Munc13-2 Differentially Affects Hippocampal Synaptic Transmission and Plasticity

The short-term dynamics of synaptic communication between neurons provides neural networks with specific frequency-filter characteristics for information transfer. The direction of short-term synaptic plasticity, that is, facilitation versus depression, is highly dependent on and inversely correlated to the basal release probability of a synapse. Amongst the processes implicated in shaping the release probability, proteins that regulate the docking and priming of synaptic vesicles at the active zone are of special importance. Here, we found that a member of the Munc13 protein family of priming proteins, namely Munc13-2, is essential for normal release probability at hippocampal mossy fiber synapses. Paired pulse and frequency facilitation were strongly increased, whereas mossy fiber long-term potentiation was unaffected in the absence of Munc13-2. In contrast, transmission at 3 other types of hippocampal synapses, Schaffer-collateral, associational-commisural, as well as inhibitory synapses onto CA3 pyramidal neurons was unaffected by the loss of Munc13-2.

Keywords: hippocampus, mossy fiber, presynaptic, release probability, synaptic plasticity, synaptic transmission

Introduction

The majority of neuronal communication takes place at specialized contact sites, the synapses. The way in which incoming signals are transduced at synapses is dynamically dependent on the recent activation history and displays various forms of plasticity on short- and long-term time scales (Zucker and Regehr 2002; Malenka and Bear 2004).

Short-term facilitation denotes the enhancement of synaptic transmission over a period of hundreds of milliseconds, and several mechanistic explanations have been put forth to account for this presynaptically mediated phenomenon. The best experimental support exists for the so-called residual Ca$^{2+}$ hypothesis, which states that Ca$^{2+}$ that has entered the presynaptic terminal after a first action potential causes the enhancement of synaptic transmission upon subsequent activations (Thomson 2000; Zucker and Regehr 2002). The site of action of this residual Ca$^{2+}$ or the role of saturation of presynaptic Ca$^{2+}$ buffers (Blatow et al. 2003) is still under debate and may vary between different types of synapses. Additionally, in some preparations an enhanced Ca$^{2+}$ influx into the presynaptic terminal, through Ca$^{2+}$-current facilitation (Borst and Sakmann 1998), action potential broadening (Geiger and Jonas 2000), or presynaptic autoreceptors (Kamiya et al. 2002; Lauri et al. 2003), may participate in short-term facilitation of transmission.

At many synaptic connections, the release probability of a synapse and the direction of short-term plasticity are inversely related, that is, synapses with a low initial release probability tend to show short-term facilitation, whereas high release probability synapses display short-term depression (Thomson 2000; Zucker and Regehr 2002), but see Hefft et al. (2002) for an example of an independence of release probability and short-term depression at an inhibitory synapse. The release probability of a synaptic connection in turn depends on many factors such as the amount of Ca$^{2+}$ influx through Ca$^{2+}$ channels, the spacing between Ca$^{2+}$ channels and the sensor for triggering vesicle release, the buffering kinetics for Ca$^{2+}$ in the presynaptic terminal and, very importantly, the number of docked and primed vesicles (Atwood and Karunanithi 2002).

Of paramount importance in the priming process of synaptic vesicles are the members of the Munc13 protein family, which consists of the orthologues Munc13-1, -2, and -3 (Augustin, Rosenmund, et al., 1999; Varioqueaus et al. 2002). Munc13-1 is expressed throughout the brain, whereas the other 2 members show mutually exclusive expression patterns, with Munc13-3 being restricted to the cerebellum and Munc13-2 dominating in the forebrain. Using cultured neurons from deletion mutant (KO [knockout]) mice, it was found that Munc13-1 is absolutely essential for vesicle release in 90% of glutamatergic terminals. The remaining 10% of glutamatergic synapses were unaffected by the loss of Munc13-1 (Augustin, Rosenmund, et al., 1999; Varioqueaus et al. 2002) and probably rely on Munc13-2, because in Munc13-1/2 double knockout (DKO) mice release at all glutamatergic synapses is silenced. g-aminobutyric acid (GABA)-releasing synapses are capable of using either Munc13-1 or Munc13-2 as a priming factor because only in Munc13-1/2 DKO mice both evoked and spontaneous release at GABAergic terminals was blocked (Varioqueaus et al. 2002). Absence of either Munc13-1 or Munc13-2 alone left GABAergic transmission unaltered.

