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Opposing Roles of Transient and Prolonged Expression of p25 in Synaptic Plasticity and Hippocampus-Dependent Memory

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Summary

While deregulation of cyclin-dependent kinase 5 (Cdk5) has been implicated in neurodegenerative diseases, its precise role in synaptic plasticity and memory remains elusive. Proteolytic cleavage of p35, a regulatory subunit of Cdk5, by calpain results in the generation of the truncated p25 protein, which causes hyperactivation of Cdk5. Using region-specific and inducible transgenic mice, we show that transiently increased p25 expression in the hippocampus enhanced long-term potentiation (LTP) and facilitated hippocampus-dependent memory. Moreover, p25 expression increased the number of dendritic spines and synapses. Importantly, enhanced memory achieved by a transient expression of p25 followed by its repression did not cause neurodegeneration. In contrast, prolonged p25 production caused severe cognitive deficits, which were accompanied by synaptic and neuronal loss and impaired LTP. Our data suggest a role for p25 in synaptic plasticity, synaptogenesis, learning, and memory and provide a model whereby deregulation of a plasticity factor can contribute to neurodegeneration.

Introduction

Cdk5 is a protein serine/threonine kinase that is critical for the development of the central nervous system (Ohshima et al., 1996). Two related neuron-specific proteins, p35 and p39, are necessary and sufficient to activate Cdk5 upon direct binding (Dhavan and Tsai, 2001). In addition to its function during development, there is emerging evidence for a role of Cdk5 in the adult brain. It has been shown that Cdk5 activity within the nucleus accumbens counteracts the effects of cocaine in mice (Bibb et al., 2001). Cdk5 activity has also been linked to dopamine signaling within the striatum (Bibb et al., 1999). In addition, pharmacological inhibition of septo-hippocampal Cdk5 activity impairs associative learning in mice (Fischer et al., 2002) and affects LTP (Li et al., 2001). Furthermore, a number of synaptic substrates of Cdk5 have been identified (for review see Fischer et al., 2003). Several studies implicated Cdk5 with synaptic-vesicle endocytosis (Tan et al., 2003; Tomizawa et al., 2003; Lee et al., 2004), while phosphorylation of PSD-95 by Cdk5 is implicated in NMDA receptor clustering (Morabito et al., 2004). Taken together, these results reveal a potential role of Cdk5 in synaptic plasticity and learning and memory in the adult.

Cdk5 activity is increased in postmortem brain samples of AD patients (Lee et al., 1999), probably due to the conversion of the Cdk5 activator p35 to the truncated p25 protein. Consistently elevated p25 levels are observed in postmortem brain samples of human AD patients (Patrick et al., 1999; Swatton et al., 2004; Tseng et al., 2002). Under neurotoxic conditions, p25 is generated through the cleavage of p35 by the cysteine protease calpain (Kusakawa et al., 2000; Lee et al., 2000; Nath et al., 2000). p25 causes cell death in cultured neurons (Nath et al., 2000; Patrick et al., 1999), and inducible p25 transgenic (CK-p25) mice that express p25 in the postnatal forebrain exhibit profound neurodegeneration, astrogliosis, and tau-associated pathology (Cruz et al., 2003).

Given that neurodegeneration is often preceded by cognitive deficits, we employed CK-p25 mice to analyze the effects of aberrant Cdk5 activity on synaptic plasticity and learning and memory. We found that prolonged p25 expression dramatically impaired hippocampal LTP and memory with accompanying synaptic and neuronal loss. Surprisingly, we show that transient p25 expression profoundly enhanced hippocampal LTP and facilitated learning and memory. In addition, transient p25 production increased dendritic spine density and the number of synapses. Notably, facilitated learning induced by transient p25 expression did not cause neurodegeneration. Thus, a prolonged expression of p25 may turn a physiological action of Cdk5 into a pathological one.

Results

Transient p25 Expression Facilitates while Prolonged p25 Expression Impairs Associative Learning

We utilized bitransgenic mice in which inducible GFP-tagged human *p25* expression is mediated by the CamKII promoter (CK) regulated tet-off system. In bitransgenic Ck-p25 mice, p25 expression is repressed in the presence of doxycycline. We reported that these mice exhibit neurodegeneration and tau-associated pathology upon p25 induction (Cruz et al., 2003). However, the initial clinical manifestations observed in neurodegenerative diseases such as AD are often cognitive impairments. Similarly, several mouse models for neurodegenerative diseases display cognitive deficits in learning paradigms such as the Morris water maze test (Ashe, 2001) and fear conditioning (Dineley et al., 2002). These results prompted us to test learning and memory in mice that overexpress p25 for different lengths of time.

Robust p25 expression and elevated hippocampal Cdk5 activity was observed 1–2 weeks after induction

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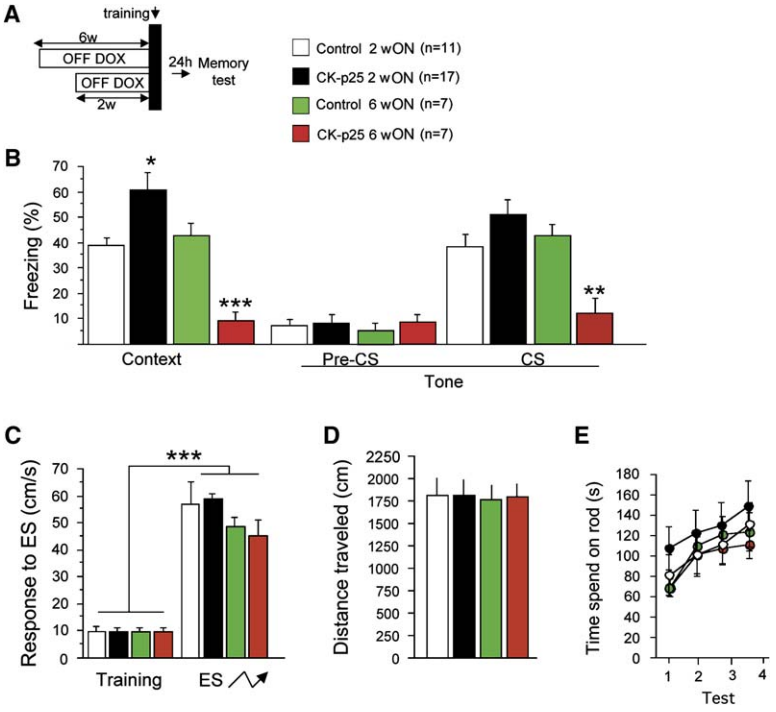

Figure 1. p25 Initially Enhances but Finally Impairs Fear Conditioning without Affecting Sensory-Motor Functions

(A) Experimental design. (B) Freezing behavior of 2-week (2wON) and 6-week (6wON) induced CK-p25 and control mice (6 w: n = 7/group; 2 w: CK-p25, n = 17; control, n = 11) during the context- and tone-dependent memory test (6 w: context; $t_{1,12}$ = 7.434; tone; $t_{1,12}$ = 4.316 vs. control). (C) Total activity during the training and the increased activity reflecting the escape response to the electric foot shock applied during the training procedure. (D) Distance traveled during the initial 3 min exposure to the training box. (E) After completing fear conditioning, the same mice were tested for motor coordination in the rotarod test. **p < 0.001 versus control group; ***p < 0.0001 versus control group; pre-CS, exposure to a novel context; CS, exposure to the conditioned stimulus (tone) in a novel context; ES, electric foot shock; w, weeks of p25 induction. Error bars indicate SEM.

(see Figure S1A in the Supplemental Data available online). Increased immunoreactivity for glial acid fibrillary protein (GFAP), indicating astrogliosis that often accompanies neuronal loss, was first observed 4 weeks after p25 induction within the hippocampus and cortex (Figure S1B). Significant brain atrophy and loss of hippocampal neurons was, however, first observed 6 weeks after p25 induction (Figures S1C and S1D). Because significant neurodegeneration was observed in mice with 6 weeks of p25 induction but not in those with 2 weeks of induction, we decided to analyze the learning behavior of the CK-p25 mice after induction for 2 and 6 weeks, respectively.

