Synaptic effects of mutations in neuronal Cav2.1 calcium channels

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Reduced ACh Release at Neuromuscular Synapses of Heterozygous *Leaner* Mice

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Abstract

Episodic ataxia type 2 (EA2) is a rare autosomal-dominant neurological disorder caused by truncation and single amino acid change mutations in CACNA1A-encoded $Ca_V2.1$ (P/Q-type) channels. The main role of $Ca_V2.1$ channels is mediation of neurotransmitter release, both in the central and peripheral nervous system. The EA2 clinical phenotype is thought to result from loss-of-function of $Ca_V2.1$ channels, which at synapses most likely results in reduced neurotransmitter release. Heterozygous *Leaner* (*Ln*/wt) mice, which carry a truncation mutation in the *Cacna1a* gene, as well as heterozygous $Ca_V2.1$ knock-out (KO/wt) mice may model some synaptic aspects of EA2, and could be used for drug studies. We, therefore, investigated acetylcholine (ACh) release at their neuromuscular junctions (NMJs), which is almost completely mediated by $Ca_V2.1$ channels. KO/wt mice did not show any ACh release abnormalities, not even at older age (12-14 months). However, *Ln*/wt mice had ~25% reduced spontaneous uniquantal ACh release and ~10% reduced low rate nerve-stimulation evoked release. There was no compensatory non-$Ca_V2.1$ channel contribution to ACh release at the KO/wt or *Ln*/wt NMJ, as indicated by an ω-agatoxin-IVA sensitivity similar to wild-type. These results indicate that *Ln*-mutated $Ca_V2.1$ channels are either normally inserted in the presynaptic membrane but have reduced function, or that they reduce the amount of functional wild-type channels via inhibition of mechanisms underlying their expression, trafficking or membrane insertion. Most EA2 cases can be successfully managed with the drug acetazolamide. It may be that the therapeutic effect of acetazolamide is due to a direct influence on (mutant) $Ca_V2.1$ channel function, restoring possible synaptic dysfunction. We, therefore, tested the effects of acetazolamide on ACh release at the NMJs of wild-type, KO/wt and *Ln*/wt mice. Acetazolamide did not affect ACh release at any of the three genotypes tested and, therefore, apparently does not act directly on (*Ln*-mutated) $Ca_V2.1$ channels.

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Introduction

Episodic ataxia type 2 (EA2) is a rare neurological disorder characterized by symptoms including ataxia, nystagmus, dysarthria, vertigo, diplopia and general weakness (OMIM #108500; for reviews, see Kullmann, 2002; Pietrobon, 2002). EA2 is caused by mutations in CACNA1A, the gene encoding high voltage-activated $Ca_V2.1$ (P/Q-type) Ca^{2+} channels, and shows broad allelic heterogeneity (Ophoff et al., 1996; Jen et al., 2004a). Whereas most mutations are missense, resulting in truncated $Ca_V2.1$ channels without function, also single amino acid changes exist that are mainly located in regions important for channel gating (Mantuano et al., 2004). The neurological symptoms are thought to result from dominant-negative effects of these CACNA1A mutations on (cerebellar) neurotransmission (Jeng et al., 2006).

Presynaptic $Ca_V2.1$ channels control neurotransmitter secretion both in the central (CNS) and peripheral (PNS) nervous system (Uchitel et al., 1992; Wheeler et al., 1995). In the vertebrate PNS, $Ca_V2.1$ channels are mainly restricted to the neuromuscular junction (NMJ), where they mediate acetylcholine (ACh) release from motor nerve terminals (Uchitel et al., 1992), explaining the neuromuscular abnormalities found in some EA2 patients (Jen et al., 2001; Maselli et al., 2003a).

Natural *Leaner* (*Ln*) mutant mice carry a mutation in their *Cacna1a* gene, resulting in a truncated $Ca_V2.1$ protein that lacks a large part of the cytoplasmic C-terminus (Fletcher et al., 1996; Doyle et al., 1997). Homozygous mice display severe ataxia and epilepsy and die prematurely at 3-4 weeks of age. We recently described that *Ln* mice have ~50% reduced nerve stimulation-evoked and spontaneous ACh release (chapter 7). $Ca_V2.1$-deficient *null*-mice ($Ca_V2.1$-KO) show a remarkably similar neurological phenotype to *Ln* and have similarly reduced ACh release at the NMJ (chapter 7). The situation in heterozygous *Ln* (*Ln*/wt) and heterozygous $Ca_V2.1$-KO (KO/wt) mice genetically resembles the situation in EA2, and it has previously been suggested on the basis of CNS studies that *Ln* and $Ca_V2.1$-KO mice may serve as models for EA2 (Pietrobon, 2005b; cf. chapter 7) and, therefore, might be useful for drug studies. Both *Ln*/wt and KO/wt mice do not exhibit any overt neurological phenotype. However, there are recent reports of mildly impaired oculomotor function (Katoh et al., 2004) and nociception (Luvisetto et al., 2004) in these mice.

