

β 1-Integrins Are Required for Hippocampal AMPA Receptor-Dependent Synaptic Transmission, Synaptic Plasticity, and Working Memory

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Integrins comprise a large family of cell adhesion receptors that mediate diverse biological events through cell–cell and cell–extracellular matrix interactions. Recent studies have shown that several integrins are localized to synapses with suggested roles in synaptic plasticity and memory formation. We generated a postnatal forebrain and excitatory neuron-specific knock-out of β 1-integrin in the mouse. Electrophysiological studies demonstrated that these mutants have impaired synaptic transmission through AMPA receptors and diminished NMDA receptor-dependent long-term potentiation. Despite the impairment in hippocampal synaptic transmission, the mutants displayed normal hippocampal-dependent spatial and contextual memory but were impaired in a hippocampal-dependent, nonmatching-to-place working memory task. These phenotypes parallel those observed in animals carrying knock-outs of the GluR1 (glutamate receptor subunit 1) subunit of the AMPA receptor. These observations suggest a new function of β 1-integrins as regulators of synaptic glutamate receptor function and working memory.

Key words: integrins; AMPA receptors; basal synaptic transmission; LTP; working memory; synaptic plasticity

Introduction

A key feature underlying nervous system function is the intricate coordination of signaling at synapses. Dynamic changes in the strength of signaling between the presynaptic and postsynaptic terminals in response to experience underlie memory formation through the actions of a plethora of molecules. Cell adhesion molecules, which are involved in mediating cell–cell and cell–extracellular matrix interactions, have been implicated in synaptic function, synaptic plasticity, and memory formation (for review, see Benson et al., 2000; Dityatev and Schachner, 2003; Yamagata et al., 2003).

One family of important cell adhesion receptors is the integrins. Integrins are transmembrane heterodimers of α - and β -subunits (Giancotti and Rouslahti, 1999; Coppelino and Dedhar, 2000; Schwartz, 2001; van der Flier and Sonnenberg, 2001). Studies of integrins from tissues other than the nervous system have revealed several unique features of these molecules. First,

integrins transduce biochemical signals bidirectionally. In the “outside-in” signal transduction pathway, integrins can activate a number of cytoplasmic tyrosine kinases in response to extracellular ligand binding, including FAK (focal adhesion kinase), Src, and PI3 (phosphatidylinositol 3-kinase), thus leading to the activation of several dominant signaling pathways such as the MAPK (mitogen-activated protein kinase) and Akt/protein kinase B (PKB) cascades. In the “inside-out” pathway, physiological events within a cell can modulate the affinity of integrins for their ligands through an induced, transmembrane conformational change (Tadokoro et al., 2003). Second, integrins interact physically with extracellular molecules and the cytoskeleton, thus providing a physical link between the external environment of the cell and intracellular components that dictate its architecture. Although these remarkable features are not fully understood mechanistically, they provide enormous potential power to these molecules for regulating synaptic functions that underlie animal behavior.

Indeed, accumulating evidence has shown that many integrins are localized to synapses (Einheber et al., 1996; Pinkstaff et al., 1999; Prokop, 1999; Chan et al., 2003) and that they are involved in synaptic function and memory formation. For example, *in vitro* studies have shown that integrin activation produces rapid changes in spontaneous electrical activity and voltage-gated Ca^{2+} currents on *Lymnaea* motoneurons (Wildering et al., 2002) or enhancement of fast excitatory transmission in rat hippocampal slices (Kramar et al., 2003). Long-term potentiation (LTP) generated at the hippocampal CA3:CA1 synapses becomes unstable in the presence of integrin antagonists (Bahr et al., 1997;

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Staubli et al., 1998; Chun et al., 2001; Kramar et al., 2002). A direct role of integrins in memory formation was first demonstrated in the *Drosophila* olfactory memory mutant, *Volado*, which is impaired in the expression of an α -integrin (Grotewiel et al., 1998). Furthermore, mice triply heterozygous at the $\alpha 3$ -, $\alpha 5$ -, and $\alpha 8$ -integrin genes are defective in paired-pulse facilitation (PPF), LTP, and spatial memory (Chan et al., 2003). Despite the broad support for the integrin family of molecules in synaptic function and behavior, the roles for individual integrin subunits in different aspects of neuronal physiology and types of memory remain unclear.

In the present study, we examined the effects of a postnatal and forebrain-specific knock-out of the $\beta 1$ -integrin gene on synaptic physiology and memory formation. Our results show that basal excitatory synaptic transmission through AMPA receptors and LTP in the hippocampus are impaired in these $\beta 1$ -integrin mutants, but PPF remains intact. Behavioral studies revealed a selective deficit in a hippocampal-dependent working memory task but not in multiple variations of hippocampal-dependent spatial memory or conditioned fear memory tasks. Interestingly, a similar dissociation between LTP and spatial memory and a parallel deficit in working memory have also been observed in knock-outs of the AMPA receptor subunit glutamate receptor subunit 1 (GluR1), suggesting a regulatory link between integrin signaling and function of the AMPA receptor.

Materials and Methods

Mice and genotyping

The floxed $\beta 1$ -integrin and α -calcium/calmodulin-dependent protein kinase II–cAMP response element (CaMKII–Cre) mice were kindly provided by E. Fuchs (Rockefeller University) and S. Tonegawa (Massachusetts Institute of Technology, Cambridge, MA), respectively. Animals were maintained as outcrossed stocks to C57BL/6 (The Jackson Laboratory, Bar Harbor, ME). Floxed $\beta 1$ -integrin knock-outs and the control littermates were obtained by crossing Cre/+; f($\beta 1$)/+ to +/+; f($\beta 1$)/+ animals. PCR genotyping of tail DNA used the following primers: CreF2 (5'-GGCGTTTCTGAGCATACCTGGAA-3') and CreR2 (5'-CACCATTGCCCTGTTTCACTATC-3') for Cre recombinase; $\beta 1$ -5 forward (For) (5'-CGCAGAACAATAGGTGCTGAAATTAC-3') and $\beta 1$ -5 reverse (Rev) (5'-TGACACTGAGAACCACAAACGGC-3') for the 5' loxP insertion in the $\beta 1$ -integrin gene; $\beta 1$ -3For (5'-CGGCTCAAAGCAGAGTGTCAGTC-3') and $\beta 1$ -3Rev (5'-CCACAACCTTCCAGTTAGCTCTC-3') for 3' loxP insertion in the gene.

Mice were housed in conventional animal cages and maintained on a 12 h light/dark cycle. All animals were handled and treated during the experiments in ways approved by the Baylor College of Medicine Institutional Animal Care and Use Committee and according to national regulations and policies.

Western blotting and immunohistochemistry

For Western blotting, freshly dissected hippocampi were homogenized in buffer containing 137 mM NaCl, 20 mM Tris-HCl, pH 8.0, 10% glycerol, 1% NP40, 0.1% SDS, and 0.1% sodium desoxycholate. Twenty-five micrograms of the hippocampal protein extract was fractionated by 7.5% SDS-PAGE. The separated proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA). The membrane was incubated with a 1:500 dilution of anti-phospho-GluR1 [serine 831 (S831)] antibody (Upstate Biotechnology, Lake Placid, NY), 1:500 anti-phospho-GluR1 [serine 845 (S845)] antibody (Upstate Biotechnology), 1:400 anti-GluR1 antibody (Chemicon, Temecula, CA), 1:200 anti-GluR2/3 antibody (Upstate Biotechnology), or 1:100 anti-actin antibody (Chemicon) for 1 h at room temperature, washed three times, and then incubated for 30 min at room temperature with a horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA). Each blot was developed with ECL Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, UK). Signals detected in Western

blots were scanned and quantified using Scion Image software (Scion, Frederick, CA). The net signal (total signal – background) for each animal was normalized against the corresponding signal for actin, and the average signal was calculated for each genotype. The relative expression level of each AMPA receptor subunit was expressed as the ratio of the average signal for each genotype relative to the Cre; +/+ control animals.

