Reelin supplementation enhances cognitive ability, synaptic plasticity, and dendritic spine density

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Apolipoprotein receptors belong to an evolutionarily conserved surface receptor family that has intimate roles in the modulation of synaptic plasticity and is necessary for proper hippocampal-dependent memory formation. The known lipoprotein receptor ligand Reelin is important for normal synaptic plasticity, dendritic morphology, and cognitive function; however, the in vivo effect of enhanced Reelin signaling on cognitive function and synaptic plasticity in wild-type mice is unknown. The present studies test the hypothesis that in vivo enhancement of Reelin signaling can alter synaptic plasticity and ultimately influence processes of learning and memory. Purified recombinant Reelin was injected bilaterally into the ventricles of wild-type mice. We demonstrate that a single in vivo injection of Reelin increased activation of adaptor protein Disabled-1 and cAMP-response element binding protein after 15 min. These changes correlated with increased dendritic spine density, increased hippocampal CA1 long-term potentiation (LTP), and enhanced performance in associative and spatial learning and memory. The present study suggests that an acute elevation of in vivo Reelin can have long-term effects on synaptic function and cognitive ability in wild-type mice.

[Supplemental material is available for this article.]

Reelin is a large extracellular matrix protein that plays a pivotal role in embryonic neuronal migration. Reelin is produced by GABAergic interneurons in the adult brain and physically associates with the postsynaptic density, dendritic spines, and axons throughout the hippocampus and cortex (Pesold et al. 1999). Reelin activates a number of neuronal signal transduction pathways in the adult central nervous system (CNS) that subsequently modulate synaptic function and plasticity. Interneurons expressing Reelin are widely distributed in the adult mammalian brain at a period long after the decrease in Cajal-Retzius cells (D’Arcangelo et al. 1997; Pesold et al. 1998, 1999; Rodriguez et al. 2000; Pappas et al. 2001; Kubo et al. 2002). Disruption of either Reelin expression or the two known receptors of Reelin, Apolipoprotein E Receptor 2 (ApoER2) and Very-Low-Density Lipoprotein Receptor (VLDLR), results in associative and spatial learning defects, impairment of hippocampal long-term potentiation (LTP), and alterations in dendritic spine morphology (Trommsdorff et al. 1999; Weeber et al. 2002a). Organotypic hippocampal cultures of mutant Reeler mice have decreased spine density, which is rescued with recombinant Reelin application in a lipoprotein-dependent manner (Niu et al. 2008). Conversely, transgenic mice that overexpress Reelin present with hypertrophy of dendritic spines in the hippocampus (Pujadas et al. 2010). Wild-type organotypic hippocampal cultures chronically treated with Reelin (>5 d) results in increases of dendritic spine density and increased AMPA receptor insertion. Acute Reelin application (<20 min) enhances LTP in acute hippocampal slices from wild-type mice, an effect dependent on the presence of both ApoER2 and VLDLR (Weeber et al. 2002a). Reelin-dependent enhancement of LTP is associated with increased Ca²⁺ currents in CA1 pyramidal neurons and increased N-methyl-D-aspartic acid receptor (NMDAR) phosphorylation (Beffert et al. 2005; Qiu et al. 2006b). Extended Reelin exposure (>20 min) increases α-amino-3-hydroxyl-5-methyl-4-isoxazole-propioniate receptor (AMPAR)-mediated synaptic responses through Phosphoinositide-3-kinase (PI3K)-dependent increases in AMPAR insertion, which is associated with a significant reduction of silent synapses (Qiu et al. 2006b).

Taken together, these findings support a role that Reelin signaling is important in overall synaptic function and can influence neuronal activity and cellular mechanisms underlying memory formation. The interpretation of in vitro experimentation makes it difficult to determine the long-term consequences of enhanced Reelin signaling in vivo on complicated processes such as cognitive ability. The present studies focus on acute in vivo activation of Reelin signaling and determine the long-lasting changes to
Results

To determine if Reelin could be transported from the ventricle into the hippocampus, a single bilateral injection of Reelin or saline was given to 4-mo-old adult mice. We previously showed that perfusion of 5 nM Reelin onto acute hippocampal slices increased LTP (Weeber et al. 2002a). Thus, the injection volume of Reelin was determined to produce an average total hemisphere concentration of 5 nM. Immunohistochemical staining for Reelin revealed rapid uptake by the hippocampus 15 min following Reelin injection. Reelin levels remained elevated 3 h following Reelin injection throughout the entire hippocampus (Fig. 1). Although robust initially, Reelin levels were comparable to saline controls 5 d post-injection, suggesting that exogenous Reelin is maintained transiently.

