Munc18-1 redistributes in nerve terminals in an activity- and PKC-dependent manner

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Munc18-1 is a soluble protein essential for synaptic transmission. To investigate the dynamics of endogenous Munc18-1 in neurons, we created a mouse model expressing fluorescently tagged Munc18-1 from the endogenous munc18-1 locus. We show using fluorescence recovery after photobleaching in hippocampal neurons that the majority of Munc18-1 trafficked through axons and targeted to synapses via lateral diffusion together with syntaxin-1. Munc18-1 was strongly expressed at presynaptic terminals, with individual synapses showing a large variation in expression. Axon–synapse exchange rates of Munc18-1 were high: during stimulation, Munc18-1 rapidly dispersed from synapses and reclustered within minutes. Munc18-1 reclustering was independent of syntaxin-1, but required calcium influx and protein kinase C (PKC) activity. Importantly, a PKC-insensitive Munc18-1 mutant did not recluster. We show that synaptic Munc18-1 levels correlate with synaptic strength, and that synapses that recruit more Munc18-1 after stimulation have a larger releasable vesicle pool. Hence, PKC-dependent dynamic control of Munc18-1 levels enables individual synapses to tune their output during periods of activity.

Introduction

Synaptic vesicle fusion is executed by Sec1/Munc18 (SM) proteins and the multisubunit SNARE protein complex of synaptobrevin-2/VAMP2, syntaxin-1, and SNAP25 (Jahn and Scheller, 2006; Jahn and Fasshauer, 2012; Rizo and Südhof, 2012). The SM protein Munc18-1 is a soluble protein initially found as an interacting partner of syntaxin-1 (Hata et al., 1993; Garcia et al., 1994; Pevsner et al., 1994). Deletion of munc18-1 completely arrests synaptic transmission (Verhage et al., 2000), whereas increased Munc18-1 levels result in a larger readily releasable vesicle pool (RRP) and increased synaptic efficacy (Toonen et al., 2006b). Mutations in munc18-1 are found in patients with epilepsy and intellectual disability, and Munc18-1 dysregulation is implicated in Alzheimer’s disease and schizophrenia (Jacobs et al., 2006; Saitsu et al., 2008; Hamdan et al., 2009; Milh et al., 2011; Vatta et al., 2012; Mastrangelo et al., 2013; Urigüen et al., 2013). Hence, Munc18-1 is required for normal brain function and its global expression levels scale with synaptic strength. However, the molecular mechanisms that control its synaptic levels are largely unknown.

Munc18-1 binds syntaxin-1 with high affinity, clamping syntaxin-1 in a conformation unable to bind other SNARE proteins (Dulubova et al., 1999; Misura et al., 2000). This interaction may stabilize both proteins and support their trafficking, as abrogated Munc18-1 expression results in aberrant targeting of syntaxin-1 in heterologous cells (Rowe et al., 2001; Medine et al., 2007; McEwen and Kaplan, 2008) and reduced expression levels of syntaxin-1 in neurons (Verhage et al., 2000; Voets et al., 2001; Zhou et al., 2013). In contrast, Munc18-1 levels are reduced in syntaxin-1 knockdown neurons (Zhou et al., 2013).

In developing neurons, syntaxin-1 is transported on vesicles via pathways dependent on the kinesin adaptor proteins syntabulin (Su et al., 2004) and FEZ1 (Chua et al., 2012). Munc18-1 is present in syntaxin–FEZ1–kinesin transport complexes (Chua et al., 2012). At this stage, the syntaxin-1/Munc18-1 dimer may...
function as a co-chaperone complex aiding transport and preventing premature breakdown of its constituents. However, in mature neurons syntaxin-1 is mainly transported via lateral diffusion along the plasma membrane (Mitchell and Ryan, 2004; Ribrault et al., 2011), and it is unknown whether at this stage Munc18-1 depends on syntaxin-1 for targeting to fusion sites.

To investigate the dynamics of endogenous Munc18-1 in neurons, we created mice expressing fluorescently tagged Munc18-1 from the endogenous munc18-1 locus. We characterized Munc18-1 dynamics using FRAP in cultured hippocampal neurons at rest, during stimulation, and upon application of several active compounds. Munc18-1 trafficked through axons and to synapses with membrane-bound syntaxin-1. During stimulation, synaptic Munc18-1 rapidly dispersed from synapses and reclustered within minutes. This was independent of syntaxin-1 and synaptic vesicle fusion but required calcium influx and PKC activity. Hence, the presence of Munc18-1 in synapses is tightly regulated; during periods of activity Munc18-1 becomes more dynamic and reclusters at synapses in a phosphorylation-dependent way. Finally, we show that increased Munc18-1 recruitment correlates with increased strength of individual synapses.

Results

Munc18-1-Venus mice as reporters of endogenous Munc18-1

Munc18-1-Venus knock-in mice were generated with Venus cDNA, replacing the stop codon of exon 20 of munc18-1 (Fig. 1 A) via homologous recombination in embryonic stem cells (Fig. 1 B). Wild-type (WT; +/+) Munc18-1-Venus heterozygous (+/m), and homozygous (m/m) genotypes were detected by PCR (Fig. 1 C). Total brain protein levels of Munc18-1-Venus in m/m mice were indistinguishable from Munc18-1 in +/+ littermates (Fig. 1, D and F), although Munc18-1-Venus levels were slightly reduced in +/+ littermates (Fig. 1 D and S1), as was found in Munc18-1-YFP mice (Kalla et al., 2006). Munc18-1-Venus localization was comparable with Munc18-1 in WT brain with high expression in axonal fibers and mossy fiber terminals of the stratum lucidum in the hippocampus (Fig. 1 G).

The morphology and synapse number of hippocampal neurons from Munc18-1-Venus mice at 8 and 14 d in vitro (DIV) was similar to WT littermates (Fig. S1 and Fig. 2, A–D). Excitatory postsynaptic current (EPSC) amplitudes (Fig. 2 E), short-term plasticity (Fig. 2, H and K), spontaneous miniature EPSC frequency and amplitude (Fig. 2 H), and RRP size and recovery (Fig. 2, I–K) of Munc18-1-Venus autaptic hippocampal cultures were similar to WT. Hence, Munc18-1-Venus neurons can be used as functional reporters of Munc18-1 dynamics in living cells.

