Synaptobrevin 1 mediates vesicle priming and evoked release in a subpopulation of hippocampal neurons.

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The core machinery of synaptic vesicle fusion consists of three SNARE proteins, the two t-SNAREs at the plasma membrane (SNAP-25, Syntaxin 1), and the vesicle bound v-SNARE, synaptobrevin 2 (VAMP2). Formation of the trans-oriented four-α-helix bundle between these SNAREs brings vesicle and plasma membrane in close proximity and prepares the vesicle for fusion. The t-SNAREs are thought to be necessary for vesicle fusion. Whether the v-SNAREs are required for fusion is still unclear, as substantial vesicle priming and spontaneous release activity remains in mammalian mass cultured synaptobrevin/cellubrevin-deficient neurons. Using the autaptic culture system from synaptobrevin 2 knockout neurons of mouse hippocampus, we found that the majority of cells were devoid of any evoked or spontaneous release and had no measurable readily-releasable pool. A small subpopulation of neurons, however, displayed release, and their release activity correlated with the presence and amount of v-SNARE synaptobrevin 1 expressed. Comparison of synaptobrevin 1 and 2 in rescue experiments demonstrate that synaptobrevin 1 can substitute for the other v-SNARE, but with a lower efficiency in neurotransmitter release probability. Release activity in synaptobrevin 2-deficient mass cultured neurons was massively reduced by a knockdown of synaptobrevin 1, demonstrating that synaptobrevin 1 is responsible for the remaining release activity. These data support the hypothesis that both t-and v-SNAREs are absolutely required for vesicle priming and evoked release and that differential expression of SNARE paralogs can contribute to differential synaptic coding in the brain.
Keywords: SNARE; neurotransmitter release; spontaneous release; release probability; short-term plasticity

Introduction

Release of neurotransmitters in the mammalian brain is mediated by fusion of synaptic vesicles (SVs) with the neuronal plasma membrane. This process has to be both fast and reliable and is performed by evolutionarily conserved protein machinery (Rizo and Rosenmund 2008; Sudhof 2013). Three proteins from the soluble N-ethylmaleimide-sensitive-factor attachment receptor (SNARE) family are considered to bring the two membranes in close proximity and carry out membrane fusion (Sollner et al. 1993; Weber et al. 1998). A v-SNARE (anchored in the SV membrane) and the t-SNAREs syntaxin (Bennett et al. 1992) and synaptosomal-associated protein 25 (SNAP-25) (Oyler et al. 1989) (both anchored in the plasma membrane) zipper up from their N- to C-terminus and thus form a four-α-helix bundle called the trans-SNARE complex (Poirier et al. 1998; Sorensen et al. 2006; Sutton et al. 1998). Whether fusion of SVs is possible without SNARE proteins is still an open question. The major v-SNARE paralog on SVs in the mammalian brain is synaptobrevin 2 (syb2), also known as vesicle-associated membrane protein 2 (VAMP2) (Elferink et al. 1989). Loss of the syb2 homolog in drosophila abolishes evoked release entirely and reduces spontaneous release significantly (Deitcher et al. 1998). Elimination of the two v-SNARE isoforms present in C. elegans leads to severe defects in synaptic transmission (Nonet et al. 1998). In mouse the syb2 knockout has been studied in high-density cultures of hippocampal neurons (Schoch et al. 2001). Even though Ca$^{2+}$-mediated evoked release is virtually absent without syb2, the pool of readily-
releasable vesicles (RRP) is only reduced to around 10% of wildtype levels and spontaneous fusion of SVs can still be observed. The additional depletion of cellubrevin (VAMP3) in these cells revealed no aggravated phenotype, which speaks against a compensatory effect of this v-SNARE in the syb2 KO background (Deak et al. 2006). In this study we addressed the question of how syb2-deficient hippocampal neurons can still promote fusion. We used the autaptic culture system (Bekkers and Stevens 1991) to study release from individual cells rather than sampling input from several neurons as done in mass culture. We characterize synaptobrevin 1 (syb1, VAMP1) as a v-SNARE in synaptic vesicle exocytosis by a molecular rescue approach and found that this paralog of syb2 is expressed in a subset of hippocampal neurons in our syb2 KO culture. We could correlate the size of the RRP of individual neurons to their syb1 expression levels, and showed that knocking down syb1 dramatically decreases the RRP of mass culture syb2 KO neurons.

