Figure 5d). Further, menin-ChIP assays demonstrate menin occupancy of the *p35* promoter region, where *Men1* deletion dramatically reduced menin occupancy at the *p35* promoter locus (Figure 5e).

We next mapped the *p35* promoter region for a *Men1*-responsive regulatory element. A 1,277 bp genomic fragment was cloned from mouse genomic DNA, spanning the *p35* 5' UTR from +59 bp to −1219 bp with respect to the ATG (+1). We then constructed a *p35* promoter truncation series comprising various regions of the *p35* promoter fused to a downstream luciferase reporter (Luc), and co-transfected these constructs into N2a cells together with a menin overexpression construct or empty vector (Figure 5f-g). The minimal *p35* promoter region required to enhance downstream luciferase expression comprised the +59 to −748 region with *Men1* co-transfection in N2a (Figure 5g) and primary neurons (Figure 5h). Endogenous *Men1* downregulation using *Men1*-targeting shRNAs in primary neuron dramatically decreased transcriptional activity from the *p35* promoter region (Figure 5h). Moreover, menin truncation constructs lacking the nuclear localization sequence failed to drive *p35* promoter-mediated luciferase activity (Figure 5i). Since expression of p35 increases during neuronal maturation, we determined whether menin expression correlates with p35 levels during neuronal development. Notably, menin protein expression increased during hippocampal neuronal maturation *in vitro*, during which, p35 protein expression and *p35* promoter luciferase activity also increased (Figure 5j). Luciferase activity in p35 promoter constructs also significantly deceased in $Men1^{-/-}$ neurons compared to controls. Furthermore, protein stability assays revealed no significant difference in p35 degradation with menin overexpression (Figure S5). Taken together, these data suggest that menin binds to the *p35* promoter region to regulate *p35* transcription.

Restoring p35 levels in CcKO mice rescues cognitive impairment and synaptic deficits associated with menin deletion

To test whether p35 expression could rescue synaptic deficits and impaired learning and memory defects observed in CcKO mice, we specifically generated an adeno-associated virus type 2 (AAV2) construct where *p35* is expressed using a CamKII promoter (*AAV-p35-T2A-GFP)* (Figure 6a). We next injected *AAV-p35-T2A-GFP (AAV-p35-GFP)* or an *AAV-GFP* control bilaterally into ventricles of newborn *Men1* CcKO mice (P0) to specifically restore *p35* expression in neurons *in vivo*. Behavioral tests were subsequently performed 60 days after injection (Figure 6b). We confirmed *p35* expression in the cortex and hippocampus of injected animals by immunostaining (Figure S6a), and we observed that although p35 overexpression had no effects on neuronal survival in CcKO mice, p35 induces neuronal apoptosis in Ctrl mice (Figure S6b-c). Given that Cdk5 hyperactivity has been shown to promote neuronal death (Su and Tsai, 2011), neuronal death in Ctrl mice with p35 overexpression could be anticipated. Next, we found that *AAV-CamKII-Cre*- mediated *p35* expression rescued escape latency deficits during training, and improved time spent in the

target quadrant in Morris water maze tests (Figure 6c-d). Deficits in contextual freezing time in CcKO mice were also rescued with p35 overexpression (Figure 6e). Additionally, LTP deficits observed in CcKO mice were also restored by *AAV-p35-GFP* expression (Figure 6f-g), and synaptic spines and dendritic complexity compromised in CcKO mice were also rescued by increased *AAV-p35-GFP* expression *in vivo* (Figure 6h-j). Similarly, overexpression of p35 also increased neuron dendritic complexity and total dendritic length in primary *Men1*$^{-/-}$ neurons derived from *Men1* deficient mice *in vitro* (Figure 6k-m). These results indicate that menin-associated learning and memory deficits were caused by attenuated p35 expression, and restoration of p35 levels can normalize menin-dependent cognitive function.

Discussion

Deficits in neuronal development such as dendritic and synaptic abnormalities can lead to impairment of neuronal function, which is causal to brain dysfunction in various neurological diseases(Kazim and Iqbal, 2016). While the importance of bidirectional communication between the synapse and nucleus during neuronal development and plasticity is well established (Cohen and Greenberg, 2008), the role of nuclear signaling components in synaptic development and plasticity remain largely unknown.

Menin is a nuclear scaffold protein and interacts with a variety of transcriptional proteins to regulate various cellular processes by modulating the transcriptome. Although menin has been well documented as a tumor suppressor in endocrine organs, lung, prostate and breast (Matkar et al., 2013), the physiological role of menin in the central nervous system remains largely unknown. We found that menin is highly expressed in the central nervous system, especially in the hippocampus; ablation of menin in postmitotic neurons led to a reduced number of dendritic spines. In contrast to its function as a tumor-suppressor, our results here reveal an unexpected role for menin in mediating dendritic spine formation and neuronal plasticity.