Munc18-1 transport in axons depends on syntaxin-1

In cultured neurons, membrane-bound syntaxin-1 diffuses laterally through the axon (Ribrault et al., 2011). Munc18-1 colocalizes with syntaxin-1 throughout the axon, and both proteins may be transported as a binary complex (Garcia et al., 1995; Ribrault et al., 2011). To test this, we imaged hippocampal neurons of Munc18-1-Venus mice at 14 DIV, when synaptic contacts are functional, and compared Munc18-1-Venus dynamics in axons with soluble GFP and membrane-bound syntaxin-1–enhanced YFP (Stx-YFP) using FRAP analysis. We bleached extended regions of single axons (Fig. 3, A–C) to discriminate fast recovery of freely diffusing GFP (Fig. 3 A) from relatively slow recovery typical for membrane-bound molecules like Stx-YFP (Fig. 3 B). Fluorescence recovery of Stx-YFP was gradual and bidirectional, but markedly different in speed and extent from soluble GFP (Fig. 3, A, B, and E). Bleaching of axonal Munc18-1-Venus resulted in similar bidirectional and gradual recovery (Fig. 3, C and D). Mean Munc18-1-Venus recovery was similar to Stx-YFP and significantly slower than GFP (Fig. 3 E). In developing neurons Munc18-1 is bound to transport vesicles (Chua et al., 2012). We observed Munc18-1-Venus puncta entering the bleached area in only 16% of Munc18-1-Venus axons (Fig. S2), with a minimal effect on total fluorescence recovery. Hence, the majority of Munc18-1 in axons of 14 DIV neurons, in contrast to developing neurons, does not bind transport vesicles. Munc18-1 also does not diffuse freely like GFP, but may move through axons by interacting with slowly diffusing proteins, like syntaxin-1.

To test this, we expressed botulinum neurotoxin C (BoNT/C) light chain in Munc18-1-Venus neurons 6–12 h before FRAP experiments. BoNT/C cleaves syntaxin-1 at the C terminus just above the transmembrane region (Blasi et al., 1993; Schiavo et al., 1995), detaching the remaining protein from the membrane and arresting synaptic vesicle fusion (de Wit et al., 2006). Fluorescence recovery of axonal Munc18-1-Venus in cells expressing BoNT/C was significantly faster than control cells (Fig. 3 F). BoNT/C expression does not affect electrical properties of neurons or neuronal morphology at the ultrastructural level (de Wit et al., 2006), making it unlikely that indirect effects of syntaxin-1 cleavage contributed to the observed Munc18-1 transport phenotype. Together, these results show that the majority of Munc18-1 does not diffuse freely but binds to syntaxin-1 for transport through the axon via lateral diffusion in the membrane.

Synaptic Munc18-1 dynamics are only partially syntaxin-1 dependent

Munc18-1 is expressed in synapses (Toonen and Verhage, 2007). Munc18-1-Venus fluorescence was high in presynaptic terminals identified by synapsin-mCherry (Fig. 4 A). The synapse-to-axon ratio of Munc18-1-Venus was significantly higher than a membrane-targeted Venus protein (mVenus, Fig. 4 B), which suggests that Munc18-1-Venus is retained at synapses. To test this, we used FRAP of Munc18-1-Venus, Stx-YFP, and GFP on single synapses (Fig. 4 C). A focused laser spot aimed at a single synapsin-mCherry punctum (Fig. 4 D, open arrowhead) bleached the synaptic region, and fluorescence recovery was followed over time (Fig. 4 D). The initial (at 10 and 30 s) recovery of synaptic Munc18-1-Venus was markedly slower than GFP and not significantly different from Stx-YFP, although the mobile fraction of Munc18-1-Venus was slightly smaller than Stx-YFP (Fig. 4 E). We then compared FRAP of Munc18-1-Venus in the presence or absence of BoNT/C. Mean fluorescence

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Figure 1. Generation and confirmation of Munc18-1-Venus (M18V) mouse. (A) Generation of M18V knockin gene. Diagrams indicate WT munc18-1 gene, targeting vector, M18V-neo-knock-in gene, and Cre-recombined M18V gene. Exons are indicated by gray boxes and numbered. Black and gray horizontal bars indicate probes used for Southern blot analysis and PCR products used for genotyping, respectively. A 5-kbp section is not shown (arrowheads). LoxP, loxP sites; Venus, Venus cDNA; AvrII, restriction enzyme site; NEO, neomycin resistance gene; TK, thymidine kinase promoter; pGEM-T Easy, targeting vector. (B) Southern blot analysis of mouse tail DNA from heterozygous (+/m) and WT (+/+) mice. DNA was AvrII-digested. m, M18V-neo-knock-in gene (8.2 kbp); +, munc18-1 gene (6.3 kbp). (C) Agarose gel of PCR products from +/+, +/m, and m/m mouse DNA. M18V, munc18-1-Venus gene PCR product, 449 bp; M18, munc18-1 gene PCR product, 195 bp. (D) Western blot of brain lysate of +/+, +/m, and m/m mice stained for M18V. Actin was used as a loading control. The lines indicate that intervening lanes have been spliced out. (E) Western blot of brain region lysate of m/m and +/+ mice at E18 stained for M18V. Valosin-containing protein (VCP) was used as a loading control. (F) Quantification of M18V expression levels in +/+, +/m, and m/m brains were analyzed by SDS-PAGE (10 μg of protein per lane) and Western blotting with Munc18-1 antibodies. M18V protein levels were normalized to VCP protein levels for each mouse (n = 6). Error bars indicate mean ± SEM. (G) Hippocampal localization of M18V fluorescence in m/m (M18V homozygote) brain slice (left) and of antibody-stained Munc18-1 in WT (+/+ ) mice (right) compared with synaptic marker VAMP and dendritic marker MAP2. CA3, hippocampal CA region; Str, striatum; arrowheads, mossy fiber terminals of the stratum lucidum. Bars: (overview images) 100 μm; (enlarged panels) 25 μm.
Synaptic Munc18-1 dynamics and levels change upon neuronal activity

Next, we tested whether Munc18-1 dynamics are modulated by activity by stimulating Munc18-1–Venus neurons while bleach- ing synaptic fluorescence during stimulation. Initial Munc18-1–Venus fluorescence recovery (after 10 and 30 s) in synapses stimulated with 600 action potentials (AP) at 20 Hz was indistinguishable from control synapses (Fig. 5 A). However, the mobile fraction (measured at 160 s) was significantly larger in

recovery of synaptic Munc18-1–Venus in BoNT/C-expressing cells was significantly larger than in control cells (Fig. 4 F) and resulted in an increased mobile fraction of Munc18-1–Venus (Fig. 4 G). Hence, deletion of syntaxin-1 increases the dynamics of synaptic Munc18-1–Venus, which indicates that synaptic retention of Munc18-1 is largely dependent on syntaxin-1. A small immobile Munc18-1–Venus pool remained upon BoNT/C treatment (Fig. 4 G), which suggests that this pool is retained by interactions with other synaptic proteins.