Here we characterized ACh release at NMJs of *Ln*/wt and KO/wt NMJs. In order to assess the possibility of a progressive phenotype, we also made recordings at NMJs of aged KO/wt mice (12-14 months of age). Furthermore, we tested the effect of acetazolamide (AZA), a drug used to treat EA2 symptoms (for reviews, see Kullmann, 2002; Pietrobon, 2002). The pharmacotherapeutic mechanism of action of AZA in EA2 has not yet been conclusively elucidated, but may include direct modulation of presynaptic (mutated) $Ca_V2.1$ channels, thereby influencing transmitter release.

Materials and Methods

All animal experiments were in accordance with national legislation, the USA National Institutes of Health recommendations for the humane use of animals, and were approved by the Leiden University Animal Experiments Committee.

Ln/wt mice were originally purchased from Jackson Laboratories (Bar Harbor, ME, USA), KO/wt mice were generated in our laboratory as described previously (chapter 7). Animals were maintained at the Leiden University Medical Centre vivarium on a 12 h light/dark cycle, with food and water available *ad libitum*.

For basal characterization, *Ln*/wt mice were tested at 5 weeks of age, KO/wt mice were studied at 2-3 months of age and at 12-14 months of age, as indicated. Wild-type littermates were used as controls wherever possible, otherwise age-matched controls were used. Mice for our pharmacological study investigating the possible effects of AZA on ACh release were 5 weeks of age (*Ln*/wt and littermate controls) or 5 months of age (KO/wt and littermate controls). Litters were genotyped after weaning as described previously (chapter 7). All experiments were carried out with the investigator blinded for genotype. Before experiments, body weights were noted. No significant differences between genotypes were found (data not shown).

For experiments, mice were killed by carbon dioxide inhalation. Phrenic nerve-hemidiaphragms were dissected and mounted in standard 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 and continuously bubbled with 95% O_2 / 5% CO_2. Using standard microelectrode equipment, we made intracellular recordings at NMJs at 28º C, as described previously (chapter 7). We recorded the spontaneous depolarizing events resulting from uniquantal acetylcholine release (miniature endplate potentials, MEPPs) and those resulting from action potential-evoked ACh release (endplate potentials, EPPs). At least 40 MEPPs and 30 endplate EPPs were recorded per endplate, sampling at least 7 NMJs per experimental condition. In order to prevent muscle action potentials, muscle Na^+ channels were blocked by 3 μM of the selective blocker μ-conotoxin-GIIIB (Scientific Marketing Associates, Barnet, Herts, UK).

EPPs were recorded following low-rate (0.3 Hz) and high-rate (40 Hz) supramaximal stimulation of the phrenic nerve. The amplitudes of EPPs and MEPPs were normalized to –75 mV, assuming 0 mV as the reversal potential for ACh-induced current (Magleby and Stevens, 1972). The normalized EPP amplitudes were corrected for non-linear summation according to (McLachlan and Martin, 1981) with an *f* value of 0.8. Quantal content, i.e. the number of ACh quanta released per nerve impulse, was calculated by dividing the normalized and corrected mean EPP amplitude by the normalized mean MEPP amplitude.

In order to assess the contribution of $Ca_V2.1$ channel to ACh release, EPPs and MEPPs were also measured in the presence of 200 nM of the specific $Ca_V2.1$ channel blocker ω-agatoxin-IVA (Scientific Marketing Associates, Barnet, Herts., U.K.) Measurements were made following a 15 min pre-incubation with the toxin. The possible effects of AZA (Sigma-Aldrich, Zwijndrecht, The Netherlands) on neurotransmitter release were investigated in presence of 50 μM of the drug. AZA was dissolved in dimethylsulfoxide to obtain a 50 mM stock solution. The final solution in Ringer's medium contained 0.1% dimethylsulfoxide. In the control condition before AZA incubation, electrophysiological measurements were made in Ringer's medium with 0.1% dimethylsulfoxide added. AZA was pre-incubated for 1 h before starting the measurements.