Among the menin-regulated genes identified with potential function in neuronal development, p35 and dysregulation of the Cdk5 pathway seemed to be associated with menin-dependent neuronal development. Cdk5 and its cognate p35 activator subunit have been well documented to be vital in brain development, dendritic spine formation and higher brain functions such as synaptic plasticity, learning and memory (Dhavan and Tsai, 2001; Fischer et al., 2002; Hawasli et al., 2007; Li et al., 2001; Su and Tsai, 2011). Cdk5 is activated by regulatory subunits, of which p35 is the most abundant. As the main activator of Cdk5, p35 null mice or inducible conditional knockout mice show defective spatial learning (Ohshima et al., 2005). Physiological regulation of Cdk5 is vital during brain development, while, cleavage of p35 to p25 induced aberrant activation of Cdk5 is reported to promote neuronal death (Fischer et al., 2005; Ris et al., 2005) and pathogenesis of neurodegenerative disease, such as Alzheimer's disease (AD) (Han et al., 2005; Su and Tsai, 2011; Zoltowska et al., 2017). Thus, precise regulation of p35 expression is essential, as alterations of p35 expression may lead to developmental brain defects or neurodegenerative onset. Cdk5 activation has been comprehensively characterized; however, transcriptional regulation of *p35* during brain development or neurodegeneration remains largely unknown. Here, we

identify menin as an important regulator for p35 expression, resulting in sustained Cdk5 kinase activity (Figure 4h). In addition to its role in neuronal development, dysregulation of menin may be also involved in neurodegenerative pathogenesis.

We also note that genes associated with synaptic function such as Synaptotagmin 1 (Syt1), Synaptotagmin 6 (Syt6) and neurexin 1(Nrxn1) were significantly up-regulated with Men1 depletion. Synaptotagmins play key roles in regulating neurotansmitter release through Ca2+-triggered synaptic vesicle exocytosis (Sudhof, 2012). As synaptic adhesion molecules, presynaptic neurexins and postsynaptic neuroligins form complexes to maintain physiological synaptic structure and function (Sudhof, 2008). Elevations in Syt1/6 and Nrxn1 levels may be derived from compensatory effects seen with Men1 deletion; although this possibility is of significant interest, mechanisms underlying this phenomenon warrant further investigation.

Epigenomic methylation of histone H3-lysine 4(H3K4) and the trimethylated form (H3K4me3) enriched at gene-proximal promoters and transcription start sites (Barrera et al., 2008; Guenther et al., 2007) alters histone tail modifications and consequent target gene transcription (Huang et al., 2012). As a component of the histone methyltransferase complex, menin recruits H3K4-specific methyltransferase MLL1 to specified gene promoter loci. H3K4me3 modification is highly regulated throughout development in the mammalian cerebral cortex (Cheung et al., 2010). The alterations in H3K4me3 and reductions in p35 expression have been observed in cortical neurons from subjects diagnosed with schizophrenia and neurodegenerative disease (Engmann et al., 2011; Huang et al., 2007; Ohshima et al., 2005). Psychological stress and histone modifications are also closely linked to the pathogenesis of neurodegenerative diseases such as AD (Chai et al., 2017; Fischer et al., 2010; Futch et al., 2017). Our data indicate that menin occupancy coincides with transcriptional activation of *H3* modification at *p35* promoter loci, resulting in enhanced *p35* expression (Figure 5). Interestingly, neuron-specific *Mll1* knockout mice exhibit synaptic plasticity and working memory deficits (Jakovcevski et al., 2015). Additionally, genetic ablation of *Mll1* from a subset of neural stem cells (NSCs) (GFAP-Cre;$Mll1^{F/F}$) exhibited growth retardation and early postnatal lethality at P30 (Lim et al., 2009), similar to our observations in *Men1*-NcKO mice. Combined with the data presented here, the menin-MLL1 complex may be involved in higher brain function such as learning and memory. Detailed mechanisms underlying the regulatory effects of menin on *p35* transcription warrants further investigation.

In summary, we show that menin—a gene closely related to MEN1 syndrome and organ tumors—is highly expressed in hippocampus and other brain regions and plays a key role in neuronal development and cognition. Menin promotes *p35* expression by directly binding to the p35 promoter, thereby facilitating *p35* expression and maintaining Cdk5 activation during brain development. Our study provides further insight into the transcriptional regulation of the Cdk5/p35 complex, which has a fundamental role in the developing nervous system and the pathogenesis of neurological disorders.

Experimental Procedures

Animals and generation of *Men1* conditional knockout mice

All mice were reared on a 12/12 light/dark cycle. All experimental procedures involved were performed according to protocols approved by the Institutional Animal Care and Use Committee at Xiamen University. The *Men1* floxed mouse strain was obtained from Prof. Guanghui Jin and Prof. Xianxin Hua (Libutti et al., 2003). *CamKIIα-Cre* mice (Tsien et al., 1996) and *Nestin-Cre* mice (Tronche et al., 1999) were provided by Dr. Zengqiang Yuan at the Institute of Biophysics of the Chinese Academy of Science. NcKO mice were obtained by crossing *Men1* floxed mouse lines with *Nestin-Cre* mice; CcKO mice were obtained by crossing the *Men1* floxed mice with *CamKIIα-Cre* lines. 2 month old litter/age-matched male mice were exclusively used for behavioral tests, electrophysiology or Golgi to exclude interfering effects derived from estrogen.

ChIP (Chromatin Immunoprecipitation)

ChIP procedures were performed followed the manufacturer's instructions (Millipore, #17–295). Briefly, primary neurons from Ctrl and NcKO mice (dissected at E16.5) were cultured for 8–12 days and fixed for 10 min at room temperature with media containing 1% formaldehyde, and quenched with 125 mM glycine for 5 min. Fixed homogenates were washed twice using ice-cold PBS containing protease inhibitors. Fixed nuclei were pelleted at 4 minutes at 2000 rpm and re-suspended in SDS Lysis Buffer (Cat#20–163), where chromatin was sheared using a SCIENTZ ultrasonic apparatus set to 28% power for 14 cycles of a 4.5 s sonication and a 9.0 s resting stage on ice. The sonicated cell supernatant was diluted 10-fold in ChIP dilution buffer (Cat#20–153), and pre-cleared using protein A Agarose/Salomon Sperm DNA (Cat#16–157). After brief centrifugation, ChIP was performed using 3 μg menin antibody (Bethyl, Cat:#A300–105A), H3K4me3 (Millipore, cat #17–614), or normal rabbit IgG (Santa Cruz, Cat #H2615) antibody incubated overnight, followed by enrichment using protein A sepharose beads for 4 h. Beads were washed 3 times with 4 different buffers respectively (Low salt immune complex wash buffer, High salt immune complex wash buffer; LiCl immune complex wash buffer), and one wash with TE (50 mM Tris HCl, 10 mM EDTA). Chromatin was eluted by agitation at 65 °C for 20 min in TES (TE plus 1% SDS), and reverse crosslinked overnight at 65 °C. Chromatin was subjected to RNase and proteinase K treatment, followed by DNA purification by phenol chloroform extraction and ethanol precipitation. DNA pellets were resuspended in 10 mM Tris and subjected to qPCR (Roche 480).