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stimulated neurons (Fig. 5, A and B). We also probed Stx-YFP mobility using FRAP and found, in line with Ribrault et al. (2011), that initial recovery of Stx-YFP fluorescence was not affected by stimulation (Fig. 5 C). However, the mobile fraction (measured at 160 s) was increased (Fig. 5, C and D), similar to Munc18-1-Venus (Fig. 5, A and B). Thus, strong stimulation increases synaptic Munc18-1 and syntaxin-1 dynamics in hippocampal neurons.

Stimulation causes dispersion of several soluble synaptic proteins (Chi et al., 2001; Sankaranarayanan et al., 2003; Star et al., 2005; Denker et al., 2011), vesicle SNAREs (Li and Murthy, 2001; Fernández-Alfonso et al., 2006), and target SNAREs (Degtyar et al., 2013). To test whether Munc18-1 disperses from the synapse during stimulation, we measured intensity changes upon stimulation in Munc18-1-Venus neurons. Munc18-1-Venus was enriched in synapses identified by synapsin-mCherry before stimulation (Fig. 6 A). During stimulation, fluorescence of Munc18-1-Venus and synapsin-mCherry at synapses decreased (Fig. 6 A): Munc18-1-Venus fluorescence dispersed from the start of stimulation (Fig. 6 B), similar to stimulated neurons (Fig. 5, A and B). We also probed Stx-YFP mobility using FRAP and found, in line with Ribrault et al. (2011), that initial recovery of Stx-YFP fluorescence was not affected by stimulation (Fig. 5 C). However, the mobile fraction (measured at 160 s) was increased (Fig. 5, C and D), similar to Munc18-1-Venus (Fig. 5, A and B). Thus, strong stimulation increases synaptic Munc18-1 and syntaxin-1 dynamics in hippocampal neurons.

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fluorescence (Fig. 6 C). Stx-YFP also dispersed from synapses (Fig. 6 B), although significantly less than Munc18-1-Venus (Fig. 6 C). Dispersion of Munc18-1-Venus and synapsin-mCherry required calcium influx and was impaired by superfusion of P/Q-, L-, and N-type calcium channel blockers (Fig. S5). To

Figure 4. Syntaxin-1 binding is the major determinant of an immobile Munc18-1 pool at synapses. (A) Munc18-1-Venus (M18V) axon expressing synapsin-mCherry. Overlay of M18V and synapsin-mCherry fluorescence (Composite) shows Munc18-1-Venus expression at presynaptic sites. Bar, 4 µm. (B) Normalized fluorescence intensities of Stx-YFP and mVenus in WT neurons and M18V measured from the center of synapsin-mCherry positive puncta up to 4 µm into the axon (left; see scale bar in A). Intensity was normalized to axon intensity at 4 µm. Right, synapse (0 µm)-to-axon ratio (4 µm) of M18V, Stx-YFP, and mVenus (K-W test: ***, P < 0.001; n.s., P > 0.05; M18V, n = 10 field of views, n = 72 synapses; Stx-YFP, n = 15 field of views, n = 58 synapses; mVenus, n = 12 field of views, n = 52 synapses). (C) Diagram illustrating FRAP experiments shown in D–G. M18V neurons (green) expressing synapsin-mCherry as a synapse marker (not depicted) were cultured together with WT neurons (gray) in a 1:50 ratio. Fluorescence in synapses was photobleached (arrowheads and box) at t = 0 s, and fluorescence recovery was followed over time (FRAP, right). (D) M18V axon with three synapses (arrowheads) identified by synapsin-mCherry. Inverted greyscale images show time points before (pre), immediately after photobleaching (bleach, open arrowheads), and during fluorescence recovery (post t = 22 s and post t = 123 s). Bottom right, normalized intensity of bleached synapse over time. Bar, 5 µm. (E) FRAP analysis of synaptic M18V, GFP, and Stx-YFP. (Inset) Statistical significance (M-W test with FDR [6] corrections: n.s., P > 0.05; *, P < 0.05; M18V, n = 14 field of views, n = 37 synapses; Stx-YFP, n = 9 field of views, n = 23 synapses; GFP, n = 9 field of views, n = 28 synapses). The broken line indicates a biexponential fit of M18V recovery with a fast mobile fraction of 0.32 and a tau of 4.6 s, and a slow mobile fraction of 0.36 and a tau of 59.8 s. (F) FRAP analysis of synaptic M18V (see E) and M18V + BoNT/C. (Inset) Statistical significance (M-W test with FDR [6] corrections: ***, P < 0.001; *, P < 0.05; M18V + BoNT/C, n = 6 cells, n = 16 synapses). (G) Mobile fraction of synaptic M18V compared with M18V + BoNT/C calculated from F at t = 180 s (M-W test: ***, P < 0.001). Error bars indicate mean ± SEM.

synapsin-mCherry (Chi et al., 2001; Fig. 6 B, inset). The intensity changes represented Munc18-1-Venus movement and not changes in Venus fluorescence due to proton-induced quenching (Nagai et al., 2002), as the fluorescence decrease of an mVenus was ninefold (P < 0.001) lower than the decrease in Munc18-1-Venus fluorescence (Fig. 6 C). Stx-YFP also dispersed from synapses (Fig. 6 B), although significantly less than Munc18-1-Venus (Fig. 6 C). Dispersion of Munc18-1-Venus and synapsin-mCherry required calcium influx and was impaired by superfusion of P/Q-, L-, and N-type calcium channel blockers (Fig. S5). To
test whether Munc18-1 dispersion required active vesicle release and syntaxin-1, we expressed BoNT/C. This did not affect Munc18-1-Venus dispersion (Fig. 6, D and E). Thus, stimulation-induced calcium influx triggers the dispersion of a fraction of synaptic Munc18-1-Venus into the axon. This dispersion is largely independent of syntaxin-1 and does not require synaptic vesicle release.

