Syntaxin-1 N-peptide and H_{abc}-domain perform distinct essential functions in synaptic vesicle fusion

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Among SNARE proteins mediating synaptic vesicle fusion, syntaxin-1 uniquely includes an N-terminal peptide ('N-peptide') that binds to Munc18-1, and a large, conserved H_{abc}-domain that also binds to Munc18-1. Previous *in vitro* studies suggested that the syntaxin-1 N-peptide is functionally important, whereas the syntaxin-1 H_{abc}-domain is not, but limited information is available about the *in vivo* functions of these syntaxin-1 domains. Using rescue experiments in cultured syntaxin-deficient neurons, we now show that the N-peptide and the H_{abc}-domain of syntaxin-1 perform distinct and independent roles in synaptic vesicle fusion. Specifically, we found that the N-peptide is essential for vesicle fusion as such, whereas the H_{abc}-domain regulates this fusion, in part by forming the closed syntaxin-1 conformation. Moreover, we observed that deletion of the H_{abc}-domain but not deletion of the N-peptide caused a loss of Munc18-1 which results in a decrease in the readily releasable pool of vesicles at a synapse, suggesting that Munc18 binding to the H_{abc}-domain stabilizes Munc18-1. Thus, the N-terminal syntaxin-1 domains mediate different functions in synaptic vesicle fusion, probably via formation of distinct Munc18/SNARE-protein complexes.

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Introduction

Synaptic vesicle fusion, like most intracellular membrane fusion, is mediated by the concerted action of two protein families, namely the SNARE (for 'soluble NSF attachment

protein receptors') and SM (for 'Sec1-Munc18 like') protein families (reviewed in Rizo and Rosenmund, 2008; Sørensen, 2009; and Südhof and Rothman, 2009). In presynaptic terminals, the SNARE proteins syntaxin-1, SNAP-25, and synaptobrevin/VAMP form a tight complex composed of a four-helical bundle that forces membranes into close proximity, and that is acted upon by Munc18-1 to catalyze fusion (Südhof and Rothman, 2009). All SNARE proteins include a SNARE motif (an ~60 residue conserved sequence) that forms the SNARE complex and a membrane anchor (usually a transmembrane region) that attaches the SNARE proteins to membranes. Syntaxin-1 is remarkable among synaptic SNARE proteins in that it additionally contains a large N-terminal region that accounts for ~60% of its total sequence. The syntaxin-1 N-terminal region is composed of two protein motifs: (i) a short N-terminal peptide ('N-peptide') that binds to Munc18-1 (Dulubova *et al*, 2007; Hu *et al*, 2007; Rickman *et al* 2007) and (ii) a larger H_{abc}-domain that consists of an autonomously folded three-helical bundle (Fernandez *et al*, 1998; Antonin *et al*, 2002; Bracher and Weissenhorn, 2004). The H_{abc}-domain folds back onto the SNARE motif of syntaxin-1 to produce the 'closed' conformation of syntaxin-1 that also binds to Munc18-1 independent of the N-peptide (Hata *et al*, 1993; Dulubova *et al*, 1999; Misura *et al*, 2000; Rickman *et al* 2007). Thus, the two N-terminal syntaxin-1 sequence motifs both separately bind to Munc18-1—which is essential for synaptic vesicle fusion (Verhage *et al*, 2000)—by two different and independent mechanisms.

Many *in vitro* and *in vivo* studies demonstrated that Munc18-1 binding by the N-peptide of syntaxin-1 is essential for fusion (Khvotchev *et al*, 2007; Shen *et al*, 2007; Tareste *et al*, 2008; Deák *et al*, 2009; Johnson *et al*, 2009; Diao *et al*, 2010; Rathore *et al*, 2010; Schollmeier *et al*, 2011; Shi *et al*, 2011). A recent study, however, concluded that Munc18-1 binding to the syntaxin-1 N-peptide is not important because Munc18-1 mutants that show diminished binding to the N-peptide were able to support fusion (Meijers *et al*, 2012). Although the mutants examined in that study still partly bind to the N-peptide, which may complicate the interpretation of the results, the Meijers *et al* (2012) paper illustrates that the role of the Munc18-1 binding to the syntaxin-1 N-peptide remains incompletely understood despite a large number of studies on its role.

In contrast to the N-peptide, the physiological function of the H_{abc}-domain and of the closed conformation of syntaxin-1 that it forms has not been examined closely. The closed conformation of syntaxin-1 is formed by an intramolecular interaction of the H_{abc}-domain of syntaxin-1 with its SNARE motif, thereby occluding the SNARE motif. The closed conformation has to open prior to SNARE-complex formation, suggesting that the closed conformation inhibits fusion. Consistent with this notion, a point mutation in syntaxin-1

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(the 'LE mutation'; Dulubova *et al*, 1999) that shifts the default conformation of syntaxin-1 from 'closed' to 'open' causes an increase in spontaneous release and in the vesicular release probability (Gerber *et al*, 2008). Moreover, this mutation partly bypasses the requirement for Munc13 (an active zone protein unrelated to Munc18-1) in synaptic vesicle priming in *C. elegans* (Richmond *et al*, 2001), which agrees with the hypothesis that Munc13 functions by opening syntaxin-1 (Ma *et al*, 2011). Finally, the LE mutation decreases the levels of Munc18-1 *in vivo* and produces a decrease in the readily releasable pool (RRP) of vesicles, suggesting that Munc18-1 binding to the closed conformation of syntaxin-1 stabilizes Munc18-1 (Gerber *et al*, 2008), similarly to the stabilization of syntaxin-1 by Munc18-1 (Verhage *et al*, 2000). These *in vivo* studies therefore reveal an important but incompletely defined role for the closed conformation of syntaxin-1 in regulating synaptic fusion. However, they do not reveal whether the H_{abc}-domain performs additional functions in fusion independent of its formation of the closed conformation of syntaxin-1. The only available information on this question is provided by *in vitro* studies documenting that the H_{abc}-domain is dispensable for fusion as such (Rathore *et al*, 2010; Shen *et al*, 2010), but these studies do not show whether the H_{abc}-domain has a physiological function of its own.