Bitransgenic CK-p25 mice were kept on doxycycline food continuously until 6 weeks after birth. In the first set of experiments, doxycycline was withdrawn for an additional 6 weeks before the mice (n = 7/group) were subjected to context- and tone-dependent fear conditioning (Figure 1A). When tested 24 hr later, the CK-p25 mice showed dramatically reduced context (p < 0.0001, $t_{1,12}$ = 7.434) and tone-dependent (p = 0.0010, $t_{1,12}$ = 4.316) freezing behavior when compared to control mice (Figure 1B). This reduction in freezing was not due to impaired pain sensation, as the response to the electric foot shock during the training was similar in CK-p25 and control mice (Figure 1C). Moreover, the explorative behavior during exposure to the novel context was not different among groups (Figure 1D). Notably, CK-p25 mice showed no deficits in motor coordination, as the performance in the rotarod test was not significantly different from that of control mice (Figure 1E). Thus, 6 weeks of p25 expression severely impairs the ability of mice to form new memories.

In the second set of experiments, p25 expression was induced for only 2 weeks before the mice were subjected to context- and tone-dependent fear conditioning (Figure 1A). Surprisingly, the CK-p25 mice (n = 17) showed significantly enhanced context-dependent freezing when compared to the control group (n = 11; p = 0.0036, $t_{1,26}$ = −3.206). Tone-dependent freezing behavior was not significantly different between the two groups (Figure 1B). As pain sensation (Figure 1C), explorative behavior (Figure 1D), and motor coordination (Figure 1E) were not different among groups, these data suggest that p25 production for 2 weeks significantly facilitates associative learning and memory. It is noteworthy that the enhanced context-dependent memory was more obvious in the female (p = 0.002 versus control) than the male CK-p25 mice (p = 0.048 versus control; data not shown). A small but significant deficit in associative memory was observed when animals were subjected to fear conditioning 4 weeks after doxycycline withdrawal (Figure S2). At this time point, astrogliosis was detected, but neuronal loss was not significant (Figures S1B and S1C).

Similar biphasic (increase followed by decrease) changes in fear memory were also observed in a separate line of bitransgenic CK-p25 mice expressing p25-GFP at lower levels (the CK-$p25_{LE}$ line, where LE stands for "low expression"; see Figure S3). Notably, CK-single, p25-single, wild-type (wt), and noninduced CK-p25 mice that were continuously fed with doxycycline displayed similar context- and tone-dependent acquisition of fear memories, demonstrating that the insertion of the CK, p25 transgene, or both in doxycyline-fed CK-p25 mice did not alter learning and memory (Figure S4A), suggesting that the phenotypes observed in induced CK-p25 mice were due to p25 production. Furthermore, baseline anxiety (Figure S4B) was not significantly altered 2 or 6 weeks after p25 induction.

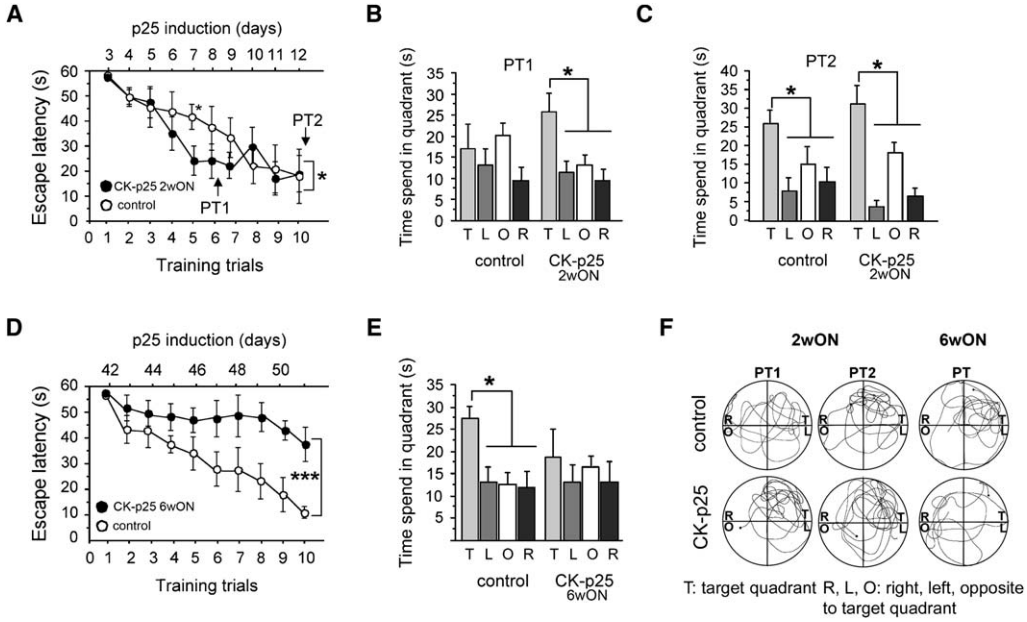

Figure 2. p25 Expression Initially Facilitates but Finally Impairs Spatial Learning

(A) Three days after p25 induction, CK-p25 (n = 7) and control mice (n = 7) were trained in the water maze paradigm for ten consecutive days (2wON group). The hidden platform was located in the target quadrant (T). Escape latencies of CK-p25 mice improved significantly faster than control mice throughout the training($F_{1,138}$ = 10.220; p = 0.015). (B) In the first probe test (PT) performed after six training trials (PT1), CK-p25 ($F_{3,24}$ = 8.691; p = 0.0249) but not control mice spent significantly more time in the target quadrant. (C) In the second probe test performed after ten trials (PT2), CK-p25 and control mice spent equal time in the target quadrant. (D) CK-p25 (n = 7) and control mice (n = 7) were subjected to water maze learning 6 weeks after p25 induction (6wON group). Escape latencies of CK-p25 mice were significantly worse than control mice ($F_{1,138}$ = 15.746; p < 0.0001). (E) In contrast to control mice ($F_{3,24}$ = 10.723; p < 0.012), CK-p25 mice showed no preference for the target quadrant during the probe test. (F) Representative images showing the swimming path of mice during the probe tests. *p < 0.05, ***p < 0.001 versus control. Error bars indicate SEM.

Transient p25 Expression Facilitates while Prolonged p25 Expression Impairs Spatial Learning and Memory

To further test the effects of transient and prolonged p25 expression, we employed the Morris water maze paradigm to examine hippocampal-dependent spatial learning. As the water maze test requires multiple trainings in a period of 10–14 days and neurodegeneration progresses rapidly in the CK-p25 mice, control (n = 7) and bitransgenic mice (n = 7) were trained 3 days after doxycycline withdrawal in order to assess the learning behavior after acute p25 induction. Notably, the escape latency of CK-p25 mice was significantly enhanced on training day 5 when compared to control mice (p = 0.0364, $t_{1,12}$ = 3,182; Figure 2A). Furthermore, when the platform was removed during the probe test performed after 6 days of training, CK-p25 mice spent significantly more time swimming in the target quadrant (p = 0.0249; $F_{3,24}$ = 8.692, target quadrant versus other quadrants; Figures 2B and 2F). At this time point, control mice did not show a significant preference for the target quadrant. In the second probe test, performed after 10 days of training, both control and CK-p25 mice spent more time swimming in the target quadrant than in the other quadrants, showing that both groups eventually learned to locate the hidden platform (Figures 2C and 2F, probe test 2). When comparing the escape latency of CK-p25 and control mice throughout the 10 days of training by repeated measurements, CK-p25 mice performed significantly better than control mice (p = 0.015, $F_{1,138}$ = 10.220). These data demonstrated that a transient phase of p25 production enhanced spatial learning and memory.

To test the spatial learning of CK-p25 mice at the onset of neurodegeneration, 6-week induced bitransgenic and control mice (n = 7/group) were trained for 10 consecutive days. Whereas the escape latency of control mice significantly improved over the 10 days of training trials, no such improvement was observed for CK-p25 mice (p < 0.0001; $F_{1,\ 138}$ = 15,746; Figure 2D). Moreover, in contrast to control mice ($F_{3,24}$ = 10.723, p < 0.05, target quadrant versus other quadrants), CK-p25 mice showed no preference for the target quadrant during the probe test performed at the conclusion of the 10-day training (Figures 2E and 2F). The escape latency was not different among groups when the platform was visible (data not shown), further supporting the idea that the CK-p25 mice are not impaired in the sensory-motor processing. Together these findings suggest that spatial learning and memory are initially enhanced by transient p25 induction but are eventually impaired when p25 levels are chronically elevated.