Immunohistochemical experiments were performed as described previously (Chan et al., 2003). Animals were anesthetized with isoflurane and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer for 10 min. The dissected brain was separated into two hemispheres and post-fixed in 4% paraformaldehyde for an additional 1 h at 23°C. Cryosections of the brain were then incubated in 7.5% goat serum in PBS [(in mM) 137 NaCl, 2.7 KCl, 10 phosphate buffer, pH 7.2] containing 0.15% Triton X-100 for 1–2 h at 23°C and then with 150 μ l of diluted primary antisera [1:100 anti-GluR1 antibody (Chemicon), 1:100 anti-GluR2/3 antibody (Upstate Biotechnology), or 1:500 anti-NR1 antibody (Upstate Biotechnology)] overnight at 4°C. Sections were washed twice at 23°C with PBS plus 0.2% Triton X-100, pH 7.4 (PBST) for 10 min and then incubated with a biotinylated secondary antibody for 1 h. The sections were washed twice in PBST and finally incubated with Vectastain ABC reagent (Vector Laboratories, Burlingame, CA) with high salt (0.5 M NaCl) to reduce background staining. The immunostaining was visualized by reacting sections with 1 mg/ml diaminobenzidine/0.3% H₂O₂ for 5–10 min. After two washes in distilled water, the sections were mounted on slides with AquaMount (Lerner Laboratories, Pittsburgh, PA).

Electron microscopy of CA1 area

Animals were anesthetized with isoflurane (50 mg/kg) and perfused transcardially with 0.1 M phosphate buffer, pH 7.2, followed by a fixative containing 2.5% glutaraldehyde, 2.0% formaldehyde in 0.1 M cacodylate buffer with 2 mM CaCl₂. After postfixation of the dissected brain overnight at 4°C, the hippocampal CA1 region was dissected and fixed again with 1% OsO₄ in 0.1 M cacodylate buffer for 1 h. After washing, the tissue fragments were stained with 1% uranyl acetate for 1 h. The tissue was then dehydrated with an ethanol series and embedded in Glauert Med embedding resin (Electron Microscopy Sciences, Hatfield, PA). Ultrathin sections were cut from the stratum radiatum and visualized with a Hitachi (Yokohama, Japan) 7500 digital electron microscope.

Hippocampal slice preparation and electrophysiology

Extracellular field recordings were obtained from the area CA1 stratum radiatum. Stimulation was delivered with a bipolar Teflon-coated platinum electrode and recording was obtained with the use of a glass micro-electrode filled with artificial CSF (ACSF) [(in mM) 125 NaCl, 2.5 KCl, 1.24 NaH₂PO₄, 25 NaHCO₃, 10 D-glucose, 2 CaCl₂, and 1 MgCl₂] (resistance, 1–4 M Ω). Input/output curves were generated by single-pulse stimulation of Schaffer collateral inputs with 2.5–28.5 mA in 2.5 mA increments. The 100 Hz stimulation consisted of two trains of 100 Hz frequency stimulation for 1 s, with each train separated by a 20 s interval. The saturating LTP protocol consisted of two trains of 100 Hz frequency stimulation for 1 s, with each train separated by a 20 s interval, repeated four times with each pair of stimulations separated by 6 min. Stimulus intensities were adjusted to give population EPSPs (pEPSPs) with slopes that were ≤ 50 , 85, or 100% that of the maximum determined from an input/output curve. Potentiation was measured as the increase of the mean pEPSP after tetanic stimulation normalized to the mean pEPSP for the duration of the baseline recording. Experimental results were obtained from those slices that exhibited stable baseline synaptic transmission for a minimum of 30 min before the delivery of the LTP-inducing stimulus. Two-way ANOVA was used to determine statistical differences in synaptic transmission, PPF, and LTP. Depolarization was performed by means of the delivery of three groups of five trains of 100 Hz stimulation, with each train separated by 20 s and each group separated by 2 min. Total depolarization (the integral of the tetanic depolarization response) was calculated as the total area of high-frequency-induced depolarization during the entire 1 s application of 100 Hz stimulation.

Behavioral assays

Water maze. Briefly, mice were handled extensively for 2 weeks before training in the water maze. The animals were kept in individual cages

during training and allowed to acclimatize to the water maze room for 1 h before the start of the experiment on each day. Each trial began by placing the mouse into the water facing the wall of the maze, and the animal was allowed to search for the platform until the animal climbed onto the platform or when a maximum of 60 s had elapsed. The animal was allowed to remain on the platform for 20 s before it was returned to its cage. Four trials were performed each day with an intertrial interval (ITI) of 60 min. Trials were balanced for starting positions and began at the 12, 3, 6, or 9 o'clock position of the pool. The platform location remained constant for any individual mouse for the duration of the training, but different animals were trained with the platform in different positions to avoid quadrant bias. Animals were trained for 5 d at the same time on each day. Twenty-four hours and/or 1 week after the last training trial, a probe trial (or transfer test) was administered in which the platform was removed from the pool, and animals were placed in a quadrant opposite to the location of the training platform and allowed to swim for 60 s. The time the mice spent searching for the platform in each quadrant and the number of times the mice crossed the virtual platform location was measured. The same procedure was performed in the "pattern completion" version of the probe trial, except that three of the four extramaze cues present on the surrounding walls of the water maze room had been removed.

Fear conditioning. During training, each mouse was placed inside a conditioning chamber (26 × 22 × 18 cm; San Diego Instruments) in the presence of white light and background noise generated by a small fan for 2 min before the onset of a conditioned stimulus (CS) (an 85 dB tone), which lasted for 30 s. A 2 s unconditioned stimulus (US) footshock (0.6 mA) was delivered immediately after the termination of the CS. Each mouse remained in the chamber for an additional 60 s, followed by another CS–US pairing. Each mouse was returned to its home cage after another 30 s. The test for contextual fear memory was performed 2 and 24 h after training by measuring freezing behavior during a 5 min test in the conditioning chamber. Freezing was defined as a lack of movement for 2 s or longer in each 5 s interval. Cued fear memory was tested in the presence of red light, coconut odor, and the absence of background noise. In addition, a triangular black box was put inside the conditioning chamber, and the grid floor was covered to present context different from that experienced during training. Each mouse was placed in this novel context for 3 min, 24 h after training to record baseline freezing after which they were exposed to the CS for another 3 min. Freezing behavior was recorded and processed by San Diego Instruments Photobeam Activity System (San Diego Instruments, San Diego, CA) software throughout each testing session.

T-maze nonmatching-to-place working memory task. The working memory task was performed as described by Reisel et al. (2002). The T-maze was made of wood and was elevated to a height of 1 m from the floor. It consisted of a start arm (47 × 10 cm) and two identical goal arms (35 × 10 cm), each covered with the bedding used in the animals' home cage environment. Animals were restricted to 1 h of food access per day, which resulted in animals maintaining at 85% of their free-feeding weight. The animals were habituated to the maze and to drinking the food reward (50 μ l of 50% sweetened, condensed milk) from a small dish (2 cm diameter) for 5 d before testing.

Each testing trial consisted of a sample run followed by a choice run. On the sample run, food reward was placed ~3 cm from the end on both goal arms, but access to one of them was blocked by a wooden block. A mouse was put inside an opaque cylindrical tube and transported from the home cage to the end of the start arm, where it was released facing the experimenter end wall of the start arm. The animal was allowed to find and drink the food reward in the unblocked goal arm. The animal was then collected into the transfer tube and returned to the end of the start arm. The time required for this transfer was <5 s and represented the minimum time lapse between the sample and the choice runs but was not included in the actual timing of the delay time. For the choice run, the wooden block was removed, and the animal was released with or without delay to choose either goal arm. The animal was allowed to drink the food reward if it chose the previously unvisited arm. The animal was then returned to the home cage. Four trials with a 20 min ITI were performed on each animal per day for 18 d. The reward arm for each trial was

assigned pseudorandomly, i.e., two times for each goal arm each day but with a different order for each animal. Working memory was expressed as the ratio of correct choices over total choices binned for 16 trials. For the statistical analysis, two-way ANOVA was performed followed by *post hoc* Scheffé's test.

