Supplementary Information

Genetic Analysis of Synaptotagmin 2 in Spontaneous and Ca^{2+}-Triggered Neurotransmitter Release

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Supplemental Methods

Protein quantifications. For quantitation of total brain proteins, homogenates from 3 months old littermate mice of the denoted genotypes were analyzed by quantitative immunoblotting using ^{125}I-labeled secondary antibodies and PhosphoImager detection (Molecular Dynamics) with GDP-dissociation inhibitor (GDI) or vasolin-containing protein (VCP) as internal standards.

Expression and purification of recombinant proteins. Wildtype and I377Nmutant synaptotagmin 2 and PKC C_2-domain expression vectors in pGEX-KG were described previously (Sugita et al, 2001) or generated by mutagenesis using the quick change mutagenesis kit (Stratagene, La Jolla, California). To remove bacterial contaminants of C_2B-domains (Ubach et al, 2001), the C_2B-domain proteins present in the bacterial lysate were treated with 1,500 units/l benzonase (Novagen, Madison, Wisconsin) for 1 h at room temperature or 4 ˚C for 2-3 hours prior to purification on glutathione agarose (0.3 ml per liter culture) and washed as described (Ubach et al., 2001) in batch 2x with 15 ml 1 M NaCl in 10 mM Tris pH 8.0, 1 mM EDTA; 5x with 20 mM $CaCl_2$ in 10 mM Tris; 5x with 1 M NaCl, 10 mM Tris, 1 mM EDTA; and 3x with 10 mM Tris, 1 mM EDTA, 150 mM NaCl before elution.

Immunofluorescence labeling. Brainstems containing the MNTB were removed after decapitation, immersed immediately in fresh 4% parafomaldehyde, and incubated

overnight at 4 ºC, followed by a second overnight incubation in 30% sucrose in phosphate buffered saline. Sections (30 μm) were cut on a Leica CM3050S cryostat, incubated in primary antibodies (syt1: 41.1, 1:5 000; syt2: A320, 1:500; Synapsin: Cl10.22, 1:1 000 or E028, 1:1 000) again overnight at 4 ºC, and then stained with Alexa Fluor conjugated secondary antibodies (Molecular Probes) at a dilution of 1:400 for 2 hours at room temperature. Sections were washed, mounted, and viewed in a confocal microscope.

Brain slicing and whole-cell recordings from MNTB neurons. Preparation of slices, simultaneous whole-cell recordings of the nerve terminal and the postsynaptic neuron were performed mostly as described (Borst et al., 1995; Wu and Borst, 1999). Wildtype or synaptotagmin 2^{I377N} mice (7-9 or 14-day-old) were decapitated, parasagittal slices of 200 μm thick were cut from the auditory brainstem with a vibratome in 4 °C in a low Ca^{2+} solution containing (in mM): 125 NaCl, 2.5 KCl, 3 $MgCl_2$, 0.05 $CaCl_2$, 25 dextrose, 1.25 NaH_2PO_4, 0.4 ascorbic acid, 3 *myo*-inositol, 2 sodium pyruvate, and 25 $NaHCO_3$ pH 7.4, and gassed with 95% O_2 and 5% CO_2. 2 mM $CaCl_2$ and 1 mM $MgCl_2$ was substituted in the normal bath solution when AP-induced EPSCs were recorded (plus 50 μM D-APV). Additional bicuculline (10 μM) and strychnine (10 μM) were applied when mEPSCs were recorded. Paired pre- and postsynaptic recordings were obtained in extracellular solution containing (in mM): 105 NaCl, 20 TEA-Cl, 2.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 25 dextrose, 0.4 ascorbic acid, 3 myo-inositol, 2 sodium pyruvate, 0.001 TTX, 0.1 3,4-diaminopyridine pH 7.4 gassed with 95% O_2 and 5% CO_2. The presynaptic pipette (3.5–5 MΩ) solution contained (in mM): 125 Csgluconate, 20 KCl, 4 MgATP, 10 Na_2-phosphocreatine, 0.3 GTP, 10 HEPES, 0.5

EGTA (pH 7.2) adjusted with CsOH. Presynaptic whole-cell recordings were made with an EPC-9 amplifier (HEKA, Lambrecht, Germany). The series resistance (<15 MΩ) was compensated by 60%. Postsynaptic pipette (2–3 MΩ) solution was similar to the presynaptic solution with Cs^+ replaced by K^+. Postsynaptic whole-cell recordings were made with an Axopatch 200B amplifier (Axon Instruments Inc., Foster City, CA). The series resistance (<15 MΩ) was compensated by 98% (lag 10 μs). Both pre- and postsynaptic currents were low-pass filtered at 5 KHz and digitized at 20 KHz. Data were expressed as means ± SEM. mEPSCs were analyzed by a home-made program in Igor which automatically recognizes individual single mini events in the trace.