Transient Expression of p25 Does Not Cause Neurodegeneration but Elicits a Long-Lasting Effect on Memory

An important question is whether transient p25 expression, while initially beneficial, would eventually impair learning and lead to neurodegeneration. We induced

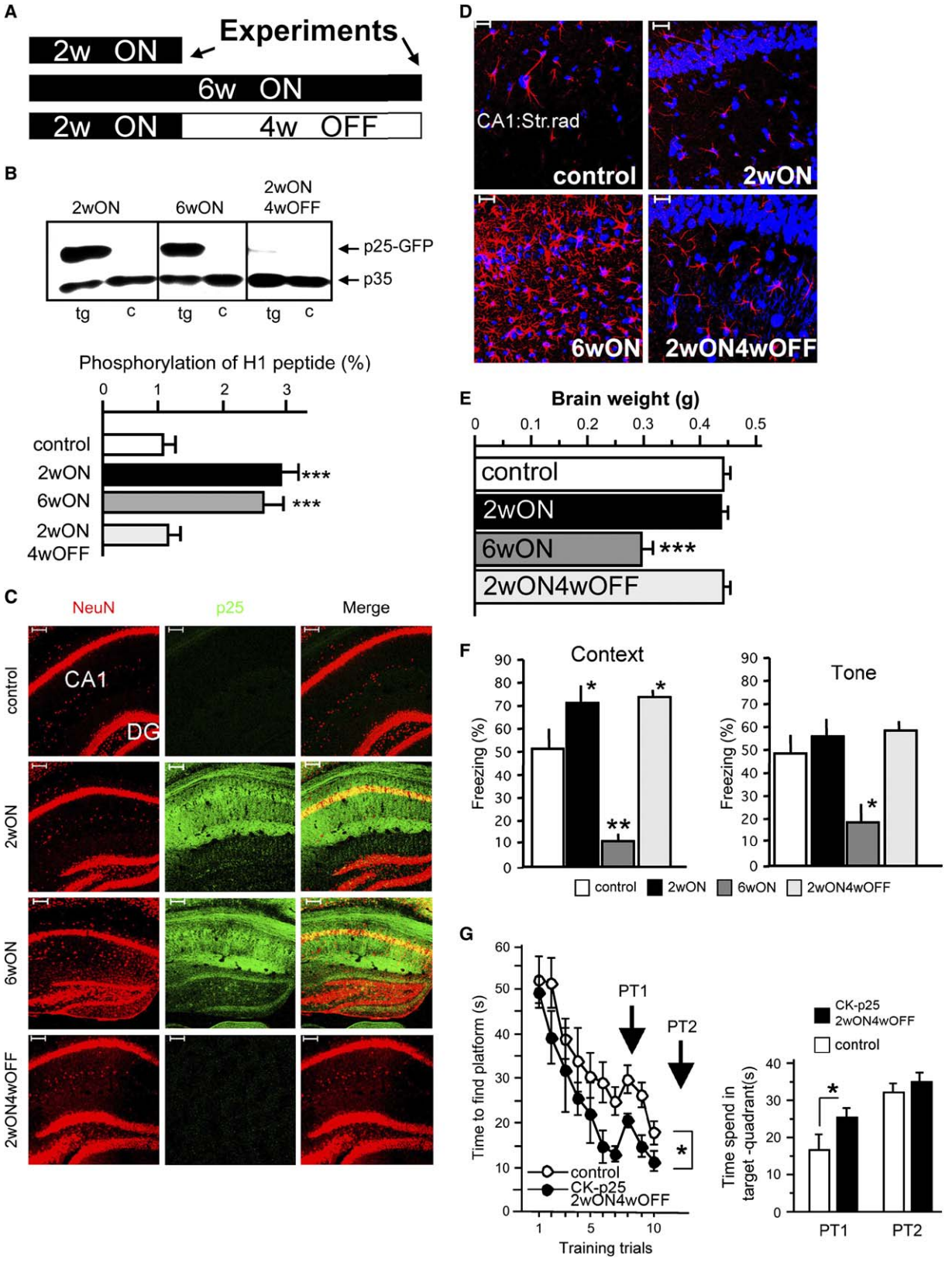

Figure 3. An Acute Phase of p25 Production Lastingly Enhances Learning without Causing Neurodegeneration

(A) Experimental design. CK-p25 mice were either induced for 2 (2wON) or 6 weeks (6wON). Another group of CK-p25 mice was induced for 2 weeks followed by 4 weeks of p25 repression (2wOn 4wOFF). Subsequently, fear conditioning, brain atrophy, neuronal loss, and astrogliosis were analyzed. ON, P25 induction; OFF, repression of p25 expression. (B) (Upper panel) The p25/p35 ratio was analyzed in hippocampal lysates of all experimental groups (n = 3/group) by immunoblotting with a C-terminal p35 antibody. (Lower panel) The same lysates were used to analyze

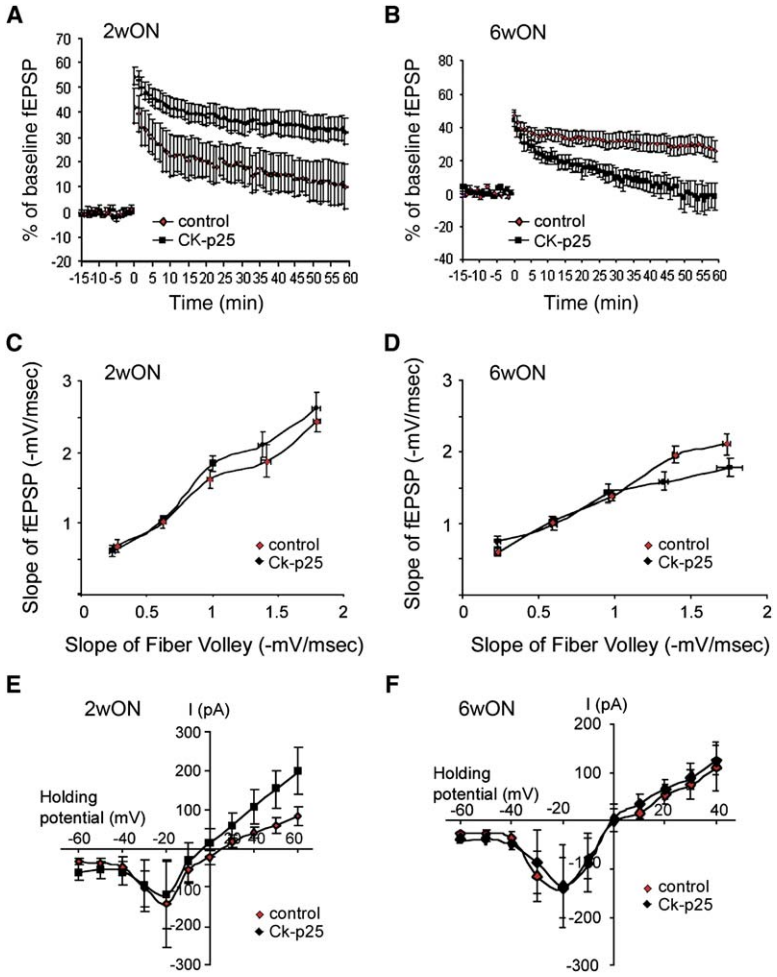

Figure 4. p25 Production Alters Hippocampal LTP

LTP was induced by a 1 s high-frequency train at time point "0" (50 Hz for [A] and 100 Hz for [B]) delivered to the CA1 synapses. (A) There was a significant increase ($p < 0.001$) in the magnitude of LTP in 2-week induced CK-p25 mice (2wON) when compared to control mice. (B) In contrast, 6-week induced CK-p25 mice (6wON) displayed markedly impaired LTP ($p < 0.001$; [B]). (C) To establish input-out relationships for synapses, the slopes of fEPSPs were plotted against the slopes of fiber volley. Basal synaptic transmission was largely normal at 2wON synapses, but slightly reduced at higher stimulation intensities at 6wON synapses (D). (E) NMDA receptor current was analyzed by whole-cell recordings of four to six cells from two to three mice per group. Larger NMDA current was detected at positive holding potential in 2wON mice. (F) No difference was observed in 6wON mice when compared to controls. Error bars indicate SEM.

p25 expression for 2 weeks and then separated mice into three different experimental groups. One group of CK-p25 mice was induced for an additional 4 weeks (6wON). The second group of mice was fed a doxycycline diet to repress p25 expression for an additional 4 weeks (2wON 4w OFF), and the last group was subjected to experimental procedures immediately after 2 weeks of p25 induction (2w ON). We subsequently analyzed p25 levels, neuronal loss, brain atrophy, and memory formation in all experimental groups (Figure 3A).

