Ducky Mouse Phenotype of Epilepsy and Ataxia Is Associated with Mutations in the Cacna2d2 Gene and Decreased Calcium Channel Current in Cerebellar Purkinje Cells

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The mouse mutant ducky, a model for absence epilepsy, is characterized by spike-wave seizures and ataxia. The ducky gene was mapped previously to distal mouse chromosome 9. High-resolution genetic and physical mapping has resulted in the identification of the Cacna2d2 gene encoding the α2β2 voltage-dependent calcium channel subunit. Mutations in Cacna2d2 were found to underlie the ducky phenotype in the original ducky (du) strain and in a newly identified strain (du2/J). Both mutations are predicted to result in loss of the full-length α2β2 protein. Functional analysis shows that the α2β2 subunit increases the maximum conductance of the α1A/β4 channel combination when coexpressed in vitro in Xenopus oocytes.

Five spontaneous autosomal recessive mouse mutations impart a phenotype that includes epileptic seizures with features similar to those occurring in human idiopathic generalized epilepsy (IGE) (Puranam and McName, 1999). Tottering (Cacna1a8, Cacna1a6–4a), slow-wave epilepsy (Slc9a1sw), lethargic (Cacnb4b), stargazer (Cacng2stg), and ducky (du) exhibit bilaterally synchronous spontaneous wave discharges (SWDs) on cortical electroencephalogram (EEG) recordings. These are accompanied by behavioral arrest and respond to the human anti-absence drug ethosuximide (Noebels et al., 1997). The electrophysiological hallmark of human absence epilepsy is 3 Hz SWDs. In mice, the frequency is usually 5–7 Hz (Noebels, 1991), except for those in Slc9a1sw (1–3 Hz) (Cox et al., 1997). Mutations in genes encoding voltage-dependent calcium channel (VDCC) subunits underlie three of these phenotypes: the genes encoding the α1A (Cacna1a), β4 (Cacnb4), and γ2 (Cacng2) subunits are mutated in tottering (Fletcher et al., 1996), lethargic (Burgess et al., 1997), and stargazer (Letts et al., 1998) mice, respectively.

Voltage-dependent Ca2+ currents have been measured in all excitable cells and are implicated in many cellular processes (Berridge et al., 1998). They have been divided on the basis of kinetics and pharmacology into L-, N-, P/Q-, R-, and T-types (Catterall, 1998). Each VDCC is composed of a pore-forming α1 subunit that may be associated with an intracellular β, a membrane-spanning γ, and a membrane-anchored, but predominantly extracellular, α2β subunit. The α1 subunit determines the main biophysical properties of the channel and is modulated by the other subunits (Walker and De Waard, 1998). Mammalian genes encoding 10 α1, four β, eight γ, and three α2β subunits have been identified (for a comprehensive list, see Ertel et al., 2000; Burgess et al. 2001).

Homozygotes for the ducky (du) allele are characterized by an ataxic, wide-based gait and paroxysmal dyskinesia (Snell, 1955). They display reduced size and a failure to breed or survive beyond 35 d. Neuropathological studies revealed dysgenesis of selective regions of the CNS, including the cerebellum, medulla, and spinal cord (Meier, 1968). Axonal dystrophy and demyelination were also reported. Heterozygotes show no obvious phenotype. The du locus was localized to mouse chromosome 9 by linkage to the phenotypic markers dilute and short ear (Snell, 1955).

To identify and characterize the du locus, a positional cloning strategy was adopted. High-resolution genetic mapping identified
the gene encoding the VDCC α2δ2 subunit as a positional and functional candidate. Mutations in this gene were identified in the original \textit{du} strain and in a new allele, \textit{du''}. This paper presents evidence that the gene underlying the ducky phenotype encodes the α2δ2 subunit and explores the effect of a mutation on Ca$^{2+}$ channel function in \textit{du/du} brain.

