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Presynaptic Ca^{2+} Influx at a Mouse Central Synapse with Ca^{2+} Channel Subunit Mutations

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Genetic alterations in Ca^{2+} channel subunits can be used to study the interaction among channel subunits and their roles in channel function. P/Q- and N-type Ca^{2+} channels reside at the presynaptic terminal and control the release of neurotransmitter at mammalian central synapses. We used fluorescence imaging techniques to investigate presynaptic Ca^{2+} currents and neurotransmitter release at hippocampal Schaffer collateral synapses in both *tottering* (*tg*, α_{1A} subunit) and *lethargic* (*lh*, β_4 subunit) mutant mice. Application of selective toxins revealed a large reduction in presynaptic P/Q-type Ca^{2+} transients, from 39% of total in +/+ mice to 6% in *tg/tg* mice, whereas the proportion of N-type increased from 35 to 68%, respectively. Neurotransmitter release in the *tg/tg* mutant relied almost exclusively on N-type channels, as shown by the complete blockade of synaptic transmission with ω-conotoxin GVIA. Remarkably, loss of β4, a subunit predicted to regulate the subcellular targeting and modulation of both P/Q- and N-type channels, resulted in no significant difference in the ratio of Ca^{2+} channel subtypes or Ca^{2+} dependence of neurotransmitter release in lethargic mice. G-protein-mediated inhibition of Ca^{2+} channels was also unaltered. These results indicate that a profound decrease in presynaptic P/Q-type currents leads to dependence of neurotransmitter release on N-type channels. In contrast, absence of β_4 appears not to compromise either P/Q- or N-type channel function at this hippocampal synapse, implicating rescue of presynaptic Ca^{2+} currents by other available β subunits. The present study reveals compensatory molecular mechanisms in the regulation of presynaptic Ca^{2+} entry and neurotransmitter release.

Key words: hippocampus; presynaptic terminal; magnesium green; transmitter release; PPF; power function; cooperativity

Voltage-gated Ca^{2+} channels play an important role in the regulation of diverse neuronal functions. At the presynaptic terminal, two major Ca^{2+} channel types, P/Q and N, are critically involved in Ca^{2+}-dependent exocytotic release of neurotransmitter (Dunlap et al., 1995). Ca^{2+} ions entering through these channels trigger quantal release in a cooperative process with other components of the vesicle fusion machinery (Dodge and Rahamimoff, 1967). Given the pivotal role of Ca^{2+} channels in controlling neurotransmitter release, defects in the structure, localization, and modulation of presynaptic Ca^{2+} channels are expected to result in aberrant synaptic signaling leading to various patterns of neural network dysfunction.

Recently, P/Q-type Ca^{2+} channel mutations have been identified in mice and humans with inherited neurological diseases (for review, see Ophoff et al., 1998; Burgess and Noebels, 1999). Mutations in the α_{1A} subunit of voltage-gated P/Q-type channels have been identified in the tottering (*tg)* mouse and its more severely affected allele *leaner* (tg^{1a}) (Fletcher et al., 1996; Doyle et al., 1997). The *tg* mutation is located in the S4–S5 linker region of the third transmembrane domain near the pore-forming region of the channel; it reduces whole-cell current density and voltage-dependent inactivation during prolonged depolarization in dissociated Purkinje cell somas (Wakamori et al., 1998). The *leaner* mutation alters the C terminus of the α_{1A} subunit and reduces both the current density and the open probability of single P/Q-type channels (Dove et al., 1998; Lorenzon et al., 1998). In contrast to α_1 subunit mutations, the lethargic (*lh)* mutant is an example of a complex Ca^{2+} channelopathy in which mutation of a single, non-pore-forming modulatory subunit has the potential to alter more than one channel subtype. Genetic analysis of the *lh* mutation indicated that the locus encodes a truncated cytoplasmic β_4 subunit, resulting in the loss of functional α_1–β_4 interactions (Burgess et al., 1997). Because β_4 subunits interact with both α_{1A} and α_{1B} transmembrane subunits, play a key role in channel assembly and localization, and possess unique modulatory sites not shared by other β subunits (Dolphin, 1998; Walker and De Waard, 1998; Walker et al., 1999), the loss of β_4 subunits in *lh* mutants could alter the function of both P/Q- and N-type Ca^{2+} channels.

Based on available data, these α_1 and β subunit mutations could directly alter synaptic transmission. However, it is not known how the mutant channels actually behave at terminals in response to physiological activation by action potentials or G-protein modulation. In the present study, we used fluorescence imaging techniques to investigate presynaptic Ca^{2+} channel function at an accessible synapse in hippocampal slices prepared from both *tg* and *lh* mutants. Schaffer axon collaterals from CA3 region pyramidal cells form excitatory synapses onto pyramidal cell dendrites in the CA1 region. Neurotransmitter release at these terminals relies on Ca^{2+} entry through N- and P/Q-type channels (Wheeler et al., 1994). We tested the effect of the α_1 and β_4 mutations on the behavior of presynaptic Ca^{2+} channels and neurotransmitter release.

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MATERIALS AND METHODS

Transverse brain slices (350-μm-thick) were prepared from hippocampi of homozygous tottering (C57BL/6J-*Cacna1a*^{tg}) and lethargic (B6EiC3H-*a*/*A*-*Cacnb4*^{lh}/+) mutant mice (aged between 5 and 9 months) and incubated at 25°C in artificial CSF containing (in mM): 124 NaCl, 3.5 KCl, 2.5 $CaCl_2$, 2 $MgCl_2$, 22 $NaHCO_3$, 1.25 NaH_2PO_4, and 10 D-glucose, gassed with 95% O_2–5% CO_2 to maintain a constant pH of 7.4. After an incubation period of at least 1 hr, slices were transferred to a submerged recording chamber mounted on an inverted microscope (Axiovert 100; Zeiss, Oberkochen, Germany) for loading with Ca^{2+}-sensitive fluorescence indicators. The chamber temperature was maintained at 30°C during all experiments.

