Aberrant Morphology and Residual Transmitter Release at the Munc13-Deficient Mouse Neuromuscular Synapse†

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In cultured hippocampal neurons, synaptogenesis is largely independent of synaptic transmission, while several accounts in the literature indicate that synaptogenesis at cholinergic neuromuscular junctions in mammals appears to partially depend on synaptic activity. To systematically examine the role of synaptic activity in synaptogenesis at the neuromuscular junction, we investigated neuromuscular synaptogenesis and neurotransmitter release of mice lacking all synaptic vesicle priming proteins of the Munc13 family. Munc13-deficient mice are completely paralyzed at birth and die immediately, but form specialized neuromuscular endplates that display typical synaptic features. However, the distribution, number, size, and shape of these synapses, as well as the number of motor neurons they originate from and the maturation state of muscle cells, are profoundly altered. Surprisingly, Munc13-deficient synapses exhibit significantly increased spontaneous quantal acetylcholine release, although fewer fusion-competent synaptic vesicles are present and nerve stimulation-evoked secretion is hardly elicitable and strongly reduced in magnitude. We conclude that the residual transmitter release in Munc13-deficient mice is not sufficient to sustain normal synaptogenesis at the neuromuscular junction, essentially causing morphological aberrations that are also seen upon total blockade of neuromuscular transmission in other genetic models. Our data confirm the importance of Munc13 proteins in synaptic vesicle priming at the neuromuscular junction but indicate also that priming at this synapse may differ from priming at glutamatergic and γ-aminobutyric acid-ergic synapses and is partly Munc13 independent. Thus, non-Munc13 priming proteins exist at this synapse or vesicle priming occurs in part spontaneously: i.e., without dedicated priming proteins in the release machinery.

Transmitter release from presynaptic terminals is mediated by the exocytotic fusion of transmitter-filled synaptic vesicles. Fusion of these vesicles is triggered by membrane depolarization and concomitant influx of Ca2+ ions and is dependent on the SNARE proteins synaptobrevin/VAMP 2, syntaxin 1, and SNAP-25, whose assembly into a highly stable SNARE complex (42) is thought to drive the fusion reaction (reviewed in references 20, 24, and 33).

Before fusion can be initiated, synaptic vesicles must be primed into a fusion-competent state (for review, see references 16, 33, and 37). Members of the Munc13 family, mammalian homologues of Caenorhabditis elegans Unc-13 (15), play an essential role during this priming reaction (2, 3, 34, 35, 38). At the molecular level, synaptic vesicle priming is thought to depend on a conformational switch of the SNARE protein syntaxin 1 from a closed conformation, which prevents SNARE complex assembly, to an open conformation, which permits it (19). It is believed that Munc13 plays an important role during this conformational switch, since the overexpression of an open syntaxin mutant in C. elegans bypassed the strict requirement for Unc-13 (35). In C. elegans, Unc-13 is essential for vesicle priming at both cholinergic and γ-aminobutyric acid-ergic (GABAergic) synapses (14, 28, 35).

In mammals, the Munc13 protein family comprises three highly homologous members, Munc13-1, bMunc13-2/ubMunc13-2 (splice variants of the Munc13-2 gene), and Munc13-3 (16), which are differentially distributed in the brain (4) and confer differential short-term plasticity characteristics to the synapses they equip (25, 38). Transmitter release from both glutamatergic and GABAergic neurons in the hippocampus is strictly dependent on Munc13 function. In the absence of Munc13-1 and Munc13-2, these neurons show neither spontaneous nor evoked synaptic release events, yet develop normal numbers of synapses which contain an electrophysiologically normal postsynaptic AMPA (α-amino-2,3-dihydro-5-methyl-3-oxo-4-isoxazole propanoic acid) and GABA receptor complement, but exhibit a broader active zone (45). These findings led to the conclusion that genesis and assembly of synapses between hippocampal nerve cells are largely independent of synaptic activity. Rather, synaptogenesis in the central nervous system may follow a default developmental program that is only modulated, stabilized, and refined by synaptic activity (45).

In many aspects, the neuromuscular synapse, which uses acetylcholine as a neurotransmitter, is similar to central synapses and therefore a widely used model for the study of synaptogenesis. The formation and maturation of the neuromuscular junction (NMJ) are known to rely in part on activity-dependent signals. Initially, evidence in support of this view was obtained in studies where the developmental role of synaptic transmission at the NMJ had been examined using anti-
cholinergic or activity-blocking drugs (reviewed in references 13 and 30). More recently, genetic studies on mutant mice lacking choline acetyltransferase (ChAT), the enzyme responsible for producing acetylcholine, provided compelling evidence for the requirement of neurotransmitter release in NMJ formation (13, 30).

