Compensatory Contribution of Ca_{2.3} Channels to Acetylcholine Release at the Neuromuscular Junction of Tottering Mice

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Kaja, Simon, Rob C. G. Van de Ven, Michel D. Ferrari, Rune R. Frants, Arn M.J.M. Van den Maagdenberg, and Jaap J. Plomp. Compensatory contribution of Ca_{2.3} channels to acetylcholine release at the neuromuscular junction of Tottering mice. J Neurophysiol 95: 2698–2704, 2006. First published December 28, 2005; doi:10.1152/jn.01221.2005. Tottering (Tg) mice carry the mutation P601L in their Cacna1a encoded Ca_{2.1} channels. Transmitter release at the wild-type neuromuscular junction (NMJ) is almost exclusively mediated by Ca_{2.1} channels, and we used this model synapse to study synaptic consequences of the Tg mutation. With electrophysiology, and using subtype-specific Ca_{2.2} channel-blocking toxins, we assessed a possible compensatory contribution of non-Ca_{2.1} channels to evoked acetylcholine (ACh) release at Tg NMJs. Release was reduced by ~75% by the Ca_{2.1} 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_{2.3} channel blocker. No Ca_{2.2} channel involvement was found. Probably, there is a small reduction in functional presynaptic Ca_{2.1} channels at Tg NMJs, which is compensated for by Ca_{2.3} channels. The remaining Ca_{2.1} channels are likely to convey enlarged Ca^{2+} flux, because evoked ACh release at Tg NMJs, at low extracellular Ca^{2+} concentration, was approximately sixfold higher than at wild-type NMJs. This is the first report of compensatory expression of non-Ca_{2.1} channels at NMJs of mice with a single amino acid change in Ca_{2.1}.

INTRODUCTION

Tottering (Tg) mice carry an amino acid change (P601L) in the pore-forming subunit of Cacna1a encoded Ca_{2.1} (P/Q-type) Ca^{2+} channels (Doyle et al. 1997; Fletcher et al. 1996), causing ataxia and epilepsy in homozygous animals. In humans, CACNA1A mutations cause familial hemiplegic migraine and other autosomal dominant neurological disorders (Imbrici et al. 2004; Jouveneau et al. 2001; Ophoff et al. 1996).

High voltage–activated neuronal Ca^{2+} channels consist of Ca_{1} (L-type), Ca_{2.1} (P/Q-type), Ca_{2.2} (N-type), and Ca_{2.3} (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_{2.2} channels are blocked by ω-conotoxin-GVIA, Ca_{2.3} channels by SNX-482, and Ca_{1} channels by dihydropyridines (Catterall 2000).

Ca_{2.1} 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 acetylcholine (ACh) release [approximately two-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_{2.1}, -2, and -3 channels act in a mutually compensatory fashion. Thus transmitter release at Ca_{2.1} (null-) mutant NMJs and central synapses relies on Ca_{2.2} and -3 channels (Cao et al. 2004; Leenders et al. 2002; Urbano et al. 2002), whereas compensatory Ca_{2.1} expression occurs in Ca_{2.2} null-mutant neurons (Takahashi et al. 2004a,b). At Tg central synapses, compensatory Ca_{2.2} channels were shown (Leenders et al. 2002; Qian and Noebels 2000). We tested the hypothesis that compensatory, non-Ca_{2.1} channels contribute to ACh release at the Tg NMJ in electrophysiological experiments using Ca_{2.2} subtype-specific blocking toxins. We also measured low-rate (0.3 Hz) evoked ACh release at Tg NMJs at low extracellular Ca^{2+} to test whether effects of the Tg mutation that are 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, 2005; Van Den Maagdenberg et al. 2004).

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). Animals were genotyped as described previously (Plomp et al. 2000). Homozygous Tg and wild-type mice were used at 6 wk of age, with the investigator blinded for genotype.

