Chapter 3

Tyrosine phosphorylation of Munc18-1 determines synaptic strength

Marieke Meijer1, Ruud F. Toonen1 and Matthijs Verhage1

1Department of Functional Genomics, Center for Neurogenomics and Cognitive Research (CNCR), Neuroscience Campus Amsterdam (NCA), VU University Amsterdam and VU University Medical Center, 1081 HV, Amsterdam, The Netherlands

Abstract

Tyrosine kinases are important regulators of synaptic strength. Here we identify Munc18-1, an essential component of synaptic vesicle release machinery, as a target for neuronal tyrosine kinase n-Src. Disrupting the main Src phosphorylation site on Munc18-1 leads to severe defects in spontaneous and evoked synaptic transmission in autaptic hippocampal neurons, which can be temporarily restored by high frequency stimulation. These results reveal a novel and potent regulatory mechanism that determines synaptic strength via tyrosine phosphorylation of Munc18-1.
3.1 Introduction

Neurons communicate via the release of neurotransmitter containing vesicles from the presynapse. This process is executed by elaborate protein machinery, whose function can be temporarily and precisely regulated via post-translational modifications on individual components. The most common form is protein phosphorylation, which adds a negative phosphate groups to certain neutral amino acids. This can change the function of a protein and thereby the efficiency of the entire synaptic vesicle release machinery. For example, phosphorylation of an essential protein in the release machinery, Munc18-1, on serine residues by protein kinase C (PKC) acutely potentiates synaptic transmission [264, 278]. Tyrosine phosphorylation of its ubiquitously expressed isoform Munc18-3 by the insulin receptor kinase modifies the interaction with Syntaxin4, and is important for insulin-stimulated GLUT4 vesicle exocytosis in fat cells [4, 122]. Whether Munc18-1 can also be phosphorylated on tyrosine residues is unknown.

Tyrosine kinases have long been associated with developmental processes, but are recently also acknowledged for their function in regulating synaptic plasticity [193]. Although most focus has been on the postsynaptic side, an additional role in presynaptic neurotransmitter release is becoming evident [6, 176, 179, 232, 272], especially for the Src tyrosine kinase (SFK) family. C-Src is enriched in synaptic vesicle fractions, is up-regulated after memory training, and phosphorylates several synaptic vesicle associated proteins, like synaptophysin, synaptogyrin and synapsin [8, 121, 179, 296]. In contrast, its neuron specific isoform, n-Src, preferably associates with presynaptic membranes, indicating that n-Src might be involved in other signalling pathways than c-Src [179]. N-Src is identical to c-Src except for a 6-amino insert in the SH3 domain due to alternative splicing [157, 198]. This domain is responsible for regulating the activity and substrate recruitment of SFKs. As a consequence of the insert, n-Src displays a higher kinase activity and does not interact with several binding partners of c-Src, including Synapsin and SFK activator FAK [145, 163, 179].

Here we investigated whether Munc18-1 is a substrate for n-Src and whether tyrosine phosphorylation regulates the function of Munc18-1 in synaptic vesicle exocytosis. This study identifies Munc18-1 as a novel presynaptic target for n-Src, with Y473 as the main phosphorylation site. Mutating this site shows that tyrosine phosphorylation of Munc18-1 strongly modulates synaptic vesicle release. Autaptic hippocampal neurons solely expressing Munc18-1 with point mutations in Y473 show a severe reduction of spontaneous and evoked release. This resulted from a priming defect and a reduced vesicular release probability, but an activity-dependent component of priming was still intact. These results reveal a novel and potent mechanism to regulate synaptic strength via tyrosine phosphorylation of Munc18-1.
3.2 Results

