CHAPTER 2

FUNCTION OF TOMOSYN-1
TOMOSYN-1 CONTROLS MUNC18 LEVELS AND SUPPORTS SYNAPTIC VESICLE REPLENISHMENT

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ABSTRACT

Secretion from presynaptic vesicles involves the formation of a SNAP (Soluble NSF Attachment Protein) Receptor (SNARE) protein complex of syntaxin, SNAP25 and synaptobrevin. Tight regulation is required to prevent premature vesicle pool depletion and thereby sustain prolonged synaptic activity. Munc18 and tomosyn respectively stimulate and inhibit membrane fusion by SNARE protein interactions and their concerted action may regulate presynaptic plasticity. Here we show that tomosyn-1 (STXBP5) deletion (Tom1KO/KO) in mice led to sublethality during early embryonic development. Autaptic Tom1KO/KO hippocampal neurons from surviving E18 mice exhibited normal neurite outgrowth and synapse formation, but reduced (synaptic) Munc18 protein levels. Although spontaneous release, initial evoked release and readily releasable pool size were not significantly affected, Tom1KO/KO neurons showed decreased paired pulse ratios, enhanced depression of neurotransmitter release upon high frequency stimulation and reduced recovery from this. Overall, the data implies that tomosyn-1 regulates Munc18 protein levels and enhances synaptic vesicle replenishment. Thus, an activity-dependent balance in Munc18 or tomosyn binding to syntaxin could be required for efficient recovery of SNARE-mediated vesicle fusion during prolonged activity.

KEYWORDS: Tomosyn-1, STXBP5, Munc18, neurotransmitter release, presynaptic plasticity
INTRODUCTION

Tomosyn is a negative regulator of synaptic transmission

Docking, priming and fusion of a vesicle with its target membrane are mediated by a core complex of three SNAP (Soluble NSF Attachment Protein Receptor) proteins - syntaxin, SNAP25 and synaptobrevin - bridging the two membranes. An array of additional proteins is involved in the assembly and maturation of this key complex (Jahn and Fasshauer, 2012). One of them is tomosyn, identified as a syntaxin-interacting protein and inhibitor of SNARE complex-mediated vesicle secretion (Fujita et al., 1998; Masuda et al., 1998; Hatsuzawa et al., 2003; Yizhar et al., 2004; Cheviet et al., 2006; Zhang et al., 2006; Gladycheva et al., 2007; Yizhar et al., 2007).

Tomosyn’s function in the transmitter release from synaptic vesicles specifically has been well studied in various model systems. In vitro, overexpression of tomosyn inhibits synaptic transmission in cultured superior cervical ganglion neurons, as demonstrated by decreased excitatory postsynaptic potential (EPSP) amplitudes and increased failure rates (Baba et al., 2005). Also in the C. elegans neuromuscular junction (NMJ), overexpression of TOM-1A decreases neurotransmission and consistently, evoked postsynaptic current (ESPC) charge is enhanced upon tomosyn deletion. The increased secretion in tomosyn mutants correlates with an enhanced primed vesicle pool, estimated by application of hyperosmotic sucrose and electron microscopy (Gracheva et al., 2006; McEwen et al., 2006). Similarly, knockdown of tomosyn in Drosophila NMJs enhances evoked release and decreases paired pulse ratio, indicative of increased release probability (Chen et al., 2011).

In the mammalian nervous system, two homologous tomosyn genes are expressed. Tomosyn-1 (STXBP5) is most widely expressed in the brain, while tomosyn-2 (STXBP5L) expression seems more restricted to specific brain regions. Furthermore, based on mRNA levels, tomosyn-1 expression is stable in various developmental stages, whereas tomosyn-2 expression is highly up-regulated during development (Groffen et al., 2005). In mouse tomosyn-1 knockout (Tom1KO/KO) hippocampal mossy fiber synapses, EPSCs are enhanced and paired pulse facilitation is decreased, implying increased vesicular release probability. Furthermore, the early stage of induced mossy fiber long-term potentiation is reduced (Sakisaka et al., 2008). Thus, in line with studies in invertebrate model organisms suggesting that tomosyn negatively regulates vesicle priming, mammalian tomosyn-1 inhibits neurotransmitter release from synaptic vesicles.
Neurite outgrowth is suggested to be regulated by tomosyn
Besides modulation of transmitter release from synaptic vesicles, several studies suggest that tomosyn regulates plasmalemmal precursor vesicle fusion for directed membrane supply during neurite outgrowth. Overexpression of tomosyn’s last two exons in the Drosophila third instar larvae neuromuscular system generates pathfinding defects and fewer or abnormal synapses (Kraut et al., 2001). Its involvement in neuronal development is confirmed by tomosyn-1 overexpression and knockdown experiments in primary cultured rat hippocampal neurons and NG108 cells in vitro. Tomosyn is suggested to direct the fusion of vesicles to the leading edge of a growth cone by locally inhibiting fusion at the palm of the growth cone (Sakisaka et al., 2004). However, tomosyn knockdown in Drosophila does not hamper NMJ synapse morphology and synapse number (Chen et al., 2011). Also in C. elegans, in vivo axon morphology and synapse number are not affected in tomosyn null mutants (Gracheva et al., 2006; McEwen et al., 2006). Thus, the role of tomosyn in neurite outgrowth is still highly debated.