The procedure for loading Ca^{2+} indicators into mouse presynaptic terminals was similar to that used in previous experiments with rat brain slices (Qian et al., 1997). Briefly, the low-affinity membrane-permeant Ca^{2+} indicator Magnesium Green AM (Molecular Probes, Eugene, OR) was dissolved in DMSO solution (80% DMSO plus 20% pluronic acid). A small amount of dye solution was pressure-injected (Picospritzer II; General Valve, Fairfield, NJ) under visual control into mouse hippocampal slices in the stratum radiatum (SR) of area CA1. A small recording area with a diameter of 150 μm, ~0.8–1 mm away from the dye injection site, was excited at the wavelength of 488/20 nm; the emitted fluorescence was filtered by a bandpass filter of 535/25 nm and converted into electrical signals with a single photodiode. A bipolar tungsten electrode was positioned in the SR, and the CA3–CA1 synapses were stimulated every 20 sec with current pulses of 0.02–0.03mA/0.2 msec adjusted to elicit a submaximal response. The stimulation-induced presynaptic Ca^{2+} transient ($[Ca_{pre}]_t$) and field EPSP (fEPSP) were simultaneously sampled at 10 kHz. Three successive traces were averaged to improve the signal-to-noise ratio. Except for Fig. 1, all sample traces shown represent an average of 15 sequential responses during steady state. The maximal slope of the fEPSP was taken as the measure of synaptic transmission. For field recording, glass microelectrodes (1–5 MΩ, filled with 2 M NaCl) were positioned in the center of the optical recording area. The evoked Ca^{2+} influx was measured by the fluorescence ratio of $\Delta F/F$. Autofluorescence of the brain slice was subtracted from the total fluorescence signal. Data in each experiment were normalized to the baseline before drug application unless otherwise stated, pooled together, and expressed as a mean ± SD. Paired two-tailed *t* tests were used to determine the statistic significance in comparing data between wild type and mutants.

Pharmacological reagents. The Ca^{2+} channel toxins ω-conotoxin GVIA (ω-CgTx GVIA) and ω-conotoxin MVIIC (ω-CgTx MVIIC) were purchased from Bachem (Bubendorf, Switzerland), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and D-amino-phosphonovalerate (D-APV) from Tocris Cookson (Ballwin, MO), adenosine (AD) from Research Biochemicals (Natick, MA), and baclofen from Sigma (St. Louis, MO).

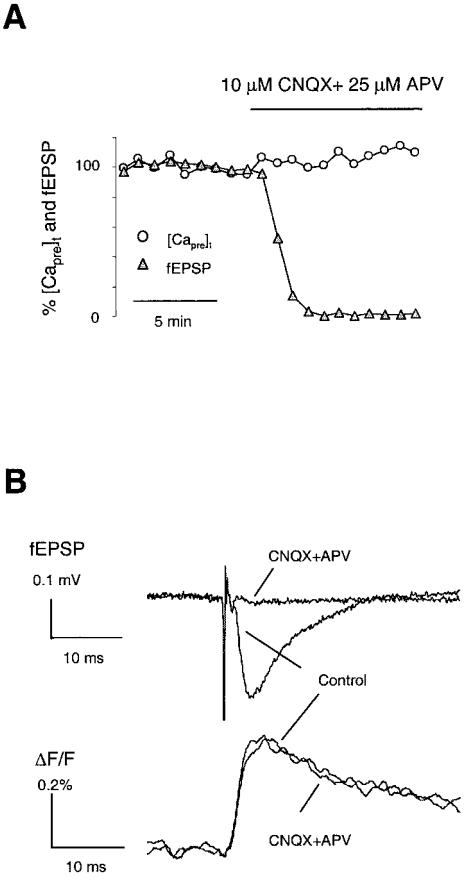


Figure 1. Selective loading of presynaptic terminals with the Ca^{2+} indicator Magnesium Green AM at the mouse hippocampal synapse. *A*, Time courses of mean amplitudes of presynaptic Ca^{2+} transient ($[Ca_{pre}]_t$) and fEPSPs under control saline conditions and during application of glutamate receptor antagonists CNQX and D-APV in a typical experiment. Blockade of the fEPSP did not alter the evoked fluorescence signal, indicating a presynaptic origin. *B*, Superimposed sample traces of the optical Ca^{2+} $\Delta F/F$ signal and the fEPSP taken during the steady-state response period in control saline and after application of the glutamate receptor antagonists. The Ca^{2+} $\Delta F/F$ trace is an average of three consecutive samples. CNQX and D-APV abolished the fEPSP but did not change the $\Delta F/F$, indicating that the measured Ca^{2+} signal was purely presynaptic.

RESULTS

Presynaptic calcium transients in mouse CA3–CA1 synapses in hippocampal slices

The selective presynaptic loading of Schaffer axon collaterals with Ca^{2+} indicator in the mouse hippocampal slice was verified by applying the glutamate receptor antagonists CNQX (10 μM) and D-APV (25 μM) as shown in Figure 1. The glutamate receptor antagonists did not alter the optical signal, $\Delta F/F$, but completely blocked the fEPSP, indicating a pure presynaptic origin of the optical signals.

Reduced P/Q-type Ca^{2+} influx at the hippocampal CA3–CA1 synapse in *tg*/*tg* but not in *lh*/*lh* mouse mutants

Previous studies of synaptic transmission at the rat hippocampal CA3–CA1 synapse indicate that both N-type and P/Q-type channels are involved in the release of neurotransmitter (Wheeler et al., 1994). Based on results obtained in dissociated Purkinje cells in which P/Q-type channels mediate the majority of postsynaptic calcium currents (Dove et al., 1998), mutation of the α_{1A} subunit in the *tg*/*tg* mouse would be anticipated to result in a reduced P/Q-type current at presynaptic terminals. Thus, less reliance of neurotransmitter release on P/Q-type channels and more reliance on N-type channels would be expected.