Based on our observations in the central nervous system (45) and the fact that Munc13-deficient mice are completely paralyzed, we expected to find a total blockade of transmitter release at the NMJ in the absence of Munc13s. We report here the unexpected finding that neuromuscular synaptic transmission is not entirely abolished in the absence of Munc13s. Nevertheless, the morphology of the NMJ shows abnormalities comparable to those seen in CHAT deletion mutant mice. We characterize the features of the neuromuscular apparatus in Munc13-deficient NMJs and discuss the role of different types of synaptic activity in regulating synaptogenesis at NMJs and the function of Munc13s at peripheral and central synapses.

MATERIALS AND METHODS

Mouse lines. Single-deletion mutant mice lacking Munc13-1, Munc13-2, or Munc13-3 were published previously (5, 6, 45). Double- and triple-mutant (DKO and TKO, respectively) mice were obtained by interbreeding of the single-mutant lines. Prior to experiments, mice heterozygous for the lethal Munc13-1 deletion and heterozygous or homozygous for the Munc13-2 deletion, and in some cases also for the Munc13-3 deletion, were mated for 24 h (embryonic day 0 [E0]). At E18.5, the pregnant mothers were sacrificed by cervical dislocation. Embryos were recovered by hystereotomy and further processed on ice. Homozygous Munc13-1/2 double-mutant and Munc13-1/2/3 triple-mutant embryos were easily recognizable in the litter due to their complete paralysis and exhibited identical phenotypes in all subsequent experiments. Embryos heterozygous for the Munc13-1 and Munc13-2 deletions or heterozygous for the Munc13-1 deletion and homozygous for the Munc13-2 deletion were indistinguishable from wild-type animals (not shown) and served as littermate controls in all subsequent analyses.

Animal preparation. For spinal cord preparations, E18.5 embryos were fixed by perfusion with 4% paraformaldehyde in phosphate buffer. The spinal cord (cervical levels 3 to 5) was then dissected out under a binocular microscope. For diaphragm preparations, E18.5 embryos were decapitated and the ribcage was quickly isolated and fixed by immersion (2 to 12 h). Subsequently, the diaphragm muscle was dissected and fixed by immersion (2 to 12 h). In some cases also for the Munc13-3 deletion, the embryos were fixed for 16 h at room temperature until red, 5% acetic acid, and 93% ethanol. After washes in water, samples were kept stained for 10 days in a solution containing 0.015% alcian blue, 0.005% alizarin red, and 0.5% aprotinin; and centrifuged at 1,000 g. The diaphragm muscles from 20 newborn mice were dissected out under a binocular microscope, and flash-frozen in liquid nitrogen. Diaphragms were then thawed; homogenized by Western blotting of muscle membranes that were prepared as follows. The diaphragm muscles from 20 newborn mice were dissected out under a binocular microscope. Fifty-micrometer-thick free-floating vibratome sections were used as whole mounts.

Immunocytochemistry. Fifty-micrometer-thick free-floating vibratome sections of the spinal cord were made at the cervical level and either stained for the vesicular acetylcholine transporter (vAChT) with a rabbit polyclonal antibody (1:500; Chemicon) or Nissl stained. Free-floating diaphragms from mutant and control mice were incubated with α-bungarotoxin–Alexa 568 (1:2,000; Molecular Probes) or with antibodies against synapsin (rabbit polyclonal, 1:500; Synaptic Systems) or S-100 (mouse monoclonal, 1:500; DAKO), to visualize acetylcholine receptors, presynaptic terminals, and Schwann cells, respectively. Acetylcholines- terase activity was visualized histochemically by incubation of the fixed diaphragms for 30 min at 37°C in 0.5 mM S-bromoiodoacetate (23). All preparations were used as whole mounts.

RESULTS

Macroscopic phenotype of Munc13-1/2 double-deficient mutants. Munc13-2 and Munc13-3 single-mutant mice are viable, fertile, and show no abnormalities (5, 45), while Munc13-1-deficient mice die within a few hours after birth (6).