3.2.1 Src phosphorylates Munc18-1 on Y473

We first tested whether Munc18-1 is a target for tyrosine kinases. For this, we used HEK293T cells to express Munc18-1 either alone or together with the tyrosine kinase n-Src, a neuronal Src isoform associated to presynaptic membranes [179]. Tyrosine phosphorylation was quantified by immunoblotting using an antibody that specifically recognises phosphorylated tyrosine residues. Since this antibody recognizes all tyrosine-phosphorylated proteins, Munc18-1 was first immunoprecipitated from whole cell lysate. In the absence of n-Src, tyrosine phosphorylation of Munc18-1 could not be observed (Fig. 3.1a). However, a clear band at the height of Munc18-1 was visible when n-Src was present (Fig. 3.1a). To identify the phosphorylation site, PhosphoSitePlus (www.phosphosite.org) was used to screen for identified sites in Munc18-1, which revealed a phosphorylation site at Tyr473, found in a large-scale screen in murine brain [7]. Y473 is of specific interest since the predicted kinase for this site was Src (scansite.mit.edu). Mutating Y473 to alanine severely reduced tyrosine phosphorylation of Munc18-1 (Fig. 3.1a-b), indicating that this site is the major site on Munc18-1 that is phosphorylated by n-Src. Y473 is located on the outer surface of domain3 in Munc18-1 [164], away from known interaction sites for Syntaxin and Mint and potentially accessible for kinases. The phosphorylation site is highly conserved across species and in the mammalian Munc18-1 isoform Munc18-2, but not Munc18-3 (Fig. 3.1c).

3.2.2 Mutating Y473 leads to strong defects in basal synaptic transmission

To test the functional importance of Munc18-1 phosphorylation at Y473, munc18-1 null neurons were rescued with Lentivirus expressing a non-phosphorylatable mutant, M18Y473A, or a phospho-mimicking mutant, M18Y473D. Munc18-1 null neurons expressing Munc18-1 were used as a control. Neurons rescued with M18Y473A or M18Y473D expressed Munc18-1 at similar levels in the synapse and were morphologically identical to control neurons (Fig. 3.1d), as determined from confocal images using an automated image analysis routine [220].

Synaptic transmission was assessed in autaptic neurons (neurons grown in isolation on glial islands), a reduced model system commonly used to study changes in release kinetics and short-term presynaptic plasticity [13, 48]. Whole-cell patch clamp experiments on these neurons revealed a profound effect on synaptic transmission. Neurons expressing M18Y473A showed a more than five-fold reduction in evoked post-synaptic current (EPSC) amplitude compared to control neurons, and neurons expressing M18Y473D hardly responded at all (Fig. 3.1e). A reduction in synaptic transmission can be caused by a reduced number of primed vesicles, comprising the readily releasable pool (RRP), or by a reduced release probability
per vesicle (Pvr). To further investigate this, neurons were subjected to a hypertonic sucrose application to release the total RRP in a calcium independent manner [210]. Consistent with the effect on EPSC size, neurons expressing M18Y473D showed a large reduction in RRP size and neurons expressing M18Y473A showed a trend towards smaller sucrose responses (Fig. 3.1f). Pvr can be calculated by dividing the EPSC charge by the charge of the RRP. Both mutant groups showed a large reduction in Pvr (Fig. 3.1g). Our results thus indicate that the severe effect on synaptic transmission is a result of a reduction in RRP size and Pvr.

Spontaneous release in neurons expressing M18Y473A and M18Y473D was almost absent (Fig. 3.1h). The frequency of spontaneous events (mEPSCs) was reduced over seventy-fold compared to controls (Fig. 3.1i). mEPSC amplitude was not affected, arguing against a postsynaptic cause for the reduced evoked and spontaneous synaptic transmission. The decay kinetics of mEPSCs in neurons expressing M18Y473D however were slower (Fig. 3.1i). To summarise, these

