Animal Model

Cerebellar Ataxia, Seizures, Premature Death, and Cardiac Abnormalities in Mice with Targeted Disruption of the Cacna2d2 Gene

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Cacna2d2 is a putative tumor suppressor gene located in the human chromosome 3p21.3 region that shows frequent allelic imbalances in lung, breast, and other cancers. The α2δ-2 protein encoded by the gene is a regulatory subunit of voltage-dependent calcium channels and is expressed in brain, heart, and other tissues. Here we report that mice homozygous for targeted disruption of the Cacna2d2 gene exhibit growth retardation, reduced life span, ataxic gait with apoptosis of cerebellar granule cells followed by Purkinje cell depletion, enhanced susceptibility to seizures, and cardiac abnormalities. The Cacna2d2<sup>−/−</sup>NCIF null phenotype has much in common with that of Cacna1a mutants, such as cerebellar neuro-degeneration associated with ataxia, seizures, and premature death. A tendency to bradycardia and limited response of null mutants to isoflurane implicate α2δ-2 in sympathetic regulation of cardiac function. In summary, our findings provide genetic evidence that the α2δ-2 subunit serves <i>in vivo</i> as a component of P/Q-type calcium channels, is indispensable for the central nervous system function, and may be involved in hereditary cerebellar ataxias and epileptic disorders in humans. (Am J Pathol 2004, 165:1007–1018)

Voltage-dependent calcium channels (VDCCs)1 play a role in generating the rhythmic membrane potential behavior of many excitable cells and also provide a pathway through which Ca<sup>2+</sup> enters cells to serve as a second messenger that regulates a diversity of cellular processes.1–3 VDCCs consist minimally of an α1 protein that forms the channel pore and voltage-sensor. Core α1 subunits are used for dividing VDCCs into three major phylogenetic types, which are supported by pharmacological and functional classification: T-type (α1<sub>1G</sub>, α1<sub>1H</sub>, and α1<sub>1I</sub>); L-type (α1<sub>1C</sub>, α1<sub>1D</sub>, α1<sub>1E</sub>, and α1<sub>1G</sub>); and the presynaptic channels-type (α1<sub>1A</sub>, α1<sub>1B</sub>, and α1<sub>1E</sub>).4 A variety of auxiliary regulatory subunits, including α2δ, β, and γ, associate with the α1-subunits, forming different heteromeric functional VDCCs.5–7 The members of the α2δ family include α2δ-1, which is ubiquitously expressed; α2δ-2, which is expressed in brain, heart, and other organs; and α2δ-3, whose transcripts are found mostly in the brain.8–10 α2δ subunits consist of two proteins that are encoded by a single gene, the product of which is post-translationally cleaved from a single polypeptide precursor.10,11 The discovery of worm and fly orthologs of α2δ-encoding genes12 demonstrates their conservation.

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over billions of years of evolution, suggesting that the α2δ protein is of fundamental importance in cellular function.

In humans, α2δ-2 is encoded by CACNA2D2 (GenBank Accession No. AF040709), which is contained, along with seven other genes, in a 120-kb region of chromosome 3p21.3 that is frequently deleted in lung, breast, and other cancers. Reduction or absence of CACNA2D2 expression in non-small cell lung cancer cell lines suggests that α2δ-2 itself may be a tumor suppressor protein. α2δ-2 is predicted to be a heavily glycosylated 175-kd protein whose single-pass transmembrane δ piece anchors the α2 protein to the membrane.

Electrophysiological studies demonstrated that α2δ-2 might function as an auxiliary component of a variety of different VDCCs: L-type [Ca,1.2 (α1C)]; P/Q-type [Ca,2.1 (α1A)]; N-type [Ca,2.2 (α1B)]; R-type [Ca,2.3 (α1G)]; and T-type [Ca,3.1 (α1H) and Ca,3.2 (α1G)]. Co-expression of α2δ-2 with each of these α1 subunits enhanced currents through the heteromeric channels. While these experiments characterized α2δ-2 as electrophysiologically versatile, its biological significance remained uncertain. Here we report that the Cacna2d2 null mice show ataxia, growth retardation, increased susceptibility to seizures, and a reduced life span. Phenotypic similarity with Cacna1a null mice and spontaneous Cacna1a mutants implicates Cacna2d2 in regulation of P/Q-type currents as a component of Cav2.1 in human hereditary cerebellar ataxias and epilepsies. We also provide evidence that α2δ-2 expression in the central nervous system, dorsal sympathetic ganglia, and cardiac conductive tissue may be related to the observed disturbances in null mutants’ heart rate regulation.