Functional competition of Munc18 and tomosyn
Munc18-1 is essential for secretion from synaptic vesicles (Verhage et al., 2000) and important for the stabilization and plasma membrane localization of syntaxin (Rowe et al., 2001). In hippocampal neurons heterozygous for Munc18-1, a normal first evoked response is accompanied by increased synaptic depression during 10 Hz stimulation, caused by a reduced readily releasable pool (RRP) size (Toonen et al., 2006). This suggests that for activity-dependent priming, Munc18 is rate-limiting. A function for Munc18 in sustained release could involve its phosphorylation via the PKC pathway (Sassa et al., 1996; Wierda et al., 2007), reducing syntaxin binding affinity (Fujita et al., 1996; de Vries et al., 2000; Barclay et al., 2003). Functional interactions between Munc18 and tomosyn are suggested by independent studies. Munc18 and tomosyn compete for syntaxin binding (Fujita et al., 1998; Gladycheva et al., 2007), which explains the enhanced membrane localization of UNC-18 (Munc18) in C. elegans tomosyn null mutants and vice versa (Gracheva et al., 2010). The amount of tethering and docking of synaptic vesicles is thereby balanced by the negative regulator tomosyn and UNC-18 as a positive regulator. In line with this, knockout of tomosyn in unc-18 nematodes partially rescues priming. Unlike UNC-18, C. elegans tomosyn is not required for the stability of syntaxin. Overall UNC-18 levels on the other hand seem slightly decreased in tom-1 mutants, despite enhanced membrane-localized Munc18 (Gracheva et al., 2010). Taken together, tomosyn and Munc18 are likely to compete for membrane-bound syntaxin and thereby regulate vesicle tethering or priming.
In this study we investigated the function of mammalian tomosyn-1 in cultured autaptic hippocampal neurons from Tom1\(^{KO/KO}\) mice (Sakisaka et al., 2008). We report that tomosyn-1 was important for early embryonic development, but dispensable for neuronal development. EPSC amplitudes in Tom1\(^{KO/KO}\) neurons were not significantly enhanced, but repeated stimulation revealed reduced sustained release. Also, reduced Munc18 protein levels were observed. These results provide important new insight into the mechanism by which tomosyn and Munc18 co-regulate activity-dependent vesicle priming.

MATERIALS AND METHODS

Laboratory animals
Tomosyn-1 deficient C57Bl/6 / BDF1 hybrid mice have been described before (Sakisaka et al., 2008). Littermate offspring was used for electrophysiology and morphology experiments. In whole brain lysate analysis by Western blot, hybrid C57Bl/6 / DBA-1 Tom1\(^{KO/KO}\) mice from homozygous breedings were used. Wildtype C57Bl/6 mice were used as controls. E18 stage embryos were obtained by Caesarian section of pregnant females from timed matings. Animals were housed and bred according to Institutional, Dutch and U.S. governmental guidelines.