Collectively, these observations thus suggest that the N-peptide and the H_{abc}-domain of syntaxin-1 perform distinct functions in synaptic vesicle fusion, but raise two major questions: first, is Munc18-1 binding to the N-peptide of syntaxin-1 indeed essential for fusion? If so, is it essential because the N-peptide stabilizes Munc18-1, Munc18-1 directly catalyses fusion, or Munc18-1 facilitates opening of syntaxin-1 and promotes SNARE-complex assembly? Second, does the H_{abc}-domain contribute to syntaxin function only by forming the closed conformation of syntaxin-1, or does it have other independent functions in synaptic vesicle fusion? To address these questions, we have now employed syntaxin-1 knockdown (KD) experiments combined with rescue manipulations, and systematically explored the role of the N-terminal syntaxin-1 sequences in synaptic vesicle fusion. Our data establish a role in synaptic fusion for both the N-peptide mediated and the H_{abc}-domain mediated interaction of syntaxin-1 with Munc18-1, with the N-peptide being essential for fusion as such without contributing to the stabilization of Munc18-1, and the H_{abc}-domain regulating fusion at multiple junctions.

Results

Generation of syntaxin-deficient neurons

Using lentiviral delivery of shRNAs into cultured cortical neurons (Pang *et al*, 2010; Yang *et al*, 2010), we tested the efficacy of shRNAs that target conserved syntaxin-1 sequences, and measured the degree of syntaxin-1B KD by quantitative rt-PCR (Ko *et al*, 2011). These experiments identified two effective shRNAs that were then cloned into a single lentivirus that also allowed facultative co-expression of shRNA-resistant rescue constructs. We validated the KD efficiency of the lentivirus thus obtained in neurons from syntaxin-1A knockout (KO) mice (Gerber *et al*, 2008). The syntaxin-1 KD lentivirus suppressed syntaxin-1B mRNA

levels by ~90% (Figure 1A), and syntaxin-1B protein levels by >60% in cultured syntaxin-1A KO neurons (Figure 1B).

To estimate the overall impact of the syntaxin-1 deficiency on neuronal integrity and synaptic function, we measured synapse densities in control and syntaxin-deficient neurons without or with co-expression of an shRNA-resistant syntaxin-1A as a rescue protein, but detected no major changes (Figure 1C).

We then examined whether the syntaxin-1 deficiency impaired synaptic transmission and whether this phenotype is rescued by re-expression of syntaxin-1A or -1B cDNAs that have been rendered insensitive to the shRNA by introduction of silent point mutations, and that were compared with each other to ensure that the two syntaxin-1 isoforms are functionally redundant. We monitored synaptic transmission by whole-cell patch-clamp recordings of cultured cortical neurons with an extracellular concentric stimulation electrode as calibrated in detail in previous studies (Maximov and Südhof, 2005; Maximov *et al*, 2007; Pang *et al*, 2010; Kaeser *et al*, 2011).

We found that the syntaxin-1 deficiency severely impaired synaptic transmission, monitored as inhibitory postsynaptic currents (IPSCs) induced either by isolated stimuli (Figure 1D) or 10 Hz stimulus trains (Figure 1E). Both syntaxin-1A and syntaxin-1B rescued these phenotypes, confirming their functional redundancy (Figures 1D and E). Thus, for all subsequent experiments we used syntaxin-1A KO neurons with the syntaxin-1B KD to achieve a maximal suppression of both major presynaptic syntaxin-1 isoforms (syntaxin-1A and -1B), and always included two controls: (i) a lentivirus that does not express an shRNA (generic control) and (ii) a lentivirus that expresses both the syntaxin-1B shRNA and a syntaxin-1A rescue protein whose synthesis was driven by a synapsin promoter and whose mRNA was rendered insensitive to the shRNA (rescue control, to control for off-target effects of shRNAs).

The Munc18-1 binding site of the syntaxin-1 H_{abc}-domain but not of its N-peptide is essential for chaperoning Munc18-1 levels

In a first set of experiments to analyse the N-terminal domains of syntaxin-1, we quantified the levels of selected proteins in syntaxin-1-deficient neurons without and with rescue with wild-type syntaxin-1A or two different mutants of syntaxin-1. We found that the syntaxin-1 deficiency caused a selective, large decrease in Munc18-1 levels that was rescued by wild-type syntaxin-1A (Figure 2). Strikingly, the decrease in Munc18-1 levels in syntaxin-deficient neurons was also fully rescued by mutant syntaxin-1A with a deletion of the N-peptide (Synt1A^{ΔN}) which binds to and anchors Munc18-1 on assembling SNARE complexes (Figure 2A; Dulubova *et al*, 2007). However, the decrease in Munc18-1 levels was not rescued by syntaxin-1A lacking the H_{abc}-domain (Figure 2B). Thus, syntaxin-1 is required for maintaining normal Munc18-1 levels, just as Munc18-1 is required for maintaining normal syntaxin-1 levels (Verhage *et al*, 2000), and this function of syntaxin-1 requires the Munc18-1 binding site of the syntaxin-1 H_{abc}-domain (probably because this domain mediates the closed conformation of syntaxin-1; Gerber *et al*, 2008), but not the Munc18-1 binding site of the syntaxin-1 N-peptide.

