Research Articles: Cellular/Molecular

Deconstructing Synaptotagmin-1's Distinct Roles in Synaptic Vesicle Priming and Neurotransmitter Release

<https://doi.org/10.1523/JNEUROSCI.1945-21.2022>

Cite as: J. Neurosci 2022; 10.1523/JNEUROSCI.1945-21.2022

Received: 27 September 2021

Revised: 18 January 2022

Accepted: 23 January 2022

This Early Release article has been peer-reviewed and accepted, but has not been through the composition and copyediting processes. The final version may differ slightly in style or formatting and will contain links to any extended data.

Alerts: Sign up at www.jneurosci.org/alerts to receive customized email alerts when the fully formatted version of this article is published.

Copyright © 2022 Bouazza-Arostegui et al.

This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license, which permits unrestricted use, distribution and reproduction in any medium provided that the original work is properly attributed.

Deconstructing Synaptotagmin-1's Distinct Roles in Synaptic Vesicle Priming and Neurotransmitter Release

Abbreviated title: Re-analysis of Synaptotagmin-1 synaptic functions.

Boris Bouazza-Arostegui^{1,2}, Marcial Camacho^{1,2}, Marisa M. Brockmann^{1,2}, Sina Zobel^{1,2} and Christian Rosenmund^{1,2}

¹Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Institute of Neurophysiology, 10117, Berlin, Germany.

²NeuroCure Cluster of Excellence, 10117, Berlin, Germany.

Corresponding author: Correspondence should be addressed to Christian Rosenmund,
christian.rosenmund@charite.de

Number of pages: 28, Number of figures: 8, Number of Tables: 1, Number of extended data: 1, Number of words
for Abstract: 181, Number of words for introduction: 611, Number of words for discussion: 1487

Conflict of interest: The authors declare no competing financial interest.

Acknowledgements: This work was supported by grants from the German Research Council (CRC 958 and Reinhart Koselleck project). Boris Bouazza-Arostegui was supported by the NeuroCure Cluster of Excellence with a NeuroCure PhD Fellowship. We are grateful to Berit Söhl-Kielczynski, Bettina Brokowski, Katja Pötschke, Sabine Lenz and Heike Lerch for excellent technical support and to Dr. Melissa Herman for her comments and helpful discussion. We thank the services of the Charité Viral core facility for virus production and characterization. We also thank the services of Advanced Medical Bioimaging Core Facility (AMBIO), Nikon imaging core facilities at the Charité Campus Mitte (Berlin, Germany). M.C. current address: Unidad de Farmacología, Facultad de Medicina, Universidad de La Laguna, 38200 La Laguna, Tenerife, Spain. M.M.B. current address: Center of Molecular Neurobiology Hamburg, Hamburg 2051, Germany.

31 Abstract

32 Synaptotagmin-1 (SYT1) is a synaptic vesicle resident protein that interacts via its C2 domain with
33 anionic lipids from the plasma membrane (PM) in a calcium-dependent manner to efficiently trigger
34 rapid neurotransmitter (NT) release. In addition, SYT1 acts as a negative regulator of spontaneous
35 NT-release and regulates synaptic vesicle (SV) priming. How these functions relate to each other
36 mechanistically and what role other synaptotagmin (SYT) isoforms play in supporting and
37 complementing the role of SYT1 is still under intensive investigation. In this work, we analyzed
38 SYT1's three putative functions in exocytosis by systematically varying its expression in autaptic
39 hippocampal glutamatergic neurons from mice of either sex. We find that regulation of release
40 probability is most sensitive to variation of expression levels, while its impact on vesicle priming is
41 least sensitive. Also, loss of SYT1 phenotypes on spontaneous release and vesicle priming is
42 compensated in less mature synaptic cultures by redundant support from SYT7. Overall, our data
43 help resolving some controversies in SYT1 functions in exocytosis and in our understanding of how
44 SYT1 contributes to the pathophysiology underlying SYT1 related human neurologic disorders.

45 Significance statement

46 Our work clarifies the functions of Synaptotagmin-1 (SYT1) protein in synaptic vesicle priming,
47 spontaneous and calcium-evoked neurotransmitter release and analyze whether these occur at
48 different stages of synaptic responses by examining their relative sensitivity to protein concentration
49 at the synaptic terminal. We demonstrate that these synaptic functions are unequally sensitive to both
50 protein levels and neuronal stage, indicating that they operate under distinct molecular mechanisms.
51 Furthermore, we analyze how these functions are modulated by another synaptotagmin isoform
52 expression. We show that to understand the phenotype displayed by SYT1 knockout neurons (*Syt1*^{-/-})
53 is necessary to consider the interplay between SYT1 and SYT7 molecules at the presynaptic terminal.

54 Introduction

55 Calcium evoked neurotransmitter (NT) release from the presynaptic nerve terminal occurs in less than
56 one millisecond following the activation of voltage-gated calcium channels triggered by the arrival of
57 an action potential (AP) (Katz, 1969; Sabatini and Regehr, 1999). A key element in orchestrating the
58 rapid and efficient presynaptic Ca²⁺-evoked NT release is the synaptic vesicular protein

59 Synaptotagmin-1 (SYT1) (DiAntonio and Schwarz, 1994; Fernández-Chacón et al., 2001; Rizo and
 60 Xu, 2015). Biochemical studies have demonstrated that SYT1 is a phospholipid-binding machine that
 61 acts in a calcium-dependent manner via its C2A and C2B cytoplasmic domains to trigger NT release
 62 (Perin et al., 1990; Brose et al., 1992; Sutton et al., 1995; Chapman and Davis, 1998; Fernandez et
 63 al., 2001).

64 In *Drosophila* and mammalian synapses in the absence of *Syt1*, AP-evoked synchronous
 65 release is drastically impaired (Broadie et al., 1994; Geppert et al., 1994; Yoshihara and Littleton,
 66 2002; Nishiki and Augustine, 2004; Maximov and Südhof, 2005; Xue et al., 2008), but
 67 structure/function analysis of the C2B domain reveals that SYT1 is involved in two additional
 68 functions. SYT1 also acts as a “clamping factor” suppressing spontaneous release of synaptic
 69 vesicles (SVs) (DiAntonio and Schwarz, 1994; Littleton et al., 1994; Chicka et al., 2008; Xu et al.,
 70 2009) and different research groups have shown a role for SYT1 in SV docking/priming, mediating
 71 the recruitment of vesicles to the plasma membrane (PM) by the interaction with phosphatidylserine
 72 (PS) and phosphatidylinositol 4, 5-bisphosphate (PIP2) (Reist et al., 1998; Liu et al., 2009; Bacaj et
 73 al., 2015; Chang et al., 2018). However, how these functions of SYT1 mechanistically interact is
 74 currently unclear.

75 In addition to the different roles of SYT1 in the process of neurotransmitter release, other
 76 synaptotagmin isoforms have been implicated in distinct and overlapping roles. For example, the
 77 Synaptotagmin-7 (SYT7) isoform has been claimed to be a high-affinity calcium sensor for
 78 asynchronous release (Bacaj et al., 2013; Chen and Jonas, 2017), a regulator of short term plasticity
 79 (STP) (Wen et al., 2010; Jackman et al., 2016; Chen et al., 2017b; Fujii et al., 2021) and to act
 80 redundantly with SYT1 in the maintenance of the ready releasable pool (RRP) of SVs (Bacaj et al.,
 81 2015). Furthermore, the expression of synaptotagmin isoforms is regulated in a cell type specific
 82 manner. At the calyx of Held synapse and in some GABAergic neurons, the vesicular Synaptotagmin-
 83 2 (SYT2) protein, the closest relative isoform of SYT1 (Geppert et al., 1991; Marquèze et al., 1995),
 84 regulates neurotransmission redundantly with SYT1 (Pang et al., 2006; Chen et al., 2017a). Synaptic
 85 transmission depends on SYT1 in the early postnatal calyx of Held synapses but later switches to
 86 SYT2 (Kochubey et al., 2016), suggesting that dynamic changes in synaptotagmin content at
 87 synapses during development and their redundant functions in priming and release have an impact in
 88 synaptic function. Consistent with this idea, heterozygotic *de novo* mutations in the *syt1* gene

89 detected in patients have been shown to be associated with a neurodevelopmental disorder (Baker et
90 al., 2015, 2018; Bradberry et al., 2020).

91 In this study, we therefore aim to analyze the proposed distinct roles of SYT1 in vesicle
92 priming, spontaneous and evoked release and investigate these functions in context of development,
93 its interplay with SYT7 and the relative contribution of SYT1 expression levels on these functions. We
94 found that the loss of function phenotype of SYT1 depends on the maturation stage of neurons, where
95 immature neurons can compensate the loss of SYT1 with expression of SYT7 in 2 of the 3 SYT1
96 functions. Furthermore, by systematically investigating the three key functions of SYT1 as a function
97 of protein levels we define distinct sensitivity of these processes arguing a distinct role of SYT1 in
98 these different functions.

99 **Materials and Methods**

100 **Animals**

101 In this study, we used embryonic day (E)18-19 *Syt1*^{+/+}, *Syt1*^{+/-} and *Syt1*^{-/-} embryos on C57BL/6
102 background of either sex generated by interbreeding *Syt1* heterozygous mice as described previously
103 (Xue et al., 2008). All procedures and animals used were handled according to the regulations of the
104 Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific
105 purposes and approved by the Berlin state authorities under the license number G-Project 106/20 and
106 the animal welfare committee of Charité – Universitätsmedizin Berlin.

107 **Lentiviral constructs and production**

108 Lentiviruses were generated by the Viral Core Facility of the Charité – Universitätsmedizin Berlin. All
109 lentiviral particles were produced as described in previous work (Lois et al., 2002). Lentiviral *Syt1*
110 construct used for rescue and overexpression experiments was generated from mouse *Syt1* cDNA
111 (NCBI reference sequence: NM_001252341). The cDNA was cloned into a lentiviral vector (FUGW)
112 with a human synapsin1 promoter and following a nuclear localization signal (NLS)-RFP-P2A
113 expression cassette for identification of infected cells. A lentivirus expressing only NLS-RFP-P2A
114 controlled by the human synapsin1 promoter served as control. For shRNA mediated knockdown
115 experiments of SYT1 protein, an shRNA cassette containing a 19bp target sequence of *Syt1*
116 (5'AGTCTTCCTGCTGCCCCGAC-3') was cloned downstream of a U6 promoter that also contained a
117 ubiquitin promoter driven RFP expression cassette (f(U6)shRNA-Syt1.Ubi-RFP.WPRE). For shRNA
118 mediated knockdown of SYT7 a 21bp targeting sequence KD607 from (Bacaj et al., 2013) was used

under the control of U6 promoter expression cassette within a lentivirus that also contained a human synapsin1 promoter to control the nuclear RFP expression cassette (f(U6) shRNA-Syt7.Syn-NLS-RFP.WPRE). A scramble RNA (*scRNA*) sequence served in both shRNA constructs as control (Watanabe et al., 2014). The lentiviral titer for all generated constructs was estimated by quantification of hippocampal neurons in mass culture expressing a fluorescent marker after DIV7.

Autaptic neuronal culture and viral infection

For electrophysiology recordings and immunocytochemistry experiments autaptic glutamatergic hippocampal neurons were prepared as previously reported by (Bekkers and Stevens, 1991). Briefly, hippocampal neurons derived from *Syt1*^{+/+}, *Syt1*^{+/-} and *Syt1*^{-/-} embryonic mice (E18-19) of either sex were used and plated at a density of 300 cells/cm² on 30 mm coverslips containing astrocytic microislands. Astrocytes were obtained from cerebral cortices of postnatal day (P) 0-2 C57BL/6N mice and plated at a density of 5000 cells/cm² on the micropattern coverslips 1 week before of the preparation of the neurons. Autaptic neuronal cultures were infected with the appropriate viral construct 24–48 h after plating and maintaining at 37 °C and 5 % CO₂.

Electrophysiology

Whole-cell patch-clamp recordings in autaptic neurons were performed at 11-21 days *in vitro* (DIV). All electrophysiological recordings were done at room temperature with a Multiclamp 700B amplifier (Molecular Devices) controlled by Clampex 10 software (Molecular Devices). Data were digitally sampled at 10 kHz and were filtered using a low-pass Bessel filter at 3 kHz. Series resistance was compensated up to 70%. Autaptic cultures during recordings were immersed in an extracellular solution that contained the following (in mM): 140 NaCl, 2.4 KCl, 10 HEPES, 10 glucose, 2 CaCl₂ and 4 MgCl₂. Borosilicate glass patch pipettes were pulled using a multistep puller (P-1000, Sutter Instruments). Pipettes with resistance (3–5 MOhms) were filled with KCl-based intracellular solution containing (in mM): 136 KCl, 17.8 HEPES, 1 EGTA, 4.6 MgCl₂, 4 ATP-Na₂, 0.3 GTP-Na₂, 12 creatine phosphate and 50 U/ml phosphocreatine kinase. Both internal and extracellular solutions were adjusted to 300 mOsm and pH 7.4. Neurons were clamp at -70 mV during recordings. Exclusion criteria were established for patched cells with a leak current higher than -200 pA. Single APs were evoked by a 2 ms depolarization step to 0 mV and excitatory postsynaptic currents (EPSCs) were recorded. To measure the synchronicity of synaptic responses we inverted the EPSC charge and integrate the signal. Then, we fitted a two components function provided by Axograph X (Axograph

Scientific), from which we calculated their relative contribution to the total charge released. The first component represents the fast and synchronous release part of the EPSC charge and the second component the slow and asynchronous release part of the EPSC charge.