**Munc18-1 reclusters at synapses after synaptic activity**

Synapsin-1a fully reclusters to synapses within 10 min after synaptic activity (Chi et al., 2001). To test whether Munc18-1-Venus also reclusters at synapses, we followed synaptic Munc18-1-Venus fluorescence after stimulation. We observed a large variation in synaptic Munc18-1-Venus intensity changes (Fig. 7, A and B) that did not correlate with initial Munc18-1-Venus levels (Fig. S4). However, the mean response of all synapses showed dispersion during the stimulus, leading to a net increase in Munc18-1-Venus levels at t = 160 s (Fig. 7 C). This reclustering was much faster than synapsin-mCherry and was not observed for mVenus (Fig. S3). We defined two sets of synapses, one with initial dispersion (ΔF/F0 < 0 at t = 10 s) followed by a net increase of fluorescence (ΔF/F0 > 0 at t = 160 s, called Δ+) and one with initial dispersion (ΔF/F0 < 0 at t = 10 s) followed by a net decrease of fluorescence (ΔF/F0 < 0 at t = 160 s, called Δ−). Nearly half of all synapses fell in the Δ+ subset and over one-third in the Δ− subset (Fig. 7 C). Unlike the increase observed for Munc18-1-Venus, Stx-YFP mean fluorescence remained below initial levels after dispersion with <25% Δ+ synapses (Fig. 7 D). Reclustering was also slower than Munc18-1-Venus (Fig. 7 D). This suggests that Munc18-1-Venus reclusters faster than syntaxin-1 (and synapsin-mCherry), and independently of syntaxin-1. Indeed, synaptic Munc18-1-Venus fluorescence increased faster upon BoNT/C expression (Fig. 7, E and F) and resulted in higher levels at t = 160 s than in control neurons. Also, the percentage of Δ+ synapses was higher upon BoNT/C (Fig. 7 E). Hence, reclustering of synaptic Munc18-1 does not require syntaxin-1, and is even enhanced in the absence of syntaxin-1. Munc18-1 reclustering was absent in neurons incubated with calcium channel blockers (Fig. 7, G and H; and Fig. S5, A–C). Hence, calcium influx as a result of synaptic activity increases Munc18-1 mobility and redistributes synaptic Munc18-1 levels such that on average synapses contain more Munc18-1 after a strong stimulus.

**PKC-dependent phosphorylation of Munc18-1 is necessary for synaptic Munc18-1 reclustering**

PKC-dependent phosphorylation of Munc18-1 is essential for DAG-induced potentiation of synaptic transmission (Wierda et al., 2007) and vesicle pool replenishment in chromaffin cells (Nili et al., 2006). To test if PKC activity modulates synaptic Munc18-1 dynamics, we applied the DAG analogue PMA to Munc18-1-Venus neurons. This did not significantly affect synaptic Munc18-1 levels (Fig. 8, A and B). PMA application during electrical stimulation did not further increase Munc18-1 recruitment after stimulation (Fig. 8, C and D) or its dynamics (Fig. S5). Hence, PMA application does not have an additive effect when PKC activity is triggered by calcium influx. However, application of the specific PKC inhibitor Ro 31-8220 (Davis et al., 1989) resulted in significantly less dispersion during stimulation and an almost complete block of Munc18-1-Venus reclustering after stimulation (Fig. 8, E and F). Ro 31-8220 application significantly reduced the number of synapses and mean recruitment in the Δ+ subset (Fig. S5). Hence, PKC activity is required for reclustering of Munc18-1 after strong stimulation. Finally, to test whether PKC-dependent phosphorylation of Munc18-1 itself is required for its reclustering, we expressed a fully functional but PKC-insensitive mutant (PKCi) of Munc18-1-EGFP (Wierda et al., 2007) in munc18-1

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Figure 5. Activity increases Munc18-1 and Syntaxin-1 mobility at synapses. (A) FRAP analysis of Munc18-1-Venus (M18V) in control synapses and synapses stimulated with 600 AP at 20 Hz starting at t = −15 s (black bar). [inset] Statistical significance (M-W test with FDR [3] corrections: n.s., P > 0.05; *, P < 0.05; M18V, n = 8 field of views, n = 19 synapses; M18V + stimulation, n = 8 field of views, n = 16 synapses). (B) Mobile fraction of synaptic M18V, control compared with stimulation calculated from A at t = 160 s (M-W test: **, P < 0.01). (C) FRAP analysis of Stx-YFP in control synapses and synapses stimulated with 600 AP at 20 Hz starting at t = −15 s (black bar). [inset] Statistical significance (M-W test with FDR [3] corrections: n.s., P > 0.05; Stx-YFP, n = 6 field of views, n = 16 synapses; Stx-YFP + stimulation, n = 6 field of views, n = 13 synapses). (D) Mobile fraction of synaptic Stx-YFP control compared with stimulation calculated from C at t = 160 s (M-W test: *, P < 0.05). Error bars indicate mean ± SEM.
**Munc18-1 levels correlate with synaptic strength**

