Diseases of the nervous system associated with calcium channelopathies

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3

CONDITIONAL INACTIVATION OF THE *CACNA1A* GENE IN TRANSGENIC MICE

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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 that of 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.

Keywords: $Ca_v2.1$, P/Q-type Ca^{2+} channels, knockout, neuromuscular junction, Cre-recombinase

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

Neuronal $Ca_v2.1$ (P/Q-type) calcium channels are abundantly expressed throughout the central nervous system (CNS), where they are crucial for neurotransmitter release (*Westenbroek et al., 1995*; *Mintz et al., 1995*). In the peripheral nervous system (PNS), $Ca_v2.1$ channels are mainly expressed at the neuromuscular junction (NMJ), mediating presynaptic ACh release (*Uchitel et al., 1992*). 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 (*Ophoff et al., 1996*; *Zhuchenko et al., 1997*; *Kors et al., 2001*). $Ca_v2.1$ channels are involved in various important (patho)physiological processes such as cortical spreading depression (*Ayata et al., 2000*; *van den Maagdenberg et al., 2004*), nociception (*Ebersberger et al., 2004*), and neurogenic vasodilatation (*Akerman et al., 2003*).

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*) (*Meier & MacPike, 1972*; *Oda, 1973*; *Jun et al., 1999*; *Fletcher et al., 1999*; *Zwingman et al., 2001*; *Pagani et al., 2004*). Analysis of these mice has shown that aberrant $Ca_v2.1$ function can be compensated for by specific upregulation of other calcium channel subtypes (*Qian & Noebels, 2000*; *Kaja et al., 2006*), suggesting a prominent cell-specific role in these neurological phenotypes (*Campbell & Hess, 1999*). Natural and $Ca_v2.1$ KO mice have provided valuable insights into the consequences of calcium channel dysfunction and the pathophysiology of epilepsy, ataxia, and dystonia (*Pietrobon, 2005*). However, further research on the underlying pathophysiological mechanisms of $Ca_v2.1$-associated diseases is seriously hampered by the fact that (1) the $Ca_v2.1$ KO mice die at an early age; (2) ablation of $Ca_v2.1$ channels occurs throughout the brain, because *Cacna1a* is broadly expressed in the CNS; and (3) 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 by highly specialized techniques, such as microiontophoresis (*Shields et al., 2005*), 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 (*Randall & Tsien, 1995*). To circumvent these problems, we generated a conditional mouse for the spatiotemporal inactivation of the *Cacna1a* gene using the Cre/lox system.

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 (*Lakso et al., 1996*). Consequently, we were able to obtain mice without the *neo* cassette, leaving only two loxP sites flanking exon 4. Thus, we generated an allele the for conditional inactivation of the *Cacna1a* gene (i.e. *Cacna1a*^{flox} allele) (*Fig. 1A*). Correct homologous recombination and