Materials and Methods:

Neuronal culture

All animals were treated according to protocols by our institution and German animal law. 18.5-day-old male and female mouse embryos (E18.5) were delivered by C-section. Hippocampal neurons from syb2 KOs (Schoch et al. 2001) and wildtype littermates were dissected out and plated on astrocyte feeder islands as described previously (Xue et al. 2007). Electrophysiological recordings were performed after 9-16 days in vitro (DIV).
**Lentiviral constructs and virus production**

Sequences of murine syb1 and -2, respectively, were cloned into a lentiviral shuttle vector under the control of a human synapsin-1 promoter. To enable identification of infected cells the expression cassette of synaptobrevin was fused to a nuclear localization sequence-tagged red fluorescent protein (NLS-RFP) via a self-cleaving P2A peptide (Kim et al. 2011). For syb1 KD a murine syb-1 specific siRNA target sequence (5’ – CAG GCG GTT ACA GCA GAC C-3’) was obtained using Genscript siRNA Target Finder (https://www.genscript.com/ssl-bin/app/rnai) and cloned as shRNA into a lentiviral shuttle vector under the control of a U6 promoter. To identify infected neurons, the shuttle vector contained a human synapsin-1 promoter, which drives the expression of a nuclear-targeted red fluorescent protein (NLS-RFP). For knockdown experiments neurons were either transduced with the syb1 shRNA construct or with a scrambled control.

Lentiviral particles were prepared as described in Lois et al. (2002). For transduction, about 1x10^6 infectious particles per 35 mm-diameter well were pipetted onto 1 DIV hippocampal neurons.

**Electrophysiology**

Whole-cell patch clamp recordings were performed in a standard extracellular solution containing the following (in mM): 140 NaCl, 2.4 KCl, 10 HEPES (Merck, Darmstadt, Germany), 10 glucose (Carl Roth, Karlsruhe, Germany), 2 CaCl₂, (Sigma-Aldrich, St. Louis, USA), 4 MgCl₂ (Carl Roth, Karlsruhe, Germany); 300 mOsm; pH 7.4. To block glutamatergic or GABAergic responses 10 µM 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[j]quinoxaline-7-sulfonamide (NBQX) (Tocris Biosci-
ence, Bristol, UK) and 30 µM bicuculline (Tocris Bioscience, Bristol, UK), respectively, were added to the extracellular solution. In mass culture experiments voltage gated sodium channels were blocked by adding 0.5 µM tetrodotoxin (TTX) (Tocris Bioscience, Bristol, UK). To assess the size of the readily-releasable pool the extracellular solution was made hypertonic by adding 500 mM sucrose (Sigma-Aldrich, St. Louis, USA) (described in Rosenmund and Stevens (1996)). Internal solution contained the following (in mM): 136 KCl, 17.8 HEPES, 1 EGTA (Carl Roth, Karlsruhe, Germany), 4.6 MgCl₂, 4 Na₂ATP, 0.3 Na₂GTP (Sigma-Aldrich, St. Louis, USA), 12 creatine phosphate (Calbiochem, Darmstadt, Germany), and 50 U/ml phosphocreatine kinase (Sigma-Aldrich, St. Louis, USA); 300 mOsm; pH 7.4. In some experiments, 5 mM fixable Cascade Blue (Life Technologies, Darmstadt, Germany) was added to the internal solution. Borosilicate glass pipettes (Science Products, Hofheim, Germany) had a resistance of 2 - 3.5 MΩ. All recordings were performed with a Multiclamp 700B amplifier under control of Clampex 10.0 (both Molecular Devices, Sunnyvale, USA). Data was acquired at 10 kHz and filtered at 3 kHz. Excitatory (EPSC) and inhibitory (IPSC) postsynaptic currents were recorded after a 2 ms depolarization from -70 mV to 0 mV. The vesicular release probability was calculated by dividing the charge of the EPSC by the charge of the RRP. The 10 Hz protocol consisted of 50 depolarization stimuli within 5 s and the responses were normalized to the first EPSC. For background noise subtraction, miniature postsynaptic currents were recorded in presence and absence of 10 µM NBQX and 30 µM bicuculline, respectively. Analysis was performed after filtering data at 1 kHz and then running a tem-
plate-based detection algorithm implemented in AxoGraph X 1.5.4 (AxoGraph, Berkeley, USA).