Stereotactic injection of adeno-associated virus

AAV2-*CamkII*-p35-T2A-GFP-WPRE (virus titer: 4.75×10^{12} per ml) and AAV2-CamkII-T2A-GFP-WPRE (virus titer: 4.91×10^{12} per ml) were purchased from BrainVTA (Wuhan, China). Packaged viruses were stereotactically injected into the bilateral ventricles of Ctrl mice or CcKO mice respectively as described previously (Wang et al., 2013; Zhao et al., 2015). The injection site was located two-fifths of the distance along a line defined between each eye and the lambda intersection of the skull. To confirm region-specific over-expression of p35 in mouse brains, mice were anesthetized and sacrificed two months after injection,

whereupon brain tissues were rapidly removed and analyzed by histological immunofluorescence staining.

Detailed experimental procedures can be found in “Supplemental information”

Statistical analysis

All data represent mean ± SEM. Statistical analyses were performed using Graphpad Prism 6, Clampfit or SigmaStat 4 statistical software. For statistical significance, experiments with two groups were analyzed using two-tailed Student’s tests and one-way ANOVA. Experiments with more than two groups were subjected to one-way ANOVA and two-way ANOVA. *P<0.05, **P<0.01, ***P<0.001. Detailed statistical analysis methods for each comparison are indicated in the figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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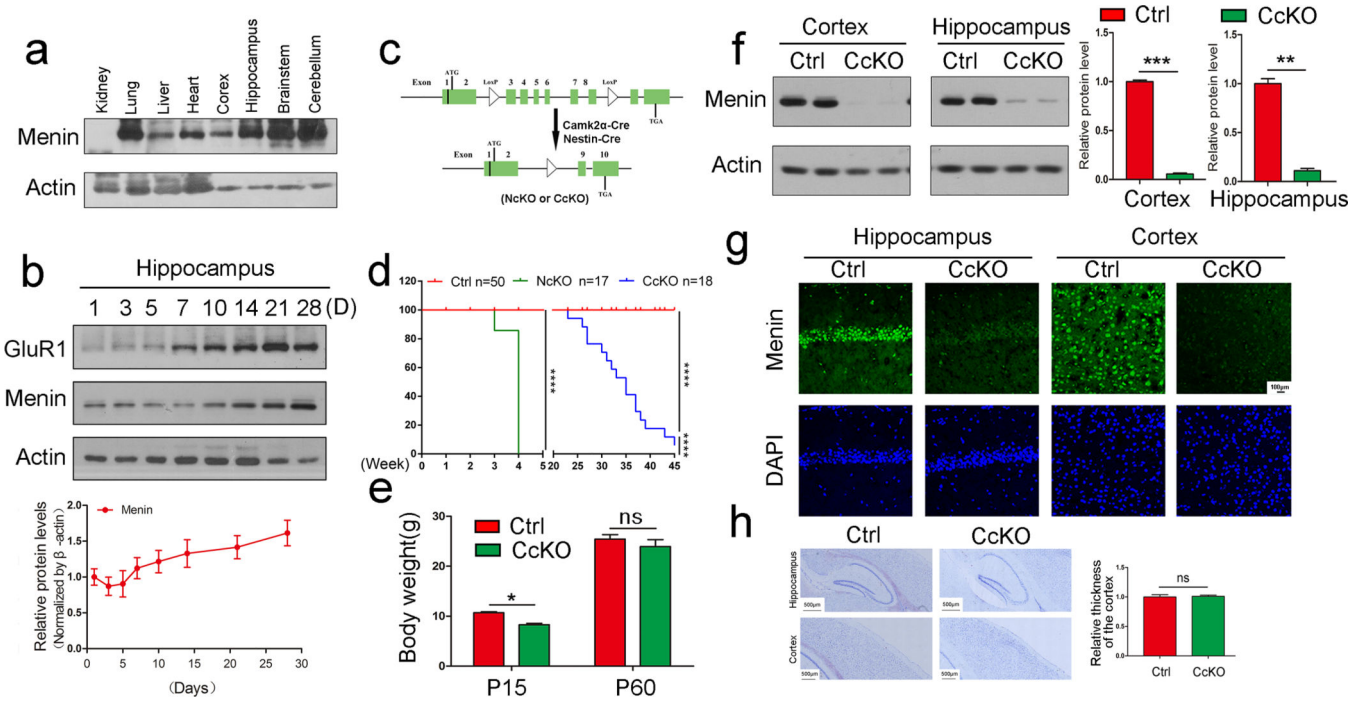

Figure 1. Generation of *Men1* neuron-specific knockout mice

(a) Menin levels were detected by western blot in organs from adult mice as indicated.