Munc18-1 expression levels in neurons control synaptic strength by modulating the number of release-ready vesicles (Toonen et al., 2006b). To determine whether activity-dependent modulation of synaptic Munc18-1 levels affects presynaptic strength on a synapse level we probed vesicle release using FM4-64 uptake during 30 AP at 1 Hz, before and after high-frequency stimulation (Fig. 9 A). Individual synapses in naive neurons showed a large variation in Munc18-1-Venus levels and FM4-64 levels (Fig. 9 B). Interestingly, and in line with our previous results in Munc18-1-overexpressing neurons (Toonen et al., 2006b), synaptic Munc18-1-Venus levels showed a significant correlation with FM4-64 loading (Spearman’s correlation $r_s = 0.27$, $P < 0.001$; Fig. 9 C), but not with synapse size (Fig. S4). Next, we tested whether a change in synaptic Munc18-1-Venus levels after high-frequency stimulation correlated with concomitant modulation in synaptic strength. We compared the change in FM4-64 uptake before and after 600 AP high-frequency stimulation between synapses with a
Synaptic activity increases Munc18-1-Venus mobility and recruits Munc18-1 to synapses independent of syntaxin-1. (A) Fluorescence and greyscale images of Munc18-1-Venus (M18V) axons expressing synapsin-mCherry. Greyscale images show the same axon before (pre), during (t = 12 s), and after stimulation (t = 198 s, M18V only) with 600 AP at 20 Hz (start t = 0 s). Bar, 5 µm. (B) Relative M18V intensity changes (ΔF/F0) in synapses marked by arrowheads in A. Black bars indicate the period of stimulation. Some synapses, after initial dispersion during stimulation, increase fluorescence above initial levels (broken line), whereas others remain low after initial dispersion or do not change during measurement. (C) Relative M18V intensity changes of all synapses (green line, n = 23 field of views, n = 510 synapses) compared with subsets (gray lines) of synapses with net positive change (Δ+, 46.3% of all synapses) and synapses with net negative change (Δ−, 37.8%) at t = 160 s. (D) Relative Stx-YFP intensity changes of all synapses (blue line, n = 10 field of views, n = 125 synapses) and of subsets (gray line) of synapses with net positive change (Δ+, 22.4% of all synapses) or a net negative change (Δ−, 36.0%) at t = 160 s. (E) Relative M18V intensity changes of all synapses (green, n = 5 cells, n = 553 synapses) and M18V ± BoNT/C synapses (yellow, n = 5 cells, n = 290 synapses). (Inset) Percentage of synapses with net positive change (−BoNT/C, 38.6%; +BoNT/C, 44.1%) and synapses with net negative change (−BoNT/C, 31.9%; +BoNT/C, 19.3%) at t = 160 s (Pearson χ² test: ***, P < 0.001). (F) Relative M18V intensity change at t = 10 s (left) and t = 160 s (right) at control synapses (green) and synapses superfused with calcium channel blockers (brown) calculated from E (M-W test with FDR [2] corrections: ***, P < 0.001). (G) Relative M18V and synapsin-mCherry intensity changes of control synapses (green, n = 6 cells, n = 250 synapses) and synapses superfused with calcium channel blockers to prevent calcium influx (brown, n = 6 cells, n = 252 synapses). Synapsin-mCherry intensity at t = 30 s, control versus calcium channel blockers, M-W test: ***, P < 0.001). (H) Relative M18V intensity change at t = 10 s (left) and t = 160 s (right) at control synapses (green) and synapses superfused with calcium channel blockers (brown) calculated from G (M-W test with FDR [2] corrections: n.s., P > 0.05; ***, P < 0.001). Note that although average dispersion (t = 10 s) in control synapses is lower compared with E, Fig. S5 shows that dispersion in Δ+ and Δ− control synapses is significantly higher than in synapses with calcium channel blockers. The horizontal broken line indicates no change.
Discussion

Here, we investigated the distribution and dynamics of endogenous Munc18-1 in axons and synapses using Munc18-1-Venus knockin mice. In line with its essential role in exocytosis, we found that Munc18-1 is highly expressed in synapses. The majority of Munc18-1 traffics through axons via lateral diffusion together with syntaxin-1. Exchange rates of Munc18-1 at individual synapses are high compared with active zone components Bassoon and Munc13-1 (Kalla et al., 2006; Tsuriel et al., 2009).

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During periods of synaptic activity, Munc18-1 becomes even more dynamic, rapidly disperses, and subsequently reclusters at synapses. This requires calcium influx and PKC-dependent phosphorylation of Munc18-1, but is independent of syntaxin-1 interaction. Importantly, synaptic Munc18-1 levels scale with release efficiency, and higher Munc18-1 levels after stimulation increase synaptic strength.

Munc18-1 axonal transport in mature neurons is largely syntaxin-1 dependent

During neuronal development, Munc18-1/syntaxin-1 and syntaxin-1 are transported by FEZ1–KIF5C (Chua et al., 2012) and syntabulin–KIF5B (Su et al., 2004; Cai et al., 2007) transport complexes. Our FRAP experiments in axons (Fig. 3) showed identical recovery of Munc18-1–Venus and Stx–YFP and increased Munc18-1–Venus recovery after cleavage of syntaxin-1, which indicates that lateral diffusion via membrane-bound syntaxin-1 rather than active transport of organelles is the main mode of delivering Munc18-1 to release sites in mature neurons. This is in line with a study on syntaxin-1 dynamics in mature rat neurons (Ribault et al., 2011), underscoring the notion that after initial membrane delivery via fusion of transport vesicles, Munc18-1–syntaxin-1 complexes diffuse through the axonal membrane. Only rarely were moving Munc18-1–Venus puncta observed, which suggests that vesicular transport of Munc18-1–syntaxin-1 complexes does continue in mature neurons but that diffusion dominates. Hence, during neuronal maturation, the mode of syntaxin/Munc18 transport switches from predominantly vesicular transport (Cai et al., 2007; Chua et al., 2012) to lateral diffusion.

Munc18-1 is highly dynamic at individual synapses

Munc18-1–Venus showed a strong synaptic preference (Fig. 4). Our FRAP measurements revealed that synaptic Munc18-1 is very mobile. Munc18-1–Venus exchange is best described with a fast (τ = 4.6 s) and a slow component (τ = 60 s), comparable to syntaxin-1 (Ribrault et al., 2011), whereas Bassoon (Tsuriel et al., 2009; Schröder et al., 2013), Munc13-1 (Kalla et al., 2006), liprin-α2 (Spangler et al., 2013), and synapsin-1 (Tsuriel et al., 2006) turnover at synapses is much slower, with exchange rates in the order of minutes to hours. Exchange rates of the total synaptic vesicle pool are even slower (Orenbuch et al., 2012b), although individual synaptic vesicle exchange between boutons is relatively fast (Darcy et al., 2006; Staras et al., 2010; Herzog et al., 2011). Synaptic activity increased the mobility of Munc18-1–Venus and Stx–YFP (Fig. 5), in line with increased mobility of synapsin (Chi et al., 2001; Tsuriel et al., 2006) and Rab3 (Star et al., 2005). In contrast, Munc13-1 and Bassoon mobility are not affected by acute stimulation (Kalla et al., 2006; Tsuriel et al., 2009). Hence, synapses exchange their components at different speeds. Proteins of the secretory machinery are more mobile than active zone scaffolding proteins and become even more dynamic during increased activity. The scaffolding proteins may therefore provide synapses with stability to maintain synapse integrity (Tsuriel et al., 2009),
whereas intersynaptic exchange of presynaptic molecules and vesicles provides plasticity during increased network activity.