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich, St. Louis, USA) for 10 minutes after 14 DIV or directly after patch-clamp recordings. Primary antibodies were purchased from Synaptic Systems (Göttingen, Germany) (anti-syb2, catalogue number 104211, anti-synaptophysin 1, catalogue number 101004) and abcam (Cambridge, UK) (anti-syb1, catalogue number ab3346). Cells were incubated with primary antibodies at 4° C over night. Fluorescently labeled secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, USA). Images were acquired on an Olympus (Tokyo, Japan) IX81 epifluorescent microscope with a UPLSAPO 60x water immersion objective and MetaMorph software (Molecular Devices, Sunnyvale, USA). To correlate electrophysiological data with immunolabeling we recorded only one cell per coverslip and filled it with fixable Cascade Blue dye. Thus, recorded cells could be easily identified by fluorescence in the 405 nm channel. Ratiometric imaging was performed with an imageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, USA) macro by choosing synaptophysin 1 positive synapses and dividing the fluorescence intensities of syb1 by those of synaptophysin 1.

Western blot

Hippocampal neurons were plated at 30000 cm⁻² on astrocyte-free coverslips and lysed after 14 DIV using 50 mM Tris/HCl (Merck, Darmstadt, Germany), pH 7.9, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate,
250 µM phenylmethylsulfonyl fluoride (Sigma-Aldrich, St. Louis, USA), 1%

Nonidet P-40, and a tablet of Complete Protease Inhibitor (Roche Diagnostics, Berlin, Germany). After separation by SDS-PAGE proteins were transferred to nitrocellulose membranes and incubated with primary and secondary antibodies (primary antibodies: anti-syb1 and anti-syb2 see above, anti-tubulin III (catalogue number T8660) from Sigma-Aldrich (St. Louis, USA); secondary antibodies were horseradish peroxidase-conjugated (Jackson ImmunoResearch West Grove, USA). Secondary antibodies were detected with ECL Plus Western Blotting Detection Reagents (GE Healthcare Biosciences, Freiburg, Germany) in a Vilber (Eberhardzell, Germany) Lourmat Fusion FX7 detection system.

Statistics

Bar graphs show mean ± standard error of the mean (SEM). Two-tailed Student's t-test (two groups) or two-way ANOVA (more than two groups) followed by Tukey's multiple comparisons test as well as correlation analysis with Pearson's correlation coefficient was performed using GraphPad Prism version 6.0d for Mac, GraphPad Software, La Jolla California USA, www.graphpad.com. The null hypothesis was rejected if p≤0.05. For correlation analysis the syb1 overexpression data were excluded, because they represent averages over all recorded cells.
Results

A subpopulation of neurons exhibits release in syb2 KO autaptic culture.

To study the nature of release activity in the absence of syb2 we grew murine hippocampal neurons from syb2 KO (Schoch et al. 2001) and wildtype (WT) littermates on glial microislands and performed whole-cell patch clamp experiments on autaptic neurons. Consistent with previous findings (Guzman et al. 2010) excitatory evoked release was virtually absent in the KO group (Figure 1).

While the WT control group showed robust evoked glutamatergic current responses (3.4 ± 0.5 nA (SEM)) in 54 out of 55 neurons, we detected a single cell out of 59 KO neurons that displayed a quantifiable evoked response (0.64 nA) (Fig. 1A,B). We next measured the readily-releasable vesicle pool (RRP) using pulsed application of hypertonic solution (Rosenmund and Stevens 1996). Vesicles that are fusion competent are forced to fuse within 3-4 seconds after the neuron is exposed to external solution containing additional 500 mM sucrose. Surprisingly, we found that 47 out of 54 cells lacking syb2 exhibited no measurable RRP, and again all but one WT cell had robust responses (Fig. 1C,D). The pool size of those 7 KO cells responding to sucrose was 56 ± 35 pC on average compared to 366 ± 50 pC in WT (n=55). Only one KO neuron had a WT-sized pool (see “Cell 1” in Fig. 2A). The lack of readily-releasable vesicles in most syb2 KO neurons was also evident when we examined spontaneous fusion events (“minis”) (Fig. 1E). Only 3 out of 47 cells in the KO showed detectable miniature excitatory postsynaptic currents (mEPSCs) compared to 46 out of 48 in WT (Fig. 1F). The average amplitude of detected minis in KO and WT was 12.5 ± 1.7 pA.
and 24.7 ± 1.0 pA, respectively (Fig. 1G). It should be mentioned that we also
found two inhibitory neurons in our syb2 KO culture with considerable evoked
current responses (6.8 and 0.9 nA) and pool sizes (3.4 and 1.8 nC). Due to low
numbers of GABAergic neurons in our culture we did not perform any further
statistical analysis on those cells.