The readily releasable pool (RRP) of synaptic vesicles was assessed by the application for 5 s of external solution containing 500 mM sucrose (Rosenmund and Stevens, 1996). Evoked-sucrose currents were recorded and the RRP size were estimated by integrating the area of the evoked-sucrose current with the steady-state current set as the baseline. Vesicular release probability (Pvr) was calculated as the ratio between the charge of the EPSC and the evoked-sucrose charge.

We calculated the paired-pulsed ratio (PPR) by dividing the second EPSC amplitude (EPSC2) by the first (EPSC1). A train of AP stimulation at 20 Hz for 5 s was recorded to assess short term plasticity. The synaptic responses from the train of AP were normalized to the first EPSC peak amplitude.

To analyze mini excitatory postsynaptic currents (mEPSC), electrophysiological traces were filtered at 1 kHz and the range of parameters for inclusion of selected events using a conventionally defined template algorithm in Axograph X (Axograph Scientific) were 5-200 pA, 0.15-1.5 ms rise-time and 0.5-5 ms half-width. False-positive events were excluded by subtracting events detected from traces in the presence of AMPA receptor antagonist, NBQX (3 μ M). Spontaneous release rate was calculated by dividing the mEPSC frequency by the number of synaptic vesicles in the RRP. The number of synaptic vesicles in the RRP was calculated by dividing the RRP size by the mEPSC charge. Off-line analysis was performed using Axograph X (Axograph Scientific).

Immunocytochemistry and image acquisition

Autaptic hippocampal neurons were immunostained as previously reported by (Xue et al., 2007). Briefly, neurons were fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich) for 10 min at 11 and 16 DIV for Figure 3 and 15-21 DIV for Figures 2, 5, 6, 7 and 8. Primary antibodies monoclonal mouse anti-Synaptotagmin-1 (1:1000; Synaptic System), polyclonal guinea pig anti-VGLUT1 (1:4000; Synaptic System) and polyclonal rabbit anti-Synaptogamin 7 (1:500; Synaptic System) were used. Secondary antibodies (1:500) conjugated with Alexa Fluor 405, 488 or 647 (Jackson ImmunoResearch) were utilized to visualize fluorescence. For quantitative assessment, all groups compared in one experiment were processed in parallel using identical antibodies solutions and other reagents.

Single neurons on the astrocytic micro-islands were imaged by using a Nikon Scanning Confocal A1Rsi+ with a 60x, 1.4 NA oil-immersion objective. For acquiring all the images, we used the same microscope and camera with identical acquisition settings for all experimental groups. Overexposure and photobleaching was avoided by checking the fluorescence signal saturation in synaptic boutons. Z stacks of neurons were set with a 0.3 μm inter-stack interval and total z axis range of 5–6 μm , and a sum of intensity projection was further used for analyses. The images were obtained at 1024 x 1024 pixels resolution and at the pixel size of 0.2 μm . During acquisition, laser settings were applied identically to all coverslips quantified in all experimental groups compared.

The analysis was performed using ImageJ software (US National Institutes of Health) by drawing ROIs of 50 synapses per neuron. Excitatory synapses (region of interest, ROI) were defined by staining for the synaptic vesicle (SV) marker VGLUT1. SYT1 or SYT7 fluorescence intensity signal for each synapse was measured within the defined ROI. Five to ten autaptic neurons were collected per condition per culture and at least three independent cultures were analyzed per experiment. Relative expression level of SYT1 among groups was calculated by normalizing the measured intensities of SYT1 to that of VGLUT1. The data were normalized to the control of each experiment.

Experimental design and statistics

For electrophysiology and immunocytochemistry experiments, we recorded and imaged approximately the same number of autaptic neurons from each experimental group each day to reduce data variability. For each parameter analyzed, the number of neurons used (n number) and the number of independent cultures (N) is indicated in the figure, specifically within the bar graph (n/N). Data were acquired from at least three independent hippocampal autaptic cultures generated from three different animals (N=3) to minimize culture-culture variation. For Figure 7 we chose the nonlinear regression model standard Hill equation. We did not constrain any parameter of the model to a constant value. We performed a global nonlinear regression; we specify that parameters are shared to fit all data sets. All data points are weighted equally in the model.

Data from each experimental group were pooled together except for Figure 2, 7 and 8 which were normalized to the mean value of the control group. Statistical analyses were performed using Prism 8 (GraphPad). All data were first subjected to Pearson omnibus K2 normality testing. Two-tailed unpaired t test or one-way ANOVA test for normally distributed data and Mann–Whitney test or Kruskal–Wallis ANOVA test for non-normally distributed data were then carried out. Post hoc multiple

209 comparison methods were used following ANOVA test. Significance and p values were calculated and
210 are shown in the corresponding Figure or Table.

211 Results

212 Phenotype of Synaptotagmin-1 loss in synaptic vesicle priming and spontaneous release is 213 sensitive to neuronal maturation state

214 Given the controversy over SYT1 involvement in clamping and priming SVs, the observation of
215 developmentally dependent effects of SYT2 in the calyx of Held (Kochubey et al., 2016) and the
216 discovery of new point mutations in *SYT1* gene associated with a neurodevelopmental disorder
217 (Baker et al., 2018), we aimed to re-analyze the roles of Synaptotagmin-1 in SV priming,
218 synchronized and spontaneous release as a function of neuronal culture age. We recorded from
219 autaptic hippocampal neurons derived from *Syt1^{+/+}* or *Syt1^{-/-}* mice across a ten-day time period (11-21
220 days in vitro (DIV)), and subsequently grouped our results into three different classes (11-12, 15-16
221 and 20-21 DIV).

222 To assess SV priming, we measured the readily releasable pool (RRP) of SVs using 5 s
223 application of 500 mOsm hypertonic solution (Rosenmund and Stevens, 1996) and integrated the
224 transient component of the evoked inward current. While the RRP charge of *Syt1^{+/+}* and *Syt1^{-/-}*
225 autaptic neurons at DIV11-12 was indistinguishable, at both later culturing stages (DIV15-16) and
226 (DIV20-21) we detected significant ~40-50% reduction in RRP charge in the *Syt1^{-/-}* group (Figure 1A;
227 (RRP, pC): DIV11-12: *Syt1^{+/+}* 135 ± 18 , $n=60/3$ and *Syt1^{-/-}* 148 ± 17 , $n=57/3$, $p=0.26$; DIV15-16: *Syt1^{+/+}*
228 477 ± 54 , $n=58/3$ and *Syt1^{-/-}* 247 ± 33 , $n=47/3$, $p=0.0004$; DIV20-21: *Syt1^{+/+}* 759 ± 89 , $n=44/3$ and *Syt1^{-/-}*
229 360 ± 54 , $n=44/3$, $p<0.0001$; Mann-Whitney test). Next, we analyzed spontaneous neurotransmitter
230 release. We observed that loss of SYT1 led to no significant changes in spontaneous release rates for
231 the early DIV group while spontaneous release rate was enhanced in the two older DIV groups
232 (Figure 1B; (Spontaneous rate, s^{-1}): DIV11-12: *Syt1^{+/+}* 0.0028 ± 0.0003 , $n=56/3$ and *Syt1^{-/-}*
233 0.0034 ± 0.0003 , $n=48/3$, $p=0.0544$; DIV15-16: *Syt1^{+/+}* 0.0029 ± 0.0005 , $n=42/3$ and *Syt1^{-/-}*
234 0.0065 ± 0.0009 , $n=43/3$, $p<0.0001$; DIV20-21: *Syt1^{+/+}* 0.0019 ± 0.0004 , $n=44/3$ and *Syt1^{-/-}*
235 0.0052 ± 0.0012 , $n=44/3$, $p<0.0001$; Mann-Whitney test), emphasizing that the impact of SYT1 loss on
236 spontaneous release, like the impact on RRP size, is dependent on the maturation state of the
237 neuron.

238 In contrast, Ca^{2+} -evoked release was massively affected regardless of culture age as indicated by the
 239 reduction in EPSC amplitude and charge in all three DIV groups (**Figure 1C**; (EPSC amplitude, nA):
 240 DIV11-12: $\text{Syt1}^{+/+}$ -1.17 ± 0.19 , $n=60/3$ and $\text{Syt1}^{-/-}$ -0.069 ± 0.007 , $n=58/3$, $p < 0.0001$; DIV15-16: $\text{Syt1}^{+/+}$ $-$
 241 3.2 ± 0.5 , $n=47/3$ and $\text{Syt1}^{-/-}$ -0.097 ± 0.012 , $n=43/3$, $p < 0.0001$; DIV20-21: $\text{Syt1}^{+/+}$ -4.19 ± 0.58 , $n=44/3$
 242 and $\text{Syt1}^{-/-}$ -0.085 ± 0.012 , $n=44/3$, $p < 0.0001$; Mann-Whitney test). Moreover, the vesicular release
 243 probability (Pvr), as calculated by dividing the EPSC charge by the RRP charge, was similarly
 244 reduced by ~60% in all three DIV groups measured (**Figure 1D**; (Pvr, %): DIV11-12: $\text{Syt1}^{+/+}$ 7.7 ± 0.7 ,
 245 $n=60/3$ and $\text{Syt1}^{-/-}$ 2.6 ± 0.3 , $n=57/3$, $p < 0.0001$; DIV15-16: $\text{Syt1}^{+/+}$ 7.4 ± 0.9 , $n=46/3$ and $\text{Syt1}^{-/-}$ 2.9 ± 0.6 ,
 246 $n=43/3$, $p < 0.0001$; DIV20-21: $\text{Syt1}^{+/+}$ 6.7 ± 0.7 , $n=44/3$ and $\text{Syt1}^{-/-}$ 2.3 ± 0.3 , $n=44/3$, $p < 0.0001$; Mann-
 247 Whitney test).

248 Lentiviral mediated SYT1 rescue experiments showed fully restored SV priming, and
 249 spontaneous and AP induced release efficacy in stages DIV15-21 (**Figure 2**; (RRP norm.): $\text{Syt1}^{+/+}$
 250 1 ± 0.11 , $n=39/3$, $\text{Syt1}^{-/-}$ 0.40 ± 0.05 , $n=31/3$, $p < 0.001$ and $\text{Syt1}^{-/-}_{+SYT1}$ 0.93 ± 0.15 , $n=29/3$, $p = \text{ns} < 0.05$;
 251 (Spontaneous rate norm.): $\text{Syt1}^{+/+}$ 1 ± 0.13 , $n=37/3$, $\text{Syt1}^{-/-}$ 2.53 ± 0.53 , $n=30/3$, $p < 0.01$ and $\text{Syt1}^{-/-}_{+SYT1}$
 252 0.96 ± 0.12 , $n=26/3$, $p = \text{ns} < 0.05$; (EPSC charge norm.): $\text{Syt1}^{+/+}$ 1 ± 0.16 , $n=42/3$, $\text{Syt1}^{-/-}$ 0.34 ± 0.05 ,
 253 $n=34/3$, $p < 0.01$ and $\text{Syt1}^{-/-}_{+SYT1}$ 1.05 ± 0.2 , $n=29/3$, $p = \text{ns} < 0.01$; (Pvr norm.): $\text{Syt1}^{+/+}$ 1 ± 0.10 , $n=37/3$,
 254 $\text{Syt1}^{-/-}$ 0.57 ± 0.09 , $n=26/3$, $p < 0.05$ and $\text{Syt1}^{-/-}_{+SYT1}$ 1.16 ± 0.15 , $n=28/3$, $p = \text{ns} < 0.01$; (PPR 40Hz, norm.):
 255 $\text{Syt1}^{+/+}$ 1 ± 0.06 , $n=32/3$, $\text{Syt1}^{-/-}$ 2.15 ± 0.20 , $n=29/3$, $p < 0.0001$ and $\text{Syt1}^{-/-}_{+SYT1}$ 0.97 ± 0.07 , $n=24/3$,
 256 $p = \text{ns} < 0.0001$; Kruskal-Wallis test), indicating that the loss of function phenotype of SYT1 at older
 257 cultures was due to the loss of the protein and not due to developmental processes. Expression levels
 258 of exogenously expressed SYT1 were near wildtype levels as indicated by relative SYT1
 259 immunofluorescence intensity from VGLUT1 marker positive compartments (**Figure 2A and B**;
 260 (VGLUT1 norm.): $\text{Syt1}^{+/+}$ 1 ± 0.06 , $n=29/3$, $\text{Syt1}^{-/-}$ 1.07 ± 0.06 , $n=23/3$, $p = \text{ns}$ and $\text{Syt1}^{-/-}_{+SYT1}$ 0.89 ± 0.04 ,
 261 $n=26/3$, $p = \text{ns/ns}$; (SYT1/VGLUT1 norm.): $\text{Syt1}^{+/+}$ 1 ± 0.05 , $n=29/3$, $\text{Syt1}^{-/-}$ 0.16 ± 0.01 , $n=23/3$, $p < 0.0001$
 262 and $\text{Syt1}^{-/-}_{+SYT1}$ 1.02 ± 0.05 , $n=26/3$, $p = \text{ns} < 0.0001$; Kruskal-Wallis test).