Ex vivo NMJ electrophysiology

Mice were killed by carbon dioxide inhalation. Hemi-diaphragms with phrenic nerve were dissected and kept in Ringer medium (in mM: 116 NaCl, 4.5 KCl, 2 CaCl_{2}, 1 MgSO_{4}, 1 NaH_{2}PO_{4}, 23 NaHCO_{3}, 11 glucose, pH 7.4) at room temperature. Intracellular...
recordings of MEPPs and EPPs were made at NMJs at 28°C using standard microelectrode equipment. At least 30 MEPPs and EPPs were recorded at each NMI, and 5–10 NMJs were sampled per experimental condition per muscle. Muscle action potentials were blocked by 3 μM α-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. 2000). EPPs and MEPPs were also measured in presence of the specific Ca²⁺ channel blockers ω-agatoxin-IVA (Ca₂,1, 200 or 400 nM, as indicated), ω-conotoxin-GVIA (Ca₂,2, 2.5 μM), and SNX-482 (Ca₂,3, 1 or 2 μM, as indicated), after a 20-min preincubation period. Toxins were from Scientific Marketing Associates. We also probed for the presence of Ca₂⁺ channels more distant from the Ca²⁺ 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 Ca²⁺/5.5 mM Mg²⁺-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 are presented as means ± SE.

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) Ca²⁺ concentration was the same at Tg and wild-type NMJs, in line with earlier observations (Plomp et al. 2000). The quantal content was ~32 (Fig. 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; Fig. 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; Fig. 1A). ω-Agatoxin-IVA decreased EPP amplitude by 93% (from 22.8 ± 1.1 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; Fig. 1B). In the presence of ω-agatoxin-IVA, EPP failure on 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; Fig. 1B and 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 Ca₂,1 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 IC₅₀ for wild-type quantal content, as determined in our laboratory (~75 nM, unpublished data). No extra reduction was observed compared with 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; Fig. 1A), excluding the possibility of a reduced toxin sensitivity of Tg-mutated Ca₂,1 channels.

Ca₂,3 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-Ca₂,1 channels is likely. Both Ca₂,2 and Ca₂,3 channels are known to mediate neurotransmitter release (Reid et al. 2003) and both partially compensate for the loss of Ca₂,1 channels at NMJs of Cacna1a null-mutant mice (Urbano et al. 2003; S. Kaja and J. J. Plomp, unpublished observations). To study compensatory involvement of Ca₂,2,2 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 (Fig. 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 Ca₂,2 channels are localized more distantly from the Ca²⁺ 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 Ca²⁺/5.5 mM Mg²⁺ 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 (~37-fold) in both genotypes (Fig. 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; Fig. 1E). These results further indicated absence of compensatory expression of Ca₂,2 channels at Tg NMJs, even at distant sites.

Next, we tested the Ca₂,3 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; Fig. 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; Fig. 1F). Together, Ca₂,1 and Ca₂,3 channels mediate ~90% (~75% and ~15%, respectively) of the 0.3-Hz evoked ACh release. Thus the apparent reduction of Ca²⁺ influx through ω-agatoxin-IVA-sensitive channels at the Tg NMJ is almost fully compensated for by Ca²⁺ influx through SNX-482-sensitive channels, i.e., Ca₂,3.

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because Cav2.1 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 Cav2.1 channels by creating a (low-affinity) receptor site in the Cav2.1 protein instead of indirectly inducing the expression of compensatory Cav2.3 channels. In that case, the 15% reduction of quantal content by 1 μM SNX-482 at Tg NMJs could be regarded as a suboptimal inhibition of SNX-482–sensitive Tg-mutated Cav2.1 channels. Although this possibility is not very likely in view of the distant localizations of the Tg mutation in Cav2.1 (in P-loop of repeat II) and the SNX-482 binding site in Cav2.3 (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 with that observed on incubation with 1 μM SNX-482 (n = 3–4 muscles, 10 NMJs per muscle, P = 0.85; Fig. 1F), indicating that it is very unlikely that the Tg mutation rendered the Cav2.1 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; Fig. 1F).
However, at Tg content was 1.7