All data is given as mean ± S.E.M.. Statistical significance was assessed using paired or unpaired Student's *t*-tests, where appropriate, on the grand mean values with n as the number of mice tested, and with 7-15 NMJs tested per muscle. In all cases *P*-values <0.05 were considered to be statistically significant.

Results

Normal ACh release at KO/wt synapses

We first investigated ACh release at diaphragm NMJs of KO/wt, and compared it with wild-type mice. No abnormalities were found. Spontaneous uniquantal release, measured as

MEPP frequency, was similar in both genotypes (1.13 ± 0.11 and 1.02 ± 0.11 s^{-1} in wild-type and KO/wt, respectively, n=5, *P*=0.53; Figure 1A). Quantal content upon low-rate (0.3 Hz) supramaximal stimulation of the phrenic nerve was similar in KO/wt mice and wild-type (31.4 ± 4.2 and 34.2 ± 2.1, respectively, n=5, *P*=0.57; Figure 1B). MEPP amplitudes, half-widths and rise and decay times did not differ between genotypes (Figure 1C, data not shown). EPP amplitudes were 22.4 ± 1.0 and 21.1 ± 1.8 mV in wild-type and KO/wt mice, respectively (n=5, *P*=0.32). Typical examples of MEPPs and EPPs recorded are shown in Figure 1C. At the wild-type NMJ, ACh release is mediated exclusively by $Ca_V2.1$ channels. However, homozygous $Ca_V2.1$-KO mice show a complex pattern of compensatory non-$Ca_V2.1$ channel contribution, in that ACh release at the NMJ becomes jointly dependent on Ca_V1, $Ca_V2.2$ and $Ca_V2.3$ channels (Uchitel et al., 1992; Urbano et al., 2003; chapter 7). In order to assess, whether neurotransmitter release in KO/wt mice remained fully dependent on $Ca_V2.1$ channels, we tested the effect of 200 nM of the specific $Ca_V2.1$ channel blocker ω-agatoxin-IVA. MEPP frequency became reduced by ω-agatoxin-IVA by ~50% (n=4, *P*<0.05), equally in both genotypes (n=4, *P*=0.97; Figure 1A). Quantal content decreased by ~90% (n=4, *P*<0.01), again equally in both genotypes (n=4, *P*=0.80; Figure 1B). This indicates that ACh release at KO/wt NMJs retains a normal dependency on $Ca_V2.1$ channels, in contrast to the homozygous $Ca_V2.1$-KO NMJ.

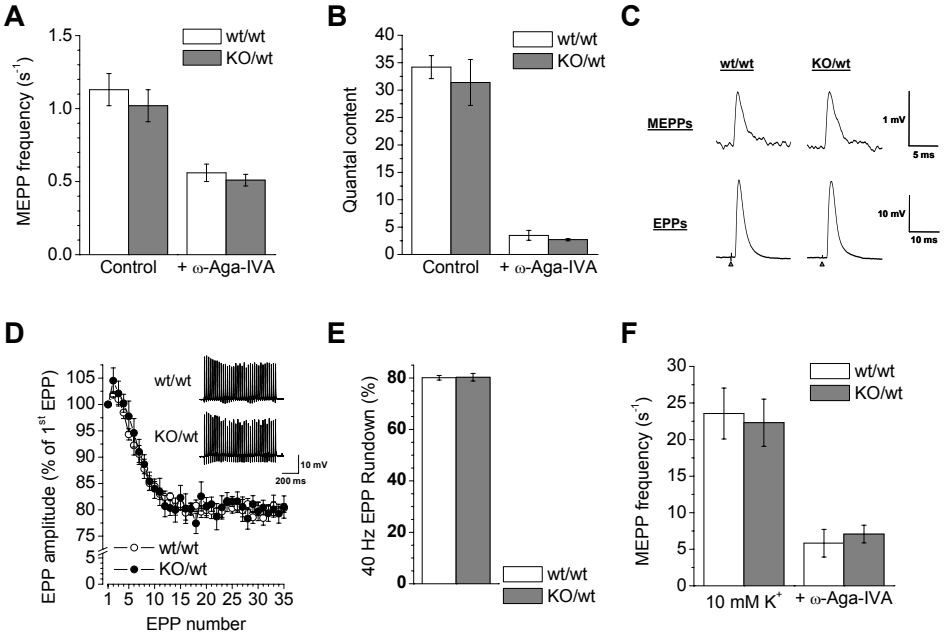

Figure 1. Unaltered ACh release at KO/wt NMJs.

