Transgenic mouse models in migraine

Ven, R.C.G. van de

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CHAPTER 6

Conditional inactivation of the *Cacna1a* gene in transgenic mice

R.C.G. van de Ven,[1†] B. Todorov,[1†] S. Kaja,[2,3,$] L.A.M. Broos,[1] J.S. Verbeek[1], J.J. Plomp,[2,3] M.D. Ferrari,[3] R.R. Frants,[1] A.M.J.M. van den Maagdenberg,[1,3]

[†]Authors contributed equally

Department of [1]Human Genetics, [2]Molecular Cell Biology - Group Neurophysiology and [3]Neurology, Leiden University Medical Centre, Leiden, The Netherlands

[$]Present address: Michael Smith Laboratories, The University of British Columbia, Vancouver, Canada

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Abstract

$Ca_v2.1$ (P/Q-type) voltage-gated calcium channels play an important role in neurotransmitter release at many brain synapses and at the neuromuscular junction. Mutations in the *CACNA1A* gene, encoding the pore forming α_1 subunit of $Ca_v2.1$ channels, are associated with a wide spectrum of neurological disorders. Here we generated mice with a conditional, floxed, *Cacna1a* allele without any overt phenotype. Deletion of the floxed *Cacna1a* allele resulted in ataxia, dystonia, and lethality during the fourth week, a severe phenotype similar to conventional $Ca_v2.1$ knockout mice. Whereas neurotransmitter release at the neuromuscular junction was not affected in the conditional mice, homozygous deletion of the floxed allele caused an ablation of $Ca_v2.1$ channel-mediated neurotransmission that was accompanied by a compensatory upregulation of $Ca_v2.3$ (R-type) channels at this synapse. Pharmacological inhibition of $Ca_v2.1$ channels is possible, but the contributing cell-types and time windows relevant to the different $Ca_v2.1$-related neurological disorders can only be reliably determined using *Cacna1a* conditional mice.

Abbreviations

ACh - acetylcholine; CNS - central nervous system; KO - knockout; EPP - endplate potential; MEPP - miniature endplate potential; *neo*, neomycin; NMJ - neuromuscular junction; RT-PCR - reverse transcription polymerase chain reaction

Introduction

Neuronal $Ca_v2.1$ (P/Q-type) calcium channels are abundantly expressed throughout the central nervous system, where they are crucial for neurotransmitter release.[1,2] In the peripheral nervous system, $Ca_v2.1$ channels are mainly expressed at the neuromuscular junction (NMJ), mediating presynaptic ACh release.[3] The pore-forming α_1-subunit of $Ca_v2.1$ channels is encoded by the *CACNA1A* gene. Mutations in *CACNA1A* result in a wide spectrum of neurological disorders, such as familial hemiplegic migraine, epilepsy, cerebral oedema in response to mild head trauma and episodic and progressive ataxia.[4-6] $Ca_v2.1$ channels are involved in various important (patho)physiological processes such as cortical spreading depression[7,8], nociception[9] and neurogenic vasodilatation.[10]

Natural mutants and conventional knockout (KO) mice of $Ca_v2.1$-α_1 exist with phenotypes ranging from severe ataxia, dystonia and premature death (*leaner*, $Ca_v2.1$ KO) to ataxia and/or epilepsy (*tottering*, *rolling Nagoya*, and *rocker*).[11-16] Analysis of these mice has shown that aberrant $Ca_v2.1$ function can be compensated for by specific upregulation of other calcium channel subtypes[17,18], suggesting a prominent cell-specific role in these neurological phenotypes.[19] Natural and $Ca_v2.1$-α_1 KO mice have provided valuable insights into the consequences of calcium channel dysfunction and pathophysiology of epilepsy, ataxia and dystonia.[20] However, further research towards the underlying pathophysiological mechanisms of $Ca_v2.1$-associated diseases is seriously hampered by the fact that, i) the $Ca_v2.1$-α_1 KO mice die at an early age, ii) ablation of $Ca_v2.1$ channels occurs throughout the brain, because *Cacna1a* is broadly expressed in CNS, and iii) ablation is already effective during gestation and thus may influence neuronal development. Although *in vivo* pharmacological blocking of $Ca_v2.1$ channels may in principle be possible using specific blockers in combination with local application using highly specialised techniques, such as microiontophoresis[21], such applications will never meet the true objective of cell type- or tissue-specific $Ca_v2.1$ channel inhibition. Moreover, the efficiency and specificity of the blocker is concentration dependent.[22] To circumvent these problems, we generated a conditional mouse for spatiotemporal inactivation of the *Cacna1a* gene using the Cre/lox system.