Expression assay for surface receptors. Surface biotinylation and pull-down of cell surface proteins were performed as described previously (Thomas-Crusells et al., 2003). Freshly dissected hippocampal slices (400 μ m) were placed in mesh chambers, washed twice in ice-cold ACSF for 5 min, and incubated with 100 μ M sulfo-succinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate (NHS-SS-biotin; Sigma, St. Louis, MO) in ACSF for 45 min. The biotinylated slices were then washed twice with 1 μ M lysine (Sigma) in ACSF for 5 min to block the excess nonbound NHS-SS-biotin. To extract total proteins, two biotinylated slices were homogenized in 200 μ l of cell lysis buffer [1% Triton X-100, 0.1% SDS, 1 mM EDTA, 50 mM NaCl, 20 mM Tris, pH 7.5, and protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN)] and incubated with rotation at 4°C for 30 min. The extract was then centrifuged for 5 min at 13,800 × g to remove extra debris. To precipitate the surface-biotinylated proteins, 400 μ g of protein extract from the supernatant was incubated with 40 μ l of UltraLink Immobilized Streptavidin beads (Pierce, Rockford, IL) in a total 800 μ l of ACSF/protease inhibitor volume overnight at 4°C with gentle mixing. The precipitates were pulled down and washed twice with 500 μ l of ACSF/protease inhibitors by brief centrifugation and, finally, resuspended in 40 μ l of SDS-PAGE buffer. To compare the relative amount of surface biotinylated proteins, one-tenth of the input extract (40 μ g) and precipitates (4 μ l) were loaded for Western blotting analysis, as described above.

Results

Generation of forebrain-specific, knock-out of the β 1-integrin gene

To define the role of β 1-integrin in adult CNS function, we generated a β 1-integrin postnatal and spatial knock-out [$f(\beta$ 1)KO] by crossing a forebrain and excitatory neuron-specific Cre transgene, driven by the α -CaMKII promoter, into the genome of animals homozygous for a floxed β 1-integrin allele [$f(\beta$ 1)]. Cre-induced deletion of exon 2, which was flanked by *loxP* sites, predicted early termination of translation (Fig. 1A) (Tsien et al., 1996; Raghavan et al., 2000). We reasoned that β 1-integrin would be a good candidate for studying integrin function in the CNS, because it is the only known subunit partner for several α -integrins, including α 3, α 5, and α 8. PCR analyses of genomic DNA isolated from the hippocampus of these and control animals confirmed the occurrence of Cre-dependent recombination at exon 2 in the $f(\beta$ 1)KO animals (Fig. 1B). The presence of PCR products representing the nonrecombined floxed β 1-integrin allele was also observed in the knock-outs because glial cells, which do not express the Cre protein, make up >30% of the cell population of the hippocampus (Geisert et al., 2002). β 1-Integrin expression in the forebrain is extremely low and cannot be reliably detected using standard protocols for RNA *in situ* hybridization (Pinkstaff et al., 1998) (data not shown).

We did not observe any alterations in CNS gross anatomy in mice deficient in β 1-integrin (data not shown) (see Fig. 7A). To determine whether the postnatal loss of β 1-integrins produces structural defects in synapses, we studied >700 axospinous and axodendritic synaptic contacts from the stratum radiatum of the hippocampal CA1 region using electron microscopy. No detectable morphological differences were observed comparing synapses of $f(\beta$ 1)KO [Cre/+; $f(\beta$ 1)/ $f(\beta$ 1)] animals with Cre (Cre/+; +/+) control animals (Fig. 2A). We compared the distribution of synapse size, determined by the length of the postsynaptic density, as well as the density of synapses in a standardized area for animals 6 months of age for the two groups. There was no

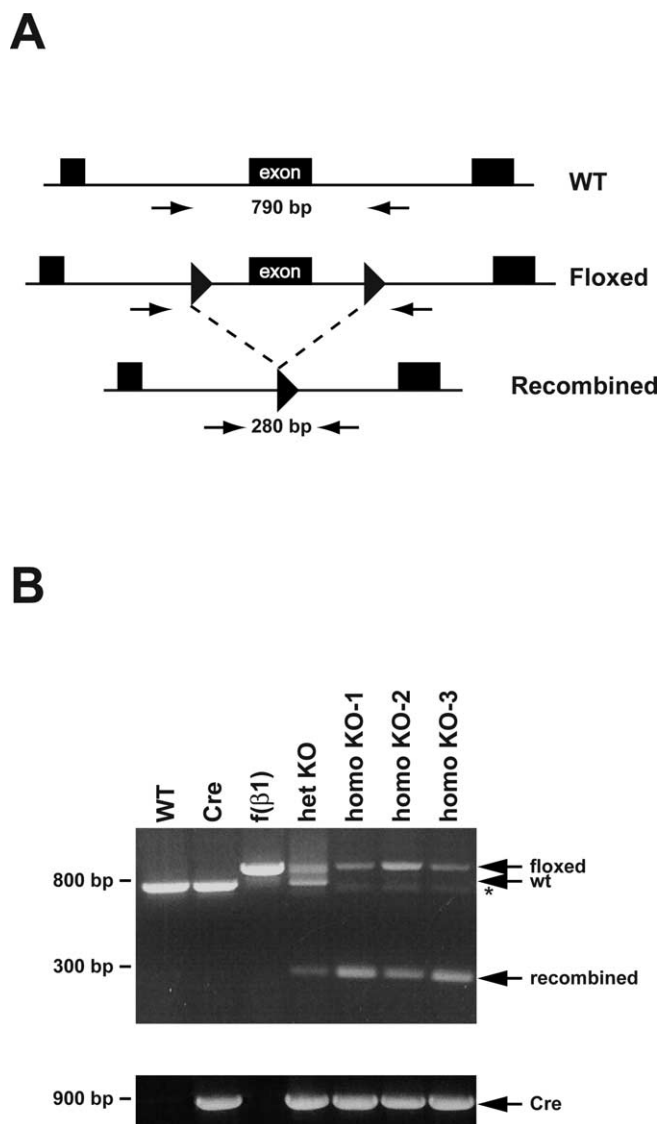


Figure 1. Cre-dependent recombination of the floxed $\beta 1$ -integrin allele. **A**, Three alleles of the $\beta 1$ -integrin gene are depicted. WT, Wild-type allele; Floxed, allele with loxP sequences flanking exon 2 in the same orientation; Recombined, allele with the sequences between the two loxP sites, including exon 2, deleted as a result of Cre-induced recombination. Triangles, loxP sequences; arrows, PCR primers used to assay for the deletion event. **B**, PCR analyses of hippocampal genomic DNA isolated from WT, Cre (Cre/+; +/+), f(β1) [+/+; f(β1)/f(β1)], heterozygous KO (het KO) [Cre/+; f(β1)/+], and three different homozygous KO (homo KO) [Cre/+; f(β1)/f(β1)] animals. The arrows indicate the identity of the PCR products from the corresponding $\beta 1$ -integrin alleles depicted in **A** or from the Cre transgene. The asterisk indicates the DNA heteroduplex formed from the floxed allele PCR product and the deleted allele PCR product. The PCR conditions were adjusted so that there was little or no competition during PCR between the products of the various alleles; note the approximately equal molar representation of the floxed and recombined alleles in het KO after PCR, taking into account the increased mass of the larger products, which leads to increased fluorescence. With 30% or more of the genomic DNA contributed by glial or nonexcitatory neurons, the relative strengths of the PCR products representing the floxed and recombined alleles of the homozygous KOs indicate that the gene was deleted in the majority of excitatory neurons.

statistical difference between the two groups in either the density [Cre/+, 3.22 ± 0.33 synapses/ $10 \mu\text{m}^2$; f(β1)KO, 3.19 ± 0.29 synapses/ $10 \mu\text{m}^2$; $p = 0.89$, t test] or the length [Cre/+, $0.23 \pm 0.003 \mu\text{m}$; f(β1)KO, $0.22 \pm 0.003 \mu\text{m}$; $p = 0.35$, t test] of the synapses (Fig. 2B), indicating that synaptic structures remain intact in the f(β1)KO animals.