263 Overall, our data confirm that loss of SYT1 leads to loss of synchronized and efficient AP
 264 triggered release, but also that the impairment of RRP and clamping of spontaneous release only
 265 appears in more mature neurons. One possible explanation could be that the loss of SYT1 at early
 266 maturation stages may be compensated by other synaptotagmin isoform, which would be

downregulated over neuronal development as similarly shown at the calyx of Held synapses (Kochubey et al., 2016). These findings may provide an explanation for findings in autaptic glutamatergic neurons where no significant changes in RRP size were reported when recordings were pooled over extended times in vitro (e.g., Liu et al. 2009 DIV12-17). We therefore limited in the remaining part of the experiments our experiments to neurons that were DIV15 or older.

Partial functional redundancy between Synaptotagmin-1 and Synaptotagmin-7

Recent genetic and functional analysis indicates that SYT7 plays a redundant role in SV priming function (Bacaj et al., 2015). Moreover, the expression of the Synaptotagmin1/2 paralogs are developmentally regulated at a murine auditory synapse, contributing to maturation dependent variation in synaptic phenotypes in synaptotagmin loss of function mouse models (Kochubey et al., 2016). We therefore explored whether SYT7 expression in less mature hippocampal neurons contributes to the milder phenotype of SYT1 loss by knocking-down SYT7 protein expression in *Syt1*^{-/-} neurons. First, we examined SYT1 and SYT7 protein levels in presynaptic compartments at early (DIV11-12) or intermediate stages (DIV15-16) in *Syt1*^{+/+} and *Syt1*^{-/-} neurons and subsequently measured at both stages the presynaptic SYT7 reduction affected by lentiviral expression of a SYT7 *shRNA* construct in *Syt1*^{-/-} autaptic neurons (**Figure 3**). We utilized VGLUT1 protein expression to spatially define presynaptic compartments and to serve as reference signal for SYT1 and SYT7 expression levels. In *Syt1*^{+/+} neurons, SYT1 protein levels gradually increased with time of DIV but stayed relatively constant in comparison to VGLUT1. In contrast, SYT7 protein expression levels remained constant and, consequently, SYT7/VGLUT1 signal decreased over time in culture (**Figure 3A and B**; (VGLUT1 norm.): *Syt1*^{+/+}_{DIV11} 1±0.12, n=21/3 and *Syt1*^{+/+}_{DIV16} 3.2±0.4, n=21/3, p<0.0001; Two-tailed unpaired t test; (SYT1 norm.): *Syt1*^{+/+}_{DIV11} 1±0.10, n=21/3 and *Syt1*^{+/+}_{DIV16} 2.9±0.4, n=21/3, p<0.0001; Two-tailed unpaired t test; (SYT7 norm.): *Syt1*^{+/+}_{DIV11} 1±0.14, n=21/3 and *Syt1*^{+/+}_{DIV16} 1.3±0.2, n=21/3, p=ns; Mann-Whitney test; (VGLUT1 norm.): *Syt1*^{-/-}_{DIV11} 1±0.12, n=27/3 and *Syt1*^{-/-}_{DIV16} 2.3±0.36, n=28/3, p<0.001; Mann-Whitney test). We tested how effective the SYT7 *shRNA* reduced the presynaptic SYT7 protein expression and found an average reduction of app. 75% across all DIVs tested using *Syt1*^{-/-} autaptic neurons (**Figure 3C**; (VGLUT1 norm.): *Syt1*^{-/-}_{+RNAi(SYT7)DIV11} 1±0.14, n=21/3 and *Syt1*^{-/-}_{+RNAi(SYT7)DIV16} 3.5±0.47, n=17/3, p<0.0001; Mann-Whitney test; (SYT7DIV11 norm.): *Syt1*^{-/-} 1±0.21, n=27/3 and *Syt1*^{-/-}_{+RNAi(SYT7)} 0.30±0.15, n=21/3, p<0.001; Mann-Whitney test;

(SYT7DIV16 norm.): $Syt1^{-/-}$ 1 ± 0.13 , $n=28/3$ and $Syt1^{-/-+RNAi(Syt7)}$ 0.34 ± 0.09 , $n=17/3$, $p<0.001$; Mann-Whitney test; (SYT1DIV11 norm.): $Syt1^{+/+}$ 1 ± 0.20 , $n=21/3$, $Syt1^{-/-}$ 0.08 ± 0.04 , $n=27/3$, $p<0.0001$ and $Syt1^{-/-+RNAi(Syt7)}$ 0.1 ± 0.096 , $n=21/3$, $p<0.0001$; Kruskal-Wallis test; (SYT1DIV16 norm.): $Syt1^{+/+}$ 1 ± 0.096 , $n=21/3$, $Syt1^{-/-}$ 0.11 ± 0.02 , $n=28/3$, $p<0.0001$ and $Syt1^{-/-+RNAi(Syt7)}$ 0.07 ± 0.06 , $n=17/3$, $p<0.0001$; Kruskal-Wallis test).

Reduction of SYT7 protein expression in $Syt1^{-/-}$ autaptic neurons reduced RRP size and increased mEPSC rates at 11-12 DIV and 15-16 DIV (**Figure 4A and B**; (RRP, pC): DIV11-12: $Syt1^{+/+}$ 165 ± 23 , $n=48/4$, $Syt1^{-/-}$ 170 ± 21 , $n=40/4$, $p=ns$ and $Syt1^{-/-+RNAi(Syt7)}$ 34 ± 6 , $n=40/4$, $p<0.0001/0.0001$; DIV15-16: $Syt1^{+/+}$ 567 ± 69 , $n=44/4$, $Syt1^{-/-}$ 279 ± 43 , $n=41/4$, $p<0.01$ and $Syt1^{-/-+RNAi(Syt7)}$ 50 ± 9 , $n=30/4$, $p<0.0001/0.0001$; Kruskal-Wallis test; (Spontaneous rate, s^{-1}): DIV11-12: $Syt1^{+/+}$ 0.003 ± 0.0004 , $n=48/4$, $Syt1^{-/-}$ 0.0043 ± 0.0005 , $n=33/4$, $p=ns$ and $Syt1^{-/-+RNAi(Syt7)}$ 0.012 ± 0.0015 , $n=38/4$, $p<0.0001/0.01$; DIV15-16: $Syt1^{+/+}$ 0.0016 ± 0.0002 , $n=44/4$, $Syt1^{-/-}$ 0.0054 ± 0.0012 , $n=41/4$, $p<0.01$ and $Syt1^{-/-+RNAi(Syt7)}$ 0.013 ± 0.001 , $n=24/4$, $p<0.0001/0.01$; Kruskal-Wallis test). These results suggest that SYT7 compensates for the loss of SYT1 dependent synaptic vesicle priming and clamp of spontaneous release (**Figure 4A and B**). Next, we examined whether SYT7 expression contributes evoked NT release and the probability of vesicle fusion of $Syt1^{-/-}$ neurons. While knocking-down of SYT7 expression levels in $Syt1^{-/-}$ neurons had an impact on the amplitude and charge of the Ca^{2+} -evoked release at both stages, the changes were proportional to the observed for the RRP size, and thus did not lead to a further significant effect in the Pvr (**Figure 4C and D**; (EPSC charge, pC): DIV11-12: $Syt1^{+/+}$ -11.1 ± 1.5 , $n=47/4$, $Syt1^{-/-}$ -3.8 ± 0.5 , $n=43/4$, $p<0.01$ and $Syt1^{-/-+RNAi(Syt7)}$ -1.1 ± 0.2 , $n=40/4$, $p<0.0001/0.001$; DIV15-16: $Syt1^{+/+}$ -36 ± 5 , $n=40/4$, $Syt1^{-/-}$ -5.3 ± 1 , $n=44/4$, $p<0.0001$ and $Syt1^{-/-+RNAi(Syt7)}$ -1.4 ± 0.3 , $n=32/4$, $p<0.0001/0.01$; (Pvr, %): DIV11-12: $Syt1^{+/+}$ 8.5 ± 1 , $n=47/4$, $Syt1^{-/-}$ 3.2 ± 0.5 , $n=40/4$, $p<0.0001$ and $Syt1^{-/-+RNAi(Syt7)}$ 3.3 ± 0.6 , $n=39/4$, $p<0.0001/ns$; DIV15-16: $Syt1^{+/+}$ 7.4 ± 0.8 , $n=38/4$, $Syt1^{-/-}$ 3.5 ± 0.6 , $n=38/4$, $p<0.001$ and $Syt1^{-/-+RNAi(Syt7)}$ 2.9 ± 0.8 , $n=30/4$, $p<0.0001/ns$; Kruskal-Wallis test) of $Syt1^{-/-}$ autaptic neurons in any stages tested.

All together these results suggest that while SYT1 is essential for evoked neurotransmitter release, SYT7 is well capable of substituting in the SYT1's role in clamping spontaneous release and enabling SV priming, which results in weakening of SYT1 deficiency of these two phenotypes in less mature neurons.

325 Synaptotagmin-1 haploinsufficiency affects release efficiency and spontaneous release rate

326 Our results on SV priming and spontaneous clamping suggest that the underlying phenotypes are
 327 dependent on both SYT1 and SYT7 protein expression levels. Since monoallelic mutations in *SYT1*
 328 lead to neurological disorders (Baker et al., 2015, 2018), it raises the question of whether and which
 329 of the three SYT1 functions in NT release is impaired by reduced SYT1 protein amount. We first
 330 quantified SV priming, clamping, and calcium-evoked release in *Syt1^{+/+}*, *Syt1^{+/-}*, and *Syt1^{-/-}* at a
 331 maturation stage where SYT1 dominates the synaptic phenotype (DIV15-21).

332 Immunocytochemistry experiments showed that the amount of SYT1 protein at presynaptic
 333 terminals of *Syt1^{-/-}* neurons was ~50% reduced compared with *Syt1^{+/+}* neurons (Figure 5A and B;
 334 (*VGLUT1 norm.*): *Syt1^{+/+}* 1 ± 0.07 , $n=26/4$, *Syt1^{+/-}* 1.18 ± 0.09 , $n=33/4$, $p=ns$ and *Syt1^{-/-}* 1.03 ± 0.09 ,
 335 $n=31/4$, $p=ns/ns$; (*SYT1/VGLUT1 norm.*): *Syt1^{+/+}* 1 ± 0.06 , $n=26/4$, *Syt1^{+/-}* 0.62 ± 0.04 , $n=33/4$, $p<0.01$
 336 and *Syt1^{-/-}* 0.18 ± 0.02 , $n=31/4$, $p<0.0001/<0.0001$; Kruskal-Wallis test). As expected, the complete
 337 absence of SYT1 in *Syt1^{-/-}* neurons resulted in a ~40% reduction in the pool size (Figure 5C).
 338 However, we found no significant differences in the RRP size between *Syt1^{+/+}* and *Syt1^{+/-}* neurons
 339 (Figure 5C; (*RRP, pC*): *Syt1^{+/+}* 330 ± 24 , $n=92/4$, *Syt1^{+/-}* 382 ± 26 , $n=87/4$, $p=ns$ and *Syt1^{-/-}* 180 ± 15 ,
 340 $n=91/4$, $p<0.0001/<0.0001$; Kruskal-Wallis test). This indicates that loss of >50% of SYT1 protein is
 341 required to impair SYT1's role in SV priming. A significant increase in the spontaneous release rate of
 342 synaptic vesicles was observed in *Syt1^{+/-}* autaptic neurons (Figure 5D; (*Spontaneous rate, s⁻¹*) *Syt1^{+/+}*
 343 0.002 ± 0.0002 , $n=61/4$, *Syt1^{+/-}* 0.004 ± 0.0004 , $n=68/4$, $p<0.05$ and *Syt1^{-/-}* 0.007 ± 0.0007 , $n=77/4$,
 344 $p<0.0001/<0.0001$; Kruskal-Wallis test).

