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Compensatory Contribution of $Ca_V2.3$ Channels to Acetylcholine Release at the Neuromuscular Junction of *Tottering* Mice

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Abstract

Tottering (*Tg*) mice carry the mutation P601L in their *Cacna1a* encoded $Ca_V2.1$ channels. Transmitter release at the wild-type neuromuscular junction (NMJ) is almost exclusively mediated by $Ca_V2.1$ channels, and we use this model synapse to study synaptic consequences of the *Tg* mutation. With electrophysiology, and using subtype-specific Ca_V2 channel-blocking toxins, we assessed a possible compensatory contribution of non-$Ca_V2.1$ channels to evoked acetylcholine (ACh) release at *Tg* NMJs. Release was reduced by ~75% by the $Ca_V2.1$ channel blocker ω-agatoxin-IVA, which was less than the ~95% reduction observed in wild-type. Release at *Tg* NMJs, but not at wild-type synapses, was reduced by ~15% by SNX-482, a $Ca_V2.3$ channel blocker. No $Ca_V2.2$ channel involvement was found. Probably, there is a small reduction in functional presynaptic $Ca_V2.1$ channels at *Tg* NMJs, which is compensated for by $Ca_V2.3$ channels. The remaining $Ca_V2.1$ channels are likely to convey enlarged Ca^{2+} flux, because evoked ACh release at *Tg* NMJs, at low extracellular Ca^{2+} concentration, was ~6-fold higher than at wild-type NMJs. This is the first report of compensatory expression of non-$Ca_V2.1$ channels at NMJs of mice with a single amino acid change in $Ca_V2.1$.

Acknowledgements

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Introduction

Tottering (*Tg*) mice carry an amino acid change (P601L) in the pore-forming subunit of *Cacna1a* encoded $Ca_V2.1$ (P/Q-type) Ca^{2+} channels (Fletcher et al., 1996; Doyle et al., 1997), causing ataxia and epilepsy in homozygous animals. In humans, *CACNA1A* mutations cause familial hemiplegic migraine and other autosomal dominant neurological disorders (Ophoff et al., 1996; Jouvenceau et al., 2001; Imbrici et al., 2004).

High voltage-activated neuronal Ca^{2+} channels consist of Ca_V1 (L-type), $Ca_V2.1$ (P/Q-type), $Ca_V2.2$ (N-type) and $Ca_V2.3$ (R-type) channels (Catterall, 2000). P- and Q-type channels are splice variants (Bourinet et al., 1999) with different sensitivities to ω-agatoxin-IVA (Stea et al., 1994). $Ca_V2.2$ channels are blocked by ω-conotoxin-GVIA, $Ca_V2.3$ channels by SNX-482, and Ca_V1 channels by dihydropyridines (Catterall, 2000).

$Ca_V2.1$ channels mediate neurotransmitter release at many central synapses and at the peripheral neuromuscular junction (NMJ), where they govern >90% of release (Uchitel et al., 1992). At mouse NMJs, synaptic effects of *Cacna1a* mutations can be studied with relative ease (Plomp et al., 2000; Van Den Maagdenberg et al., 2004). Previously, we showed abnormal spontaneous ACh release (~2-fold increased miniature endplate potential [MEPP] frequency) at *Tg* NMJs, as well as reduced high-rate (40 Hz) evoked release (increased endplate potential [EPP] amplitude rundown). However, low-rate (0.3 Hz) evoked release was unchanged (Plomp et al., 2000).

$Ca_V2.1$, -2 and -3 channels act in a mutually compensatory fashion. Thus, transmitter release at $Ca_V2.1$ (*null*-)mutant NMJs and central synapses becomes to rely on $Ca_V2.2$ and –3 channels (Leenders et al., 2002; Urbano et al., 2002; Cao et al., 2004), whereas compensatory $Ca_V2.1$ expression occurs in $Ca_V2.2$ *null*-mutant neurons (Takahashi et al., 2004a; Takahashi et al., 2004b). At *Tg* central synapses, compensatory $Ca_V2.2$ channels were shown (Qian and Noebels, 2000; Leenders et al., 2002). We tested the hypothesis that compensatory, non-$Ca_V2.1$ channels contribute to ACh release at the *Tg* NMJ in electrophysiological experiments using Ca_V2 subtype-specific blocking toxins. We also measured low-rate (0.3 Hz) evoked ACh release at *Tg* NMJs at low extracellular Ca^{2+}, in order to test whether effects of the *Tg* mutation not visible at normal Ca^{2+} concentration can be unmasked, similar to our recent finding in mice carrying the human familial hemiplegic migraine-associated mutations R192Q and S218L (Kaja et al., 2004; Van Den Maagdenberg et al., 2004; Kaja et al., 2005).

Materials and Methods

Mice

All animal experiments were in accordance with national legislation, Leiden University guidelines and the *American Physiological Society's* Guiding Principles in the Care and Use of Animals. *Tg* mice were raised from original breeder pairs obtained from Jackson Laboratories (Bar Harbor, ME, USA). Animals were genotyped as described previously (Plomp et al., 2000). Homozygous *Tg* and wild-type mice were used at 6 weeks of age, with the investigator blinded for genotype.