Methods

Generation of transgenic mice

Mouse genomic DNA clones were derived from pPAC4 library (129/SvevTACfBr strain). A PGK-*Neomycin* (*neo*) cassette flanked by directly orientated LoxP sites was cloned

into the *Eco*RV site downstream of exon 4. A third LoxP site, in the same orientation, was introduced at the *Eco*RI site 1 kb upstream of exon 4. The linearised construct was electroporated into E14 embryonic stem cells of 129Ola background. Correctly recombined embryonic stem cell colonies were selected using Southern blot analysis with external probes as well as PCR using primer sets for the *neo* cassette (primers P1: 5'-TACCGGTGGATGTGGAATG-3`, P2: 5`-CGGGACGGAGTTTGACGTAC-3`) and the upstream LoxP site (primers P3: 5`-AGTTTCTATTGGACAGTGCTGGT-3`, P4: 5`-TTGCTTAGCATGCACAGAGG-3`).

Two correctly targeted clones harbouring the *Cacna1a*^{neo} allele (Fig. 1) were used to generate chimeric mice and establish a colony of mice after germline transmission. To subsequently delete the *neo* cassette, female *Cacna1a*^{neo} mice were crossed with male EIIA-driven Cre-deleter mice[23], resulting in mice with the conditional *Cacna1a*^{flox} allele. Correct deletion of the *neo* cassette was confirmed by Southern blot analysis with restriction enzyme *Apa*I. Digestions yielded bands of 10.7 and 8.0 kb after removal of the *neo* cassette as detected by 5´ and 3´ external probes, respectively (Fig. 1B). All animal experiments were performed in accordance with the guidelines of the respective Universities and national legislation.

RNA analysis

Total RNA was isolated from brain tissue using RNA Instapure (Eurogentec, Seraing, Belgium). For RT-PCR, first-strand cDNA was synthesized using random primers, and subsequent PCR was performed using *Cacna1a* and *Cyclophilin* specific primers. PCR products of *Cacna1a* were used to probe the Northern blot using standard conditions.

Protein analysis

All steps were carried out on ice, and all buffers contained protease inhibitor cocktail (Roche, Mannheim, Germany). Brains from the various genotypes were processed simultaneously. Membrane protein extraction from homogenized cerebella was performed as described earlier.[8] Western blotting was done according to the enhanced chemiluminescence protocol (Amersham Biosciences). For Western blotting equal amounts of protein were loaded in each lane as demonstrated by β-actin immunostaining.

Histology

Brains were obtained after perfusion with phosphate buffered saline, followed by 4% buffered paraformaldehyde. For immunohistochemistry coronal section of 40µm were processed using the free floating method. In brief, antigen retrieval was performed for 30 min. at 80ºC in 25mM citrate buffer (pH 8.75). Sections were incubated in 10%

heat-inactivated NHS / 0.5% TX100 in TBS for 1h followed by incubation with rabbit polyclonal $Ca_v2.1$-α_1 antibody (#AB5152, Chemicon, Temecula, CA, USA), 1:200 diluted in 2% heat-inactivated NHS / 0.4% TX100 in TBS for 72hrs at 4ºC. Secondary biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA, USA) was applied in 1:200 dilution in the same buffer for 2hrs at room temperature. Finally, for detection, sections were incubated with avidin-biotin complex (Vector Laboratories, Burlingame, CA, USA) for 1h at room temperature, washed, and developed in 0.1 mg/ml diaminobenzidine with 0.005% H_2O_2.Paraffin embedded sagittal cerebellar sections (5 μm) were processed for Klüver-Barrera staining.