345 In agreement with previous work (Geppert et al., 1994; Nishiki and Augustine, 2004; Xue et
 346 al., 2008), autaptic *Syt1^{-/-}* neurons showed severe desynchronization of evoked release, which is
 347 reflected in the drastic reduction in peak EPSC amplitude (Figure 5E). Furthermore, integrating the
 348 charge of the EPSC over 1s past AP triggering, we also observed a ~75% decrease in the EPSC
 349 charge (Figure 5F). In contrast, we detected no significant difference in *Syt1^{+/-}* EPSC amplitude and
 350 charge when compared with *Syt1^{+/+}* autaptic neurons (Figure 5E and F; (*EPSC amplitude, nA*):
 351 *Syt1^{+/+}* -3.0 ± 0.2 , $n=91/4$, *Syt1^{+/-}* -2.8 ± 0.2 , $n=98/4$, $p=ns$ and *Syt1^{-/-}* -0.06 ± 0.006 , $n=94/4$,
 352 $p<0.0001/<0.0001$; Kruskal-Wallis test; (*EPSC charge, pC*): *Syt1^{+/+}* -24 ± 2 , $n=91/4$, *Syt1^{+/-}* -23 ± 2 ,
 353 $n=98/4$, $p=ns$ and *Syt1^{-/-}* -5 ± 0.5 , $n=94/4$, $p<0.0001/<0.0001$; Kruskal-Wallis test). We then examined
 354 whether 50% loss of SYT1 protein leads to some changes in EPSC kinetics in the *Syt1^{+/-}* neurons.

355 The EPSC rise time of *Syt1*^{+/-} neurons was like wild-type, while the rise time was ~3 fold slower for
 356 *Syt1*^{-/-} neurons (**Figure 5G**; (EPSC rise time, ms): *Syt1*^{+/+} 0.9 ± 0.04 , $n=83/4$, *Syt1*^{+/-} 0.9 ± 0.04 , $n=83/4$,
 357 $p=ns$ and *Syt1*^{-/-} 2.8 ± 0.3 , $n=65/4$, $p<0.0001/<0.0001$; Kruskal-Wallis test). The decay time of EPSC
 358 was analyzed by a two-exponential fit, and we found that between the *Syt1*^{+/+} and *Syt1*^{+/-} neurons,
 359 both the fast and slow component was unaltered in their relative amplitudes and time constants
 360 (**Figure 5H**). In contrast, in *Syt1*^{-/-} neurons time constant increased, and the fractional contribution of
 361 the slow component increased from ~20% to 80 % (**Figure 5H**; (EPSC components): *Syt1*^{+/+} (slow)
 362 0.15 ± 0.02 (fast) 0.85 ± 0.02 , $n=71/4$, *Syt1*^{+/-} (slow) 0.18 ± 0.01 (fast) 0.82 ± 0.01 , $n=68/4$, $p=0.1705$ and
 363 *Syt1*^{-/-} (slow) 0.85 ± 0.05 (fast) 0.15 ± 0.05 , $n=78/4$, $p<0.0001/<0.0001$; Kruskal-Wallis test).

364 The reduction of EPSC charge in *Syt1*^{-/-} exceeds the reduction in RRP size, indicating that
 365 complete loss of SYT1 leads to a reduction in vesicular release probability. We thus measured
 366 vesicular release probability in *Syt1*^{+/-} neurons and surprisingly found a significant reduction in Pvr
 367 when compared with *Syt1*^{+/+} autaptic neurons (**Figure 5I**; (Pvr, %): *Syt1*^{+/+} 8.6 ± 0.4 , $n=86/4$, *Syt1*^{+/-}
 368 7.5 ± 0.6 , $n=86/4$, $p<0.05$ and *Syt1*^{-/-} 4.4 ± 0.5 , $n=90/4$, $p<0.0001/<0.0001$; Kruskal-Wallis test). The
 369 reduced release probability was corroborated by analysis of short-term plasticity experiments, in
 370 which we applied sequential pairs of action potentials with an interpulse interval of 25, 50, or 100 ms.
 371 Consistent with the Pvr results, *Syt1*^{-/-} neurons displayed a significant increase of the paired-pulsed
 372 ratio (PPR) at 10, 20 and 40 Hz compared with *Syt1*^{+/+} and a significant increase of the PPR at 20 and
 373 40Hz compared with *Syt1*^{+/-} (**Figure 5J**; (10Hz): *Syt1*^{+/+} 0.91 ± 0.02 , $n=47/4$, *Syt1*^{+/-} 0.98 ± 0.03 , $n=62/4$,
 374 $p=ns$ and *Syt1*^{-/-} 1.38 ± 0.09 , $n=41/4$, $p<0.0001/<0.0001$; Kruskal-Wallis test; (20Hz): *Syt1*^{+/+} 0.90 ± 0.02 ,
 375 $n=53/4$, *Syt1*^{+/-} 0.99 ± 0.02 , $n=50/4$, $p<0.05$ and *Syt1*^{-/-} 1.51 ± 0.13 , $n=20/4$, $p<0.0001/<0.0001$; Kruskal-
 376 Wallis test; (40Hz): *Syt1*^{+/+} 1.09 ± 0.03 , $n=86/4$, *Syt1*^{+/-} 1.28 ± 0.05 , $n=81/4$, $p<0.01$ and *Syt1*^{-/-}
 377 3.46 ± 0.37 , $n=78/4$, $p<0.0001/<0.0001$; Kruskal-Wallis test). In addition, we performed 20 AP at 20 Hz
 378 train-pulses experiment. *Syt1*^{-/-} neurons strongly facilitated, and even *Syt1*^{+/-} neurons showed
 379 significantly less depression compared to *Syt1*^{+/+} neurons (**Figure 5K**; *Syt1*^{+/+} 0.55 ± 0.03 , $n=53/4$,
 380 *Syt1*^{+/-} 0.70 ± 0.04 , $n=50/4$, $p<0.01$ and *Syt1*^{-/-} 4.7 ± 0.6 , $n=20/4$, $p<0.0001$; Kruskal-Wallis test).

381 Together, these results demonstrate that the 50% reduced SYT1 protein at *Syt1*^{+/-} presynaptic
 382 terminals did not affect the RRP size, but modestly impaired release efficiency and the rate of
 383 spontaneous release. Although isn't clear whether the patients carrying a heterozygous mutation in

the *SYT1* gene may have an altered SYT1 presence at the glutamatergic synapses, these findings may be relevant to understand how a 50% deficiency in SYT1 expression could contribute to develop an aberrant neurological phenotype.

Synaptotagmin-1 is a limiting factor for vesicle fusion release efficiency during high-frequency stimulation

Our analysis with the *Syt1*^{+/-} neurons showed that 50% reduced SYT1 expression affects synaptic properties such as spontaneous release rate and efficiency of the release, indicating the relative sensitivity of SYT1 protein levels on release efficacy. We extended this analysis to overexpression of the SYT1. We used a dose of lentivirus to mediate SYT1 overexpression in *Syt1*^{+/-} autaptic neurons (DIV15-21), which led to an overexpression of ~75% of WT levels in SYT1 content in presynaptic terminals (**Figure 6A**; (*VGLUT1* norm.): *Syt1*^{+/-} 1 ± 0.04 , $n=30/3$ and *Syt1*^{+/-}_{+SYT1} 1.06 ± 0.04 , $n=30/3$, $p=0.29$; (*SYT1/VGLUT1* norm.): *Syt1*^{+/-} 1 ± 0.05 , $n=30/3$ and *Syt1*^{+/-}_{+SYT1} 1.78 ± 0.06 , $n=30/3$, $p<0.0001$; Two-tailed unpaired *t* test). RRP size (**Figure 6B**; (*RRP*, *pC*): *Syt1*^{+/-} 549 ± 85 , $n=33/3$ and *Syt1*^{+/-}_{+SYT1} 500 ± 57 , $n=36/3$, $p=0.67$; Mann-Whitney test), spontaneous release rate (**Figure 6C**; (*Spontaneous rate*, *s*⁻¹): *Syt1*^{+/-} 0.003 ± 0.0005 , $n=31/3$ and *Syt1*^{+/-}_{+SYT1} 0.0017 ± 0.0002 , $n=34/3$, $p=0.45$; Mann-Whitney test) and the EPSC amplitude (**Figure 6D**; (*EPSC amplitude*, *nA*): *Syt1*^{+/-} -2.99 ± 0.43 , $n=34/3$ and *Syt1*^{+/-}_{+SYT1} -4.10 ± 0.47 , $n=37/3$, $p=0.084$; Mann-Whitney test) were statistically not significant compared to *Syt1*^{+/-} neurons (DIV15-21). Also computing Pvr in the SYT1 overexpressing neurons did not show any significant change (**Figure 6E**; (*Pvr*, %): *Syt1*^{+/-} 7.0 ± 1.1 , $n=33/3$ and *Syt1*^{+/-}_{+SYT1} 8.4 ± 1.0 , $n=35/3$, $p=0.19$; Mann-Whitney test). However, SYT1 overexpression caused more depression of the second response during paired-pulse experiments (**Figure 6F**; (*40Hz*): *Syt1*^{+/-} 1.26 ± 0.06 , $n=34/3$ and *Syt1*^{+/-}_{+SYT1} 0.98 ± 0.04 , $n=33/3$, $p=0.0005$; Mann-Whitney test) compared to *Syt1*^{+/-}, emphasizing that release efficacy is more sensitive to changes in SYT1 levels than its function in regulating RRP size.

Manipulation of endogenous expression levels of Synaptotagmin-1 reveals different functional thresholds

Does SYT1 regulate NT-release and recruitment of SVs to the presynaptic PM in a protein concentration dependent manner? To address this question, we examined the impact of titrating endogenous SYT1 protein expression in autaptic neurons at DIV15-21 using *RNA interference* (*RNAi*)

technology, which led to a graded reduction of SYT1 levels in presynaptic terminals down to 10-25% of $Syt1^{+/+}$ SYT1 expression levels (**Figure 7A**; (VGLUT1 norm.): $Syt1^{+/+}$ 1 ± 0.06 , $n=19/3$, $Syt1^{+/+}_{1xRNA(Syt1)}$ 1.32 ± 0.1 , $n=20/3$, $p=ns$, $Syt1^{+/+}_{2xRNA(Syt1)}$ 1.26 ± 0.09 , $n=24/3$, $p=ns$ and $Syt1^{+/+}_{4xRNA(Syt1)}$ 1.33 ± 0.10 , $n=27/3$, $p=ns$; (SYT1/VGLUT1 norm.): $Syt1^{+/+}$ 1 ± 0.05 , $n=19/3$, $Syt1^{+/+}_{1xRNA(Syt1)}$ 0.25 ± 0.04 , $n=20/3$, $p<0.001$, $Syt1^{+/+}_{2xRNA(Syt1)}$ 0.13 ± 0.02 , $n=24/3$, $p<0.0001$ and $Syt1^{+/+}_{4xRNA(Syt1)}$ 0.096 ± 0.01 , $n=27/3$, $p<0.0001$; Kruskal-Wallis test).