Figure Legends for Supplemental Data

Figure S1. Synaptic protein quantification from brain and spinal cord homogenates from wildtype and synaptotagmin-2^{I377N} mice. Protein levels were quantitated using immunoblots with iondinated secondary antibodies and phosphoimager detection. All data are from three pairs of littermate mice. As listed in the table 1, the expression level of synaptotagmin-2 in both cerebellum and spinal cord decreased to ~20% as compared to wildtype littermates. At the same time, synaptotagmin-1 expression level increased 30-40% in cerebellum and spinal cord. The overall expression level of synaptotagmin-1 in lower compared to cerebral cortex. Sixteen other brain specific proteins did not show obvious changes in all three regions tested (see Suppl. Table 1). See note.

Figure S2. Presynaptic Ca^{2+} current in MNTB from wildtype and synaptotagmin-2^{I377N}. In addition to a role in Ca^{2+}-triggering of release, synaptotagmin-1 has been implicated in regulating

Ca^{2+}-channels because it is co-immunoprecipitated with N- and P/Q-type Ca^{2+}-channels (Bennett et al., 1992), participates in a Ca^{2+}-dependent interaction with a GST-fusion protein derived from a cytosolic sequence of the N-type channel (Leveque et al. 1992; Wiser et al., 1997), and upon oocyte expression modifies the kinetic properties of N-type Ca^{2+}-channels (Cohen et al. 2003). However, we observed no change in Ca^{2+}-currents in the mutant synapses, suggesting that synaptotagmin-2 is not a major regulator of Ca^{2+}-channels in the Calyx. ***A.*** Superimposed representative of Ca^{2+} current from Calyces of wildtype (red trace) and synaptotagmin-2^{I377N} (blue trace) mice. Presynaptic stimulation protocol is shown in gray line above the traces. ***B.*** Pooled data showing no changes in peak Ca^{2+} current, and also in total charge transfer during 50 ms depolarization (***C.***).

Supplementary Table 1. Protein quantitation in wild-type and synaptotagmin 2^{I377N} mice. Varies proteins from Forebrain, Cerebellum (including brainstem) and Spinal cord of 3 months old littermate mice of the denoted genotypes were quantified with ^{125}I-labeled secondary antibodies and PhosphoImager detection. GDP-dissociation inhibitor (GDI) or vasolin-containing protein (VCP) was used as internal standard. Synaptotagmin 2 (Syt 2) was not detected in cortex, abundant Syt 2 was seen in cerebellum and spinal cord in wild-type animals but largely decreased (~20-30% of that of wild-type) in Synaptotagmin 2^{I377N} mice. Synaptotagmin 1 (Syt 1) was moderate upregulated in caudal brain regions. Other synaptic vesicle proteins or synaptic proteins detected show no obvious change. Abbreviations listed under the table. Number of animals used listed in the parenthesis. * $p<0.05$