*Figure 3.1: Figure legend on next page*
Figure 3.1: (See previous page) Tyrosine phosphorylation of Munc18-1 disrupts basal synaptic transmission in autaptic neurons. (A) Munc18-1 is phosphorylated by n-Src at Y473. Munc18-1 was immunoprecipitated (IP) from cell lysate of HEK293T cells expressing M18<sub>WT</sub> or M18<sub>Y473A</sub> together with n-Src or an empty vector as control. Munc18-1 was then immunoblotted for tyrosine phosphorylation using the 4G10 antibody. The total amount of Munc18-1 was detected after stripping and reblotting for Munc18-1. (B) Data were collected from three independent experiments and quantified as the ratio of tyrosine phosphorylated Munc18-1 over total Munc18-1 precipitated (M18<sub>WT</sub>: 1.0 ± 0.16, n=3; M18<sub>Y473A</sub>: 0.26 ± 0.06, n=3; Paired t-test, p=0.0116). (C) Alignment of amino acid sequence surrounding Y473 of mouse Munc18 isoforms and of Munc18-1 across different species. Highlighted residues are identical to mouse Munc18-1. (D) Rescued hippocampal neurons were stained for the dendritic marker MAP2, synapse marker VAMP2 and Munc18-1. Mean synaptic Munc18-1 intensity (M18<sub>WT</sub>: 389 ± 85 a.u., n=21; M18<sub>Y473A</sub>: 321 ± 48 a.u., n=20; M18<sub>Y473D</sub>: 261 ± 49 a.u., n=20; Kruskal-Wallis, p=0.269). Mean synapse number (M18<sub>WT</sub>: 129 ± 14, n=21; M18<sub>Y473A</sub>: 159 ± 16, n=20; M18<sub>Y473D</sub>: 159 ± 14, n=20; ANOVA, p=0.228). Mean dendrite length (M18<sub>WT</sub>: 0.433 ± 0.028 mm, n=21; M18<sub>Y473A</sub>: 0.461 ± 0.037 mm, n=20; M18<sub>Y473D</sub>: 0.454 ± 0.037 mm, n=20; ANOVA, p=0.834). (E) Evoked release from rescued autaptic hippocampal neurons upon a single action potential stimulation. Mean EPSC amplitude (M18<sub>WT</sub>: 7.96 ± 0.94 nA, n=30; M18<sub>Y473A</sub>: 1.44 ± 0.34 nA, n=33; M18<sub>Y473D</sub>: 0.12 ± 0.05 nA, n=30; Kruskal-Wallis, p<0.001). Typical responses are depicted on the right. (F) Evoked release from rescued autaptic hippocampal neurons upon hyperosmotic sucrose application (500mM, 3.5s.) was used to assess the RRP. Mean RRP charge (M18<sub>WT</sub>: 1.68 ± 0.64 nC, n=13; M18<sub>Y473A</sub>: 0.27 ± 0.08 nC, n=13; M18<sub>Y473D</sub>: 0.08 ± 0.02 nC, n=10; Kruskal-Wallis, p<0.001). Typical responses are depicted on the right. (G) The vesicular release probability per neuron was calculated by dividing EPSC charge by RRP charge. Mean P<sub>v</sub>r (M18<sub>WT</sub>: 6.75 ± 0.77%, n=13; M18<sub>Y473A</sub>: 1.98 ± 0.72%, n=13; M18<sub>Y473D</sub>: 0.24 ± 0.20%, n=10; Kruskal-Wallis, p<0.001). (H) Example traces of spontaneous release of single vesicles (mEPSCs) from rescued autaptic hippocampal neurons. (I) Mean mEPSC frequency (M18<sub>WT</sub>: 20.18 ± 5.18 Hz, n=15; M18<sub>Y473A</sub>: 0.48 ± 0.15 Hz, n=20; M18<sub>Y473D</sub>: 0.28 ± 0.13 Hz, n=17; Kruskal-Wallis, p<0.001). Mean mEPSC amplitude (M18<sub>WT</sub>: 26.0 ± 1.7 pA, n=15; M18<sub>Y473A</sub>: 24.5 ± 1.6 pA, n=20; M18<sub>Y473D</sub>: 27.8 ± 2.2 pA, n=17; ANOVA, p=0.4454). Mean mEPSC decay time (M18<sub>WT</sub>: 1.27 ± 0.05 ms, n=15; M18<sub>Y473A</sub>: 1.51 ± 0.10 ms, n=20; M18<sub>Y473D</sub>: 1.84 ± 0.17 ms, n=17; Kruskal-Wallis, p=0.011).

Results suggest that tyrosine phosphorylation of Munc18-1 might control both spontaneous and evoked synaptic transmission in naive neurons, and that the loss-of-function phenotype of the phospho-mimicking mutant is more severe than the non-phosphorylatable mutant.