The Munc13-1/2 double-deletion mutant mice (Munc13-1/2-DKO) and Munc13-1/2/3 triple-deletion mutant mice (Munc13-1/2/3-TKO) studied here showed even stronger phenotypic alterations, whereas Munc13-2/3 double-mutant mice (Munc13-2/3-DKO) were viable and fertile, indicating a dom-
nant role of Munc13-1 in mice. Munc13-1/2-DKO and Munc13-1/2/3-TKO mice were morphologically indistinguishable from each other, and had identical phenotypes with respect to neuromuscular synaptic structure and function (see below). This could be due to the fact that NMJ axon terminals contain Munc13-1 and ubMunc13-2, but neither bMunc13-2 nor Munc13-3, as determined by Western blot analysis of muscle membrane preparations (Fig. 1A). Therefore, results obtained from Munc13-1/2-DKO and Munc13-1/2/3-TKO mice were pooled and subsequently referred to as “Munc13-1/2-DKO” for clarity. More distantly related Munc13 homologues are either faintly detectable (Munc13-4; molecular mass, 112 kDa) or not detectable (BAP3; molecular mass, 125 kDa) in muscle membrane preparations (Fig. 1A). Therefore, results obtained from Munc13-1/2-DKO and Munc13-1/2/3-TKO mice were pooled and subsequently referred to as “Munc13-1/2-DKO” for clarity. More distantly related Munc13 homologues are either faintly detectable (Munc13-4; molecular mass, 112 kDa) or not detectable (BAP3; molecular mass, 125 kDa) in muscle membrane preparations (Fig. 1A). Because Munc13-1/2-DKO mice were often born dead, all experiments were carried out on E18.5 embryos, whose central and peripheral nervous systems are developed extensively and which can be recovered alive upon hysterectomy. Munc13-2-KO and Munc13-2/3-DKO littermates were used as controls as they were indistinguishable from wild-type animals with respect to neuromuscular synaptic transmission.

Munc13-1/2-DKO embryos were completely paralyzed, did not breathe or respond to tactile stimulation, and had a very fragile appearance. They had a hunched posture (Fig. 1B) and often showed hematomas along the spinal cord and on the skull. In the Munc13-1/2-DKO mouse (Fig. 1C), no developmental defect of the skeleton was detectable after in toto staining for bone and cartilage. However, the rib cage appeared larger and the vertebra more compact at the cervical level, probably reflecting a permanent paralysis of the embryo throughout development.

The total paralysis we observed in Munc13-1/2-DKO embryos indicated a profound defect at the NMJ in addition to the central nervous system dysfunction seen in these mice (6, 45). To examine this in more detail, we investigated the structure and function of the NMJ using the well-characterized phrenic nerve/diaphragm muscle preparation as a model system.

Muscle morphology in Munc13-1/2 double-deficient mutants. The fragile appearance of the Munc13-1/2-DKO embryo was paralleled by an abnormally thin musculature. In E18.5 embryos, the diaphragm muscle appeared fully developed along its rostro-caudal axis, but its outermost edges in-

FIG. 1. Munc13 isoforms at the neuromuscular junction and phenotypic alterations in the Munc13-1/2-DKO mouse mutant. (A) Immunoblot analysis of muscle membrane extract (M) with anti-Munc13-1, -b/ubMunc13-2, -Munc13-3, -Munc13-4 and -BAP3 isoform-specific antibodies. Brain (lanes B) or lung (lanes L) homogenates were used as positive control. (B and C) E18.5 Munc13-1/2-DKO mutant and control littermate mice gross morphology (B) and skeleton (C; bones are stained in blue and cartilage in pink). A white arrow points to a broadened rib cage, and a black arrow points to a stiffened neck and a compacted spinal cord. Scale bar, 3 mm.
dicated an impaired lateral extension of myotubes (Fig. 2A). Moreover, muscle fibers were not strictly aligned and formed intermingled bundles (Fig. 2A). Muscle cells were more loosely attached to each other, on average smaller than in diaphragms of control littermates (muscle cell area, $389 \pm 25 \mu m^2$, $n = 104$, control, versus $136 \pm 5 \mu m^2$, $n = 227$, Munc13-1/2-DKO), and exhibited centrally localized nuclei that had apparently not migrated to the cell periphery (Fig. 2B), indicating a maturation delay or defect of some of the myotubes. In addition, many mostly oversized blood vessels ran throughout the diaphragm (Fig. 2B), possibly due to a lack of muscle tone.

**Diaphragm innervation in Munc13-1/2 double-deficient mutants.** The diaphragm is innervated by the left and right phrenic nerves, each of which branches and forms synapses that are typically organized in a discrete endplate band within the central region of the respective hemidiaphragm. Using a whole-mount enzymatic staining for acetylcholinesterase, which is particularly abundant at the synaptic cleft, we observed regularly distributed synapses in both hemidiaphragms of control mice (Fig. 3). In Munc13-1/2-DKO mice, however, the phrenic nerves exhibited an abnormally extensive branching throughout the muscle and its terminal arborizations covered a much broader surface of the muscle (Fig. 3). The acetylcholinesterase staining intensity in Munc13-1/2-DKO diaphragms, which correlates with the amount of acetylcholinesterase present at a given synapse, was slightly reduced as compared to control levels.