To determine whether in vivo application of Reelin can result in specific Reelin signaling, we examined the activation state of the obligate downstream adaptor protein Disabled-1 (Dab1) at 15 min, 3 h, and 5 d following injection. Reelin induces tyrosine phosphorylation at Y220 by Src family tyrosine kinases (SFKs) in vitro (Keshvara et al. 2001; Ballif et al. 2003). Reelin injected mice showed pronounced increases in Dab1 phosphorylation at Tyrosine-220 (Y220) throughout the hippocampus at 15 min and 3 h (Fig. 2). Importantly, increases in Dab1 phosphorylation were less robust at 3 h than 15 min, which is consistent with rapid degradation of tyrosine phosphorylated Dab1 following Reelin stimulation (Arnaud et al. 2003).

Reelin enhances Ca$^{2+}$ entry through NMDA receptors and results in increased Ser 133 phosphorylation and nuclear translocation of CREB (Befort et al. 2006). Once in the nucleus, CREB promotes transcription of genes important for the formation of new synaptic connections and long-term memories (Huang et al. 1996; Martin et al. 1997; Pang and Lu 2004; Zhao et al. 2005). We detected active CREB pSer-33 was elevated in CA1, CA3, and dentate gyrus at 15 min and 3 h post-injection (Fig. 3). Similar to Dab1, phosphorylation levels of CREB were noticeably lower at 3 h and returned to baseline levels at 5 d post-injection. Increased CREB phosphorylation was also seen with quantitative Western blot analysis (Supplemental Fig. 1). To determine ApoER2 dependence of Reelin induced increases in Dab1 and CREB phosphorylation states, we injected ApoER2 KO with Reelin. No differences were detected to Dab1 or CREB phosphorylation compared to saline injected ApoER2 KO animals (data not shown). These findings confirm that in vivo application of purified recombinant Reelin can activate downstream signaling pathways similar to that seen with in vitro Reelin application.

Considering chronic application of Reelin to hippocampal cultures increases spine density, we determined whether Reelin injection influenced dendritic spine formation. Spine density on the apical oblique (AO) and basal shaft (BS) dendrites of CA1 pyramidal neurons was quantified at 3 h and 5 d post-injection. Spine density was significantly increased in 5 d Reelin-injected mice compared to 3 h (Fig. 4). To determine if the presence of ApoER2 is required for Reelin-induced increases in spine density, ApoER2 KO mice were injected with Reelin bilaterally into the ventricles 5 d post-injection when the Reelin-dependent effect on spine density was observed. All Reelin-injected experimental groups were normalized to their respective saline control groups. At 5 d post-injection, we found that both AO and BS spine densities were significantly increased in Reelin-injected wild-type mice compared to both saline-injected wild-type and Reelin-injected ApoER2 KO mice (Fig. 5). The modest increase of spine density in Reelin-injected ApoER2 KO mice may be attributed to Reelin–VLDLR interactions and signaling. Taken together, these findings are consistent with Reelin application in cultured hippocampal slices and correlate increased spine density with the activation of Dab1 and CREB.
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Levels. We found that Reelin injection had no effect on the mice to explore either the open field or EPM (Fig. 7A,B). These results indicate that Reelin injection has no effect on anxiety levels in these mice. Furthermore, any significant differences found in spatial or associative learning through Reelin injection would not be attributable to alterations in anxiety, but rather cognitive function.