Primary neuron culture
Hippocampi were isolated from E18 stage mouse brains in Hanks Buffered Salt Solution (HBSS; Sigma) plus 10 mM HEPES (Invitrogen) at room temperature. After removal of the meninges, hippocampi were incubated in HBSS/HEPES plus 0.25% trypsin (Invitrogen) for 20 min at 37°C. The hippocampi were washed and triturated to obtain a single cell suspension with a fire polished pipette tip. Cultured neurons were maintained in Neurobasal medium (Invitrogen) supplemented with 2% B-27 (Invitrogen), 1.8% HEPES, 0.25% glutamax (Invitrogen) and 0.1% penicillin/streptomycin (Invitrogen). Autaptic cultures were generated by plating neurons on microdot islands of glia cells. Such microislands were prepared by plating 6K/well freshly prepared rat glia on etched glass coverslips, coated with 0.15% w/v agarose and dots of 0.1 mg/ml poly-D-lysine (Sigma) / 0.7 mg/ml rat tail collagen (BD Biosciences) substrate (adapted from Segal and Furshpan, 1990). 2K/well (12-well plate) neurons were plated to ensure single neurons per island of glia. Network cultures used for Western blotting were obtained by plating 150K/well hippocampal neurons on a confluent layer of frozen rat glia grown on etched glass coverslips.
Immunohistochemistry and morphological analysis
For immunohistochemistry, cultures were fixed with 3.7% formaldehyde (Electron Microscopy Sciences). Cells were permeated with 0.1% Triton X-100 (Sigma) in phosphate-buffered saline (PBS) for 5 min at room temperature. Next, aspecific antibody binding was prevented by incubation with blocking solution containing 2% normal goat serum and 0.05% Triton X-100 in PBS for 20 min. Primary antibody incubation was done for 2 h at room temperature. After washing with PBS, cells were incubated for 1 h at room temperature with Alexa dye conjugated secondary antibodies (Molecular Probes, 1:1000). Antibodies were diluted in blocking solution. After washing again, cells were mounted using Dabco (Sigma) - Mowiol (Calbiochem). Imaging was done with a confocal LSM510 microscope (Carl Zeiss). Neurite length, complexity, synapse number and (synaptic) protein levels were assessed with automated neurite tracking software SynD (Schmitz et al., 2011). For intensity measures correlated to distance from soma, a cut-off value of 100 µm was used, corresponding to the smallest cell. Hippocampal autaptic neurons were fixed at DIV (days in vitro) 14 and stained with an antibody recognizing MAP2 (Abcam, 1:1000) for dendritic analysis and VAMP2 (synaptobrevin, Synaptic systems, Cl69.1, 1:2000) for analysis of synapses. Munc18 (Synaptic systems, 2701, 1:1000) and syntaxin-1 (Synaptic systems, I379, 1:1000) protein levels were estimated by fluorescence intensity.

Western blot
Brain lysate for Western blot analysis was prepared by direct brain homogenization in 1 ml Laemmli sample buffer per 0.1 g brain (one year old animals) and boiling for 15 min at 100°C. After wet protein transfer to a PVDF membrane for 2 h at 350 mA at 4°C, aspecific antibody binding to the membrane was prevented by incubation of the membrane with blocking solution (5% w/v milk powder and 0.2% Tween-20 in Tris-buffered saline (TBS)) for 1 h at 4°C. Primary antibody incubation was done overnight at 4°C. After washing with TBS, the membrane was stained with secondary antibody conjugated with alkaline phosphatase (AP; DAKO, 1:5000) for 1 h at 4°C. After washing again, the AP conjugated antibody was visualized using ECF substrate (GE Healthcare). The membrane was scanned with a Fujifilm FLA-5000 Reader. Primary antibodies that were used were anti-Munc18 (Synaptic systems, 2701, 1:5000) and anti-actin (Chemicon, 1:2000).

Electrophysiology
Hippocampal autaptic neuron whole-cell voltage clamp electrophysiology was performed at room temperature with 2-4 MΩ borosilicate glass pipettes. Cells that had been in culture for 14 to 18 days were used. Extracellular solution contained
10 mM HEPES, 10 mM glucose, 140 mM NaCl, 2.5 mM KCl, 4 mM MgCl₂ and 4 mM CaCl₂. Intracellular solution consisted of 125 mM K⁺-gluconic acid, 10 mM NaCl, 4.6 mM MgCl₂, 4 mM K₂-ATP, 15 mM creatine phosphate, 10 U/ml phosphocreatine kinase and 1 mM EGTA. The pH of both solutions was set to 7.3 and buffers were ensured to have an osmolarity of 300 mOsm. Cells were stimulated and signals were recorded with a Multiclamp 700B amplifier and Digidata 1440A (Axon Instruments). Electrical stimulation was done by depolarization from -70 to 30 mV for 0.5 ms. 75% series resistance (R_{SER}) compensation was used when indicated. Only cells with a series resistance below 10 MΩ, a leak current below 500 pA and EPSC amplitudes of at least 500 pA were used for analysis. Analysis was done with Clampfit 10 (Axon Instruments), Mini Analysis (synaptosoft) and Matlab (Mathworks).

Statistics
Statistical analysis was performed using SPSS. Mendelian distribution was assessed by chi-square test. Kolmogorov-Smirnov was used to test normality of data distribution. A Mann-Whitney test for two independent samples was applied when groups were not normally distributed. Else, independent sample Student t-tests were performed, taking into account Levene’s test for equality of variances. Repeated measures or two-way ANOVA tests were used when indicated. A significance level of p<0.05 was used.

RESULTS
Skewed Mendelian distribution in Tom1^{WT/KO} interbreedings
Tom1^{WT/KO} interbreedings gave rise to a highly distorted genetic distribution as depicted in table 1. Already at embryonic stage E18, only 2.5% out of the expected 25% Tom1^{KO/KO} mice were observed ($\chi^2$(2)=67.5, p<0.0001; n=241). A similar Mendelian skew appeared in a population of three week old mice ($\chi^2$(2)=31.1, p<0.0001; n=90), indicating tomosyn-1 is mainly important in early development. Although Tom1^{KO/KO} mice were born at a low frequency, surviving homozygous mice appeared healthy by gross anatomical inspection.