At synapses, ~40% of Munc18-1-Venus resided in an immobile fraction. Cleavage of syntaxin-1 resulted in a larger mobile Munc18-1-Venus fraction, but did not fully abolish the immobile fraction. This suggests that Munc18-1 also interacts with other binding partners at the synapse. Here, Munc18-1 may be released when syntaxin-1 interacts with other SNAREs, which lowers the binding affinity of Munc18-1 for syntaxin-1 (Xu et al., 2010). Strong interaction between Munc18-1 and syntaxin-1 in the axon may ensure proper delivery of Munc18-1 to synapses and protect syntaxin-1 from nonspecific interactions. A more dynamic interaction at the synapse provides plasticity to modulate SNARE complex formation and interactions of Munc18-1 with other proteins.

Synaptic activity acutely reorganizes synaptic Munc18-1 levels

Synaptic activity triggers synapsin dispersion from synapses (Chi et al., 2001; Fig. 6). In the same synapses, we observed significant Munc18-1-Venus dispersion during stimulation. Stx-YFP also dispersed, although to a smaller extent, which is in line with recent results (Degtyar et al., 2013). Dispersion of Munc18-1-Venus was not affected by BoNT/C expression (Fig. 6). Thus, calcium influx triggers dispersion irrespective of vesicle fusion, thereby increasing the concentration of soluble and dynamic Munc18-1.

Munc18-1 repopulated the synapse within 2 min after stimulation, much faster than synapsin (Chi et al., 2001; Orenbuch et al., 2012a) and Rab3a (Star et al., 2005), and reclustering already started during the stimulation train. On average, synapses contained more Munc18-1 after stimulation. Interestingly, post-stimulation changes in Munc18-1-Venus levels were highly variable: some synapses showed an ~20% increase in fluorescence, whereas others showed an ~10% net decrease (Fig. 7). Such distinct and acute changes in synaptic levels have not been reported before. As Munc18-1 expression levels correlate with synaptic strength and stamina (Toonen et al., 2006b), this provides a mechanism that helps individual synapses to quickly tune their output and maintain vesicle release during periods of high demand. The wide range in initial synaptic Munc18-1-Venus levels is in line with the notion that individual synapses can have very different release probabilities (Rosenmund et al., 1993; Murthy et al., 1997; Matz et al., 2010; Ariel et al., 2012). This presynaptic mechanism adds to other well-known factors that tune synaptic output like postsynaptic target identity (Reyes et al., 1998; Sylwestrak and Ghosh, 2012) and synaptic position on the dendrite (de Jong et al., 2012), and may partly underlie the effects of neuronal activity (Branco et al., 2008) and calcium influx (Ermolyuk et al., 2012) on synaptic output.

Reclustering of Munc18-1-Venus is PKC dependent and correlates with synaptic strength

Activation of the DAG–PKC pathway potentiates synaptic transmission (Malenka et al., 1986; Shapira et al., 1987) and is essential for post-tetanic potentiation (Brager et al., 2003; Fioravante et al., 2011). Furthermore, we have shown that DAG–PKC-dependent synaptic potentiation requires Munc18-1 phosphorylation, and that Munc18-1 phosphorylation is essential for maintaining synaptic release during repetitive stimulation and for synaptic recovery after stimulation (de Vries et al., 2000; Wierda et al., 2007). Here we show that Munc18-1-Venus re-clustering after stimulation is calcium-dependent and almost absent after PKC inhibition. Moreover, a PKC-insensitive mutant Munc18-1 showed strongly impaired re-clustering after intense stimulation. Thus, PKC-dependent phosphorylation of Munc18-1 is required for its re-clustering and consequent changes in synaptic levels. Together, this provides a plausible mechanism for the fact that efficacy of synaptic vesicle release depends on Munc18-1 expression levels (Toonen et al., 2006b) and PKC activity (Majewski and Iannazzo, 1998; Stevens and Sullivan, 1998; Francis et al., 2002). We have previously shown that the PKC–Munc18-1 pathway translocates synaptic vesicles to the active zone (Wierda et al., 2007) and that Munc18-1 overexpression promotes membrane delivery of secretory vesicles in chromaffin cells (Toonen et al., 2006a). Hence, PKC-dependent recruitment of Munc18-1 temporarily increases its synaptic levels, thereby facilitating synaptic vesicle recruitment. Importantly, FM4-64 loading correlated with Munc18-1-Venus expression levels in individual synapses (Fig. 9). Moreover, synapses that recruited more Munc18-1 after high-frequency stimulation had a larger releasable vesicle pool. Hence, at the single synapse, Munc18-1 expression levels correlate with synaptic strength.

Together, our data support a model in which soluble Munc18-1 is dependent on plasma membrane–bound syntaxin-1 for delivery to the synapse at rest (Fig. 10, 1). At the synapse, Munc18-1 resides with syntaxin-1 and SNAP25 in microdomains at the plasma membrane (Lang et al., 2002; Sieber et al., 2006; Pertsinidis et al., 2013). Munc18-1 also interacts with other binding partners, and a fraction of Munc18-1 may transfer from syntaxin-1 (Fig. 10, 2) to these partners at some point after arrival in the synapse. Calcium influx temporarily increases Munc18-1 dynamics, which are independent of vesicle release or syntaxin-1 binding (Fig. 10, 3). Soluble Munc18-1 can disperse or may bind syntaxin-1 (Fig. 10, 4). After stimulation, PKC controls re-clustering by phosphorylation of Munc18-1 (Fig. 10, 5). PKC-dependent re-clustering of Munc18-1 temporarily increases the release of the synaptic vesicle pool.