values are means ± SE. Effect of specific Ca2+ blocking toxins on spontaneous ACh release (measured as MEPP frequency) at both wild-type and Tg NMJs. Neither the selective Ca2,2 channel blocker ω-conotoxin-GVIA (2.5 μM), nor the Ca2,3 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,2,1 blocker ω-agatoxin-IVA reduced MEPP frequency ~50% in both genotypes. MEPP, miniature endplate potential.

showing that ACh release at Tg NMJs is governed exclusively by Ca,2,1 and Ca,2,3 channels.

Increased 0.3-Hz evoked ACh release at Tg NMJs in low Ca2+

We studied 0.3-Hz evoked ACh release at Tg NMJs in low (0.2 mM) extracellular Ca2+. 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 approximately sixfold higher (10.7 ± 0.9, n = 4 muscles, 7–10 NMJs per muscle, P < 0.01; Fig. 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; Fig. 2, B and D). In 0.2 mM Ca2+, EPP failure upon a nerve 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; Fig. 2, B and 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 Ca2+ 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 (Fig. 2E).

Ca,2,3 channels do not contribute disproportionally to rundown of EPP amplitude during 40-Hz nerve stimulation

Previously, at normal extracellular Ca2+ 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 1st EPP, was ~8% lower; Plomp et al. 2000). Specific Ca,2,3 channel behavior, for instance a relatively large use-dependent inhibition, might underlie such increased rundown. To test this hypothesis, we recorded and quantified 40-Hz EPP rundown at Tg NMJs in the presence of either no channel

**TABLE 1. Effect of specific Ca2+ channel blocking toxins on MEPP frequency**

<table>
<thead>
<tr>
<th>Wild-Type</th>
<th>Tg</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.96 ± 0.12 s⁻¹</td>
</tr>
<tr>
<td>+ ω-agatoxin-IVA (200 nM)</td>
<td>0.49 ± 0.05 s⁻¹†</td>
</tr>
<tr>
<td>Control</td>
<td>0.90 ± 0.11 s⁻¹</td>
</tr>
<tr>
<td>+ ω-conotoxin-GVIA (2.5 μM)</td>
<td>0.89 ± 0.13 s⁻¹</td>
</tr>
<tr>
<td>Control</td>
<td>0.96 ± 0.16 s⁻¹</td>
</tr>
<tr>
<td>+ SNX-482 (1 μM)</td>
<td>0.93 ± 0.15 s⁻¹</td>
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![Fig. 2](http://jn.physiology.org/)

A: 0.3-Hz evoked ACh release at Tg NMJs at low (0.2 mM) extracellular Ca2+ concentration. A: at Tg NMJs, quantal content was approximately sixfold higher than at wild-types (n = 4 muscles, 7–10 NMJs per muscle, P < 0.01). B: representative examples of 10 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 approximately fivefold higher at Tg NMJs (n = 4 muscles, 7–10 NMJs per muscle, P < 0.001). E: Ca,2,3 blocker SNX-482 (1 μM) did not affect quantal content at Tg NMJs at low Ca2+ (n = 4 muscles, 10 NMJs per muscle, P = 0.41), whereas Ca,2,1 blocker ω-agatoxin-IVA (200 nM) almost completely inhibited it (n = 2 muscles, 5 NMJs per muscle). †P < 0.05.
blocks, ω-agatoxin-IVA (200 or 400 nM), SNX-482 (1 or 2 μM), or both toxins in combination at normal Ca²⁺ level (Fig. 3, A and B). Normalized EPP rundown in the presence of ω-agatoxin-IVA and in the presence of SNX-482 was similar (to ~77% of the 1st EPP in the train, n = 3–6 muscles, 7–10 NMJs per muscle, P = 0.65; Fig. 3C), which does not differ from the control condition without toxins. This indicates that Ca₂,3 channels do not contribute disproportionally to EPP rundown at Tg NMJs.