Ex vivo *NMJ electrophysiology*

Mice were euthanized by carbon dioxide inhalation. Hemi-diaphragms with phrenic nerve were dissected and kept in Ringer's medium (in mM: NaCl 116, KCl 4.5, $CaCl_2$ 2, $MgSO_4$ 1, NaH_2PO_4 1, $NaHCO_3$ 23, glucose 11, pH 7.4) at room temperature. Intracellular recordings of MEPPs and EPPs were made at NMJs at 28 °C using standard micro-electrode equipment.

At least 30 MEPPs and EPPs were recorded at each NMJ, and 5-10 NMJs were sampled per experimental condition per muscle. Muscle action potentials were blocked by 3 μM μ-conotoxin-GIIIB (Scientific Marketing Associates, Barnet, UK). For EPP recording, the nerve was stimulated at 0.3 or 40 Hz. Procedures for analysis of MEPPs and EPPs and calculation of quantal contents, i.e. the number of ACh quanta released per nerve impulse, have been described before (Van Den Maagdenberg et al., 2004). EPPs and MEPPs were also measured in presence of the specific Ca^{2+} channel blockers ω-agatoxin-IVA ($Ca_V2.1$, 200 or 400 nM, as indicated), ω-conotoxin-GVIA ($Ca_V2.2$, 2.5 μM) and SNX-482 ($Ca_V2.3$, 1 or 2 μM, as indicated), following a 20 min pre-incubation period. Toxins were from Scientific Marketing Associates. We also probed for the presence of $Ca_V2.2$ channels more distant from the Ca^{2+} sensor at release sites by testing the effect of ω-conotoxin-GVIA on the quantal content in the presence of 50 μM of the K^+ channel blocker 4-aminopyridine (4-AP, Sigma-Aldrich, Zwijndrecht, The Netherlands), in a 0.5 mM Ca^{2+}/5.5 mM Mg^{2+}-Ringer medium, according to the methods described by Urbano et al. (2003).

Statistical analysis

Paired or unpaired Student's *t*-tests were used where appropriate, on grand mean values with *n* as the number of mice tested, and 5-10 NMJs tested per muscle, per condition. $P<0.05$ was considered to be statistically significant. Data is presented as mean ± S.E.M.

Results

Reduced ω-agatoxin-IVA sensitivity of evoked ACh release at Tg NMJs

Total 0.3 Hz stimulation-evoked ACh release at normal (2 mM) Ca^{2+} concentration was the same at *Tg* and wild-type NMJs, in line with earlier observations (Plomp et al., 2000). The quantal content was ~32 (Figure 1). ω-Agatoxin-IVA (200 nM) reduced quantal content at NMJs of wild-type muscles by 94%, from 32.5 ± 1.6 to 1.8 ± 0.8 (n=4 muscles, 7-10 NMJs per muscle, $p<0.001$; Figure 1A). However, at *Tg* NMJs it decreased only by 73% (from 33.9 ± 1.9 to 9.0 ± 2.0, n=4 muscles, 7-10 NMJs per muscle, $p<0.001$; Figure 1A). ω-Agatoxin-IVA decreased EPP amplitude by 93% (from 22.8 ± 1.1 mV to 1.6 ± 0.7 mV) in wild-type, but only by 69% (from 27.2 ± 0.8 to 8.3 ± 1.0 mV) at *Tg* NMJs (n=4 muscles, 7-10 NMJs per muscle, $p<0.01$; Figure 1B). In the presence of ω-agatoxin-IVA, EPP failure upon a stimulus was frequently observed at wild-type but almost never at *Tg* NMJs (29.8 ± 11.8 and 1.5 ± 1.5 % of the stimuli, respectively, n=4 muscles, 7-10 NMJs per muscle, $p<0.05$; Figures 1B, C). MEPP amplitudes and kinetics were unaffected by ω-agatoxin-IVA (data not shown). MEPP frequency was reduced by ~50% at both *Tg* and wild-type NMJs (Table 1), as shown previously (Plomp et al., 2000).

The possibility exists that the *Tg* mutation renders the $Ca_V2.1$ channel less sensitive to ω-agatoxin-IVA, although such an effect is not very likely in view of the distant localizations of the *Tg* mutation (amino acid 601, P-loop of repeat II) and the ω-agatoxin-IVA binding site (amino acid 1658, C-terminal end of S3, repeat IV, Bourinet et al., 1999; Winterfield and Swartz, 2000). However, such an effect could explain the lesser reduction of quantal content by ω-agatoxin-IVA, compared with wild-type NMJs. We, therefore, experimentally tested this possibility by measuring ACh release at wild-type and *Tg* NMJs also in the presence of a higher ω-agatoxin-IVA concentration (400 nM), which is more than five times the IC_{50} for wild-type quantal content, as determined in our laboratory (~75 nM, unpublished data). No extra reduction was observed, compared to that in the presence of 200 nM toxin (n=3-4 muscles, 10 NMJs per muscle, $p=0.85$ in wild-type and $p=0.34$ in *Tg*, Figure 1A), excluding the possibility of a reduced toxin-sensitivity of *Tg*-mutated $Ca_V2.1$ channels.