We found that the number of fusion-competent vesicles, as measured by sucrose application, was only significantly reduced when the SYT1 protein expression level was below 10% (**Figure 7B**; (RRP norm.): $Syt1^{+/+}$ 1 ± 0.11 , $n=51/4$, $Syt1^{+/+}_{1xRNA(Syt1)}$ 0.97 ± 0.13 , $n=35/4$, $p=ns$, $Syt1^{+/+}_{2xRNA(Syt1)}$ 0.83 ± 0.11 , $n=37/4$, $p=ns$ and $Syt1^{+/+}_{4xRNA(Syt1)}$ 0.61 ± 0.08 , $n=39/4$, $p<0.05$; Kruskal-Wallis test). On the other hand, spontaneous release rate was affected by the gradual reduction in SYT1 expression levels, showing progressive unclamping with reduction of SYT1 expression, reaching a ~3-fold increase when SYT1 levels were close to those of $Syt1^{-/-}$ (**Figure 7C**; (Spontaneous rate norm.): $Syt1^{+/+}$ 1 ± 0.11 , $n=55/4$, $Syt1^{+/+}_{1xRNA(Syt1)}$ 1.54 ± 0.16 , $n=40/4$, $p<0.05$, $Syt1^{+/+}_{2xRNA(Syt1)}$ 1.67 ± 0.23 , $n=35/4$, $p<0.05$ and $Syt1^{+/+}_{4xRNA(Syt1)}$ 2.4 ± 0.47 , $n=41/4$, $p<0.0001$; Kruskal-Wallis test). Processes involved in evoked release showed an even higher sensitivity to SYT1 expression levels (**Figure 7D-G**). The EPSC charge as well as the fast synchronous component of release were significantly reduced with all levels of SYT1 knock-down tested (**Figure 7D and E**; (EPSC charge norm.): $Syt1^{+/+}$ 1 ± 0.11 , $n=57/4$, $Syt1^{+/+}_{1xRNA(Syt1)}$ 0.58 ± 0.1 , $n=36/4$, $p<0.01$, $Syt1^{+/+}_{2xRNA(Syt1)}$ 0.36 ± 0.06 , $n=39/4$, $p<0.0001$ and $Syt1^{+/+}_{4xRNA(Syt1)}$ 0.32 ± 0.05 , $n=43/4$, $p<0.0001$; Kruskal-Wallis test; (EPSC components): $Syt1^{+/+}$ (slow) 0.28 ± 0.02 (fast) 0.72 ± 0.02 , $n=56/4$, $Syt1^{+/+}_{1xRNA(Syt1)}$ (slow) 0.61 ± 0.06 (fast) 0.39 ± 0.06 , $n=29/4$, $p<0.0001$, $Syt1^{+/+}_{2xRNA(Syt1)}$ (slow) 0.66 ± 0.04 (fast) 0.34 ± 0.04 , $n=39/4$, $p<0.0001$ and $Syt1^{+/+}_{4xRNA(Syt1)}$ (slow) 0.65 ± 0.04 (fast) 0.35 ± 0.04 , $n=40/4$, $p<0.0001$; One-way ANOVA test). Additionally, the release efficacy was significantly reduced in all knocked-down groups, demonstrated by a reduced Pvr and increased paired-pulse ratio compared with $Syt1^{+/+}$ (**Figure 7F and G**; (Pvr norm.): $Syt1^{+/+}$ 1 ± 0.08 , $n=51/4$, $Syt1^{+/+}_{1xRNA(Syt1)}$ 0.73 ± 0.12 , $n=30/4$, $p<0.01$, $Syt1^{+/+}_{2xRNA(Syt1)}$ 0.52 ± 0.07 , $n=31/4$, $p<0.0001$ and $Syt1^{+/+}_{4xRNA(Syt1)}$ 0.5 ± 0.07 , $n=39/4$, $p<0.0001$; (PPR40Hz, norm.): $Syt1^{+/+}$ 1 ± 0.05 , $n=51/4$, $Syt1^{+/+}_{1xRNA(Syt1)}$ 1.84 ± 0.18 , $n=34/4$, $p<0.001$, $Syt1^{+/+}_{2xRNA(Syt1)}$ 2.16 ± 0.19 , $n=33/4$, $p<0.0001$ and $Syt1^{+/+}_{4xRNA(Syt1)}$ 2.67 ± 0.35 , $n=39/4$, $p<0.0001$; Kruskal-Wallis test). To exclude that

these results might be due to the number of viral particles used, we manipulated SYT1 endogenous protein expression in *Syt1^{+/-}* neurons (DIV15-21) using the same *Syt1* RNAi construct (**Figure 8**). We used half of the amount of viral infection units to reduce the expression to approximately the same levels as in the titration experiments in the *Syt1^{+/+}* neurons (**Figure 8A**; (*VGLUT1* norm.): *Syt1^{+/-}* 1 ± 0.08 , $n=20/3$, *Syt1^{+/-}* $1xRNA(Syt1)$ 1.2 ± 0.16 , $n=17/3$, $p=ns$ and *Syt1^{+/-}* $2xRNA(Syt1)$ 0.93 ± 0.14 , $n=15/3$, $p=ns$; (*SYT1/VGLUT1* norm.): *Syt1^{+/-}* 1 ± 0.07 , $n=20/3$, *Syt1^{+/-}* $1xRNA(Syt1)$ 0.32 ± 0.03 , $n=17/3$, $p<0.0001$ and *Syt1^{+/-}* $2xRNA(Syt1)$ 0.22 ± 0.02 , $n=15/3$, $p<0.0001$; Kruskal-Wallis test). All electrophysiological parameters measured showed the same trend as our experiments performed on *Syt1^{+/+}* neurons (**Figure 8B-G**; (*RRP* norm.): *Syt1^{+/+}* 1 ± 0.16 , $n=21/3$, *Syt1^{+/+}* $1xRNA(Syt1)$ 0.72 ± 0.14 , $n=22/3$, $p=ns$ and *Syt1^{+/+}* $2xRNA(Syt1)$ 0.5 ± 0.08 , $n=19/3$, $p<0.05$; (*Spontaneous rate* norm.): *Syt1^{+/+}* 1 ± 0.25 , $n=15/3$, *Syt1^{+/+}* $1xRNA(Syt1)$ 1.70 ± 0.39 , $n=18/3$, $p=ns$ and *Syt1^{+/+}* $2xRNA(Syt1)$ 3.21 ± 1.09 , $n=15/4$, $p<0.05$; (*EPSC charge* norm.): *Syt1^{+/+}* 1 ± 0.15 , $n=29/3$, *Syt1^{+/+}* $1xRNA(Syt1)$ 0.35 ± 0.06 , $n=23/3$, $p<0.001$ and *Syt1^{+/+}* $2xRNA(Syt1)$ 0.29 ± 0.05 , $n=21/4$, $p<0.0001$; (*EPSC components*): *Syt1^{+/+}* (slow) 0.27 ± 0.04 (fast) 0.73 ± 0.04 , $n=21/3$, *Syt1^{+/+}* $1xRNA(Syt1)$ (slow) 0.62 ± 0.08 (fast) 0.38 ± 0.08 , $n=18/3$, $p<0.01$ and *Syt1^{+/+}* $2xRNA(Syt1)$ (slow) 0.64 ± 0.07 (fast) 0.36 ± 0.07 , $n=20/3$, $p<0.001$; (*Pvr* norm.): *Syt1^{+/+}* 1 ± 0.12 , $n=22/3$, *Syt1^{+/+}* $1xRNA(Syt1)$ 0.62 ± 0.11 , $n=21/3$, $p<0.05$ and *Syt1^{+/+}* $2xRNA(Syt1)$ 0.57 ± 0.09 , $n=20/3$, $p<0.01$; (*PPR40Hz*, norm.): *Syt1^{+/+}* 1 ± 0.09 , $n=27/3$, *Syt1^{+/+}* $1xRNA(Syt1)$ 2.47 ± 0.28 , $n=22/3$, $p<0.0001$ and *Syt1^{+/+}* $2xRNA(Syt1)$ 2.41 ± 0.2 , $n=17/3$, $p<0.0001$; Kruskal-Wallis test).

Our previous results suggest different relationships between synaptic functions and SYT1 expression levels. To understand these relationships in greater detail, we used our wide range of SYT1 protein levels measurements at the presynaptic terminals of glutamatergic neurons (DIV15-21) to construct dose-response curves for different release properties: RRP size, spontaneous release rate, EPSC charge and Pvr (**Figure 7 H-K**) and fitted our experimental data with a standard Hill equation (see [Materials and Methods](#)). To illustrate how SYT7 may potentially modulate SYT1's synaptic functions and to illustrate putative redundant function between SYT1 and SYT7, we included the normalized data from the SYT7 knock-down experiments performed on *Syt1^{-/-}* neurons from Figure 4 (DIV15-16). However, the SYT7 data point was excluded from the curve fitting of the SYT1 expression-function relation.

First, the function of SYT1 in synaptic vesicle priming was least sensitive to protein loss (**Figure 7H**), since even an 85% decrease in SYT1 protein expression at the synapse still displayed

normal priming function. The best-fit value K_d for this parameter of 0.02 demonstrates that only when SYT1 is almost absent from the synapse, RRP size decreases (**Figure 7H**). SYT1's function as a regulator of spontaneous NT-release (**Figure 7I**) showed a higher sensitivity to protein levels, leading to increased spontaneous release activity when the expression levels were reduced by 50% or more. Evoked release was the most sensitive to the variations of SYT1 expression, as reflected both by assessing EPSC charge and Pvr (**Figure 7J and K**). The similarity of the EPSC charge and Pvr functions is not surprising since both reflect SYT1's role as calcium sensor for evoked release. The K_d value (0.47) for the EPSC charge fit indicates that genetic modification of SYT1 expression associated with allelic loss or gene duplication may lead to more pronounced impact on evoked neurotransmitter release compared to SYT1 functions in vesicle priming or suppression of spontaneous release. The difference in the sensitivity and shape of the dose-function relationships (**Figure 7H-I**) may also indicate that SYT1 performs its distinct functions with different molecular stoichiometries.

Discussion

In this study, we iterated the existence of three different synaptic functions performed by SYT1 using an autaptic primary neuronal culture model. Previous results concerning the ability of SYT1 to promote SV docking/priming (Geppert et al., 1994; Jorgensen et al., 1995; Reist et al., 1998; Nagy et al., 2006; Liu et al., 2009; Bacaj et al., 2013, 2015; Imig et al., 2014; Chang et al., 2018; Huson et al., 2020) and spontaneous release clamping (DiAntonio and Schwarz, 1994; Geppert et al., 1994; Littleton et al., 1994; Mackler et al., 2002; Yoshihara and Littleton, 2002; Chicka et al., 2008; Liu et al., 2009; Xu et al., 2009; Bacaj et al., 2013; Wierda and Sørensen, 2014) have been contradictory. Here, we demonstrated that SYT1 has a role in SV priming and clamping spontaneous release, which becomes essential over neuronal maturation. More mature SYT1-lacking hippocampal glutamatergic neurons showed a deficit in the RRP size and an increase in the spontaneous release rate, while early autaptic cultures did not (Table 1). Therefore, the time point and neuronal maturation stage at which experiments were performed could explain some discrepancies in previous work. What factor could compensate for SYT1 loss at early stages? We found that SYT7 has partially overlapping functions to SYT1 (Figure 4 and 7), largely consistent with previous results (Bacaj et al., 2013, 2015), and we conclude that at an early neuronal stage endogenous SYT7 protein could compensate for the loss of SYT1 in calcium-independent SV functions, such as priming and clamping of spontaneous

502 release. When both proteins were missing at the presynaptic terminal, regardless of the culture stage,
 503 the pool of SVs was consistently reduced and spontaneous release was significantly increased.

504 To dissect the specific interaction of the different mechanisms of NT-release and to better
 505 understand SYT1's functions beyond that in synchronous release, we investigated the impact of
 506 systematically varying SYT1 expression levels on SV priming, clamping, and evoked release. SV
 507 priming was impaired only after a major drop in SYT1 concentration, suggesting that this process,
 508 while ultimately affected by the presence of SYT1, was relatively insensitive to changes in the number
 509 of SYT1 molecules. Varying SYT1 protein concentration also had a rather moderate impact on
 510 spontaneous release compared to the graded impact of varying SYT1 concentration on calcium
 511 triggered release processes. Accordingly, heterozygotic *Syt1*^{+/-} excitatory hippocampal neurons with a
 512 50% reduction in SYT1 protein expression in the presynaptic terminal showed no effect on RRP size
 513 compared to WT SYT1 protein levels, while calcium-dependent release efficacy was impaired by the
 514 50% SYT1 protein reduction in *Syt1*^{+/-} neurons. Hence, when we applied a dose-response model to
 515 illustrate the sensitivity of the priming function to SYT1 expression the curve described has a
 516 hyperbolic-like shape, where small amounts of SYT1 protein expression are sufficient to preserve
 517 maximum priming activity. Our overall analysis of the sensitivity of synaptic processes to SYT1 protein
 518 levels renders a rank where the least sensitive function is SV priming, followed by clamping of
 519 spontaneous and, finally, calcium-evoked release. Do the differences in sensitivity to SYT1 protein
 520 levels of these three synaptic processes indicate that SYT1 is involved in three consecutive and
 521 independent pathways? Due to the clear difference in sensitivity to expression levels for priming
 522 compare to evoked release we think that SYT1 is affecting at least two pathways, in addition to its
 523 possible roles in endocytosis (Poskanzer et al., 2003; Yao et al., 2012; Liang et al., 2017). One
 524 hypothesis is that SYT1 undergoes different conformational stages to regulate different synaptic
 525 processes. Indeed, it has been suggested that SYT1 C2B domain-dependent oligomerization
 526 provides the molecular basis for SYT1 control of spontaneous and asynchronous release, and upon
 527 influx of calcium SYT1 oligomers undergo a conformational change from an SV clamping mode to a
 528 mode which allows the triggering of synchronous release (Bello et al., 2018; Tagliatti et al., 2020).

529 SYT1 protein level titration experiments not only revealed to us the distinct roles of SYT1, but
 530 may also could contribute to the understanding of the pathophysiological mechanisms underlying
 531 SYT1-associated neurodevelopmental disorders (Baker et al., 2015, 2018; Bradberry et al., 2020). In

our study, we show that haploinsufficiency of SYT1 resulted in an aberrant spontaneous release phenotype and decreased release probability with additional consequences in short-term plasticity characteristics. If patients with heterozygous SYT1 mutations that has been described to produce a SYT1 loss-of-function (Bradberry et al., 2020), should occur a reduced SYT1 protein level, this could lead to an enhance spontaneous release or a decreased release probability and, ultimately, contribute to network disfunction. It has been reported that at least one of the de novo SYT1 mutations (*SYT1*_{M303K}) is expressed at lower level than endogenous wild-type protein and failed to localize at nerve terminals (Baker et al., 2018). Furthermore, two of the variants (*SYT1*_{D304G} and *SYT1*_{D366E}) failed to efficiently relocalize to nerve terminals following stimulation (Baker et al., 2018). While allelic expressivity may not explain the whole pathophysiology of the SYT1-associated neurodevelopmental disorder it could exacerbate synaptic manifestations of individual SYT1 variants (Baker et al., 2018; Bradberry et al., 2020). In fact, a recent article revealed aberrant spontaneous NT release with some mutations from SNAP25-associated encephalopathies, indicating that when this form of release is affected it could result in developmental and epileptic encephalopathies (Alten et al., 2021). Conversely, our findings may indicate that the patient's pathophysiology that could derive from changes in the SYT1 expression levels are unlikely to be related to the priming of synaptic vesicles function at the presynaptic terminal.