**DISCUSSION**

We studied compensatory contribution of non-Ca₂,1 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 Ca₂,1 channels (Giovannini et al. 2002; Kaja et al. 2005; Uchitel et al. 1992). The lesser inhibition found at Tg NMJs (only ~75%) suggests compensatory involvement of non-Ca₂,1 channels. The experiments with SNX-482, a selective blocker of Ca₂,3 channels, show that ~15% of the total release at Tg NMJs is mediated by Ca₂,3 channels. ACh release at NMJs of Cacna1a null-mutant mice, which lack Ca₂,1 channels and die at ~3 wk of age, becomes dependent on Ca₂,3 as well as Ca₂,2 channels (Urbano et al. 2003). However, we could exclude compensatory involvement of Ca₂,2 channels at Tg NMJs by showing insensitivity of quantal content to ω-conotoxin-GVIA, even in a protocol testing for Ca₂,2 channels localized more distantly from the Ca²⁺ sensor at release sites. Thus we showed that Ca₂,3 channels are recruited first as compensatory channels at Tg NMJs. This is the first report of compensatory expression of non-Ca₂,1 channels at NMJs of mice with a single amino acid change in Ca₂,1. On the basis of the results of this 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 Ca₂,1 channels, but in their absence, become occupied by Ca₂,3 channels (Urbano et al. 2003). This would suggest a small reduction in the presynaptic membrane expression of Ca₂,1 channels at Tg NMJs, leaving slots available for Ca₂,3 channels. However, although reduced Tg-mutated Ca₂,1 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 twofold 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 Ca₂,1 channel expression is masked by increased Ca²⁺ flux through the remaining channels, because of a mutation-induced shift of their activation voltage toward more negative values, as proposed by us earlier (Plomp et al. 2000). Extra Ca²⁺ influx through Tg-mutated Ca₂,1 channels was further substantiated here by the finding that ACh release in low extracellular Ca²⁺ was approximately sixfold increased at Tg NMJs. This increase was solely caused by the Tg-mutated Ca₂,1 channels and not due to compensatory Ca₂,3 channels, because 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 sim-
ilar increases in MEPP frequency and low-Ca\textsuperscript{2+} quantal content at those NMJs (Kaja et al. 2004, 2005; Van Den Maagdenberg et al. 2004). Voltage-clamp measurements in (transfected) primary cultured neurons 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\textsubscript{a,2.1} channels at NMJs may have a negatively shifted activation voltage. However, enigmatically, Wakamori et al. (1998) showed normal activation voltage for Tg channels in Purkinje cell bodies and transfected baby hamster kidney cells. It may, however, be that extrapolation of data obtained at cell body channels to behavior 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 after a mutation-induced change of interaction with factors such as calmodulin or G proteins (Catterall 2000; Lee et al. 1999), leading to increased Ca\textsuperscript{2+} influx. 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\textsubscript{a,2.3} 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\textsuperscript{2+} influx caused by Tg Ca\textsubscript{a,2.1} mutation. Alternatively, membrane insertion may normally fail because of absence of available slots at ACh release sites, but become successful when Tg Ca\textsubscript{a,2.1} mutation results in some free slots.