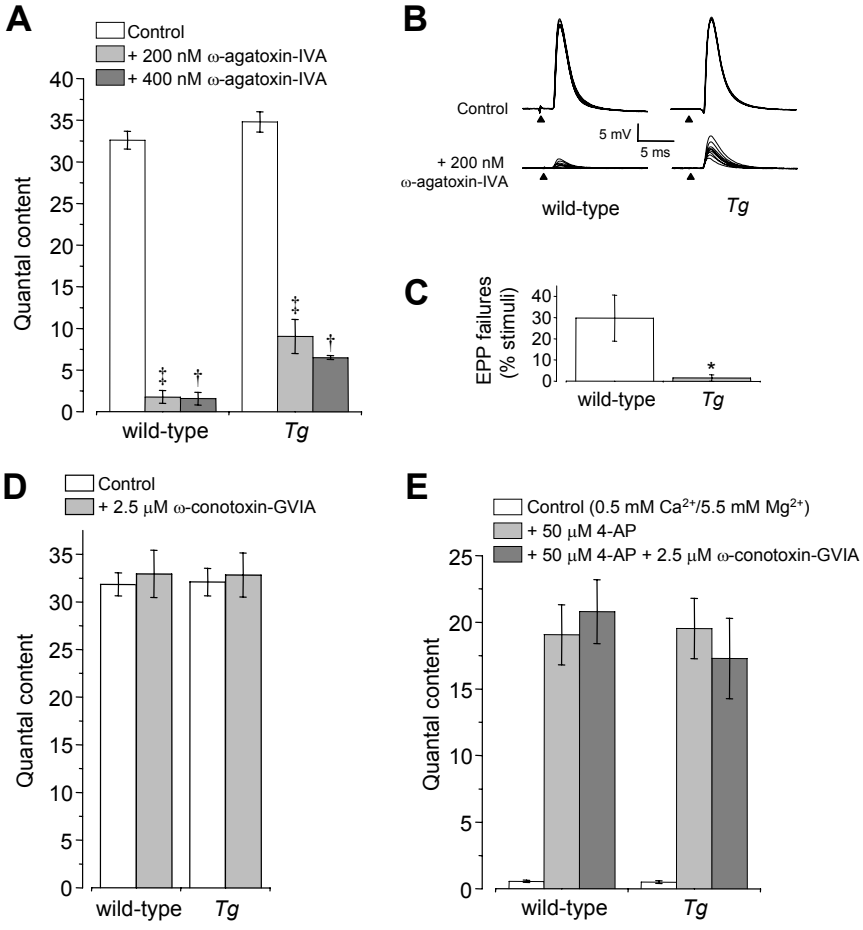

Figure 1. Effect of selective Ca_V2 channel blockers on 0.3 Hz evoked ACh release at the *Tg* diaphragm NMJ, as determined with electrophysiological recording of synaptic signals.
(**A**) $Ca_V2.1$ blocker ω-agatoxin-IVA (200 nM) reduced quantal content by ~95% at wild-type NMJs (n=4 muscles, 7-10 NMJs per muscle, $p<0.001$), but only by ~75% at *Tg* NMJs (n=4 muscles, 7-10 NMJs per muscle, $p<0.001$), which was a statistically significantly smaller reduction ($p<0.05$). The quantal content reductions induced by a higher ω-agatoxin-IVA concentration (400 nM) were similar. (**B**) Representative examples of nine superimposed 0.3 Hz evoked EPPs, before (top panel) and after (bottom panel) application of ω-agatoxin-IVA. Arrowheads indicate moment of nerve stimulation. (**C**) Quantification of EPP failures during 0.3 Hz stimulation in the presence of 200 nM ω-agatoxin-IVA, which were much more common at wild-type NMJs. (**D**) $Ca_V2.2$ blocker ω-conotoxin-GVIA (2.5 μM) did neither affect quantal content at wild-type (n=4 muscles, $p=0.73$) nor *Tg* (n=4 muscles, 7-10 NMJs per muscle, $p=0.71$) NMJs. (**E**) Lack of sensitivity of quantal content to ω-conotoxin-GVIA (2.5 μM) in the presence of 0.5 mM Ca^{2+}/5.5 mM Mg^{2+}-Ringer and 50 μM 4-AP. (**F**) $Ca_V2.3$ blocker SNX-482 (1 and 2 μM) reduced quantal content at *Tg* NMJs by ~15% (n=3-4 muscles, 7-10 NMJs per muscle, $p<0.05$). Subsequent addition of 400 nM ω-agatoxin-IVA almost completely blocked evoked ACh release (n=3 muscles, 10 NMJs per muscle, $p<0.01$). * $p<0.05$, † $p<0.01$, ‡ $p<0.001$.