While SYT1's function as a calcium sensor for NT release has been extensively proven, the role for SYT7 is less clear. SYT7 has been suggested as the calcium sensor for asynchronous release (Maximov et al., 2008; Bacaj et al., 2013; Luo et al., 2015; Turecek and Regehr, 2018). SYT7 has also been proposed as a mediator of short-term facilitation of transmission during repetitive stimulation (Wen et al., 2010; Jackman et al., 2016; Chen et al., 2017b; Fujii et al., 2021), involving a mechanism of SYT7's concerted action with SYT1 on the fusion energy barrier (Schotten et al., 2015; Chen et al., 2017b; Jackman and Regehr, 2017; Huson et al., 2020). Supporting its role as a calcium sensor, SYT7 contributes to regulated exocytosis in chromaffin and pancreatic cells (Sugita et al., 2001; Shin et al., 2002; Schonn et al., 2008; Bendahmane et al., 2020). Additionally, it has been reported that SYT7 functions as a Ca²⁺-sensor for synaptic vesicle replenishment (Liu et al., 2014; Silva et al., 2021). In our study, we found no direct evidence that SYT7 is involved in regulating calcium-dependent release. However, we showed that suppressing SYT7 expression on *Syt1*^{-/-} neurons further decreased evoked neurotransmitter release, and the analysis of RRP size reveals

that the reduction in evoked response is rather due to the priming action of SYT7, and not due to an effect on calcium triggered release. It is quite possible that putative competition between SYT1 and SYT7 may contribute to the observed modulation of calcium triggered NT release.

This study could be framed within a series of works that aim to understand how synapses respond to the relative changes in the expression of different presynaptic components and to study the consequences on neurotransmission (Arancillo et al., 2013; Zarebidaki et al., 2020). Although mammalian and invertebrate loss-of-function mutants of presynaptic proteins continues to provide deep insights of their role in neurotransmitter release (Geppert et al., 1994; Schulze et al., 1995; Deng et al., 2011), a more sophisticated model where there is a control of protein amount has been proven to be a powerful approach. For instance, examining the Munc13-1 concentration dependency of priming showed that RIM boosts Munc13-1's priming function (Zarebidaki et al., 2020). Another example comes from the study of Syntaxin-1 (Stx1), where to titrate down Stx1's protein expression levels revealed that priming and vesicle fusion are likely governed by highly related mechanisms (Arancillo et al., 2013). Here, therefore we advocate for this experimental approach as a useful way to obtain a quantitative understanding of how the different elements of the presynaptic release machinery differently regulate the steps involved in the process of synaptic transmission.

Overall, to precisely understand the distinct functions of SYT1, variations in the expression and balance between SYT1 and SYT7 at SVs and the PM over neuronal stages should be taken into consideration. Also, our data demonstrated that both SYT1 and SYT7 are capable of SV priming and clamping. Likely the efficient execution of these functions relies on their intermolecular interactions with both SNAREs and phospholipids from the PM (Perin et al., 1990; Chapman et al., 1995; Li et al., 1995; Sugita et al., 2002; Zhou et al., 2017). Further study of how the interplay between these proteins affects the intrinsic speed of neurotransmitter release is needed. Moreover, we emphasize that when studying disorders associated with presynaptic protein mutations, all these parameters could be conditioning the quantitative aspects of the cellular phenotype and, in turn, the pathophysiology that derives from it.

References

- Alten B, Zhou Q, Shin OH, Esquivies L, Lin PY, White KI, Sun R, Chung WK, Monteggia LM, Brunger AT, Kavalali ET (2021) Role of Aberrant Spontaneous Neurotransmission in SNAP25-Associated Encephalopathies. *Neuron* 109(1):59-72.e5.

- 592 Arancillo M, Min S, Gerber S, Mu A, Wu Y, Herman M, Trimbuch T, Rah J, Ahnert-hilger G, Riedel D, Su TC
 593 (2013) Titration of Syntaxin1 in Mammalian Synapses Reveals Multiple Roles in Vesicle Docking, Priming,
 594 and Release Probability. *J Neurosci* 33:16698–16714.
- 595 Bacaj T, Wu D, Burré J, Malenka RC, Liu X, Südhof TC (2015) Synaptotagmin-1 and -7 Are Redundantly
 596 Essential for Maintaining the Capacity of the Readily-Releasable Pool of Synaptic Vesicles. *PLoS Biol*
 597 13(10):e1002267.
- 598 Bacaj T, Wu D, Yang X, Morishita W, Zhou P, Xu W, Malenka RC, Südhof TC (2013) Asynchronous Phases of
 599 Neurotransmitter Release. *Neuron* 80:947–959.
- 600 Baker K, Gordon SL, Melland H, Bumbak F, Scott DJ, Jiang TJ, Owen D, Turner BJ, Boyd SG, Rossi M, Al-
 601 Raqad M, Elpeleg O, Peck D, Mancini GMS, Wilke M, Zollino M, Marangi G, Weigand H, Borggraefe I,
 602 Haack T, Stark Z, Sadedin S; Broad Center for Mendelian Genomics, Tan TY, Jiang Y, Gibbs RA,
 603 Ellingwood S, Amaral M, Kelley W, Kurian MA, Cousin MA, Raymond FL (2018) SYT1-associated
 604 neurodevelopmental disorder: A case series. *Brain* 141:2576–2591.
- 605 Baker K, Gordon SL, Grozeva D, Van Kogelenberg M, Roberts NY, Pike M, Blair E, Hurles ME, Chong WK,
 606 Baldeweg T, Kurian MA, Boyd SG, Cousin MA, Raymond FL (2015) Identification of a human
 607 synaptotagmin-1 mutation that perturbs synaptic vesicle cycling. *J Clin Invest* 125:1670–1678.
- 608 Bekkers JM, Stevens CF (1991) Excitatory and inhibitory autaptic currents in isolated hippocampal neurons
 609 maintained in cell culture. *Proc Natl Acad Sci U S A* 88:7834–7838.
- 610 Bello OD, Jouannot O, Chaudhuri A, Stroeve E, Coleman J, Volynski KE, Rothman JE, Krishnakumar SS (2018)
 611 Synaptotagmin oligomerization is essential for calcium control of regulated exocytosis. *Proc Natl Acad Sci*
 612 *U S A* 115:E7624–E7631.
- 613 Bendahmane M, Morales A, Kreutzberger AJB, Schenk NA, Mohan R, Bakshi S, Philippe JM, Zhang S, Kiessling
 614 V, Tamm LK, Giovannucci DR, Jenkins PM, Anantharam A (2020) Synaptotagmin-7 enhances calcium-
 615 sensing of chromaffin cell granules and slows discharge of granule cargos. *J Neurochem* 154:598–617.
- 616 Bradberry MM, Courtney NA, Dominguez MJ, Lofquist SM, Knox AT, Sutton RB, Chapman ER (2020) Molecular
 617 Basis for Synaptotagmin-1-Associated Neurodevelopmental Disorder. *Neuron* 107:52-64.
- 618 Brodie K, Bellen HJ, DiAntonio A, Littleton JT, Schwarz TL (1994) Absence of synaptotagmin disrupts
 619 excitation-secretion coupling during synaptic transmission. *Proc Natl Acad Sci U S A* 91:10727–10731.
- 620 Brose N, Petrenko AG, Südhof TC, Jahn R (1992) Synaptotagmin: A calcium sensor on the synaptic vesicle
 621 surface. *Science* 256:1021–1025.
- 622 Chang S, Trimbuch T, Rosenmund C (2018) Synaptotagmin-1 drives synchronous Ca²⁺-triggered fusion by C2
 623 B-domain-mediated synaptic-vesicle-membrane attachment. *Nat Neurosci* 21:33–42.
- 624 Chapman ER, Davis AF (1998) Direct interaction of a Ca²⁺-binding loop of synaptotagmin with lipid bilayers. *J*
 625 *Biol Chem* 273:13995–14001.

- 626 Chapman ER, Hanson PI, An S, Jahn R (1995) Ca^{2+} regulates the interaction between synaptotagmin and
 627 syntaxin 1. *J Biol Chem* 270:23667–23671.
- 628 Chen C, Arai I, Satterfield R, Young SM, Jonas P (2017a) Synaptotagmin 2 Is the Fast Ca^{2+} Sensor at a Central
 629 Inhibitory Synapse. *Cell Rep* 18:723–736.
- 630 Chen C, Jonas P (2017) Synaptotagmins: That's Why So Many. *Neuron* 94:694–696.
- 631 Chen C, Satterfield R, Young SM, Jonas P (2017b) Triple Function of Synaptotagmin 7 Ensures Efficiency of
 632 High-Frequency Transmission at Central GABAergic Synapses. *Cell Rep* 21:2082–2089.
- 633 Chicka MC, Hui E, Liu H, Chapman ER (2008) Synaptotagmin arrests the SNARE complex before triggering fast,
 634 efficient membrane fusion in response to Ca^{2+} . *Nat Struct Mol Biol* 15:827–835.
- 635 de Wit H, Walter AM, Milosevic I, Gulyás-Kovács A, Riedel D, Sørensen JB, Verhage M (2009) Synaptotagmin-1
 636 Docks Secretory Vesicles to Syntaxin-1/SNAP-25 Acceptor Complexes. *Cell* 138:935–946.
- 637 Deng L, Kaeser PS, Xu W, Südhof TC (2011) RIM proteins activate vesicle priming by reversing autoinhibitory
 638 homodimerization of munc13. *Neuron* 69:317–331.
- 639 DiAntonio A, Schwarz TL (1994) The effect on synaptic physiology of synaptotagmin mutations in drosophila.
 640 *Neuron* 12:909–920.
- 641 Fernández-Chacón R, Königstorfer A, Gerber SH, García J, Matos MF, Stevens CF, Brose N, Rizo J,
 642 Rosenmund C, Südhof TC (2001) Synaptotagmin I functions as a calcium regulator of release probability.
 643 *Nature* 410:41–49.
- 644 Fernandez I, Araç D, Ubach J, Gerber SH, Shin OH, Gao Y, Anderson RGW, Südhof TC, Rizo J (2001) Three-
 645 dimensional structure of the synaptotagmin 1 C2B-domain: Synaptotagmin 1 as a phospholipid binding
 646 machine. *Neuron* 32:1057–1069.
- 647 Fujii T, Sakurai A, Littleton JT, Yoshihara M (2021) Synaptotagmin 7 switches short-term synaptic plasticity from
 648 depression to facilitation by suppressing synaptic transmission. *Sci Rep* 11:1–9.
- 649 Geppert M, Archer BT, Südhof TC (1991) Synaptotagmin II: A novel differentially distributed form of
 650 synaptotagmin. *J Biol Chem* 266:13548–13552.
- 651 Geppert M, Goda Y, Hammer RE, Li C, Rosahl TW, Stevens CF, Südhof TC (1994) Synaptotagmin I: a major
 652 Ca^{2+} sensor for transmitter release at a central synapse. *Cell* 79:717–727.
- 653 Huson V, Meijer M, Dekker R, Veer M Ter, Ruiter M, van Weering J, Verhage M, Cornelisse LN (2020) Post-
 654 tetanic potentiation lowers the energy barrier for synaptic vesicle fusion independently of synaptotagmin-1.
 655 *Elife* 9:1–56.
- 656 Imig C, Min SW, Krinner S, Arancillo M, Rosenmund C, Südhof TC, Rhee JS, Brose N, Cooper BH (2014) The
 657 Morphological and Molecular Nature of Synaptic Vesicle Priming at Presynaptic Active Zones. *Neuron*
 658 84:416–431.
- 659 Jackman SL, Regehr WG (2017) The Mechanisms and Functions of Synaptic Facilitation. *Neuron* 94:447–464.