Figure 1 Characterization of syntaxin-1 knockdown (KD) neurons. (A) Summary graph of syntaxin-1A and -1B mRNA levels measured by quantitative rt-PCR in cortical neurons cultured from newborn wild-type or syntaxin-1A knockout (KO) mice. Syntaxin-1A KO neurons were infected at DIV5–7 with a control lentivirus (Control) or with lentiviruses expressing shRNAs to syntaxin-1 without (Synt KD) or with co-expression of shRNA-resistant syntaxin-1A mRNA (KD + Synt1A). Neurons were analysed at DIV13–16, and RNA levels were normalized to those of wild-type neurons (ND = non-detectable). (B) Representative immunoblot (left) and summary quantification (right) of syntaxin-1 protein levels in neurons obtained as described in (A). Reactive proteins were visualized by enhanced chemiluminescence (left), but quantified with 125 I-labelled secondary antibodies and phosphorimager detection (right). (C) Representative images (left) and quantification of synapse density (right) in control, Synt KD, and KD + Synt1A neurons analysed by double immunofluorescence labelling for MAP2 (blue) and synapsin (red). (D) Representative traces (left) and summary graphs of the amplitudes (right) of inhibitory postsynaptic currents (IPSCs) evoked by isolated action potentials in cortical neurons cultured from syntaxin-1A KO mice and infected with control lentivirus (Control), or lentiviruses expressing only syntaxin-1 shRNAs (Synt KD) or syntaxin shRNAs plus either wild-type syntaxin-1A (KD + Synt1A) or wild-type syntaxin-1B (KD + Synt1B). (E) Representative traces (left) and summary graphs of the total synaptic charge transfer (right) of IPSCs evoked by 10 stimuli applied at 10 Hz in neurons described in (D). Data shown are means $\pm$ SEMs. In (A, B), three independent neuronal cultures were examined; in (C–E), numbers of experiments/neuronal cultures (C) or patched neurons/neuronal cultures (D, E) analysed are shown in the bars; statistical assessments were performed by Student's *t*-test (* P < 0.05, ** P < 0.01, and *** P < 0.001, all versus control). Source data for this figure is available on the online supplementary information page.

The syntaxin-1 N-peptide is essential for fusion

To test whether the N-peptide of syntaxin-1 is functionally important, we further characterized the impairment in neurotransmitter release produced by the syntaxin-1 deficiency, and examined whether mutant syntaxin-1A lacking the N-peptide can rescue the impairment in neurotransmitter release in syntaxin-1-deficient neurons. As above, neurotransmitter release was measured by monitoring synaptic transmission in cultured neurons using an approach

described in detail previously (Maximov and Südhof, 2005; Maximov *et al*, 2007; Pang *et al*, 2010; Kaeser *et al*, 2011).

We initially studied spontaneous release. The syntaxin-1 deficiency severely impaired the frequency of spontaneous release events at both excitatory and inhibitory synapses (monitored as miniature excitatory postsynaptic currents (mEPSCs) and inhibitory postsynaptic currents (mIPSCs), respectively). Wild type—but not N-terminally deleted syntaxin-1A (Synt1^{ΔN}; Figure 3A)—was able to rescue this

Figure 2 Syntaxin-1 H_{abc}-domain but not N-peptide is required for maintenance of Munc18-1. (A) Representative immunoblots (left) and quantification of the levels (right) of proteins from cortical neurons cultured from syntaxin-1A KO mice and infected with control lentivirus (Control) or lentiviruses expressing the syntaxin-1 KD alone (Synt1 KD) or together with wild-type syntaxin-1A (KD + Synt1A) or N-terminally truncated syntaxin-1A (KD + Synt1A^{ΔN}; see Figure 3A). Blots were probed with antibodies to the indicated proteins (VCP, p97/vasolin-containing protein; GDI, guanosine diphosphate dissociation inhibitor; S25, SNAP-25; Cpx, complexin; Syb-2, synaptobrevin-2), and quantified after visualization with ¹²⁵I-labelled secondary antibodies. (B) Same as (A), except that the effect of the H_{abc}-domain deletion in syntaxin-1A (KD + Synt1A^{ΔHabc}; see Figure 5A) was analysed. Expression of H_{abc}-domain deficient syntaxin-1A cannot be visualized because the antibody epitope for syntaxin-1 is in the H_{abc}-domain. Data shown are means ± SEMs (*n* = 3 independent neuronal cultures); statistical assessments were performed by Student's *t*-test (**P* < 0.05, ****P* < 0.001, all versus control). Source data for this figure is available on the online supplementary information page.

impairment (Figures 3A–C). No significant changes in mEPSC or mIPSC amplitudes were noted. Moreover, the syntaxin-1 deficiency decreased the size of the RRP of vesicles at inhibitory synapses, monitored as the IPSCs induced by application of hypertonic sucrose (Rosenmund and Stevens, 1996; Figure 3D). Again, wild-type syntaxin-1A nearly completely rescued the phenotype, whereas N-terminally deleted syntaxin-1A did not.

We then went on to measure evoked neurotransmitter release, probed as IPSCs induced by isolated action potentials. Similarly to spontaneous release, the syntaxin-1 deficiency impaired release evoked by isolated action potentials (Figure 4A). Again, this phenotype was rescued by wild-type but not by N-peptide-deficient syntaxin-1A. In addition, the syntaxin-1 deficiency produced a striking change in the kinetics and reliability of evoked IPSCs, suggesting that it delays the speed of neurotransmitter release and desynchronizes release (Figure 4B). The rise times of IPSCs were decreased almost two-fold, and the standard deviation (SD) of the rise times of IPSCs (which serves as a measure of variability between events) was increased >4-fold (Figure 4B). This phenotype was also rescued by wild-type but not by N-peptide-deficient syntaxin-1A, consistent with the essential role of the N-peptide in fusion.

Finally, we measured the effect of the syntaxin-1 deficiency on IPSCs induced by a 1-s 10 Hz stimulus train. We found that

the total charge transfer during the train was again severely impaired; this phenotype was also rescued by wild-type but not by N-peptide-deficient syntaxin-1A (Figure 4C; Supplementary Figure S1). The impairment in evoked release during a stimulus train induced by the syntaxin-1 deficiency extended to delayed release, and we observed no syntaxin-1-dependent change in the ratio of delayed release to total release induced by the stimulus train (Figure 4D). Together, these findings demonstrate that the Munc18-1 binding site of the syntaxin-1 N-peptide is essential for synaptic membrane fusion, confirming and extending previous conclusions that binding of Munc18-1 to the N-terminal sequence of syntaxin-1 plays a crucial role in membrane fusion (Khvotchev *et al*, 2007; Shen *et al*, 2007, 2010; Tareste *et al*, 2008; Deák *et al*, 2009; Rathore *et al*, 2010; however, see Meijers *et al*, 2012 for an opposing view).