Pool size in syb2 KO neurons correlates to syb1 expression levels.

An earlier study in syb2 KO high-density culture (Schoch et al. 2001) reported an
overall approximately 90% decrease in RRP size and mEPSC frequency in excita-
tory neurons. However, in the experimental conditions of high-density neuronal
culture, postsynaptic neurons sample input from several presynaptic neurons. In
syb2 KO autaptic culture, where each neuron receives input only from itself, we
found that most of the cells are incapable of releasing any neurotransmitter and
only a small subpopulation showed release (Figure 1). This result may be ex-
plained by the presence of another paralog of syb2 in the neurotransmitter-
releasing subpopulation, such as synaptobrevin 1 (syb1). While the initial study
had no evidence for syb1 expression (Schoch et al., 2001), we reevaluated the
putative role of this paralog by correlating syb1 expression with the ability of
individual cells to be release competent. To examine whether syb1 may be re-
sponsible for vesicle priming in the absence of syb2, we correlated RRP size with
syb1 protein levels. Individual syb2 KO neurons were recorded and labeled with
a fixable fluorescent dye present in the patch pipette solution. After recording,
the neurons were fixed and stained with a syb1 antibody (Fig. 2A). We identified
the recorded cells and plotted the pool size against the levels of syb1 protein
This analysis exhibited a strong correlation between syb1 levels and RRP size ($r=0.91$) (Fig. 2B), indicating that syb1 may be responsible for remaining release in syb2 KO neurons.

**Release properties differ between syb1 and syb2.**

To provide evidence that syb1 expression is capable of mediating vesicle priming, we performed syb1 rescue experiments in syb2-deficient neurons. We expressed syb1 in syb2 KO autaptic neurons using a lentiviral expression system (Lois et al. 2002). We compared the electrophysiological parameters to WT as well as syb2 KO neurons where we expressed syb2 with the same shuttle vector as syb1. Both syb paralogs robustly rescued the deficiency in evoking synaptic responses (Figure 3). We noted however that the EPSC amplitude size for syb1 rescues was significantly reduced ($2.2 \pm 0.3$ nA) compared to the syb2 rescues ($3.6 \pm 0.6$ nA) (Fig. 3A,B). The reduction in EPSC size was not due to a smaller RRP as determined by pulsed sucrose application (Fig. 3C,D). Instead, we observed a decreased vesicular release probability in the syb1 rescue compared to syb2 and WT (Fig. 3E). This was confirmed by two additional experiments. First, comparative analysis of response amplitudes during repetitive 10 Hz stimulation experiments showed that syb1-rescued neurons displayed less depression than the syb2 rescue group (Fig. 3F), a hallmark for reduced release probability (Zucker and Regehr 2002). Second, the frequency of spontaneous release was significantly reduced in the syb1 rescue compared to syb2 rescue and WT (Fig. 3G,H), indicating a higher energy barrier for fusion competent vesicles. mEPSC
amplitudes were unaltered (syb1 rescue: 20.5 ± 1.5 pA (n=43); syb2 rescue: 21.0 ± 1.0 pA (n=56); WT: 21.8 ± 1.3 pA (n=28)). While it is possible that the change in release probability is caused by a change in relative expression levels of the two synaptobrevin paralogs, we consider it unlikely as both constructs rescue vesicle priming equally well. Furthermore, synaptic function in our system appears relatively insensitive to changes in expression levels, as syb2 heterozygous (het) deficient neurons do not display significant changes in vesicle release, priming or release probability (EPSC amplitudes: WT 6.4 ± 1.2 nA, het 6.1 ± 1.1 nA; RRP size: WT 1.0 ± 0.2 nC, het 0.9 ± 0.2 nC; Pvr: WT 6.0 ± 0.7%, het 5.6 ± 0.6%; n=22 each from 2 cultures). Taken together, the rescue experiments demonstrate that syb1 can substitute for loss of syb2 in terms of maintenance of neurotransmitter release in central synapses. However, syb1 promotes evoked and spontaneous release less efficiently compared to syb2.