To further analyze the neuromuscular connectivity in Munc13-1/2-DKO mice, combined immunostainings for synapsin-containing presynaptic terminals, $\alpha$-bungarotoxin-binding acetylcholine receptors, and S-100-expressing Schwann cells were carried out. In control as well as in Munc13-1/2-DKO diaphragms, all axon terminals were juxtaposed to acetylcholine receptor clusters, and vice versa, and all terminals were ensheathed by Schwann cells (Fig. 4). However, acetylcholine receptor and synapsin stainings showed that motor endplate units in the Munc13-1/2-DKO diaphragm were only poorly aligned along the midline of the diaphragm and no longer confined to it, but rather distributed as a large array of clusters (Fig. 4). Quantitative analyses showed that the area occupied by endplates (as defined by $\alpha$-bungarotoxin-labeled acetylcholine receptor clusters) at the midline of the diaphragm was larger in the Munc13-1/2-DKO mice than in the control mice ($4,984 \pm 1,150 \mu m^2$ per $0.2 mm^2$, $n = 5$, in Munc13-1/2-DKO mice versus $2,144 \pm 436 \mu m^2$ per $0.2 mm^2$, $n = 5$, in control mice, $P < 0.05$). In addition, the number of synapses was clearly increased in the Munc13-1/2-DKO diaphragm mouse (not shown).

**Cytoarchitecture of the spinal cord and morphology of the phrenic nerve in Munc13-1/2 double-deficient mutants.** The phrenic motor neuron cell bodies that innervate the diaphragm are located at cervical levels C3 to C5 of the spinal cord and typically undergo massive apoptosis around E15 to -17 in the rat and mouse embryo (1, 22). We analyzed the number of the large cell body phrenic motor neurons in control and Munc13-1/2-DKO mice in Nissl-stained vibratome sections. We found...
that at all cervical levels these motor neuron groups in the ventral horn were larger in Munc13-1/2-DKO mice and contained more cells than the corresponding areas in control sections (Fig. 5A). No sign of degeneration was detectable in dorsal root ganglia (not shown). Motor neuron somata receive a specific recurrent cholinergic innervation, which we visualized by immunostaining for the vAChT. Cholinergic terminals in the ventral horn of cervical levels C3 to C5 in the Munc13-1/2-DKO mice showed a density that was comparable to that in control sections but covered a larger area of the ventral horn, again indicating an abnormally large population of motor neurons in the mutant (Fig. 5B). Low-magnification ultrastructural analysis of phrenic nerves showed that as a result of the increased motor neuron number in Munc13-1/2-DKO mice, the nerves were larger and contained more axons (367 ± 27, n = 6, in Munc13-1/2-DKO mice versus 213 ± 16, n = 8, in controls, P < 0.001) (Fig. 5C and D). This mutant phenotype was accompanied by an increased number of Schwann cell bodies (44 ± 5.8, n = 3, in Munc13-1/2-DKO mice versus 27 ± 1.5, n = 3, in controls, P < 0.05), but the extents of axon myelinization were similar in control and Munc13-1/2-DKO nerves (Fig. 5C).

Electrophysiological properties of neuromuscular synaptic transmission in Munc13-1/2-DKO mice. We investigated the characteristics of synaptic transmission at the NMJ of Munc13-1/2-DKO and Munc13-1/2/3-TKO mice. As predicted by the lack of Munc13-3 immunoreactivity in NMJ terminals, our analyses showed no difference between Munc13-1/2-DKO (n = 13) and Munc13-1/2/3-TKO (n = 3) mice. Therefore, as for morphological observations, data from Munc13-1/2-DKO and Munc13-1/2/3-TKO mice were subsequently pooled.

Surprisingly, and in contrast to glutamatergic and GABAergic synapses in hippocampal neurons, intracellular recordings of MEPPs revealed that neurotransmitter release is not com-

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**FIG. 3.** Impaired branching and endplate distribution of the Munc13-1/2-DKO phrenic nerve terminating onto the diaphragm surface. (A and B) Detail of the left (A) and right (B) hemidiaphragms of Munc13-1/2-DKO mutant and control littermate, stained for acetylcholinesterase. Scale bar, 180 μm.