The N-peptide remains functionally essential in constitutively open syntaxin-1

At least three hypotheses could account for the essential role of the Munc18-binding N-peptide of syntaxin-1 in fusion. The N-peptide may be involved in stabilizing Munc18-1 levels, it may be required because Munc18-1 binding to syntaxin enables a direct function of Munc18-1 in membrane fusion, or because Munc18-1 opens the closed conformation of syntaxin-1 (to which Munc18-1 also binds, independent of

the N-peptide; Hata *et al*, 1993; Dulubova *et al*, 1999), which controls the speed of SNARE-complex assembly (Gerber *et al*, 2008). The data in Figure 2A already rule out the first hypothesis. To differentiate between the two remaining hypotheses, we tested whether the functional requirement for the N-peptide of syntaxin-1 was abolished by constitutively opening the closed conformation of syntaxin-1. To open syntaxin-1, we used the 'LE mutation' that shifts the default 'closed' conformation of syntaxin-1A into a preferentially 'open' state (Dulubova *et al*, 1999; Figure 5A).

We found that LE-mutant syntaxin-1A was fully active in fusion, consistent with previous results (Gerber *et al*, 2008). However, deletion of the N-peptide from syntaxin-1 impaired

fusion even when the LE mutation was present and when syntaxin-1 was constitutively open (Figures 5A and B). Thus, opening syntaxin-1 does not render the N-peptide non-essential, and Munc18-1 likely does not simply function to 'open' syntaxin-1 during fusion *in vivo*. However, the LE mutation did rescue the slow, desynchronized kinetics of release in syntaxin-deficient synapses expressing N-peptide deleted syntaxin-1A (Figure 5C), probably because it itself accelerates SNARE-complex assembly and fusion.

To further validate the conclusion that the N-peptide of syntaxin-1 is essential for synaptic membrane fusion, we also examined EPSCs evoked by isolated action potentials. We found that the syntaxin-1 deficiency dramatically decreased the amplitude of evoked EPSCs similar to IPSCs, and that this phenotype was also rescued by wild-type syntaxin-1A or by LE-mutant syntaxin-1A (Figure 5D). However, deletion of the N-peptide from either wild-type or LE-mutant syntaxin-1A again blocked its ability to rescue the syntaxin-1 deficiency phenotype, documenting the general requirement for the N-peptide in synaptic membrane fusion.

The syntaxin-1 H_{abc}-domain performs multiple functions in synaptic vesicle fusion

We next analysed the *in vivo* role of the H_{abc}-domain of syntaxin-1 in fusion to resolve the puzzling observation that despite its evolutionary conservation and size, the H_{abc}-domain is dispensable for fusion *in vitro* (Rathore *et al*, 2010; Shen *et al*, 2010). However, in rescue experiments with syntaxin-1-deficient neurons, we found that syntaxin-1 lacking the H_{abc}-domain but still containing the N-peptide (Figure 6A) was unable to rescue the decrease in spontaneous release that is induced by the syntaxin-1 deficiency (Figures 6B and C). Moreover, syntaxin-1A lacking the H_{abc}-domain did not rescue the decrease in the RRP in syntaxin-deficient neurons (Figure 6D), consistent with the inability of syntaxin-1A lacking the H_{abc}-domain to reverse the decrease in Munc18-1 levels in syntaxin-1-deficient neurons (Figure 2B). Since vesicle priming depends on Munc18-1 (Gulyás-Kovács *et al*, 2007; Deak *et al*, 2009), the loss of Munc18-1 could account for the decrease in the RRP observed with mutant syntaxin-1 lacking the H_{abc}-domain, but it does not explain the decrease in spontaneous mini release (see Discussion).

Figure 3 Syntaxin-1 N-peptide is essential for spontaneous synaptic vesicle fusion and for maintenance of the readily releasable pool (RRP) of vesicles. (A) Domain structures and N-terminal sequences of wild-type (top; Synt1A) and mutant syntaxin-1A with a deletion of the N-peptide (bottom; Synt1A^{ΔN}). (B, C) Representative traces (left) and summary graphs of the frequency (middle) and amplitudes (right) of miniature excitatory (mEPSCs; B) and miniature inhibitory postsynaptic currents (mIPSCs; C) in cortical neurons cultured from syntaxin-1A KO mice and infected with control lentivirus (Control), or lentiviruses expressing only syntaxin-1 shRNAs (Synt KD) or syntaxin shRNAs plus either wild-type syntaxin-1A (KD+Synt1A) or N-terminally truncated syntaxin-1A (KD+Synt1A^{ΔN}). (D) Representative traces (left) and summary graphs of the total synaptic charge transfer (right) of IPSCs evoked by a 30-s application of 0.5 M hypertonic sucrose to induce exocytosis of the RRP of vesicles, monitored in cortical neurons as described for (B). Data shown in summary graphs are means ± SEMs; numbers of cells/independent cultures analysed are listed in the bars. Statistical assessments were performed by Student's *t*-test comparing a test condition to the control (**P*<0.05; ***P*<0.01).

Figure 4 Syntaxin-1 N-terminal peptide is essential for evoked synaptic vesicle fusion. (A) Representative traces (left) and summary graphs of the charge transfer (right) of inhibitory postsynaptic currents (IPSCs) evoked by isolated action potentials in cortical neurons obtained as described for Figure 2B. (B) Summary graphs of the IPSC rise times (left) and the variability of IPSC rise times (expressed as the standard deviation of rise times; right). (C) Representative traces (left) and summary graphs of the total synaptic charge transfer (right) of IPSCs evoked by 10 stimuli applied at 10 Hz. (D) Summary graphs of delayed release (left) and the ratio of delayed to total release (right) measured in the experiments described in (C). Delayed release is defined as the synaptic charge transfer occurring 100 ms after the last train stimulus (Maximov and Südhof, 2005). Data shown are means $\pm$ SEMs; numbers of cells/independent cultures analysed are shown in the bars. Statistical assessments were performed by Student's *t*-test comparing a condition to the control (***P* < 0.01; ****P* < 0.001).