Immunoblotting revealed that p25 levels were diminished in the 2wON 4wOFF mice (Figure 3B). In agreement, hippocampal Cdk5 activity in this group was indistinguishable from control mice (Figure 3B), and increased phosphorylation of Cdk5 substrates NR2A, PAK, and PSD-95 were reversed (see Figure S6). Moreover, staining for the neuronal marker NeuN (Figure 3C) and the glial marker GFAP (Figure 3D) demonstrated that in contrast to the 6wON mice, no obvious neuronal loss or astrogliosis was observed in the 2wON 4wOFF group. Consistently, while 6 weeks of p25 expression resulted in significant brain atrophy ($p < 0.0001$), the brain weights of the 2wON mice and the 2wON 4wOFF mice were indistinguishable from control mice (Figure 3E). We also examined CK-p25 mice that were induced for 6 weeks followed by 6 weeks of p25 repression. These mice displayed significantly reduced brain atrophy compared to those induced for 12 weeks (data not shown), suggesting that continuous p25 expression is required for maintaining the course of pathogenesis.

Cdk5 activity. (C) Representative images of hippocampal brain sections from mice group (n = 3/group) immunostained for the neuronal marker NeuN and p25-GFP. Note the severe brain atrophy in 6wON CK-p25 mice. (D) Sections used in (B) were immunostained for GFAP to detect astrogliosis. Representative pictures show GFAP immunoreativitiy (red) in the hippocampus CA1 stratum radiatum. Blue, Hoechst dye. Scale bar, 20 μm. (E) Brain weights of mice used in this experiment. Note the significant brain atrophy in 6wON CK-p25 mice. (F) Experimental groups were subjected to fear conditioning ($n_{control} = 8$, $n_{2wON} = 7$, $n_{6wON} = 7$, $n_{2wON4wOFF} = 8$). Context- and tone-dependent memory acquisition were analyzed 24 hr later (Context: CK-$p25_{6wON}$ versus control $t_{1,13}$=3.183; CK- $p25_{2wON}$ versus control $t_{1,13} = -2.304$; CK-$p25_{2wON\$wOFF}$ versus control $t_{1,14} = -3.104$; Tone: CK-$p25_{6wON}$ versus control $t_{1,13} = 3.071$). (G) Spatial learning was analyzed in 2wON 4wOFF CK-p25 and control mice by employing the water maze test (n = 7/group). (Left panel) When compared to control, CK-p25 mice improved significantly faster in their ability to find the hidden platform throughout the training trials ($F_{1,138} = 11.777$, $p < 0.008$). (Right panel) During the first probe test (PT1) performed after seven training trials, CK-p25 mice spent significantly more time swimming in the target quadrant ($t_{1,12} = -2.167$, $p < 0.0412$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$ versus control. CA1:Str.rad, hippocampal subfield CA1; str.rad, stratum radiatum. Error bars indicate SEM.

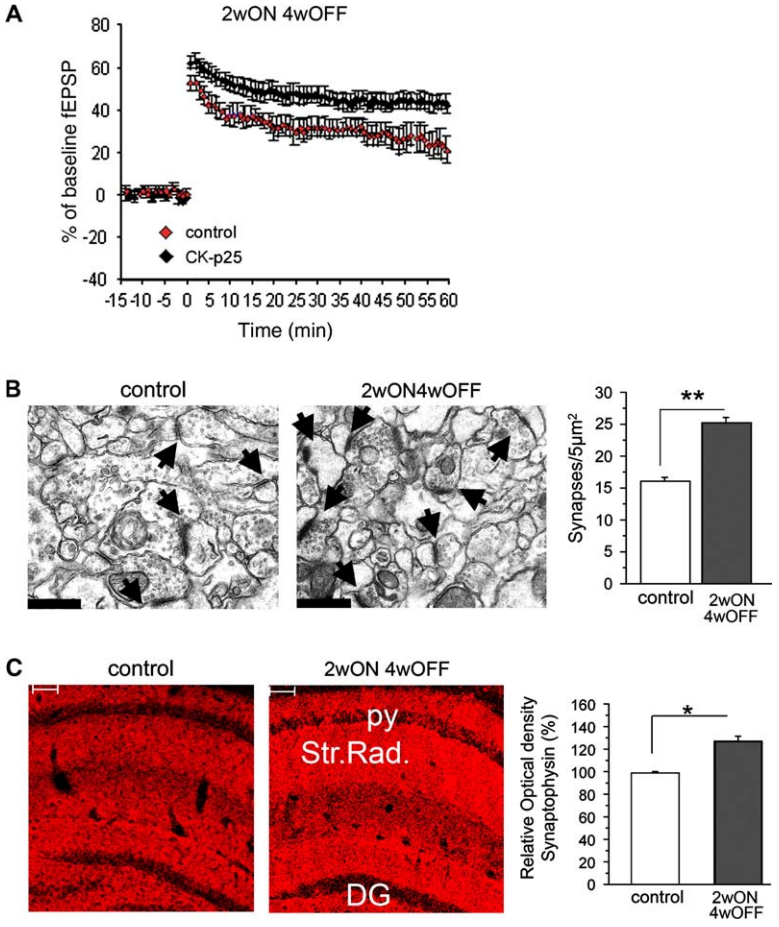

Figure 5. Enhanced Synaptic Plasticity in CK-p25 Mice that Were Induced for 2 Weeks followed by 4 Week p25 Repression

(A) There was a significant increase in the magnitude of LTP in 2wON 4wOFF mice when compared to control mice. (B) Brains (n = 3/group) from control or 2wON 4wOFF mice were prepared for electron microscopy (EM). Depicted are representative images from the hippocampal CA1 stratum radiatum region. Arrows point to synapses identified by the electron-dense postsynaptic density (PSD). The number of synapses was significantly increased in 2wON 4wOFF mice ($p < 0.001$). (C) Brain sections (n = 3/group) from control or 2wON 4wOFF mice were immunostained for the presynaptic marker protein synaptophysin. Representative images show the hippocampal CA1 region. Scale bar, 100 μm. Semiquantitative analysis revealed that synaptophysin immunoreactivity was significantly enhanced in 2wON 4wOFF mice ($p < 0.05$). py, pyramidal cell layer; Str. Rad., hippocampal CA1 stratum radiatum; DG, dentate gyrus. *$p < 0.05$ versus control; **$p < 0.001$ versus control. Error bars indicate SEM.

Consistent with our earlier observation, when trained in the fear-conditioning paradigm, the 6wON mice (n = 7) showed significantly impaired memory consolidation ($p = 0.0086$, $t_{1,13} = 3,183$), whereas the 2wON mice (n = 7) displayed significantly enhanced freezing behavior in the context-dependent memory test ($p = 0.0370$, $t_{1,13} = -2,304$ versus control; Figure 3F). Remarkably, the ability to acquire context-dependent fear memories was still enhanced in the 2wON 4wOFF mice (n = 8; $p = 0.0348$, $t_{1,14} = -3,104$; Figure 3F). Similar to our previous findings, tone-dependent fear conditioning was impaired in the 6wON mice but was not affected in the other groups (Figure 3F). In addition, spatial learning using the water maze paradigm was facilitated in CK-p25 mice (n = 7/group) that were induced for 2 weeks followed by p25 repression. As memory consolidation in this paradigm does not occur in a well-defined time window, the training procedure was performed between 3–5 weeks of p25 repression. Over the 10 days of training trials, the escape latency of the 2wON 4wOFF mice improved significantly faster compared to control mice ($p < 0.008$, $F_{1, 138} = 11.777$; Figure 3G). Moreover, when the probe test was performed after seven training trials, the 2wON 4wOFF mice spent significantly more time in the target quadrant than control mice ($t_{1,12} = -2.167$, $p < 0.0412$; Figure 3G).

Collectively, these data indicate that continuous p25 expression is required to induce astrogliosis, neuronal loss, and cognitive decline. Moreover, transient p25 expression is sufficient to enhance the ability to acquire new memories for a prolonged time period.

Effects of Transient and Prolonged p25 Expression on Hippocampal Synaptic Plasticity

To determine the cellular basis for the diametrically opposing effects of transient and prolonged p25 expression on hippocampus-dependent memories, we performed electrophysiological recordings with the use of hippocampal slices. First, we examined the effects of p25 expression on LTP. Field EPSPs (fEPSP) were evoked at the CA1 synapses by stimulating Schaffer collaterals at a low frequency (1 per min) to establish a stable baseline. In order to reveal both facilitating and inhibitory effects, a slightly weaker tetanic stimulation (50 Hz, 1 s) was used to induce LTP. A transient expression of p25 enhanced LTP. The CK-p25 mice induced for 7–9 days exhibited a significant increase in the magnitude of LTP when compared to control mice (Figure 4A). Synaptic potentiation measured 60 min after application of tetanus was 11.25% ± 8.59% above baseline (n = 6 slices) in control slices but 33.48% ± 4.59% (n = 8 slices) in the 2wON slices (Student's t test, $p < 0.001$). Remarkably, when compared to control littermates, LTP was still significantly enhanced in the 2wON 4wOFF mice (Figure 5A). A stronger tetanus (100 Hz, 1 s) was applied to the 6wON mice to reveal their inhibitory effect. Severe impairment in LTP was seen in these mice when compared to the control mice (Figure 4B). At 60 min after

tetanus, the fEPSP slope was 33.57% ± 6.08% (n = 13 slices) above baseline in control but only 3.66% ± 8.47% (n = 10 slices) in 6wON slices ($p < 0.001$).