Table 1. Genotype distribution of Tom1^{WT/WT}, Tom1^{WT/KO} and Tom1^{KO/KO} mice in Tom1^{WT/KO} interbreedings did not follow Mendelian ratios at ages E18 and P21 (***,p<0.0001).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Expected</th>
<th>Observed E18***</th>
<th>Observed P21***</th>
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<tr>
<td>Tom1^{WT/WT}</td>
<td>25%</td>
<td>36.5%</td>
<td>43.3%</td>
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<td>Tom1^{WT/KO}</td>
<td>50%</td>
<td>61.0%</td>
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<td>Tom1^{KO/KO}</td>
<td>25%</td>
<td>2.5%</td>
<td>2.2%</td>
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Munc18 levels are decreased in Tom1\(^{KO/KO}\) neurons

Previous literature points towards a role of tomosyn in neurite outgrowth (Kraut et al., 2001; Sakisaka et al., 2004), whereas no effects on neuronal morphology and synapse formation is observed in other studies (Gracheva et al., 2006; McEwen et al., 2006; Chen et al., 2011). In our experiments, autaptic hippocampal Tom1\(^{KO/KO}\) neurons showed no changes in neuronal development compared to Tom1\(^{WT/WT}\) neurons, as assessed by neurite outgrowth, complexity and the number of synapses (Figure 1A-D). Evident however was a clear reduction in Munc18 staining intensity, both in synapses (Figure 1E, 26% reduction; Mann-Whitney U=237, z=-3.561, p<0.001, r=-0.449) and overall in the neurites (Figure 1H; two-way ANOVA F(1, 1281)=131.332, p<0.001 for genotype, n.s. for interaction between genotype and distance from soma F(20,1281)=0.383, p=0.994). This was not caused by general decreased synaptic protein levels, since both syntaxin and VAMP2 protein levels were unaffected (Figure 1F,G,I). These immunocytochemistry results were confirmed by Western blot analysis of whole brain lysate, in which actin normalized Munc18 protein level in Tom1\(^{KO/KO}\) brains was reduced by 27% (Figure 1J; t(30)=3.746, p=0.001). Thus, although tomosyn-1 was not involved in the development of cultured hippocampal neurons, it contributed importantly to the regulation of Munc18 protein levels.

Figure 1 (next page). Tomosyn-1 deficiency decreased neuronal Munc18 levels, without affecting cellular morphology in hippocampal autaptic neurons. (A) Hippocampal dendrites were stained with an antibody against MAP2 (cyan in typical examples), while synapses were detected using a VAMP2 antibody (red). Munc18 and Syntaxin-1 protein levels were assessed with protein specific antibodies (green). (B) Mean total dendrite length and (C) branching was unaffected in Tom1\(^{KO/KO}\) neurons and also (C) synapse number was normal. Munc18 intensity in (E) synaptic puncta, as well as in (H) the neurites was lower in Tom1\(^{KO/KO}\) neurons. This was not the case for both (F, I) syntaxin and (G) VAMP2. Tom1\(^{WT/WT}\) and Tom1\(^{KO/KO}\) littermates were used. (J) Actin normalized Munc18 protein level was 27% lower for Tom1\(^{KO/KO}\) whole brain lysate compared to Tom1\(^{WT/WT}\) (pooled samples of three one-year-old mouse brains per group). Data is depicted as mean ± S.E.M. with n-numbers shown in the bars; ***p<0.001).
Figure 1 (legend on previous page).
Tomosyn-1-enhanced synaptic recovery

Since tomosyn has been implicated in the regulation of SNARE complex formation during vesicular neurotransmitter release, we performed electrophysiological experiments to assess synaptic release characteristics of Tom1$^{\text{KO/KO}}$ cultured hippocampal autaptic neurons, in which pre- and postsynaptic effects can be discriminated and spontaneous single vesicle fusion assessed. In contrast to the enhanced synaptic transmission at hippocampal mossy fiber synapses of Tom1$^{\text{KO/KO}}$ mice in slice preparations (Sakisaka et al., 2008), we found no significant effects on basal synaptic transmission in cultured hippocampal Tom1$^{\text{KO/KO}}$ neurons, as indicated by normal first evoked EPSC amplitudes (Figure 2A,B). EPSC decay time was somewhat prolonged in Tom1$^{\text{KO/KO}}$ neurons (table 2; Mann-Whitney U=1244, z=-1.991, p=0.047, r=-0.187). Spontaneous release was not significantly affected (Figure 2C,D and table 2; release frequency Mann-Whitney U=178, z=-1.223, p=0.221, r=-0.187).