Materials and Methods

Construction of Targeting Vector and Generation of Cacna2d2tm1NCIF Mice

A replacement-type Cacna2d2-targeting vector was based on a mouse genomic clone isolated from a 129/SvJ lambda FIXII library (Stratagene, La Jolla, California). Cacna2d2 exons were identified by Southern hybridization, partial sequencing, and BLAST analysis. Mouse EST clones represented in GenBank by Accession No. AA000341 (Research Genetics, Huntsville, AL) was used as a probe. A 2629-bp fragment containing eight exonic sequences, respectively. The neo-gene with the phosphorothioate linker was integrated into the construct as 5' and 3' flanking sequences. The neo-gene with the phosphorothioate linker was integrated into the construct as 5' and 3' flanking sequences.
CA) according to the manufacturer’s protocol. Two primer sets were used for RT-PCR. One of them, 5’-GCATAAATATGATGTCACAC-3’ and 5’-GGGGCTTGAAAATATAACCA-3’, represented a Caca2d2 cDNA fragment with positions 1742–2862. The other pair, 5’-TCTACTCTGTGTCCTCATTG-3’ and 5’-TGAGTCTAGGGACGTGGGG-3’, corresponded to positions 3113–4149 (GenBank Accession No. AF247139). Comparative PCR on human cardiovascular MTN cDNA (Clontech, Palo Alto, CA) was performed as suggested by the manufacturer with human primers 5’-GCATAAATATGATGTCACAC-3’ and 5’-GGGGCTTGAAAATATAACCAACCG-3’ corresponding to the first set of mouse primers. Northern analysis and RT-PCR were performed on mouse brain mRNA isolated with a FastTrack 2.0 kit (Invitrogen). A Caca2d2 cDNA EST clone (GenBank Accession No. AA000341, positions 3237–5498 in AF042792) purchased from Genome Systems, Inc. (St. Louis, MO) was used as a 3’ probe, while the RT-PCR product obtained with the first set of primers (see above) was used as a 5’ probe. The same mouse EST clone was also used to generate 35S-labeled RNA probes for in situ hybridization on mouse tissues. These probes were synthesized using MAXiScribe (Ambion Inc., Austin, TX) in vitro transcription kit and two primers. Primer 5’-TAATAGCCTCCTACTGAGGGAACATCAGGACTGCGG-3’ contained T7 RNA-polymerase promoter shown in uppercase and was used to produce sense probe. Primer 5’-AATTACCTCCACTAAGGGGACCTCGAAGGAGG-3’ contained T3 RNA-polymerase promoter and was used to generate antisense probe. DNA template used in the RNA synthesis was eliminated with DNase I following the in vitro transcription reaction. For in situ hybridization, tissues of postnatal mice or embryos at stages 13.5 and 15 dpc were perfused and fixed as described. Briefly, the samples were hydrated, paraffin-embedded, and sectioned at 4 mm onto microscope slides. For pre-hybridization, the slides were deparaffinized in xyleno, hydrated through a series of graded ethanol/DEPC-saline, put through a microwave RNA retrieval procedure, and the RNA was further unmasked by permeabilization with pronase. For hybridization, a riboprobe 750,000 cpm was added to the hybridization buffer, denatured at 95°C for 5 minutes, and incubated with the slides for 14 hours at 55°C. Slides were washed in the washing buffer, incubated with RNase A, dehydrated in graded ethanol rinses, and dried under vacuum. Dried slides were immersed into twofold-diluted K5 nuclear emulsion (Polysciences, Warrington, PA), slowly dried at room temperature, and exposed for 14 days. The slides were then developed using D19 reagent (Eastman Kodak, Rochester, NY), and latent images fixed using Kodak Fixer. The slides were finally thoroughly rinsed, counterstained with hematoxylin (Richard-Allen, Kalamazoo, MI), dehydrated, and glass-covered using permanent mounting media. Visualization of signal was done on a Leitz Laborlux-S (Wetzlar, Germany) microscope.

**Animal Testing**

Animals used in these studies were maintained in facilities fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and all procedures were performed under protocols approved by the NIH (Publication No. 86–23, 1985) and NCI Animal Care and Use Committees in strict compliance with the Guide for the Care and Use of Laboratory Animals of the National Research Council (National Academy Press, Washington, DC; http://www.nap.edu/readingroom/books/labrats).