To investigate whether the reduced RRP size could be assigned to an upstream docking defect, synapses from autaptic cultures were assessed at ultra-structural level (Fig. 3.2a). The number of vesicles docked at the active zone was smaller in M18<sub>Y473A</sub> while the total amount of vesicles was similar (Fig. 3.2b-c). This docking defect however is unlikely to be fully responsible for the much larger reduction in RRP size. In addition, secretion defective M18<sub>Y473D</sub> expressing neu-
Figure 3.2: synaptic vesicles are halted in pre-primed state. Autaptic excitatory neurons expressing M18\textsubscript{WT}, M18\textsubscript{Y473A} or M18\textsubscript{Y473D} were analysed with electron microscopy. (A) Electron micrographs of typical synapses. (B) Neurons expressing M18\textsubscript{Y473A} have less synaptic vesicles (SV) docked at the active zone. Average number of docked SV (M18\textsubscript{WT}: 7.63 ± 0.15 SVs; M18\textsubscript{Y473A}: 5.71 ± 0.28 SVs; M18\textsubscript{Y473D}: 8.45 ± 0.20 SVs; Multilevel ANOVA, p<0.001). (C) Total number of SVs is not significantly different. Average total number of SVs (M18\textsubscript{WT}: 142 ± 11 SVs; M18\textsubscript{Y473A}: 120 ± 12 SVs; M18\textsubscript{Y473D}: 148 ± 15 SVs; Multilevel ANOVA, p=0.052). (D) The active zone is longer in neurons expressing M18\textsubscript{Y473D} compared to M18\textsubscript{Y473A}. Average active zone (AZ) length (M18\textsubscript{WT}: 561 ± 24 nm; M18\textsubscript{Y473A}: 501 ± 21 nm; M18\textsubscript{Y473D}: 646 ± 59 nm; Multilevel ANOVA, p=0.009). (E) Neurons expressing M18\textsubscript{Y473A} have less SVs docked per AZ length (M18\textsubscript{WT}: 0.0140 ± 0.0006 docked SVs/nm AZ; M18\textsubscript{Y473A}: 0.0117 ± 0.0005 docked SVs/nm AZ; M18\textsubscript{Y473D}: 0.0141 ± 0.0009 docked SVs/nm AZ; Multilevel ANOVA, p=0.016). M18\textsubscript{WT}: N=6 neurons, n=137 synapses; M18\textsubscript{Y473A}: N=8 neurons, n=136 synapses; M18\textsubscript{Y473D}: N=8 neurons, n=116 synapses.

Neurons are fully functional in docking (Fig. 3.2b-c), confirming a post-docking role of Munc18-1. Neurons expressing M18\textsubscript{Y473D} have a larger active zone compared to M18\textsubscript{Y473A} expressing neurons (Fig. 3.2d), but this does not explain the difference in docking efficiency between the mutants (Fig. 3.2e). These findings suggest the non-phosphorylatable mutant M18\textsubscript{Y473A} is defective in docking, priming and release of synaptic vesicles. In contrast, the phospho-mimicking M18\textsubscript{Y473D} mutant shows an almost complete loss-of-function phenotype in steps downstream docking.
3.2.3 Synaptic transmission is temporarily restored during sustained stimulation

Since the loss-of-function phenotype of both mutants includes reduced vesicular release probability, short-term plasticity is likely to be altered. Indeed, facilitation of EPSC sizes could be observed in both mutants, resulting in a build-up of synchronous release during prolonged stimulation (Fig. 3.3a,c). Within these stimulation trains, neurons expressing M18<sub>Y473A</sub> showed a large increase in EPSC size up to 4-fold depending on the stimulation frequency (Fig. 3.3b,d), reaching similar EPSC sizes as control neurons (Fig. 3.3a,c). Even in neurons expressing M18<sub>Y473D</sub>, who hardly showed initial release, synchronous release was partly restored during sustained stimulation, albeit more slowly than in the non-phosphorylatable mutant (Fig. 3.3a,c). This up-regulation of secretion was transient and did not persist after two minutes of rest normally given to cells between measurements. These results indicate that although tyrosine phosphorylation of Munc18-1 is critical for basal synaptic transmission, synaptic transmission can be temporarily restored during periods of stimulation.