In the present study, we took advantage of the fact that, in contrast to Munc13-1 KOs and Munc13-1/2 DKO s, Munc13-2 KO mice are viable after birth. They can therefore serve as a source of acute slice preparations from young adult developmental stages, which can be used to study the functional role of Munc13-2 in synaptic plasticity in different types of synapses within a functional network. We focused our analysis on the hippocampus because it contains a rich repertoire of diverse types of synapses with different short-term and long-term plasticity characteristics. We found that the absence of Munc13-2 leads to a decrease in release probability at mossy fiber synapses, which was accompanied by an increase in short-term facilitation, and to a remarkable reduction of asynchronous transmitter release. In contrast, both Schaffer-collateral and associational-commisural (AC) synapses functioned normally without Munc13-2.
Materials and Methods

In Situ Hybridization
Paraformaldehyde-fixed 12-μm-thick sagittal cryostat sections prepared from freshly frozen adult mouse brains were made and processed as described (Augustin, Betz, et al. 1999). Several 45-pb-long antisense oligonucleotides specific for mouse bMunc13-2 or ubMunc13-2 were chosen as probes based on the rat sequences (bMunc13-2: GenBank accession no. AADI1190; bp 230, 745, and 1456; ubMunc13-2: GenBank accession no. U24071; bp 7770) and corrected for mismatches upon alignment against the mouse genome. They were labeled with terminal transferase by using 35S-ATP. In control experiments, hybridizations were performed with a 1000-fold excess of the respective unlabeled oligonucleotides. Sections were exposed to Kodak (Sigma-Aldrich, Hamburg, Germany) Biomax films for 1-3 weeks. All oligonucleotides were used alone or in combination for a given Munc13-2 isoform and gave strictly identical labeling patterns.

Immunoblotting
For quantitative analysis, proteins were separated on 7.5% (RIM1) and 12% (Rab3A, Calbindin, and Glut 6/7) polyacrylamide gels and blotted to nitrocellulose using standard procedures. For detection we used monoclonal mouse antibodies against Rab3A (1:2000, Synaptic Systems, Göttingen, Germany), RIM (1:1000, BD Transduction Laboratories, San Jose, CA), Calbindin (1:1000, SWANT, Bellinzona, Switzerland), and GluR 6/7 (1:10 000, Millipore, Schwalbach, Germany). By using fluorescently labeled secondary antibodies signal intensities were estimated on an Odyssey Infrared Imaging System (Li-cor Biosciences, Bad Homburg, Germany). Expression levels were normalized to β-tubulin (1:10 000, Sigma, Hamburg, Germany), which served as loading controls. All Western blots were done twice and statistical significance was tested using Student’s t-test.

Acute Slice Preparation
All experiments were performed according to the animal welfare guidelines of the Charité, Universitätmedizin, Berlin. For field potential, whole-cell and calcium imaging experiments hippocampal slices were prepared from littermate wild-type (WT) and Munc13-2 KO mice, aged between postnatal days P 18-35 and the majority of experiments were performed in a double-blind fashion. The generation of Munc13-2−/− mice was described before (Varoqueaux et al. 2002). In brief, the animals were anesthetized with diethyl ether, decapitated, and the brains removed. Tissue blocks containing the subicular area and hippocampus were mounted on a Vibratome (Vibraslice MA752, Campden Instruments, Loughborough, UK or Leica VT1200 S, Leica, Wetzlar, Germany) in a chamber filled with ice-cold artificial cerebrospinal fluid (ACSF), containing (in mM): 50 NaCl, 150 sucrose, 2.5 NaHCO3, 1 KCl, 1 NaH2PO4, 0.5 CaCl2, 7 MgCl2, 10 glucose; pH 7.4. Saggital slices were cut to 7.3 with KOH for recording of glutamatergic excitatory postsynaptic currents (EPSCs). For the measurement of GABAergic inhibitory postsynaptic currents (IPSCs), pipettes were filled with K-gluconate 35, KCI 100, MgCl2 2, EGTA 0.1, NaATP 2, HEPES 10, pH adjusted to 7.2 with KOH. IPSCs were isolated by blocking a-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (APMA) and N-methyl-D-aspartate-mediated currents with 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzof[1]quinoxaline-7-sulfonamide disodium salt (NBQX) (25 μM) and d-(-)-2-amino-5-phosphonopentanoic acid (d-APV) (25 μM), respectively. Series resistances were continuously checked during the recordings and not allowed to vary more than 25% during the course of the experiment. No series resistance compensation was used. Field potential recordings were performed with low-resistance patch pipettes filled with external solution. All recordings were performed at room temperature.

Fluorescence Measurements
Mossy fibers were locally labeled with a pressure stream of the low-affinity calcium indicator Magnesium Green (Molecular Probes, Eugene, OR) (Regehr and Tank 1991; Breustedt et al. 2003), dissolved in 20% pluronic/dimethyl sulfoxide. Recordings were started 2-3 h after labeling. Epifluorescence was measured with a single photodiode from a spot more than 200 μm away from the loading site with an Olympus LumPlan FL 60× 0.9NA water immersion objective (Olympus Deutschland GmbH, Hamburg, Germany). The filter set used was for excitation HQ480/40, Dicrhoic Q505, and for emission HQ520LP (AHF Analysetechnik, Tübingen, Germany). The signals from the photodiode were low-pass filtered with 1 kHz with a 4-pole Bessel filter at 5 kHz, and captured with Igor Pro software (WaveMetrics Inc., OR). The change in fluorescence intensity (ΔF) relative to the baseline intensity of fluorescence (F) was calculated as ΔF/F.