(**A**) Spontaneous, uniquantal ACh release, measured as MEPP frequency, was similar at wild-type (wt/wt) and KO/wt NMJs (n=5 muscles, 10-15 NMJs per muscle, *P*=0.53). The selective $Ca_V2.1$ channel blocking toxin ω-agatoxin-IVA (200 nM) reduced MEPP frequency by ~50% (n=4 muscles, 8-15 NMJs per muscle, *P*<0.05), similarly in both genotypes. (**B**) Low-rate nerve stimulation-evoked ACh release, quantal content, was not different between KO/wt and wild-type (n=5 muscles, 10-15 NMJs per muscle, *P*=0.57). ACh release was dependent exclusively on $Ca_V2.1$ channels, as shown by the sensitivity of ACh release to 200 nM ω-agatoxin-IVA, which reduced quantal content in both genotypes by ~90% (n=4 muscles, 8-15 NMJs per muscle, *P*<0.01). (**C**) Representative recording traces of MEPPs (top panel) and low-rate stimulation-evoked EPPs (bottom panel). Triangle indicates moment of nerve stimulation. (**D**) ACh release at high rate nerve stimulation was unchanged. Representative EPP trains (40 Hz stimulation, 1 s traces) are shown (inset). (**E**) Rundown of EPP amplitudes during 40 Hz stimulation trains was similar at KO/wt synapses and wild-type (n=5 muscles, 10-15 NMJs per muscle, *P*=0.90). (**F**) MEPP frequency increased similarly in both genotypes upon 10 mM K^+ depolarization (n=4 muscles, 10 NMJs per muscle, *P*=0.67). ω-Agatoxin-IVA reduced high K^+-induced MEPP frequency equally at KO/wt and wild-type NMJs (n=4 muscles, 10 NMJs per muscle, *P*<0.05).

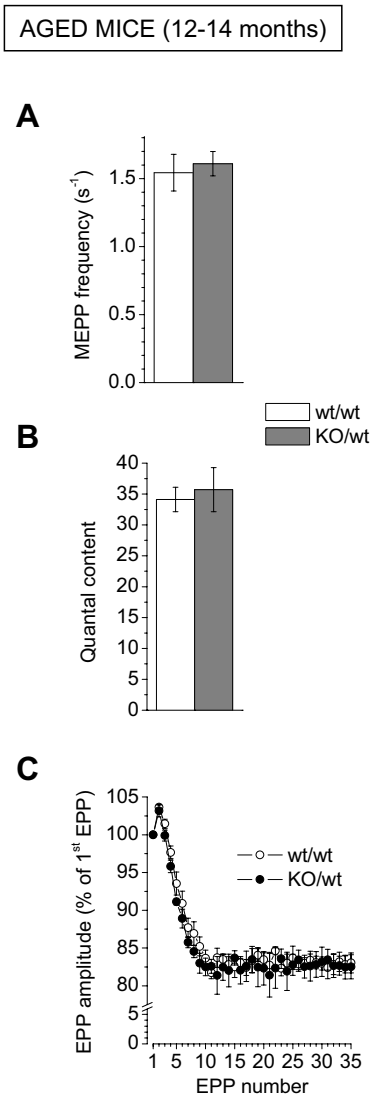

Figure 2. No long-term changes in ACh release at KO/wt NMJs of aged (12-14 months old) mice.

(**A**) MEPP frequency (n=4 muscles, 7-10 NMJs per muscle, *P*=0.70), (**B**) low rate evoked (n=4 muscles, 7-10 NMJs per muscle, *P*=0.71) and (**C**) high rate, 40 Hz, nerve stimulation-evoked ACh release (n=4 muscles, 7-10 NMJs per muscle, *P*=0.86) measured at KO/wt NMJs were not different from wild-type (wt/wt).

Some changes in channel function may only become apparent upon high-intensity use. We, therefore, investigated ACh release at high-rate (40 Hz) stimulation. During a 1s stimulus train, rundown of EPP amplitudes was similar in both genotypes. EPP rundown levels (mean amplitude of EPPs 21-35 of the stimulus train expressed as percentage of the amplitude of the first EPP) were 80.1 ± 0.9 and 80.3 ± 1.5 at wild-type and KO/wt NMJs, respectively (n=5, *P*=0.90; Figures 1D, E).