The lack of compensatory involvement of Ca\textsubscript{a,2.2} channels at Tg NMJs, as opposed to Cacna1a null-mutant NMJs, may be explained by the presence of remaining Ca\textsubscript{a,2.1} channels. For instance, expression of syntaxin-1A is dependent on selective Ca\textsubscript{a,2.1}-mediated Ca\textsuperscript{2+} influx (Sutton et al. 1999). This presynaptic protein can inhibit the function of Ca\textsubscript{a,2.1} and Ca\textsubscript{a,2.2} channels (Bezprozvanny et al. 1995) but not that of Ca\textsubscript{a,2.3} channels. Thus if altogether present at the NMJ, Ca\textsubscript{a,2.2} channels in the Tg presynaptic membrane may be silenced. The situation at central synapses seems different. Compensatory Ca\textsubscript{a,2.2} 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 (Kaja et al. 2005; Van Den Maagdenberg et al. 2004): increased spontaneous ACh release and evoked release that is normal at physiological extracellular Ca\textsuperscript{2+} but strongly increased, compared with wild-type, at low Ca\textsuperscript{2+}. However, clear differences exist. There is no compensatory involvement of Ca\textsubscript{a,2.3} channels at R192Q NMJs, because \(\omega\)-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 with wild-type (Plomp et al. 2000). It may be that Ca\textsubscript{a,2.3} channel behavior contributes to this phenomenon. Normal rundown at wild-type NMJs is likely caused by a combination of Ca\textsubscript{a,2.1} channel inactivation, its recovery, and the replenishment of releasable ACh vesicles. A relatively large degree of use-dependent inhibition of Ca\textsubscript{a,2.3}, compared with that of Tg Ca\textsubscript{a,2.1} channels, e.g., induced by faster inactivation (Williams et al. 1994), might add disproportionately to the EPP rundown at Tg NMJs. However, normalized EPP rundown at Tg NMJs in the presence of either SNX-482 or \(\omega\)-agatoxin-IVA did not differ. This indicates that the contribution of use-dependent inhibition of Ca\textsubscript{a,2.3} channels to EPP rundown is either similar to that of Tg Ca\textsubscript{a,2.1} channels or that use-dependent inhibition of Ca\textsubscript{a,2} channels is not at all a factor contributing to rundown at normal Ca\textsuperscript{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\textsuperscript{2+} concentration at Tg as well as R192Q NMJs is unclear. The Ca\textsuperscript{2+} influx through mutant channels at physiological extracellular Ca\textsuperscript{2+} may be of such magnitude that presynaptic sensors saturate. Alternatively, Ca\textsuperscript{2+}/calmodulin-dependent Ca\textsubscript{a,2.1} inactivation (Lee et al. 1999) may be increased at mutant synapses, because of increased Ca\textsuperscript{2+} influx or, although not very likely (as discussed above), a direct change in Ca\textsubscript{a,2.1} modulatory characteristics. Alternatively, the localization of the different types of Ca\textsubscript{a} channels relative to the Ca\textsuperscript{2+} sensor of the neuroexcytotoxic mechanism may play a role (Urbano et al. 2003; Wu et al. 1999). Tg-mutated Ca\textsubscript{a,2.1} 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 with wild-type, at normal extracellular Ca\textsuperscript{2+} concentration. Similarly, a closer localization of Tg-Ca\textsubscript{a,2.1} channels to release sites than Ca\textsubscript{a,2.3} channels may explain the lack of contribution of Ca\textsubscript{a,2.3} channels to evoked ACh release under the condition of low extracellular Ca\textsuperscript{2+}.

It is unclear whether compensatory Ca\textsubscript{a,2.3} 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\textsubscript{a,2.3} channels after partial down-regulation of Ca\textsubscript{a,2.1} channels, as shown in cerebellar Purkinje cells (Pinto et al. 1998). However, total genetic Ca\textsubscript{a,2.1} ablation results in unaltered or reduced Ca\textsubscript{a,2.3} current density in cerebellar neurons (Fletcher et al. 2001; Jun et al. 1999). The unidentified residual Ca\textsuperscript{2+} current shown at Tg hippocampal synapses after blocking Ca\textsubscript{a,2.1} and -2 channels (Qian and Noebels 2000) may be due to Ca\textsubscript{a,2.3} channel expression. It would be interesting to cross-breed Tg with Caenale mice (Wilson et al. 2000) to test Ca\textsubscript{a,2.3} channel involvement.

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