Drugs and Solutions
D-(-)-2,3-diaminopropionic acid (D-APV) (25 μM), 6-imino-3-(4-methoxyphenyl)-1(6H)-pyridazinobutane acid hydrobromide (Gabazine), and NBQX were obtained from Tocris International (via Biotrend, Cologne, Germany). All other chemicals were obtained from Sigma-Aldrich (Munich, Germany).

Analysis and Statistics
Average values are given as mean ± SEM throughout the text. For statistical analysis an unpaired Student’s t-test was used (Prism software package, Graph Pad; El Camino Real, CA). Asynchronous EPSCs evoked in ΔF/F were detected with an amplitude threshold algorithm (Strathclyde Electrophysiology Software, Strathclyde University, UK), with the threshold set to 5 pA, which was kept constant throughout the experiment. Latencies were measured from the beginning of the stimulation artifact to the peak amplitude of the EPSC. Data in Figure 5E for calcium imaging were fitted according to a bimolecular binding process with the following equation: y = ((Bmax1 × x)/(K1 + x)) + ((Bmax2 × x)/(K2 + x)) (Microcal Origin, Northampton, MA).

Results
Expression of Munc13-2 in the Murine Brain
As a complement to previous studies in rats we investigated the distribution of Munc13-2 mRNA in brains of mice. Both the brain-specific bMunc13-2 (Augustin, Betz, et al. 1999) and the ubiquitous ubMunc13-2 (Song et al. 1998) N-terminal splice variants of Munc13-2 were detected at the mRNA level. The distribution pattern for bMunc13-2 (Fig. 1A, 2) is in full agreement with that previously reported in the rat brain (Augustin, Betz, et al. 1999), as bMunc13-2 mRNA was predominantly detected in the olfactory bulb, the cortex, the collicular region, and the hippocampus, where both pyramidal neurons and dentate gyrus granule cells express the protein. Cerebellar Purkinje cells, as well as neurons from the centrolateral thalamic nucleus, also abundantly expressed bMunc13-2 mRNAs. Signals observed in

1110 1110 Munc13-2 in Synaptic Transmission · Breustedt et al.
Impaired Mossy Fiber Synaptic Transmission in Mice Lacking Munc13-2

In order to characterize potential effects of the lack of Munc13-2 on hippocampal synaptic transmission, we first performed input-output analyses. To this end, presynaptic afferents were stimulated and the corresponding afferent fiber volley (input) and field excitatory postsynaptic potentials (fEPSPs, output) were recorded. Stimulating the Schaffer collaterals and recording in stratum radiatum of area CA1 revealed no difference in the input-output behavior of synaptic transmission between WT and Munc13-2 KO mice (Fig. 1B, WT: n = 5–7 slices, 4 mice vs. KO: n = 5–7 slices, 4 mice, P > 0.05). In contrast, stimulation of the mossy fiber pathway, which connects granule cells of the dentate gyrus with pyramidal cells in CA3, showed a considerable and significant reduction in synaptic transmission of about 40% in Munc13-2 KO mice (Fig. 1C, WT: n = 3–6 slices, 4 mice, and KO n = 4–8 slices, 6 mice, P < 0.05). The selectivity of stimulating mossy fiber inputs was assessed by the application of the group II metabotropic glutamate receptor (mGluR) agonist DCG IV (1 μM) at the end of each experiment throughout this study (see also Figs 3 and 6).

To further investigate transmission at mossy fiber synapses, we performed whole-cell recordings of CA3 pyramidal neurons while selectively eliciting mossy fiber EPSCs. In many cases it turned out to be difficult to obtain postsynaptic responses in those recordings from slices of Munc13-2 KO mice. Collectively, we observed a significantly higher incidence of transmission failures in Munc13-2 KO mice as compared with WT animals (Fig. 2A, WT-failure rate 2.7 ± 1.3%, n = 10 cells, 8 mice and KO-failure rate 29.7 ± 9.4%, n = 10 cells, 8 mice, P < 0.05). From the experiments presented in Figures 1 and 2 we conclude that synaptic transmission at mossy fiber synapses is severely compromised in Munc13-2 KOs, whereas transmission is normal at Schaffer-collateral synapses (please note also the unaltered short-term plasticity at Schaffer-collateral synapses in the Supplementary Fig. 1).