Intense stimulation at 40Hz leads to a rapid depression of EPSC size in neurons expressing M18<sub>WT</sub>. At this frequency, neurons expressing M18<sub>Y473A</sub> and M18<sub>Y473D</sub> hardly build-up synchronous release before depression sets in (Fig. 3.3e, typical traces). However, during such intense stimulation synaptic transmission quickly switched from synchronous to asynchronous release. When we analysed the total transferred charge instead of the EPSC peak amplitude, a large increase of charge was observed in neurons expressing M18<sub>Y473A</sub> and M18<sub>Y473D</sub> (Fig. 3.3e), which was even stronger than the build-up of synchronous release during less intense stimulation (Fig. 3.3a,c). For neurons expressing M18<sub>Y473A</sub>, the total charge transferred during a 40Hz train was identical to neurons expressing M18<sub>WT</sub>. These results indicate that the loss-of-function phenotype resulting from mutating the key residue for tyrosine phosphorylation in Munc18-1 can be restored by sustained stimulation.

3.2.4 Increased synaptic transmission after HFS is due to increased RRP size and Pvr

To gain further insight in the mechanism that restores release in the phosphotyrosine mutants, synaptic transmission after high frequency stimulation (HFS) was assessed in more detail. First, the size of a single EPSC was tested two seconds after HFS. While neurons expressing M18<sub>WT</sub> showed minor depression of EPSC size after HFS, EPSC size was strongly augmented by HFS in neurons expressing M18<sub>Y473A</sub> (Fig. 3.4a). Furthermore, this augmentation was dependent on the stimulation frequency, with higher frequencies leading to stronger augmentation. Therefore, we focussed on the stimulation frequency that gave the most pronounced EPSC size augmentation, 40Hz stimulation. Neurons were probed with either a single action potential or a hypertonic sucrose solution to assess
Figure 3.3: Figure legend on next page
Figure 3.3: (See previous page) Synaptic transmission is restored during sustained stimulation. (A) Autaptic excitatory neurons expressing M18\textsubscript{WT}, M18\textsubscript{Y473A} or M18\textsubscript{Y473D} were subjected to stimulation trains of 100 pulses at different frequencies. (A) Absolute EPSC amplitudes during a 5Hz train. (B) EPSC amplitudes during a 5Hz train normalized to the first EPSC. Neurons expressing M18\textsubscript{Y473D} were not included since synchronous release is almost absent in the first EPSC. (C) Absolute EPSC amplitudes during a 10Hz train. (D) EPSC amplitudes during a 10Hz train normalized to the first EPSC. Neurons expressing M18\textsubscript{Y473D} were not included since synchronous release is almost absent in the first EPSC. (E) Example traces of the currents evoked by 40Hz stimulation. Transferred charge between pulses is depicted on the right. Insert shows total charge transferred during stimulation.

RRP size and Pvr under basal conditions and two seconds after HFS. Neurons expressing M18\textsubscript{Y473A} or M18\textsubscript{Y473D} showed a pronounced increase in EPSC charge after HFS, while neurons expressing M18\textsubscript{WT} did not (Fig. 3.4b). The same effect, but less pronounced, was observed for RRP size (Fig. 3.4c). HFS enhanced Pvr in all groups, but while Pvr was strongly decreased in mutant neurons compared to control cells under basal conditions, Pvr was similar among groups directly after HFS. These results suggest that HFS leads to a larger increase in Pvr in neurons expressing M18\textsubscript{Y473A} or M18\textsubscript{Y473D}.

What effect would the increased RRP and Pvr have on short-term plasticity? To answer that question, we gave a second 10Hz stimulation train two seconds after a first one. While synaptic transmission in neurons expressing M18\textsubscript{Y473A} was strikingly different during the first train (Fig. 3.3c), it was similar to control neurons during the second train (Fig. 3.3e) (Fig. 5a). The same trend was observed in neurons expressing M18\textsubscript{Y473D}, although EPSC sizes remain smaller (Fig. 3.5a). Nonetheless, both mutants showed less depression of EPSC size compared to control neurons (Fig. 3.5b-c). These results show that a single train of 100 pulses at 10Hz does not completely rescue synaptic transmission in the tyrosine mutants. Taken together, these results suggest that synaptic transmission is temporarily restored during HFS due to an increase in the amount and release probability of vesicles in the RRP.