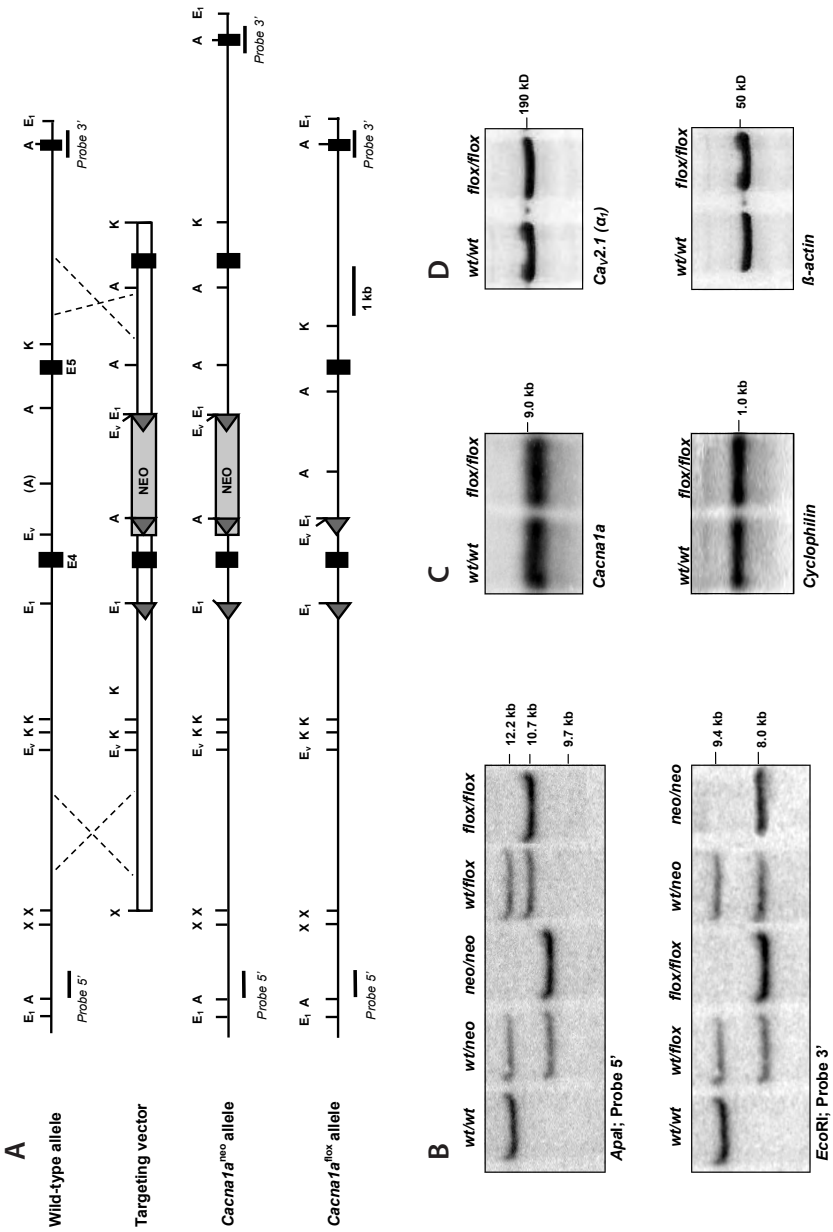

Figure 1. Generation of conditional Cacna1a mouse (A) Schematic representation of the genomic structure of the relevant part of the *Cacna1a* wild-type allele, the targeting vector, the allele after homologous recombination (*Cacna1a*^{neo} allele) and the conditional *Cacna1a*^{flox} allele after partial Cre-mediated deletion. Black boxes indicate exons (E). Probes for Southern analysis are indicated. Restriction sites: E_1, *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: *Apa*I- and *Eco*RI-digested genomic DNA from the different genotypes probed with either the 5' or the 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.

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*). 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 the 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 (*Fig. 2A, 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 (*Fig. 2B, E*). The expression pattern of $Ca_v2.1$ channels was also without abnormalities in other brain regions such as the hippocampal and cortical regions (*Fig. 2C, 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

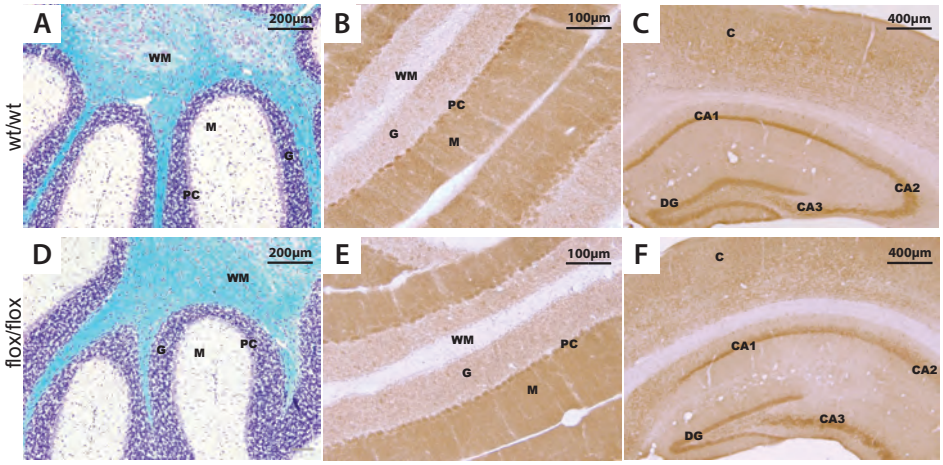

Figure 2. Histology and expression of $Ca_v2.1$ in wild-type and *Cacna1a*^{flox} mice (A, D) Klüver-Barrera stained sagittal sections from the cerebellum. **(B, E)** Immunostaining on cerebellar coronal sections. **(C, F)** Relatively high $Ca_v2.1$ expression observed in the hippocampus, whereas lower in cortical regions. No apparent overall structural abnormalities or differences in $Ca_v2.1$-α1 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. regions of the hippocampus; C, cerebral cortex.

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 (*Jun et al., 1999*). 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 the 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 was identical to that of conventional $Ca_v2.1$ KO mice (*Jun et al., 1999*; *Fletcher et al., 2001*). 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 NMJs (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 nM ω-Agatoxin-IVA (*Fig. 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 (*Urbano et al., 2003*; *Kaja & Plomp, unpublished data*; *Pardo et al., 2006*). However, in conventional $Ca_v2.1$ KO mice, neuromuscular transmission becomes for a large part dependent on compensatory $Ca_v2.3$ channels (*Urbano et al., 2003*). Application of 1 μM 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 $Ca_v2.1$ KO NMJs (*Urbano et al., 2003*).