Surprisingly but fully consistent with the *in vitro* fusion data, H_{abc}-domain deleted syntaxin-1A was able to reverse the decrease in the size of evoked EPSCs and IPSCs induced by the syntaxin-1 deficiency (Figures 7A and B). Moreover, H_{abc}-domain deleted syntaxin-1A rescued the deceleration and desynchronization of release in syntaxin-1-deficient synapses (Figure 7C). Furthermore, when we probed the effect of the H_{abc}-domain deletion on release induced by a 10-Hz stimulus train (Figures 7D and E), we again observed that the H_{abc}-domain deleted syntaxin-1A fully rescued the impairment in release, although it did lead to increase short-term synaptic depression and a change in paired-pulse depression (Supplementary Figures S1 and S2). Thus, deletion of the H_{abc}-domain of syntaxin-1A does not impair action potential-evoked release, but blocks the ability of syntaxin-1A to rescue the loss of spontaneous release and the decrease in the RRP. We also tested whether the N-peptide was still required for the ability of H_{abc}-domain deleted syntaxin-1 to support evoked neurotransmitter release, as it is for wild-type syntaxin-1 and for LE-mutant syntaxin-1. Strikingly, we found that deletion of the

N-peptide in addition to the H_{abc}-domain blocked its ability to rescue release (Supplementary Figure S3). This finding provides further evidence for the hypothesis that the Munc18-binding N-peptide of syntaxin-1 is invariably required for the function of syntaxin-1 in membrane fusion. Together, these findings support the overall notion that syntaxin-1 always acts in a complex with Munc18-1 in fusion, and confirm previous results showing that the H_{abc}-domain is not essential for syntaxin-1 function in fusion as such (Rathore *et al*, 2010; Shen *et al*, 2010).

Finally, we examined a syntaxin-1A mutant that contained—in addition to the H_{abc}-domain deletion—asparagine substitutions in a conserved string of five aspartate residues at the N-terminus of syntaxin-1A (Figure 6A). The asparagine substitution mutant was examined to test the possibility that the aspartate residues might function as a Ca²⁺-binding site that controls asynchronous release (Sun *et al*, 2007). It was tested in the context of the H_{abc}-domain deletion because expression of the same substitution mutant on the background of wild-type syntaxin-1A appeared to cause lower expression levels. The asparagine substitutions

Figure 5 Opening up syntaxin-1 rescues the impaired kinetics but not the decreased magnitude of IPSCs induced in the presence of syntaxin-1A lacking the N-peptide. (A) Domain structures and N-terminal sequences of wild-type (top; Synt1A) and mutant syntaxin-1A with the LE mutation that renders syntaxin-1 constitutively open (Dulubova *et al*, 1999) without (Synt1A^{LE}) and with an additional deletion of the N-peptide (Synt1A^{LE+ΔN}). (B) Representative traces (left) and summary graphs of the synaptic charge transfer (right) of IPSCs evoked by isolated action potentials in cortical neurons cultured from syntaxin-1A KO mice and infected with control lentivirus (Control) or lentivirus expressing only syntaxin-1 shRNAs (Synt KD), or syntaxin-1 shRNAs plus either wild-type syntaxin-1A (KD+Synt1A), LE mutant constitutively open syntaxin-1A (KD+Synt1A^{LE}; see Dulubova *et al*, 1999), or N-terminally truncated, LE-mutant syntaxin-1A (KD+Synt1A^{LE+ΔN}). (C) Analysis of IPSC rise times (left) and the variability of rise times expressed as the standard deviation of rise times (right). Data are from the same experiments as those shown in (A). (D) Representative traces (left) and summary graphs of the amplitudes (right) of EPSCs evoked by isolated action potentials in cortical syntaxin-1A KO neurons infected with control lentivirus (Control) or lentivirus expressing only syntaxin-1 shRNAs (Synt KD), or syntaxin-1 shRNAs plus either wild-type syntaxin-1A (KD+Synt1A), N-terminally truncated syntaxin-1A (KD+Synt1A^{ΔN}), LE-mutant constitutively open syntaxin-1A (KD+Synt1A^{LE}), or N-terminally truncated, LE-mutant syntaxin-1A (KD+Synt1A^{LE+ΔN}). Data shown are means ± SEMs; numbers of cells/independent cultures analysed are shown in the bars. Statistical assessments were performed by Student's *t*-test comparing a condition to the control (**P* < 0.05; ***P* < 0.01; ****P* < 0.001).

had no effect on the ability of H_{abc}-domain deleted syntaxin-1A to rescue evoked release in syntaxin-1-deficient synapses, or its inability to rescue spontaneous release or the RRP

(Figures 6 and 7). This result indicates that the poly-aspartate sequence is unlikely to represent a Ca²⁺-sensor motif of major physiological significance.

Discussion

In the present study, we used a molecular replacement approach to examine the function of the N-terminal domains of syntaxin-1. We knocked down syntaxin-1B in syntaxin-1A KO neurons, and replaced the eliminated syntaxin-1 with wild-type and mutant forms of syntaxin-1A. The N-terminal sequences of syntaxin-1 include two identifiable domains: the short N-peptide and the longer H_{abc}-domain (Fernandez *et al*, 1998; Dulubova *et al*, 2007; Shen *et al*, 2007, 2010). Interestingly, both N-terminal domains bind to Munc18-1, but by independent mechanisms. The N-peptide binds directly to Munc18-1 similar to the binding of the N-peptide of other syntaxins to their cognate SM proteins (Yamaguchi *et al*, 2002; Dulubova *et al*, 2003, 2007; Khvotchev *et al*, 2007; Shen *et al*, 2007, 2010), whereas the H_{abc}-domain binds independent of the N-peptide when the H_{abc}-domain is folded back onto the SNARE motif in the closed conformation of syntaxin-1 (Dulubova *et al*, 1999; Misura *et al*, 2000).