Ex vivo neuromuscular junction electrophysiology

Ex vivo NMJ electrophysiology was performed in diaphragm nerve-muscle preparations, as described previously.[27] At each NMJ, 40 miniature endplate potentials (MEPPs; spontaneous uniquantal ACh release) and 30 endplate potentials (EPPs) at 0.3 Hz nerve stimulation were recorded. Muscle action potentials were blocked by 3 μM μ-conotoxin-GIIIB. The quantal content at each NMJ, i.e. the number of ACh quanta released per nerve impulse, was calculated from EPP and MEPP amplitudes. EPPs and MEPPs were also measured in the presence of either 200 nM ω-Agatoxin-IVA (blocks $Ca_v2.1$ channels) or 1 μM SNX-482 (blocks $Ca_v2.3$ channels) during a 45 min measuring period, following a 15 min pre-incubation with the toxin. All toxins were obtained from Scientific Marketing Associates, Barnet, Herts, UK. Data is given as group mean values ± SEM. Statistical significance was assessed on group mean values with *n* as the number of mice tested, with 6-10 NMJs sampled per muscle per condition, using paired or unpaired Student's t-tests, where appropriate.

Results

As a first step we generated *Cacna1a*^{neo} mice that, in addition to the LoxP site upstream of exon 4, also contain a *neo* cassette flanked by LoxP sites (Fig. 1A). Heterozygous and homozygous *Cacna1a*^{neo} mice are fertile and show no overt phenotype. To delete the *neo* cassette, we crossed the mice with transgenic mice expressing Cre recombinase under the control of the adenovirus EIIA early promoter.[23] Consequently, we were able to obtain mice without the *neo* cassette leaving only two loxP sites flanking exon 4. Thus, we generated an allele for conditional inactivation of the *Cacna1a* gene (i.e. *Cacna1a*^{flox} allele) (Fig. 1A). Correct homologous recombination and deletion of the *neo* cassette was confirmed by Southern blot (Fig. 1B) and PCR analysis. The presence of both remaining LoxP sites in the *Cacna1a*^{flox} allele was confirmed by direct sequencing (data not shown).

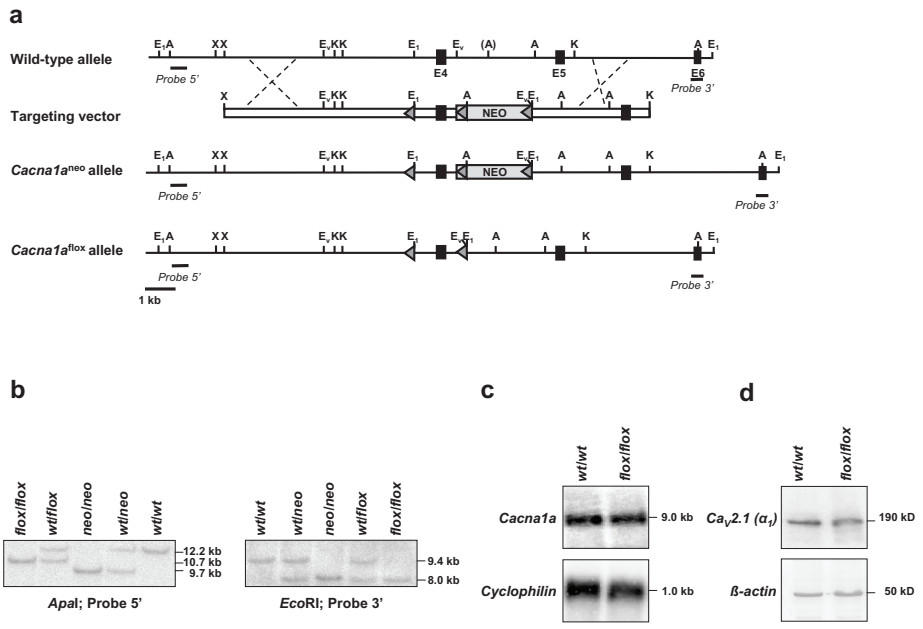

Figure 1. Generation of conditional Cacna1a mouse. (**A**) Schematic representation of the genomic structure of the relevant part of the *Cacna1a* wild-type allele, the targeting vector, the allele after homologous recombination (*Cacna1a*^{neo} allele) and the conditional *Cacna1a*^{flox} allele after partial Cre-mediated deletion. Black boxes indicate exons (E). Probes for Southern analysis are indicated. Restriction sites: E_I, *Eco*RI; A, *Apa*I; E_V, *Eco*RV; K, *Kpn*I; X, *Xba*I; (A), polymorphic *Apa*I site between the construct and the wild-type; (**B**) Southern blot: ApaI- and EcoRI-digested genomic DNA from the different genotypes probed either with the 5′ or 3′ probe; (**C**) Northern blot of cerebellar total RNA isolated from wild-type or homozygous conditional mice, probed either for *Cacna1a* or *Cyclophilin*; (**D**) Qualitative Western blot of cerebellar membrane protein extracts from wild-type or homozygous conditional mice, probed with $Ca_v2.1$-α_1 and ß-actin antibody.