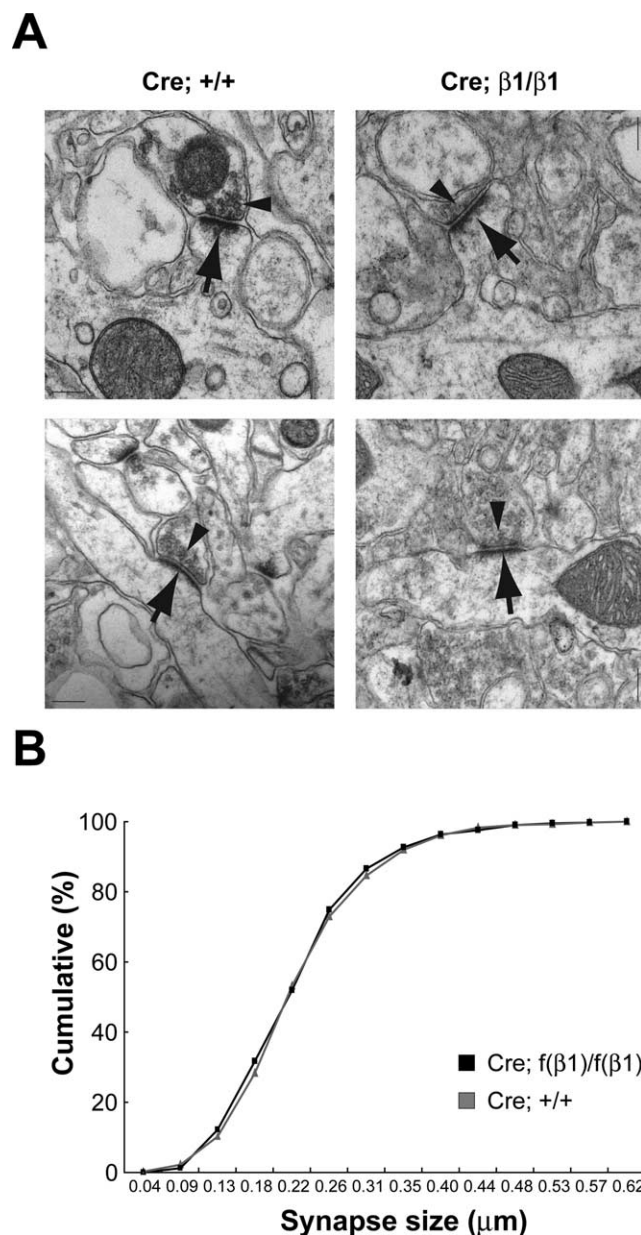


Figure 2. Normal synaptic ultrastructure in $\beta 1$ -integrin knock-outs. **A**, Representative electron micrographs of axosynaptic (top panels) and axodendritic (bottom panels) synaptic contacts in the hippocampal CA1 stratum radiatum region of Cre control (left column) and f(β1)KO (right column) animals. No detectable morphological difference in either type of contact was detected between the two genotypes at either presynaptic (arrowheads) or postsynaptic (arrows) terminals. Scale bars, $0.3 \mu\text{m}$. **B**, Cumulative frequency plot of synapse size in the CA1 stratum radiatum, measured as the length of the postsynaptic density. Synapse size distribution of f(β1)KOs was indistinguishable from the Cre control animals.

Impaired basal synaptic transmission in f(β1)KO mice

We began physiological studies on the roles of $\beta 1$ -integrin in the adult CNS by examining the basal synaptic transmission in the Shaffer collateral pathway of the hippocampus using field recordings of hippocampal slices (Fig. 3). The f(β1)KO slices showed a significant reduction in evoked field EPSPs (fEPSPs) across a range of stimulus intensities (Fig. 3A). In contrast, when measured in the same fEPSP trace, there was no difference in fiber volley amplitude in the mutant mice compared with the two parental controls genotypes, indicating an equivalent transfer of the presynaptic stimulus into an axonal depolarization (Fig. 3B).

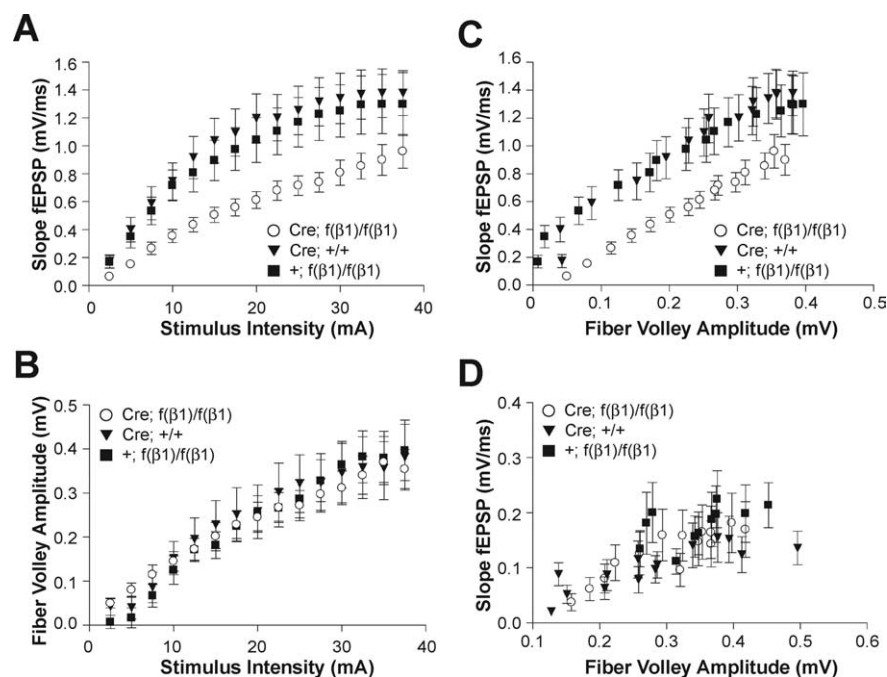


Figure 3. Impaired AMPA receptor synaptic transmission in $\beta 1$ -integrin knock-outs. **A**, The fEPSP plotted against the intensity of stimulus applied to the Schaffer collateral. Loss of $\beta 1$ -integrin had a significant effect on slope of the fEPSP (postsynaptic measurement) at increasing stimulus intensities [Cre; $f(\beta 1)/f(\beta 1)$, $n = 29$; Cre; $+/+$, $n = 22$ and $+/+$; $f(\beta 1)/f(\beta 1)$, $n = 24$; $F_{(2,1076)} = 21.29$; $p < 0.0001$; ANOVA with repeated measures]. **B**, No difference between $f(\beta 1)$ KO [Cre; $f(\beta 1)/f(\beta 1)$, $n = 29$] and the two control genotypes [Cre; $+/+$, $n = 22$ and $+/+$; $f(\beta 1)/f(\beta 1)$, $n = 24$] was observed in the amplitude of the fiber volley at varying stimulus intensities, indicating equivalent transfer and conversion of the electrical stimulus into axonal depolarization. **C**, A significant decrease in overall synaptic transmission was detected as the slope of the fEPSP plotted against the amplitude of the fiber volley at increasing stimulus intensities (2.5–28.5 mA). **D**, NMDA receptor-mediated field potentials assessed by blocking AMPA receptors with 5 mM CNQX, a selective AMPA receptor antagonist, and measuring potentials in ACSF containing no Mg^{2+} . No significant change was observed between the genotypes in NMDA receptor-mediated synaptic transmission under these conditions [Cre; $f(\beta 1)/f(\beta 1)$, $n = 8$; Cre; $+/+$, $n = 9$ and $+/+$; $f(\beta 1)/f(\beta 1)$, $n = 7$]. Data represent mean \pm SEM.

These results, combined as the slope of the fEPSP versus the fiber volley amplitude (Fig. 3C), clearly indicate that the loss of $\beta 1$ -integrin results in a significant decrease in the overall basal synaptic transmission in hippocampal slices across a broad range of stimulus intensities. Moreover, this reduced synaptic transmission appears to be the result of a decrease in the evoked postsynaptic fEPSP. For instance, a fiber volley produced with a stimulation of 20 mA in the control slices elicited an fEPSP with a slope of ~ 1.1 mV/ms, whereas the fEPSP elicited by the same fiber volley amplitude in the $f(\beta 1)$ KO slices was approximately one-half of this value. Because the fiber volley is one measure of neuron presynaptic function, these results are consistent with a deficit of the $\beta 1$ -integrin mutants in postsynaptic responses to equal presynaptic stimuli. Because the majority of the fast excitatory synaptic transmission under the recording conditions is AMPA receptor mediated, these results also suggest that AMPA receptor function may be compromised by the absence of $\beta 1$ -integrins. As an additional test of this idea, we also measured the fEPSPs in the mutant and control slices in the absence of Mg^{2+} to release the NMDA receptor block and in the presence of CNQX to block AMPA receptor potentials. Under these recording conditions, no significant differences were observed in overall synaptic transmission between the $f(\beta 1)$ KO and the control animals (Fig. 3D), suggesting that synaptically evoked NMDA receptor-mediated currents were normal in area CA1 of mutant mice. Overall, then, the most straightforward interpretation of our results is that the decrement in synaptic connectivity is attributable to a decrease in

AMPA receptor function and not a decrease in synaptic glutamate release.