**Seizure Susceptibility Testing**

Twelve- to 24-week-old mice were used. Pentylenetetrazol (PTZ) (Sigma-Aldrich, St. Louis, MO), in 0.9% NaCl solution, was administered by subcutaneous injection into a loose fold of skin on the right belly. Mice were observed for 30 minutes. Animals exhibiting a clonic spasm of at least 5 seconds duration were scored as positive for seizure occurrence. To determine the 50% convulsive dose (CD50) a range of PTZ doses were used spanning the CD50 value. Each mouse received only a single injection and the animal was immediately euthanized after exhibiting convulsions or at the end of the 30-minute observation period. In a separate series of experiments the behavioral seizure score was determined with increasing cumulative doses of PTZ. Mice (18 to 24 weeks old) were injected intraperitoneally with a 10 mg/kg dose of PTZ and observed for 30 minutes. Additional doses of 20, 40, and 60 mg/kg were administered at 30-minute intervals until tonic hind limb extension occurred, at which time the mice were immediately euthanized. PTZ typically induced a sequence of behaviors that were scored in each of the 30-minute intervals after dosing according to the following scale: 0, normal behavior; 1, behavioral arrest; 2, vocalization and limb twitches; 3, sustained forelimb clonus; 4, wild running and jumping; and 5, tonic hind limb extension.

**Inverted Screen Test**

A modification of the horizontal screen test was used as a measure of overall motor function. Mice were placed on a horizontally oriented grid (consisting of parallel 1.5-mm diameter rods situated 1 cm apart), and the grid was inverted. The time the animal remained on the underside of the grid was recorded. Mice that fell from the grid within <15 seconds were considered to have reduced strength or coordination.

**Footprint Pattern Test**

Mice with hind paws dipped in non-toxic ink were placed at one end of a dark tunnel measuring 10 cm × 10 cm × 50 cm, the bottom of which was lined with white paper. Mice walked down the tunnel, and their footprints were used to assess the ability to walk in a straight line, irregularities, and orientation of the hind paws.

**Echocardiography**

Trans-thoracic echocardiography was performed using an Acuson Sequoia equipped with a 15-MHz linear trans-
dor (15L8) in a phased-array configuration. Animals were placed on a heating pad (37°C) and imaged under isoflurane anesthesia in the left lateral decubitus position. Two-dimensional images were obtained from parasternal long- and short-axis views with optimized gain and depth settings. M-mode images were obtained from short-axis views at the papillary muscle level at a sweep speed of 100 mm/s. Two-dimensional and M-mode images were digitized and stored for off-line analysis. Cardiac measurements were performed off-line from the M-mode images using a leading edge-to-leading edge technique according to the American Society of Echocardiography guidelines. Measurements from five cardiac cycles were averaged to obtain the following parameters in diastole and systole: interventricular septum (IVSd, IVSs); posterior wall (PWd, PWs); and left ventricular internal diameter (LVIDd, LVIDs). The percentage of LV fractional shortening (LVFS) was calculated as \((LVIDd - LVIDs) / LVIDd \times 100\). LV mass was calculated according to the uncorrected cube method using the equation \(LV mass = 1.05((IVSd+LVIDd+PWd)^3 - (LVIDd)^3)\), as previously reported.

**ECG Recordings**

ECGs were recorded in conscious and sedated mice with different Cacna2d2 status using a recently developed noninvasive ECG recording device and software (Mouse Specifics, Boston, MA), as described previously. Conscious or isoflurane-sedated animals were positioned on the ECG platform with gel-coated electrodes embedded in its base. The amplified signals were digitized with 16-bit precision at a sample rate of 2000 samples/second. Only continuous recordings (>10 to 25 ECG complexes) generated from mice with three paws in contact with three electrodes were analyzed. The following parameters were measured: HR and RR interval, PR interval, QRS duration and maximum height, QT and corrected QT intervals.

**Blood Pressure Measurements and Clinical Chemistry**

Noninvasive blood pressure and serum electrolytes were measured on each of four null and five wild-type animals by Phenotyping Service of the Jackson Laboratory (West Sacramento, CA). Growth hormone, T3, T4, and IGF-1 mRNAs showed expression of the full-length transcript in male and female mice from 22 days to 70 days of age using a leading edge-to-leading edge technique according to the American Society of Echocardiography guidelines. Northern blot analysis on brain mRNA samples from each of four null and four wild-type animals depicted an mRNA pattern (64:130:62) that was very close to the expected Mendelian distribution (64:130:62) that was very close to the expected Mendelian distribution. Northern blot analysis on brain mRNAs showed expression of the full-length transcript in heterozygotes but not null-mutant animals (Figure 1, C). While Cacna2d2m1NCIF mice appeared healthy, had normal life spans, and were not distinguishable from their littermates in any assay, null-mutants could not survive beyond 70 days of age.

**Pathology**

Cacna2d2+/+, Cacna2d2+/−, and Cacna2d2−/− male and female mice from 22 days to 70 days of age were completely necropsied. All tissues were fixed in neutral buffered formalin, embedded in paraffin, sections cut at 4 to 6 µm, and stained with hematoxylin and eosin (H&E). Selected tissues were stained with luxol fast blue and Bodian stains. For immunohistochemistry of the brain, antibodies to glial fibrillary acidic protein (GFAP) (DAKO, Carpinteria, CA), Ki-67 (polyclonal, Novocastra, Newcastle on Tyne, UK), calbindin (D-28K, Sigma), and caspase-3 (Promega, Madison, WI) were used. The TUNEL method (ApopTag® In Situ Apoptosis Detection kit, Intergen, Purchase, NY) was used to identify apoptotic cells.