We also measured spontaneous ACh release upon mild depolarization by 10 mM K^+ Ringer's medium. MEPP frequency increased ~12-fold in both genotypes (n=4, *P*=0.67), averaging 23.6 ± 3.5 and 22.3 ± 3.2 s^{-1} in wild-type and KO/wt mice, respectively (n=4, *P*=0.80; Figure 1F). ω-Agatoxin-IVA (200 nM) subsequently decreased MEPP frequency by ~75% in both genotypes (n=4, *P*<0.05), to almost equal levels at wild-type and KO/wt NMJs (5.8 ± 1.9 and 7.1 ± 1.2 s^{-1}, respectively, n=4, *P*=0.60; Figure 1F).

KO/wt mice may develop a neuromuscular synaptic phenotype with age. We, therefore, also measured ACh release at NMJs of 12-14 months old mice. However, all investigated parameters were not different from wild-type control. MEPP frequencies were 1.54 ± 0.14 and 1.61 ± 0.09 s^{-1} at wild-type and KO/wt NMJs, respectively (n=4, *P*=0.70; Figure 2A). Quantal contents at 0.3 Hz stimulation remained at ~35 (34.1 ± 2.0 and 35.7 ± 3.6, respectively, n=4, *P*=0.71; Figure 2B). Furthermore, rundown of EPP amplitudes was not altered in aged KO/wt mice. The mean EPP rundown levels were 83.1 ± 1.0% in wild-type and 82.7 ± 1.7% in KO/wt mice (n=4, *P*=0.86; Figure 2C).

Reduced ACh release at Ln/wt NMJs

We next studied ACh release at *Ln*/wt NMJs. Spontaneous release was reduced by ~25% at *Ln*/wt synapses compared with wild-type. MEPP frequencies were 0.88 ± 0.07 and 0.65 ± 0.04 s^{-1} at wild-type and *Ln*/wt NMJs, respectively (n=12, *P*<0.01; Figure 3A). MEPP amplitude was reduced by 12% (1.04 ± 0.05 and 0.91 ± 0.04 mV, n=12, *P*<0.05).