Upon repetitive stimulation however, Tom1$^{\text{KO/KO}}$ release deviated from Tom1$^{\text{WT/WT}}$. Paired pulse ratios were decreased in Tom1$^{\text{KO/KO}}$ neurons at all stimulation intervals tested (Figure 2E,F; two-way ANOVA for genotype F(1,534)=68.596, p<0.001, n.s. for interaction between genotype and interstimulus interval F(4,534)=0.656, p=0.623). During a 40 Hz stimulation protocol of 2.5 s duration, overall release was reduced (Figure 2G). Synchronous release kinetics were not significantly affected, as assessed by a bi-exponential fit on EPSC amplitude rundown (Figure 2H-J; fast decay $\tau$ Mann-Whitney U=634, z=-1.58, p=0.114, r=-0.18). The rundown of the total charge however was significantly faster in Tom1$^{\text{KO/KO}}$ neurons (Figure 2K-M; fast decay $\tau$ from bi-exponential fit Mann-Whitney U=522, z=-2.659, p=0.008, r=-0.30; slow decay $\tau$ from bi-exponential fit Mann-Whitney U=567, z=-2.226, p=0.026, r=-0.25). RRP size (Rosenmund and Stevens, 1996) and replenishment can be estimated by back-extrapolation of the total current released in a 40 Hz stimulation paradigm (Figure 2N; Schneggenburger et al., 1999). Interestingly, Tom1$^{\text{KO/KO}}$ neurons exhibited reduced vesicle replenishment (Figure 2P; Mann-Whitney U=155, z=-2.032, p=0.042, r=-0.306), which is likely to cause the overall release deficit during intense train stimulation, without an effect on RRP size (Figure 2O). Last, recovery from a 40 Hz stimulation protocol is slower in Tom1$^{\text{KO/KO}}$ neurons (Figure 2Q; Repeated measure ANOVA for the interaction between time and genotype with Greenhouse-Geisser correction of degrees of freedom F(2.422, 188.949)=3.212, p=0.033). Thus, tomosyn-1 appears to function in synaptic recovery without affecting RRP size.
Figure 2 (legend on next page).
Figure 2 (previous page). Decreased synaptic recovery in hippocampal autaptic Tom1\(^{\text{KO/KO}}\) neurons. (A) Averaged EPSC traces. (B) First evoked current amplitude and kinetics (Table 2), as well as (C,D) spontaneous release frequency and kinetics (Table 2) were not significantly affected in Tom1\(^{\text{KO/KO}}\) neurons. Typical example traces are shown in (C). (E) Averaged traces of a paired stimulation protocol with various interstimulus intervals. (F) Quantification of paired pulse amplitudes shows an overall decreased paired pulse ratio in Tom1\(^{\text{KO/KO}}\) neurons. (G) Averaged release during a 2.5 s 40 Hz stimulation. (H) Normalized EPSC amplitudes during the first 250 ms of this stimulation protocol. EPSC amplitude rundown kinetics during the whole train were characterized by a bi-exponential fit with a (I) fast and (J) slow decay time constant \(\tau\). (K) Normalized total charge released during the first 500 ms of a 40 Hz train stimulation protocol. Rundown kinetics during the whole train were characterized by a bi-exponential fit with a (L) fast and (M) slow decay time constant \(\tau\). (N) Average cumulative charge released during a 40 Hz train stimulation and back-extrapolation of the last 40 stimuli to estimate (O) RRP size and (P) replenishment after RRP depletion. (Q) Recovery from a 40 Hz train stimulation was reduced in Tom1\(^{\text{KO/KO}}\) neurons. Tom1\(^{\text{WT/WT}}\) and Tom1\(^{\text{KO/KO}}\) C57Bl/6 littermates were used. Bars represent mean ± S.E.M with n-numbers shown in the bars; *p<0.05, **p<0.01, ***p<0.001.