3.3 Discussion

Here, we investigated whether Munc18-1 is a substrate for tyrosine kinases and what the potential effect of tyrosine phosphorylation on synaptic transmission would be. We found that Munc18-1 can indeed be phosphorylated in vivo by the neuronal tyrosine kinase n-Src, mainly on site Tyr473. To study the consequence of tyrosine phosphorylation at this site, a non-phosphorylatable mutant (M18\textsubscript{Y473A}) and a phospho-mimicking mutant (M18\textsubscript{Y473D}) were expressed in autaptic hippocampal munc18-1 null neurons. Both spontaneous and evoked synaptic transmission were severely affected in these neurons compared to neurons
Figure 3.4: Figure legend on next page
Figure 3.4: (See previous page) HFS increases both Pvr and RRP. Synaptic transmission was assessed after HFS in autaptic excitatory neurons expressing M18\textsubscript{WT}, M18\textsubscript{Y473A} or M18\textsubscript{Y473D}. (A) A single pulse was given two seconds after stimulation trains of different frequencies (100 pulses). EPSC augmentation is calculated by dividing the EPSC amplitude of the single pulse by the amplitude of the first EPSC within the preceding stimulation train. Mean augmentation after 5Hz train (M18\textsubscript{WT}: 0.54 ± 0.05, n=8; M18\textsubscript{Y473A}: 2.88 ± 0.47, n=10; Unpaired t-test with Welch correction, p=0.0008). Mean augmentation after 10Hz train (M18\textsubscript{WT}: 0.70 ± 0.10, n=8; M18\textsubscript{Y473A}: 5.39 ± 0.77, n=10; Unpaired t-test with Welch correction, p=0.0002). Mean augmentation after 40Hz train (M18\textsubscript{WT}: 0.93 ± 0.11, n=8; M18\textsubscript{Y473A}: 8.77 ± 1.52, n=10; Unpaired t-test with Welch correction, p=0.0006). Neurons expressing M18\textsubscript{Y473D} were not included since synchronous release is almost absent at the start of stimulation. (B) Absolute EPSC charge at start (s) and two seconds after (a) 40Hz stimulation. Mean EPSC charge at start (M18\textsubscript{WT}: 70.9 ± 19.5 pC, n=12; M18\textsubscript{Y473A}: 10.3 ± 4.5 pC, n=13; M18\textsubscript{Y473D}: 0.888 ± 0.342 pC, n=9; Kruskal-Wallis test, p<0.0001). Mean EPSC charge after HFS (M18\textsubscript{WT}: 75.4 ± 17.4 pC, n=12; M18\textsubscript{Y473A}: 46.2 ± 14.5 pC, n=13; M18\textsubscript{Y473D}: 25.6 ± 9.3 pC, n=9; Kruskal-Wallis test, p=0.0846). Naive versus after stimulation (M18\textsubscript{WT}: Paired t-test, p=0.4049; M18\textsubscript{Y473A}: Wilcoxon matched-pairs signed-ranks test, p=0.0005; M18\textsubscript{Y473D}: Paired t-test, p=0.0271) (C) Absolute RRP size (charge released upon hypertonic sucrose application) in basal condition (b) or two seconds after 40Hz stimulation (a). Mean basal RRP size (M18\textsubscript{WT}: 1.73 ± 0.69 nC, n=12; M18\textsubscript{Y473A}: 0.268 ± 0.075 nC, n=13; M18\textsubscript{Y473D}: 0.061 ± 0.019 nC, n=9; Kruskal-Wallis test, p<0.0001). Mean RRP size after HFS (M18\textsubscript{WT}: 1.34 ± 0.54 nC, n=12; M18\textsubscript{Y473A}: 0.439 ± 0.107 nC, n=13; M18\textsubscript{Y473D}: 0.207 ± 0.050 nC, n=9; Kruskal-Wallis test, p=0.0557). Naive versus after HFS (M18\textsubscript{WT}: Paired t-test, p=0.0557; M18\textsubscript{Y473A}: Paired t-test, 0.0100; M18\textsubscript{Y473D}: Paired t-test, p=0.0246) (D) Pvr (EPSC charge / RRP charge) in basal condition (b) and two seconds after 40Hz stimulation (a). Mean Pvr before (M18\textsubscript{WT}: 7.07 ± 0.76 %, n=12; M18\textsubscript{Y473A}: 1.98 ± 0.72 %, n=13; M18\textsubscript{Y473D}: 0.27 ± 0.22 %, n=9; Kruskal-Wallis test, p<0.0001). Mean Pvr size after HFS (M18\textsubscript{WT}: 10.6 ± 1.6 %, n=12; M18\textsubscript{Y473A}: 12.2 ± 2.8 %, n=13; M18\textsubscript{Y473D}: 10.8 ± 2.2 %, n=9; ANOVA, p=0.8500). Before vs after (M18\textsubscript{WT}: Paired t-test, p=0.0201; M18\textsubscript{Y473A}: Paired t-test, 0.0021; M18\textsubscript{Y473D}: Paired t-test, p=0.0012). (E) RRP charge two, four or eight seconds after 40Hz stimulation, normalized to basal RRP charge. Relative RRP in neurons expressing M18\textsubscript{WT} (2 seconds after: 0.75 ± 0.09, n=7; 4 seconds after: 0.78 ± 0.08, n=7; 8 seconds after: 0.63 ± 0.08, n=7). Relative RRP in neurons expressing M18\textsubscript{Y473A} (2 seconds after: 1.56 ± 0.19, n=7; 4 seconds after: 1.77 ± 0.17, n=7; 8 seconds after: 1.44 ± 0.14, n=7). Relative RRP in neurons expressing M18\textsubscript{Y473D} (8 seconds after: 3.23 ± 1.16, n=6; 4 seconds after: 2.88 ± 0.80, n=6; 8 seconds after: 3.05 ± 0.65, n=6).