**Statistics**

Data in this study were evaluated using standard analysis of variance and Student’s t-tests, repeated measures analysis of variance, profile analysis, analysis of covariance, post-hoc tests, non-parametric methods, survival analysis, and graphical descriptive techniques. Data analyzed with parametric methods were routinely tested for conformance with homogeneity of variance and covariance requirements. Kaplan-Meier plots were constructed for survival, and differences in survival distributions were tested with the log rank test.

**Results**

**Generation and Characterization of Cacna2d2m1NCIF Mice**

We constructed a Cacna2d2-targeting vector, in which a 3445-bp XbaI/HindIII fragment (positions 6593434–6596878 on NT_039477) containing seven 3′-terminal exons of the gene was replaced by a neomycin-resistance gene (Figure 1A). This replacement eliminated the sequence encoding the entire δ subunit with a single transmembrane domain plus the 87 C-terminal amino acids of the α2 responsible for binding the δ subunit. Genotyping of 256 F2 offspring by Southern hybridization (Figure 1B) revealed a Cacna2d2+/+ : Cacna2d2+/- : Cacna2d2−/− distribution (64:130:62) that was very close to the expected Mendelian distribution. Northern blot analysis on brain mRNAs showed expression of the full-length transcript in heterozygotes but not null-mutant animals (Figure 1, C and D).

**Growth Delay, Neurological Phenotype, and Premature Death in Null-Mutants**

While Cacna2d2+/- mice appeared healthy, had normal life spans, and were not distinguishable from their Cacna2d2+/- littermates in any assay, null-mutants could be reliably identified at approximately 4 weeks of age and older by dystonic episodes, ataxic waddling gait typical for cerebellar dysfunction, and reflexive clutching of hind limbs when lifted by their tails (Figure 2). Dystonic episodes usually started with characteristic extension of the hind limbs. During the next stage that lasted from a few minutes to more than one-hour, conscious animals were immobilized on their abdomen or side by whole-body extension. After recovery, the null-mutants walked in a poorly coordinated fashion, dragging their hind limbs and often listing to one side. They frequently showed myo-
clonic jerking of the limbs or whole body tremor, and tonic extension of the hind limbs. In four animals, generalized clonic seizures that lasted up to 30 minutes and affected the entire body were observed. The null-mutants developed growth delay starting at the age of 4 weeks (P < 0.0001 by analysis of covariance, profile analysis, and post-hoc t-tests). The mean body weights of Cacna2d2−/− mice were ~1.5 to 2 times less than that of their wild-type age mates (Figure 3A). Cacna2d2−/− mice also had markedly reduced life spans (P < 0.001 by the log rank test), with a median survival time of 150 days (Figure 3B). Among 50 Cacna2d2−/− mice studied, 23 (46%) died prematurely with no obvious cause of death. Most of these mice died at young ages, and no premature deaths were observed at ages older than 6 months (Figure 3B).

Evaluation of Motor Function

Motor impairment in null-mutants was evaluated with the inverted screen test, in which the animals were required to support their body weight by grasping a wire grid. None of 6 Cacna2d2−/− remained on the inverted screen for at least 15 seconds, whereas 3 of 6 of the Cacna2d2+/− and 6 of 7 of the Cacna2d2+/+ remained on the grid. The mean times on the grid were 5.5 ± 1.5 seconds, 56 ± 23 seconds, and 138 ± 83 seconds, respectively. Compared with wild-type control mice, Cacna2d2−/− mice had significant motor impairment (P = 0.006, Kolmogorov-Smirnov test). Heterozygotes were not significantly different (P = 0.713) from wild-type mice.

Cacna2d2 Expression in Mouse Tissues

In situ hybridization with mouse embryo and adult cerebellum sections showed that Cacna2d2 is expressed primarily in the central nervous system (Figure 4). In the postnatal cerebellum, a very high level of gene expression was detected in the Purkinje cell layer, while granular
produced poor signals (Figure 4, A and B). Remarkably, Cacna2d2 expression was also detected outside the central nervous system, namely, in the dorsal sympathetic ganglia (Figure 4C). While no Cacna2d2 expression was detected in mouse embryonic heart, previous reports indicated that this gene is highly expressed in both mouse and human adult hearts.8–10 Indeed, using the RT-PCR assay we demonstrated that the CACNA2D2 mRNA is expressed mainly in four cardiac regions: aortes, aorta, interventricular septum, and atrioventricular node (Figure 5). These areas represent highly innervated parts of the heart's conductive system. Limited levels of CACNA2D2 expression were also detected in the muscle tissue, such as apex and ventricles.

