

Table S1

For statistical analysis, all groups were firstly subjected to D'Agostino and Pearson omnibus normality testing and further subjected to two-sided unpaired student's t-test or one-way analysis of variance (ANOVA) followed by *post hoc* Dunnnet's multiple comparison test for normally distributed data or Mann-Whitney test and Kruskal-Wallis ANOVA followed by *post hoc* Dunn's multiple comparison test for non-normally distributed data. P values are displayed in the table. The statistical significant groups are marked in gray.

A: Number of docked vesicle (0 – 5 nm from the membrane) per 500 nm AZ
(Kruskal-Wallis ANOVA followed by *post hoc* Dunn's multiple comparison test; WT vs. mutants). n represents the number of electron micrographs (synapses) analyzed

Fig. 1I	Resting		
	Mean±SEM	n	p-value
Syt1 WT	1.92±0.11	254	-
Syt1 KO	1.24±0.10	210	< 0.0001
FL	2.01±0.14	102	> 0.9999
D309, 363, 365A	2.17±0.13	193	> 0.9999
K325, 327A	1.01±0.08	235	< 0.0001
R398, 399Q	0.89±0.08	197	< 0.0001

B: Number of normalized total SVs per μm^2
(Kruskal-Wallis ANOVA followed by *post hoc* Dunn's multiple comparison test; WT vs. mutants). n represents the number of electron micrographs (synapses) analyzed.

Fig. 1J; S3I	Resting		
	Mean±SEM	n	p-value
Syt1 WT	141±3.54	254	-
Syt1 KO	1.24±0.10	210	> 0.9999
FL	138±4.93	102	> 0.9999
D309, 363, 365A	131±3.48	193	> 0.9999
K325, 327A	139±3.27	235	> 0.9999

R398, 399Q	133±3.28	197	> 0.9999
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C: Number of vesicles (0 – 5nm from the membrane) per 500 nm AZ
(unpaired two-tailed Mann-Whitney test before vs. 10ms after AP),
n represents the number of electron micrographs (synapses) analyzed

Fig. 1L;S2A-E	before			after AP			
	Mean±SEM	n	Normalized	Mean±SEM	Normalized	n	p-value
Syt1 WT	1.92±0.11	254	1±0.06	1.45±0.13	0.76±0.07	148	0.0029
Cd ²⁺ Syt1WT	1.36±0.13	114	1±0.09	1.43±0.13	1.05±0.10	100	0.6929
Syt1 KO	1.24±0.10	210	1±0.08	1.37±0.16	1.11±0.13	94	0.5042
D309, 363, 365A	2.17±0.13	193	1±0.06	2.00±0.18	0.92±0.08	133	0.2387
K325, 327A	1.01±0.08	235	1±0.08	1.41±0.10	1.40±0.10	184	0.0017
R398, 399Q	0.89±0.08	197	1±0.09	1.48±0.14	1.67±0.16	149	0.0009
Cd ²⁺ K325,327A	1.19±0.10	152	1±0.09	1.19±0.11	0.99±0.10	154	0.7286
Cd ²⁺ R398,399Q	0.86±0.10	147	1±0.11	0.74±0.08	0.86±0.10	143	0.6006
6DA K325,327A	0.88±0.09	182	1±0.10	0.96±0.09	1.04±0.09	179	0.4184
6DA R398,399Q	0.94±0.08	144	1±0.09	1.10±0.10	1.18±0.10	148	0.4522

D: Number of vesicles (0 – 5 nm from the membrane) per 500nm AZ
(unpaired two-tailed Mann-Whitney test; 10 ms vs. before; 10 ms vs. 100 ms; 10 ms vs. 500ms after AP) n represents the number of electron micrographs (synapses) analyzed.

Fig. 2C	Mean±SEM	n	Normalized to resting	p-value
R398, 399Q	0.68±0.07	126	1.00±0.11	0.0038

10ms R398, 399Q	1.10±0.10	131	1.61±0.15	-
100ms R398, 399Q	0.79±0.08	125	1.15±0.12	0.0344
500ms R398, 399Q	0.69±0.09	127	1.01±0.13	0.0008

E: EPSC amplitudes and kinetics

(unpaired two-tailed Mann-Whitney test; 1st EPSC vs. 2nd EPSC, 100Hz)

For Fig. 3C, the paired-pulse ratio were compared between Syt1 KO and R398, 399Q. For weighted decay time constant, the Syt1 KO group was tested using unpaired two-tailed t-test. n represents the number cells analyzed.