Syb1 promotes release in syb2 KO mass culture.

To test if syb1 is responsible for the remaining release observed in syb2 KO mass cultures (Schoch et al. 2001), we genetically manipulated syb1 expression in syb2 KO cultures using shRNA knockdown (KD). Western blot analysis showed that syb1 is indeed expressed in mass cultured hippocampal neurons, and that the shRNA successfully suppresses the expression of syb1 (Fig. 4A). We subsequently assessed the size of the input RRP in three groups: WT, syb2 KO and syb2 KO+syb1 KD neurons. Since in mass cultures the input to the recorded cell can be both glutamatergic and GABAergic we added either bicuculline or NBQX to our bath solutions to determine the impact of this manipulation on both neu-
rotransmitter systems. We confirmed the reduction of RRP size in syb2 KO compared to WTs (Figure 4B,C). The syb2 KO+syb1 KD group showed an additional reduction of pool size to 0.7±0.2 % of WT levels in the presence of bicuculline and 0.3±0.1% of WT levels in NBQX (Fig. 4C). These findings indicate that syb1 is an important v-SNARE for rendering vesicles fusion competent in the syb2 KO.

Surprisingly, we detected only a slight further reduction in the frequency of mEPSCs and no change in mIPSC frequency in the syb1 knockdown (Fig. 4D).

Discussion

SNAREs are considered to be essential components of the synaptic vesicle fusion process. Yet whether v-SNAREs are absolutely required for vesicle fusion is currently unclear. In this study we used the advantages of the autaptic cell culture system to examine the loss of syb2, the major v-SNARE in hippocampal neurons. Our major findings are: 1. The majority of syb2-deficient neurons, when grown on isolated microislands, are devoid of any Ca2+-mediated evoked or spontaneous release and have no measurable readily-releasable pool (Figure 1). In a small subpopulation of neurons, however, we detected the expression of the v-SNARE paralog syb1 and found a correlation between syb1 protein levels and remaining RRP size (Figure 2). This supports the hypothesis that v-SNAREs are absolutely required for vesicle fusion. 2. In rescue experiments with syb1 and -2 we observed, that syb1 is less efficient in release, which is reflected in reduced EPSC amplitudes and a decreased release probability and mEPSC frequency (Figure 3). We interpret the remaining responses in mass cultured syb2 KO neurons (Deak et al. 2004; Deak et al. 2006; Schoch et al. 2001) to be caused by a subpopulation
of neurons that express syb1 (Figure 4). Our results clearly argue that v-SNAREs are mandatory for evoked release and the fusion competence of synaptic vesicles.

To our knowledge, syb1 has not yet been characterized in the fusion of synaptic vesicles at central synapses. Whereas syb2 is the dominant v-SNARE with a high release probability, syb1 could promote fusion in cells that depend on lower release probability and less efficient release. The usage of different v-SNARE paralogs on synaptic vesicles might thus serve as a vesicle-intrinsic determinant of release probability. Deak et al. (2004) also examined syb2 KO neurons in mass culture and found reduced release probability in those cells compared to wild type neurons. Even though the authors did not attribute these findings to residual syb1 levels at that time it fits very well with our results that syb1 possesses an intrinsically lower release probability compared to syb2. Carrying out styryl dye and HRP uptake experiments Deak et al. (2004) studied exo- and endocytosis in syb2 KO neurons. Interestingly, the authors did not report any obvious variance between individual synapses, as would be expected if syb1 mediates release in a subpopulation of cells. The discrepancy between these uptake studies and our electrophysiological analyses remains to be studied.