To examine this prediction, we tested the response of $[Ca_{pre}]_t$ and synaptic transmission in +/+ mice to the application of Ca^{2+} channel toxin ω-CgTx GVIA, a select N-type channel blocker. Similar to earlier findings in the rat, application of 1 μM ω-CgTx GVIA to +/+ mouse slices partially blocked $[Ca_{pre}]_t$, as shown in Figure 2*A*. Approximately 35 ± 3% (n = 6) of $[Ca_{pre}]_t$ was N-type, and the fEPSP was reduced to 55 ± 4% (n = 6) of baseline after N-type channels were blocked by the toxin. In the presence of ω-CgTx GVIA, subsequent exposure to a second Ca^{2+} channel toxin, ω-CgTx MVIIC, which blocks both N- and P/Q-type channels, was used to isolate and quantify the amount of residual P/Q-type Ca^{2+} current. As shown in the sample trace of Figure 2*A*, application of 2 μM ω-CgTx MVIIC further reduced the $[Ca_{pre}]_t$ by ~39 ± 3% (n = 2) of control and almost completely eliminated synaptic transmission. Therefore, at the +/+ mouse hippocampal CA3–CA1 synapse, P/Q-type channels contribute ~40% of the total Ca^{2+} influx associated with neuro-

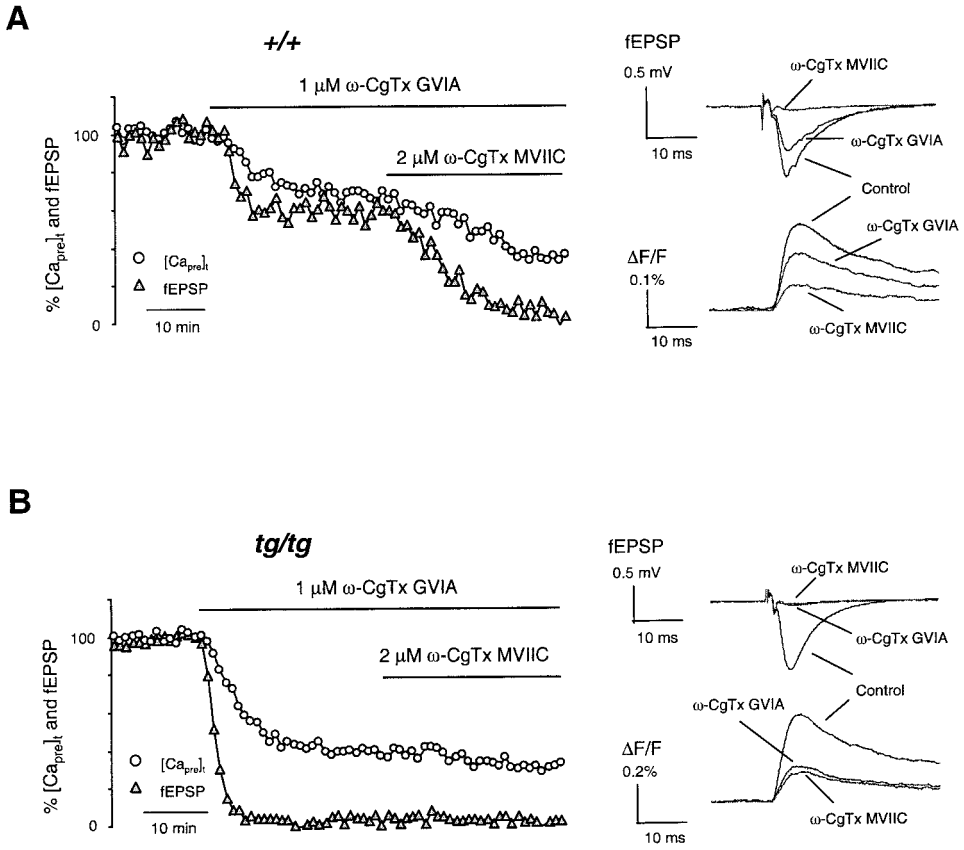


Figure 2. Differential effects of Ca^{2+} channel toxins on $[Ca_{pre}]_t$ and fEPSP in +/+ and *tg/tg* mice. *A*, Time courses of $[Ca_{pre}]_t$ and fEPSP in response to ω-CgTx GVIA and ω-CgTx MVIIC in a representative experiment from +/+ mouse. Blockade of N-type Ca^{2+} currents with 1 μM ω-CgTx GVIA partially blocked synaptic transmission (~35% of the total Ca^{2+} influx). In the presence of ω-CgTx GVIA, 2 μM ω-CgTx MVIIC further reduced $[Ca_{pre}]_t$ by ~40% and completely abolished fEPSP, indicating the involvement of P/Q-type channels in neurotransmitter release as well. *Inset* shows sample traces taken during steady-state periods in the control solution and after application of ω-CgTx GVIA and ω-CgTx MVIIC. *B*, Time courses of $[Ca_{pre}]_t$ and fEPSP in response to ω-CgTx GVIA and ω-CgTx MVIIC in a typical experiment from *tg/tg* mouse. In contrast to +/+ slices, ω-CgTx GVIA primarily reduced $[Ca_{pre}]_t$ (by ~70%) and completely eliminated synaptic transmission. Further application of ω-CgTx MVIIC revealed a small amount of P/Q-type current (~6%). This indicates that mutation of the α_{1A} subunit in *tg/tg* mice severely compromises the function of P/Q-type Ca^{2+} channels, resulting in a heavy reliance of neurotransmitter release on N-type Ca^{2+} channels. *Inset* shows sample traces taken in the steady state of control solutions and after application of ω-CgTx GVIA and ω-CgTx MVIIC.

transmitter release. We also tested the involvement of Ca^{2+} channels other than N- and P/Q-type. Application of 10 μM nifedipine, which blocks L-type channel, did not affect either $[Ca_{pre}]_t$ or fEPSP (data not shown). This indicates that the amount of residual $[Ca_{pre}]_t$, ~25%, is likely R-type.

Clear presynaptic defects were observed when the same set of experiments was performed on *tg/tg* mice. As shown in Figure 2*B*, in contrast to the small reduction of $[Ca_{pre}]_t$ by the ω-CgTx GVIA in +/+ mice, there was a large decrease of $[Ca_{pre}]_t$ (68 ± 5%; $n = 5$) when the N-type channel blocker was applied. Consistent with the finding that the $[Ca_{pre}]_t$ was predominantly N-type, ω-CgTx GVIA completely eliminated neurotransmitter release in *tg/tg* mouse. Further application of ω-CgTx MVIIC revealed a very small contribution of P/Q-type currents to the total Ca^{2+} influx (6 ± 0.1%; $n = 2$).