Table 2 (next page). Additional electrophysiology data of Tom1\(^{\text{WT/WT}}\) and Tom1\(^{\text{KO/KO}}\) neurons. For each of four independent experiments (with or without 75% series resistance compensation Rser), EPSC amplitudes are shown, as well as spontaneous release frequency, amplitude and half width, which were all not significantly affected by Tomosyn-1 deficiency (EPSC amplitude pooled data Mann-Whitney U=1869, z=-1.638, p=0.101, r=-0.142; spontaneous release frequency week 3 Mann-Whitney U=0, z=-2.249, p=0.024, r=-0.795). EPSC decay time was slightly prolonged in Tom1\(^{\text{KO/KO}}\) neurons upon pooling of data (Mann-Whitney U=1244, z=-1.991, p=0.047, r=-0.187). Cumulative total charge released during a 100 stimulus 40 Hz train was reduced in Tom1\(^{\text{KO/KO}}\) neurons compared to Tom1\(^{\text{WT/WT}}\) neurons (Week 1 t(12.43)=2.789, p=0.016, week 4 t(19)=1.677, p=0.110, week 1 and 2 pooled Mann-Whitney U=160, z=-1.1915, p=0.055, r=-0.289, week 3 and 4 pooled Mann-Whitney U=106, z=-1.759, p=0.079, r=-0.293; all weeks pooled normalized data Mann-Whitney U=513, z=-2.746, p=0.006, r=-0.307). Additionally, replenishment (slope charge) and RRP size (Y-axis intercept) were estimated by back-extrapolation of total charge released during the last 40 stimuli of this train stimulation. By this, replenishment during a 40 Hz stimulation train was reduced in Tom1\(^{\text{KO/KO}}\) neurons (Week 1 t(12.996)=2.867, p=0.013, week 4 t(19)=1.858, p=0.079, week 1 and 2 pooled Mann-Whitney U=155, z=-2.032, p=0.042, r=-0.306, week 3 and 4 pooled Mann-Whitney U=96, z=-2.076, p=0.038, r=-0.346; all weeks pooled normalized data Mann-Whitney U=485, z=-3.016, p=0.003, r=-0.337). Mean ± S.E.M (n) is shown; *p<0.05, **p<0.01.
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<th>Week 1 (R&lt;sub&gt;ser&lt;/sub&gt; compensation 0%)</th>
<th>Week 2 (R&lt;sub&gt;ser&lt;/sub&gt; compensation 0%)</th>
<th>Week 3 (R&lt;sub&gt;ser&lt;/sub&gt; compensation 75%)</th>
<th>Week 4 (R&lt;sub&gt;ser&lt;/sub&gt; compensation 75%)</th>
<th>Total</th>
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<td>Tom&lt;sup&gt;1&lt;/sup&gt; WT/WT</td>
<td>4.3 ± 0.35 (11)</td>
<td>5.3 ± 0.61 (11)</td>
<td>42.0 ± 3.88 (14)</td>
<td>1.0 ± 0.06 (11)</td>
<td>4.3 ± 0.35 (11)</td>
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<td>4.9 ± 0.61 (11)</td>
<td>5.3 ± 0.61 (11)</td>
<td>5.3 ± 0.35 (16)</td>
<td>1.0 ± 0.10 (11)</td>
<td>4.9 ± 0.61 (11)</td>
</tr>
<tr>
<td>Tom&lt;sup&gt;1&lt;/sup&gt; KO/KO</td>
<td>15.4 ± 2.23 (16)</td>
<td>13.0 ± 1.29 (26)</td>
<td>15.4 ± 0.67 (20)</td>
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<td>15.4 ± 0.67 (20)</td>
<td>13.0 ± 1.29 (26)</td>
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<td>13.0 ± 1.29 (26)</td>
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**EPSC amplitude (pA)**

- **Week 1 (R<sub>ser</sub> compensation 0%)**
  - Tom<sup>1</sup> WT/WT: 4.3 ± 0.35 (11)
  - Tom<sup>1</sup> KO/KO: 15.4 ± 2.23 (16)

**EPSC amplitude (normalized)**

- **Week 1 (R<sub>ser</sub> compensation 0%)**
  - Tom<sup>1</sup> WT/WT: 1.0 ± 0.10 (11)
  - Tom<sup>1</sup> KO/KO: 15.4 ± 2.23 (16)

**EPSC decay time (ms)**

- **Week 1 (R<sub>ser</sub> compensation 0%)**
  - Tom<sup>1</sup> WT/WT: 4.9 ± 0.61 (11)
  - Tom<sup>1</sup> KO/KO: 15.4 ± 2.23 (16)

**Spontaneous release frequency (%)**

- **Week 1 (R<sub>ser</sub> compensation 0%)**
  - Tom<sup>1</sup> WT/WT: 15.0 ± 2.23 (16)
  - Tom<sup>1</sup> KO/KO: 15.4 ± 2.23 (16)

**Spontaneous release amplitude (pA)**

- **Week 1 (R<sub>ser</sub> compensation 0%)**
  - Tom<sup>1</sup> WT/WT: 15.0 ± 2.23 (16)
  - Tom<sup>1</sup> KO/KO: 15.4 ± 2.23 (16)

**Spontaneous release half width (ms)**

- **Week 1 (R<sub>ser</sub> compensation 0%)**
  - Tom<sup>1</sup> WT/WT: 5.3 ± 0.61 (11)
  - Tom<sup>1</sup> KO/KO: 5.3 ± 0.61 (11)

**Cumulative total charge in 40Hz (pC)**

- **Week 1 (R<sub>ser</sub> compensation 0%)**
  - Tom<sup>1</sup> WT/WT: 2.0 ± 0.03 (13)
  - Tom<sup>1</sup> KO/KO: 2.0 ± 0.03 (13)