Ca_V2.3 channels contribute to evoked ACh release at Tg NMJs

Hence, in view of the reduced ω-agatoxin-IVA-sensitivity of evoked ACh release at *Tg* NMJs, compensatory involvement of non-Ca_V2.1 channels is likely. Both Ca_V2.2 and Ca_V2.3 channels are known to mediate neurotransmitter release (Reid et al., 2003), and both partially compensate for the loss of Ca_V2.1 channels at NMJs of *Cacna1a null*-mutant mice (Urbano et al., 2003; chapter 7). In order to investigate compensatory involvement of Ca_V2.2 channels, we recorded MEPPs and EPPs at NMJs of *Tg* and wild-type muscles before and after application of the selective blocker ω-conotoxin-GVIA (2.5 μM). In either genotype, the toxin affected neither evoked nor spontaneous ACh release (Figure 1D, Table 1). Quantal contents were similar (31.8 ± 1.2 and 32.9 ± 2.5 at wild-type NMJs, n=4 muscles, 7-10 NMJs per muscle, p=0.73, and 31.4 ± 1.2 and 30.0 ± 3.2 at *Tg* NMJs, n=4 muscles, 7-10 NMJs per muscle, p=0.71, before and in presence of the toxin, respectively). The possibility exists that compensatory expressed Ca_V2.2 channels are localized more distantly from the Ca^{2+} sensor at release sites, as proposed for *Cacna1a null*-mutant NMJs (Urbano et al., 2003). We tested this hypothesis using the protocol described in Urbano et al. (2003). The quantal content was first measured in Ringer medium containing 0.5 mM Ca^{2+}/5.5 mM Mg^{2+} and was similar at wild-type and *Tg* NMJs (0.58 ± 0.09, and 0.51 ± 0.09, respectively, n=5-6 muscles, 10 NMJs per muscle, p=0.64). Addition of 50 μM 4-AP increased quantal content equally (about 37-fold) in both genotypes (Figure 1E). Further addition of 2.5 μM ω-conotoxin-GVIA did not affect quantal content (19.1 ± 2.3 and 20.8 ± 2.4 before and during toxin in wild-type, n=5 muscles, 10 NMJs per muscle, p=0.18; 19.5 ± 2.3 and 17.3 ± 3.0 before and during toxin in *Tg*, n=7 muscles, 10 NMJs per muscle, p=0.24, Figure 1E). These results further indicated absence of compensatory expression of Ca_V2.2 channels at *Tg* NMJs, even at distant sites.

Next, we tested the Ca_V2.3 channel blocker SNX-482 (1 μM). Quantal content of wild-type NMJs was unchanged (29.4 ± 0.9 before and 30.0 ± 1.4 in presence of SNX-482, n=4 muscles, 7-10 NMJs per muscle, p=0.51; Figure 1F). However, at *Tg* NMJs SNX-482 reduced quantal content by ~15% (from 30.5 ± 1.6 to 25.8 ± 1.7, n=4 muscles, 7-10 NMJs per muscle, $p<0.05$; Figure 1F). Together, Ca_V2.1 and Ca_V2.3 channels mediate ~90% (~75% and ~15%, respectively) of the 0.3 Hz evoked ACh release. Thus, the apparent reduction of Ca^{2+} influx through ω-agatoxin-IVA-sensitive channels at the *Tg* NMJ is almost fully compensated for by Ca^{2+} influx through SNX-482-sensitive channels, i.e. Ca_V2.3, since Ca_V2.1 channels mediate 90-95% of 0.3 Hz evoked release at wild-type channels. SNX-482 did not statistically significantly affect spontaneous release in either genotype, although there was a tendency for reduction at *Tg* NMJs (Table 1).

It might be speculated that the *Tg* mutation brings (some) SNX-482-sensitivity onto Ca_V2.1 channels, by creating a (low-affinity) receptor site in the Ca_V2.1 protein, instead of indirectly inducing the expression of compensatory Ca_V2.3 channels. In that case, the 15% reduction of quantal content by 1 μM SNX-482 at *Tg* NMJs could be regarded as a sub-optimal inhibition of SNX-482-sensitive *Tg*-mutated Ca_V2.1 channels. Although this possibility is not very likely in view of the distant localizations of the *Tg* mutation in Ca_V2.1 (in P-loop of repeat II) and the SNX-482 binding site in Ca_V2.3 (on repeats III and IV, presumably in S3-4 regions, Bourinet et al., 2001), we nevertheless tested it by exposing *Tg* NMJs to a doubled SNX-482 concentration (2 μM). However, no extra reduction of quantal content at *Tg* NMJs occurred, compared to that observed upon incubation with 1 μM SNX-482 (n=3-4 muscles, 10 NMJs per muscle, p=0.85, Figure 1F), indicating that it is very unlikely that the *Tg* mutation rendered the Ca_V2.1 channel sensitive to SNX-482. We also added 400 nM ω-agatoxin-IVA to the *Tg* preparations that were incubated in 2 μM SNX-482, and observed an almost

complete block of quantal content (to only 2.3% of the quantal content before the toxins, n=3 muscles, 10 NMJs per muscle, $p<0.01$, Figure 1F), again showing that ACh release at *Tg* NMJs is governed exclusively by $Ca_V2.1$ and $Ca_V2.3$ channels.

Table 1. Effect of specific Ca_V2 channel blocking toxins on MEPP frequency.