Our experiments allow three major conclusions. First, the N-peptide is essential for syntaxin-1 function in fusion as such; second, the Munc18-1 binding site of the H_{abc}-domain but not the Munc18-1 binding site of the N-peptide is required for chaperoning Munc18-1 and maintaining its normal levels; and third, the H_{abc}-domain specifically regulates spontaneous vesicle fusion without being required for evoked synaptic vesicle exocytosis. In conjunction with previous studies, these conclusions suggest a functional domain map of syntaxin-1 that is shown in Figure 8.

The syntaxin-1 N-peptide

An essential role for the syntaxin-1 N-peptide interaction with Munc18-1 in synaptic vesicle fusion was previously shown using peptide-mediated disruption of this binding reaction in the calyx of Held synapse (Khvotchev *et al*, 2007), analysis of syntaxin-1 deletion mutants in liposome fusion assays based on lipid mixing (Shen *et al*, 2007; Rathore *et al*, 2010) and in *C. elegans* (McEwen and Kaplan 2008; Johnson *et al*, 2009; Rathore *et al*, 2010), and examination of Munc18-1 mutations that block Munc18-1 binding to the N-peptide (Deák *et al*, 2009). The present study extends this conclusion by demonstrating that deletion of the syntaxin-1 N-peptide also blocks syntaxin-1 function in synaptic vesicle fusion in cultured mammalian neurons, documenting this conclusion by measurements of a variety of parameters including spontaneous release (Figures 3B and C), the RRP (Figure 3D), and evoked synaptic transmission induced by isolated action potentials and action-potential trains (Figures 4A–D).

One hypothesis to explain the essential role of Munc18-1 in fusion had been that Munc18-1 binding to the N-peptide allows Munc18-1 to ‘open’ closed syntaxin-1 so that it can subsequently form SNARE complexes (reviewed in Südhof and Rizo, 2011). To test this hypothesis, we examined whether constitutively opening syntaxin-1 by the LE mutation (Dulubova *et al*, 1999) or by deletion of the entire H_{abc}-domain renders the N-peptide functionally dispensable (Figures 5A and 6A; Supplementary Figure S3). Strikingly, we find that even constitutively open syntaxin-1 or syntaxin-1 lacking the H_{abc}-domain requires an N-peptide for activity, although the LE mutation does rescue the impairment in release kinetics induced by the N-peptide deletion (Figure 5; Supplementary Figure S3). Collectively, and in agreement

with previous data (Khvotchev *et al*, 2007; Shen *et al*, 2007; Rodkey *et al*, 2008; Deak *et al*, 2009; Johnson *et al*, 2009; Diao *et al*, 2010; Rathore *et al*, 2010; Schollmeier *et al*, 2011), our results thus support an instrumental role of the N-peptide of syntaxin-1 in synaptic vesicle fusion *in vivo*.

Our results with the N-peptide—as well as those of Shen *et al* (2007), Khvotchev *et al*, (2007), Rodkey *et al* (2008), Deak *et al*, (2009), Johnson *et al* (2009), Diao *et al* (2010), Rathore *et al* (2010), and Schollmeier *et al* (2011)—led to a different conclusion than those of a recent study, which suggested that mutations in Munc18-1 that block binding to the syntaxin-1 N-peptide do not inactivate Munc18-1 function (Meijers *et al*, 2012). Our present results were obtained with a different approach from that of Meijers *et al* (2012), as we tested the function of the syntaxin-1 N-peptide in synaptic release. A possible hypothesis to account for these differences in conclusions might be that the N-peptide of syntaxin-1 binds to fusogenic factors other than Munc18-1, despite the high affinity and specificity of this binding reaction. However, other previous studies used similar approaches to Meijers *et al* (2012) but arrived at different conclusions. For example, we previously showed that mutations in Munc18-1 that disrupt binding to the SNARE complex do disrupt fusion (Deak *et al*, 2009). Furthermore, the F115E mutation in Munc18-1 that impairs its binding to the syntaxin-1 N-peptide was reported to fully support synaptic transmission in mouse cortical neurons (Meijers *et al*, 2012), but was unable to sustain synaptic transmission in *C. elegans* (Johnson *et al*, 2009). Thus, an alternative explanation for the present differences in conclusions in our view derives from the fact that all of the mutants used by Meijers *et al* (2012) still partly bind to syntaxin-1, although with diminished affinity. Since the mutant Munc18-1 molecules were overexpressed in cultured neurons but not in *C. elegans*, it is plausible that a partial loss-of-function could have been overcome in the experiments by Meijer *et al* (2012) by increased concentrations of the mutant Munc18-1 protein that still binds to the N-peptide. Thus, we feel that on balance, the evidence for a functional role of the syntaxin-1 N-peptide/Munc18-1 binding in fusion is compelling.

Syntaxin-1 chaperones Munc18-1 via the H_{abc}-domain Munc18-binding site

The syntaxin-1 deficiency produces an ~50% decrease in Munc18-1 levels (Figure 2), similarly to the opening of syntaxin-1 with the LE mutation (Gerber *et al*, 2008). The decrease in Munc18-1 levels is fully rescued with syntaxin-1 lacking the N-peptide (which does not support fusion, but still forms a closed conformation that binds Munc18-1), demonstrating that the loss of Munc18-1 levels is not secondarily due to a decrease in neurotransmitter release but represents an activity of syntaxin-1 that is independent of its function in neurotransmitter release (Figure 2A). Consistent with the requirement of the syntaxin-1 closed conformation for maintaining Munc18-1 levels (Gerber *et al*, 2008), syntaxin-1 lacking the H_{abc}-domain was unable to maintain normal Munc18-1 levels (Figure 2B), even though it still fully supported evoked neurotransmitter release (Figure 7). These results indicate that upstream of the common function of Munc18-1 and syntaxin-1 in membrane fusion, the complex formed by closed syntaxin-1 with Munc18-1 (Hata *et al*, 1993) serves to chaperone both