**FIG. 4.** Normal apposition of presynaptic, postsynaptic, and glial elements at the Munc13-1/2-DKO motor endplate. (A and B) Confocal micrographs of Munc13-1/2-DKO mutant and control littermate, double immunostained for α-bungarotoxin (α-BGT; to visualize acetylcholine receptors) and synapsin (as a marker for presynapses) (A) or S-100 (S100β; as a marker for Schwann cells) (B). Scale bars, 90 μm in panel A and 190 μm in panel B.
pletely abolished at the NMJs of Munc13-1/2-DKO mice. MEPP amplitude (2.89 ± 0.28 mV, n = 15 muscles) was not statistically significantly different from that in control embryos (2.96 ± 0.28 mV, n = 15, P = 0.86), and MEPP frequency was more than doubled in Munc13-1/2-DKO embryos (4.42 ± 0.60 per min, n = 15), as compared to that in controls (1.80 ± 0.34 per min, n = 15, P < 0.001; Fig. 6A). α-Latrotoxin elicits massive asynchronous unquantal acetylcholine release through exocytosis from all synaptic vesicles that are fusion competent and therefore are probably in a primed state. Application of this toxin revealed that this type of neurotransmitter release is strongly impaired at NMJs of Munc13-1/2-DKO embryos. MEPP frequency was 591 ± 137 per min (n = 8) in Munc13-1/2-DKO NMJs and 1,996 ± 500 per min (n = 7, P < 0.05) in control NMJs (Fig. 6B). MEPP amplitudes were similar in Munc13-1/2-DKO embryos (4.00 ± 0.6 mV, n = 6) and controls (2.81 ± 0.30 mV, n = 7, P = 0.09). Like α-latrotoxin, application of hypertonic sucrose solution, which triggers the release of fusion-competent synaptic vesicles, induced much lower MEPP frequencies in Munc13-1/2-DKO embryos (144 ± 66 per min, n = 5) as compared to controls (619 ± 100 per min, n = 7, P < 0.01) (Fig. 6C). Unexpectedly, sucrose treatment reduced MEPP amplitude to 1.33 ± 0.24 mV (n = 5) compared to 3.35 ± 0.23 mV (n = 7) in the controls. Thus, the asynchronous unquantal acetylcholine release induced by α-latrotoxin or hypertonic shock is severely reduced at Munc13-1/2-DKO NMJs.

We stimulated the phrenic nerve at 0.3 and 20 Hz through a suction electrode to evoke acetylcholine release by nerve impulses. The resulting muscle contractions were monitored visually through the microscope. We observed a robust contraction of the whole control muscle preparation, involving all muscle fibers, which was well sustained at 20 Hz. However, contraction of Munc13-1/2-DKO preparations was much weaker because not all fibers contracted and was not very well sustained at 20 Hz (for video recordings, see supplemental material). This indicated that presynaptic transmitter release can at least to some extent induce postsynaptic action potentials in these mutants (Fig. 6D). EPPs were recorded in depolarized fibers. In 77.1% ± 4.0% of the cases (n = 12), evoked stimulation failed to induce an EPP in the muscle fibers of Munc13-1/2-DKO mice, while in controls, only 0.7% ± 0.7% (n = 6) failures were observed (Fig. 6D and E). In the Munc13-1/2-DKO mice, stronger variability in the delay between the time of nerve stimulation and EPP occurrence was observed (Fig. 6D). The amplitude of the evoked EPP was significantly smaller in Munc13-1/2-DKO NMJs (5.52 ± 0.46 mV, without failures taken into account; 1.34 ± 0.26 mV, with failures taken into account; n = 12) than in control NMJs (21.40 ± 2.10 mV, n = 6, P < 0.001) (Fig. 6E). The calculated quantal content was decreased by 81%, from 6.09 ± 0.74 (n = 6) in control NMJs to 0.56 ± 0.20 (n = 6) in the Munc13-1/2-DKO NMJ (Fig. 6G). Thus, nerve impulse-evoked acetylcholine release is dramatically reduced at Munc13-1/2-DKO NMJs.

Ultrastructural characteristics of NMJs in Munc13-1/2 double-deficient mutants. At the ultrastructural level, well-formed synapses were observed in both control animals and Munc13-1/2-DKO mice. Synapses in the mutants tended to contain more boutons than control synapses, which may be a correlate of the increased complexity of innervation and the larger endplate size observed in the mutants at the light microscopic level (Fig. 4). Synaptic boutons at NMJs of Munc13-1/2-DKO mice contained normal-sized small synaptic vesicles but also dense core and clathrin-coated vesicles (Fig. 7). Boutons in mutant synapses were aligned with postsynaptic densities in muscle cells and exhibited clusters of small synaptic vesicles that were occasionally observed along the plasma membrane or docked at the active zone (Fig. 7, insert). Pre- and postsynaptic membranes were continuously juxtaposed on each other and separated by a well-developed basal lamina. In the control samples, many synapses showed junctional folds reflecting a normal maturation process. In contrast, small invaginations, but no deep folds, were observed at the postsynaptic membranes of NMJs in Munc13-1/2-DKO mice (Fig. 7, insert).