	Wild-type	*Tg*
Control	$0.96 \pm 0.12\ s^{-1}$	$2.04 \pm 0.32\ s^{-1}$
+ ω-agatoxin-IVA (200 nM)	$0.49 \pm 0.05\ s^{-1}$ †	$1.04 \pm 0.17\ s^{-1}$ *#
Control	$0.90 \pm 0.11\ s^{-1}$	$2.01 \pm 0.20\ s^{-1}$
+ ω-conotoxin-GVIA (2.5 μM)	$0.89 \pm 0.13\ s^{-1}$	$1.98 \pm 0.26\ s^{-1}$
Control	$0.96 \pm 0.16\ s^{-1}$	$1.88 \pm 0.20\ s^{-1}$
+ SNX-482 (1 μM)	$0.93 \pm 0.15\ s^{-1}$	$1.57 \pm 0.24\ s^{-1}$

Values are means ± SEM. Effect of specific Ca_V2 blocking toxins on spontaneous ACh release (measured as MEPP frequency) at both wild-type and *Tg* NMJs. Neither the selective $Ca_V2.2$ channel blocker ω-conotoxin-GVIA (2.5 μM), nor the $Ca_V2.3$ blocker SNX-482 (1 μM) significantly affected spontaneous ACh release. N=4 muscles, 7-10 NMJs per muscle.
† Different from control, $p<0.01$; * different from control, $p<0.05$; # as described previously (Plomp et al., 2000), $Ca_V2.1$ blocker ω-agatoxin-IVA reduced MEPP frequency ~50% in both genotypes.

Increased 0.3 Hz evoked ACh release at Tg NMJs in low Ca^{2+}

We investigated 0.3 Hz evoked ACh release at *Tg* NMJs in low (0.2 mM) extracellular Ca^{2+}. At wild-type NMJs, quantal content was 1.7 ± 0.4 (n=4 muscles, 7-10 NMJs per muscle). However, at *Tg* NMJs it was ~6-fold higher (10.7 ± 0.9, n=4 muscles, 7-10 NMJs per muscle, $p<0.01$; Figure 2A). EPP amplitudes were 1.8 ± 0.6 and 9.1 ± 0.8 mV at wild-type and *Tg* NMJs, respectively (n=4 muscles, 7-10 NMJs per muscle, $p<0.01$; Figures 2B, D). In 0.2 mM Ca^{2+}, EPP failure upon a stimulus was regularly observed at wild-type but not *Tg* NMJs (48.7 ± 5.7 and 1.3 ± 1.3% of the stimuli, respectively, n=4 muscles, 7-10 NMJs per muscle, $p<0.001$; Figures 2B, C). MEPP amplitudes did not differ between genotypes (1.00 ± 0.10 and 0.96 ± 0.05 mV at wild-type and *Tg* NMJs, respectively, n=4 muscles, 7-10 NMJs per muscle, p=0.73).

In a separate experimental series, the quantal content at *Tg* NMJs at low Ca^{2+} was unaffected by SNX-482 (7.6 ± 1.7 before and 7.2 ± 1.5 in presence of the toxin, n=4 muscles, 10 NMJs per muscle, p=0.41), but was reduced by 98%, to 0.2 ± 0.1 (n=2 muscles, 5 NMJs per muscle) by ω-agatoxin-IVA (Figure 2E).

$Ca_V2.3$ channels do not contribute disproportionally to rundown of EPP amplitude during 40 Hz nerve stimulation

Previously, at normal extracellular Ca^{2+} concentration, we have shown that rundown of EPP amplitude during tetanic (40 Hz) nerve stimulation is somewhat more pronounced at *Tg* NMJs (the rundown plateau level, expressed as percentage of the first EPP, was ~8% lower, Plomp et al., 2000). Specific $Ca_V2.3$ channel behaviour, for instance a relatively large use-dependent inhibition, might underlie such increased rundown. In order to test this hypothesis we recorded and quantified 40 Hz EPP rundown at *Tg* NMJs in the presence of either no channel blockers, ω-agatoxin-IVA (200 or 400 nM), SNX-482 (1 or 2 μM) or both toxins in combination, at normal Ca^{2+} level (Figures 3, B). Normalized EPP rundown in the presence of ω-agatoxin-IVA and in the presence of SNX-482 was similar (to about 77% of the first EPP in the train, n=3-6 muscles, 7-10 NMJs per muscle, p=0.65, Figure 3C), which does not differ from the control condition without toxins. This indicates that $Ca_V2.3$ channels do not contribute disproportionally to EPP rundown at *Tg* NMJs.