Materials and methods

Generation of Munc18-1-Venus knockin mice

A Venus cDNA fragment from pVenus-N1 (Nagai et al., 2002) was subcloned using restriction enzymes [SmaI–NotI and SmaI, respectively] into pUC21 [Vieira and Messing, 1991], resulting in pUC21VENUS. A loxNE-Olox cassette [containing neomycin resistance gene and thymidine kinase promoter flanked by two LoxP sites] was subcloned using SalI–XbaI into pUC21VENUS in an antisense orientation 3’ of the Venus stop codon. The left cloning arm, a genomic sequence 5’ of, and including, exon 20 of the munc18-1 gene from 129/Sv embryonic stem (ES) cell DNA, was subcloned using PCR into pGEM-T Easy (Promega), resulting in pGEM-T Easy-Left-Arm. The right cloning arm, a genomic sequence 3’ of exon 20 of the munc18-1 gene from 129/Sv ES cell DNA, was subcloned using PCR into pGEM-T Easy-Right-Arm. The right cloning arm was subcloned into pGEM-T Easy-Left-Arm using KpNI–AvrII and KpNI–Spel.
Venom and the laxNLOxR cassette from pUC21:VENUS were subcloned between the left and right arm, with Venom replacing the stop codon of exon 20 in frame, creating the targeting vector (Fig. 1 A). Mice carrying the munc18-1-venus-neo gene were generated by homologous recombination in ES cells and identified by Southern blotting (Fig. 1 B) or PCR (Fig. 1 C).

Heterozygous mice (+/m) were crossed with EIIa-cre mice, which express Cre recombinase in early embryonic stages (Lasko et al., 1996), to remove the neomycin resistance cassette. All imaging experiments were conducted on hippocampal m/m neurons of +/m × +/-m crosses. Munc18-1 null mutant mice [deleting exon 2–6] have been described previously (Verhage et al., 2000). All animals were housed and bred according to the institutional and Dutch governmental guidelines for animal welfare.

Neuronal cultures and transfection

Dissociated hippocampal cultures were obtained from embryonic day 18 (E18) m/m mice as described previously (Meijer et al., 2012). In brief, hippocampi were dissected in Hapes-buffered HBBS (Invitrogen) and digested with 0.25% trypsin (Invitrogen) at 37°C for 20 min. After washing and trituration, cells were plated at a density of 25,000 cells/well for low-density cultures on top of a pregrown rat glia feeder layer (Bekkers and Stevens, 1991) in Neurobasal medium supplemented with 2.5% FCS, 1% GlutaMax-I (Invitrogen), and 1% penicillin/streptomycin. Cultures were maintained at 37°C/5% CO2 for 2 h. After three washes with neurobasal medium supplemented with 2% FCS, 1% GlutaMax-I, and 1% penicillin/streptomycin, the medium was replaced once every week in low-density cultures.

Where applicable, Munc18-1-Venus neurons were cultured together with WT neurons in a 1.5 ratio to image single fluorescent neurons. Neurons were used for experiments at 14–21 DIV.

For imaging experiments on WT neurons, syntaxin-1-EYFP, GFP, mVenus, and synapom-mCherry were transfected using calcium phosphate precipitate at 6–10 DIV as described previously (Köhmann et al., 1999). For FM4-64 loading experiments in Munc18-1-Venus neurons, synapsin-ECFP was transfected using calcium phosphate precipitate at 6–10 DIV. For all other imaging experiments on Munc18-1-Venus neurons, synapsin-mCherry was expressed by adding lentiviral particles before plating. After trituration, lentiviral particles were added and cells were incubated at 37°C/5% CO2 for 2 h. After three washes with neurobasal medium supplemented with 10% fetal bovine serum to inactivate the lentiviral particles, cells were plated on glia feeder layers. BoNT/C-ECFP was expressed using Semliki viral particles 6–12 h before imaging in autaptic cultures.

ECFP signal in the soma of a single neuron on a micro island ensured BoNT/C expression in the imaged synapse.

For imaging of Munc18-1-EYFP and PKC-insensitive Munc18-1-EYFP on munc18-1 null mutant neurons (Verhage et al., 2000), E18 embryos were obtained from timed munc18-1 heterozygous null mutant matings and processed for WT neurons. Dissociated null mutant neurons were infected in solution before plating on glia micro islands with lentiviral particles expressing Munc18-1-EYFP WT or PKC-insensitive mutant together with synapsin-mCherry particles and placed in a culture cell incubator for 1.5 h. After two washing steps in MEM with 10% FCS, neurons were counted and plated at a density of 2,000 cells/18 mm coverslip.

Figure 10. Munc18-1 transport to and behavior in the synapse. In mature neurons, Munc18-1 (shown in green) is transported to synapses via lateral diffusion with membrane-bound syntaxin-1 (shown in light gray). At the synapse, Munc18-1 interacts with syntaxin-1 (individually or in microdomains) or dissociates and interacts with other binding partners (X-Y). Calcium influx triggers dissociation from binding partners (Z) and temporarily increases free Munc18-1 concentrations in the synapse (A).

Electrophysiological recordings

Electrophysiological recordings were performed on autaptic glutamatergic hippocampal neurons at room temperature (21–24°C) as described previously (Weber et al., 2010). In brief, isolated hippocampal neurons were plated on astrocyte micro islands (Bekkers and Stevens, 1991) in neurobasal medium supplemented with B-27 (Invitrogen), 17.3 mM Hapes, 1% GlutaMax-I (Invitrogen), and 1% penicillin/streptomycin (Invitrogen). Autaptic cells between 10 and 14 DIV were used for experiments. The patch-pipette solution included 135 mM potassium glutamate, 10 mM Hapes, 1 mM EGTA, 4.6 mM MgCl2, 4 mM Na-ATP, 15 mM creatine phosphate, 50 U/ml phosphocreatine kinase, and 300 mMosM, pH 7.3. The standard extracellular medium consisted of 140 mM NaCl, 2.4 mM KCl, 10 mM glucose, 4 mM CaCl2, 4 mM MgCl2, and 300 mOsm, pH 7.3. Cells were maintained at 70 mV using an EPC-9 amplifier (HEKA) under control of Pulse 8.80 software (HEKA). Currents were low-pass filtered at 1 or 5 kHz and stored at either 10 or 20 kHz. Pipette resistance ranged from 4 to 6 MΩ. The series resistance was compensated for 75%. Only cells with series resistances <20 MΩ were analyzed. EPSCs were evoked by depolarizing the cell from −70 to 0 mV for 2 ms.