The mouse neuromuscular junction (NMJ) has already been shown to depend on syb1 in Ca\(^{2+}\)-triggered exocytosis (Liu et al. 2011). The authors found a reduction in spontaneous and evoked neuro transmitter release as well as lower initial vesicular release probability in syb1 KO NMJs compared to WT. In contrast to our findings, they did not find a decrease in pool size when syb1 levels are reduced. Instead, they report a reduced sensitivity and cooperativity to calcium in the KO neurons. However, due to the relatively high expression of syb2 in the NMJ it is
rather difficult to directly compare the two studies, as the number of syb pro-
teins present is supposedly very different in the two systems. We find it remark-
able though, that the mouse neuromuscular junction, consisting of thousands of
unreliable single vesicle release sites (Meriney and Dittrich 2013), depends at
least partially on syb1 – a v-SNARE that we show to mediate release with lower
release probability.
Interestingly, rescue studies with cellubrevin (VAMP3), also closely related to
syb2, reported a complete compensation of the loss of syb2 in neurons (Deak et
al. 2006) and chromaffin cells (Borisovska et al. 2005). The sequence homology
between syb1 and -2 is 76% with the largest differences in the N-terminus. How-
ever, cellubrevin, which can completely compensate the loss of syb2, does not
possess a sizable N-terminus beyond the SNARE motif and shares only 74% se-
quence homology with syb2. So it remains to be studied which part of the se-
quence is responsible for the reduced release efficiency of syb1.
Our findings that syb1 has a significantly lower release probability compared to
syb2 might explain why the majority of syb2 KO neurons in our autaptic culture
that still had a measurable RRP did not show evoked responses. Studies from
syntaxin hypomorphs have shown that a reduction in the expression of the t-
SNARE syntaxin 1a/1b leads to drastic reductions in both RRP size as well as
release probability (Arancillo et al. 2013). Combining this with the intrinsically
lower release probability of syb1 would even further reduce the likelihood of
Ca²⁺-evoked fusion, causing it to drop below detection levels.
Whereas RRP size in the syb2 KO can be correlated to syb1 expression, the
knockdown of syb1 did not affect miniature events to the same extent. There are
two different possible explanations: The mini frequency in syb2 KO mass culture
is already quite low (0.6 to 1Hz (compare also (Deak et al. 2006; Schoch et al. 2001)). It is conceivable that spontaneous fusion at this low rate can still be maintained with the reduced RRP that we saw in the syb1 knockdown. Alternatively, spontaneously fusing vesicles may originate from a different pool than the RRP (Ramirez et al. 2012; Sara et al. 2005). In the scenario when syb2 is absent, syb1 drives fusion of the RRP whereas an additional v-SNARE may exclusively drive spontaneous fusion. A possible candidate could be the non-canonical SNARE Vps10p-tail-interactor-1a (vti1a), which has been shown to promote spontaneous release in the absence of syb2 (Ramirez et al. 2012). Similarly, in *drosophila* Ca$^{2+}$-evoked release is thought to be driven by the v-SNARE n-syb, whereas spontaneous release persists even in the absence of n-syb (Deitcher et al. 1998; Yoshihara et al. 1999). Studying a complete genetic deletion of syb1 and -2 could help to differentiate between the two explanations. Taken together, our results support the hypothesis that v-SNAREs are absolutely required for evoked release and vesicle priming at central synapses. Furthermore, differential expression of SNARE paralogs can contribute to making vesicle release probability and short-term plasticity characteristics more diverse among different synapses.
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Disclosures:

The authors declare no conflicts of interest, financial or otherwise.
References


Figure 1. A small subpopulation of neurons exhibits release in syb2 KO autaptic culture. A, Example traces of evoked responses of a WT and two syb2 KO autaptic neurons after 2 ms depolarization. Stimulations are indicated by an open square; artifacts and action potentials are blanked. B, Plot of average EPSC amplitudes in WT and syb2 KO autaptic neurons. Grey squares represent individual KO neurons. Note that we detected only a single evoked glutamatergic response in the KO cultures (lower trace in Fig 1A). C, Example traces of a WT and two syb2 KO neurons after application of 500 mM sucrose for 5s. The majority of cells (47 out of 54) had no measurable RRP. D, Plot of average RRP size in WT and syb2 KO autaptic neurons. E, Example traces of mEPSCs recorded in a WT and two syb2 KO neurons. Note that only 3 out of 47 neurons showed spontaneous release. F, Plot of mEPSC frequencies and (G) amplitudes. Bar graphs show mean ± SEM; *p<0.05, **p<0.01, ***p<0.001. Cell numbers per number of independent cultures are indicated in graphs.
Figure 2. RRP size is correlated to syb1 expression levels in syb2-deficient neurons. A, RRP traces of two exemplary syb2 KO autaptic neurons that have been filled with Cascade Blue dye during recordings (left) and their corresponding post-hoc immunostainings for syb1 (right). Evoked response of “Cell 1” is shown in Fig 1A. Scale bar 20 µm. B, Plot of RRP size against the intensity of syb1 fluorescence normalized to the fluorescence intensity of synaptic marker synaptophysin 1 (syp1). Cells 1 and 2 shown in (A) are indicated in red and grey, respectively. Mean RRP size and syb1 expression levels of the syb1 rescue (Fig. 3D) are labeled with “syb1 OE” (error bars show SEM). Pearson’s correlation coefficient r=0.91, p<0.001 (data point for syb1 OE was excluded from the correlation analysis (see Materials and Methods section).
Figure 3. Syb1 rescues the loss of syb2 but has different release properties. A, Exemplary EPSC amplitudes of WT and syb2 KO autaptic neurons rescued with either syb1 or -2. Stimulations are indicated by an open square; artifacts and action potentials are blanked. B, Plot of mean EPSC amplitudes. C, 500 mM sucrose application for 5 s and (D) quantification of RRP size. E, Plot of mean vesicular release probability. F: 10 Hz train comparing the short-term plasticity in WT, syb1 and syb2 rescues, normalized to first response. G, Example traces of mEPSCs in WT, syb1 and syb2 rescues. H, Plot of mean mEPSC frequencies in the syb1 rescue compared to WT and syb2 rescue. Bar graphs show mean ± SEM; *p<0.05, **p<0.01, ***p<0.001. Cell numbers per number of independent cultures are indicated in graphs.
Figure 4. Syb1 expression levels determine synaptic responses in absence of syb2 in mass cultures. A, Western blot of protein lysates from WT (left lane) and syb2 KO (center and right lane) hippocampal mass cultures after 14 DIV. Antibodies against syb1 and tubulin. Note that syb1 levels were drastically reduced in the syb1 knockdown (right lane). B, Exemplary responses to sucrose application of WT and syb2 KO mass culture neurons infected with a scrambled shRNA (black) and syb2 KO neurons infected with a shRNA against syb1 (grey) in the presence of 30 µM bicuculline. Note the different scale bars for WT and syb2 KO. C, Plot of glutamatergic and GABAergic RRP size in syb2 KO cultures with and without syb1 knockdown, normalized to WT RRP. D, Plot of mean mPSC frequencies normalized to syb2 KO frequencies. Bar graphs show mean ± SEM; *p<0.05, **p<0.01, ***p<0.001. Cell numbers per number of independent cultures are indicated in graphs.
**A**

WT syb2 KO

![Graph showing EPSC amplitude with 58 cells for WT and 1 cell for syb2 KO.](image)

**B**

EPSC amplitude

![Bar graph showing EPSC amplitude with WT and syb2 KO conditions.](image)

**C**

WT syb2 KO

![Graph showing readily releasable pool with 47 cells for WT and 7 cells for syb2 KO.](image)

**D**

Readily releasable pool

![Bar graph showing readily releasable pool with WT and syb2 KO conditions.](image)

**E**

WT syb2 KO

![Graph showing mEPSC frequency with 44 cells for WT and 3 cells for syb2 KO.](image)

**F**

mEPSC frequency

![Bar graph showing mEPSC frequency with WT and syb2 KO conditions.](image)

**G**

mEPSC amplitude

![Bar graph showing mEPSC amplitude with WT and syb2 KO conditions.](image)
Correlation of syb1 expression to RRP size

A

Cell 1
500 mM sucrose
0.5 nA
1 s

Cell 2
500 mM sucrose

B

Correlation of syb1 expression to RRP size

syb1 OE

Cell 1

syb1 OE

Cell 1

syb1 OE
mEPSCs mIPSCs

0.0 0.5 1.0 norm. to syb2 KO

59/3 39/2

56/3 41/2

mPSC frequency

syb2 KO +syb1 KD

500 mM sucrose

0.5 s

0.5 nA

mass culture

WT

0.2 nA

500 mM sucrose

0.5 s

500 mM sucrose

5% of WT RRP size

syb1 KD -/- +/+ - -
syb2 KO +syb1 KD syb2 KO +syb1 KD

52/3 31/2

31/2 28/2

syb1 KD

52/3 25/2

20/2

Tubulin

Syb1

Readily-releasable pool

% of WT RRP size

glutamatergic GABAergic

syb2 KO

51/3 52/3

syb2 KO +syb1 KD 31/2

0

1

2

3

4

5

mPSC frequency

norm. to syb2 KO

59/3 39/2

56/3 41/2

syb2 KO