In contrast, presynaptic Ca^{2+} influx was apparently unaffected by the β_4 subunit mutation of the *lh/lh* mouse. As shown in Figure 3*A*, application of ω-CgTx GVIA resulted in a significant $[Ca_{pre}]_t$ reduction (37 ± 4%; $n = 7$), comparable with that observed in +/+ slices, although the fEPSP in *lh/lh* was more reduced by the toxin than in +/+ slices. On average, the remaining fEPSP was ~43 ± 5% ($n = 7$) of baseline after blocking N-type channels. The optical recording of $[Ca_{pre}]_t$ is insensitive to this small (~10%) difference in the fEPSP because this amount of variability in the fEPSP amplitude would arise from only a 3–4% change of $[Ca_{pre}]_t$, which is equivalent to the size of the noise signal. Application of ω-CgTx MVIIC in the presence of ω-CgTx GVIA further decreased $[Ca_{pre}]_t$ by 34 ± 3% ($n = 2$) in *lh/lh* mouse. Figure 3*B* summarizes the experimental data with ω-CgTx GVIA and ω-CgTx MVIIC on +/+, *tg/tg*, and *lh/lh* mutant mice. These results indicate that the mutation in the pore-forming region of the α_{1A} subunit in *tg/tg* mouse severely compromises the function of presynaptic P/Q-type channels. However, the loss of the β_4 subunit function in *lh/lh* mouse does not significantly change the Ca^{2+} influx profile at the hippocampal CA3–CA1 synapse.

Relationship between $[Ca_{pre}]_t$ and synaptic transmission at the mouse hippocampal synapse

The relationship between presynaptic Ca^{2+} influx and neurotransmitter release has been thought to reflect the Ca^{2+} cooperativity of synaptic vesicle exocytosis. Patch-clamp studies of this relationship have found that neurotransmitter release at mammalian central synapses is a power function of the presynaptic Ca^{2+} current with a power number between 3 and 4 (Borst and Sakmann, 1996; Wu et al., 1998). A very similar power relationship between optically measured $[Ca_{pre}]_t$ and postsynaptic responses has been also obtained at central synapses in the rat cerebellum and hippocampus (Mintz et al., 1995; Qian et al., 1997). In the present experiments, application of ω-CgTx GVIA primarily reduced $[Ca_{pre}]_t$ but only partially blocked the neurotransmitter release (Fig. 2*A*), resulting in a power number of 1.4 ± 0.2 ($n = 6$) for the N-type channel in presynaptic terminals of wild-type mice. The lower power number for the N-type channel has also been observed in other species and is thought to be attributable to a loose coupling between the channel and vesicle release machinery (Mintz et al., 1995; Qian and Saggau, 1999; Wu et al., 1999). The *tg/tg* mouse provides a valuable opportunity to directly test the interaction between N-type channels and vesicle release machinery, because the loss of P/Q-type channel function results in the shift to the N-type channel population as the predominant Ca^{2+} source for neurotransmitter release. We studied the rela-

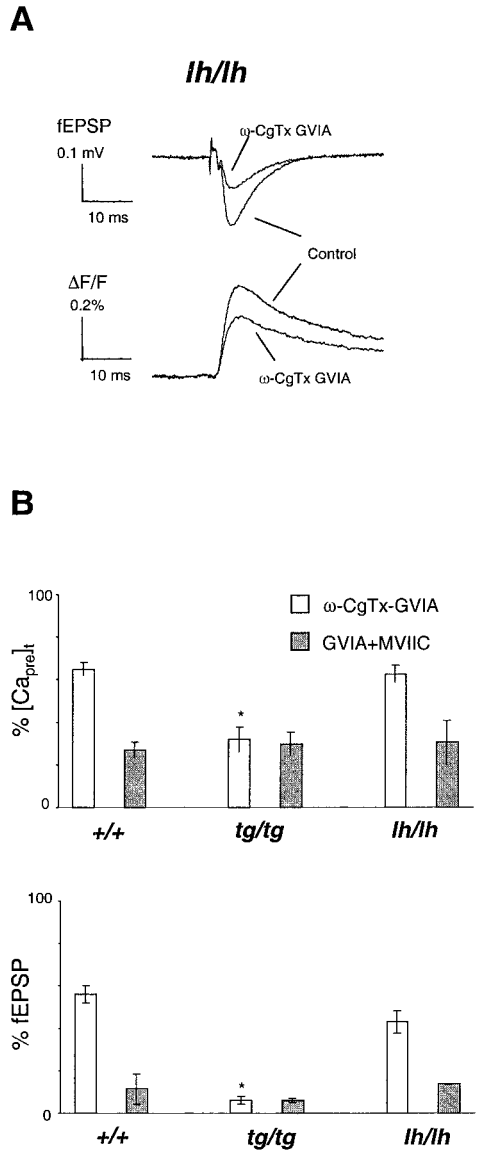


Figure 3. Presynaptic effects of Ca^{2+} channel toxins on $[Ca_{pre}]_t$ and fEPSP are similar in *lh/lh* and +/+ mice. *A*, Sample traces of $[Ca_{pre}]_t$ and fEPSP in response to ω-CgTx GVIA in a typical experiment from a *lh/lh* mouse. ω-CgTx GVIA (1 μM) decreased the $[Ca_{pre}]_t$ to the level similar to that in +/+ slices, and fEPSP was partially reduced. *B*, Summary data for the effects of Ca^{2+} channel toxins on $[Ca_{pre}]_t$ and fEPSP from +/+, *tg/tg*, and *lh/lh* mice. There was a significant difference in the presynaptic Ca^{2+} influx profile between +/+ and *tg/tg* mice (for both N–$[Ca_{pre}]_t$ and P/Q–$[Ca_{pre}]_t$; paired two-tailed *t* test; $p < 0.005$) but not between +/+ and *lh/lh* mice.