(b) Menin, GluR1 and actin (as a loading control) expression levels in hippocampal lysates from C57BL/6 mice at different postnatal days analyzed by western blot.

(c) Generation of conditional *Men1* knockout mice by crossing $Men1^{F/F}$ mice with *CamkIIα*-Cre mice or *Nestin*-Cre mice.

(d) Survival curve of Ctrl, CcKO and NcKO mice. Ctrl mice: n=50, CcKO mice: n=18; NcKO mice=17; ***$P < 0.001$, survival is analyzed for significance by Log-rank (Mantel-Cox) test.

(e) Body weights of Ctrl and CcKO mice at P15 and P60. ns: not significant *$P < 0.05$, one-way ANOVA with Tukey post hoc test.

(f) Menin protein expression is significantly decreased in hippocampus and cortex of CcKO mice compared to Ctrl mice.

(g) Representative cortical and hippocampal brain sections from Ctrl and CcKO mice stained with menin (green) antibody. Section was counterstained with DAPI (blue). Scale bar, 100μm.

(h) Hippocampal size and cortical thickness show little difference in CcKO mice compared with Ctrls. scale bar, 500μm. Data represent the mean ± SEM. n = 3, ns: not significant, one-way ANOVA with Tukey post hoc test.

See also Figure S1.

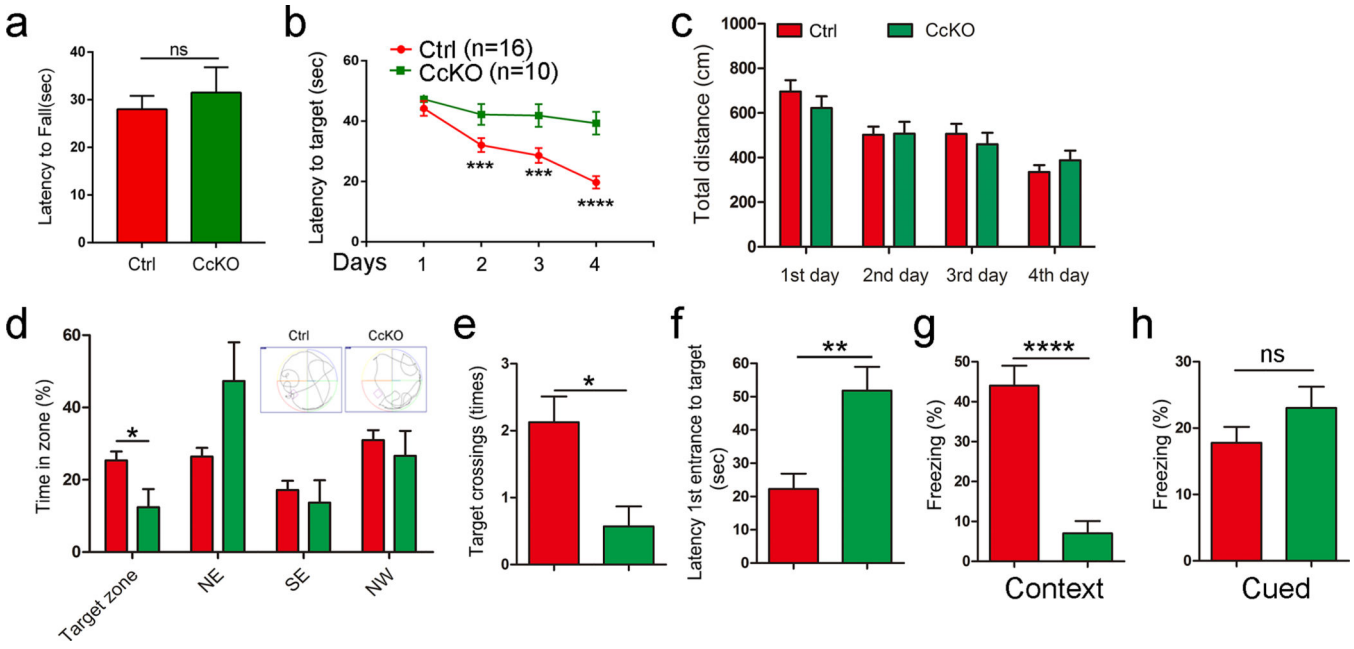

Figure 2. Cognitive deficits in CcKO knockout mice

Male litter and age-matched control and CcKO mice were used for behavior tests.

(a) CcKO and control mice behavior in Rotarod tests. CcKO: n=20; Ctrl: n=23

(b-f) During Morris water maze tests, Ctrl mice (n=16) and CcKO mice (n=10) were analyzed for escape latency during a 4-day training period (b). Total distance traveled was also recorded (c). On the next day, mice were analyzed for time spent in the Target zone and other quadrants (NE, SE and NW) (d), number of target crossings (e) and time required from entrance to the target platform (f).

(g-h) During fear conditioning tests, mice were trained and analyzed for freeze response under contextual (g), and cued stimuli (h), respectively. Freezing response is shown as a percentage of the total time duration.

Data represent mean ± SEM. ns: not significant, *$P<0.05$, **$P<0.01$, ****$P< 0.0001$, one-way ANOVA with Tukey post hoc test.