Assessment of Cardiac Function

To explore the possibility of heart failure due to the α2δ-2 deficiency we evaluated null mutants' heart function. First, histo-pathological study of the null mutants' hearts revealed no signs of cardiomyopathy, apoptosis, or any other abnormality (data not shown). Second, heart function was assessed in vivo by surface electrocardiography (ECG) and trans-thoracic echocardiography. A trend to bradycardia was revealed in conscious null-mutants as compared to the Cacna2d2+/− and Cacna2d2++/++ control group (747 ± 53 versus 714 ± 73, control versus knockout, P = 0.07, Table 1). No significant differences were observed in other major ECG parameters. Remarkably, isoflurane anesthesia produced different effects on heart rates in two cohorts. While in both cases isoflurane reduced heart rate, the null-mutants' response was less profound than that of the control cohort (186 ± 37 versus 102 ± 44, control versus knockout, P = 0.01, Table 1). Consistent with the previous histopathological evaluation, echocardiographic study showed no evidence of systolic abnormalities in the null mutants' echocardiograms, and
may be caused by cerebellar impairment, we compared

tion of disturbance in coordinated motor activity, which

and had little fat. Because ataxia is a clinical manifesta-

significant; NS, not significant; MS, marginally

control mice were analyzed; for isoflurane study, 6 null and 6

QTc, corrected QT interval; for baseline measurements, 29 null-mutants

/littermates, indicating that the null mutants

HRiso, decrease in heart rate in response to isoflurane; p-values computed with Student’s t test;

Mean (standard deviation); p-values computed with Student’s t test;

p-values computed with Student’s t test;

Table 1. Major ECG Parameters in Wild-Type and

Cacna2d2/-/- Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Null</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (bpm)</td>
<td>747 (53)</td>
<td>714 (73)</td>
<td>MS (0.07)</td>
</tr>
<tr>
<td>RR interval (ms)</td>
<td>81 (7)</td>
<td>86 (10)</td>
<td>MS (0.07)</td>
</tr>
<tr>
<td>PR interval (ms)</td>
<td>27 (3)</td>
<td>26 (3)</td>
<td>NS</td>
</tr>
<tr>
<td>QRS duration (ms)</td>
<td>11 (1)</td>
<td>11 (1)</td>
<td>NS</td>
</tr>
<tr>
<td>QT interval (ms)</td>
<td>42 (5)</td>
<td>43 (4)</td>
<td>NS</td>
</tr>
<tr>
<td>QTc interval (ms)</td>
<td>46 (3)</td>
<td>46 (2)</td>
<td>NS</td>
</tr>
<tr>
<td>ΔHRiso</td>
<td>186 (37)</td>
<td>102 (44)</td>
<td>0.01</td>
</tr>
<tr>
<td>ΔRRiso</td>
<td>27 (9)</td>
<td>14 (7)</td>
<td>0.04</td>
</tr>
</tbody>
</table>

no significant differences in IVSd, IVSs, PWd, PWs, LVIDd, LVIDs, LV%FS, and LV mass were found between
null mutants and wild-type animals (6 null-mutants and 6
wild-type animals; data not shown). No significant differ-
ences were detected in blood pressure and serum elec-
trolyte concentrations between Cacna2d2/-/- mice and a
control group (potassium, sodium, magnesium, and cal-
cium measurements on five wild-type and four homozy-
gous mutant mice; data not shown).

PTZ Seizure Test

As noted, Cacna2d2/-/- mice exhibited sporadic behav-
ioral seizures. To further pursue the possibility of an ep-
ileptic phenotype, seizure susceptibility was determined with
the convulsant PTZ. For all genotypes, there was a
dose-dependent increase in the fraction of animals ex-
hibiting clonic seizures with PTZ doses in the range of 10
to 80 mg/kg (Figure 6A). However, the dose-response
curve for Cacna2d2/-/- mice was significantly shifted to
the left compared with the curves for Cacna2d2+/- and
Cacna2d2+/- littermates, indicating that the null mutants
have greater seizure susceptibility. In a separate series of
experiments examining the behavioral seizure score with
cumulative dosing of PTZ, all genotypes exhibited a
dose-dependent increase in the seizure score with PTZ
doses in the range 10 to 60 mg/kg, and all showed tonic
hind limb extension at PTZ doses of 60 mg/kg or less. As
shown in Figure 6B, null mutant mice exhibited higher
seizure scores than their littermates at 10 and 20 mg/kg
cumulative doses of PTZ, indicating that they experience
more severe seizures at these doses. Both studies indi-
cate that the null mutants have enhanced seizure sus-
cceptibility.