Increased Mossy Fiber Short-Term Plasticity in the Absence of Munc13-2

To further investigate the altered functionality of mossy fiber synaptic transmission in Munc13-2 KOs, we analyzed several parameters of short-term plasticity. A prominent feature of mossy fiber synapses is their remarkably high paired-pulse facilitation, in which the second of 2 closely spaced stimuli leads to a larger postsynaptic response than the first (Salin et al. 1996). We found this paired-pulse facilitation to be larger in Munc13-2 KO than in WT mice over a range of interstimulus intervals in fEPSP measurements (Fig. 3A, WT-PPR: 2.5 ± 0.1, n = 11 slices, 5 mice vs. KO-PPR: 3.4 ± 0.2, n = 15 slices, 5 mice; P < 0.01 for 50-ms interstimulus interval). Whole-cell recordings of mossy fiber EPSCs confirmed this result (Fig. 3B, WT-PPR: 2.6 ± 0.2, n = 7 cells, 5 mice vs. KO-PPR: 6 ± 1.4, n = 7 cells, 6 mice, P < 0.05 at 50-ms interstimulus interval). Similarly, application of 5 pulses with a stimulation frequency of 25 Hz showed a much more pronounced increase of the fifth over the first
response of the train in Munc13-2 KO mice than in WT controls (Fig. 3C, WT: 5.3 ± 0.3, n = 11 slices, 6 mice vs. KO: 8.3 ± 0.5, n = 14, 7 mice, P < 0.0001, amplitude of the fifth normalized to the first response). Another characteristic phenomenon observed at mossy fiber synapses is the so-called frequency facilitation. This index of short-term plasticity also revealed a much more pronounced facilitation in Munc13-2 KO mice (Fig. 3D, frequency facilitation ratio for stimulus number 30: WT 5.3 ± 0.3, n = 19 slices, 7 mice vs. KO 7.4 ± 0.4, n = 18 slices, 6 mice, P < 0.0001).

As already mentioned, the selectivity of each recording of mossy fiber responses was confirmed by suppression of transmission through application of DCG IV (1 μM), which activates group II mGluRs that are expressed at presynaptic mossy fiber terminals. This procedure reliably separates selective mossy fiber stimulation from contamination with inputs from other sources, such as AC which also contact CA3 pyramidal neurons (Nicoll and Schmitz 2005). The amount of suppression of synaptic transmission by DCG IV was of the same magnitude in both genotypes (Fig. 3E and F, WT: 92.4 ± 1.6% of control, n = 11 cells, 8 mice vs. KO: 93.3 ± 1.8%, n = 10 cells, 9 mice, P = 0.7). This finding also shows that the modulation of the presynaptic release machinery via G-protein-coupled receptors is not affected by the lack of Munc13-2. In summary, the absence of Munc13-2 leads to an enhanced short-term plasticity in mossy fiber synaptic transmission.

**Intact Short-Term Plasticity of IPSCs and AC-EPSCs in the Absence of Munc13-2**

Almost all excitatory synapses rely on Munc13-1 as a necessary priming factor, because in the absence of Munc13-1 roughly 90% of excitatory synapses in cultured hippocampal neurons do not show any synaptic transmission, that is, Munc13-2 alone is not capable of supporting transmission in these synapses (Augustin, Rosenmund, et al., 1999). In contrast, at inhibitory synapses of cultured neurons either Munc13-1 or Munc13-2 is sufficient to maintain normal synaptic transmission, and only if both proteins are absent inhibitory transmission is completely shut down (Varoqueaux et al. 2002). Therefore, we wanted to address the question as to whether the absence of Munc13-2 has any influence on inhibitory synaptic transmission onto CA3 pyramidal neurons in the more intact hippocampal slice preparation. We did not detect any significant differences in the amplitude or frequency of spontaneous IPSCs between WT and Munc13-2 KO mice (for frequency WT: 6.3 ± 0.8 Hz, n = 5 cells vs. KO 7.5 ± 1.1 Hz, n = 4 cells, P = 0.38 and for amplitude WT: −46.1 ± 13.6 pA vs. KO: −49.1 ± 6.9 pA, P = 0.86, the holding potential was −70 mV). Short-term depression of stimulus evoked IPSCs was also similar in both genotypes for the interstimulus intervals tested (Fig. 4A and B, WT-PPR: 0.68 ± 0.04, n = 7 cells, 3 mice vs. KO-PPR: 0.7 ± 0.05, n = 7 cells, 3 mice, P = 0.36 for 50-ms interstimulus interval).

To further examine synapse specificity of the effects of Munc13-2 KO on synaptic transmission we investigated AC synapses in whole-cell recordings from CA3 pyramidal cells. This synapse distinguishes itself from the mossy fiber synapse by a much lower magnitude of paired-pulse and frequency facilitation (Salin et al. 1996), as well as by an insensitivity to group II mGluR agonists. Firstly, we did not find any significant differences in short-term facilitation between WT and Munc13-2 KO mice at AC synapses (Fig. 4C, WT-PPR: 1.15 ± 0.07, n = 11 cells, 5 mice vs. KO-PPR: 1.19 ± 0.1, n = 12 cells, 7 mice, P = 0.76). Next, in order to test for potential differences in basal release probability between WT and Munc13-2 KO mice at this synapse, we made use of the low-affinity competitive AMPA receptor antagonist γ-DGG. A higher release probability,
leading to a larger glutamate transient in the synaptic cleft that competes for α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) binding with the γ-DGG would result in a smaller reduction of the EPSC by γ-DGG (Wadiche and Jahr 2001). The opposite holds true for the condition of a lower release probability, where the γ-DGG effect on the EPSC should be stronger. Application of γ-DGG in a concentration of 0.5 mM reduced the EPSCs in both