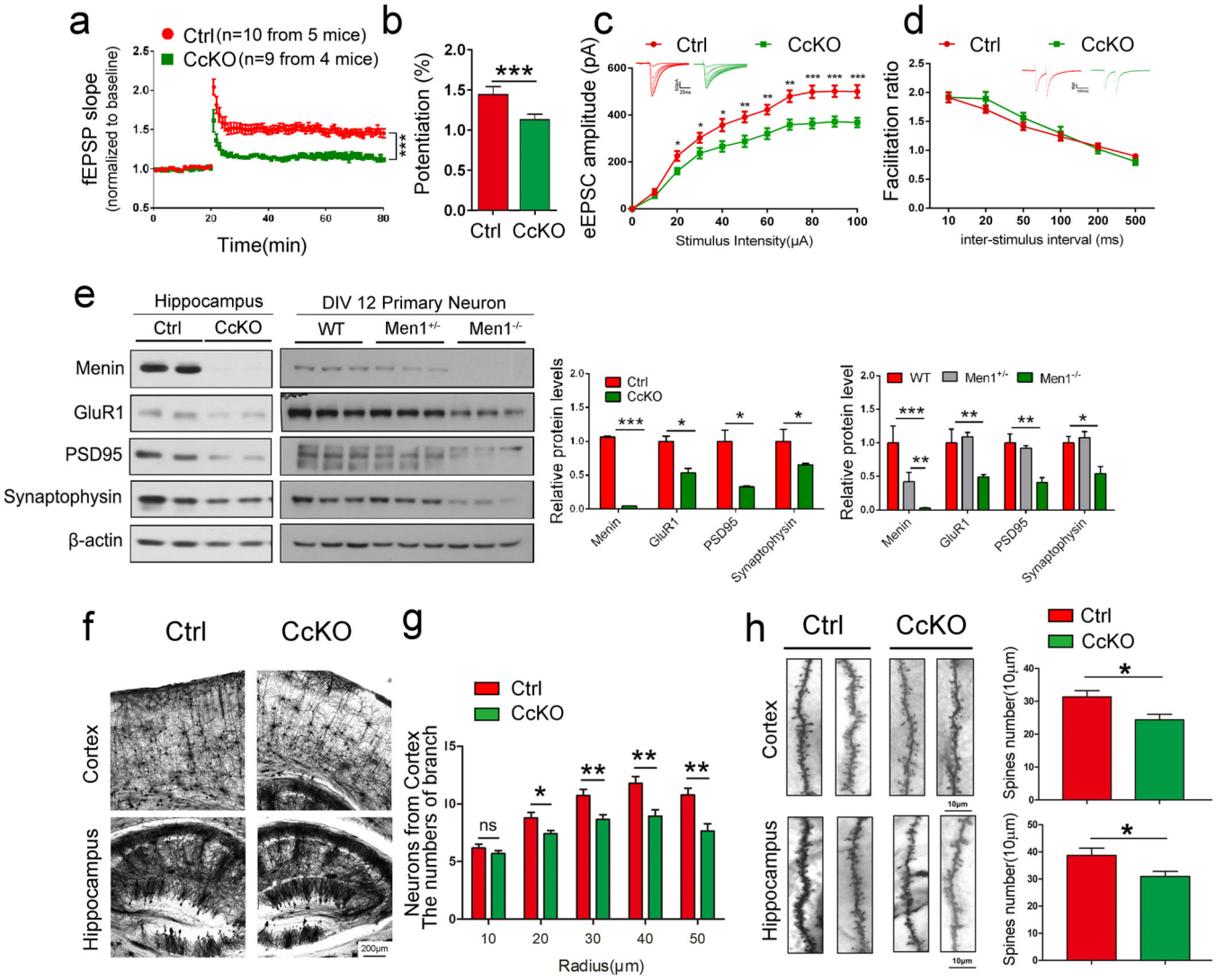

Figure 3. Impaired synaptic functions in CcKO mice

(a-b) LTP recordings from male litter and age-matched control and CcKO mouse brain. fEPSP potentiation was quantified during the last 10 min of recording, and significance was determined by repeated-measures ANOVA with Bonferroni's post hoc analysis.

(c) CcKO show decreased basal synaptic transmission as reflected in input–output curve from whole-cell recordings, Ctrl: n=17 from 6 mice; CcKO: n=20 from 7 mice.

(d) Paired-pulse facilitation ratios were obtained from whole-cell recordings in CA1 pyramidal neurons. Ctrl: n=19 from 6 mice; CcKO: n=21 from 7 mice. Data represent the mean ± SEM. and analyzed by one way ANOVA followed with Dunnett's test.

(e) GluR1, Synpatophysin, and PSD95 are significantly decreased in CcKO hippocampus or *Men1* knockout neurons. Data represent the mean ± SEM. n = 3 mice per group. $^{**}P < 0.01$, $^{***}P < 0.001$, one-way ANOVA with Tukey post hoc test.

(f) Two month-old Ctrl and CcKO mice were subjected to Golgi staining. Representative Golgi staining from cortex and hippocampal CA1 regions are shown. Scale bar, 200μm.

(g) Men1-deficient neurons from CcKO mice display significantly reduced dendritic arborization.

(h) Representative projection images of dendritic spines from cortical and hippocampal neurons from Ctrl and CcKO mice are shown in the left panel. A summary plot of dendritic spine density is shown in the adjacent graphs (right), scale bar, 10μm. Data represent the mean ± SEM. $n = 20$ neurons from 3 mice per group. $*P < 0.05$, $**P < 0.01$, one-way ANOVA with Tukey post hoc test and two-way ANOVA.

See also Figure S2.