Pathology, Histology, and
Immunohistochemistry

At necropsy, the null mice were runty as compared to
Cacna2d2+/- or Cacna2d2+/- littermates, dehydrated,
and had little fat. Because ataxia is a clinical manifesta-
tion of disturbance in coordinated motor activity, which
may be caused by cerebellar impairment, we compared
cerebellums isolated from Cacna2d2/-/- and wild-type
mice. Necropsy of mutant mice revealed no significant
differences in the overall gross cerebellum anatomy or
Purkinje cell layer architecture (Figure 7). Consistent le-
sions in the cerebellum, however, were detected in the
granular layer. Apoptosis and thinning (loss of granule
neurons) in the granule cell layers, especially in the ver-
mis, was detected starting from day 22, becoming more
profund later. Apoptosis in the granule cell layer was
initially demonstrated with hematoxylin staining (Figure 8,
A and B) and then verified with the TUNEL method and
caspase-3 antibody (Figure 8, C and D, respectively). At
P70 and later GFAP, immunoreactive glial cell processes
came prominent in the granule cell layer (Figure 8, E
and F). No apoptotic bodies were seen in Cacna2d2+/-
or Cacna2d2+/- mice (data not shown). While no Purkinje

Figure 6. Cacna2d2 null mice show increased seizure susceptibility to the
convulsant pentylenetetrazol (PTZ). A: Dose-response curves for induction
of clonic seizures demonstrate a greater PTZ sensitivity of Cacna2d2/-/-
mice compared to wild-type and heterozygous littermates. The curves show
logistic fits to the data. The CD50 value obtained by the Spearman-Karber
method for the null mutant is 26.6 mg/kg (95% confidence limit (CL): 21.4 to
33.1) compared with 52.0 mg/kg (95%CL: 47.7 to 56.8) and 55.8 mg/kg (95%
CL: 49.25 to 63.2), respectively, for Cacna2d2+/- and Cacna2d2+/- ani-
mals. For each genotype, groups of three to six animals were tested at three
to four doses. B: Seizure score values with increasing cumulative doses of
PTZ. Cacna2d2/-/- mice showed significantly greater behavioral seizure
score values at the 10 and 20 mg/kg doses compared with Cacna2d2+/- and
Cacna2d2+/- mice (P < 0.008, Kruskal-Wallis analysis of variance); eight
animals were tested for each genotype. See “Experimental Procedures” for
seizure score scale.
cells depletion was observed in Cacna2d2−/− mice at the age of about 1 month (Figure 7B), first signs of Purkinje cells depletion were noticed at P70 (Figure 8, A and B) and became more prominent later, especially in aging mice (> 1-year-old, data not shown). Since mice became clinically ill when these lesions appeared and were sacrificed or died soon thereafter, we could not determine the natural course of the lesions except in one 10-week-old mouse that exhibited reactive gliosis of Bergman glia in H&E stained sections. Lesions in other tissues, including severe thymic atrophy and suppurative rhinitis, were sometimes found in null mice. Pathological evaluation of Cacna2d2−/− hearts revealed no obvious abnormalities.

Discussion

Cerebellar Degeneration and Increased Seizure Susceptibility

The results of our study unequivocally implicate α2δ-2 deficiency in cerebellar degeneration and ataxia, seizure susceptibility, and poor survival. Comparative analysis of this first α2δ-type subunit knockout model, the spontaneous ducky mutant that was recently shown to carry a Cacna2d2 gene rearrangement, and other previously characterized calcium neurological models facilitated identification of cells, currents, and mechanisms related to α2δ-2 involvement in cerebellar ataxia. Homozygous inactivation of the Cacna2d2 gene performed in this study produced a phenotype that had much in common with ducky and other epileptic models, such as tottering, rolling Nagoya, and Cacna1a