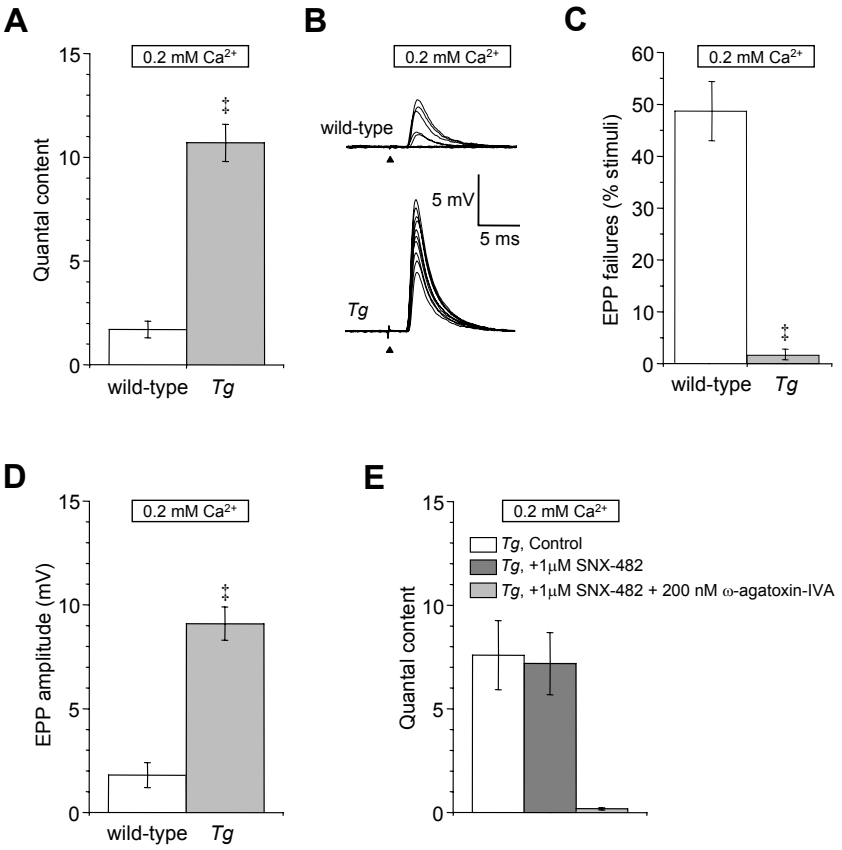

Figure 2. Evoked (0.3 Hz) ACh release at *Tg* NMJs at low (0.2 mM) extracellular Ca^{2+} concentration.
(**A**) At *Tg* NMJs, quantal content was ~6-fold higher than at wild-types (n=4 muscles, 7-10 NMJs per muscle, $p<0.01$). (**B**) Representative examples of ten superimposed 0.3 Hz evoked EPPs from wild-type and *Tg* NMJs. Arrowheads indicate moment of nerve stimulation. (**C**) Quantification of EPP failures during 0.3 Hz stimulation, which were much more common at wild-type NMJs. (**D**) Mean EPP amplitude was ~5-fold higher at *Tg* NMJs (n=4 muscles, 7-10 NMJs per muscle, $p<0.001$). (**E**) $Ca_V2.3$ blocker SNX-482 (1 μM) did not affect quantal content at *Tg* NMJs at low Ca^{2+} (n=4 muscles, 10 NMJs per muscle, $p=0.41$), while $Ca_V2.1$ blocker ω-agatoxin-IVA (200 nM) almost completely inhibited it (n=2 muscles, 5 NMJs per muscle). ‡ $p<0.001$.

Discussion

We investigated compensatory contribution of non-$Ca_V2.1$ channels to evoked ACh release at *Tg* NMJs. At wild-type mouse NMJs, ω-agatoxin-IVA reduces quantal content by >90%, indicating that ACh release is almost exclusively mediated by $Ca_V2.1$ channels (Uchitel et al., 1992; Giovannini et al., 2002; Kaja et al., 2005). The lesser inhibition found at *Tg* NMJs (only ~75%) suggests compensatory involvement of non-$Ca_V2.1$ channels. The experiments with SNX-482, a selective blocker of $Ca_V2.3$ channels, show that ~15% of the total release at *Tg* NMJs is mediated by $Ca_V2.3$ channels. ACh release at NMJs of *Cacna1a null*-mutant mice, which lack $Ca_V2.1$ channels and die at ~3 weeks of age, becomes dependent on $Ca_V2.3$ as well as $Ca_V2.2$ channels (Urbano et al., 2003). However, we could exclude compensatory involvement of $Ca_V2.2$ channels at *Tg* NMJs by showing insensitivity of quantal content to ω-conotoxin-GVIA, even in a protocol testing for $Ca_V2.2$ channels localized more distantly from the Ca^{2+} sensor at release sites. Thus, we show that $Ca_V2.3$ channels are recruited first as compensatory channels at *Tg* NMJs. This is the first report of compensatory expression