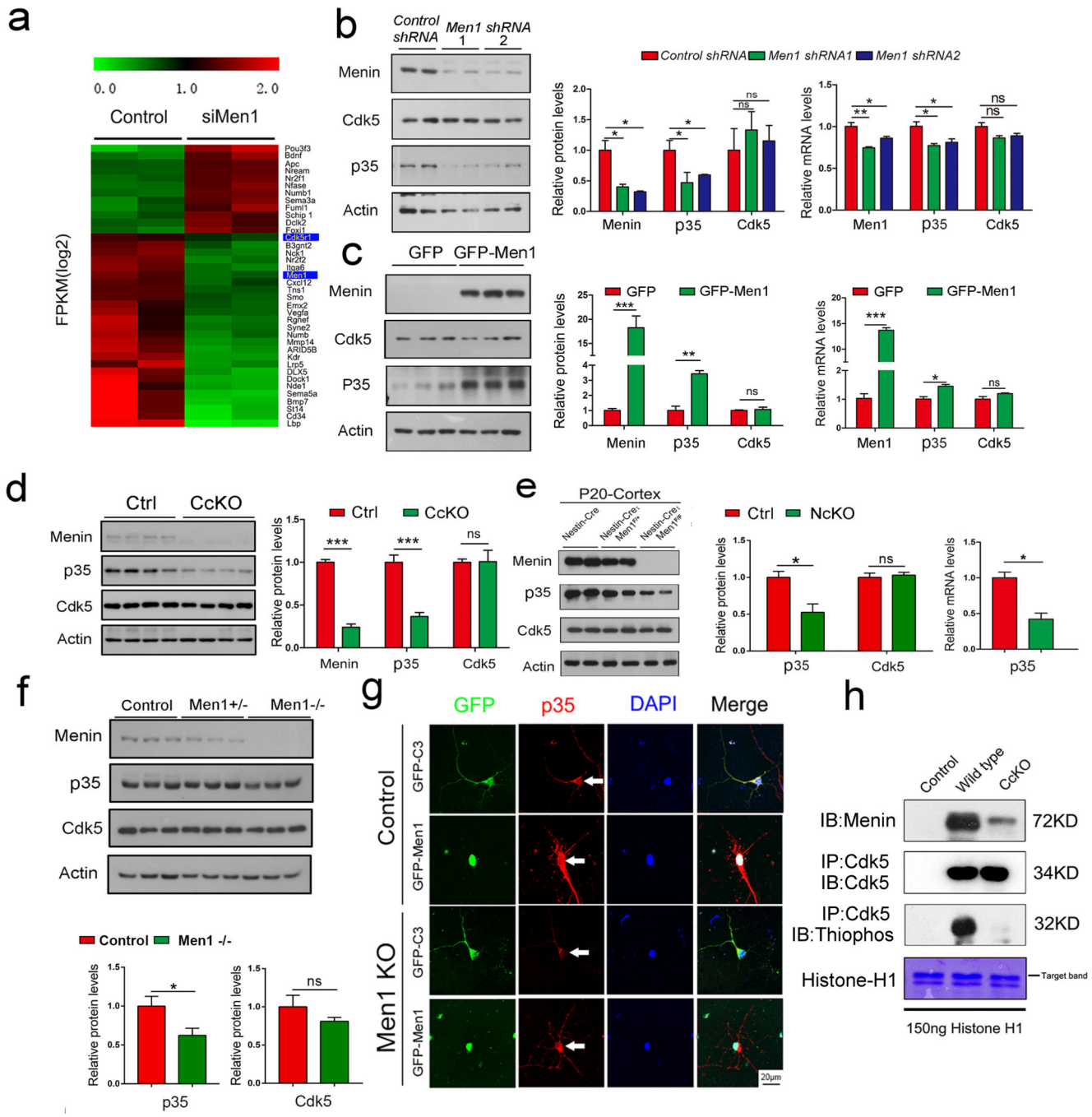

Figure 4. Loss of menin suppresses *p35* expression and Cdk5 activity

(a) Microarray-based transcriptome analysis of N2a cells treated with *Men1*-siRNA or control siRNA. Heat map of genes associated with development of the nervous system derived from microarray screening is shown. The Cdk5 activator *p35*, in addition to *Men1* was found to demonstrate attenuated expression with *Men1* knockdown (blue rectangle). (b) p35/Cdk5 protein and mRNA levels in N2a cells transfected with two *Men1* shRNAs and control shRNAs.

(c) p35/Cdk5 protein and mRNA levels were measured in N2a cells transfected with GFP-Menin and control GFP.

(d-e) p35/Cdk5 protein levels and mRNA levels were detected in CcKO and NcKO mouse brains.

Data represent the mean ± SEM. n = 3, ns: not significant, $*P < 0.05$, $**P < 0.01$, $***P<0.001$, one-way ANOVA with Tukey post hoc test.

(f) p35 protein levels are reduced in DIV12 cultured cortical neurons from embryonic 16.5 *Men1*$^{F/F}$ (*Men1*$^{+/+}$), *CamkIIα-Cre; Men1*$^{F/+}$ (*Men1*$^{+/-}$), and *CamkIIα-Cre; Men1*$^{F/F}$ (*Men1*$^{-/-}$) mice.

(g) *Men1* KO or WT primary cortical neurons were cultured from CcKO or WT mice brain. After the indicated plasmid transfection, p35 was stained on DIV 12. Scale bar, 20μm.

(h) *In vitro* Cdk5 kinase activity assay. Menin expression was measured in cortical lysate of Ctrl and CcKO mice brain. The brain lysates were then co-immunoprecipitated by Cdk5 antibody and followed anti-Thiophosphate Ester antibody assay with Histone-H1 as substrate. The levels of Histone-H1 were visualized by Coomassie blue staining.

See also Figure S3 and S4.