Both heterozygous and homozygous *Cacna1a*^{flox} mice are viable, breed normally, and do not show any overt phenotype. Northern blot analysis revealed normal levels of expression of *Cacna1a* RNA (Fig. 1C). The LoxP sites do not alter splicing of exon 4 as was assessed by sequencing RT-PCR products of cerebellar cDNA of mutant *Cacna1a*^{flox} mice (data not shown). Qualitative Western blot analysis revealed similar expression levels for $Ca_v2.1$-α_1 protein in *Cacna1a*^{flox} and wild-type cerebellar extracts (Fig. 1D). No apparent cytoarchitectural abnormalities were observed in Klüver-Barrera stained sections of wild-type and *Cacna1a*^{flox} brains (Figs. 2A and D). We focussed mainly on the cerebellum because of its high expression of $Ca_v2.1$ channels. Immunohistochemistry revealed a normal expression pattern of $Ca_v2.1$-α_1 protein for both the *Cacna1a*^{flox} and wild-type mice with a high expression in the Purkinje cell and molecular layer (Figs. 2B and E). The expression pattern of $Ca_v2.1$ channels was also without abnormalities in other brain regions such as the hippocampal and cortical regions (Figs. 2C and F).

To exclude the possibility that introduction of LoxP sites had a major consequence on

the function of $Ca_v2.1$ channels, we investigated the evoked ACh release at the diaphragm NMJs with *ex vivo* electrophysiological methods. The quantal content in NMJs did not significantly differ between the two genotypes: 28.9 ± 1.0 for wild-type and 32.9 ± 2.8 for *Cacna1a*^{flox} mice (n = 4 muscles, 6-10 NMJs per muscle, p=0.23) (Fig. 3). Application of 200 nM of the specific $Ca_v2.1$ blocker ω-Agatoxin-IVA reduced the quantal content by >90% in both genotypes (p<0.01) (Fig. 3), clearly indicating that the presence of LoxP sites in the genomic sequence of *Cacna1a* does not alter the function of $Ca_v2.1$ channels at this synapse.

Integration of a *neo* cassette in exon 4 of the *Cacna1a* gene resulted in *loss-of-function* and ablation of $Ca_v2.1$-α1 in $Ca_v2.1$ KO mice.[12] Here we generated and investigated mice lacking exon 4 (*Cacna1a*$^{\Delta E4}$) by breeding our *Cacna1a*^{flox} mice with EIIA-driven Cre-deleter mice (Fig. 4A). Cre recombination resulting in deletion of floxed sequences in the *Cacna1a*^{flox} allele was confirmed by PCR and Southern blot analysis (data not shown). *Cacna1a*$^{\Delta E4}$ mice exhibit progressively severe ataxia and dystonia starting around P10-12, and died at P20-22 if left unaided. At P20, *Cacna1a*$^{\Delta E4}$ mice were significantly smaller than their littermate controls. The observed phenotype is identical to that of conventional $Ca_v2.1$-α1 KO mice.[11,12] Analysis of neurotransmitter release at the NMJ revealed a significantly decreased (~40%, p<0.05) quantal content of 15.7 ± 3.0 at *Cacna1a*$^{\Delta E4}$ NMJs, compared to wild-type (26.7 ±1.2, n = 3 muscles, 6-10 NMJs per muscle) (Fig. 4B). ACh release at *Cacna1a*$^{\Delta E4}$ NMJs appeared insensitive to 200