	1 st EPSC		2 nd EPSC		
Fig. 3B Amplitudes (nA)	Mean±SEM	n	Mean±SEM	n	p-value
Syt1 KO	-0.14±0.17	46	-0.50±0.60	46	< 0.0001
R398, 399Q	-0.22±0.21	64	-1.09±0.93	64	< 0.0001
Fig. 3C Paired-pulse ratio (PPR)					
Syt1 KO	3.70±0.18	45			-
R398, 399Q	5.61±0.27	63			< 0.0001
Rise time (ms)					
Syt1 KO	5.50±0.63	46	3.40±0.28	41	0.0061
R398, 399Q	2.92±0.39	62	1.28±0.11	58	< 0.0001
Half width (ms)					
Syt1 KO	23.97±2.63	46	38.00±4.19	46	0.0009
R398, 399Q	25.02±4.54	64	14.08±0.96	64	0.0009
Fig. 3E Weighted decay (ms)					
Syt1 KO	109.3±10.28	46	98.42±7.25	46	0.3894
R398, 399Q	98.46±9.64	64	48.59±2.84	64	< 0.0001

F: Decay kinetics of 2nd EPSC
(unpaired two-tailed Mann-Whitney test; Syt1 KO vs. R398, 399Q)
n represents the number cells analyzed.

Fig. 3E	Mean±SEM	n	
	Fast tau (ms)		
Syt1 KO	41.16±5.54	46	-
R398, 399Q	12.17±1.26	64	< 0.0001
	Percentage		
Syt1 KO	0.59±0.05	46	-
R398, 399Q	0.73±0.01	64	0.313
	Slow tau (ms)		
Syt1 KO	228.9±20.84	46	-
R398, 399Q	147.0±6.49	63	0.002
	Percentage		
Syt1 KO	0.41±0.05	46	-
R398, 399Q	0.27±0.01	64	0.3786

G: mEPSC amplitudes and kinetics
(unpaired two-tailed Mann-Whitney test; Syt1 KO vs. R398, 399Q)
n represents the number cells analyzed

Amplitudes (pA)	Mean±SEM	n	p-value
Syt1 KO	-25.31±1.14	47	-
R398, 399Q	-27.77±1.24	64	0.1624
Rise time (ms)			
Syt1 KO	0.47±0.01	47	-
R398, 399Q	0.44±0.01	64	0.0376
Half width (ms)			

Syt1 KO	3.05±0.05	47	-
R398, 399Q	2.87±0.08	64	0.0041
Decay time constant (ms)			
Syt1 KO	2.32±0.05	44	-
R398, 399Q	2.17±0.07	59	0.0035

H: Synchronicity ratio (EPSC2/EPSC1) (unpaired two-tailed Mann-Whitney test; 10 ms vs. 25/100/500 ms after AP) n represents the number cells analyzed

Fig. 3G	Normalized	n	p-value
10 ms R398, 399Q	1.95±0.10	34	-
25 ms R398, 399Q	1.78±0.07	51	> 0.3413
100 ms R398, 399Q	1.20±0.04	49	< 0.0001
500 ms R398, 399Q	1.18±0.05	47	< 0.0001

I: Paired-pulse ratio (EPSC2/EPSC1) (unpaired two-tailed student's t test; 10 ms vs. 25/100/500 ms after AP) n represents the number cells analyzed

Fig. 3G		n	p-value
10 ms R398, 399Q	5.81±0.27	34	-
25 ms R398, 399Q	4.01±0.18	51	< 0.0001
100 ms R398, 399Q	1.88±0.06	49	< 0.0001
500 ms R398, 399Q	1.48±0.05	47	< 0.0001

J: Decay kinetics of 2nd EPSC in 1/2/4/10 mM Ca²⁺ in R398, 399Q.
(unpaired two-tailed Mann-Whitney test; 10 ms 1Ca²⁺ vs. 2Ca²⁺/4Ca²⁺/10Ca²⁺)
n represents the number cells analyzed.

Fig. 3I	Normalized	n	p-value
1 Ca ²⁺ R398, 399Q	54.52±8.03	36	-
2 Ca ²⁺ R398, 399Q	51.46±5.90	39	0.7881
4 Ca ²⁺ R398, 399Q	28.33±2.59	41	< 0.0001
10 Ca ²⁺ R398, 399Q	23.55±2.26	27	< 0.0001

K: Decay kinetics of 2nd EPSC in 1/2/4/10 mM Ca²⁺ in wild-type FL.
(unpaired two-tailed Mann-Whitney test; 10 ms 1Ca²⁺ vs. 2Ca²⁺/4Ca²⁺/10Ca²⁺)
n represents the number cells analyzed.