tionship between $[Ca_{pre}]_t$ and neurotransmitter release at the hippocampal CA3–CA1 synapse in *tg/tg* mice. In a series of experiments, we measured $[Ca_{pre}]_t$ and fEPSP while varying extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) from 2.5 (control) to 1.5 and 0.75 mM. The extracellular Mg^{2+} level was raised to maintain a constant size of the presynaptic fiber volley. Figure 4*A* shows the time course of the $[Ca_{pre}]_t$ and fEPSP during a typical experiment in +/+ slices. As expected, the mean amplitude of normalized fEPSPs was a power function of the normalized $[Ca_{pre}]_t$, as illustrated in a double-log plot (Fig. 4*B*). Table 1 summarizes the results of the fEPSP/$[Ca_{pre}]_t$ power number for +/+, *tg/tg*, and *lh/lh* mice. The *tg/tg* mouse exhibits similar Ca^{2+} cooperativity of neurotransmitter release except for larger mean power numbers than +/+ mice (paired two-tailed *t* test; $p < 0.05$ for both 1.5 and 0.75 mM $[Ca^{2+}]_o$). We also measured the relationship of $[Ca_{pre}]_t$ and neurotransmitter release in *lh/lh* mice. In those experiments, no significant difference in the mean power number between +/+ and *lh/lh* mice was found. These findings demonstrate that mutation of the β_4 subunit, despite its potential for altering the interaction between the Ca^{2+} channel and exocytotic release machinery, does not have a significant impact on the Ca^{2+} cooperativity of neurotransmitter release at the CA3–CA1 synapse.

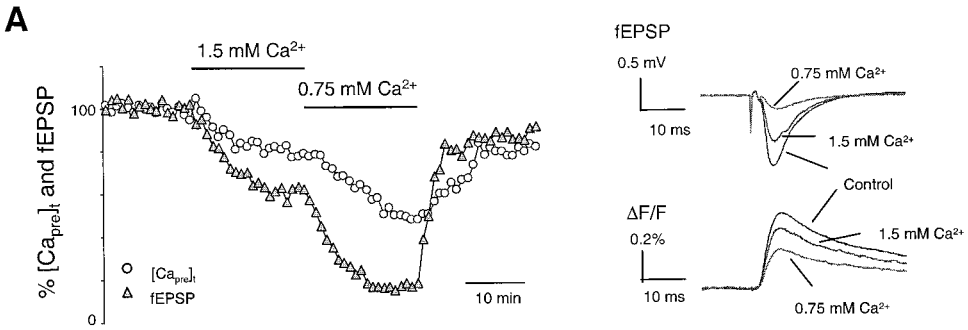
Increased paired-pulse facilitation in *tg/tg* mouse

Because the optical signal in the present study arises from a fiber population, the absolute value of presynaptic Ca^{2+} influx in single mutant terminals was not available. However, paired-pulse facilitation (PPF), which is sensitive to the amount of Ca^{2+} entry, was investigated to determine whether the reduction of P/Q-type current in the *tg/tg* mouse changes the overall Ca^{2+} influx and thereby affects the dynamics of neurotransmitter release. A pair of stimuli separated by an interval of 40 msec was delivered every 30 sec to evoke a paired response of fEPSPs. The stimulation intensity was adjusted to a range in which slopes of both fEPSPs varied linearly with stimulation intensity. The average ratio of the slope of the second fEPSP versus the first within this range was taken as the value of PPF at the synapse. Figure 5, *A* and *B*, shows sample traces of fEPSPs in response to a paired stimulus. As summarized in Figure 5*C*, the slope of the second fEPSP in +/+ mice was facilitated by ~76 ± 21% ($n = 16$). A significantly larger PPF (118 ± 10%; $n = 15$; paired two-tailed *t* test; $p < 0.005$) was observed in *tg/tg* mice. This suggests that the rate of action potential-evoked neurotransmitter release at the CA3–CA1 synapse in *tg/tg* mice was smaller than in +/+ mice, a result most likely attributable to the reduced Ca^{2+} influx through mutant P/Q-type channels. The PPF in *lh/lh* mice was ~86 ± 24% ($n = 12$), a value which was very similar to the +/+ mice.

Modulation of presynaptic Ca^{2+} channels in *lh/lh* mice is similar to +/+ mice

β subunits compete for binding to a site in the linker region between transmembrane domains I and II of Ca^{2+} channel α_1 subunits that also contains a G-protein binding site (De Waard et al., 1997). Thus, loss of functional β_4 subunits in *lh/lh* mice could alter competitive interactions among the remaining β subunits, or between β subunits and G-proteins, and affect channel modulation. Because β_4 is a component of several Ca^{2+} channel subtypes, including N- and P/Q-type, measurement of the presynaptic Ca^{2+} influx profile in *lh/lh* slices with selective channel toxins may not demonstrate a change in Ca^{2+} influx if both N- and P/Q-type channels are equally affected by the mutation. However, Ca^{2+} channel modulation by G-proteins could reveal a latent effect of the β_4 mutation on presynaptic Ca^{2+} channels, because the β subunit has been shown to attenuate the G-protein inhibition of Ca^{2+} currents (Campbell et al., 1995; Roche et al., 1995). A larger G-protein-mediated inhibition of presynaptic Ca^{2+} channels in *lh/lh* mouse would be expected if presynaptic Ca^{2+} channels lack β subunits.

We tested the G-protein-mediated inhibition of presynaptic Ca^{2+} channels by applying adenosine, a neuromodulator that has been demonstrated previously to inhibit presynaptic Ca^{2+} channels at the guinea pig and rat hippocampal CA3–CA1 synapse (Wu and Saggau, 1994; Qian et al., 1997). The presynaptic aden-



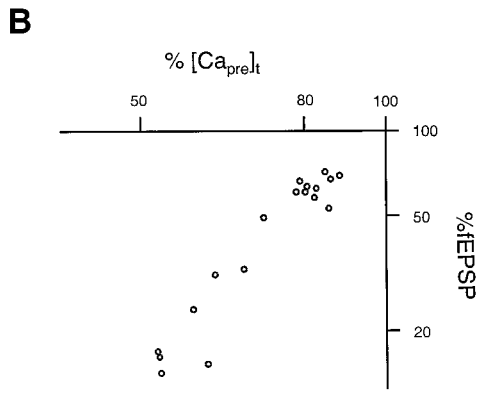


Figure 4. Ca^{2+} cooperativity of neurotransmitter release at the mouse CA3–CA1 synapse. *A*, Time courses of $[Ca_{pre}]_t$ and fEPSP in response to the manipulation of extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) in a typical experiment from a +/+ mouse. *Inset* shows sample traces taken at steady state in solutions containing 2.5 (control), 1.5, and 0.75 mM $[Ca^{2+}]_o$. *B*, Log–log plot summarizing the relationship between corresponding $[Ca_{pre}]_t$ and fEPSP responses from a +/+ mouse. The normalized fEPSP was a power function of the normalized $[Ca_{pre}]_t$ with power numbers ranging between 2 and 3.