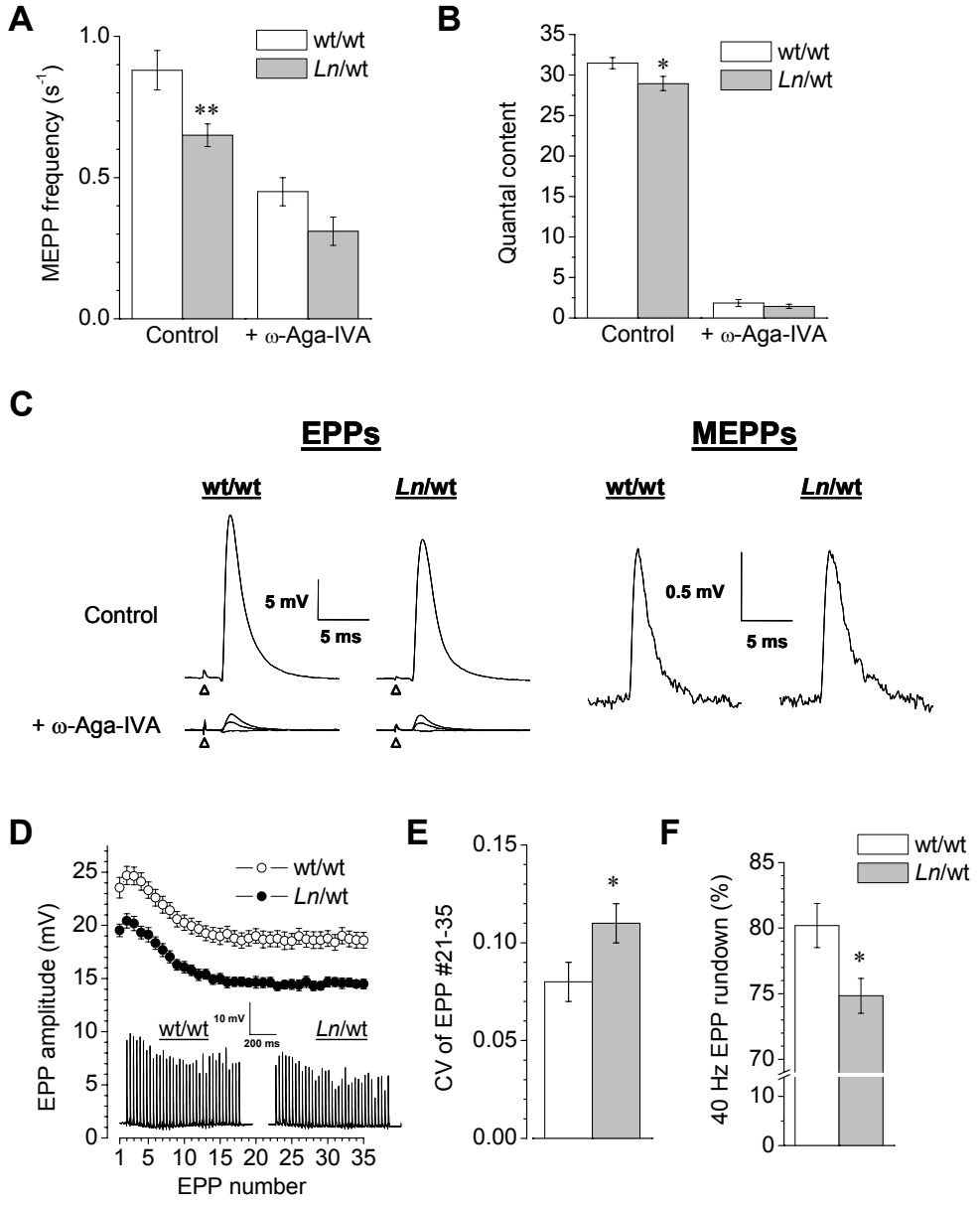

Figure 3. Mild ACh release reduction at *Ln*/wt synapses.
(**A**) MEPP frequency was reduced by ~25% at *Ln*/wt NMJs, compared with wild-type (wt/wt; n=12 muscles, 8-15 NMJs per muscle, $P<0.01$). ω-Agatoxin-IVA (200 nM) reduced MEPP frequency similarly in both genotypes (n=5-7 muscles, 7-10 NMJs per muscle, $P<0.05$). (**B**) Quantal content was ~10% lower at *Ln*/wt synapses, compared with wild-type (n=12 muscles, 8-15 NMJs per muscle, $P<0.05$). ACh release remained fully dependent on (*Ln*-)$Ca_V2.1$ channels as ω-agatoxin-IVA reduced quantal content by ~95% in both genotypes (n=5-7 muscles, 7-10 NMJs per muscle, $P<0.01$). (**C**) Example traces of EPPs measured before and during application of ω-agatoxin-IVA (left, triangle indicates moment of nerve stimulation) and MEPPs (right). Under control conditions, EPPs were ~15% smaller in *Ln*/wt compared with wild-type (n=12 muscles, 8-15 NMJs per muscle, $P<0.001$). (**D**) High rate (40 Hz) nerve stimulation-evoked ACh release was reduced at *Ln*/wt synapses, compared with wild-type. Example traces of 1 s 40 Hz trains are shown (inset). (**E**) EPPs during the plateau phase (EPPs #21-35) at *Ln*/wt NMJs showed a larger coefficient of variance (CV) than wild-type (n=8 muscles, 8-10 NMJs per muscle, $P<0.05$). (**F**) The rundown level, i.e. the mean amplitude of EPPs #21-35 expressed as percentage of the amplitude of the first EPP of the train, was somewhat lower at *Ln*/wt NMJs (n=12 muscles, 7-10 NMJs per muscle, $P<0.05$). * $P<0.05$, ** $P<0.01$.

When we measured nerve stimulation-evoked release, we found EPP amplitudes reduced by 15%, averaging 22.9 ± 0.7 mV at wild-type and 19.4 ± 0.5 mV at *Ln*/wt NMJs (n=12, $P<0.001$). Quantal content was 31.5 ± 0.7 in wild-type and 28.9 ± 0.9 in *Ln*/wt mice, corresponding to reduction by 8 ± 3% (n=12, $P<0.05$; Figure 3B). Representative traces of EPPs and MEPPs are shown in Figure 3C.

We investigated whether any non-$Ca_V2.1$ channel contribution to ACh release at the NMJ was present in *Ln*/wt mice. Spontaneous ACh release was reduced >60% by ω-agatoxin-IVA at both wild-type and *Ln*/wt NMJs. MEPP frequencies were reduced from 1.01 ± 0.09 to 0.45 ± 0.05 s^{-1} (n=7, $P<0.001$) at wild-type, and from 0.65 ± 0.07 to 0.31 ± 0.05 s^{-1} (n=5, $P<0.05$) at *Ln*/wt NMJs (Figure 3A). Nerve stimulation-evoked release was reduced by ~95%, equally in both genotypes. Quantal contents reduced from 29.8 ± 1.5 to 1.8 ± 0.4 (n=7, $P<0.01$) and from 27.4 ± 1.6 to 1.4 ± 0.3 (n=5, $P<0.01$) at wild-type and *Ln*/wt NMJs, respectively (Figure 3B). Example traces of EPPs measured in the presence of ω-agatoxin-IVA are shown in Figure 3C.