- 660 Jackman SL, Turecek J, Belinsky JE, Regehr WG (2016) The calcium sensor synaptotagmin 7 is required for
661 synaptic facilitation. *Nature* 529:88–91
- 662 Jorgensen EM, Hartweg E, Schuske K, Nonet ML, Jin Y, Horvitz HR (1995) Defective recycling of synaptic
663 vesicles in synaptotagmin mutants of *Caenorhabditis elegans*. *Nature* 378:196–199.
- 664 Katz B (1969) The release of neural transmitter substances (Book, 1969). Springfield, Ill, Thomas.
- 665 Kedar GH, Munch AS, van Weering JRT, Malsam J, Scheutzw A, de Wit H, Houy S, Tawfik B, Söllner TH,
666 Sørensen JB, Verhage M (2015) A post-docking role of synaptotagmin 1-C2B domain bottom residues
667 R398/399 in mouse chromaffin cells. *J Neurosci* 35:14172–14182.
- 668 Kochubey O, Babai N, Schneggenburger R (2016) A Synaptotagmin Isoform Switch during the Development of
669 an Identified CNS Synapse. *Neuron* 90:984–999.
- 670 Li C, Ullrich B, Zhang JZ, Anderson RGW, Brose N, Südhof TC (1995) Ca^{2+} -dependent and -independent
671 activities of neural and non-neural synaptotagmins. *Nature* 246:170.
- 672 Liang K, Wei L, Chen L (2017) Exocytosis, endocytosis, and their coupling in excitable cells. *Front Mol Neurosci*
673 10:1–10.
- 674 Littleton JT, Stern M, Perin M, Bellen HJ (1994) Calcium dependence of neurotransmitter release and rate of
675 spontaneous vesicle fusions are altered in *Drosophila* synaptotagmin mutants. *Proc Natl Acad Sci U S A*
676 91:10888–10892.
- 677 Liu H, Bai H, Hui E, Yang L, Evans CS, Wang Z, Kwon SE, Chapman ER (2014) Synaptotagmin 7 functions as a
678 Ca^{2+} -sensor for synaptic vesicle replenishment. *Elife* 3:1–18.
- 679 Liu H, Dean C, Arthur CP, Dong M, Chapman ER (2009) Autapses and networks of hippocampal neurons exhibit
680 distinct synaptic transmission phenotypes in the absence of synaptotagmin I. *J Neurosci* 29:7395–7403
- 681 Lois C, Hong EJ, Pease S, Brown EJ, Baltimore D (2002) Germline transmission and tissue-specific expression
682 of transgenes delivered by lentiviral vectors. *Science* 295:868–872.
- 683 Luo F, Bacaj T, Südhof TC (2015) Synaptotagmin-7 is essential for Ca^{2+} -triggered delayed asynchronous
684 release but not for Ca^{2+} -dependent vesicle priming in retinal ribbon synapses. *J Neurosci* 35:11024–
685 11033.
- 686 Mackler J, Drummond J, Loewen C, Robinson I, Reist N (2002) The C(2)B Ca^{2+} -binding motif of synaptotagmin
687 is required for synaptic transmission in vivo. *Nature* 418:340–344.
- 688 Marquère B, Boudier JA, Mizuta M, Inagaki N, Seino S, Seagar M (1995) Cellular localization of synaptotagmin I,
689 II, and III mRNAs in the central nervous system and pituitary and adrenal glands of the rat. *J Neurosci*
690 15:4906–4917.
- 691 Maximov A, Lao Y, Li H, Chen X, Rizo J, Sørensen JB, Südhof TC (2008) Genetic analysis of synaptotagmin-7
692 function in synaptic vesicle exocytosis. *Proc Natl Acad Sci U S A* 105:3986–3991.
- 693 Maximov A, Südhof TC (2005) Autonomous function of synaptotagmin 1 in triggering synchronous release

- 694 independent of asynchronous release. *Neuron* 48:547–554.
- 695 Nagy G, Kim JH, Pang ZP, Matti U, Rettig J, Südhof TC, Sørensen JB (2006) Different effects on fast exocytosis
696 induced by synaptotagmin 1 and 2 isoforms and abundance but not by phosphorylation. *J Neurosci*
697 26:632–643.
- 698 Nishiki TI, Augustine GJ (2004) Synaptotagmin I synchronizes transmitter release in mouse hippocampal
699 neurons. *J Neurosci* 24:6127–6132.
- 700 Pang ZP, Sun J, Rizo J, Maximov A, Südhof TC (2006) Genetic analysis of synaptotagmin 2 in spontaneous and
701 Ca²⁺-triggered neurotransmitter release. *EMBO J* 25:2039–2050.
- 702 Perin MS, Fried VA, Mignery GA, Jahn R, Südhof TC (1990) Phospholipid binding by a synaptic vesicle protein
703 homologous to the regulatory region of protein kinase C. *Nature* 345:260–263.
- 704 Poskanzer KE, Marek KW, Sweeney ST, Davis GW (2003) Synaptotagmin I is necessary for compensatory
705 synaptic vesicle endocytosis in vivo. *Nature* 426:559–563.
- 706 Reist NE, Buchanan J, Li J, DiAntonio A, Buxton EM, Schwarz TL (1998) Morphologically docked synaptic
707 vesicles are reduced in synaptotagmin mutants of *Drosophila*. *J Neurosci* 18:7662–7673.
- 708 Rizo J, Xu J (2015) The Synaptic Vesicle Release Machinery. *Annu Rev Biophys* 44:339–367.
- 709 Rosenmund C, Stevens CF (1996) Definition of the readily releasable pool of vesicles at hippocampal synapses.
710 *Neuron* 16:1197–1207.
- 711 Sabatini BL, Regehr WG (1999) Timing of synaptic transmission. *Annu Rev Physiol* 61:521–542.
- 712 Schonh JS, Maximov A, Lao Y, Südhof TC, Sørensen JB (2008) Synaptotagmin-1 and -7 are functionally
713 overlapping Ca²⁺ sensors for exocytosis in adrenal chromaffin cells. *Proc Natl Acad Sci U S A* 105:3998–
714 4003.
- 715 Schotten S, Meijer M, Walter AM, Huson V, Mamer L, Kalogreades L, Ter Veer M, Ruiter M, Brose N,
716 Rosenmund C, Sørensen JB, Verhage M, Cornelisse LN (2015) Additive effects on the energy barrier for
717 synaptic vesicle fusion cause supralinear effects on the vesicle fusion rate. *Elife* 2015:1–25.
- 718 Schulze KL, Broadie K, Perin MS, Bellen HJ (1995) Genetic and electrophysiological studies of *drosophila*
719 syntaxin-1A demonstrate its role in nonneuronal secretion and neurotransmission. *Cell* 80:311–320.
- 720 Shin OH, Rizo J, Südhof TC (2002) Synaptotagmin function in dense core vesicle exocytosis studied in cracked
721 pc12 cells. *Nat Neurosci* 5:649–656.
- 722 Silva M, Tran V, Marty A (2021) Calcium-dependent docking of synaptic vesicles. *Trends Neurosci.* 44(7):579-
723 592.
- 724 Sugita S, Han W, Butz S, Lao Y, Liu X, Fera R, Su TC, Hines H, Na B (2001) Synaptotagmin VII as a plasma
725 membrane Ca(2+) sensor in exocytosis. *Neuron* 30:459–473.
- 726 Sugita S, Shin OH, Han W, Lao Y, Südhof TC (2002) Synaptotagmins form a hierarchy of exocytotic Ca²⁺
727 sensors with distinct Ca²⁺ affinities. *EMBO J* 21:270–280.

- 728 Sutton RB, Davletov BA, Berghuis AM, Sudhof TC, Sprang SR (1995) Structure of the first C2 domain of
 729 synaptotagmin I: A novel Ca²⁺/phospholipid-binding fold. *Cell* 80:929–938.
- 730 Tagliatti E, Bello OD, Mendonça PRF, Kotzadimitriou D, Nicholson E, Coleman J, Timofeeva Y, Rothman JE,
 731 Krishnakumar SS, Volynski KE (2020) Synaptotagmin 1 oligomers clamp and regulate different modes of
 732 neurotransmitter release. *Proc Natl Acad Sci U S A* 117:3819–3827.
- 733 Turecek J, Regehr WG (2018) Synaptotagmin 7 mediates both facilitation and asynchronous release at granule
 734 cell synapses. *J Neurosci* 38:3240–3251.
- 735 Watanabe S, Trimbuch T, Camacho-Pérez M, Rost BR, Brokowski B, Söhl-Kielczynski B, Felies A, Davis MW,
 736 Rosenmund C, Jorgensen EM (2014) Clathrin regenerates synaptic vesicles from endosomes. *Nature*
 737 515:228–233.
- 738 Wen H, Linhoff MW, McGinley MJ, Li GL, Corson GM, Mandel G, Brehm P (2010) Distinct roles for two
 739 synaptotagmin isoforms in synchronous and asynchronous transmitter release at zebrafish neuromuscular
 740 junction. *Proc Natl Acad Sci U S A* 107:13906–13911.
- 741 Wierda KDB, Sørensen JB (2014) Innervation by a GABAergic neuron depresses spontaneous release in
 742 glutamatergic neurons and unveils the clamping phenotype of synaptotagmin-1. *J Neurosci* 34:2100–2110.
- 743 Xu J, Pang ZP, Shin OH, Südhof TC (2009) Synaptotagmin-1 functions as a Ca²⁺ sensor for spontaneous
 744 release. *Nat Neurosci* 12:759–766.
- 745 Xue M, Ma C, Craig TK, Rosenmund C, Rizo J (2008) The Janus-Faced Nature of the C 2 B Domain Is
 746 Fundamental for Synaptotagmin-1 Function. *Nat Struct Mol Biol* 15:1160–1168.
- 747 Xue M, Reim K, Chen X, Chao H, Deng H, Rizo J, Brose N, Rosenmund C (2007) Distinct domains of complexin
 748 I differentially regulate neurotransmitter release. *Nat. Struct Mol Biol.* 14:949–958.
- 749 Yao J, Kwon SE, Gaffaney JD, Dunning FM, Chapman ER (2012) Uncoupling the roles of synaptotagmin I during
 750 endo-and exocytosis of synaptic vesicles. *Nat Neurosci* 15:243–249.
- 751 Yoshihara M, Littleton JT (2002) Synaptotagmin functions as a calcium sensor to synchronize neurotransmitter
 752 release. *Neuron* 36:897–908.
- 753 Zarebidaki F, Camacho M, Brockmann MM, Trimbuch T, Herman MA, Rosenmund C (2020) Disentangling the
 754 roles of RIM and Munc13 in synaptic vesicle localization and neurotransmission. *J Neurosci* 40:9372–9385.
- 755 Zhou Q, Zhou P, Wang AL, Wu D, Zhao M, Südhof TC, Brunger AT (2017) The primed SNARE – complexin –
 756 synaptotagmin complex for neuronal exocytosis. *Nature* 548:420–425.

757 **Figures/Extended data/Table legends**

758 **Figure 1. Electrophysiological characterization of *Synaptotagmin-1* knockout hippocampal glutamatergic autaptic**
 759 **neurons at different time points. (A)** Representative sucrose-evoked current traces (left) and summary bar graphs (right) of
 760 total current charge of *Syt1*^{+/+} (black) and *Syt1*^{-/-} (red) neurons evoked by 0.5 M sucrose solution for 5 s at early (DIV11–12),

intermediate (DIV15-16) and late (DIV20-21) culture stages. **(B)** Example traces of spontaneous release events (right) and summary bar graphs of spontaneous release rate (left) calculated by dividing the mEPSC frequency by the number of primed synaptic vesicles at different neuronal culture stages. **(C)** Representative EPSC traces (left) and summary bar graphs of EPSC amplitudes (right) evoked by a 2 ms depolarization in 2 mM extracellular Ca^{2+} from autaptic neurons at the three neuronal stages from *Syt1*^{+/+} (black) and *Syt1*^{-/-} (red). Action potentials were blanked for better EPSC illustration and substituted by arrows. **(D)** Plot of vesicular release probability (Pvr) in % calculated by dividing the evoked EPSC charge by the sucrose charge at the different neuronal stages. Data are mean $\pm$ SEM. Statistical significance and p values were calculated using the Mann-Whitney *U* test (****P*≤0.001, *****P*≤0.0001, ns = not significant).

Figure 2. Evaluation of the synaptic SYT1 functions in *Syt1*^{-/-} neurons rescued with SYT1. All immunocytochemistry and electrophysiological experiments were done from *Syt1*^{+/+} and *Syt1*^{-/-} hippocampal glutamatergic autaptic neurons at DIV15-21. **(A)** Representative images of hippocampal glutamatergic autaptic neurons of *Syt1*^{-/-} infected with a lentivirus containing a nuclear GFP vector (-) or *Syt1*. **(B)** Summary bar graph showing SYT1/VGLUT1 fluorescence normalized to *Syt1*^{+/+} (right). Summary bar plot of VGLUT1 fluorescence (left) and normalized summary bar plot of SYT1/VGLUT1 fluorescence intensity. **(C)** Bar plot of RRP size estimated by application of a hypertonic solution of *Syt1*^{-/-} neurons rescued with SYT1 normalized to the *Syt1*^{+/+} control. **(D)** Summary bar plot of spontaneous release rate from *Syt1*^{-/-} neurons rescued with SYT1 normalized to the *Syt1*^{+/+} control. **(E)** Sample traces of EPSCs (right) and summary bar plot of the quantification of total EPSC charge transfer (left) from *Syt1*^{-/-} neurons rescued with SYT1 normalized to the *Syt1*^{+/+} control. Artifacts and/or action potentials were blanked and substituted by arrows. **(F)** Bar plot of Pvr from *Syt1*^{-/-} neurons rescued with SYT1 normalized to *Syt1*^{+/+} control. **(G)** Graph of the paired-pulse ratio. All data shown represent mean $\pm$ SEM. Statistical analysis was applied by Kruskal-Wallis test (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001, ns = not significant). Scale bars = 5 μm .