Figure 3. Mossy fiber short-term plasticity is increased in the absence of Munc13-2. (A) The graph displays the average paired-pulse ratio of mossy fiber EPSPs for interstimulus intervals of 40, 50, and 100–500 ms. In Munc13-2 KO mice, this ratio is significantly increased for all intervals. Traces above the graph are overlaid averages of 5 responses for interstimulus intervals between 50 and 500 ms. (B) Paired-pulse ratio in whole-cell recording of mossy fiber EPSCs at an interstimulus interval of 50 ms. The summary graph shows the increased ratio in KO mice. Sample traces are averages of 10 consecutive sweeps. (C) Summary bar graph of EPSP responses elicited with a stimulation paradigm consisting of 5 pulses delivered at 25 Hz. The amplitude of the fifth response is normalized to the first in the train. (D) The graph shows the averaged time course of frequency facilitation for WT and Munc13-2 KO mice. Starting with a baseline stimulation rate of 0.05 Hz for 10 stimuli, the stimulation frequency was raised to 1 Hz for 20 pulses (numbers 11–30) and afterwards returned to the baseline frequency. A more pronounced increase in frequency facilitation can be observed in slices from KO animals. (E) The averaged time course of inhibition of mossy fiber EPSCs by the group II metabotropic glutamate receptor agonist DCG IV (1 μM) for n = 11 (WT) and n = 10 (Munc13-2 KO) cells. (F) The bar graph summarizes the maximum suppression of EPSC amplitudes by application of DCG IV for both genotypes. Traces above the graph are averages of 5 sweeps from representative experiments showing responses in control condition and in the presence of DCG IV, respectively.
Figure 4. Short-term plasticity of GABAergic and AC synapses onto CA3 pyramidal cells as well as presynaptic calcium influx into mossy fiber terminals is normal in the absence of Munc13-2. (A) Whole-cell recording of stimulus-evoked IPSCs in CA3 pyramidal neurons. The bar graph shows the average paired-pulse depression at an interstimulus interval of 50 ms. Traces above the graph are averages of 5 sweeps for WT (left) and Munc13-2 KO (right). (B) The graph plots the IPSC paired pulse ratio for interstimulus intervals of 50, 100, 400, and 1500 ms for WT (n = 7 to 4 cells, open circles) and Munc13-2 KO (n = 7 to 5 cells, black circles). There is no statistically significant difference for any interstimulus interval. Please note the logarithmic time-axis. (C) The bar graph summarizes the paired-pulse facilitation of AC-evoked EPSCs in CA3 pyramidal neurons for WT (n = 11 cells, open circles) and Munc13-2 KO mice (n = 12 cells, black circles) for 50-ms interstimulus interval. Traces on top are averages of 5 sweeps each. (D) Application of the low-affinity AMPA receptor antagonist γ-DGG leads to a decrease in EPSC amplitude at AC synapses. The decrease in amplitude was not significantly different in WT and Munc13-2 KO experiments as seen in this summary plot of n = 4 experiments each. Representative traces on top depict overlays of averages of 5 traces each during control and γ-DGG conditions. (E) Single photodiode recordings of calcium transients in mossy fiber terminals. The graph depicts the paired-pulse ratio of calcium transients in WT (n = 3 slices, open circles) and Munc13-2 KO mice (n = 7 slices, black circles) for interstimulus intervals of 50-500 ms. Facilitation of Ca²⁺ transients was determined by subtracting a single-pulse from the paired-pulse experiment. No difference between the genotypes could be observed. (F) Reduction of the external calcium concentration led to a relative inhibition of calcium transients in mossy fiber terminals, which was not significantly different in WT (n = 3 slices) and Munc13-2 KO (n = 4 slices) animals. Peak calcium influx was normalized to the standard extracellular calcium concentration of 2.5 mM. Curves were fitted according to a double-exponential binding reaction (see Methods).
genotypes to the same extent (Fig. 4D, WT 56.8 ± 4% of control, n = 4 cells, 2 mice vs. KO 51.0 ± 8%, n = 4 cells, 2 mice, P = 0.56), indicating that the basal release probability is unchanged by the loss of Munc13-2 at the AC synapse.