References

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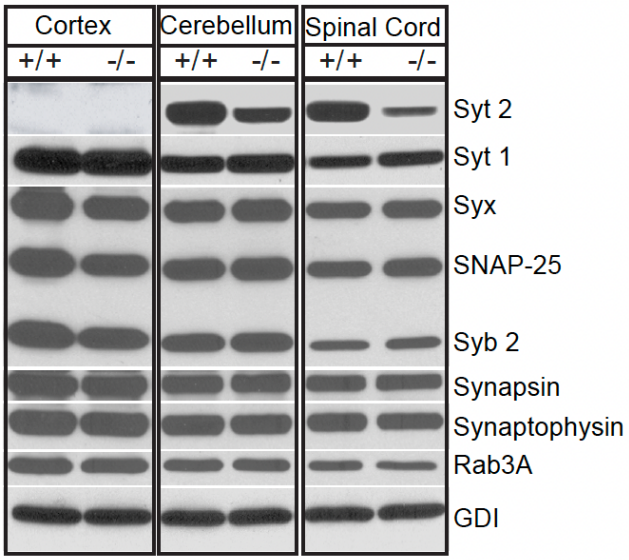
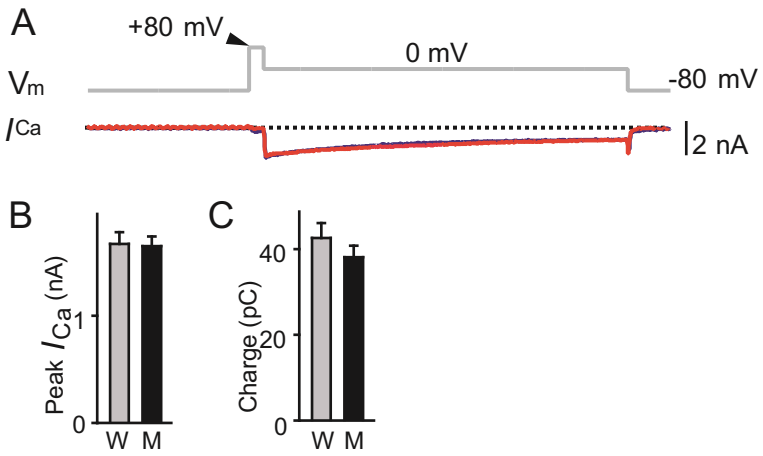


(Suppl. Figure S1, Pang et al.)



(Suppl. Figure S2, Pang et al.)

Suppl. Table 1. Protein quantitation in wildtype and synaptotagmin 2 ^{I377N} mice

	Cortex		Cerebellum		Spinal Cord	
Protein	Wildtype	Mutant	Wildtype	Mutant	Wildtype	Mutant
Syt 2 (n=3)	n.d.	n.d.	100±1.3	20.1±1.1*	100±5.8	28.9±3.2 *
Syt 1 (n=6)	100±2.0	106.2±5.8	100±4.7	132.1±3.1*	100±2.6	141.3±6.7*
Syntaxin (n=3)	100±7.1	90.1±2.6	100±6.0	98.8±9.4	100±1.7	103.6±1.4
SNAP 25 (n=3)	100±3.0	90.2±5.7	100±2.1	106.7±2.5	100±10.4	112.9±10.9
Syb (n=3)	100±3.1	85.5±5.2	100±3.4	108.3±5.7	100±3	110.9±17.6
Rab 3 (n=3)	100±3.3	87.6±4.7	100±5.9	98.3±2.7	100±4.9	93.2±1.3
Syp (n=3)	100±0.3	93.2±3.2	100±6.5	96.0±8.6	100±3.2	98.6±9.1
Synapsin (n=3)	100±5.2	95.8±9.2	100±3.6	113.6±8.6	100±3.2	90.4±7.7
Conplexin (n=3)	100±1.4	97.1±3.0	100±1.9	100.5±10.1	100±12.3	87.7±6.5
Munc 18 (n=3)	100±4.8	104.6±5.0	100±3.8	97.1±1.5	100±9.2	100±6.0
PSD95 (n=3)	100±1.3	98.6±6.4	100±6.9	104.1±2.6	100±4.1	99.3±3.0
SCAMP (n=3)	100±3.6	98.0±4.6	100±3.8	87.4±3.2	100±2.5	101.2±1.6
CASK (n=3)	100±5.7	100±2.7	100±2.4	88.5±6.2	100±10.3	86.8±2.7
Synuclein (n=3)	100±6.6	95.9±2.8	100±1.7	94.7±8.1	100±2.8	85.2±4.9
CSP (n=3)	100±3.4	100.8±9.8	100±4.1	81.5±4.7	100±3.3	96.2±1.0
Mint1 (n=3)	100±1.9	97.9±2.0	100±8.1	97.6±8.6	100±2.5	94.3±4.0
Liprin (n=3)	100±3.4	86.6±6.5	100±4.1	127±3.1	100±9.7	86.0±7.5
ERC1 (n=3)	100±6.2	80±14.6	100±10.2	114±9.5	100±16.7	91.8±4.4

CASK: calmodulin-associated serine/threonine kinase; CSP: cysteine-string protein; ERC:ELKS-Rab6-interacting protein-CAST; Mint1: munc18-interacting-protein 1; SCAMP: secretory carrier membrane proteins; Syb: synaptobrevin 2; Syp: synaptophysin; Syt1: synaptotagmin-1;Syt2: synaptotagmin-2. Numbers of samples were listed in the parenthesis. * indicates $p<0.05$.