**Cumulative total charge in 40Hz (normalized)**

- **Week 1 (R<sub>ser</sub> compensation 0%)**
  - Tom<sup>1</sup> WT/WT: 2.0 ± 0.03 (13)
  - Tom<sup>1</sup> KO/KO: 2.0 ± 0.03 (13)

**RBP: Y-axis intercept from 40Hz back-extrapolation (pC)**

- **Week 1 (R<sub>ser</sub> compensation 0%)**
  - Tom<sup>1</sup> WT/WT: 2.0 ± 0.03 (13)
  - Tom<sup>1</sup> KO/KO: 2.0 ± 0.03 (13)

**RBP: Y-axis intercept from 40Hz back-extrapolation (normalized)**

- **Week 1 (R<sub>ser</sub> compensation 0%)**
  - Tom<sup>1</sup> WT/WT: 2.0 ± 0.03 (13)
  - Tom<sup>1</sup> KO/KO: 2.0 ± 0.03 (13)

**Replenishment: Slope from 40Hz back-extrapolation (pC/s)**

- **Week 1 (R<sub>ser</sub> compensation 0%)**
  - Tom<sup>1</sup> WT/WT: 2.0 ± 0.03 (13)
  - Tom<sup>1</sup> KO/KO: 2.0 ± 0.03 (13)

**Replenishment: Slope from 40Hz back-extrapolation (normalized)**

- **Week 1 (R<sub>ser</sub> compensation 0%)**
  - Tom<sup>1</sup> WT/WT: 2.0 ± 0.03 (13)
  - Tom<sup>1</sup> KO/KO: 2.0 ± 0.03 (13)
DISCUSSION

Tom1\textsuperscript{KO/\textsubscript{KO}} mice were used to evaluate the function of tomosyn-1 in neuronal development and synaptic function. We found that, in line with previous observations (Sakisaka et al., 2008), Tom1\textsuperscript{WT/\textsubscript{KO}} interbreedings gave rise to a severely skewed Mendelian genetic distribution. Hippocampal neuron cultures of surviving E18 Tom1\textsuperscript{KO/\textsubscript{KO}} mice developed normally as assessed by neurite length, dendritic complexity and synapse number. However, (synaptic) Munc18 levels were decreased. A concomitant functional deficit in synaptic plasticity was observed, affecting short-term paired pulse ratio as well as vesicle replenishment during prolonged stimulation. Together this indicates that tomosyn-1 is involved in Munc18 protein stability and synaptic recovery of vesicular neurotransmitter release.

Tomosyn-1 is important for early development

Given the low incidence of Tom1\textsuperscript{KO/\textsubscript{KO}} animals in Tom1\textsuperscript{WT/\textsubscript{KO}} interbreedings at embryonic stage E18, while postnatal life expectancy appeared normal, tomosyn-1 contributes importantly to early development. This is consistent with tomosyn-1 mRNA expression, which, compared to tomosyn-2, is already high in early stages and stable during development. An early developmental mechanism requiring tomosyn-1 may be found in oocyte polarity and could be similar to regulation of asymmetric cell division in \textit{Drosophila} neuroblasts by the tomosyn homolog Lgl (Albertson and Doe, 2003). The yeast tomosyn homolog Sro7p is a secretory regulator (Lehman et al., 1999) that interacts directly with the Par-1 kinase homolog Kin2 (Elbert et al., 2005). Par-1 is a known oocyte polarity gene in \textit{C. elegans}, \textit{Drosophila} and mice (Guo and Kemphues, 1995; Shulman et al., 2000; Moore and Zernicka-Goetz, 2005). Interestingly, polarized localization of a \textit{Drosophila} Par-1 target, oskar mRNA (Shulman et al., 2000), requires the Munc18 homolog Rop (Ruden et al., 2000). Thus, concerted actions of tomosyn-1 and Munc18 proteins might regulate early oocyte development. It would be interesting also to elucidate what exactly makes surviving individuals resistant to tomosyn-1 deficiency in early development. They must have developed a coping mechanism, for instance by compensatory expression of the tomosyn-2 isoform.

Tomosyn-1 is not essential for neurite outgrowth

Based on previous studies, the role of tomosyn in neurite outgrowth remains controversial (Kraut et al., 2001; Sakisaka et al., 2004; Gracheva et al., 2006; McEwen et al., 2006; Chen et al., 2011). In our Tom1\textsuperscript{KO/\textsubscript{KO}} study we found no evidence for involvement of tomosyn in neuronal development. Possibly, a phenotype was masked by tomosyn-2 redundancy. Likewise, remaining tomosyn protein after incomplete knockdown could be sufficient for normal neuronal development (Chen
et al., 2011). In *C. elegans* studies, tomosyn mutants lack expression of full-length TOM-1A/C isoforms, but the short TOM-1B isoform is predicted to be expressed (Gracheva et al., 2006; McEwen et al., 2006), which could function to control neurite outgrowth. To verify whether tomosyn-2 expression in Tom1KO/KO neurons is enough to preserve neurite outgrowth, it will be informative to repeat these experiments in double knockout neurons.