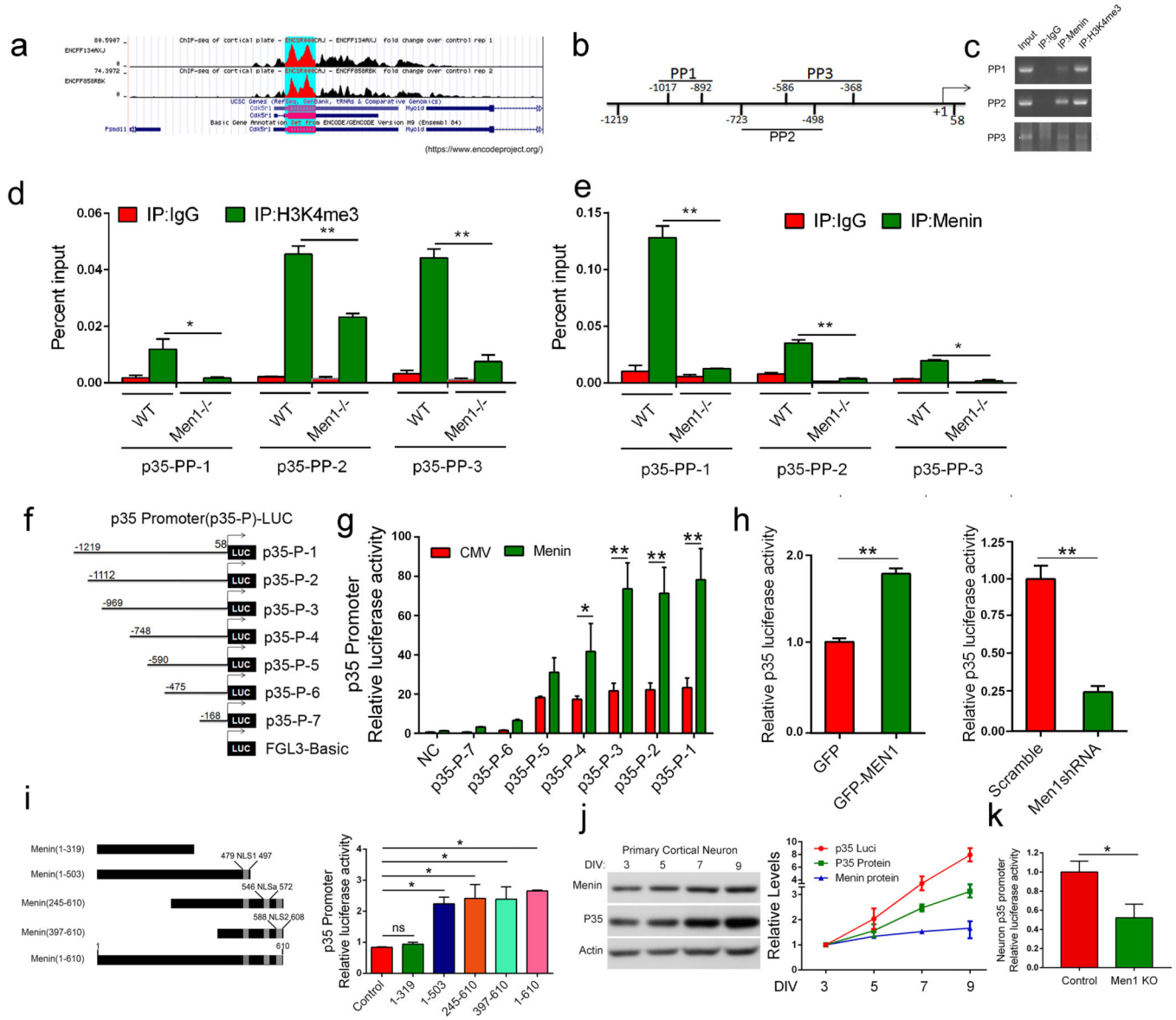

Figure 5. Menin binds to the *p35* promoter region and to activate transcription

(a) *H3K4me3*-binding distribution at the *p35* locus derived from ChIP-Seq data from the cortical plate. Red regions represent regions that show enrichment for *H3K4me3*-binding.

(b) Schematic representation of mouse *p35* and primer pairs (PP) used for ChIP assays;

(c) ChIP-qPCR bands for pp1, pp2 and pp3.

(d-e) ChIP assays using antibodies against H3K4me3 (d) or menin (e) were performed in cultured WT or *Men1* knockout neurons on DIV12. Data represent mean ± SEM. n = 5, $*P < 0.05$, $**P < 0.01$, one-way ANOVA with Tukey post hoc test.

(f-g) *p35* promoter region and *p35* promoter truncations were cloned and fused to a luciferase reporter (Luc). (g) Menin overexpression enhances transcription from the 5′ flanking region of the mouse *p35* gene.

(h) Menin overexpression increases the *p35* promoter-dependent luciferase activity, whereas *Men1* knockdown by shRNA decreases the *p35* promoter-dependent luciferase activity in primary cortical neurons.

(i) Menin truncations were constructed as shown in left panel; the effects of *p35* promoter truncation on luciferase activity were determined (right panel). Data represent mean ± SEM. n = 6, $*P < 0.05$, as determined by one-way ANOVA with Tukey post hoc test.

(j) Luciferase activity from *p35* promoter activation correlated with menin and p35 levels were determined in primary cortical neurons at DIV 3, 5, 7, 9 as indicated.

(k) Luciferase activity from p35 promoter constructs are significantly deceased in $MEN1^{-/-}$ neurons (Men1 KO) compared to Controls.

See also Figure S5.