To determine if Reelin supplementation enhances spatial learning and memory, mice were tested in the hidden platform water maze (HPWM) task. Mice were trained using a spaced experimental paradigm of four trials per day with an intertrial interval of 1 h. Reelin-injected mice required less time to find the hidden platform on training days 1, 3, and 4, showing that Reelin injection improved spatial learning in the HPWM (Fig. 8A). Of particular interest was the significant difference exhibited by Reelin-injected animals on day 1. Closer examination of each trial on day 1 revealed Reelin-injected mice had significantly shorter latencies on trials 3 and 4 compared to the first trial (Fig. 8B). Furthermore, by trials 3 and 4 of day 1, Reelin-treated mice found the platform in the same amount of time as the saline-treated mice during cued testing (data not shown).

Of particular interest was the effect of Reelin injection on memory retention in the HPWM. Reelin-injected mice had a significantly greater amount of target quadrant entries compared to saline-injected mice during the probe trial conducted on day 5, indicating enhanced memory retention of platform location (Fig. 8C). Reelin-injected mice retrained to find a different platform location (opposite quadrant) on day 6 continued to show a significant decrease in latency to the platform on the second day of training (Fig. 8A). Taken together, Reelin-injected mice learned more quickly, retained memory more efficiently, and relearned more quickly.

We next examined hippocampal-dependent associative fear conditioned learning and memory. Animals were trained with a

Figure 3. Reelin injection enhanced CREB phosphorylation throughout the entire hippocampus. Activation of CREB was determined with p-Ser133 specific antibody. Immunohistochemical analysis of CREB activation revealed that Reelin injection resulted in increased Ser133 phosphorylation of CREB at the 15-min time point, remained elevated through 3 h, and returned to baseline after 5 d post-injection (scale bar: 50 μm).

Our previous report of increased hippocampal LTP following acute application of Reelin is likely a result of Reelin-dependent changes in NMDAR regulation, as well as localized signal transduction activation (Qiu et al. 2006b). However, 5 d following Reelin injection, post-translational modifications of ligand-gated ion channels and Reelin-activated signaling systems would be expected to return to homeostatic levels. It is possible that the observed altered spine density following the single injection of Reelin is sufficient to change overall synaptic function in the hippocampus. Thus, we determined if synaptic transmission or plasticity in area CA1 is altered 5 d following Reelin injection. Reelin-injected mice showed significantly enhanced theta-burst stimulation (TBS)-induced LTP (Fig. 6A–B). Examination of basal synaptic transmission revealed no significant differences of field excitatory post-synaptic potential (fEPSP) slopes between groups in response to equivalent amounts of input (Fig. 6C) or alteration in fiber volley amplitude (data not shown). Also, no changes in presynaptic function were supported by quantifying short-term plasticity evaluated by paired-pulse facilitation (PPF). No significant changes were observed in PPF testing between experimental groups (Fig. 6D), further suggesting that changes in LTP are primarily due to postsynaptic modification following TBS.

Initially, mice were tested in the open field and elevated plus maze (EPM) to determine if Reelin injection alters somatosensory input and anxiety levels. We found that Reelin injection had no effect on the mice to explore either the open field or EPM (Fig. 7A,B). These results indicate that Reelin injection has no effect on anxiety levels in these mice. Furthermore, any significant differences found in spatial or associative learning through Reelin injection would not be attributable to alterations in anxiety, but rather cognitive function.

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Figure 4. Reelin increased spine density in pyramidal cells in area CA1. Mouse brains were Golgi stained and CA1 were imaged (n = 4 mice/group). (A) Representative AO dendrites at 3 h and at 5 d per group. (B) Averaged AO spine densities for each group at 3 h (saline: 15.21 ± 0.79, n = 25; Reelin: 15.51 ± 0.79, n = 28) and at 5 d (saline: 13.5 ± 0.60, n = 25; Reelin: 19.2 ± 0.86, n = 34). Reelin significantly increased AO spine density in area CA1 at 5 d but not 3 h post-injection. (C) Representative BS dendrites at 3 h and at 5 d for each group. (D) Averaged BS spine densities for each group at 3 h (saline: 15.75 ± 0.76, n = 25; Reelin: 15.30 ± 0.71, n = 33) and at 5 d (saline: 13.64 ± 0.85, n = 28; Reelin: 17.64 ± 0.70, n = 28). Reelin significantly increased BS spine density in area CA1 at 5 d but not 3 h post-injection. (Data expressed as mean ± SEM; *P < 0.0005.)
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Discussion