**Possible mechanisms of tomosyn-1-dependent Munc18 regulation**

Munc18 and tomosyn have opposite effects on synaptic release and compete for membrane-bound syntaxin (Fujita et al., 1998; Gladycheva et al., 2007; Gracheva et al., 2010). Our data showed decreased (synaptic) Munc18 protein levels in Tom1KO/KO neurons. A similar trend of about 20% overall reduction in Munc18 levels is observed in tom-1 *C. elegans* mutants, in which the membrane-bound fraction of UNC-18 is increased by lack of competition with tomosyn (Gracheva et al., 2010). Tomosyn-mediated regulation of Munc18 protein levels may occur at the level of transcription, translation or Munc18 protein stability/degradation. The lack of tomosyn-1 could also affect the balance between different states of the protein: as syntaxin/Munc18 dimers, SNARE complex-bound and cytosolic Munc18. Possibly, Munc18 stability is affected by these changes. We speculate that the absence of tomosyn-1 may lead to the accumulation of non-productive syntaxin/Munc18 complexes, associated with an increased degradation. In line with previous data (Gracheva et al., 2010), tomosyn-1 deficiency did not affect syntaxin levels. Thus, tomosyn appears to regulate Munc18 levels specifically.

**Tomosyn-1-regulated Munc18 levels might affect presynaptic plasticity**

Previous literature clearly points towards a role of tomosyn in the regulation of RRP size. However, we observed a normal evoked release amplitude and RRP size in autaptic hippocampal Tom1KO/KO neurons. Possibly, tomosyn-2 redundancy masks a role for tomosyn-1 in synaptic release in this model system. Upon repetitive stimulation however, tomosyn-1 deficiency becomes rate-limiting. This was observed in paired pulse ratio, but also on a longer timescale during a 40 Hz rundown protocol and recovery from this. Interestingly, a similar phenotype has been observed in Munc18-1 heterozygous autaptic hippocampal neurons, suggesting that Munc18 levels become limiting during sustained release. Also in these neurons, initial evoked release is normal, while increased synaptic depression in Munc18-1 heterozygous neurons is evident during a 10 Hz rundown protocol. This is attributed to a decreased hypertonic sucrose estimated RRP size. Recovery from a 40 Hz stimulus train and spontaneous release frequency are not affected in Munc18-1 heterozygous neurons, but increase upon Munc18 overexpression
(Toonen et al., 2006). Interestingly, tomosyn depletion leads to reduced Munc18 levels, but, unlike Munc18 depletion by heterozygosity, does not seem to affect RRP size. Possibly, tomosyn deletion additionally enhances RRP size, as is suggested before (Gracheva et al., 2006; McEwen et al., 2006) in a Munc18 independent manner, thereby cancelling out a reduction in RRP size by lower levels of Munc18. Thus, the increased synaptic depression observed in Tom1\textsuperscript{KO/KO} neurons may be mediated mainly by decreased Munc18 levels.

**Activity-induced phosphorylation of Munc18 and tomosyn could be involved in RRP replenishment**

Munc18-dependent sustained release is mediated by activity-dependent phosphorylation of Munc18 via the PKC pathway (Sassa et al., 1996; Wierda et al., 2007), reducing syntaxin binding (Fujita et al., 1996; de Vries et al., 2000; Barclay et al., 2003). Hereby, progression of SNARE complex formation by Munc13 may be facilitated. In addition, cAMP-dependent synaptic enhancement by forskolin is decreased by tomosyn knockdown in Drosophila NMJ (Chen et al., 2011). This is regulated by PKA-induced phosphorylation of tomosyn, resulting in decreased syntaxin binding (Baba et al., 2005). Possibly, this facilitates tomosyn replacement by synaptobrevin. Also, reduced competition for syntaxin binding by PKA-induced tomosyn phosphorylation could increase the number of Munc18/closed-syntaxin complexes and enhance vesicular tethering (Gracheva et al., 2010). Although the RhoA/ROCK pathway has mainly been implicated in structural plasticity, phosphorylation of syntaxin by RhoA/ROCK can further shift the balance between Munc18 and tomosyn binding (Sakisaka et al., 2004). Various activity-dependent signaling pathways involving concerted actions of syntaxin-bound Munc18 and tomosyn are thus likely to act synergistically to inhibit neurotransmitter secretion during rest, and uphold synaptic strength during episodes of high activity.

All in all, we conclude that tomosyn-1 regulates Munc18 levels and is important for sustained release upon prolonged demand. The activity-dependent competitive state of concerted Munc18 and tomosyn-1 binding to syntaxin could regulate presynaptic plasticity by controlling SNARE complex availability for sustained release.