A number of experiments were performed to determine the potential mechanisms underlying the increase and decrease in LTP observed in 2- and 6-week induced CK-p25 mice, respectively. First, we measured basal synaptic transmission by input-out curves, generated by plotting fEPSP slopes against the slopes of fiber volley. There was a small decrease in basal synaptic transmission in synapses of the 6wON animals, particularly when the presynaptic afferents were stimulated at higher intensities (Figure 4D). This is consistent with the finding that the total levels of AMPA receptors are reduced in these mice (data not shown). This small deficit, however, is less likely to account for the marked impairment in LTP. Second, we have examined several different forms of short-term plasticity. Synaptic response to a brief, high-frequency stimulation (HFS, 100 Hz, 20 pulses), which depletes all the readily releasable pool of transmitters or vesicles docked at presynaptic membrane, was measured. The rate of depletion of docked vesicles, as reflected by the ratio of the 20th and the 1st EPSP slopes, was the same in control and the 6wON mice (Figure S5B). Synaptic response to a prolonged, low-frequency stimulation (LFS, 12 Hz, 300 pulses) was also not changed, suggesting that the reserved pool of vesicles is normal in the 6wON mice (Figure S5D). Paired-pulse facilitation (PPF), which reflects the probability of presynaptic transmitter release, was slightly increased (Figure S5F). This again cannot explain the reduction in LTP. Finally, we measured NMDA receptor currents by employing whole-cell recordings in the presence of GABA (bicuculline, 10 μM) and AMPA receptor (CNQX, 20 μM) antagonists. The current-voltage relationships (I-V plots) of NMDA receptor currents for control of 6wON mice were indistinguishable (Figure 4F). Thus, the LTP deficit induced by prolonged p25 expression appears to be due to deficits downstream of NMDA receptor.

Similar experiments were performed using 2wON mice. The input-output curves were similar between control and 2wON groups (Figure 4C), suggesting that basal synaptic transmission is normal. The HFS response and PPF were also normal (Figure S5A and S5E). Interestingly, synaptic response to LFS was consistently lower in slices derived from 2wON mice, compared to control (Figure S5C). Finally, we examined the effect of acute p25 expression on NMDA receptor. While the I-V curves were very similar at the negative holding potentials, NMDA currents were larger at positive holding potentials at the CA1 synapses of 2wON mice, compared to control (Figure 4E). While these data suggest that the NMDA receptors are more active in the 2wON synapses, how much this contributes to the increase in LTP remains to be investigated.

Dendritic Spine Density Is Increased in CK-p25 Mice

It was previously reported that pharmacological inhibition of Cdk5 activity attenuates cocaine-mediated increased dendritic spine formation in striatal neurons (Norrholm et al., 2003). To explore whether aberrant activation of Cdk5 influences dendritic spine formation, we investigated the spine density of CA1 pyramidal neurons

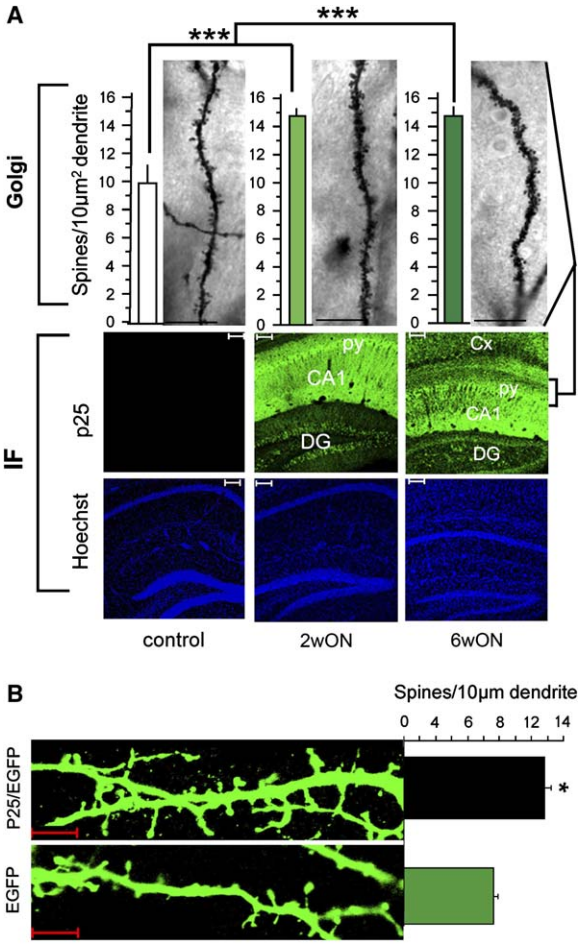

Figure 6. p25 Induction Increases Dendritic Spine Density

(A) (Top) Representative images from Golgi-stained brains of control, 2wON, and 6wON mice depicting apical dendrites of CA1 pyramidal neurons. The number of dendritic spines was significantly increased in 2wON and 6wON mice. Scale bar, 10 μm. (Bottom) Representative images from 2wON, 6wON, and control mice immunostained for p25 and Hoechst. Scale bar, 100 μm. (B) Representative images of primary hippocampal neurons transfected with p25/EGFP at DIV13 and fixed 16–18 hr later. Neurons transfected with p25/EGFP displayed significantly more spines than EGFP-transfected neurons. Scale bar, 5 μm. *$p < 0.05$ versus control; ***$p < 0.0001$ versus control. Error bars indicate SEM.

in CK-p25 mice by Golgi impregnation. The number of spines per dendrite was upregulated in 2-week induced CK-p25 mice when compared to control mice (Figure 6A; n = 3/group). In contrast to our behavioral and electrophysiological data showing impaired learning and decreased LTP, the CK-p25 mice induced for 6 weeks still exhibited a higher number of spines per dendrite. However, these mice displayed significant neuronal loss, as revealed by Hoechst dye staining (Figure 6A, n = 3/group), brain weight, and neuronal counts (Figures S1C and S1D). It should be noted that the majority of hippocampal CA1 pyramidal neurons in either 2wON or 6wON mice express high levels of p25 (Figure 6A, see also Figure 3C).

To further verify whether the increased spine density in the CK-p25 mice was an immediate consequence of p25 expression, we transfected 13-day-old dissociated

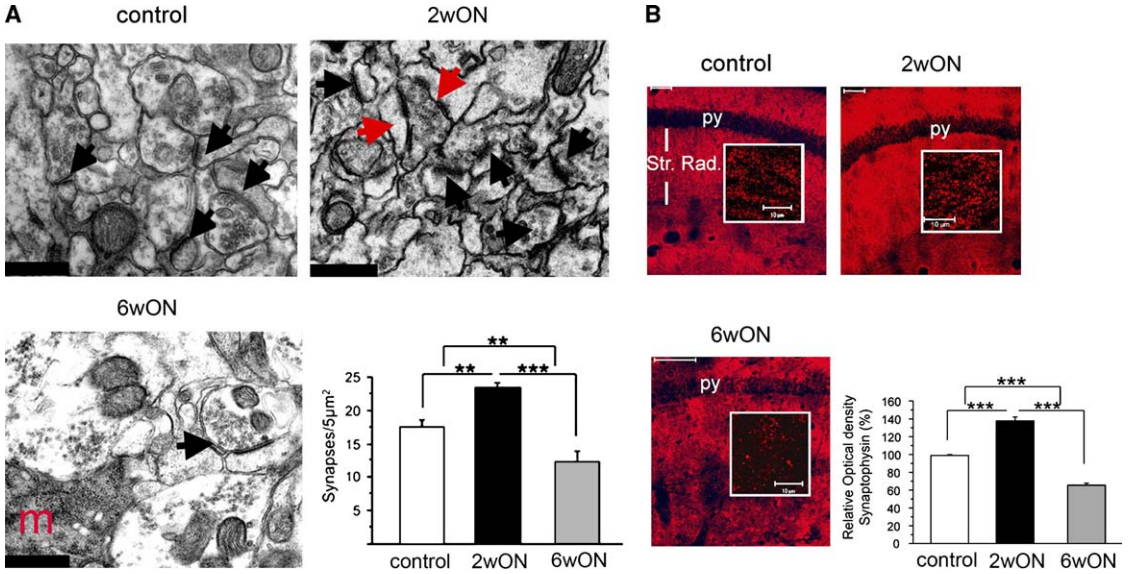

Figure 7. p25 Production Initially Increases but Finally Decreases the Number of Synapses