Figure 6 Syntaxin-1 H_{abc}-domain is essential for spontaneous synaptic vesicle fusion and for maintenance of the RRP. (A) Domain structures of wild-type (top) and H_{abc}-domain deleted syntaxin-1A (bottom; Synt1A^{ΔHabc}). The sequence alignment below the diagram depicts the N-terminal sequences of wild-type syntaxin-1A, H_{abc}-domain deleted syntaxin-1A, and H_{abc}-domain deleted syntaxin-1A which contained additional asparagine substitutions in the putative Ca²⁺-binding sequence of syntaxin-1A (the poly-aspartate region; Synt1A^{ΔHabc+DN}). (B, C) Representative traces (left) and summary graphs of the frequency (middle) and amplitudes (right) of mEPSCs (B) and mIPSCs (C) in cortical neurons cultured from syntaxin-1A KO mice and infected with control lentivirus (Control) or lentivirus expressing only syntaxin-1 shRNAs (Synt KD), or syntaxin shRNAs plus wild-type syntaxin-1A (KD + Synt1A), H_{abc}-domain deleted syntaxin-1A (KD + Synt1A^{ΔHabc}), or H_{abc}-domain deleted syntaxin-1A with an additional substitution of the poly-aspartate residues for poly-asparagine residues (KD + Synt1A^{ΔHabc+DN}). (D) Representative traces (left) and summary graphs of the total synaptic charge transfer for poly-asparagine residues evoked by a 30-s application of 0.5 M hypertonic sucrose to induce exocytosis of the RRP, monitored in cortical neurons as described for (B). Data shown are means ± SEMs; numbers of cells/independent cultures analysed are shown in the bars. Statistical assessments were performed by Student's *t*-test comparing a condition to the control (***P* < 0.01; ****P* < 0.001).

molecules, as also suggested by the finding that a loss of one of the two molecules or of the closed conformation of syntaxin-1 results in a decrease in the levels of the other (Verhage *et al*, 2000; Gerber *et al*, 2008; Figure 2). Thus, Munc18-1 is not simply a syntaxin-1 chaperone, nor does syntaxin-1 serve only as a Munc18-1 chaperone.

The syntaxin-1 H_{abc}-domain

Both the H_{abc}-domain deletion and the LE mutation of syntaxin-1 generate an 'open' syntaxin-1 conformation, produce a loss of Munc18-1, and result in a decrease in the RRP, with these three events likely acting as a cause-effect series (Gerber *et al*, 2008). However, the two syntaxin-1 mutations differ in one major phenotype: the H_{abc}-domain deletion causes a DECREASE in spontaneous release, whereas the LE mutation results in an INCREASE in spontaneous release (Figure 6). The decrease in spontaneous release produced by the H_{abc}-domain deletion of syntaxin-1 cannot be explained by the decrease in the RRP because the LE mutation causes the same decrease in the RRP but results in an increase in spontaneous release (Gerber *et al*, 2008). Thus, our data suggest that the H_{abc}-domain of syntaxin-1 performs at least one additional function in synaptic vesicle fusion besides

chaperoning Munc18-1 and maintaining the RRP: It is essential for spontaneous miniature release (Figure 8).

Why is spontaneous release decreased upon deletion of the H_{abc}-domain of syntaxin-1, and why is evoked release retained despite the decrease in spontaneous release and in the RRP? As regards the first question, accumulating evidence suggests that spontaneous and evoked release are mechanistically distinct and differentially regulated in central synapses (Sara *et al*, 2005; reviewed in Sutton and Schuman, 2009 and Ramirez and Kavalali, 2011). Our results suggests that the H_{abc}-domain may be involved in the differential regulation of spontaneous and evoked release, in addition to mediating the closed conformation of syntaxin-1, although the molecular mechanism by which the H_{abc}-domain mediates this differential regulation remains unknown. As regards the second question, it seems likely that the loss of the closed conformation in syntaxin-1 lacking an H_{abc}-domain accelerates and enhances fusion, similar to what was previously shown for LE-mutant syntaxin-1B (Gerber *et al*, 2008). Thus, a decrease in the RRP is compensated by an increased refilling rate of the RRP and an increased fusion speed. An important general implication of our findings is that spontaneous release cannot be simply considered as a

Figure 7 Syntaxin-1 H_{abc}-domain is not essential for evoked synaptic vesicle fusion. (A) Representative traces (left) and summary graphs of the amplitude (right) of EPSCs evoked by isolated action potentials in cortical neurons obtained as described for Figure 5B. (B) Representative traces (left) and summary graph of the synaptic charge transfer (right) of IPSCs evoked by isolated action potentials in cortical neurons obtained as described in (A). (C) Analysis of IPSC rise times (left) and the variability of IPSC rise times (expressed as the standard deviation of rise times; right), calculated from the experiments shown in (B). (D) Representative traces (left) and summary graphs of the total synaptic charge transfer (right) of IPSCs evoked by 10 stimuli applied at 10 Hz. (E) Summary graphs of delayed release (left) and the ratio of delayed to total release (right) measured in the experiments described in (D). Data shown are means $\pm$ SEMs; numbers of cells/independent cultures analysed are shown in the bars. Statistical assessments were performed by Student's *t*-test comparing a condition to the control (**P* < 0.05; ***P* < 0.01; ****P* < 0.001).

Figure 8 Functional domain structure of syntaxin-1. The attributions of the various functions are based on the present study (for the N-peptide and the H_{abc}-domain) and previous *in vitro* and *in vivo* studies (Dulubova *et al*, 1999, 2007; Misura *et al*, 2000; Khvotchev *et al*, 2007; Shen *et al*, 2007; Deák *et al*, 2009; Rathore *et al*, 2010).

readout of synapse numbers, RRP size, or release probability, but rather represents the combined end result of many processes that include those mentioned above, and additionally involves other processes specific for this mode of release.