Immunohistochemistry

Cultured neurons from 1 to 14 DIV were fixed in 3.7% formaldehyde in PBS, pH 7.4, for 20 min at RT. Neurons were washed three times in PBS, permeabilized in 0.5% Triton X-100 (Sigma-Aldrich) in PBS for 5 min, and incubated with blocking solution (PBS containing 2% normal goat serum, 2% bovine serum albumin, and 0.1% Triton X-100) for 30 min at RT. Neurons were then incubated with primary antibodies in blocking solution for 1 h at RT, washed three times with PBS, and incubated with secondary antibodies conjugated to Alexa Fluor in blocking solution for 1 h at RT (1:1,000; Invitrogen). Primary antibodies used were chicken polyclonal MAP2 (1:20,000; Abcam), mouse monoclonal AnkyrinG (1:1,000; SC-12719; Santa Cruz Biotechnology, Inc.), Smi-312 (1:500; Covance), and polyclonal syntaxin-1b (1:1,000; Invitrogen). For secondary antibodies, Alexa Fluor 488, Alexa Fluor 546, and Alexa Fluor 647 were used. All imaging was performed using a confocal microscope (LSM 510 Meta; Carl Zeiss) with a 63× oil objective lens, NA 1.4, and analyzed in MATLAB (MathWorks).
For labeling of brain slices, P14–P21 WT and Munc18-1-Venus mice were perfused transcardially with 4% PFA in PBS, then brains were removed and post-fixed in 4% PFA in PBS overnight. Subsequently, brains were submerged in 30% sucrose in PBS for 3 d before cryosectioning. Before incubation in blocking solution (5% normal goat serum, 2.5% BSA, and 0.2% Triton X-100 in PBS) for 1 h, 35-µm cryosections were incubated in 1% H2O2 for 30 min and rinsed with PBS. Sections were incubated with primary antibody in blocking solution overnight at 4°C on a shaker. Primary antibodies used were rabbit polyclonal Munc18-1:1b [1:200; Synaptic Systems], chicken polyclonal MAP2 [1:200; Abcam], and mouse monoclonal VAMP2 [1:500; Synaptic Systems]. Cryosections were then washed four times in PBS and incubated with secondary antibody (Alexa Fluor; Invitrogen) diluted in blocking solution for 2 h on a shaker. Finally, cryosections were washed twice in 2.5% DABCO (Invitrogen) in Mowiol on glass slides. All steps were performed at RT, unless otherwise stated. Images were acquired on a confocal microscope (LSM 510 Meta; Carl Zeiss) with either a 10x air objective lens or 0.7x mechanical zoom (Fig. 1 G, top), or a 40x objective (1.3 NA) and 0.7x mechanical zoom (Fig. 1 G, bottom).

Live imaging

All live imaging experiments, except FM4-64 synapse labeling (Fig. 9), were conducted on a custom-built tandem illumination microscope (TIM; Olympus) consisting of an inverted imaging microscope (IX81; Olympus) and an upright laser scanning microscope. The inverted microscope part was used for imaging fluorescence using an MT20 light source (Olympus), appropriate filter sets (Semrock), and a 60x oil objective lens [NA 1.49] with or without a 1.6x additional magnification on an EM charge-coupled device (CCD) camera [C9100-02; Hamamatsu Photonics]. Xcellence RT imaging software (Olympus) was used for controlling the microscope and recording the images.

FM4-64 synapse labeling. Experiments were conducted on an inverted microscope [Axio Observer Z; Carl Zeiss] for imaging fluorescence, using an illumination unit [Polychrome V; Till Photonics], appropriate filter sets [Chroma and Semrock], and a 40x oil objective lens (NA 1.3) equipped with automated focus adjustment [Definite Focus; Carl Zeiss] on an EM-CCD camera [C9100-02; Hamamatsu Photonics]. Synaptic vesicle pools were labeled by field stimulation for 30 s at 1 Hz in the presence of 10 µM FM4-64 [Invitrogen]. FM4-64, dissolved in imaging solution, was applied locally using a barrel pipette 30 s before, during, and 1 min after stimulation to ensure complete labeling of all recycling vesicles. The cells were subsequently washed by applying dye-free imaging solution for 15 min via the barrel pipette. The FM4-64 labeling experiments consisted of several sequential steps: a baseline of 10 frames each [Synapsin-mCherry, Munc18-1-Venus, FM4-64]; FM4-64 synapse labeling and wash with 1 frame each every 2 min; baseline of 10 frames each; 30 s of 600 AP at 20 Hz, or 600 AP at 2 Hz with 30 s of 0.2 Hz imaging + 5 s 1 frame every 30 s after Munc18-1-Venus re-clustering; baseline of 10 frames each; FM4-64 synapse labeling and wash with 1 frame each every 2 s; baseline of 10 frames each; 30 s of 600 AP at 20 Hz, and baseline of 10 frames each. Coverslips were placed in an imaging chamber and perfused with imaging solution [Tyrode’s: 2 mM CaCl2, 2.5 mM KCl, 119 mM NaCl, 2 mM MgCl2, 20 mM glucose, and 25 mM Hepes, pH 7.4]. Electrical field stimulation by parallel platinum electrodes was applied by a Master-8 system [A.M.P.I.], a stimulus isolator (A385RC; World Precision Instruments) delivering 30-mA, 1-ms pulses, and in the presence of 50 µM [2R]-alpha-aminoadipic acid (AP5; Tocris Bioscience) and 10 µM 6,7-dinitroquinoxaline-2,3-dione (DNQX; Tocris Bioscience) to block network activity by inhibiting glutamatergic transmission. PMA (Sigma-Aldrich) was bath applied. All imaging experiments were performed at RT [21–24°C].
References

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