DISCUSSION

The functional relevance of differential Munc13 protein expression at the NMJ. Munc13-1, -2, and -3 are essential for synaptic vesicle priming in central synapses. Data on the priming activity of N-terminally truncated Munc13-1 fragments (3) indicate that the evolutionarily conserved domain structure in Munc13-1, -2, and -3 and the related Munc13-4 (27) and BAP3 (39) proteins, which consists of two Munc13 homology domains flanked by two C2 domains, acts as the minimal priming module. This module covers most of the C-terminal two-thirds of Munc13-1/2/3 (16) and is thought to mediate Munc13 priming activity by binding to (10) and regulating the function of (36) syntaxins. Differences between members of the Munc13 protein family with respect to their priming activity or the fusion reaction they regulate could be due to the type of syntaxin-like SNARE protein their minimal priming module interacts with.

We found the murine NMJ to contain Munc13-1 and ub-Munc13-2, but not bMunc13-2, Munc13-3, or the more distantly related BAP3, and only trace amounts of Munc13-4. The two Munc13 isoforms expressed at the NMJ are the most closely related Munc13 variants. In contrast to other family members, they do not only share the highly conserved C-terminal...
minal region but also have highly homologous N-terminal regions which contain a C2 domain that binds the active zone components RIM1 and RIM2 (11) and a calmodulin binding site (25). Thus, Munc13-1 and ubMunc13-2 may interact with the same protein partners and have similar basic functions, and mutual compensation upon loss of one of the two isoforms is highly likely at the NMJ, as was also reported for hippocampal GABAergic synapses (45). Nevertheless, Munc13-1 and ubMunc13-2 differentially modulate short-term plasticity at hippocampal synapses (25, 38), and their coexpression at the NMJ may allow for the tuning of presynaptic molecular mechanisms over a wide range of synaptic activity rates in order to guarantee high fidelity of synaptic transmission.

Using the diaphragm NMJ as a model system, we found that upon genetic deletion of Munc13s (Munc13-1, -2, and -3) and in the absence of significant levels of the related Munc13-4 and BAP3 proteins, evoked synaptic transmission is strongly reduced while spontaneous release persists, and the NMJ system exhibits all classical developmental aberrations that are typically observed upon complete block of spontaneous and evoked synaptic transmission (13, 30). In the light of previous studies on the function of Munc13s at central synapses, two of our findings at the NMJ are very unexpected: (i) synaptic vesicle priming in the NMJ appears to be partially independent of bona fide Munc13s (Munc13-1, -2, and -3); and (ii) despite the quite large spontaneous transmitter release activity at

FIG. 7. Immature but well-formed neuromuscular synapses in the Munc13-1/2-DKO mutant. Electron micrographs of representative motor endplates in Munc13-1/2-DKO mutant and control littermate. At low magnification (A), Munc13-1/2-DKO motor endplates are always composed of more presynaptic elements, containing numerous small synaptic vesicles, than the littermate ones. In either case, magnified areas of the synaptic active zone (B) allow recognition of small synaptic vesicles docked at the active zone membrane, large dense-core vesicles, clathrin-coated vesicles, and an intact basal lamina. However, the postsynaptic membrane of the Munc13-1/2-DKO muscle cell fails to develop secondary folds that normally accompany the maturation process of neuromuscular synapses (arrow in control). Scale bars: 700 nm in upper panels and 220 nm in lower panels.