Table 1. Power relationship between $[Ca_{pre}]_t$ and fEPSP in +/+ control, *tg*/*tg*, and *lh*/*lh* mutants

Power number	1.5 mM $[Ca^{2+}]_o$	0.75 mM $[Ca^{2+}]_o$
+/+	2.4 ± 0.6, $n = 11$	2.9 ± 0.4, $n = 8$
tg/*tg*	3.5 ± 0.5, $n = 6$ (*)	3.7 ± 0.7, $n = 6$ (*)
lh/*lh*	2.5 ± 0.5, $n = 6$	3.1 ± 0.3, $n = 6$

* $p < 0.05$, significantly different from +/+.

osine receptor acts in a convergent pathway with other neuromodulators, such as Neuropeptide Y and muscarine, to inhibit presynaptic Ca^{2+} channels (Qian et al., 1997). Because the size of the presynaptic fiber volley changed during application of adenosine under our experimental conditions, all experiments were performed in the presence of 10 μM CNQX and 25 μM APV to isolate the presynaptic fiber volley from postsynaptic currents activated by the stimulus. Figure 6*A* shows the time course of the $[Ca_{pre}]_t$ and the size of the fiber volley from a typical experiment. Application of a saturating concentration of adenosine (100 μM) induced a reversible reduction of $[Ca_{pre}]_t$, and the size of fiber volley also decreased slightly. Figure 6*B* summarizes the results from +/+ and *lh*/*lh* mice. The size of the fiber volley during application of adenosine was 82 ± 4% of baseline in +/+ ($n = 4$) and 84 ± 1% of baseline in *lh*/*lh* slices ($n = 4$), respectively. The corresponding optical signal $\Delta F/F$ was reduced to 49 ± 6% of baseline in +/+ and 46 ± 4% of baseline in *lh*/*lh* mice. There was no significant difference in the reduction of the fiber volley or the presynaptic optical signal between +/+ and *lh*/*lh* slices. We also tested the modulation of presynaptic Ca^{2+} entry by baclofen, a neuromodulator known to activate presynaptic $GABA_B$ receptors and to inhibit presynaptic Ca^{2+} channels at this synapse in guinea pig and rat (Wu and Saggau, 1995; Qian et al., 1997). Figure 5*C* summarizes the effects of 50 μM baclofen on the $[Ca_{pre}]_t$ (+/+, $n = 4$, 52 ± 4%; *lh*/*lh*, $n = 3$, 55 ± 1%) and the size of fiber volley (+/+, 85 ± 5%; *lh*/*lh*, 79 ± 6%). Similar to the action of adenosine, no significant difference in the modulation of Ca^{2+} channel by baclofen between +/+ and *lh*/*lh* mouse was detected. This indicates that G-protein modulation of presynaptic Ca^{2+} channels at the hippocampal CA3–CA1 synapse of lethargic mutants is not affected.

DISCUSSION

We have investigated the effect of Ca^{2+} channel subunit mutations on the function of presynaptic Ca^{2+} channels and neurotransmitter release at a mouse central synapse. Our results demonstrate molecular compensatory mechanisms in the regulation of presynaptic Ca^{2+} entry that allow preservation of exocytotic release. Absence of the observed mechanism in some neuronal circuits may give rise to distinct, circuit-specific neurological phenotypes in tottering and lethargic mutants.

Mutation of P/Q-type Ca^{2+} channels in the *tg* mutant shifts the reliance of neurotransmitter release from P/Q-type channels to N-type channels

Mutation of the pore-forming Ca^{2+} channel α_{1A} subunit severely compromises the function of P/Q-type calcium currents at the presynaptic terminal in tottering neurons, as indicated by the very small fraction of P/Q-type $[Ca_{pre}]_t$ detectable at the *tg*/*tg* mouse hippocampal CA3–CA1 synapse. Consistent with the optical measurement showing preserved total presynaptic Ca^{2+} entry, neurotransmitter release at the synapse exhibits a virtually complete reliance on N-type Ca^{2+} channels, despite the presence of residual presumed R-type currents. These results are consistent

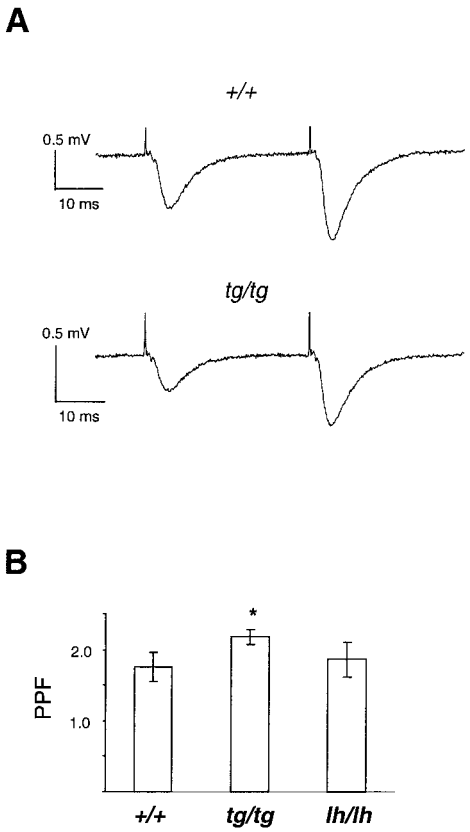


Figure 5. Increased paired-pulse facilitation in *tg/tg* mouse. *A*, Representative sample traces of the fEPSP in response to paired-pulse stimulation in +/+ and *tg/tg* mice. *B*, Summary PPF data from +/+, *tg/tg*, and *lh/lh* mouse slices. PPF was similar in *lh/lh* and +/+ mice. PPF in *tg/tg* mice was significantly larger than in +/+ mice, suggesting a reduced level of neurotransmitter release at *tg/tg* synapses.

with the major reduction of P/Q-type current that has been measured at the soma of *tg/tg* neurons (Wakamori et al., 1998) and demonstrate the ability of the N-type release machinery in the presynaptic compartment to develop and function despite the decreased calcium flux through a channel that normally participates in synaptic transmission.