Hippocampal synaptic plasticity in $f(\beta 1)$ KO mice

We next investigated the effects of $\beta 1$ -integrin knock-out on synaptic plasticity. PPF is a transient form of presynaptic-dependent short-term plasticity believed to be the result of enhanced probability of synaptic vesicle release. This phenomenon occurs with the facilitation of a closely timed second stimulus primarily as a result of residual calcium remaining from the first stimulation (Wu and Saggau, 1994). PPF in the $f(\beta 1)$ KO mice was normal (Fig. 4A), consistent with normal presynaptic function observed in the unaltered fiber volley amplitude at increasing stimulus intensity (Fig. 3B). In contrast, NMDA receptor-dependent LTP in the $f(\beta 1)$ KO mice was reduced to approximately one-half of the level found in the controls using two trains of high-frequency stimulation (HFS) (100 Hz) at a stimulus intensity that generated 50% of the maximum synaptic response (Fig. 4B). This reduction of LTP was not attributable to a deficit in the total amount of postsynaptic depolarization induced by HFS. Comparisons of the total depolarization envelope during successive 100 Hz/s stimulus trains showed no significant difference between $f(\beta 1)$ KO and control slices (Fig. 4C). This shows that the total conductance through all ion channels during HFS was similar between the mutant and control.

In addition, the deficit in LTP remained in the $f(\beta 1)$ KOs even when saturating amounts of HFS (8 trains of 100 Hz stimulation) were used to generate the LTP at stimulus intensities that elicited equal fEPSPs (controls, 50%; $(\beta 1)$ KOs, 85% of max) (Fig. 4D). Together, these results indicate that $f(\beta 1)$ KOs have a deficit in NMDA receptor-dependent LTP. It remains unclear, however, whether this deficit is attributable to an impairment in the molecular machinery underlying hippocampal LTP induction or whether the deficit is a consequence of the impaired synaptic transmission we observed in our studies of baseline synaptic connectivity. Additional studies will be required to distinguish between these two possibilities. Nevertheless, these studies indicate that hippocampal synaptic plasticity deficits occur in $\beta 1$ -integrin-deficient animals.

Hippocampal-dependent spatial learning and fear memory are normal in $f(\beta 1)$ KO mice

Subsequently, we examined the memory capabilities of $f(\beta 1)$ KO mice using a battery of behavioral tests. We first measured the behavior of the $f(\beta 1)$ KO animals in rotarod and open-field tests and found no detectable difference in initial performance or improvement over multiple trials, indicating that the $f(\beta 1)$ KOs had normal locomotor and exploratory activities as well as normal motor learning (data not shown). Subsequently, we investigated spatial memory performance in the water maze using a training protocol consisting of four training trials per day. The $f(\beta 1)$ KO animals were indistinguishable from control animals for escape

latency (Fig. 5A) or path lengths (data not shown) and improvement with training in this hippocampal-dependent task. Memory examined 24 h or 1 week after the last acquisition trial in a probe trial was identical for all three genotypes (Fig. 5B,C). Recently, certain mutants have been reported as deficient in a “pattern completion” version of the probe trial, in which all but one of the obvious spatial cues present during training were removed, but normal when all of the cues were present (Nakazawa et al., 2002). When tested under this more difficult condition, the $f(\beta 1)$ KOs still showed memory indistinguishable from the control animals (Fig. 5D).

We next examined the performance of $f(\beta 1)$ KOs along with controls in a pavlovian fear conditioning paradigm. Animals were presented with two tone–foot shock pairings inside a sound proof chamber. Memory of the context, a hippocampal-dependent memory, was measured by placing animals inside the same chamber in which they had received the training 2 or 24 h later and recording the percentage of time spent in a fearful posture (freezing). The $f(\beta 1)$ KOs and the control animals exhibited a high and equivalent level of freezing responses at both 2 and 24 h after training (Fig. 5E). To measure memory of the tone cue, which is hippocampal independent, we placed the animals after training into a chamber with distinct contextual cues and measured their fear response to the tone that was paired with shock during training. The percent time that the $f(\beta 1)$ KOs and the controls spent in a fearful posture before the tone onset during the memory test (pre-CS) was low, with no significant differences between groups (Fig. 5F). In addition, the percentage of time that all genotypes spent freezing during the tone presentation was high, with no significant differences between groups. Therefore, despite a deficit in basal hippocampal synaptic transmission and LTP, the $f(\beta 1)$ KO animals surprisingly have intact hippocampal-dependent spatial as well as contextual and cued fear memories.

Working memory is impaired in $f(\beta 1)$ KO mice

The impaired basal synaptic transmission in the hippocampus of the $f(\beta 1)$ KOs suggests that the function of synaptic AMPA receptors may be compromised in the absence of $\beta 1$ -integrins. Interestingly, knock-out mice of the GluR1 subunit of the AMPA receptors have been reported to exhibit normal spatial memory and deficient LTP, similar to the phenotypes exhibited by the $\beta 1$ -integrin animals discussed above (Zamanillo et al., 1999). Moreover, a recent study has shown that GluR1-deficient mice are impaired in working memory in a nonmatching-to-place T-maze test (Reisel et al., 2002). Because of the similar behavioral and physiological phenotypes of the GluR1 and the $f(\beta 1)$ KO animals, we tested the $f(\beta 1)$ KOs in the same, nonmatching-to-place T-maze test used previously for GluR1 KOs.

In this nonmatching-to-place T-maze assay, each trial consists of two runs: a sample run and a choice run (Fig. 6A). For the