FIG. 6. Strongly reduced acetylcholine release evoked by nerve impulses, α-latrotoxin, or hypertonic medium at Munc13-1/2-DKO NMJs. Bar graphs display the group mean values ± standard error of the mean (n = 5 to 15 embryos, 1 to 21 NMJs sampled per muscle). (A) Spontaneous uniquantal acetylcholine release, MEPPs, recorded in normal Ringer’s medium. Superimposed example traces show the MEPPs observed during a 135-s measuring period. (B) MEPPs recorded in the presence of 2.5 nM α-latrotoxin. (C) MEPPs recorded in the presence of 0.5 M sucrose-Ringer’s medium. (D) Examples of nerve stimulation-evoked responses. The moment of phrenic nerve stimulation is indicated with a black triangle. At relative hyperpolarized membrane potentials, a full-size muscle action potential is elicited in control muscle (upper left), leading to contraction that is visible as an artifact on the signal (indicated by open triangle). At Munc13-1/2-DKO NMJs, subthreshold and delayed EPPs and failures were observed (upper right), sometimes leading to delayed muscle action potentials. Subsequent traces (0.3-Hz stimulation) have been superimposed. At depolarized muscle fibers, EPPs become unmasked. At control NMJs, no failures were observed at 0.3-Hz stimulation (bottom left), while at Munc13-1/2-DKO NMJs there were many failures and very small, delayed EPPs. (E) Percentage of stimuli leading to failures. (F) EPP amplitude, normalized to −75 mV membrane potential, failures taken into account. (G) Quantal content (i.e., the number of acetylcholine quanta released upon a single nerve impulse).
Munc13-1/2-DKO NMJs, the innervation of the diaphragm exhibits the same developmental aberrations that are also observed in the complete absence of NMJ synaptic transmission (13, 30).

Munc13-independent synaptic vesicle priming at the NMJ. Synaptic transmission at glutamatergic and GABAergic synapses of murine hippocampal neurons is strictly dependent on the presence of Munc13-1 and -2. Munc13-3, Munc13-4, or BAP3 does not functionally replace Munc13-1 and -2 in these synapses (45). Likewise, spontaneous and evoked transmitter release at the cholinergic NMJ in C. elegans is entirely blocked in worms carrying the complete loss-of-function allele of unc-13, unc-13(s69) (35), although an Unc-13 homologue similar to BAP3 and Munc13-4 (27) is most likely present. In contrast, at murine NMJs lacking Munc13s, spontaneous transmitter release persists and some evoked transmitter release is elicitable. It is unlikely that trace amounts of Munc13-4 or BAP3 mediate the residual synaptic vesicle priming at these mutant NMJs because even robust levels of BAP3 are not sufficient to ameliorate the Munc13-deficient mutant phenotype in hippocampal synapses (45) and Munc13-4 does not bind to RIM (27). Apart from Munc13-4 and BAP3, CAPS proteins (i.e., CAPS1 and CAPS2 in mammals) have been proposed to be priming proteins (9, 27, 32, 43). However, CAPS proteins do not compensate for the loss of Munc13s from hippocampal neurons in spite of strong expression at their synaptic terminals (41, 45) and are therefore also unlikely to support synaptic vesicle priming in NMJs.

As the murine and human genomes do not contain any additional genes with homology to Munc13s, our findings indicate that some vesicle priming at the NMJ occurs in the absence of Munc13s and that therefore either non-Munc13 priming proteins must exist or vesicle priming at the NMJ can occur in part spontaneously without priming proteins. Vesicle priming independent of Munc13-1, -2, and -3 has previously been suggested to occur in chromaffin cells (3). According to the current molecular model, Munc13s mediate synaptic vesicle priming by stabilizing the open conformation of the SNARE syntaxin 1, thereby allowing the formation of SNARE dimers containing syntaxin 1 and SNAP25 or of trans SNARE complexes (16, 20, 36). Constitutive SNARE-mediated intracellular and secretory membrane fusion reactions do not require a Munc13-like priming step; Saccharomyces cerevisiae does not express homologues of Munc13-1, -2, -3, and -4 or BAP3 (27); and bona fide Munc13s first appear during evolution in organisms with a central nervous system (27), indicating that Munc13-independent spontaneous SNARE priming does occur. It is likely that synaptic vesicle exocytosis at the mouse NMJ involves in part a syntaxin variant or other SNARE complex components such as SNAP23 that are less dependent on Munc13s stabilizing the open conformation of syntaxin, with the consequence that some vesicle priming indeed occurs spontaneously.

The SNARE protein complement of murine NMJs is only partially known. NMJs lacking SNAP25 exhibit increased spontaneous transmitter release but lack evoked release (48). It is possible that spontaneous release in SNAP25 KO mutants is due to the presence of SNAP23, which can partly replace SNAP25 but has strikingly different functional features (40). Essentially, our data and the published account in the literature are best compatible with a scenario according to which Munc13-mediated vesicle priming is essential for a majority of synaptic vesicles at NMJs, while a small subpopulation of vesicles can undergo spontaneous priming. This would explain why in the absence of Munc13-1, -2, and -3 evoked transmitter release is strongly reduced while spontaneous release persists.

The increase in the frequency of spontaneous release events seen in SNAP25 KO (48) and Munc13-1/2-DKO NMJs may then simply be due to the increased number of synapses formed in these mutant NMJs.