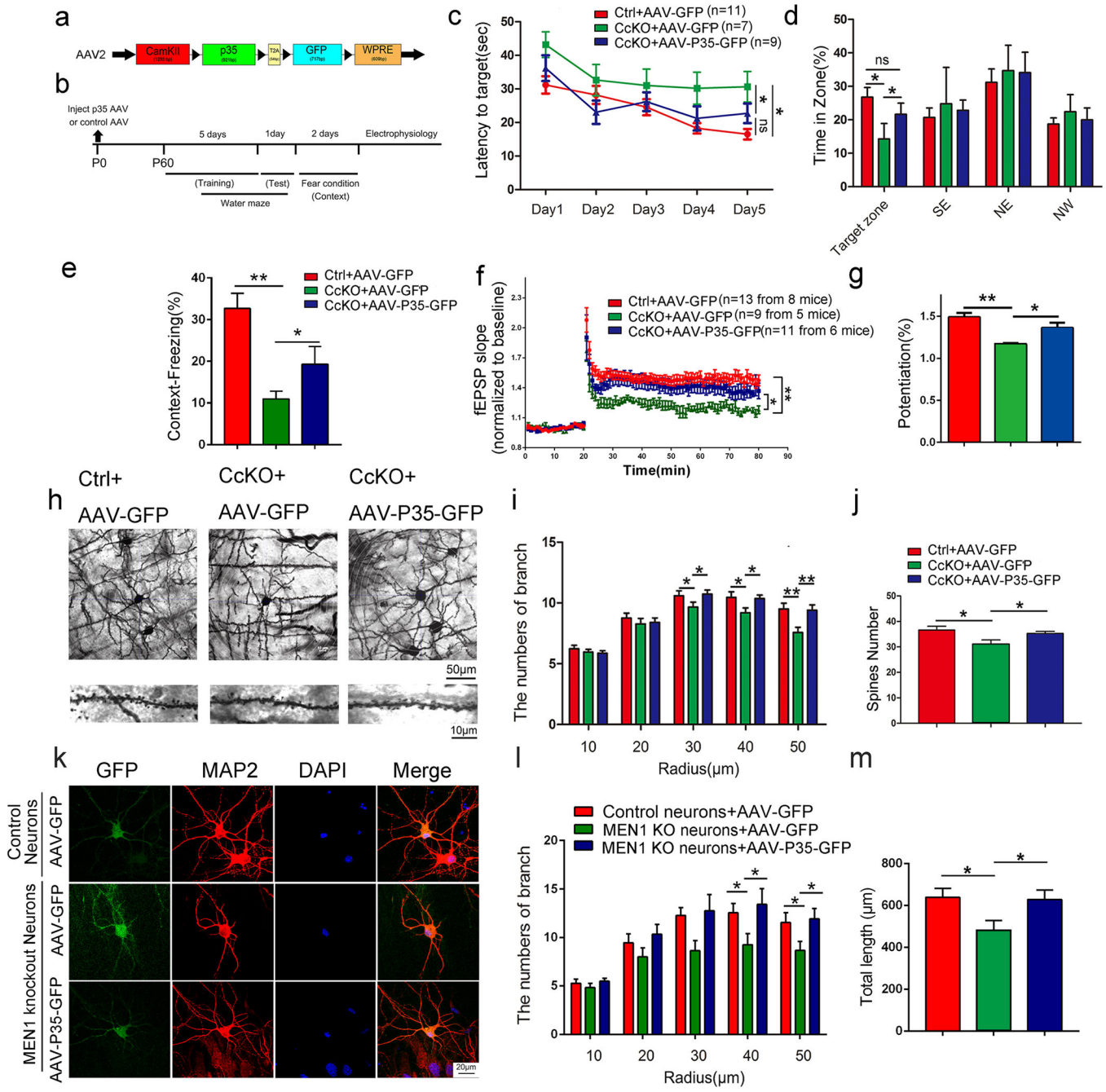

Figure 6. Exogenous *p35* expression rescues cognitive impairment and synaptic deficits in CcKO mice

(a) Schematic illustration of *CaMKII*-driven *p35* with *T2A-GFP* AAV constructs.

(b) Workflow of stereotactic AAV injection/rescue experiments.

(c-e) The effects of *AAV-p35-GFP* injection on cognitive deficits in 3-month-old CcKO mice in Morris water maze tests (c and d) and context fear condition (e). Data represent mean ± SEM. *$P < 0.05$, **$P < 0.01$, determined by one-way ANOVA with Tukey's post hoc analysis.

(f-g) The effects of AAV virus injection above on CA1 LTP in Ctrl and CcKO mice, Data represent mean ± SEM. *$P < 0.05$, **$P < 0.01$, as determined by repeated-measures ANOVA with Bonferroni's post hoc analysis during the last 10 min of recording.
(h-j) Cortical region of Ctrl and CcKO mice injected with AAV particles above were subjected to Golgi staining, and representative images of dendritic spines derived from cortical neurons are shown. Scale bar, 50μm or 10μm respectively (h). (i) and (j) represent summary plots of dendritic arborization and spine density *(i; $F2,200=9.992$, $P<0.0001$,* two-way ANOVA*).* n = 20 neurons from 3 mice per group. *$P < 0.05$, **$P < 0.01$, as determined by one-way ANOVA with Turkey post hoc analysis and two-way AVONA analysis.
(k-m) Cortical neurons from Ctrl and Men1-deficient mice were cultured *in vitro*, and transduced with AAV-GFP or AAV-p35-GFP on DIV7. Confocal projection images of Map2 stained neurons (red) on DIV 12 are shown. Summary of dendritic arborization and total length are presented in panel l and m. Scale bar: 20μm, 20 neurons in each condition. Values represent the mean±SEM. *$P < 0.05$, one-way ANOVA with Turkey post hoc test.
See also Figure S6.