Impairments in Reelin signaling have been implicated in several human cognitive disorders including schizophrenia, Alzheimer’s disease, and autism spectrum disorders (Impagnatiello et al. 1998; Andres 2002; Ballmaier et al. 2002; Costa et al. 2002; Botella-Lopez et al. 2006; Chin et al. 2007; Durakoglugil et al. 2009). However, the extent to which Reelin can be tested as a therapeutic agent has been limited by our lack of understanding in vivo actions of Reelin in the adult CNS. The present studies sought to determine in vivo effects of Reelin signaling on synaptic plasticity and cognitive function in adult wild-type mice. To reduce caveats associated with long-term Reelin application, a single-injection strategy was used. This strategy allowed us to track Reelin entry into the hippocampus, activation of signal transduction, and provided a time point from which to compare specific changes to spine density, LTP, and cognitive function.

The detection of Reelin in human cerebrospinal fluid suggests Reelin can be transported throughout the CNS (Ignatova et al. 2004). The fast dissemination of Reelin from the ventricle into the hippocampus further suggests that Reelin can be readily transported throughout the CNS. Furthermore, Reelin dissemination was associated with robust activation of Dab1 and CREB at the 15-min post-injection time point. These results confirm that our purified recombinant Reelin is biologically active and that increased Reelin signaling precedes changes observed in hippocampal plasticity and CA1 dendritic spine morphology 5 d post-injection.

Absence of Reelin or Dab1 results in reduced dendritic arborization and dendritic spine density in adult mice (Niu et al. 2004). Conversely, chronic in vitro Reelin supplementation can rescue the reduction in dendritic spines exhibited in neuronal cultures lacking Reelin (Qiu et al. 2006a). The present study is the first to report the observation that Reelin can alter in vivo dendritic spine density in hippocampal area CA1 of wild-type mice. Interestingly, a single injection of Reelin has the capability of increasing dendritic spine density. New spine growth is often associated with synapse formation (Knott et al. 2002; Holtmaat et al. 2006) and spines that persist over a week are likely to last for standard two-shock protocol as previously described (Weeber et al. 2002a). Three separate groups of mice were tested for freezing to the context at either 1, 24, or 72 h after training. Reelin-injected mice exhibited enhanced context-dependent freezing at both 24-h and 72-h time points, but not at the 1-h time point (Fig. 9). Taken together, these data demonstrate that Reelin supplementation in wild-type mice enhances both associative and spatial learning and memory with a single injection of Reelin 5 d prior to the start of training.

Figure 5. Reelin significantly increased spine density in wild-type mice compared to ApoER2 KO mice 5 d post-injection in area CA1. Spine increases are expressed as a percentage of experimental saline controls. (A) Representative AO dendrites of both Reelin-treated groups at 5 d post-injection. (B) Averaged AO percent dendritic spine increase for each group (wild-type saline: 100.0 ± 4.4%; wild-type Reelin: 142.3 ± 6.5%; ApoER2 KO saline: 100 ± 4.3%; ER2 KO Reelin: 113.6 ± 3.7%). Reelin significantly increased AO spine density in area CA1 compared to saline- or Reelin-injected ER2 −/− mice (**P < 0.01; ***P < 0.001). Reelin significantly increased BS spine density in area CA1 compared to saline- or Reelin-injected ER2 −/− mice (**P < 0.01; ***P < 0.001).