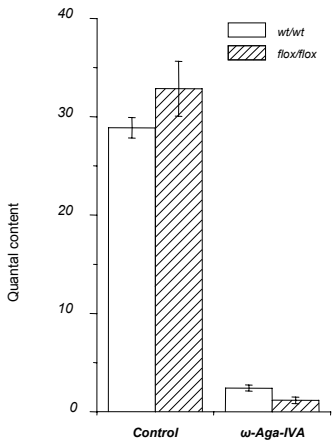

Figure 3. Neurotransmitter release at the NMJ in $Ca_v2.1$ conditional mice. Neurotransmitter release is not altered at NMJs of conditional *Cacna1a*^{flox} mice. Quantal content (0.3 Hz stimulation) is not significantly different from wild-type (n=4 muscles, 6-10 NMJs per muscle, $p=0.23$); application 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).

Here, we 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 KO was obtained by Cre recombinase-mediated deletion of exon 4; *Cacna1a*$^{\Delta E4}$ mice displayed a phenotype identical to that described for the conventional $Ca_v2.1$ KO mice (*Jun et al., 1999*;

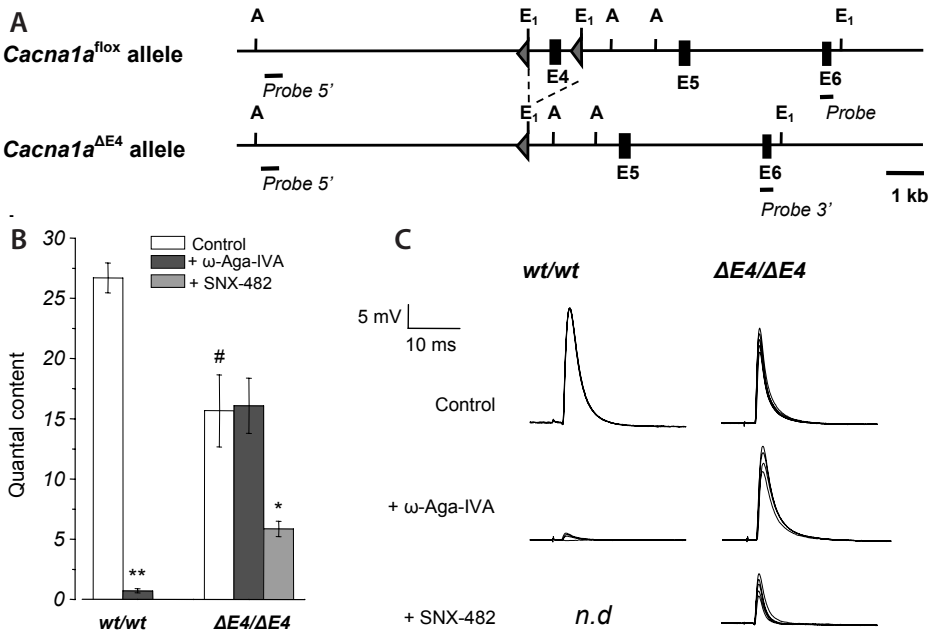

Figure 4. Deletion of exon 4 functionally results in a knockout. (A) Schematic representation of Cre-recombinase-mediated deletion of exon 4 (*Cacna1a*$^{\Delta E4}$ allele). **(B)** Quantal content (0.3 Hz stimulation) was reduced by 41% at NMJs of *Cacna1a*$^{\Delta E4}$ knockout mice (n=3 muscles, 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.

Fletcher et al., 1999). We showed that *Cacna1a*$^{\Delta E4}$ have no $Ca_v2.1$ channel-mediated ACh release at the NMJ. Furthermore, in agreement with earlier experiments on conventional $Ca_v2.1$ KO mice (*Urbano et al., 2003*), $Ca_v2.3$ 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 edema. The increasing availability of transgenic mouse lines with spatial-temporal expression of Cre-recombinase in the brain (*Morozov et al., 2003*) makes such $Ca_v2.1$ studies feasible.

EXPERIMENTAL PROCEDURES

Generation of transgenic mice.

Mouse genomic DNA clones were derived from a 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 linearized 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 harboring 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 (*Lakso et al., 1996*), 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, under 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 (*van den Maagdenberg et al., 2004*). Western blotting was done according to the enhanced chemiluminescence protocol (*Amersham Biosciences, Roosendaal, The Netherlands*). 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 PBS, followed by 4% buffered paraformaldehyde. For immunohistochemistry, 40 µm coronal sections of were processed using the free floating method. In brief, antigen retrieval was performed for 30 min at 80°C in 25 mM 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*), 1:200 diluted in 2% heat-inactivated NHS / 0.4% TX100 in TBS, for 72hrs at 4°C. Secondary biotinylated goat anti-rabbit antibody (*Vector*

Laboratories, Burlingame, CA) 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*) 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 (*Plomp et al., 1992*). 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 are 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.

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