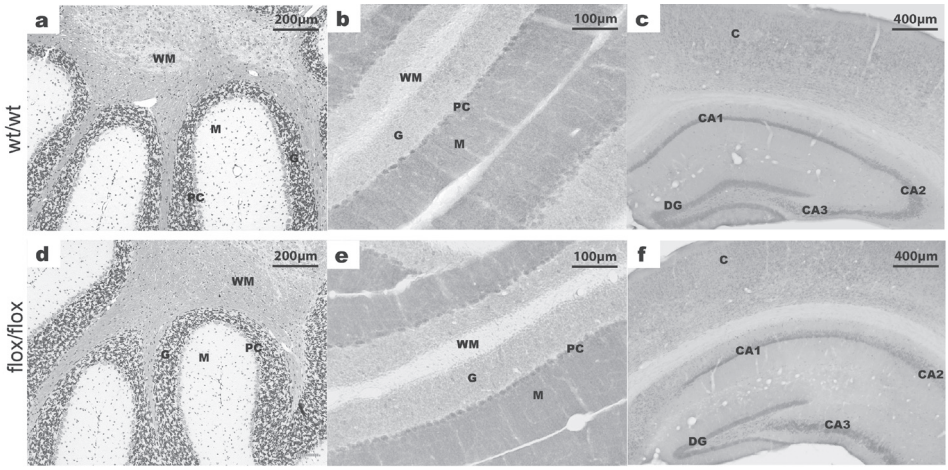

Figure 2. Histology and expression of $Ca_v2.1$ in wild-type and Cacna1a^{flox} mice. (**A,D**) Klüver-Barrera stained sagittal sections from the cerebellum; (**B,E**) Immunostaining on cerebellar coronal sections (**C,F**) Relatively high $Ca_v2.1$ expression observed in the hippocampus, whereas lower in cortical regions. No apparent overall structural abnormalities or differences in $Ca_v2.1$-α_1 expression level and pattern were observed. WM, white matter; G, granule cell layer; M, molecular cell layer; PC, Purkinje cell layer; WM, white matter; DG, dentate gyrus; CA1-3, Cornu Ammonis regions of the hippocampus; C, cerebral cortex.

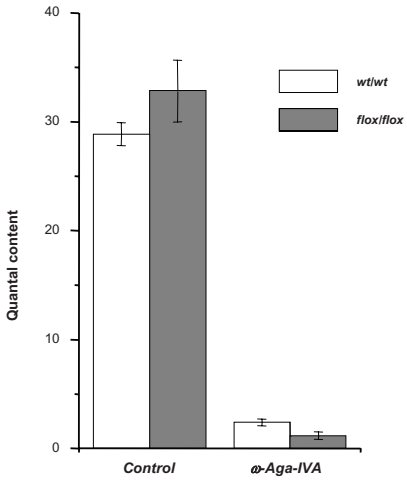

Figure 3. Neurotransmitter release at the NMJ in $Ca_v2.1$ conditional mouse. Neurotransmitter release is not altered at NMJs of conditional *Cacna1a*^{flox} mice. Quantal content (0.3 Hz stimulation) is not significantly different from wild-type (n=4 muscles, 6-10 NMJs per muscle, p=0.23); application of $Ca_v2.1$ specific blocker ω-Agatoxin-IVA (200 nM) causes a reduction of >90% of the quantal content in both wild-type and *Cacna1a*^{flox} NMJs (n=4 muscles, 6-10 NMJs per muscle).

nM ω-Agatoxin-IVA (Figs. 4B, C). $Ca_v2.3$ (R-type) channels do not mediate transmitter release at the wild-type NMJ, as demonstrated by an insensitivity of the quantal content to 1 μM of the $Ca_v2.3$ channel blocker SNX-482 (Kaja and Plomp, unpublished data).[24,25] However, in conventional $Ca_v2.1$-α_1 KO mice neuromuscular transmission becomes for a large part dependent on compensatory $Ca_v2.3$ channels.[25] Application of 1μM of the $Ca_v2.3$ channel blocker SNX-482 to *Cacna1a*$^{\Delta E4}$ NMJ preparations in the present study revealed a similar compensatory $Ca_v2.3$ channel contribution, since quantal content was reduced by 63% (p<0.05, Fig. 4B, D). The remaining portion of transmitter release is most likely predominantly mediated by $Ca_v2.2$ channels, as shown at conventional $Ca_v2.1$-α1 KO NMJs.[25]