(A) Brains (n = 3/group) from control or CK-p25 mice 2 (2wON) and 6 weeks (6wON) after induction were prepared for electron microscopy (EM). Depicted are representative images from the hippocampal CA1 stratum radiatum region. Arrows point to synapses identified by the electron-dense postsynaptic density (PSD). Scale bar, 200 nm. The number of synapses was significantly enhanced in 2wON mice ($p < 0.001$) but decreases in 6wON mice ($p < 0.001$). Red arrows indicate perforated synapses. (B) Brain sections (n = 3/group) from control mice or 2wON and 6wON mice were immunostained for the presynaptic marker protein synaptophysin. Representative images show the hippocampal CA1 region. Scale bar, 100 μm. Insets show magnification. Scale bar, 10 μm. Semiquantitative analysis revealed that synaptophysin immunoreactivity was significantly enhanced in 2wON mice ($p < 0.0001$) but decreased in 6wON mice ($p < 0.0001$). **$p < 0.001$, ***$p < 0.0001$; py, pyramidal cell layer; Str. Rad., hippocampal CA1 stratum radiatum; m, macrophage. Error bars indicate SEM.

cultures of rat hippocampal neurons (13DIV) with EGFP or EGFP/p25. As reported previously (Patrick et al., 1999), neurotoxicity was evident 24–36 hr after p25 expression in dissociated neurons (data not shown). Cells were fixed 16–18 hr after transfection, and the number of dendritic spines was analyzed. A significantly higher spine/dendrite ratio was observed in p25/EGFP-transfected neurons compared to cells transfected with EGFP alone (Figure 6B). Transfection of p35 in primary hippocampal neurons did not lead to increased spine density, supporting the notion that p25 is a more potent Cdk5 activator (data not shown). Together, these observations suggest that increased spine density is a cell-autonomous effect of p25 expression.

To further investigate the role of Cdk5 in dendritic spine formation, we analyzed CA1 pyramidal neurons in homozygous and heterozygous p35-deficient mice. Spine density was significantly reduced in both p35 null and heterozygous deficient mice. Moreover, contextual fear conditioning was severely impaired in $p35^{+/-}$ or $p35^{-/-}$ mice when compared to control littermates (Figure S7). Thus, Cdk5 activity plays a critical role in dendritic spine development.

Altered Number of Synapses in CK-p25 Mice

To further determine the effect of p25/cdk5 activity on the number of synapses, we performed transmission electron microscopy to analyze the synapse number in the CA1 stratum radiatum. A synapse is defined as the presence of the electron-dense postsynaptic density juxta-opposed to the presynaptic terminal containing synaptic vesicles. According to these criteria, the number of synapses was significantly higher in 2wON mice when compared to control mice ($p < 0.001$; Figure 7A). In addition, more perforated synapses were observed in the transgenic mice. By contrast, significantly fewer synapses were observed in 6wON mice ($p < 0.0001$; Figure 7A). Interestingly, the 2wON 4wOFF mice (see Figure 3) still exhibited an elevated number of synapses (Figure 5B). We also performed immunohistochemistry for the presynaptic protein synaptophysin in the CA1 stratum radiatum. Synaptophysin is a synaptic vesicle protein and has been widely used as a molecular and functional marker of synapses in rodents and humans (Honer, 2003). In the 2wON mice (n = 3/group), a brighter synaptophysin immunoreactivity (SVP-IR) was observed (Figure 7B). In contrast, after 6 weeks of p25 induction, markedly reduced SVP-IR was detected (Figure 7B), indicating a reduction of presynaptic terminals at this time point. Brighter SVP-IR was also observed in the 2wON 4wOFF mice (Figure 5C). Together, these data suggest that 2wON mice display an increased number of synapses, whereas 6 weeks after p25 induction, the number of functional synaptic connections is reduced.

Discussion

p25 Production Initially Facilitates but Eventually Impairs Learning

Previous work established the specific involvement of the hippocampus in context-dependent fear conditioning, whereas the amygdala is believed to be crucial for tone-dependent fear conditioning (Fendt and Fanselow,

1999). We demonstrated that transient p25 production selectively enhanced hippocampus-dependent associative memory, measured by context-dependent fear conditioning, without affecting amygdala-dependent memory. These findings are in agreement with a previous report showing that the acquisition of context-dependent fear memories is impaired by intrahippocampal injection of the Cdk5 inhibitor butyrolactone I (Fischer et al., 2002). In CK-p25 mice, p25 levels are highest within the hippocampal formation but are low in the amygdala (Figure 3, data not shown), which may account for the lack of significantly enhanced tone-dependent fear conditioning. The enhancement of hippocampus-dependent memory by transient p25 expression was further supported by the Morris water maze test. CK-p25 mice performed significantly better throughout the training period and in the first probe test performed after 6 days of training. Importantly, transient p25 expression for 2 weeks followed by repression for 4 weeks still facilitated fear conditioning and spatial learning without causing neurodegeneration. Thus, a brief phase of p25 expression causes long-lasting changes in synaptic plasticity that facilitate learning and memory.

By contrast, context-dependent fear conditioning and spatial learning were eventually impaired by prolonged p25 expression. Interestingly, tone-dependent fear conditioning was also impaired in 4- and 6-week induced CK-p25 mice, most likely reflecting pathological events such as astrogliosis and the significant loss of neurons within cortical brain regions that may play a role in the processing and retrieval of tone-dependent fear memories (Milad et al., 2004). Importantly, impaired learning correlated with the onset of astrogliosis and neurodegeneration. This is in contrast to other animal models of neurodegenerative diseases where impaired learning often precedes histopathological changes (Richardson et al., 2003; Saura et al., 2004) and may in part be due to the rapid development (within 6 weeks of p25 production) of neuronal loss in CK-p25 mice.

Taken together these data indicate that transient p25 production facilitated learning, whereas the ability to form new memories was impaired by prolonged p25 expression.

Transient p25 Expression Facilitates while Prolonged p25 Expression Impairs Synaptic Plasticity

On the cellular level, facilitated learning mediated by transient p25 production was accompanied by enhanced LTP at the CA3-CA1 synapse. This finding is in agreement with a previous study showing that the Cdk5 inhibitor roscovitine impairs hippocampal LTP (Li et al., 2001). In many cases, increased NMDA receptor function correlates with enhanced LTP and learning and memory (Tang et al., 1999). Notably, p25/Cdk5-mediated phosphorylation of NR2A on S1232 increases NMDA receptor channel conductance (Wang et al., 2003). Interestingly, we found that $NR2A_{1232}$ phosphorylation (see Figure S6A) and NMDA receptor current were increased in 2-week induced CK-p25 mice, suggesting that facilitated NMDA receptor signaling may be involved in the initially enhanced LTP and memory in CK-p25 mice.

In addition, we showed that dendritic spine density of hippocampal CA1 neurons was higher in 2-week induced CK-p25 mice, suggesting more synapses at this time point. This finding was confirmed by electron microscopic analysis of the hippocampal CA1 stratum radiatum, which revealed a higher synapse number that was accompanied by increased SVP-IR. Interestingly, the number of synapses determined by SVP-IR and electron microscopy was still elevated in CK-p25 mice induced for 2 weeks followed by 4 week of p25 repression that displayed facilitated learning and LTP. The finding that phosphorylation of NR2A was not heightened in these mice (see Figure S6B) further suggests that in addition to increased NMDA signaling other mechanisms such as synaptogenesis are likely contributing to the facilitated learning in CK-p25 mice.

Surprisingly, higher spine density was observed in CA1 pyramidal neurons of 6-week induced CK-p25 mice when neuronal loss was readily detectable. This is paradoxical to several other findings in these mice: (1) the number of synapse, as determined by electron microscopy, is decreased; (2) SVP-IR is reduced; basal synaptic transmission, measured by field recording, was also slightly reduced. These results suggest that many of the new spines formed as a consequence of prolonged p25 expression may not be functional. It may simply be a compensatory reaction in response to the overall cell loss or reduction in synaptic activity. Indeed, it has been shown that spine density is increased in primary neurons in response to decreased synaptic transmission (Kirov et al., 2004).