Figure 6. Reelin enhances hippocampal synaptic plasticity. Mice were sacrificed 5 d following single, bilateral injections for electrophysiology experiments. LTP was induced with TBS (five bursts of 200 Hz separated by 200 msec, repeated six times with 10 sec between the six trains; arrow) after 20 min of baseline recording and changes in fEPSP slope are expressed as a percentage of baseline. (A) Representative fEPSP traces from both saline (white) and Reelin (black) injected hippocampi. (B) Reelin injection enhanced LTP in area CA1. The last 5 min of fEPSPs slope recordings were averaged (bar) for both saline (n = 9) and Reelin (n = 10) injected (**P < 0.01). (C) Output field analysis following increasing field stimulation fit with nonlinear regression. There were no significant differences between experimental groups. (D) PPF was induced with the use of paired pulses given with an initial delay of 20 msec and the time to the second pulse was increased 20 msec incrementally until a final delay of 300 msec was reached. There was no significant difference between experimental groups.
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Materials and Methods

Animals
All animals were ~4 mo old and obtained from Jackson Labs (Bar Harbor, ME). Animals were housed in standard 12-h light–dark cycle and fed normal chow ad libitum. All animal care protocols used were in accordance with the Institutional Animal Care and Use Committee of the University of South Florida.

Figure 7. There is no effect of Reelin on general locomotor or anxiety levels in wild-type mice. (A) After 5 d post-injection of saline (n = 7) or Reelin (n = 6), mice underwent open-field testing as a locomotor and general anxiety control for behavioral testing. Data represent the ratio of time spent (sec) in the open field vs. the perimeter of the field. There were no significant differences between experimental groups. (B) After 5 d post-injection, mice underwent elevated plus maze testing as a general anxiety control. Data represent the ratio of time spent (sec) in the open areas vs. the closed arms of the elevated maze. There were no significant differences between experimental groups.

Figure 8. Reelin supplementation enhanced spatial learning and memory. Mice began training in the HPWM 5 d post-injection of saline (white, n = 7) or Reelin (black, n = 5). Mice were trained in the HPWM for 4 d, four trials per day. On day 5, the platform was removed and a 60-s probe trial was conducted. On trial days 6 and 7, the platform was moved to the opposite quadrant. (A) Reelin significantly reduced latency to the platform during HPWM training. (B) Analysis of day 1 training reveals Reelin reduced latencies to the platform after the second trial compared to the initial trial. Reelin significantly lowered latencies on trials 3 and 4 compared to saline-injected animals. (C) Reelin increased target quadrant entries during the probe trial compared to saline-treated animals (*p < 0.05; *p < 0.01).

Reelin purification
Recombinant Reelin was produced using HEK293 cells that were stably transfected with the full-length Reelin construct pCrl vector as previously described (Qiu and Weeber 2007).

Cannulations
Mice were anesthetized with isoflurane and placed on a Kopf stereotaxic surgery apparatus (Stoelting Co.). To insert cannulas, two holes were drilled through the skull and bilateral cannulas (Plastics One Inc., C-C distance = 1.9 mm) were implanted into the lateral ventricles (A.P. = 0.35 from bregma, L ± 0.75 mm from the sagittal suture, and V = 2.5 mm from flat skull surface) under aseptic conditions. The cannulas were secured with locite and dental cement, and the incisions were sutured. Cannulated mice were observed 2 h post-operatively in individual cages on a warm heating pad. The mice were kept in their individual cages for the duration of all behavior experiments to prevent other mice from tampering with the cannulas. Rectal temperatures were measured daily to monitor for infectious or inflammatory responses and any mouse with a temperature of 100.5 °F or higher was euthanized. Mice were fed a wet mash mixture in a Petri dish for 3 d post-operative and allowed to recover for a total of 5 d. On post-operative day 5, mice were injected with a 1-μL single injection of saline (0.9% NaCl) or Reelin (300 nM in media).

Direct bilateral injections
Mice were anesthetized as described in cannulations above. To allow direct passage of the needle (Hamilton) into the brain, two holes were drilled through the skull and 0.5 μL of solution containing saline (0.9% NaCl) or Reelin (210 μM in media) was injected (A.P. = −0.24 from bregma, L ± 0.75 mm from the sagittal suture, and V = −2.5 mm from flat skull surface) using a Quintessential Stereotaxic Injector (Kopf, Stoelting Co.) at a rate of 1.0 μL/min and was left in place for 45 sec. The needle was then removed and the holes were sealed with dental cement and the incisions sutured. Mice were treated post-operatively as described above in cannulations.