Here, we have generated a conditional $Ca_v2.1$ mouse model that will be useful to study the consequences of temporal and spatial ablation of $Ca_v2.1$ channels. We did not find evidence that insertion of LoxP sites into the *Cacna1a* gene alters gene expression or $Ca_v2.1$ channel function. A complete functional knockout was obtained by Cre recombinase-mediated deletion of exon 4; *Cacna1a*$^{\Delta E4}$ mice displayed a phenotype identical to that described for the conventional $Ca_v2.1$-α_1 KO mouse models.[11,12] We showed that *Cacna1a*$^{\Delta E4}$ have no $Ca_v2.1$ channel-mediated ACh release at the NMJ. Furthermore, in agreement with earlier experiments in conventional $Ca_v2.1$-α_1 KO mice[25], $Ca_v2.3$ channels partly compensate for the loss of $Ca_v2.1$ channels. Our data clearly show that *Cacna1a*$^{\Delta E4}$ is a functional *null* allele. The ability to spatially and/or temporally ablate $Ca_v2.1$ channels in a non-invasive way using the $Ca_v2.1$ conditional mouse provides a much needed tool to further study the pathogenesis of migraine, epilepsy, ataxia and trauma-induced oedema. The increasing availability of transgenic mouse lines with spatial-temporal expression of Cre-recombinase in brain[26] makes such $Ca_v2.1$ studies feasible.

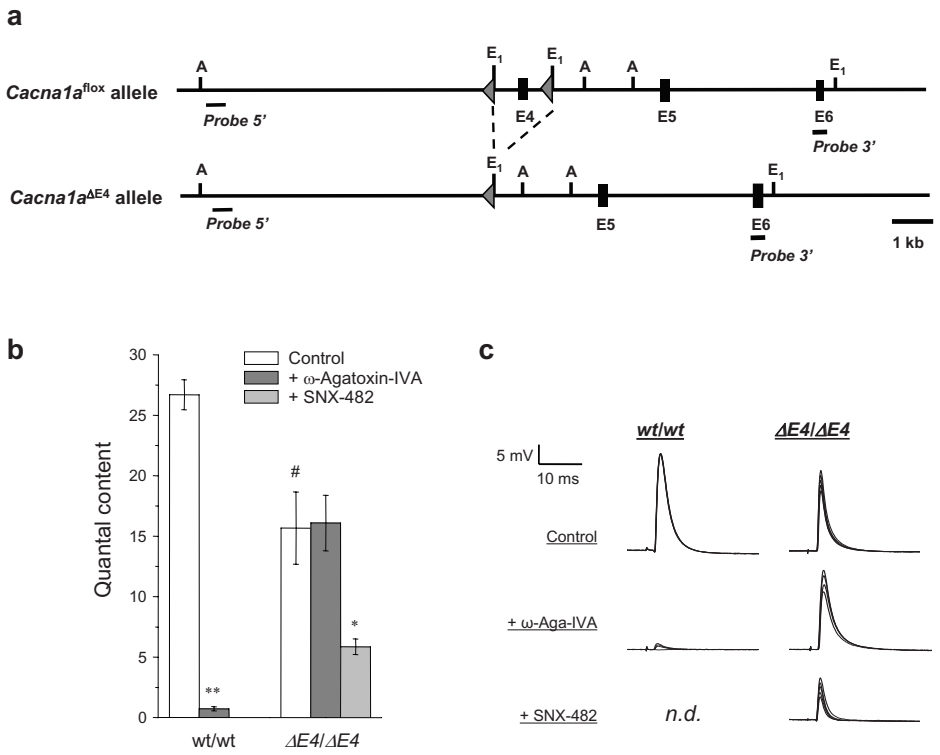

Figure 4. Deletion of exon 4 functionally results in a knockout. (**A**) Schematic representation of Cre-recombinase-mediated deletion of exon 4 (*Cacna1a*$^{\Delta E4}$ allele); (**B**) Quantal content (0.3 Hz stimulation) was reduced by 41% at NMJs of *Cacna1a*$^{\Delta E4}$ knockout mice (n=3 muscles, 6-10 NMJs per muscle; $p<0.05$) compared to wild-type mice. Neurotransmitter release at NMJs in *Cacna1a*$^{\Delta E4}$ knockout mice becomes partially dependent on $Ca_v2.3$ channels, as shown by application of the selective blocker SNX-482, causing ~63% reduction of quantal content. (**C**) Superimposed example traces of 0.3 Hz EPPs recorded at wild-type and *Cacna1a*$^{\Delta E4}$ NMJs, before and after application of toxins. *$p<0.05$, **$p<0.01$, compared to control without toxin; #$p<0.05$, compared to wild-type.

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