In summary, our data indicate that the complex domain structure of syntaxin-1 is required for a multifaceted functional role of syntaxin-1 in different types of synaptic vesicle fusion that includes both evoked and spontaneous fusion (Figure 8). The high degree of conservation among syntaxins, including within their H_{abc}-domains, indicates that similar functions may also apply to other isoforms in other types of fusion reactions.

Materials and methods

Neuronal cultures

Neuronal cultures were obtained from cortex of newborn mice as described (Yang *et al*, 2010). Neurons were infected with lentiviruses at DIV5–7, and analysed at DIV13–16.

Plasmid construction and production of lentiviruses

Lentiviral expression vector and three helper plasmids (pRSV-REV, pMDLg/pRRE and vesicular stomatitis virus G protein expression vector) were co-transfected into HEK293T cells (ATCC, VA), at 6, 2, 2, and 2 µg of DNA per 25 cm² culture area, respectively (Pang *et al*, 2010), using FuGENE 6 transfection reagent (Roche, UK) or calcium phosphate, and cell-culture supernatants containing the viruses were collected 48 h after transfection and directly used for infection of neurons. All steps were performed under level II biosafety conditions.

Development and validation of syntaxin-1 shRNAs

Syntaxin-1A and -1B cDNA sequences were aligned and two conserved regions were chosen as targets of shRNAs (AGAGCAGCTGGAGATCAC and GATCATCATTTGCTGTGTG). Synthetic shRNA oligonucleotides (sense sequences: TCGACCAGG CAGCTGGAGATCACTTCAAGAGA GTGATCTCCAGCTGCCTCTGGTT TTTTGGAAAT and CGCGCCGATCATCATTTGCT GTGTGT TCAAG AGACACACAGCAAATGATGATCATTTTGGAAA) were inserted into L309 vector (Pang *et al*, 2010) using *XhoI*-*XbaI* and *AscI*-*RsrII* sites respectively. The KD effects of the syntaxin-1 shRNAs were validated in wild-type cortical neurons by quantitative RT-PCR.

Immunocytochemistry

Neurons were fixed and permeabilized at –20°C in 100% methanol, incubated with anti-MAP2 (mouse monoclonal; Sigma) and anti-synapsin (rabbit polyclonal; E028) primary antibodies in PBS with 4% BSA and 1% goat serum, washed, and stained with monoclonal anti-MAP2 and polyclonal anti-synapsin and visualized using

Alexa Fluor 633 goat anti-mouse and Alexa Fluor 546 goat anti-rabbit secondary antibodies (Molecular Probes). Images were acquired by using a Leica TCS2 confocal microscope equipped with ×63 oil-immersion objective with numerical aperture of 1.32. Identical settings were applied to all samples in each experiment. Stacks of z-section images were acquired and converted to maximal projection images by using Leica Confocal Software, and analysed blindly with a custom Matlab program as described (Nam and Chen, 2005). Images were thresholded by intensity to exclude the diffuse/intracellular pool, and then puncta were quantified by counting the number of supra-threshold areas of sizes between 0.25 and 4 µm². Representative images were merged using ImageJ 1.44p software. Presynaptic terminals (visualized via synapsin staining) were presented in red, and dendrites (MAP2 staining) in blue.

Protein quantifications

Proteins from cultured neurons harvested in cold SDS sample buffer at DIV14–16 were resolved by 10–12% SDS-PAGE, transferred onto nitrocellulose membranes, and reacted with antibodies to syntaxin-1 (polyclonal; 438B), Munc18 (monoclonal; BD Biosciences), Syt1 (monoclonal; CL41.1), Rab3 (polyclonal; T957), SNAP-25 (polyclonal; P913), complexins (polyclonal; L668 and L669), synaptobrevin-2 (polyclonal; P939), VCP (polyclonal; 433B), and GDI (monoclonal; Synaptic Systems). Signals were quantified using ¹²⁵I-labelled secondary antibodies and Phosphorimager detection.

Electrophysiological recordings

Electrophysiological recordings were performed in whole-cell patch-clamp mode using concentric extracellular stimulation electrodes (Yang *et al*, 2010). Evoked synaptic responses were triggered by a bipolar electrode placed 100–150 µm from the soma of neurons recorded. Patch pipettes were pulled from borosilicate glass capillary tubes (Warner Instruments) using a PC-10 pipette puller (Narishige). The resistance of pipettes filled with intracellular solution varied between 3 and 5 MOhm. After formation of the whole-cell configuration and equilibration of the intracellular pipette solution, the series resistance was adjusted to 8–10 MOhm. Synaptic currents were monitored with a Multiclamp 700B amplifier (Molecular Devices). The frequency, duration, and magnitude of the extracellular stimulus were controlled with a Model 2100 Isolated Pulse Stimulator (A-M Systems, Inc.) synchronized with Clampex 10 data acquisition software (Molecular devices). The whole-cell pipette solution contained (in mM) 120 CsCl, 10 HEPES, 10 EGTA, 0.3 Na-GTP, 3 Mg-ATP and 5 QX-314 (pH 7.2, adjusted with CsOH). The bath solution contained (in mM) 140 NaCl, 5 KCl, 2 MgCl₂, 2 CaCl₂, 10 HEPES-NaOH, and 10 glucose (pH 7.4). IPSCs and EPSCs were pharmacologically isolated by adding the AMPA and NMDA receptor blockers CNQX (20 µM) and AP-5 (50 µM) or the GABA_A-receptor blockers picrotoxin (50 µM) to the extracellular solution. Spontaneous mIPSCs were monitored in the presence of tetrodotoxin (TTX; 1 µM) to block action potentials. Miniature events were analysed in Clampfit 10 (Molecular Devices) using the template matching search and a minimal threshold of 5 pA and each event

was visually inspected for inclusion or rejection by an experimenter blind to the recording condition. Sucrose-evoked release was triggered by a 30-s application of bath solution to which 0.5 M sucrose were added and which also contained AP-5, CNQX, and TTX.

Statistical analyses

Statistical analyses were performed with Student's *t*-tests or two-way ANOVA tests comparing test to control samples analysed in the same experiments.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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