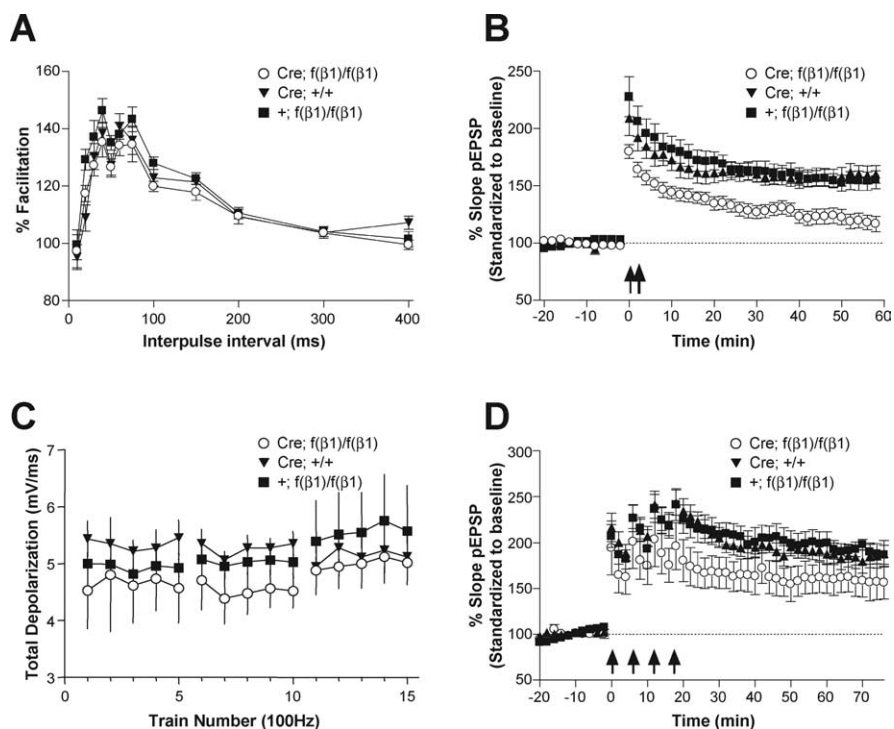


Figure 4. Synaptic plasticity in $\beta 1$ -integrin knock-outs. **A**, PPF in $f(\beta 1)$ KOs [Cre; $f(\beta 1)/f(\beta 1)$, $n = 26$] and controls [Cre; $+/+$, $n = 24$ and $+/+$; $f(\beta 1)/f(\beta 1)$, $n = 24$]. The loss of $\beta 1$ -integrin expression had no effect on short-term plasticity measured by PPF at any interpulse interval. **B**, Long-lasting plasticity was measured as LTP generated with two trains of 100 Hz stimulation (arrows) at a stimulus intensity that generated 50% of the maximum synaptic response. The $f(\beta 1)$ KOs [Cre; $f(\beta 1)/f(\beta 1)$, $n = 26$] showed significantly lower LTP than the controls [Cre; $+/+$, $n = 21$ and $+/+$; $f(\beta 1)/f(\beta 1)$, $n = 18$; $F_{(2,1919)} = 186$; $p < 0.0001$; ANOVA with repeated measures]. **C**, The total depolarization envelope was measured as the integral of the depolarization during successive trains of 100 Hz HFS. All three genotypes [Cre; $f(\beta 1)/f(\beta 1)$, $n = 4$; Cre; $+/+$, $n = 4$ and $+/+$; $f(\beta 1)/f(\beta 1)$, $n = 4$] showed a similar level of total depolarization during each 1 s of stimulation. **D**, The deficit in LTP remained when saturating amounts of HFS (8 trains at 100 Hz) were used to induce the maximum LTP [Cre; $f(\beta 1)/f(\beta 1)$, $n = 7$; Cre; $+/+$, $n = 9$ and $+/+$; $f(\beta 1)/f(\beta 1)$, $n = 11$; $F_{(2,819)} = 67$, $p < 0.001$]. Data represent mean \pm SEM.

sample run, one of two target arms of the T-maze is randomly blocked so that the animal is forced to obtain a food reward from the unblocked target arm. For the choice run, both target arms are unblocked, and the animal is free to move into either arm but is rewarded only if the previously unsampled arm is chosen. After initiating training, all animals performed at the random (50%) level. The animals were then trained to a criterion of 75% correct choices over 16 trials in the choice run, with no delay between the two runs. After reaching criterion, they were tested with no delay between the sample and choice runs, or with delays imposed between the two runs. The animals must remember the arm chosen during the sample run over the delay to obtain the food reward and to be scored as having made a correct choice. We expressed working memory as the percentage of correct choices the animal makes during the choice runs.

The $f(\beta 1)$ KO animals were able to learn and perform as well as the controls, reaching the criterion after 6 d of training like the controls. After reaching criterion, they also performed equivalently to the controls when the choice run was made with no delay after the sample run (Fig. 6B). However, the $f(\beta 1)$ KOs performed significantly poorer than the control animals with a 10 s delay. This performance was also significantly different from their performance with no delay. The controls, in contrast, showed no decrease in performance with a 10 s delay. When we extended the delay to 1 min, all groups performed near the random choice level, which was significantly lower than their performance with no delay, indicating the transient nature of this type

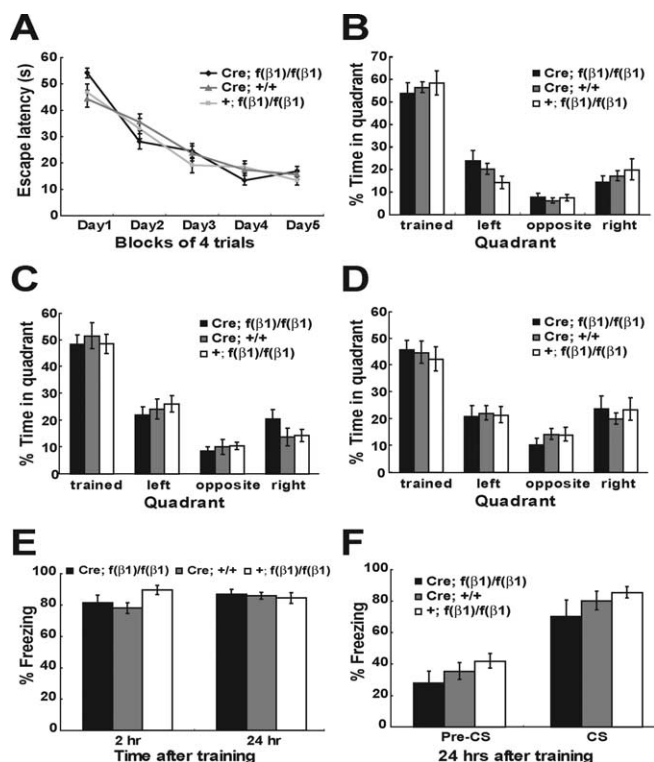


Figure 5. $\beta 1$ -integrin knock-outs exhibited normal spatial memory and conditioned fear memory. **A**, Acquisition of spatial memory plotted as escape latency as a function of training day in the hidden version of the water maze. No significant difference was observed between the $f(\beta 1)$ KO [Cre; $f(\beta 1)/f(\beta 1)$, $n = 13$] and the control groups [Cre; +/+, $n = 10$; +; $f(\beta 1)/f(\beta 1)$, $n = 11$; $F_{(2,31)} = 0.112$; $p = 0.89$; ANOVA with repeated measures]. **B**, Percentage of time spent in each quadrant during the probe trial at 24 h after the last training trial, 1 week after the last training trial (**C**), and in the “pattern completion” version of the water maze test (**D**). In all three tests, the $\beta 1$ -integrin knock-out and the control groups spent significantly more time in the trained quadrant than in the other three quadrants ($p \leq 0.01$ for each genotype; Scheffé’s *post hoc* comparison). However, no significant difference was found between the knock-out and the control groups in any of the quadrants ($p \geq 0.23$ for each quadrant). **E**, Performance at 2 and 24 h after contextual fear conditioning with two CS–US pairings. No significant difference in conditioned response was observed between the $\beta 1$ -integrin knock-out and the control groups at either time point ($p \geq 0.32$). **F**, Performance at 24 h after cued fear conditioning with two CS–US pairings. Both the $f(\beta 1)$ KO and the control groups exhibited a conditioned response during the 3 min presentation of the CS ($p \leq 0.01$ for each genotype). However, no significant difference in conditioned response was observed between the $\beta 1$ -integrin knock-out and the control groups either before ($p \geq 0.94$) or during ($p \geq 0.26$) the presentation of the CS.

of working memory. It is noteworthy that there was no significant difference between the performance of the $f(\beta 1)$ KOs with a 10 s and 1 min delay. These results, which were replicated in a second independent experiment with a different set of animals, along with those of Zamanillo et al. (1999), show that working memory is more labile in the absence of the $\beta 1$ -integrins and the GluR1 subunit of the AMPA receptor. They therefore support the hypothesis that $\beta 1$ -integrins regulate AMPA receptor functions for synaptic transmission, synaptic plasticity, and working memory.

Expression of AMPA and NMDA receptor subunits is normal in the $f(\beta 1)$ KOs

To investigate whether the expression of the AMPA receptors is altered by the loss of $\beta 1$ -integrins, we performed immunohistochemistry to study the distribution and abundance of GluR1 and GluR2/3 subunits in the $f(\beta 1)$ KO brains (Fig. 7A). We also looked at the distribution of the NR1 subunit of the NMDA