The importance of synaptic activity for NMJ formation. The phenotypic alterations seen at the Munc13-1/2-DKO NMJ are similar to those reported for the ChAT-deficient mouse NMJ, in which synaptic vesicles are no longer loaded with acetylcholine (13, 30). The same types of abnormal nerve arborizations and disorganized termination areas with more or larger synaptic endplates are also seen in SNAP25-deficient NMJs (48).

Functionally, the Munc13-1/2/3 and SNAP25 KO mice differ from ChAT KO mutants in various aspects. In the ChAT KO, transmission is blocked at the NMJ, while motor neuron cell bodies receive functionally normal synaptic inputs. In the Munc13-1/2-DKO and SNAP25 KO, synaptic transmission at spinal cord synapses is either abolished (not shown) or strongly reduced. This situation is similar to that described for Munc18-1 deletion mutant mice, which are characterized by a complete lack of spontaneous and evoked transmitter release at the NMJ as well as at central synapses (12, 23, 46). In these mutants, motor neurons form synaptic contacts very transiently in early embryonic life and soon thereafter degenerate and retract axonal processes (23). This phenomenon, which is not seen in ChAT KO mice (13, 30) or Munc13-1/2-DKO mice, was explained by the lack of synaptic signaling onto motor neurons in Munc18-1 mutants (23). However, given the fact that in Munc13-1/2-DKO mice no motor neuron degeneration is seen despite a complete shutdown of synaptic transmission onto these neurons, this explanation may be wrong. Rather, the role of Munc18-1 may extend to the regulation of intracellular membrane trafficking events necessary for neuronal survival or of developmentally earlier and more general secretory events, whose impairment dramatically compromises neuronal survival (e.g., neurotrophin signaling). As a consequence, Munc18-1 may exert a more stringent control of exocytosis at the NMJ, abolishing not only fast synaptic transmission but also the secretion of neuroactive peptides, neurotrophic factors, or hormones. In contrast, Munc13-1/2/3 deletion still allows for the fusion of a small population of synaptic and peptidergic vesicles, and ChAT deletion still permits the release of transmitter-deficient vesicles that contain neuroactive or neurotrophic peptides. These may influence synapse formation, synapse maintenance, and neuronal survival (13, 30).

The increased number of motor neurons in the spinal cord of Munc13-DKO mice is presumably due to a cessation of their apoptosis, which occurs normally around E15 to E17 (1, 22). As mentioned above, this blockade of apoptosis is unlikely to result from a block of synaptic signaling in the spinal cord. Instead, it might be due to the local malfunction of the NMJ, thus influencing the well-described process of embryonic synapse elimination that usually leads to the consolidation of only one axon/endplate per muscle fiber (17, 18, 26, 29).

Spontaneous action potentials, which typically occur in mo-
tor neurons during development (21), are likely to be important for shaping nerve-muscle connectivity. They originate from either the spiking of premotor interneurons or the coordinated quantal neurotransmitter release from motor neurons, which is unlikely to occur in Munc13-1/-2-DKO embryos. Thus, muscle action potentials and contractions driven by action potentials in motor neurons are unlikely to take place in vivo in the Munc13-DKO embryo, despite the fact that stimulation of the cut phrenic nerve can elicit action potentials and evoke some contractile response. Similarly, ChaT and SNAP25 deficiencies presumably lead to the elimination of muscle activation driven by motor neuron action potentials. Given that all three mutants exhibit the same phenotype with respect to motor neuron survival and refinement of NMJ connectivity, we conclude that the proper development of motor neurons and NMJs does not depend on a trophic action of spontaneous quantal acetylcholine release. Rather, successful and reliable action potential-driven postsynaptic depolarizations, which can even occur spontaneously during development, appear to be necessary to regulate motor neuron survival and shape mature NMJs.

It is likely that retrograde signaling from the muscle to the innervating motor neuron is involved in the effects of these action potential-driven synthetic events on motor neuron number and NMJ morphology. Interestingly, a pattern of connectivity similar to the one found in Munc13-1/-2-DKO NMJs is seen in MyoD-deficient mice. Here, the abnormal branching must be due to an impaired retrograde signaling because MyoD is a muscle-specific transcription factor (47). In addition, the phenotypes of Rapsyn- and MuSK-deficient mice, which show the same aberrant motor neuron survival seen in Munc13-1/-2-DKO and ChaT KO mice (7, 44), indicate a role of retrograde signaling from muscle in motor neuron survival. By analogy, it is possible that impaired transmission at the Munc13-1, ChaT-, or SNAP25-deficient NMJ affects muscle electrical activity, thereby influencing the levels of myogenic regulatory factors or other signaling molecules and, in turn, retrograde signaling.

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