CONDITIONAL INACTIVATION OF THE CACNA1A GENE IN TRANSGENIC MICE

Boyan Todorov, Rob C.G. van de Ven, Simon Kaja, Ludo A.M. Broos, Jaap J. Plomp, Michel D. Ferrari, Rune R. Frants, Arn M.J.M. van den Maagdenberg

Departments of Human Genetics, Molecular Cell Biology – Group Neurophysiology, and Neurology, Leiden University Medical Centre, 2300 RC Leiden, The Netherlands; Authors contributed equally; Present address: Michael Smith Laboratories, The University of British Columbia, 301-2185 East Mall, Vancouver B.C., Canada V6T 1Z4

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ABSTRACT

Ca$_{2.1}$ (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 $\alpha_1$ subunit of Ca$_{2.1}$ 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$_{2.1}$ 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$_{2.1}$ channel-mediated neurotransmission that was accompanied by a compensatory upregulation of Ca$_{2.3}$ (R-type) channels at this synapse. Pharmacological inhibition of Ca$_{2.1}$ channels is possible, but the contributing cell types and time windows relevant to the different Ca$_{2.1}$-related neurological disorders can only be reliably determined using Cacna1a conditional mice.

Keywords: Ca$_{2.1}$, 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\textsubscript{2.1} (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\textsubscript{2.1} channels are mainly expressed at the neuromuscular junction (NMJ), mediating presynaptic ACh release (Uchitel et al., 1992). The pore-forming \(\alpha\) subunit of Ca\textsubscript{2.1} 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\textsubscript{2.1} 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\textsubscript{2.1-}\(\alpha\), exist with phenotypes ranging from severe ataxia, dystonia, and premature death (leaner, Ca\textsubscript{2.1} 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\textsubscript{2.1} 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\textsubscript{2.1} 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\textsubscript{2.1}-associated diseases is seriously hampered by the fact that (1) the Ca\textsubscript{2.1} KO mice die at an early age; (2) ablation of Ca\textsubscript{2.1} 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\textsubscript{2.1} 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\textsubscript{2.1} 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\textsuperscript{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\textsuperscript{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\textsuperscript{floxed} 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\textsuperscript{neo} allele) and the conditional Cacna1a\textsuperscript{flox} allele after partial Cre-mediated deletion. Black boxes indicate exons (E). Probes for Southern analysis are indicated. Restriction sites: E\textsubscript{II}, Eco\textsubscript{RI}; A\textsubscript{II}, Apa\textsubscript{I}; EV, Eco\textsubscript{RV}; K\textsubscript{I}, Kpn\textsubscript{I}; X\textsubscript{I}, Xba\textsubscript{I}; (A), polymorphic Apa\textsubscript{I} site between the construct and the wild-type. (B) Southern blot of cerbellar total DNA from the different genotypes probed with either the 5' or the 3' probe. (C) Northern blot of cerbellar total RNA isolated from wild-type or homozygous conditional mice probed with either Cacna1a or Cyclophilin. (D) Qualitative Western blot of cerbellar membrane protein extracts from wild-type or homozygous conditional mice probed with Ca\textsubscript{v}2.1-\(\alpha_1\) and \(\beta\)-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 \textit{Cacna1a}^{\text{flox}} allele was confirmed by direct sequencing (\textit{data not shown}). Both heterozygous and homozygous \textit{Cacna1a}^{\text{flox}} mice are viable, breed normally, and do not show any overt phenotype. Northern blot analysis revealed normal levels of expression of \textit{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 \textit{Cacna1a}^{\text{flox}} mice (\textit{data not shown}). Qualitative Western blot analysis revealed similar expression levels for Ca\textsubscript{2,1-\alpha}, protein in \textit{Cacna1a}^{\text{flox}} and wild-type cerebellar extracts (Fig. 1D). No apparent cytoarchitectural abnormalities were observed in Klüver-Barrera stained sections of wild-type and \textit{Cacna1a}^{\text{flox}} brains (Fig. 2A, D). We focussed mainly on the cerebellum because of its high expression of Ca\textsubscript{2,1} channels. Immunohistochemistry revealed a normal expression pattern of Ca\textsubscript{2,1-\alpha}, protein for both the \textit{Cacna1a}^{\text{flox}} and wild-type mice with a high expression in the Purkinje cell and molecular layer (Fig. 2B, E). The expression pattern of Ca\textsubscript{2,1} 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\textsubscript{2,1} channels, we investigated the evoked ACh release at the diaphragm NMJs with \textit{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 \textit{Cacna1a}^{\text{flox}} mice (\textit{n} = 4 muscles, 6-10 NMJs per muscle, \textit{p} = 0.23) (Fig. 3). Application of 200 nM of the specific Ca\textsubscript{2.1} blocker \textit{\omega}-Agatoxin-IVA reduced

![Image](image_url)