Limited by the axon population recording techniques used in this study, a direct comparison of absolute levels of presynaptic Ca^{2+} entry and neurotransmitter release at single +/+ and mutant terminals was not possible. However, the increase in paired-pulse facilitation at *tg/tg* synapses suggests a reduced amount of evoked neurotransmitter release accompanying the impaired P/Q-type currents in *tg/tg* mutants. This result is consistent with the reduction of evoked transmitter release during synchronous network discharges in tottering hippocampus (Helekar and Noebels, 1994) and in the thalamus after local electrical stimulation (Caddick et al., 1999) and can be explained by the reduced presynaptic P/Q-type calcium entry we have directly observed here.

Based on recordings from presynaptic terminals at the rat calyx of Held synapse (Iwasaki and Takahashi, 1998), hippocampal CA3 terminals (Qian et al., 1997), and cerebellar parallel fiber terminals (Mintz et al., 1995), the P/Q-type channel is a major source of Ca^{2+} controlling evoked neurotransmitter release at mammalian central synapses. In our experiment, the amount of P/Q-type Ca^{2+} influx at this mouse hippocampal synapse is very similar to that observed in the rat hippocampus and cerebellum (Mintz et al., 1995; Qian et al., 1997). It is expected that P/Q-type channels at mouse CA3–CA1 presynaptic terminals will have a similar contribution to neurotransmitter release as their counterparts in rat hippocampal and cerebellar synapses. Thus, a large reduction in the P/Q-type Ca^{2+} influx as observed in *tg/tg* mice should greatly diminish neurotransmitter release. Despite this prediction, synaptic transmission at *tg/tg* CA3–CA1 synapses remained primarily intact. This may reflect a situation in which more N-type Ca^{2+} channels are associated with synaptic vesicle release machinery in *tg/tg* terminals compared with +/+ terminals. The reduction of Ca^{2+} currents through P/Q-type channels may prevent them from forming a stable complex with vesicle release proteins, because some of these interactions, for example that between the Ca^{2+} channel and syntaxin, require a quite high Ca^{2+} concentration (Sheng et al., 1996). Alternatively, the tottering mutation could produce an alteration in α_{1A} protein conformation that directly disrupts interactions with synaptic proteins. In either case, N-type Ca^{2+} channels in *tg/tg* mouse could maintain the release properties of synaptic transmission at a viable level. Morphological reorganization may also contribute to strengthening the synaptic transfer function, as it does in the cerebellar cortex in which ultrastructural studies of the granule cell–Purkinje cell synapse in *tg/tg* and tg^{la}/tg^{la} mice indicate an increase in the area of synaptic contact between parallel fibers and Purkinje cell dendrites (Rhyu et al., 1999). This structural plasticity may also partially compensate for any reduction of neurotransmitter release per synaptic contact to Purkinje cells in *tg/tg* mutants caused by decreased P/Q-type currents.

Ca^{2+} cooperativity of neurotransmitter release at the mouse hippocampal synapse

We have found that neurotransmitter release at the mouse synapse depends on a nonlinear power function of the presynaptic Ca^{2+} influx. The mean power number for wild-type mouse terminals is between 2.5 and 3.0, which is slightly less than what is observed at the guinea pig and rat hippocampal synapse (Wu and Saggau, 1994; Qian et al., 1997). The different Ca^{2+} indicators used in those experiments and a possible species difference may contribute in part to this variation. Moreover, Qian and Saggau (1999) have shown that interpretation of the measured apparent power numbers also depends on the basal level of neurotransmitter release. A higher basal level of neurotransmitter release usually results in a lower apparent power number as the release machinery approaches saturation. This may also explain why a higher power number was measured in *tg/tg*, but not in *lh/lh*, when compared with +/+ presynaptic terminals, because the basal level of neurotransmitter release is likely to be reduced as a result of the decreased presynaptic P/Q-type Ca^{2+} currents in the *tg/tg* mutant.

Functional rescue of synaptic transmission at the hippocampal CA3–CA1 synapse in the absence of normal α_{1A}–β_4 interactions

In contrast to the α_1 subunit mutation, mutation of the Ca^{2+} channel β_4 subunit in the *lh/lh* mouse does not alter presynaptic activity as assessed by several criteria, including presynaptic Ca^{2+} influx profile, paired-pulse facilitation, and G-protein-mediated modulation of presynaptic Ca^{2+} channels. The simplest explanation would be that P/Q-type channels at CA3 presynaptic terminals normally lack β_4 subunits, and therefore the mutation would not exert any functional effects at this synapse. However, *in situ* hybridization studies reveal strong expression of all four β subunits in mouse CA3 pyramidal neurons, making this possibility less likely (Burgess et al., 1999). The more likely interpretation