Nevertheless, it seems that the increase in spine density at both 2-week and 6-week time points (transient and prolonged) is mediated by p25. In support of this view, we demonstrated that transfection of primary hippocampal neurons with p25 increased the dendritic spines density. In addition, the spine density was decreased in CA1 neurons of homozygous and heterozygous p35-deficient mice that exhibit severe learning deficits (see Figure S7). Moreover, previous work showed that the Cdk5 inhibitor roscovitine prevents cocaine-induced increases of spine density of striatal spiny neurons (Norrholm et al., 2003). Taken together these data suggest that Cdk5 activity regulates the formation of dendritic spines and that transient p25 production induces synaptogenesis in the adult brain.

It can be speculated that p25 production hyperactivates the cellular mechanisms underlying synaptic plasticity and synaptogenesis that are normally regulated by physiological p35/Cdk5 activity. In support of this view, it was shown that p25 expression partially rescued the phenotype of p35-deficient mice (Patzke et al., 2003), indicating that p25/Cdk5 can functionally replace p35/Cdk5 activity, at least during developmental processes. Moreover, we found that the phosphorylation of physiological Cdk5 substrates that have been implicated with synaptic plasticity, such as PAK-1, NMDA receptor 2A, and PSD-95 (see Figure S6) was increased 2- to 4-fold in CK-p25 mice. PAK-1 is a Cdk5 substrate that regulates actin dynamics (Bokoch, 2003), which is implicated in learning (Fischer et al., 2004) and is essential for spine formation and motility (Matus, 2000). Overexpression of a dominant-negative mutant of PAK-1 in the forebrain disrupted spine formation in cortical neurons and

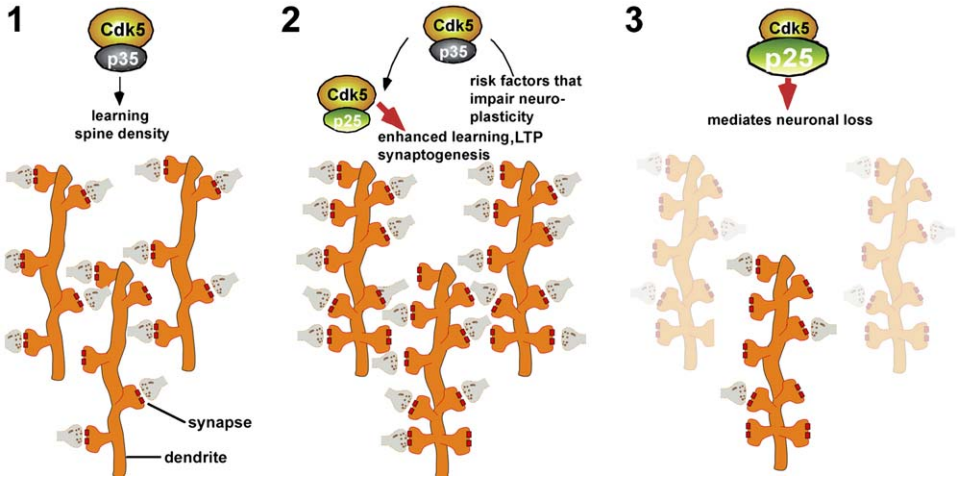

Figure 8. Model Suggesting the Involvement of p25 Production in Neurodegenerative Diseases

Under normal conditions, Cdk5/p35 activity is required for LTP, learning, and memory and may regulate the density of dendritic spines (1). Cleavage of p35 to p25 can be induced by risk factors of neurodegenerative diseases and may occur when neuroplasticity is impaired. As p25 induces synaptogenesis and facilitates learning, it can be speculated that generation of p25 is initially a phenomena to compensate for decreased neuroplasticty (2). However, chronically high p25 levels cause neuronal loss that contributes to decreased LTP and impaired learning (3).

prevented the transition of hippocampal memories to higher cortical regions (Hayashi et al., 2004). Therefore, regulation of PAK-1 by p25/Cdk5 may modulate synaptogenesis. Most likely other, possibly unidentified, Cdk5 substrates may also be involved in this process, and further experiments will be necessary to elucidate the role of p25 and Cdk5 activity in synaptogenesis within the adult brain.

Prolonged p25 expression eventually impaired LTP and memory. These phenotypes correlated with synaptic and neuronal loss. In a number of studies, p25 production was implicated in apoptotic cell death, Tau pathology, and deregulation of mitochondrial function (Cruz et al., 2003; Darios et al., 2005; Weishaupt et al., 2003), suggesting that p25-induced cell death is a pleiotrophic effect. In addition, we found here that NR2A was highly phosphorylated in 6-week induced CK-p25 mice (Figure S6A). Although NMDA receptor function is required for LTP and hippocampus-dependent memory, it can cause neuronal cell death when hyperactivated (Waxman and Lynch, 2005). Importantly, the neurotoxic effect of hyperactivated NMDA receptors is linked to calpain-mediated p25 production in vitro (Kerokoski et al., 2004), and phosphorylation of S1232 on NR2A has been implicated with ischemic cell death (Wang et al., 2003). Therefore, NR2A might be a Cdk5 substrate that contributes initially to enhanced memory and later to neurodegeneration.

Importantly, transient p25 expression that enhanced learning did not cause astrogliosis or neurodegeneration, suggesting that p25-mediated enhancement of synaptic plasticity may precede its detrimental actions leading to cell death.

p25 Production Might Be a Mechanism to Compensate for Impaired Neuroplasticity

As p25 has been implicated in neurodegeneration, it was surprising to see that transient expression of p25 enhanced LTP and facilitated learning and memory. The question arises whether p25 may elicit these novel functions under physiological conditions.

In pathological conditions such as neurodegenerative diseases, altered neuroplasticity has been observed. For example, although the total number of neurons and synapses is reduced, the synaptic contact size is increased in brain samples of AD patients (Scheff, 2003). Similarly, the expression of certain proteins related to synaptic plasticity, such as GABA and glutamate receptors, is elevated in AD brains (Armstrong et al., 2003). In addition "reactive synaptogenesis" takes place in response to brain injury (Kaplan, 1988; Scheff, 2003). These phenomena have been interpreted as compensatory responses that occur when neuroplasticity is impaired in order to retain the integrity of the neuronal network (Cotman and Anderson, 1988; Scheff, 2003). Thus it can be speculated that p25-mediated synaptogenesis may represent a compensatory response during neurodegenerative diseases (see Figure 8). As such, enhanced LTP, synaptogenesis, and memory formation may be detectable in CK-p25 mice because in this mouse model p25 is produced in the absence of pathological conditions that impair neuroplasticity.

Interestingly, p25 expression is enhanced by several risk factors, such as exitotoxicity, Aβ-peptides, and ischemia (Lee et al., 2000; Wang et al., 2003). p25 levels are elevated in human individuals that developed AD (Patrick et al., 1999; Tseng et al., 2002; Swatton et al., 2004). Although the interpretation of results from animal models for human diseases is limited, the observed hippocampal p25/p35 ratio in five of our CK-p25 mice is similar to the ratio that has been described in the hippocampus of AD patients (Tseng et al., 2002), suggesting that our CK-p25 mice offer a relevant model to investigate p25 pathology.

Our data indicate that p25 production might initially be a compensatory response to AD risk factors. Therefore, increased p25 levels are likely to be an early event in AD pathogenesis. Because p25 has only been investigated

in postmortem samples from patients with fully developed AD and substantial brain atrophy, it will be important to analyze p25 levels in patients with preclinical AD and mild cognitive impairment but no substantial neuronal loss. This will also help to clear up any discrepancies with other studies suggesting that increased p25 levels may be generated through a postmortem process (Tandon et al., 2003, Yoo and Lubec, 2001, Taniguchi et al., 2001). There is also evidence suggesting that Cdk5 activity might decease during aging. A recent study found that the p35 gene is downregulated in aged individuals (Lu et al., 2004). It is possible that p25 is produced to compensate for the loss of Cdk5 activity during aging. In this scenario, chronic exposure to AD risk factors would further increase p25 levels to a critical concentration that ultimately contributes to neuronal loss. Furthermore, under this scenario, any mutations altering Cdk5 expression or activity would also cause a compensatory increase in p25 levels that would put an individual at risk for AD. Because a single nucleotide polymorphism (SNP) has been found in the Cdk5 gene that is specific to familial AD patients (Rademakers et al., 2005), it will be interesting to analyze whether this SNP affects Cdk5 expression.