We also measured 40 Hz nerve stimulation-evoked release, which approaches the physiological firing rate of motor nerves (Figure 3D). EPP amplitudes during the plateau phase (EPPs #21-35) showed larger variation, as determined by calculation of the coefficient of variance (Figure 3E). Furthermore, 40 Hz EPP rundown level, the mean EPP amplitude during the plateau phase (EPPs #21-35) expressed as percentage of the amplitude of the first EPP in the 40 Hz train, was somewhat more pronounced at *Ln*/wt NMJs (80.2 ± 1.7% and 74.8 ± 1.3% at wild-type and *Ln*/wt NMJs, respectively; n=12, $P<0.05$; Figure 3F).

No effects of AZA on ACh release at wild-type, KO/wt and Ln/wt NMJs

We assessed whether AZA exerted an effect on ACh release at *Ln*/wt, KO/wt and wild-type NMJs. However, incubation with 50 μM AZA did not affect the spontaneous or low-rate nerve stimulation-evoked ACh release (quantal content) at NMJs of either genotype NMJs (Table 1).

Table 1. Effects of acetazolamide on ACh release at wild-type, KO/wt and *Ln*/wt NMJs.

Release parameter	wild-type (*n=8*)	KO/wt (*n=4*)	*Ln*/wt (*n=4*)
MEPP frequency (s^{-1})	+6% (*P*=0.46)	+13% (*P*=0.32)	+14% (*P*=0.13)
MEPP amplitude (mV)	+3% (*P*=0.95)	+6% (*P*=0.89)	+11% (*P*=0.53)
EPP amplitude (mV)	+1% (*P*=0.91)	-4% (*P*=0.20)	+6% (*P*=0.60)
Quantal content (0.3 Hz)	+5% (*P*=0.77)	-7% (*P*=0.65)	+3% (*P*=0.61)

Acetazolamide (50 μM) applied directly to the muscle/nerve-preparation did not affect ACh release parameters at wild-type (wt/wt), KO/wt or *Ln*/wt NMJs. MEPP frequencies, MEPP and EPP amplitudes and low rate nerve stimulation-evoked quantal content did not change significantly.

Discussion

Here we measured ACh release at NMJs of KO/wt and *Ln*/wt mice. Whereas KO/wt mice did not exhibit any synaptic abnormalities, *Ln*/wt NMJs showed slightly (10-25%) reduced ACh release. As will be elaborated below, this indicates that *Ln*/wt but not KO/wt NMJs model some synaptic aspects of EA2. The studies with AZA show that this EA2 drug does not act directly on (*Ln*-mutant) $Ca_V2.1$ channels.

Normal ACh release at KO/wt NMJs

We did not reveal any abnormalities in ACh release at the KO/wt NMJ, indicating haplosufficiency, even at NMJs of 12-14 months old mice. This suggests existence of a compensatory mechanism, increasing the expression from the one remaining wild-type allele, ensuring sufficient assembly and trafficking of wild-type $Ca_V2.1$ channels to the presynaptic membrane. Alternatively, it may be that in the normal condition wild-type $Ca_V2.1$ is expressed in excess from both wild-type alleles but that membrane insertion is limited by the amount of $Ca_V2.1$-specific 'slots', proposed to present at ACh release sites (Cao et al., 2004). In the KO/wt condition, all available slots would then simply become filled with $Ca_V2.1$ protein expressed from the one remaining wild-type allele.

Our data is compatible with the lack of any overt phenotype of KO/wt mice. However, studies of KO/wt CNS neurons have indicated reduced $Ca_V2.1$-mediated calcium current, although controversy exists. While the current density in cerebellar Purkinje and granule cells was reported to be similar to wild-type (Jun et al., 1999), that in cerebellar granule neurons of an independently generated $Ca_V2.1$-KO mouse was found reduced by ~50% (Fletcher et al., 2001). Furthermore, KO/wt show subtle oculomotor deficits and nociception abnormalities (Katoh et al., 2004; Luvisetto et al., 2004). Therefore, the lack of changes at the KO/wt NMJ observed may be site-specific, possibly caused by the specfic presynaptic environment.