**Calcium-Influx into Mossy Fiber Boutons is Unaltered in the Absence of Munc13-2**

As short-term plasticity at mossy fiber synapses displays an unusually large magnitude, additional mechanisms besides the residual Ca$^{2+}$ hypothesis have been suggested to be of significance. One such hypothesis poses that activation of presynaptic kainate autoreceptors at mossy fiber (MF) terminals leads to an enhancement of stimulus-induced Ca$^{2+}$ influx, that is, a short-term facilitation of presynaptic Ca$^{2+}$ influx (Kamiya et al. 2002). To test whether differences in presynaptic Ca$^{2+}$ influx into MF terminals may be responsible for the increased short-term facilitation of MF-EPSP/Cs in Munc13-2 KO mice we optically recorded Ca$^{2+}$ transients in MF boutons. Mossy fibers were labeled with the membrane-permeable Ca$^{2+}$ indicator dye Magnesium-Green AM and stimulus-induced Ca$^{2+}$ influxes were recorded with a single photodiode (Regehr et al. 1994; Gundlfinger et al. 2007). In both genotypes we found a moderate short-term facilitation of the Ca$^{2+}$ transients for interstimulus intervals of up to 300 ms. The magnitude of the facilitation was not significantly different in the 2 genotypes (Fig. 4E: WT: 1.14 ± 0.07, n = 5 slices, 4 mice vs. KO: 1.11 ± 0.02, n = 7 slices, 3 mice, P = 0.78 for 50-ms ISIs). Moreover, lowering the external Ca$^{2+}$ concentration did not show any differences in the relative reduction of the Ca$^{2+}$ transients (Fig. 4F).

In conclusion, the lack of Munc13-2 has no influence on short-term plasticity characteristics of inhibitory or AC excitatory PSCs on CA3 pyramidal neurons, nor can an alteration in the short-term facilitation of MF bouton Ca$^{2+}$ transients account for the increase in short-term plasticity of mossy fiber synapses.

We also tested for the expression levels of important synaptic proteins, which all have been implicated in different forms of plasticity at hippocampal synapses, that is, Rim1α (Castillo et al. 2002; Schoch et al. 2002), the kainate receptor subunits GluR 6/7 (Contractor et al. 2001; Schmitz et al. 2001; Kamiya et al. 2002; Lauri et al. 2003), Rab3A (Castillo et al. 1997), and Calbindin (Blatow et al. 2003). Specifically for the latter, protein calcium-buffer saturation has been implicated to be of importance in short-term plasticity at mossy fiber synapses. The expression of neither of these proteins was significantly altered in Munc13-2 KO mice (Fig. 5A and B), therefore most likely not causing altered short-term plasticity at mossy fibers in the KO animals.

**Reduced Release Probability at Mossy Fiber Synapses in Munc13-2 KO Mice**

A possible common cause for the increase in failures and short-term facilitation of mossy fiber synaptic transmission could be a reduction in release probability. Therefore, we again tested the release probability in both genotypes by the application of the low affinity AMPAR antagonist γ-DGG. Because the mossy fiber synapse has a comparatively low intrinsic basal release probability we had to use rather small amounts of γ-DGG. We found a concentration of 100 μM of the drug to be suitable for this purposes. In WT animals γ-DGG reversibly reduced MF-EPSCs to 65% of control, much less inhibition than in Munc13-2 KO mice, where the EPSC was decreased to 35% of control (Fig. 6, WT: 65% ± 5%, n = 7 cells, 5 mice vs. KO: 35.4% ± 7.5%, n = 6 cells, 5 mice, P < 0.01). These results indicate that there is a reduction of release probability in Munc13-2 KO mice. This reduction can lead to the observed increases in failure rate and short-term plasticity of MF transmission in Munc13-2 KO mice.

If the loss of Munc1-3-2 leads to a reduction in the average release probability this should then also be reflected in a reduction of the frequency of miniature events. Recording of miniature EPSCs in CA3 pyramidal neurons in acute slices is not straightforward, because the source of the spontaneous input is not known as it could stem from either mossy fibers, AC, or perforant path synapses. A possible way to circumvent this problem and to record pure mossy fiber mEPSCs is to look at stimulus-induced asynchronous release events in the presence of strontium. These events show the typical characteristics of mEPSCs (Bekkers and Clements 1999). Therefore, we recorded MF-EPSCs and promoted asynchronous release by replacing the extracellular Ca$^{2+}$ with 8 mM Sr$^{2+}$ (Bekkers and Clements 1999; Lawrence et al. 2004). Under these conditions, synchronous release was strongly reduced and individual asynchronous release events were monitored for 1 s after the presynaptic stimulus (Fig. 7). The spontaneous release frequency, which was determined from a 1-s recording period prior to the stimulus, was not different between WT and Munc13-2 KO mice (WT: 5.4 ± 1.3 Hz, n = 6 cells, 5 mice vs. KO: 5.5 ± 2.1 Hz, n = 6 cells, 4 mice; P = 0.98). For comparison of asynchronous release, the detected events were computed into a poststimulus latency histogram with a bin width of 10 ms. The first 10 ms after stimulation was considered to consist of synchronous release events and was therefore excluded from the histograms (Bekkers and Clements 1999). To minimize possible contamination by spontaneous release in the analysis we only compared the first 200 ms (10–210 ms) after the stimulus during which the asynchronous release frequency was at least 5 times higher than the spontaneous release frequency. We found that the average number of asynchronous release events per trial was strongly reduced in
Munc13-2 KO mice as compared with WT (Fig. 7C, WT: 14.9 ± 2 events, that is, average number of events per trial and cell within 10–210 ms after the stimulus, n = 6 cells, 5 mice and KO: 5.9 ± 1.5 events, n = 6 cells, 4 mice; P < 0.01). At the end of the recording period in Sr2⁺, the perfusion was switched back to a Ca2⁺-containing solution and the mossy fiber origin of the recording was verified, after the synchronous EPSCs had recovered, by the application of DCG IV (1 μM). This marked reduction in the frequency of release events in the presence of strontium is consistent with the role of Munc13-2 in the first stages of priming and is a further line of evidence for a reduced average release probability at mossy fiber synapses in Munc13-2 KO animals.