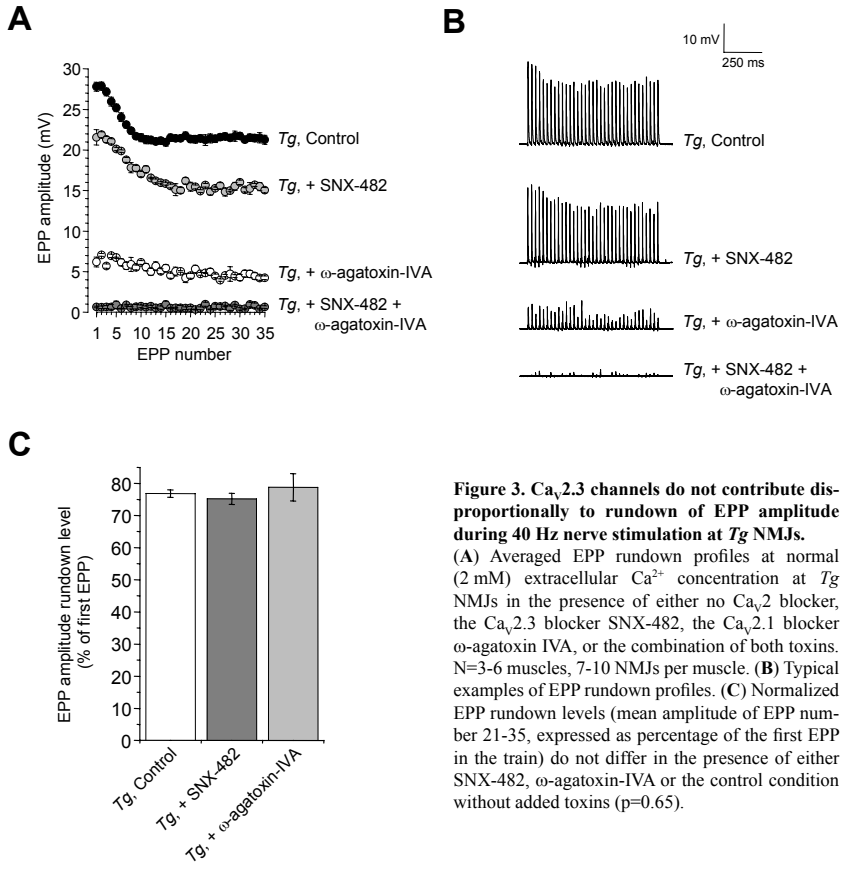

Figure 3. $Ca_V2.3$ channels do not contribute disproportionally to rundown of EPP amplitude during 40 Hz nerve stimulation at *Tg* NMJs. (**A**) Averaged EPP rundown profiles at normal (2 mM) extracellular Ca^{2+} concentration at *Tg* NMJs in the presence of either no Ca_V2 blocker, the $Ca_V2.3$ blocker SNX-482, the $Ca_V2.1$ blocker ω-agatoxin IVA, or the combination of both toxins. N=3-6 muscles, 7-10 NMJs per muscle. (**B**) Typical examples of EPP rundown profiles. (**C**) Normalized EPP rundown levels (mean amplitude of EPP number 21-35, expressed as percentage of the first EPP in the train) do not differ in the presence of either SNX-482, ω-agatoxin-IVA or the control condition without added toxins (p=0.65).

of non-$Ca_V2.1$ channels at NMJs of mice with a single amino acid change in $Ca_V2.1$. On the basis of the results of the present extensive study we have to revise our earlier, preliminary view of similar ω-agatoxin-IVA-sensitivity of evoked ACh release at wild-type and *Tg* NMJs, which was based on only a single experiment per genotype (Plomp et al., 2000).

The studies on *Cacna1a null*-mutant NMJs have led to the hypothesis that ACh release sites have 'slots' that are preferentially filled with $Ca_V2.1$ channels, but in their absence become occupied by $Ca_V2.3$ channels (Urbano et al., 2003). This would suggest a small reduction in the presynaptic membrane expression of $Ca_V2.1$ channels at *Tg* NMJs, leaving 'slots' available for $Ca_V2.3$ channels. However, although reduced *Tg*-mutated $Ca_V2.1$ channel expression at nerve terminals has indeed been proposed (Leenders et al., 2002), such a reduction was not supported by our previous finding of a 2-fold increased spontaneous ACh release at *Tg* NMJs that remains sensitive to ω-agatoxin-IVA (Plomp et al., 2000). The explanation may be that the effect of a small reduction in *Tg* $Ca_V2.1$ channel expression is masked by increased Ca^{2+}-flux through the remaining channels, due to a mutation-induced shift of their activation voltage towards more negative values, as proposed by us earlier (Plomp et al., 2000). Extra Ca^{2+} influx through *Tg*-mutated $Ca_V2.1$ channels was further substantiated here, by the finding that ACh release in low extracellular Ca^{2+} was ~6-fold increased at *Tg* NMJs. This increase was solely due to the *Tg*-mutated $Ca_V2.1$ channels and not to compensatory $Ca_V2.3$ channels, since it was unaffected by SNX-482 and almost completely inhibited by

ω-agatoxin-IVA. There is an interesting parallel between the electrophysiology of *Tg* NMJs and those of R192Q and S218L *Cacna1a*-mutated mice (knock-in models for human familial hemiplegic migraine), in that we have recently observed similar increases in MEPP frequency and low-Ca^{2+} quantal content at those NMJs (Kaja et al., 2004; Van Den Maagdenberg et al., 2004; Kaja et al., 2005). Voltage-clamp measurements in (transfected) primary cultured neurones and heterologous expression systems showed a clear negative shift of activation voltage for both R192Q- and S218L-mutated channels (Tottene et al., 2005; Van Den Maagdenberg et al., 2004). These parallels with *Tg* synapses further suggest that *Tg*-mutated $Ca_V2.1$ channels at NMJs may have a negatively shifted activation voltage. However, enigmatically, Wakamori and colleagues showed normal activation voltage for *Tg* channels in Purkinje cell bodies and transfected baby hamster kidney cells (Wakamori et al., 1998). It may, however, be that extrapolation of data obtained at cell body channels to behaviour of presynaptic channels is not justifiable because of the specific interactions of presynaptic channels with their native environment at transmitter release sites.

Besides a shift in activation voltage, *Tg* channels may have altered modulatory properties following from a mutation-induced change of interaction with factors such as calmodulin or G-proteins (Lee et al., 1999; Catterall, 2000), leading to increased Ca^{2+} flux. However, such an explanation is not very likely in view of the very different localizations of the *Tg* mutation and known binding and effector sites of modulatory factors (Zhong et al., 2001).

As yet, it is unclear how compensatory presynaptic $Ca_V2.3$ channels are recruited. At wild-type NMJs, they do not contribute to ACh release, and are undetectable with immunohistochemistry (Westenbroek et al., 1998). It may be that protein expression must first be triggered, e.g. by the changed Ca^{2+} influx due to *Tg* $Ca_V2.1$ mutation. Alternatively, membrane insertion may normally fail due to absence of available 'slots' at ACh release sites, but become successful when *Tg* $Ca_V2.1$ mutation results in some free 'slots'.