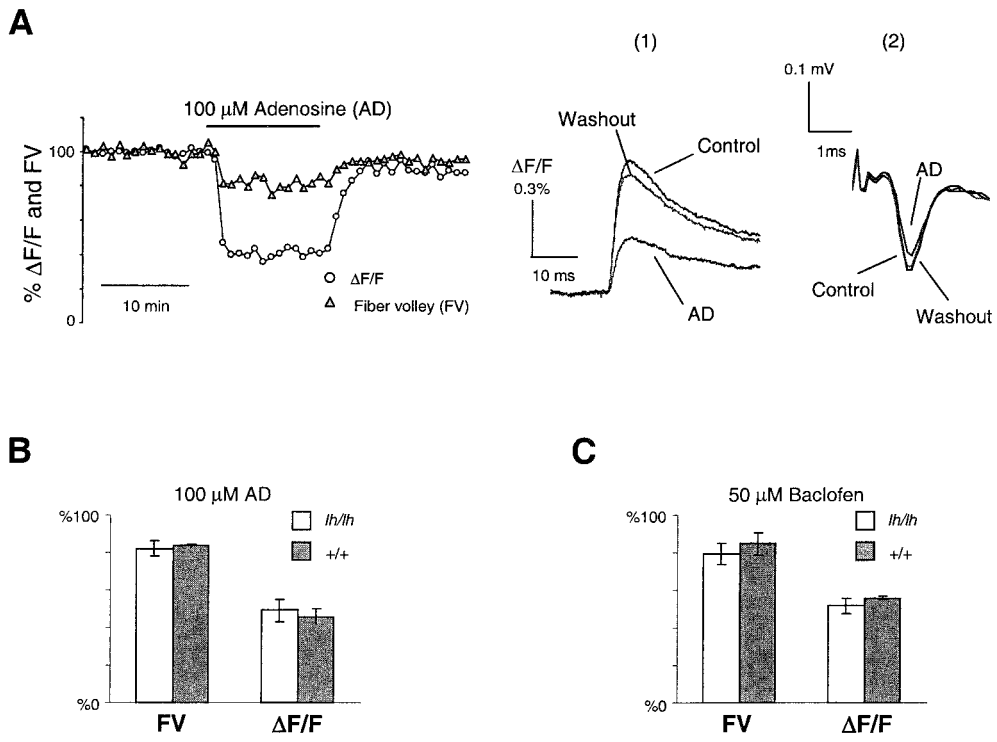


Figure 6. Modulation of presynaptic Ca^{2+} channels is equivalent in *lh/lh* and +/+ mice. *A*, Time courses of $[Ca_{pre}]_t$ and the size of the presynaptic fiber volley in response to application of the neuromodulator AD to +/+ terminals. *Inset 1* shows sample traces of $[Ca_{pre}]_t$ taken during the steady state in control solutions and after application of AD at the saturating concentration of 100 μM. AD resulted in a reversible reduction of $[Ca_{pre}]_t$. *Inset 2* shows that the size of the presynaptic fiber volley was also slightly reduced during application of AD. *B*, Summary data comparing the inhibition of $[Ca_{pre}]_t$ observed in +/+ and *lh/lh* mice after application of AD. There was no significant difference in the AD-induced reduction of the Ca^{2+} signal or in the fiber volley size between +/+ and *lh/lh* mice. *C*, Summary data comparing the inhibition of $[Ca_{pre}]_t$ observed in +/+ and *lh/lh* mice after the application of baclofen, a $GABA_B$ receptor agonist. Similar to the AD response, there was no significant difference in the baclofen-induced reduction of the Ca^{2+} signal or in the fiber volley size between +/+ and *lh/lh* mice. These results indicate that modulation of presynaptic Ca^{2+} channels by G-proteins at the hippocampal CA3–CA1 synapse in *lh/lh* mice was not altered by mutation of the Ca^{2+} channel β_4 subunit.

is that other members of the β subunit family may be able to substitute for some aspects of β_4 function, a process termed reshuffling. Recent coimmunoprecipitation studies demonstrate that α_{1A} and α_{1B} subunits show novel interactions with β_{1-3} subunits in lethargic brain (McEnery et al., 1998; Burgess et al., 1999). In cell types in which all four Ca^{2+} channel β subunits are coexpressed, such as Purkinje cells, the redundancy of β subunit expression during α_{1A} channel assembly is sufficient to rescue the current-carrying ability of P/Q-type channels (Burgess et al., 1999). In CA3 neurons, loss of functional α_1–β_4 subunit interactions may be compensated by reshuffling α_{1A} subunits with other (β_{1-3}) subtypes. Here, we extend the range of P/Q-type channel properties rescued to include three additional functions beyond the nominal restoration of current, namely, the targeted localization of the channels to presynaptic terminals, participation in the synaptic release process, and modulation by G-proteins. We also demonstrate that this rescue applies to presynaptic α_{1B} channels.

Presynaptic function and the cellular basis for neurological phenotypes in Ca^{2+} channel α_{1A} and β_4 subunit mutants

Both tottering and lethargic mice develop a stereotyped pattern of neurological deficits, including ataxia, spike-wave seizures, and paroxysmal dyskinesias of the limbs (Noebels and Sidman, 1979; Noebels, 1984; Hosford et al., 1992). The cellular basis of the neurological phenotype in these two mutants is currently being explored and may depend strongly on the coexpression profiles of α_1 and β subunits in tottering and lethargic neurons, respectively.

Our findings of compensated release at tottering synapses suggest that similar behavior will be found at other synapses in the nervous system, and the degree of functional rescue of α_{1A} will depend on the amount of the coexpressed α_{1B} subunit. Although both α_{1A} and α_{1B} are diffusely expressed and widely colocalized, our data show that circuits normally lacking N-type release could become functionally silenced by the *tg/tg* mutation. Within the hippocampus, this may be the case for certain types of inhibitory synapses. Study of rat hippocampal interneurons reveals that N- and P/Q-type channels are segregated at inhibitory terminals (Poncer et al., 1997). Some inhibitory neurons in stratum lucidum and stratum oriens use only P/Q-type channels, as indicated by the fact that inhibitory postsynaptic potentials onto pyramidal neurons are sensitive only to P/Q-type but not N-type channel blockers, whereas others are pure N-type. Thus, P/Q-type channel mutations would disproportionately affect the function of this subset of neurons and similar populations throughout the nervous system, impair inhibitory synaptic transmission, and thereby disrupt the balance of neuronal excitation and inhibition. The tottering phenotype is therefore predicted to arise from circuits containing presynaptic terminals that are unable to sustain N-type release.

In the case of lethargic mice, a similar prediction can be made on the basis of functional rescue by coexpressed β subunits. The relatively unaffected presynaptic Ca^{2+} entry and neurotransmitter release at *lh/lh* CA3–CA1 synapses predicts that functional deficits of synaptic transmission in lethargic brain will arise where presynaptic β_{1-3} subunits are not available for interaction with α_{1A} and α_{1B} subunits. Interestingly, one site in which a mismatch is particularly clear is in the thalamus, in which β_4 is strongly expressed, but there is a striking absence of β_{1-3} subunit coexpression (Tanaka et al., 1995; Burgess et al., 1999). Thalamic relay cells integrate inputs from the neocortex and cerebellum and play important roles in pathways mediating oscillatory spike-wave activity and ataxia. The remarkable similarity in the neurological phenotypes of α_{1A} and β_4 mutations in tottering and lethargic mice suggests that the functionally vulnerable synapses may overlap in these circuits.

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