Immunohistochemistry
At 15 min, 3 h, and 5 d following injection, mice were transcardially perfused with saline (0.9% NaCl) containing 0.5 × phosphatase inhibitor cocktails I and II (Sigma). Brains were hemisected and half-brains were post-fixed in 4% PFA overnight at 4°C and then cryoprotected in 30% sucrose–phosphate-buffered saline (PBS) for 24 h, embedded in OCT and serial-sectioned at 26 μm coronally using a cryostat Microm HM 550 (Thermo Scientific). Free-floating brain sections were blocked in PBS containing 10% normal donkey serum (NDS), 2% bovine serum albumin.

months (Holtmaat et al. 2005). Although Reelin levels and Reelin signaling have returned to baseline 5 d following injection, enduring changes in dendritic spine density likely contribute to the observed enhancement of synaptic plasticity and learning and memory.

Disruption of ApoER2 results in associative and spatial learning deficits and impairment of hippocampal LTP in adult mice (Trommsdorff et al. 1999). In vitro Reelin enhancement of LTP requires the presence of ApoER2 (Weeber et al. 2002a). In agreement with these findings, we found that deletion of ApoER2 prevented the Reelin-mediated increase in Dab1 and CREB activation. Importantly, the absence of ApoER2 does not appear to affect the transport of Reelin following injection. In agreement with studies using acute hippocampal slices, organotypic hippocampal cultures, and primary neuronal cultures, our in vivo single, bilateral injection of Reelin resulted in rapid signaling in an ApoER2-dependent manner.

The present studies expand the known role of Reelin from synaptic modulator through localized signal transduction to a long-lasting modulator of dendritic morphology. Furthermore, the enhancement of synaptic plasticity and learning ability for longer periods of time implicates Reelin signaling as a potential future therapeutic target for human disorders that involve cognitive decline. Conversely, this implicates that reductions in Reelin signaling may underlie impaired cognitive ability found in human disease states, such as Alzheimer’s disease, schizophrenia, and autism spectrum disorders.
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(BSA), 0.2% Triton X-100, 1 × phosphatase inhibitor cocktail 1/II (Sigma), and 0.1% sodium azide for 1 h at room temperature. For changes in Reelin, sections were incubated overnight in primary antibody solution (blocking solution minus NDS) with 1:1000 anti-Reelin antibodies (G10, MAB5364; Millipore). Changes in the phosphorylation states of Dab1 and CREB were determined using phospho-specific antibodies recognizing p-Tyr220 (Abcam, ab78200) and p-Ser133 (Cell Signal, #9198), respectively. The day after antibody incubation, sections were washed in PBS and then incubated with Alexa-conjugated secondary antibodies for 2 h at room temperature. After washing, sections were mounted on gelatin-coated slides, Prolong (P36930; Invitrogen) was applied and slides were coverslipped. Images were taken with a 10 × objective on a Zeiss upright fluorescence microscope (AxioImager.Z1) and were processed using ImageJ software (NIH). The total time spent in the open arms and closed arms were recorded and analyzed with ANY-Maze animal activity system (Stoelting Co.). Anxiety levels were assessed as the ratio of time spent in the open field vs. closed field.

Western blot analysis
We assessed P38K activity through quantitative Western blot analysis of phosphorylation changes to serine/threonine protein kinase (Akt). There were no significant changes to the phosphorylation state of Akt at 15 min or 3 h post-injection (data not shown).

Golgi staining and analysis of dendritic morphology in vivo
Hemibrains from saline-perfused mice (described in section above) were processed for Golgi staining with the FD Rapid Golgi Stain kit (FD NeuroTechnologies) as previously described (Hoe et al. 2009). Brains were sliced using a Vibratome (VT1000S, Leica) at a thickness of 150 μm. Bright-field microscopy (Axioplan 2; Zeiss) images (at 63 × magnification) were taken of CA1 pyramidal cells (n = 56 neurons per treatment). The images were coded and dendritic spines were counted in a blinded manner using the Neurulcida software (MicroBrightField).