The lack of compensatory involvement of $Ca_V2.2$ channels at *Tg* NMJs, as opposed to *Cacna1a null*-mutant NMJs, may be explained by the presence of remaining $Ca_V2.1$ channels. For instance, expression of syntaxin-1A is dependent on selective $Ca_V2.1$-mediated Ca^{2+} influx (Sutton et al., 1999). This presynaptic protein can inhibit the function of $Ca_V2.1$ and $Ca_V2.2$ channels (Bezprozvanny et al., 1995), but not that of $Ca_V2.3$ channels. Thus, if altogether present at the NMJ, $Ca_V2.2$ channels in the *Tg* presynaptic membrane may be silenced. The situation at central synapses seems different. Compensatory $Ca_V2.2$ channels have been shown at *Tg* hippocampal synapses (Qian and Noebels, 2000) and forebrain synaptosomes (Leenders et al., 2002).

The changes at *Tg* NMJs partly resemble those found at NMJs of the R192Q knock-in mouse model for familial hemiplegic migraine (Van Den Maagdenberg et al., 2004; Kaja et al., 2005): increased spontaneous ACh release and evoked release that is normal at physiological extracellular Ca^{2+} but strongly increased, compared to wild-type, at low Ca^{2+}. However, clear differences exist. There is no compensatory involvement of $Ca_V2.3$ channels at R192Q NMJs, because ω-agatoxin-IVA reduces quantal content by the same extent (>90%) as in wild-types. Furthermore, the reduction in high-rate evoked ACh release at *Tg* NMJs (Plomp et al., 2000), is more pronounced than that at R192Q NMJs (Kaja et al., 2005).

The *Tg* NMJ displays some extra rundown of high-rate evoked ACh release, compared to wild-type (Plomp et al., 2000). It may be that $Ca_V2.3$ channel behaviour contributes to this phenomenon. Normal rundown at wild-type NMJs is likely due to a combination of $Ca_V2.1$ channel inactivation, its recovery, and the replenishment of releasable ACh vesicles. A relatively large degree of use-dependent inhibition of $Ca_V2.3$, compared to that of (*Tg*-)$Ca_V2.1$

channels, e.g. induced by faster inactivation (Williams et al., 1994), might add disproportionally to the EPP rundown at *Tg* NMJs. However, normalized EPP rundown at *Tg* NMJs in the presence of either SNX-482 or ω-agatoxin-IVA did not differ. This indicates that the contribution of use-dependent inhibition of $Ca_V2.3$ channels to EPP rundown is either similar to that of (*Tg*-)$Ca_V2.1$ channels, or that use-dependent inhibition of Ca_V2 channels is not at all a factor contributing to rundown at normal Ca^{2+} level. It may be that the replenishment rate of ACh vesicles at release sites is the major determinant of EPP rundown under these conditions.

The mechanism of increased ACh release becoming unmasked at low extracellular Ca^{2+} concentration at *Tg* as well as R192Q NMJs is unclear. The Ca^{2+} influx through mutant channels at physiological extracellular Ca^{2+} may be of such magnitude that presynaptic sensors saturate. Alternatively, Ca^{2+}/calmodulin-dependent $Ca_V2.1$ inactivation (Lee et al., 1999) may be increased at mutant synapses, due to increased Ca^{2+} influx or, although not very likely (as discussed above), a direct change in $Ca_V2.1$ modulatory characteristics. Alternatively, the localization of the different types of Ca_V2 channels relative to the Ca^{2+} sensor of the neuroexocytotic mechanism may play a role (Urbano et al., 2003; Wu et al., 1999). *Tg*-mutated $Ca_V2.1$ channels might be more closely localized than wild-type channels and therefore contribute more efficiently to ACh release. This may also (partly) explain the higher MEPP frequency at *Tg* NMJs, compared to wild-type, at normal extracellular Ca^{2+} concentration. Similarly, a closer localization of *Tg*-$Ca_V2.1$ channels to release sites than $Ca_V2.3$ channels may explain the lack of contribution of $Ca_V2.3$ channels to evoked ACh release under the condition of low extracellular Ca^{2+}.

It is unclear whether compensatory $Ca_V2.3$ channel-mediated transmitter release at *Tg* CNS synapses, as present at the NMJ, influences the symptoms of ataxia and epilepsy. Central neurons can upregulate $Ca_V2.3$ channels after partial downregulation of $Ca_V2.1$ channels, as shown in cerebellar Purkinje cells (Pinto et al., 1998). However, total genetic $Ca_V2.1$ ablation results in unaltered or reduced $Ca_V2.3$ current density in cerebellar neurons (Fletcher et al., 2001; Jun et al., 1999). The unidentified residual Ca^{2+} current shown at *Tg* hippocampal presynapses after blocking $Ca_V2.1$ and -2 channels (Qian and Noebels, 2000) may be due to $Ca_V2.3$ channel expression. It would be interesting to cross-breed *Tg* with *Cacna1e* mice (Wilson et al., 2000) in order to test $Ca_V2.3$ channel involvement.