expressing wild-type Munc18-1. The diminished synaptic transmission resulted from a reduction in the number of primed vesicles and the release probability of those vesicles, while an activity-dependent priming component was still intact. Since both mutants rescued the lethal munc18-1 null phenotype, it is unlikely that the secretion defects are due to improper protein folding [99]. It is tempting to speculate that tyrosine phosphorylation of Munc18-1 might be a novel and potent regulatory mechanism to determine synaptic strength.
Figure 3.5: Short-term plasticity after HFS. Two stimulation trains of 10Hz were given with a two second interval to autaptic excitatory neurons expressing M18WT, M18Y473A or M18Y473D. (A) Depression of absolute EPSC amplitude during the second 10Hz train. (B) The rate of depression during the second 10Hz stimulation train shown as EPSC sizes normalized to the first EPSC in the second train. (C) Quantification of the rate of depression. Shown is the relative size of the last EPSC in the second train normalized to the first EPSC of the second train. Mean relative EPSC amplitude (M18WT: 0.255 ± 0.046, n=17; M18Y473A: 0.395 ± 0.057, n=19; M18Y473D: 0.550 ± 0.064, n=18; ANOVA, p<0.0027).

3.3.1 Munc18-1 functions in a priming pathway with CAPS and Munc13

While priming was clearly affected in the Y473 mutants under basal conditions, high frequency stimulation could temporarily restore priming and strongly augment EPSC sizes, indicating that an activity dependent priming mechanism was still intact. A similar phenotype was found in neurons lacking Munc13-1 or CAPS1/2 [123, 209], suggesting that these proteins are part of the same sequential pathway. For instance, tyrosine phosphorylation of Munc18-1 might recruit CAPS to the release machinery. Munc18-1 can modulate the affinity of CAPS for Syntaxin1 in vitro [49]. Recruited CAPS will then stabilize primed vesicles by preventing them from returning to an un-primed state [123]. Tyrosine phosphorylation of Munc18-1 might also aid the opening of Syntaxin1 by Munc13 [154], facilitating the priming process. Calcium influx caused by sustained activity increases the forward rate of priming [172] and could temporarily overcome the reduced priming efficiency in the non-phosphorylatable mutant. This model of tyrosine phosphorylation of Munc18-1 as a facilitator of priming under basal conditions can only be reconciled with the priming defect of the phospho-mimicking M18Y473A mutant if a cycle of tyrosine phosphorylation is required to establish a normal RRP size (Fig. 3.6a).