Reduced ACh release at Ln/wt NMJs

At the *Ln*/wt NMJ, spontaneous and nerve stimulation-evoked ACh release was reduced by ~25% and ~10%, respectively, which is less then the reduction of ~50% we observed previously in homozygous *Ln* mice (chapter 7). Reduced ACh release at *Ln*/wt NMJs may be directly due to changes of the biophysical properties of *Ln*-$Ca_V2.1$, reducing Ca^{2+} influx. Indeed, reduced open probability and a positive shift of activation voltage has been shown (Dove et al., 1998; Wakamori et al., 1998). Alternatively, *Ln*-$Ca_V2.1$ protein may in some way inhibit wild-type $Ca_V2.1$ channel expression and/or membrane insertion, without being inserted itself. However, from our previous studies at NMJs of homozygous *Ln* NMJs it appears that *Ln*-mutated $Ca_V2.1$ channels are inserted at release sites and capable of mediating transmitter release, albeit less efficiently. Hence, *Ln*-$Ca_V2.1$ channels seem to have no disadvantage in competing with wild-type $Ca_V2.1$ channels for available pre-synaptic slots. The presence of *Ln*-$Ca_V2.1$ channels apparently occludes any compensatory increases in wild-type $Ca_V2.1$ membrane insertions; otherwise we should have observed a haplosufficient phenotype, as seen in KO/wt mice.

No compensatory non-$Ca_V2.1$ channel contributions at KO/wt and Ln/wt NMJs

Previous studies showed compensatory contributions of Ca_V1, $Ca_V2.2$ and $Ca_V2.3$ channel to ACh release at the NMJ of homozygous KO mice (Urbano et al., 2003; chapter 7). At the homozygous *Ln* NMJ there is a small (~15%) contribution of $Ca_V2.3$ channels (chapter 7). However, at the KO/wt as well as *Ln*/wt NMJ, evoked ACh release was reduced by more than 90% by ω-agatoxin-IVA, as in wild-type controls, excluding compensatory non-$Ca_V2.1$ channel expression. These findings are in line with our hypothesis of increased expression of the wild-type allele in KO/wt mice and, furthermore, indicate that the presence of wild-type $Ca_V2.1$ channels at *Ln*/wt NMJs occludes compensatory $Ca_V2.3$ expression.

AZA does not act directly on (Ln-mutated) $Ca_V2.1$ channels

AZA is a carbonic anhydrase inhibitor that is frequently used to treat glaucoma, epileptic seizures and altitude sickness. Of interest, >90% of EA2 patients respond positively to AZA, which has led to the hypothesis that AZA may act on (EA2-mutated) $Ca_V2.1$ channels,

restoring their function. However, bath application of AZA at 50 μM, which is at the high end of the therapeutical plasma concentration range (Chapron et al., 1989), did not induce any significant effects on ACh release at *ex vivo*, at either wild-type, KO/wt or *Ln*/wt NMJs. This shows that AZA does not directly affect (mutated) presynaptic $Ca_V2.1$ channels, in agreement with the reported lack of effect of 10 μM AZA on wild-type and H1736L EA2-mutant $Ca_V2.1$ channels expressed in HEK cells (Spacey et al., 2004). Instead, AZA might act on accessory Ca_V channel subunits. However, this would not be detected in our experimental system, as our recent studies of the natural mutants *ducky*, *lethargic* and *stargazer*, lacking functional Ca_V-$\alpha_2\delta$-2, -β_4 and -γ_2 subunits, respectively, have revealed that these accessory subunits are redundant at the mouse NMJ (chapter 10). Alternatively, AZA may indirectly influence (mutant) $Ca_V2.1$ function by lowering the extracellular pH by its carbonic anhydrase inhibiting activity. Experiments administering AZA chronically for prolonged periods (3-6 weeks) are required to test for possible long-term (indirect) *in vivo* effects of AZA on (mutated) $Ca_V2.1$ channels.

Are KO/wt and LN/wt NMJs modeling EA2 synapses?

In muscle biopsy NMJs from EA2 patients with truncation mutations, a large (~50%) reduction of nerve stimulation-evoked ACh release has been reported (Maselli et al., 2003a), explaining the electromyographical abnormalities seen in these patients (Jen et al., 2001). On the basis of our present NMJ analyses we conclude that KO/wt mouse NMJs do not model EA2 synapses, whereas *Ln*/wt NMJs do so to some extent. Apparently, EA2 mutations have very specific effects on $Ca_V2.1$ channel function, which presumably can only be well-modeled in synapses from yet to be generated knock-in mice carrying specific human EA2 mutations.