Fig. 3I	Normalized	n	p-value
1 Ca ²⁺ FL	7.62±0.54	31	-
2 Ca ²⁺ FL	7.85±0.55	31	0.7906
4 Ca ²⁺ FL	9.96±0.77	31	0.0395
10 Ca ²⁺ FL	11.72±1.10	28	0.0039

L: Number of docked vesicles (0 nm from the membrane) per 500 nm AZ
(Kruskal-Wallis ANOVA followed by *post hoc* Dunn's multiple comparison test; WT vs. toxins). n represents the number of electron micrographs (synapses) analyzed.

Fig. 4F	Resting		
	Mean±SEM	n	p-value
Syt1 WT	1.72±0.10	254	-

BoNT/A Syt1 WT	1.30±0.09	206	0.0317
BoNT/B Syt1 WT	0.31±0.04	229	< 0.0001

M: Number of docked vesicles (0 nm from the membrane) per 500 nm AZ
(unpaired two-tailed Mann-Whitney test; before vs. after AP)
n represents the number of electron micrographs (synapses) analyzed.

Fig. 4 H, J	before		after AP		
	Mean±SEM	n	Mean±SEM	n	p-value
Syt1 WT	1.72±0.10	254	1.09±0.12	148	< 0.0001
BoNT/A Syt1 WT	1.30±0.09	206	1.32±0.08	229	0.4749
BoNT/B Syt1 WT	0.31±0.04	229	0.28±0.04	201	0.8879

N: Readily releasable pool (RRP) charge
(Kruskal-Wallis ANOVA followed by *post hoc* Dunn's multiple comparison test; WT vs. mutants). n represents the number of cells analyzed.

Fig. S4B			
Charge(nC)	Mean±SEM	n	p-value
Syt1 WT	3.16±0.37	30	-
Syt1 KO	1.23±0.20	33	<0.001
D309, 363, 365A	3.27±0.45	30	>0.05
K325, 327A	1.85±0.26	29	<0.05
R398, 399Q	1.83±0.32	27	>0.05

O: Number of Vglut1 positive synapses
(One-way ANOVA followed by Tukey's multiple comparison test; WT vs. mutants). n represents the number of images analyzed

Fig. S4D			
Counts	Mean±SEM	n	p-value
Syt1 WT	5.92±0.40	15	-
Syt1 KO	5.87±0.42	15	>0.05
D309, 363, 365A	6.25±0.49	15	>0.05
K325, 327A	6.14±0.34	15	>0.05
R398, 399Q	5.98±0.45	15	>0.05

P: Number of docked vesicle (0 – 5 nm from the membrane)
(Kruskal-Wallis ANOVA followed by *post hoc* Dunn's multiple comparison test; WT vs. mutants). n represents the number of electron micrographs (synapses) analyzed

Fig. S5E	Resting		
Counts	Mean±SEM	n	p-value
Syt1 WT	1.75±0.13	102	-
Syt1 KO	0.89±0.10	99	< 0.001
C2A 3DA	1.55±0.13	95	> 0.05
C2AB 6DA	1.82±0.13	96	> 0.05

Q: Active zone length
(Kruskal-Wallis ANOVA followed by *post hoc* Dunn's multiple comparison test; WT vs. mutants). n represents the number of electron micrographs (synapses) analyzed

Fig. S5F	Resting		
Length (nm)	Mean±SEM	n	p-value
Syt1 WT	412.6±10.89	102	-
Syt1 KO	404.3±12.24	99	> 0.05
C2A 3DA	418.2±12.09	95	> 0.05
C2AB 6DA	445.5±11.39	96	> 0.05

R: Number of docked vesicle (0 – 5 nm from the membrane) per 500 nm AZ
(Kruskal-Wallis ANOVA followed by *post hoc* Dunn's multiple comparison test; WT vs. mutants). n represents the number of electron micrographs (synapses) analyzed.

Fig. S5G	Resting		
Counts	Mean±SEM	n	p-value
Syt1 WT	2.17±0.16	102	-
Syt1 KO	1.09±0.12	99	< 0.001
C2A 3DA	1.82±0.15	95	> 0.05
C2AB 6DA	1.90±0.17	96	> 0.05

S: Normalized SNAP-25 intensity (SNAP-25/Synaptophysin)
(Kruskal-Wallis ANOVA followed by *post hoc* Dunn's multiple comparison test; WT vs. mutants). n represents the number of images analyzed.

Fig. S6B	Resting		
	Mean±SEM	n	p-value
C-terminal			
Control	1.00±0.06	21	-
12h	0.52±0.02	21	< 0.001
16h	0.41±0.01	21	< 0.001
N-terminal			
Control	1.00±0.06	19	-
12h	1.17±0.04	21	>0.05
16h	1.04±0.06	21	> 0.05