Mossy Fiber Long-term Potentiation is Not Affected by the Loss of Munc13-2

Recent data indicate that Munc13, Rim1α, and Rab3A form a tripartite molecular complex that may facilitate vesicle priming during mossy fiber long-term potentiation (LTP) (Dulubova et al. 2005). Regarding the well-established presynaptic form of mossy fiber LTP, a pivotal role has been shown for the synaptic vesicle protein Rab3A and its interacting molecule RIM1α. It was demonstrated that without RIM1α or Rab3A LTP cannot be elicited at mossy fiber synapses (Castillo et al. 1997, 2002). Given the interaction in the tripartite complex, it seemed possible that Munc13-2 is also of importance for mossy fiber LTP.

To test this possibility, we first monitored chemical potentiation of mossy fiber transmission by application of the adenylate cyclase activator forskolin (50 μM). An increase in cAMP levels and subsequent protein kinase A activity have been shown to be crucial for the establishment of mossy fiber LTP (Huang et al. 1994; Weisskopf et al. 1994). A 15-min wash-in of the drug led to an enhancement of transmission that outlasted the application period in WT and Munc13-2 KO mice with no significant difference between the 2 genotypes (Fig. 8A, WT: 389% ± 20.2% of control, n = 5 slices, 4 mice vs. KO: 423% ± 21.5% of control, n = 6 slices, 4 mice).
In a final set of experiments, LTP was electrically evoked by applying a tetanus of 125 pulses at a frequency of 25 Hz for 3 times with an interval of 20 s between the tetani. Again, the magnitude of LTP was not significantly different between WT and KO animals (Fig. 8B, WT: 235.8% ± 22.4% of control, n = 4 slices, 2 mice vs. KO: 256.0% ± 20.5%, n = 5 slices, 2 mice; P = 0.52). Thus, we conclude that Munc13-2 is dispensable for the expression of LTP at hippocampal mossy fiber synapses.

Discussion

In the present study, we analyzed the functional role of the active zone component Munc13-2 in 4 different types of
synapses in hippocampal slices. We found severely compromised synaptic release and increased short-term plasticity at mossy fiber synapses of Munc13-2 KO mice, whereas the functionality of Schaffer-collateral, AC, and inhibitory synapses onto CA3 pyramidal neurons was unaffected in the mutant mice. The absence of Munc13-2 led to a strong reduction in the input–output behavior of mossy fiber synaptic transmission in field potential recordings. This measure is sensitive to 3 important parameters of synaptic transmission, that is, quantal content ($q$), number of release sites ($n$), and release probability ($p$). Several lines of evidence, however, indicate that the drastic impairment of mossy fiber transmission in Munc13-2 KOs is caused by a strong reduction in release probability. (Note that here and in all of the following, we use the term “release probability” to refer to the average release probability ($Pr$) of a given synaptic connection.) First, an increase in paired-pulse ratio as seen in the Munc13-2 KO mice indicates a lower release probability, because of the inverse relationship that exists between release probability and short-term plasticity (Thomson 2000; Zucker and Regehr 2002). Second, we observed a higher rate of transmission failures in Munc13-2 KO than in WT mice in whole-cell recordings of CA3 pyramidal neurons. Third, we found a higher sensitivity of mossy fiber synaptic transmission to the competitive low-affinity AMPA receptor antagonist $\gamma$-DGG in Munc13-2 KO than in WT animals. (Please see Results for rationale of the argument regarding $\gamma$-DGG.) Finally, we analyzed mossy fiber specific miniature events by promoting asynchronous release through the replacement of calcium by strontium. Here, the frequency of asynchronous release events was drastically reduced in Munc13-2 KO mice. Taken together, these results support the notion that Munc13-2 is essential for the regulation of release probability at mossy fiber synaptic terminals.

Recently, it has been proposed that the active zone component Rim13a has a specific role in boosting asynchronous release in hippocampal cell culture autapses (Calakos et al. 2004). We rather explain our findings in the strontium recordings as a direct reflection of the reduced release probability in Munc13-2 KO mice and not as a special role of Munc13-2 in the asynchronous release process itself. The loss of components of the active zone implicated in docking and priming of synaptic vesicles can lead to a reduction in release probability and subsequent changes in short-term plasticity, as has been shown for 2 putative components of the tripartite Munc13/ Rab3A/Rim13a complex in the hippocampal slice preparation (Castillo et al. 1997, 2002; Schoch et al. 2002). Deletion of either Rab3A or Rim13a causes an increase in paired-pulse facilitation at Schaffer-collateral synapses, but only in receptor antagonist $\gamma$-DGG in Munc13-2 KO than in WT animals. (Please see Results for rationale of the argument regarding $\gamma$-DGG.) Finally, we analyzed mossy fiber specific miniature events by promoting asynchronous release through the replacement of calcium by strontium. Here, the frequency of asynchronous release events was drastically reduced in Munc13-2 KO mice. Taken together, these results support the notion that Munc13-2 is essential for the regulation of release probability at mossy fiber synaptic terminals.