Figure 7. Cerebellar morphology of Cacna2d2tm1NCIF mice. Cerebellums of young null mutants (P28-P38) are not different from their wt siblings by general appearance (A) or by Purkinje cells morphology as assessed by anti-calbindin staining (B, 16 cerebellar sections of each animal analyzed).
Figure 8. *Cacna2d2*−/− mice have reduced granule cell density and increased apoptosis in cerebellum. H&E-stained sections of cerebellum in null-mutant (A) and control (B) mice at P70 (magnification, ×90) show thinning of granule cell layer, deficiency of Purkinje cells, and presence of Bergmann’s glia (reactive astrocytosis). Arrowheads in (A) indicate picnotic cells or apoptotic bodies. TUNEL (C) and caspase-3 antibody (D) staining show increased amount of apoptotic cells and bodies (arrows) in null mutant. Cerebellar cortex immunostaining with GFAP antibody (brown color) indicates enhanced expression of GFAP, representing glial cell proliferation (gliosis) in *Cacna2d2*−/− (E) in comparison with wild-type (F) (magnification, ×60).
in the Purkinje layer and barely detectable in granular cells (Figure 4, A and B, and earlier observations14), the first sign of cerebellar degeneration in Cacna2d2 null mice was the depletion of the granule cell layer (P22) via caspase 3-dependent apoptosis (Figure 8). Only later was this process followed by gradual loss of Purkinje cells, with most of them gone by 52 weeks (data not shown). This observation is consistent with the view that granular cells’ survival during normal cerebellum development is largely dependent on forming proper connections with Purkinje cells26 and that perturbations in Purkinje cells during critical stages of cerebellar development often result in devastating effects on granule cells.31 Purkinje cell-dependent depletion of granular neurons was described also in another cerebellar neurodegenerative model, lurcher heterozygous mice.32 Remarkably, ducky mice showed a quite distinct pattern of cerebellar pathology that included reduction in the gross size and appearance of cerebellum, demyelination, and early abnormalities in Purkinje cells morphology.23–25,33 More severe cerebellar degeneration in ducky mutants may be associated with elimination of longer portions of the α2δ-2 protein (Table 2) that may produce more acute effect on assembling, stability, and function of the entire multiprotein channel complex. Unfortunately, different genetic backgrounds of these two mutations (du on TKDU and duJ on C57BLKS/J) complicates direct comparison of the ducky phenotypes with Cacna2d2−/− (129/SvJ;C57BL/6J background). Nevertheless, the work on du and duJ25,33 in conjunction with the present model provide strong evidence for a role of α2δ-2 in promoting cerebellar neurons survival and functional interaction between granular and Purkinje neurons.

Concurrent with the histopathological signs of cerebellar degeneration, null mice developed multiple signs of neurological dysfunction, such as ataxia, clenching reflex, and dystonia. The fact that Cacna2d2 null mice have normal serum somatotropic axis hormones (data not shown) implies that the observed growth delay is, most likely, a consequence of ataxia-associated indolent feeding behavior that causes fat depletion, dehydration, and malnutrition.

Another important feature that relates Cacna2d2-deficient mice to Cacna1a mutants is seizure-like behavior.25–29 Spontaneous behavioral seizures in our model prompted us to use the chemoconvulsant PTZ to quantitatively assess seizure susceptibility/vulnerability. The null-mutants showed a significantly reduced PTZ threshold, demonstrating their enhanced seizure susceptibility. Given the electro-physiological flexibility of α2δ regulatory subunits and insufficiency in their number as compared to VDCC core subunits (3 types versus 10), one may expect that each of three α2δ subunits may be found in vivo in more than one calcium channel type. In addition to its association with P/Q-type (CaV2.1) channels as discussed above, it appears that α2δ-2 may be the regulatory subunit of T-type (CaV3.1) Ca2⁺ channels that underlie rhythmogenesis in some central nervous system neurons involved in epileptic activity.8,9,14 However, a recent study on mice with a targeted disruption of the CaV3.1 core subunit gene Cacna1g showed normal susceptibility to seizures.34 This observation does not directly support the association of α2δ-2 with T-type channels in vivo. Further characterization of cortical calcium channels and networks with α2δ-2 involvement is required to verify whether P/Q channels may be solely responsible for increased seizure susceptibility in our model.

Most channelopathies associated with seizures result from alterations in channel function that are expected to enhance neuronal excitability.35 An exception to this seems to be Ca2⁺ P/Q channelopathies where the epileptic phenotype occurs with reduced excitatory channel function. Different mechanisms were proposed to explain how a reduction in pre-synaptic P/Q currents might underlie spontaneous spike-and-wave discharges.36,37 Based on experiments on co-expression of α2δ-2 with a variety of VDCC core subunits and recordings made on α2δ-2-deficient Purkinje cells,7,8,14,24 the lack of the α2δ-2 auxiliary subunit in Cacna2d2−/− mice would be also expected to diminish the depolarizing current contributing to neurotransmitter release. While the net effect of this alteration is difficult to predict, the epileptic phenotype is consistent with other Ca2⁺ channelopathies where a reduced Ca2⁺ current caused by defects in Ca2⁺ channel proteins does exist.38 In this context, it is interesting to note that the antiepileptic drug gabapentin binds specifically and with high affinity to α2δ-1 and α2δ-2 proteins.39 While a role for this interaction in the anticonvulsant activity of gabapentin is yet to be definitively established,40 it is nevertheless intriguing that α2δ-2 does appear to regulate seizure susceptibility, at least in the mouse mutants, suggesting that the interaction could very well be of clinical relevance.