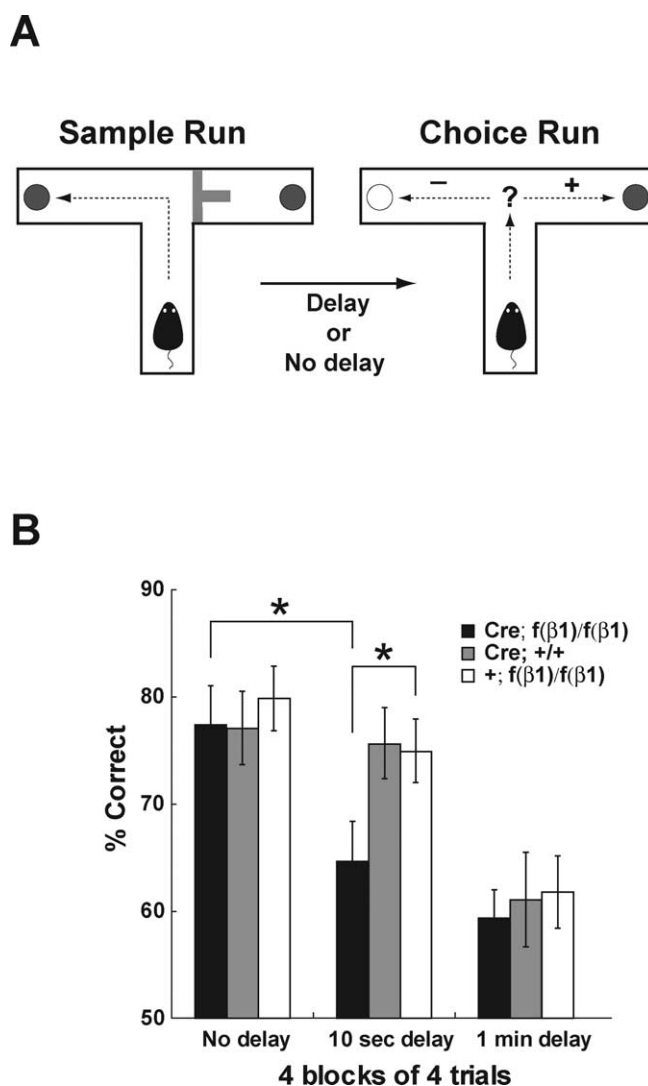


Figure 6. $\beta 1$ -integrin knock-outs exhibited impaired working memory. **A**, Schematic diagram of the nonmatching-to-place T-maze assay. Each trial consists of a sample run and a choice run. For the sample run, one of two target arms of the maze is blocked, and the animal is forced to obtain the food reward from the unblocked arm. For the choice run, after a delay or with no delay, both target arms are unblocked, and the animal is free to move into either arm. The animal is considered to have made the correct choice (+) if it visits the previously unsampled arm but incorrect (−) if it visits the previously sampled arm. **B**, Working memory expressed as the percentage of correct choices made in a nonmatching-to-place T-maze assay with the choice run performed immediately, 10 s, or 1 min after the sample run. For the statistical analysis, we performed a two-way ANOVA with the main effects of genotype and delay ($F_{(2,26)} = 1.68$, $p > 0.19$; delay $F_{(2,45)} = 13.81$, $p < 0.0001$; interaction, $F_{(4,45)} = 0.83$, $p > 0.51$), followed by *post hoc* Scheffé’s test, which showed a significant effect only between the Cre/+; $f(\beta 1)/f(\beta 1)$ vs Cre/+; +/+ ($p = 0.020$) and Cre/+; $f(\beta 1)/f(\beta 1)$ vs +/+; $f(\beta 1)/f(\beta 1)$ ($p = 0.027$) at 10 s delay and no significant genotype effect at other delays ($p > 0.9$). Similar results were obtained in an independent experiment with a second group of animals [Cre; $f(\beta 1)/f(\beta 1)$, $n = 15$; Cre; +/+, $n = 9$ and +; $f(\beta 1)/f(\beta 1)$, $n = 12$; data not shown]. The asterisks represent significant differences.

receptor, the other major excitatory neurotransmitter receptor in the hippocampus. No detectable difference was observed in either the pattern or level of the immunohistochemical signal between the $f(\beta 1)$ KO and control animals for these receptor subunits. Results showing the immunohistochemical signals in the hippocampus are shown in Figure 7A, but no difference was observed throughout the brain. These results therefore suggest that $\beta 1$ -integrin KO does not affect the abundance or distribution of AMPA or NMDA receptor subunits.

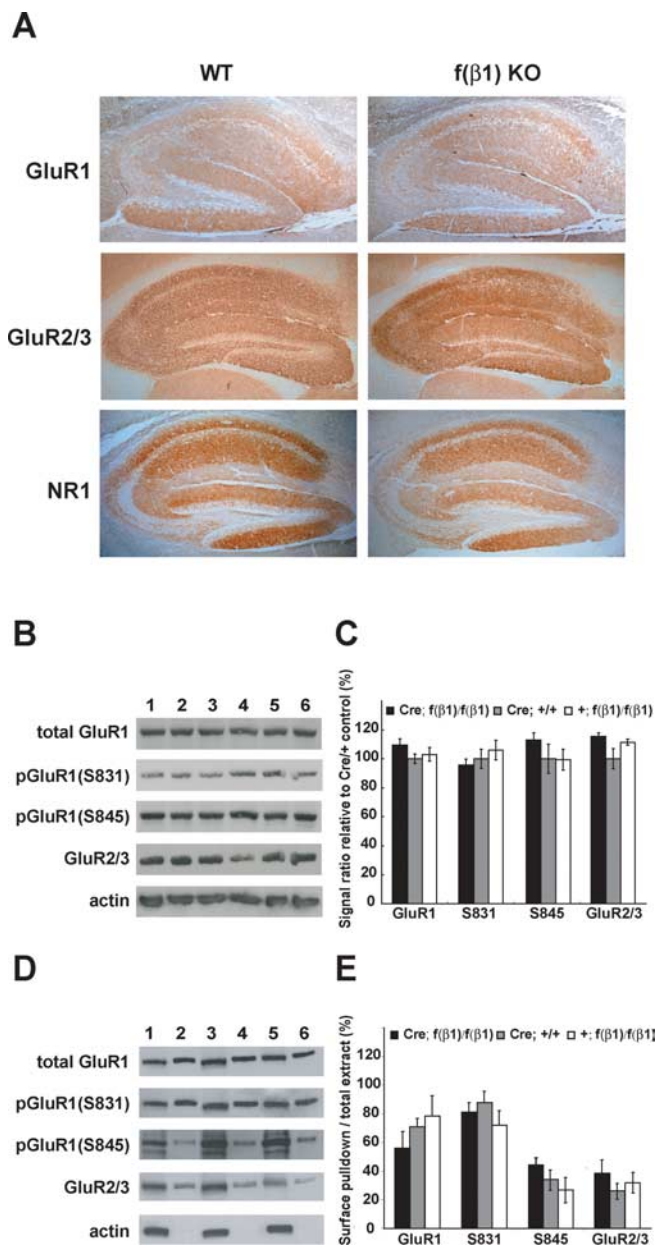


Figure 7. Expression of AMPA and NMDA receptor subunits in $\beta 1$ -integrin knock-outs. **A**, Immunohistochemistry was performed to compare the expression pattern of GluR1, GluR2/3, and NR1 subunits in the hippocampus of $f(\beta 1)$ KO and wild-type (WT) animals. No differences were observed. **B**, Immunoblots were performed to compare the expression levels of S831-phosphorylated GluR1, S845-phosphorylated GluR1, total GluR1, and GluR2/3 subunits in hippocampal synaptosomal extracts from two individual $f(\beta 1)$ KOs (lanes 3 and 6), Cre; +/- (lanes 1 and 4) and $f(\beta 1)/f(\beta 1)$ (lanes 2 and 5) animals. Expression of actin was used as an internal control for protein loading. **C**, Bar graph summarizing the relative expression levels obtained from the immunoblots in **B**. Signals from each genotype were expressed as the ratio relative to that of the Cre; +/- controls. No significant differences were detected between the $f(\beta 1)$ KOs and the controls for all subunits examined ($p \geq 0.28$ for each antibody used; Scheffé's *post hoc* comparison). **D**, Immunoblots were performed to compare the surface expression levels (lanes 2, 4, and 6) with the total biotinylated protein extract (lanes 1, 3, and 5) of total GluR1, S831-phosphorylated GluR1, S845-phosphorylated GluR1, and GluR2/3 subunits in hippocampal extracts from $f(\beta 1)$ KO (lanes 3 and 4), Cre; +/- (lanes 1 and 2), and $f(\beta 1)/f(\beta 1)$ (lanes 5 and 6) animals. Expression of actin was used as an internal control for protein loading and also for the absence of cytoplasmic proteins in the pull-down fraction. **E**, Bar graph summarizing the relative expression levels of surface proteins to those in the total biotinylated protein extracts obtained from the immunoblots in **D**. No significant differences were detected between the $f(\beta 1)$ KOs and the controls for all subunits examined ($n = 3$; $p \geq 0.56$ for each antibody used; Scheffé's *post hoc* comparison).