Open field
General locomotor activity and anxiety were measured with an open field task. Following 3-h post-single injections into the cannulas, B6C3Fe-a/a mice were placed in an open field chamber (40 × 40 × 27 cm) and allowed to free roam for 15 min under moderate room-lit conditions. Both the time and distance spent in the center and perimeter regions were recorded and analyzed with ANY-Maze animal activity system (Stoelting Co.). Anxiety levels were assessed as the ratio of time spent in the center to time spent at the perimeter.

Elevated plus maze (EPM)
General anxiety levels were measured with the use of the EPM. Following 5 h post-single injections into the cannulas, B6C3Fe-a/a mice were tested in the EPM. The EPM consists of two-well-lit open arms (30 × 5 cm) facing each other and two enclosed arms (30 × 5 × 15 cm) which faced each other. Each arm is attached to a central corner open-square platform (4.5 cm off-centre and elevated 40 cm off the floor). Mice were placed in the central open square platform facing the closed arms and allowed to explore for a 5-min period. Mouse activity was monitored by a digital camera (WV-BP330; Panasonic). The total time spent in the open arms and closed arms were recorded and analyzed with ANY-Maze animal activity system (Stoelting Co.). Anxiety levels were assessed as the ratio of time spent in the open field vs. closed field.

Fear conditioning
Following 5 d post-single cannulated injections, B6C3Fe-a/a mice wild-type mice were tested in the contextual fear conditioning as previously described (Weeber et al. 2002a,b). Briefly, an aversive stimulus was paired with an auditory conditioned stimulus (CS) within a novel environment. Animals were placed in the fear-conditioning apparatus for 5 min, then a 30-sec acoustic CS was delivered with a 0.5-mA unconditioned stimulus (US) applied to the floor grid during the last sec of the CS. Training consisted of two US paired with two CS with a 2-min interval. The mice were placed in the chamber and monitored for freezing to the context ~1 h, 24 h, and 72 h after training. Learning was assessed by measuring freezing behavior every 1 sec. Freezing behavior was recorded and processed by Freezing V1.2.00 software (ANY-Maze) throughout testing.

HPWM
Following 5 d post-single cannulated injections, 4-mo-old B6C3Fe-a/a wild-type mice were tested in the HPWM as previously described. Briefly, the mice were trained to locate an escape platform hidden beneath the water surface. The training trials lasted for 4 d, four trials per day, with an intertrail interval of 15 min. Goal latencies were obtained using automated video tracking software (ANY-maze). On day 5, the platform was removed and mice were allowed to free swim for 60 sec (probe) and time spent in quadrants and number of target platform entries calculated. On days 6 and 7, the platform was moved to the opposite quadrant and mice were retrained to locate it. Another probe test was performed on day 8.

Extracellular recordings
Following 5 d post-single direct injections, C57BL/6J mice wild-type mice were sacrificed and hippocampi were dissected out for electrophysiological experimental paradigms as previously described (Weeber et al. 2002a; Beffert et al. 2005; Peters et al. 2006). fEPSPs were obtained from area CA1 stratum radiatum with the use of a glass microeleetrode filled with ACSF (2–4 μl). fEPSPs were evoked through stimulation of the Schaffer collaterals using a 0.1-msec biphasic pulse delivered every 20 sec. After a consistent response to a voltage stimulus was established, threshold voltage for evoking fEPSPs was determined and the voltage was increased incrementally every 0.5 mV until the maximum amplitude of the fEPSP was reached (I/O curve). All other stimulation paradigms were induced at the same voltage, defined as 50% of the stimulus voltage used to produce the maximum fEPSP amplitude, for each individual slice. PPF was induced with two paired pulses given with an initial delay of 20 msec and the time to the second pulse incrementally increased 20 msec.
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Statistical analyses

Data obtained from open field, elevated plus maze, fear conditioning, and electrophysiological experiments were analyzed using the Student’s two-tailed t-tests and significance was assigned at P < 0.05 (Prism Software) unless otherwise indicated. Differences in HPWM day 1 trial latencies were analyzed by a two-way ANOVA and followed by a Student’s t-test, and significance was assigned at P < 0.05 (Prism Software).

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