Overall, our data unambiguously show that Cacna2d2, which is widely expressed in CNS (cerebellum, cortex, thalamus, spinal cord, etc) and dorsal ganglia (Figure 4 and 14), is indispensable for central nervous system function and postnatal development. Based on the similarity between Cacna1a and Cacna2d2 null phenotypes and their co-expression in Purkinje cells, it may be assumed that products of these genes serve as components of the same calcium channel, ie, CaV2.1 (P/Q). Indeed, this conclusion is supported by the reduction in the Purkinje P-type currents reported in ducky homozygotes.25

**Table 2. Comparison of Cacna2d2 Mouse Models**

<table>
<thead>
<tr>
<th>Cacna2d2 mutant</th>
<th>Exons affected</th>
<th>Protein sequence affected</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ducky2J</td>
<td>2-39 duplicated, 4-39 deleted</td>
<td>Loss of most of α2δ, the entire δ</td>
<td>25, 33</td>
</tr>
<tr>
<td>ducky2J/Tkdu</td>
<td>9-39 (a 2-bp deletion in exon 9)</td>
<td>Loss of &gt;800 C-terminal amino acids of α2δ, the entire δ</td>
<td>25, 33</td>
</tr>
<tr>
<td>Cacna2d2tmINCIF</td>
<td>exon 3</td>
<td>Extra 39 amino acids, no disulfide linkage with δ</td>
<td>50</td>
</tr>
<tr>
<td>Cacna2d2entla</td>
<td>33-39 deleted</td>
<td>Loss of the 87 C-terminal amino acids of α2δ, the entire δ</td>
<td>This study</td>
</tr>
</tbody>
</table>

**References**

For a comprehensive list of references, please refer to the full article.
Premature Death and Cardiac Function

Although the Cacna2d2−/− mice that died at early age were runty and not thriving, there were no apparent behavioral changes or lesions that would account for sudden death. Since cardiac arrest is one of the most plausible mechanisms of unexpected death at young ages and in epileptic patients,41–43 we assessed possible involvement of the Cacna2d2 product in cardiac function. Taking into consideration that α2δ-2 may serve as a regulatory subunit of multiple VDCCs,8–10 we first identified human heart areas and nervous tissue where α2δ-2 is expressed. Localization of Cacna2d2 transcripts in heart-innervating sympathetic ganglia (Figure 4A) complemented reports on α2δ-2 expression in rat root ganglia44 and suggested that α2δ-2 may be involved in autonomic regulation of cardiac function. Indeed, examination of the baseline ECG from conscious Cacna2d2 null mice revealed a tendency to bradycardia (Table 1). Isoflurane anesthesia further unveiled abnormalities in null-mutants’ heart rate regulation, suggesting that α2δ-2 deficiency compromises response to the anesthetic. Volatile anesthesia produces its effect in the mammalian cerebral cortex by reduction in excitatory synaptic transmission, which, in turn, is caused by a decreased amount of transmitter glutamate in the synaptic cleft.45 The observed difference in heart rate modulation, therefore, may be connected with the inability of Cacna2d2 null-mutants to efficiently down-regulate synaptic calcium currents and the neurotransmitter release.

In summary, while our animal model provides, for the first time, evidence on α2δ-2 involvement in cardiac rhythm regulation, no obvious cardiac mechanism was detected to explain the propensity to sudden death in the null mice. Recently, using ribonuclease protection assay Chu and Best46 identified all three α2δ subunits’ transcripts in rat heart atria. More studies into the structure and function of cardiac calcium channels are required to understand the exact roles of α2δ-1, α2δ-2, or α2δ-3 in heart and the extent of their functional redundancy. Creating double α2δ mutants may help in answering this question and further assess the significance of α2δ subunits for cardiac function.

Clinical Significance of the Cacna2d2tm1NCIF Model

In line with previously characterized Cacna2d2 ducky mutants, our model clearly implies a link between α2δ-2 and congenital neurological disorders. Mutations in the CACNA1A gene encoding the core α1A subunit of the P/Q type VDCC have been associated in humans with familial hemiplegic migraine, episodic ataxia type-2, autosomal dominant spinocerebellar ataxia (SCA6), and idiopathic generalized epilepsies.47–49 It is of utmost importance, therefore, to see if mutations in the human CACNA2D2 gene from the 3p21.3 chromosomal region can produce effects similar to those observed in our animal model. The Cacna2d2tm1NCIF model may also serve as a valuable tool for in-depth study of calcium-dependent neuronal death and development of therapies for P-type-channels-associated congenital disorders.

Note added in proof

While this manuscript was in review, a novel spontaneous Cacna2d2 mutant, Cacna2d2entel, was characterized and crossed into a C57BL/6J background.60 This mutation produced an in-frame full-length α2δ-2 protein with a 39 amino acids duplication in exon 3. The subunit was capable of incorporation into cellular membrane but failed to undergo proper processing to covalently bind the δ subunit. Cacna2d2entel homozygotes showed a neurological phenotype and cerebellar morphology similar to our model.

Acknowledgments

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