Protein phosphorylation of AMPA receptors is a critical modification that influences receptor function. Because integrins signal by activating multiple protein kinases, it seemed possible that the loss of $\beta 1$ -integrin would influence the phosphorylation state of the receptor. In particular, phosphorylation at S831 and S845 of GluR1 has been shown to potentiate the ion channel function of the receptor (Roche et al., 1996; Barria et al., 1997; Derkach et al., 1999; Banke et al., 2000). Furthermore, mutations at these two sites produced LTP deficits and normal spatial reference memory (Lee et al., 2003). We therefore measured the level of phosphorylation of these two sites in the $f(\beta 1)$ KOs by immunoblotting hippocampal protein extracts and probing these with phosphorylation site-specific antibodies against S831 and S845. As shown in Figure 7, *B* and *C*, there was no detectable difference in signal intensity from the $f(\beta 1)$ KOs compared with the controls.

Finally, we examined the level of expression of GluR1 and its level of phosphorylation at S831 and S845 on the cell surface. To do this, we conducted surface biotinylation of hippocampal slices and measured the expression levels from the biotinylated fractions by immunoblotting. As shown in Figure 7, *D* and *E*, neither the level of total GluR1 nor the level of phosphorylation at S831 and S845 showed a significant difference between the $f(\beta 1)$ KO and the control animals. Together, these results suggest that the steady-state expression level of AMPA receptor subunits and the phosphorylation level of S831 and S845 are not regulated by $\beta 1$ -integrins.

Discussion

Using a genetic approach to remove $\beta 1$ -integrin expression in the postnatal forebrain, we demonstrated that $\beta 1$ -integrins are required for three aspects of adult hippocampal function: basal synaptic transmission, synaptic plasticity, and working memory. Surprisingly, the $f(\beta 1)$ KOs did not have a deficit in hippocampal-dependent spatial or contextual memory.

In the absence of $\beta 1$ -integrins, fEPSPs were dramatically reduced at the CA3–CA1 synapses. This reduction is most consistent with a postsynaptic defect in AMPA receptor synaptic transmission because it was accompanied by normal fiber volley amplitude and normal PPF. In addition, the majority of the fast excitatory synaptic transmission is mediated by AMPA receptors. The large deficit in output responses (>50%) observed in the $f(\beta 1)$ KOs requires a change in AMPA receptor-mediated synaptic transmission. Furthermore, altered NMDA receptor function was not detected by measuring the NMDA receptor-dependent synaptic transmission in the presence of AMPA receptor blockers. These results are consistent with the observation reported recently that infusion of an integrin ligand into rat hippocampal slices causes a slow (minutes) increase in fast excitatory transmission mediated by AMPA receptors (Kramar et al., 2003). The combined physiological results therefore suggest that integrin activation causes an increase in AMPA receptor function, and integrin inactivation causes a decrease in AMPA receptor function.

How might $\beta 1$ -integrins regulate AMPA receptor functions? Our EM studies have shown that the basic synapse structure is not disrupted in the absence of $\beta 1$ -integrins. Western blotting and immunohistochemical results suggested that the loss of $\beta 1$ -integrin function does not lead to a global redistribution or loss of AMPA receptor subunits at steady state. However, it is possible that our assays do not have the resolution needed to detect subtle changes that might exist in a subset of synapses. It is also possible that such changes might occur only after integrin activation. Accumulating evidence has demonstrated that protein phosphory-

lation is critical in modulating AMPA receptor-mediated transmission by altering either channel properties (Benke et al., 1998; Banke et al., 2000; Poncer et al., 2002) or abundance at the synaptic surface through receptor trafficking (Shi et al., 1999; Hayashi et al., 2000; Lu et al., 2001). For example, phosphorylation at S831 of GluR1 by CaMKII and at S845 by PKA have been shown to potentiate the response of the receptor to glutamate (Roche et al., 1996; Barria et al., 1997; Derkach et al., 1999; Banke et al., 2000), and phosphorylation of S845 might also be important for the internalization and recycling of the receptors (Ehlers, 2000). Interestingly, activation of integrins has been shown to stimulate a variety of signaling molecules. One potential candidate for regulating AMPA receptors is the tyrosine kinase Src. In fact, direct activation of endogenous Src in rat CA1 hippocampal neurons has been reported to potentiate AMPA receptor currents through an increase in the receptor conductance (Lu et al., 1998). More recently, Kramar et al. (2003) have also shown in rat hippocampal slices that the enhancement of AMPA receptor potentials by integrin-activating peptides can be blocked by the Src inhibitor PP2. However, it remains unknown whether Src regulates AMPA receptors directly.

Recent studies have also shown that AMPA receptor functions are also regulated by receptor interaction with the cytoskeleton. Drugs that disrupt actin filaments reduce AMPA receptor-mediated synaptic transmission (Kim and Lisman, 1999), LTP (Kim and Lisman, 1999; Krucker et al., 2000), and the internalization of AMPA receptors (Zhou et al., 2001) in hippocampal neurons. Shen et al. (2000) have shown that disruption of actin filaments or the interaction between GluR1 and 4.1N, a cytoskeleton-associated protein, decreases the cell surface expression of GluR1 in heterologous cells, suggesting that 4.1N may link AMPA receptors to the actin cytoskeleton. The intimate association of integrins with actin and other cytoskeletal proteins may allow them to modulate the interaction of AMPA receptors with components of the cytoskeleton and thus the synaptic functions of the receptor.

Our results once again confirm a role for integrins in LTP at CA3–CA1 synapses. Because the expression of LTP requires AMPA receptors, our findings offer a possible integrin-dependent regulatory mechanism for AMPA receptor function in LTP. Furthermore, although some LTP was still detected in the $f(\beta 1)$ KOs, potentiation was completely abolished in the GluR1^{-/-} mutants (Zamanillo et al., 1999), suggesting that $\beta 1$ -integrins probably have a modulatory rather than an on/off, switch role for LTP.

Our behavioral results have uncovered a hitherto unknown function for integrins and revealed a notable similarity between the $f(\beta 1)$ KO and GluR1^{-/-} mice. Both mutants exhibit normal spatial reference memory in the water maze despite suffering dramatic deficits in hippocampal CA3–CA1 LTP. In fact, the same dissociation between LTP and spatial reference memory was also observed in animals heterozygous for the $\alpha 3$ -integrin null allele or simultaneously for $\alpha 3$ and $\alpha 8$ null alleles (Chan et al., 2003). In addition, the $\beta 1$ -integrin knock-out and GluR1^{-/-} animals were both impaired in the same nonmatching-to-place working memory task, which has also been shown to be hippocampus dependent (Olton et al., 1979; Deacon et al., 2002). One possible explanation is that LTP in the hippocampal CA1 area is not required for spatial reference memory but is critical for working memory. A distinct difference between these two tasks is the persistence requirement for memory. In the water maze, the mice are trained to locate a fixed platform location over a period of days. In contrast, working memory must be kept on line for

tens of seconds in the working memory task. It is conceivable that structural plasticity, which involves changes in synaptic number and morphology, accounts for the formation of spatial reference memory, whereas short-term synaptic plasticity, without associated structural plasticity, underlies working memory. Another possibility is that a requirement of NMDA receptor/ $\beta 1$ -integrin/AMPA receptor-dependent LTP can be substituted by other LTP mechanisms for spatial reference memory but not for working memory. Nevertheless, NMDA receptor/ $\beta 1$ integrin/AMPA receptor-dependent LTP is dispensable for spatial reference memory but not for working memory. It is interesting that, like the deficit in LTP, the magnitude of impairment in working memory also differs between the $f(\beta 1)$ KO and GluR1^{-/-} mice. The GluR1^{-/-} mice exhibit a complete loss of working memory even with no delay between the sample run and the choice run. The $f(\beta 1)$ KO mice, in contrast, showed a memory deficit only after a delay was imposed between the two runs. This may indicate that $\beta 1$ -integrins are involved in the stability rather than the formation of working memory. One model for the instability based on the AMPA receptor function is that working memory requires a certain level of AMPA receptor potentiation, which decays rapidly and thus is short lasting. In the absence of $\beta 1$ -integrins, a lower level of potentiation caused by the impaired AMPA receptor potentials results in a shorter time interval to fall below the threshold required for the expression of working memory.

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