Figure 3. Presynaptic SYT7 quantification of Synaptotagmin-7 in presence and absence of SYT1 and its silencing effect in *Syt1*^{-/-} hippocampal glutamatergic neurons at different neuronal stages. **(A)** Representative images and **(B)** quantification of SYT1 (green) and SYT7 (blue) protein levels at VGLUT1 (red) positive regions at DIV11 and DIV16 of *Syt1*^{+/+} and *Syt1*^{-/-} neurons. **(C)** Quantification of SYT1, SYT7 and VGLUT1 from *Syt1*^{-/-} autaptic neurons infected with lentivirus carrying either an empty vector or *RNA interference (RNAi)* for SYT7 at early and mature stages. Data are mean $\pm$ SEM. Statistical significance and p values were calculated using the Mann-Whitney *U* test (***P*≤0.01, ****P*≤0.001, *****P*≤0.0001, ns = not significant). Scale bars = 5 μm .

Figure 4. Knockdown of Synaptotagmin-7 in *Syt1*^{-/-} hippocampal glutamatergic neurons at different neuronal stages. **(A)** Representative traces at early (DIV11-12) and intermediate (DIV15-16) autaptic neuronal stages (right) and summary bar graphs (left) of the charge of sucrose evoked release of *Syt1*^{+/+} (black), *Syt1*^{-/-} (red) and *Syt1*^{-/-} neurons infected with lentivirus containing *RNA interference (RNAi)* for silencing SYT7 (purple) **(B)** Example traces of spontaneous release events (right) and summary bar graphs of spontaneous release rate (left) at the two different neuronal stages as A. **(C)** Representative EPSC traces (right) and summary bar graphs of total EPSC charge measured over an interval of 1s (left). **(D)** Summary bar graphs of vesicular release probability. Data are mean $\pm$ SEM. Statistical significance and p values were estimated by a Kruskal-Wallis test (**P*≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001, ns = not significant).

Figure 5. Evaluation of SV priming and neurotransmitter release in Synaptotagmin-1 heterozygous neurons. All immunocytochemistry and electrophysiological experiments were done from *Syt1*^{+/+}, *Syt1*^{+/+} and *Syt1*^{-/-} hippocampal

glutamatergic autaptic neurons at DIV15-21. **(A)** Sample images from *Syt1^{+/+}*, *Syt1^{+/-}* and *Syt1^{-/-}* hippocampal glutamatergic autaptic neurons double labeled with VGLUT1 (black puncta, left panel), SYT1 (black puncta middle panel) antibodies and colocalization image of SYT1 in green and VGLUT1 in red (right merge panel), demonstrating the presence of Synaptotagmin-1 at the presynaptic terminals. Scale bar, 5 μ m. **(B)** Bar plot of VGLUT1 average fluorescence intensities at presynaptic terminal for the three *Syt-1* genotypes (left), normalized to SYT1 expression levels of the *Syt1^{+/+}* neurons. Summary bar plot of SYT1 average fluorescence intensity at VGLUT1 positives puncta normalized to SYT1 average fluorescence intensity of *Syt1^{+/+}* (right). **(C)** Sample traces of sucrose responses (left) for the three *Syt-1* genotypes. Bar graph of sucrose evoked current charges of *Syt1^{+/+}* (black), *Syt1^{+/-}* (orange) and *Syt1^{-/-}* (cayenne) autaptic neurons (right). **(D)** Example traces of spontaneous release events (left) and 106 mEPSC events superimposed from *Syt1^{+/+}*, 198 mEPSC events superimposed from *Syt1^{+/-}* and 257 mEPSC events superimposed from *Syt1^{-/-}* of a recording time period of 3 seconds in a 15ms time window (center). Summary bar graph (right) of the spontaneous release rate of mEPSC events. **(E)** Representative EPSC traces (left) and summary bar graphs (right) of EPSC amplitudes. **(F)** Summary bar graph of total EPSC charge transfer. **(G)** Summary bar plot of the rise time (20-80%) of EPSCs. **(H)** Bar graphs of the relative contribution of the synchronic and asynchronic components to the EPSC charge. **(I)** Bar plot of the average vesicular release probability of *Syt1^{+/+}*, *Syt1^{+/-}* and *Syt1^{-/-}*. **(J)** Summary graph of the average pair-pulse ratio plotted against interpulse interval from 25 to 100 ms corresponding to 40, 20 and 10Hz of *Syt1^{+/+}* (black), *Syt1^{+/-}* (orange) and *Syt1^{-/-}* (cayenne) autaptic neurons. **(K)** Short-term plasticity response during high frequency (20Hz) stimulation. Representative sample traces of EPSCs resulted from the stimulation of 20 consecutives APs separated by 50 ms (right) and plots of mean EPSC amplitudes normalized to the first EPSC of *Syt1^{+/+}* (black), *Syt1^{+/-}* (orange) and *Syt1^{-/-}* (cayenne) during train stimulation. Artifacts and action potentials were blanked from the traces and substituted by arrows in E and K. Data are mean $\pm$ SEM. Statistical significances and p values were obtained by Kruskal-Wallis test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, ns = not significant). Scale bars = 5 μ m.

Figure 6. Impact of Synaptotagmin-1 overexpression on synaptic properties. All immunocytochemistry and electrophysiological experiments were done from *Syt1^{+/+}* hippocampal glutamatergic autaptic neurons at DIV15-21. **(A)** Representative images of *Syt1^{+/+}* autaptic neurons with and without overexpression of SYT1, co-immunolabeled for VGLUT1 (left), SYT1 (middle) and merge image (right), showing the presence of VGLUT1 in red and SYT1 in green. Scale bar, 5 μ m. Quantification graphs of normalized VGLUT1 fluorescence intensities and SYT1/VGLUT1 fluorescence intensity ratios of *Syt1^{+/+}* hippocampal neurons overexpressing GFP vector (black) or SYT1 (olive green) (right). **(B)** Representative sucrose-evoked current traces (left) and summary bar plot of RRP size (right). **(C)** Example traces of spontaneous release events (left) and summary bar graph (right) of spontaneous release rate. **(D)** Sample EPSC traces (left) and bar plot of EPSC amplitude (right). **(E)** Summary bar graph of average probability of vesicle release. **(F)** Bar graph of paired-pulse ratio (PPR) at 40Hz (left). Representative sample traces of EPSCs resulted from the stimulation of 2 consecutives APs separated by 25 ms (right). Artifacts and/or action potentials are blanked and substituted by arrows in **D** and **F**. All data shown represent mean $\pm$ SEM. Statistical analysis was applied Mann-Whitney *U* test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, ns = not significant). Scale bars = 5 μ m.

Figure 7. Dose dependence of Synaptotagmin-1 on neurotransmitter release functions. All immunocytochemistry and electrophysiological experiments were done from *Syt1^{+/+}* hippocampal glutamatergic autaptic neurons at DIV15-21. **(A)** Representative images immunolabeled for VGLUT and SYT1 as figure 6, from *Syt1^{+/+}* hippocampal glutamatergic autaptic

neurons infected with a lentiviral vector expressing a scramble RNA (control) or increasing amounts of a *Syt1* RNA interference (RNAi). Scale bar, 5 μ m. Summary bar graphs of VGLUT1 fluorescence intensities at presynaptic terminal (left) and SYT1/VGLUT1 fluorescence intensities ratios normalized to *Syt1*^{+/+} (right). (B) Plot of sucrose charges of *Syt1*^{+/+} control and increasingly Knockdown SYT1 autaptic neurons normalized to the average *Syt1*^{+/+} control sucrose charge. (C) Summary bar graph of spontaneous release rate normalized to control *Syt1*^{+/+}. (D) Normalized bar graphs of the effect on evoked EPSC charge of titration of SYT1. (E) Bar graphs of the relative contribution of the synchronic and asynchronic components to the EPSC total charge transfer with different amount of SYT1 expression levels. (F) Vesicular release probability (Pvr) normalized to *Syt1*^{+/+}. (G) Normalized summary graph of the pair-pulse stimulation at 40Hz. Statistical analysis was applied by Kruskal-Wallis test (*P $\leq$ 0.05, **P $\leq$ 0.01, ***P $\leq$ 0.001, ****P $\leq$ 0.0001, ns = not significant). (H-K) Plots of RRP size (H), spontaneous release rate (I), EPSC charge (J) and Pvr (K) normalized to *Syt1*^{+/+} against the SYT1/VGLUT1 fluorescence intensity ratios, obtained from the titration of SYT1 in autaptic neuronal cultures. To calculate SYT1 expression at synapses: *Syt1*^{+/+} (black), *Syt1*^{-/-} (orange) and *Syt1*^{-/-} (burgundy) data (Figure 5) and *Syt1*^{-/-} + RNAi (SYT7) (purple) data (Figure 4, DIV16) were normalized to *Syt1*^{+/+} and subtracting the *Syt1*^{-/-} expression levels in the different experiments. For *Syt1*^{+/+} with the different amounts of *Syt1* RNAi (dark grey) and *Syt1*^{+/+} + SYT1 (light grey) data (Figures 6 and 7) SYT1 expression levels were normalized to *Syt1*^{+/+}. All cells used for the model were from DIV15-21. *Syt1*^{-/-} + RNAi (SYT7) cells were excluded from the model, but the group is represented in the plot to illustrate its functional relevance. Discontinuous grey-line represent the curve-fitting of the Hill function. All data shown represent mean $\pm$ SEM. N data. Scale bars = 5 μ m. For H-K data point values, see also the table in Figure 7-1.

Figure 8. Impact of SYT1 RNAi viral titer on synaptic function in Synaptotagmin-1^{-/-} neurons. All immunocytochemistry and electrophysiological experiments were done from *Syt1*^{+/+} hippocampal glutamatergic autaptic neurons at DIV15-21. (A) Representative images of *Syt1*^{+/+} cultured hippocampal glutamatergic neurons infected with a lentiviral vector expressing either RNA interference (RNAi) or scramble RNA as control. Scale bar, 5 μ m. Summary bar graph of VGLUT1 (left) fluorescence at synaptic terminal. Summary bar graph showing SYT1/VGLUT1 fluorescence at the synaptic terminal normalized to *Syt1*^{+/+} (right). (B) Normalized bar graph of sucrose charge currents from autaptic cultures of *Syt1*^{+/+} neurons with scRNA (control) or RNA interference. (C) Summary bar graph of spontaneous release rate. (D) Normalized bar graphs of the effect of RNA interference on evoked EPSC charge. (E) Bar graphs of the relative contribution of the synchronic and asynchronic components to the EPSC total charge transfer after a single AP with different amount of SYT1 levels. (F) Vesicular release probability (Pvr) was calculated by the ratio of evoked EPSC charge and RRP size and normalized to *Syt1*^{+/+}. (G) Normalized summary graph of the pair-pulse stimulation at 40Hz. Statistical analysis was applied by Kruskal-Wallis test (*P $\leq$ 0.05, **P $\leq$ 0.01, ***P $\leq$ 0.001, ****P $\leq$ 0.0001, ns = not significant). All data shown represents mean $\pm$ SEM. Scale bars = 5 μ m.

Table 1. Summary table with the electrophysiological parameters measured in autaptic hippocampal glutamatergic neurons related to the culture stage. *Pvr tendency towards an increased value, confirmed by STP experiment.

Figure 7-1. Values corresponding to Figure 7-1(H-K).

Table 1. Summary table with the electrophysiological parameters measured in autaptic hippocampal glutamatergic neurons related to the culture stage

		SYT1-/-			SYT1-/- +SYT7KD				
		DIV			DIV		SYT1 Expression (DIV15-21)		
		11-12	15-16	20-21	11-12	15-16	<50%	50-100%	>100%
Synaptic Transmission Parameter	RRP	=	Reduced	Reduced	Reduced	Reduced	Reduced	=	=
	Spontaneous Release	=	Increased	Increased	Increased	Increased	Increased	Increased	=
	Evoked Release	Reduced	Reduced	Reduced	Reduced	Reduced	Reduced	=	=
	Pvr	Reduced	Reduced	Reduced	=	=	Reduced	Reduced	*Increased

*Pvr tendency towards an increased value, confirmed by STP experiment