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Rab3A KOs show a dissociation between paired-pulse facilitation (increase) and release probability (normal) (Schoch et al. 2002). At the mossy fiber synapse on the other hand, short-term plasticity was unaffected by the loss of either Rab3A or Rim1\(\alpha\) but long-term potentiation was abolished in both cases (Castillo et al., 1997, 2002). The data presented here show that Munc13-2 does not influence basal synaptic transmission and short-term facilitation (please see Supplementary Fig. 1) at Schaffer-collateral synapses, but plays a decisive role in regulating release probability at mossy fiber synapses.

In hippocampal autaptic cell cultures it was recently found that synapses solely driven by Munc13-2 display synaptic short-term facilitation (Rosenmund et al. 2002) using double KOs of Munc13-1 and Munc13-2 in combination with subsequent overexpression of either protein. Here, in the acute hippocampal slice preparation however, we could not detect a specific facilitatory function of Munc13-2 for the mossy fiber synapse. On the contrary, we observed an increase in short-term facilitation in the absence of Munc13-2. Short-term facilitatory phenomena would have been expected to be not present or be strongly reduced in synapses without Munc13-2 if this protein was necessary to obtain a facilitatory phenotype. We interpret the elevated facilitation to be a consequence of the reduced release probability at mossy fiber synapses. The autaptic cell cultures in the study of Rosenmund et al. (2002) most likely consisted predominantly of pyramidal neurons. Therefore, mossy fiber synapses were not analyzed, which confounds a direct comparison of those data with the present study.

The finding that Munc13-2 shapes basal release probability and short-term facilitation but does not affect long-term plasticity is in contrast to the study of Rim1\(\alpha\) at mossy fiber synapses, where long-term potentiation but not short-term facilitation was compromised (Castillo et al. 2002). One explanation for this disparity might be that even though Rab3A/Rim1\(\alpha\) and Munc13s may form a tripartite complex, the individual molecules do exert additional functions that are independent of each other (Wojcik and Brose 2007). A possible difference is that Munc13s act downstream of the other 2 partners in the priming process but, unlike Rim1\(\alpha\), may not be a phosphorylation substrate for PKA (Lonart et al. 2003) and may therefore not participate in the expression mechanism of long-term potentiation. Two other reports have found that loss of Munc13-3 leads to an increase in short-term facilitation at parallel-fiber synapses onto Purkinje cells (Augustin et al. 2001) and Golgi cells in the cerebellum, but post-tetanic potentiation was normal (Beierlein et al. 2007). Also, in the latter study the role of a Munc13 protein was confined to facilitation over short time scales.

The specific importance of Munc13-2 for mossy fiber synapses is further emphasized by the fact that transmission at Schaffer-collateral as well as AC synapses and inhibitory inputs onto CA3 pyramidal neurons were unaffected by the loss of this protein. The unchanged behavior of the inhibitory inputs was rather expected, given the functional redundancy of Munc13-1 and Munc13-2 at GABAergic terminals (Varoquaux et al. 2002). But for the hippocampal glutamatergic synapses the apparent functional specificity of Munc13-2 at mossy fiber synapses is interesting. Although, there is a strong expression of both Munc13-1 and Munc13-2 in area CA1 and CA3 as well as the dentate granule cell layer in the hippocampus (Kalla et al. 2006), only mossy fiber synapses are strongly affected by the loss of Munc13-2. Therefore, despite the ubiquitous expression of the 2 Munc13 gene products in the murine forebrain, a functional specialization at different synapses has to be taken into account. Differential functional effects at different synapses have also been described for Rab3A and Rim1\(\alpha\) (see above) (Castillo et al. 2002; Schoch et al. 2002).

To summarize our results, we could clearly demonstrate that the presynaptic active zone protein Munc13-2 differentially shapes synaptic transmission and short-term plasticity at Schaffer-collateral onto CA1 pyramidal cell synapses and GABAergic, AC, and MF synapses onto CA3 pyramidal cells. Specifically, Munc13-2 is critical for normal release probability as well as short-term facilitation at mossy fiber synapses, whereas it does not significantly modulate these parameters at the other examined hippocampal synapses. Therefore, Munc13-2 participates in fine tuning the high-pass filtering characteristics of mossy fiber synapses and strongly influences how presynaptic spike patterns are translated through this second step of the hippocampal trisynaptic circuit. This synapse-specific effect might contribute to how the network differentially copes with chronically altered neuronal activity (Kim and Tsien 2008) and thus be of particular pathological relevance.

Supplementary Material
Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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Notes
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References