Alternative explanations can also be envisioned, namely that the mutations designed to modify tyrosine phosphorylation had unintentional secondary effects.
Replacing tyrosine might have disrupted other post-translational modifications which take place at this residue, like tyrosine sulfation. In addition, replacing tyrosine with the much smaller alanine lacking an aromatic ring might have modified the local protein structure. If a model on the function of tyrosine phosphorylation of Munc18-1 is solely based on the phospho-mimicking mutant, the outcome would drastically change. In this model, phosphorylation of Munc18-1 has a strong inhibitory effect on synaptic transmission (Fig. 3.6b). Resolving the molecular pathway which activates tyrosine phosphorylation of Munc18-1 will enable the study of the effects of acute phosphorylation in live neurons, and will be crucial to test the above hypothesis.

**Figure 3.6:** Regulation of priming by tyrosine phosphorylation of Munc18-1. Alternative models on the function of tyrosine phosphorylation of Munc18-1. (A) A cycle of tyrosine phosphorylation of Munc18-1 is required for efficient priming. Munc18-1 must exist in a non-phosphorylated form in steps upstream priming. N-Src phosphorylation of Munc18-1 subsequently facilitates priming or stabilizes the primed state by the recruitment of CAPS. (B) Tyrosine phosphorylation of Munc18-1 acts as a brake on synaptic transmission, possibly by inhibiting the association of CAPS to the priming complex.

### 3.3.2 Munc18-1 as a presynaptic inhibitory target for tyrosine kinases

We show that a member of the Src family kinases (SFKs), n-Src, is capable of phosphorylating Munc18-1 in vitro. SFKs negatively regulate glutamate release from synaptosomes and cultured cerebellar granule cells [6, 176, 179], and calcium induced insulin secretion from rat pancreatic islets and INS-1 cells [42]. In addition, the SFK member Lyn blocked ethanol-induced up-regulation of dopamine release from the nucleus accumbens [77]. In hippocampal neurons, tyrosine phosphorylation of Synapsin by c-Src prevents excessive reserve pool depletion during periods of intense stimulation by recruiting vesicles to the reserve pool instead...
of the RRP [162]. These results show that several SFK family members act as a brake on synaptic transmission. The almost complete arrest of synaptic vesicle release in the tyrosine phospho-mimicking Munc18-1 mutant suggests that n-Src, like Lyn and c-Src, could act as a brake on synaptic transmission, reducing synaptic strength in excitatory hippocampal neurons.

While this study focussed on n-Src other SFK members, like Fyn or c-Src, might also target Munc18-1. Their distinctive localization suggest that individual SFKs are part of different signalling pathways [142], but some targets might overlap. In addition, there are potential candidates among other families. Ack1, a member of the activated Cdc42-associated tyrosine kinase (Ack) family, is present in axon terminals and is up-regulated by neuronal activity [259]. Activation of the presynaptic receptor tyrosine kinase TrkB by BDNF is proposed to enhance neurotransmitter release and bi-directionally increase SFK activity [107, 192, 193]. TrkB can thus potentially phosphorylate Munc18-1 either directly or indirectly via SFKs.

### 3.3.3 Tyrosine phosphorylation modulates Pvr

Not all defects caused by disrupting the tyrosine phosphorylation cycle on Munc18-1 can be explained by a priming defect. In contrast to priming deficient CAPS-1/2 DKO neurons, tyrosine mutant neurons have a decreased Pvr and a disproportionally large reduction of spontaneous release frequency compared to the RRP defect [123]. Besides regulating priming, Munc18-1 can also temporarily increase release rates upon PKC phosphorylation [255, 278]. The same holds for Munc13-1, which next to an essential role in priming is also capable of increasing release rates in response to DAG/phorbol ester binding by lowering the energy barrier for fusion [10]. Our results suggest that tyrosine phosphorylation of Munc18-1 might also regulate this energy barrier. To summarize, our results suggest that Munc18-1’s central function in synaptic vesicle release can be modulated by n-Src, which will regulate synaptic strength and short-term plasticity.