\textbf{Figure 2.} Histology and expression of Ca\textsubscript{2.1} in wild-type and \textit{Cacna1a}^{\text{flox}} mice (A, D) Klüver-Barrera stained sagittal sections from the cerebellum. (B, E) Immunostaining on cerebellar coronal sections. (C, F) Relatively high Ca\textsubscript{2.1} expression observed in the hippocampus, whereas lower in cortical regions. No apparent overall structural abnormalities or differences in Ca\textsubscript{2,1-\alpha} 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_{2.1} 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_{2.1}-\alpha_1 in Ca_{2.1} KO mice (Jun et al., 1999). Here we generated and investigated mice lacking exon 4 (Cacna1a_{\text{floxed}}) by breeding our Cacna1a_{\text{floxed}} mice with EIIA-driven Cre-deleter mice (Fig. 4A). Cre recombination resulting in the deletion of floxed sequences in the Cacna1a_{\text{floxed}} allele was confirmed by PCR and Southern blot analysis (data not shown). Cacna1a_{\text{floxed}} mice exhibit progressively severe ataxia and dystonia starting around P10-12, and died at P20-22 if left unaided. At P20, Cacna1a_{\text{floxed}} mice were significantly smaller than their littermate controls. The observed phenotype was identical to that of conventional Ca_{2.1} 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_{\text{floxed}} NMJs compared to wild-type NMJs (26.7 ±1.2, n = 3 muscles, 6-10 NMJs per muscle) (Fig 4B). ACh release at Cacna1a_{\text{floxed}} NMJs appeared insensitive to 200 nM \omega-Agatoxin-IVA (Fig. 4B, C). Ca_{2.3} (R-type) channels do not mediate transmitter release at the wild-type NMJ, as demonstrated by an insensitivity of the quantal content to 1 \mu M of the Ca_{2.3} channel blocker SNX-482 (Urbano et al., 2003; Kaja & Plomp, unpublished data; Pardo et al., 2006). However, in conventional Ca_{2.1} KO mice, neuromuscular transmission becomes for a large part dependent on compensatory Ca_{2.3} channels (Urbano et al., 2003). Application of 1 \mu M of the Ca_{2.3} channel blocker SNX-482 to Cacna1a_{\text{floxed}} NMJ preparations in the present study revealed a similar compensatory Ca_{2.3} 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_{2.2} channels, as shown at conventional Ca_{2.1} KO NMJs (Urbano et al., 2003).

Here, we generated a conditional Ca_{2.1} mouse model that will be useful to study the consequences of temporal and spatial ablation of Ca_{2.1} channels. We did not find evidence that insertion of LoxP sites into the Cacna1a gene alters gene expression or Ca_{2.1} channel function. A complete functional KO was obtained by Cre recombinase-mediated deletion of exon 4; Cacna1a_{\text{floxed}} mice displayed a phenotype identical to that described for the conventional Ca_{2.1} KO mice (Jun et al., 1999; 0
10
20
30
40
\text{Quantal content}
\text{Control} \quad \omega-\text{Aga-IVA}

Figure 3. Neurotransmitter release at the NMJ in Ca_{2.1} conditional mice. Neurotransmitter release is not altered at NMJs of conditional Cacna1a_{\text{floxed}} 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_{2.1}-specific blocker \omega-Agatoxin-IVA (200 nM) causes a reduction of >90% of the quantal content in both wild-type and Cacna1a_{\text{floxed}} NMJs (n=4 muscles, 6-10 NMJs per muscle).
We showed that Cacna1aΔE4 have no Ca v2.1 channel-mediated ACh release at the NMJ. Furthermore, in agreement with earlier experiments on conventional Cav2.1 KO mice (Urbano et al., 2003), Cav2.3 channels partly compensate for the loss of Ca v2.1 channels. Our data clearly show that Cacna1aΔE4 is a functional null allele. The ability to spatially and/or temporally ablate Ca v2.1 channels in a non-invasive way using the Cav2.1 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 EcoRV site downstream of exon 4. A third LoxP site, in the same orientation, was introduced at the EcoRI 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’-TACCGGTTGATGGAATG-3’; P2: 5’-CGGGACGGAGTTTGACGTAC-3’) and the upstream LoxP site (primers P3: 5’-AGTTTCTATTGGACAGTGTACCAGCTG-3’; P4: 5’-TTGCTCTGACGAGCAGAAGACTGG-3’).

Two correctly targeted clones harboring the Cacna1a<sup>neo</sup> 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<sup>neo</sup> mice were crossed with male EIIA-driven Cre-deleter mice (Lakso et al., 1996), resulting in mice with the conditional Cacna1a<sup>flox</sup> allele. Correct deletion of the neo cassette was confirmed by Southern blot analysis with restriction enzyme ApaI. 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<sub>2.1-α</sub>, 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% H₂O₂. 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 ω-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 Ca₂⁺, channels) or 1 μM SNX-482 (blocks Ca₃.3 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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