Elevated p25 levels were also observed in animal models of neurodegenerative diseases (Nguyen et al., 2001; Saura et al., 2004). Although, elevated p25 levels in these studies have been considered to contribute to cell death, p25 production in vivo does not always correlate with neuronal loss. For example, p25 is detected in APPswe mice that do not develop neurodegeneration (Otth et al., 2002). Furthermore, in another line of transgenic mice that constitutively express p25 under control CamKII promoter (the p25/p35 ratio is these mice is 0.33), no astrogliosis, neuronal loss, or cognitive decline is observed. Interestingly, transgenic animals performed slightly better when they were retrained in a reversal task of the water maze paradigm (Angelo et al., 2003). Thus, the fact that CK-p25 mice eventually develop neurodegeneration is most likely due to higher p25 levels expressed in these mice. However, it has to be reiterated that transient p25 expression did not cause astrogliosis or neuronal loss in CK-p25 mice induced for 2 weeks followed by 4 weeks of p25 repression. Taken together these data suggest that p25 production in vivo is not detrimental per se but can lead to neuronal cell death when p25 levels are chronically high.

In summary, it is intriguing that several studies suggest that during the pathogenesis of AD, which manifests over several years, compensatory mechanisms that initially enhance neuroplasticity eventually become maladaptive when chronically activated (Arendt, 2004; Mesulam, 1999). A similar scenario can be envisioned for p25. Thus, the present study provides evidence that p25 generation might be a compensatory phenomenon to enhance neuroplasticity. These mice may also serve as a model whereby a factor that promotes plasticity can eventually contribute to neurodegeneration (Figure 8).

Experimental Procedures

Animals

All mice were conceived and raised in the presence of doxycycline (1 mg/g in food; Bio-Serv, Frenchtown, NJ). To induce p25 expression, mice were fed a normal diet. To repress p25 production, mice were again fed a doxycycline diet. All experimental groups were identically treated and fed doxycycline diet for the same time.

Behavior

Fear Conditioning

Fear conditioning was performed as described previously (Fischer et al., 2002).

Morris Water Maze Learning

The water maze paradigm (Morris et al., 1982) was performed in a circular tank (diameter 1.2 min) filled with opaque water. A platform (11 × 11 cm) was submerged below the water's surface in the center of the target quadrant. The swimming path of the mice was recorded by a video camera and analyzed by the Videomot 2 software (TSE). For each training session, the mice were placed into the maze subsequently from four random points of the tank. Mice were allowed to search for the platform for 60 s. If the mice did not find the platform within 60 s, they were gently guided to it. Mice were allowed to remain on the platform for 15 s. During the memory test (probe test), the platform was removed from the tank, and the mice were allowed to swim in the maze for 60 s.

Elevated Plus-Maze Test

This test was used to detect anxiety-related behavior. The time spent in the open arms, closed arms, and center of the elevated plus-maze apparatus (H-10-35EPM, Coulbourne Instruments) were recorded for 5 min using the VideoMot2 system (TSE).

Rotarod

After mice became familiarized with the procedure, they were placed on the rotarod (Economex, Columbus; accelerating speed 4–40 rpm, 0.5%/s increment), and the time until the animal would fall off the rotating rod was measured in four tests performed on two consecutive days.

Immunoblot/Kinase Assay

The following antibodies were used in a 1:1000 dilution: p35 (C-19, Santa Cruz, polyclonal), NMDA receptor 2A (MAB5216, Chemicon), synaptophysin (SVP-38, monoclonal), actin (polyclonal), and GFAP (polyclonal) were from Sigma. Phospho-NR2A (1:500), phospho-PSD-95 (1:5), and phospho-PAK (1:1000) antibodies were gifts from Dr. You-Ming Lu, Dr. Maria Morabito, and Dr. Magareta Nikolic, respectively. The same lysates were used for kinase assays performed as described before (Cruz et al., 2003).

Cultivation and Transfection of Hippocampal Primary Neurons

DIV13 neurons were transfected with EGFP or p25/EGFP with the use of lifofectamin 2000. Spine density was quantified in a blind manner by using LSM5 software (Leica). Spines were defined as structures protruding from dendrites that were not longer that 2 μm.

Electrophysiology

LTP Recordings

Transverse hippocampal slices (400 μm) were preprared as described previously (Pozzo-Miller et al., 1999). Field excitatory postsynaptic potentials (EPSPs) were evoked in CA1 stratum radiatum by stimulating Schaffer collaterals with twisted bipolar nichrome electrodes and recorded with ACSF-filled glass pipettes (<5 MΩ) by using an Axoclamp-2B amplifier. Test stimuli consisted of monophasic 200 ms pulses of constant current and were delivered by stimulus isolation units. Tetanic stimulation was applied after stable baseline was established for at least 20 min. LTP was induced by either 50 Hz for 1 s or 100 Hz for 1 s. Only slices exhibiting maximum fEPSP of 3–6 mV in amplitude without superimposed population spikes were used. Stimulus intensity was adjusted to evoke 40%–50% of the maximum fEPSP, and basal synaptic transmission was monitored by the low-frequency stimulation (every 30 s) via stimulating electrode positioned at the side of the recording electrode.

Measurement of NMDA Current

Whole-cell recordings were made at −50 mV in the presence of 20 μM CNQX and 10 μM bicuculline methiodide from CA1 pyramidal neurons of hippocampal slices. Recording electrodes (5–8 MΩ) were filled with intracellular solution containing (mM): 130 K-gluconate, 10 HEPES, 2 Mg2-ATP, 0.5 Na3-GTP, 11 EGTA, 10 phosphocreatine, and 5 QX-314. Excitatory postsynaptic currents (EPSCs) were evoked at a rate of 0.05 Hz by a stimulating electrode placed in the

stratum radiatum. Access resistance was monitored throughout experiments and ranged from 10–25 MΩ. Data were discarded when access resistance changed by >15% during an experiment. NMDA current was recorded when the cells were held at the voltage from −60 to 40 mV.

Basal Synaptic Transmission

fEPSP was monitored by low-frequency stimulation (two per minute) of Schaffer collaterals. Stimulus intensity was adjusted to evoke fEPSP (from small intensity to high intensity).

Measurement of Readily Releasable Pool of Vesicles

Synaptic fatigue was induced by a 1 s train at 100 Hz. Because the 100th fEPSP was often too small to measure accurately, we used the ratio of the slope of the 20th to that of the first fEPSP to analyze the readily releasable pool of neurotransmitters.

Measurement of Reserve Pool Vesicles

Synaptic responses to a train of low-frequency stimulation (LFS) (12.5 Hz, 300 pulses) were measured in the presence of DL-APV (50 μM). The fEPSP slopes were recorded during the entire LFS, expressed as the percentage of the first fEPSP slope, and were plotted against stimulus numbers.

Paired-Pulse Facilitation

The responses of fEPSP to paired-pulse stimulation at (25–200 ms) were used to measure PPF. Ratios of the second fEPSP slopes to the first fEPSP slopes were calculated and plotted against different interpulse intervals.

Immunostaining

Immunohistochemical analysis was performed as described before (Sananbenesi et al., 2002). Antibodies were used in a 1:1000 concentration: GFP (polyclonal, Molecular probes), GFAP, and synaptophysin (SVP-38) were from Sigma.

Electronmicroscopy

Mice were perfused with 4% paraformaldehyde/glutraraldehyde in 100 mM Cacodylate buffer, pH 7.4. Hippocampal sections (1 μm) were stained with 1% Toluidine blue, further trimmed to the CA1 stratum radiatum region and stained with uracyl acetate and lead citrate. Neuropil areas were photographed using a JEOL1200EX electron microscope. Microphotographs were used to analyze the number of synapses blind to the genotype. Synapses were defined by the presence of a clear PSD facing a presynaptic structure with synaptic vesicles. For each experimental group, at least 500 μm^2 CA1 stratum radiatum neuropil was analyzed.

Golgi Impregnation

Golgi-Cox-stained brains (Ramon-Molinier, 1970) were cut to 200 μm thick cross-sections and analyzed using a Zeiss 200 Axiovert microscope and open lab software. The number of apical and basal spines on hippocampal CA1 pyramidal neurons was counted blind to the genotype. For each experimental group, a minimum of 40 cells per animal (n = 3) were analyzed. CA1 hippocampal neurons within the region −1.4 mm to −1.6 mm (relative to the bregma position) were included for the analysis.

Statistical Analysis

The data were analyzed by unpaired Student's t test. One-way ANOVA followed by post hoc Scheffe's test was employed to compare means from several groups at the same time. Data are presented as SEM. A detailed description of the statistics employed in this study can be found in DC Howell's Statistical Method for Psychology (Howell, 2002).

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/48/5/825/DC1/.

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