**MATERIALS AND METHODS**

**Genetic and physical mapping**

Mice were obtained from The Jackson Laboratory (Bar Harbor, ME). DNA was prepared from tail biopsies or liver samples by standard methods. Microsatellite markers were amplified as described previously (Dietrich et al., 1996). Recombinants were identified by agarose gel electrophoresis or PAGE or single-strand conformation polymorphism (MDB1432) analysis. Yeast artificial chromosome (YAC) clones were identified by PCR-based library screens (Haldi et al., 1996) or from a web-based database of clones (Nusbaum et al., 1999). Genomic clones were obtained from the Human Genome Mapping Project Resource Centre (Cambridge, UK).

**Candidate gene analysis**

Total RNA was prepared from frozen tissue using RNAzol B (Biogenesis, Sandown, NH) and used to prepare mRNA or cDNA using mRNA purification or First Strand cDNA synthesis kits (Amersham Pharmacia Biotech, Little Chalfont, UK). Northern blot analysis of 10 μg of cerebellar mRNA using Duralon UV nylon membrane and full-length \textit{Cacna2d2} or human β actin as probes (Stratagene, La Jolla, CA) was performed using the suggested conditions of the manufacturer to optimize the identification of the wild-type 5.5 kb \textit{Cacna2d2} transcript. This may have resulted in underestimation of the quantity of smaller transcripts (<2 kb). The full-length \textit{Cacna2d2} cDNA was assembled using degenerate primers, reverse transcription (RT)-PCR, rapid amplification of cDNA ends (RACE), and sequencing. All primer sequences are available on request. RACE was performed using the 5'3' RACE kit (Roche Diagnostics, Hertfordshire, UK). Sequencing was performed on an ABI 373XL sequencer using TaqFS chemistry (PE Applied Biosystems, Foster City, CA). Genomic DNA was embedded in agarose and subjected to pulsed field gel electrophoresis (PFGE) on a Bio-Rad (Hercules, CA) clamped homogeneous electrical field electrophoresis system.

**Electrode implantation and EEG measurements**

Homozygous \textit{du''} and control unaffected mice (8–12 weeks of age) were tested for spontaneous seizure activity. Mice were anesthetized with tribromoethanol (400 mg/kg, i.p.) and placed in a stereotaxic holder fitted with a mouse incisor bar. Burr holes were drilled (1 mm posterior to bregma, 1 mm lateral to midline) on both sides of the skull. Two Teflon-coated bipolar electrodes were implanted at 0.4–0.8 mm below the dura. Three screws were placed at the periphery of the skull to anchor the dental cap. Mice were allowed to recover for 2 d before EEG recordings were measured. The parameters for determining spike-wave discharges were described previously (Hosford et al., 1995).

In situ hybridization and immunohistochemical analyses

Mice [aged postnatal day 21 (P21) to P24] were terminally anesthetized by CO$_2$ inhalation and perfused with 4% paraformaldehyde. The brain was dissected into cold paraformaldehyde and then transferred through a sucrose gradient before embedding in OCT (Agar) and sectioning. Alternatively, the brain was removed without fixing and frozen in liquid nitrogen. Cryostat sections (10–15 μm) were cut and air-dried onto positively charged slides (BDH Laboratory Supplies, Poole, UK). cDNA fragments corresponding to \textit{α2δ2} [nucleotide (nt) 3705–4909], \textit{α2δ1} (nt 3521–3895), and \textit{α2δ3} (nt 2581–3602) were subcloned into pBluescript SK+-. Sense and antisense RNA probes were prepared using T3 or T7 polymerase and digoxigenin (DIG) RNA labeling mix and purified using Quick spin columns (Roche Diagnostics). In \textit{in situ} hybridization, probes were hybridized previously described conditions (Eisenstat et al., 1999).

Immunohistochemistry was performed on perfused tissue and isolated cells with a polyclonal calbindin D28K antibody (Chemicon, Harrow, UK) and on perfused tissue alone with a polyclonal calretinin antibody (Chemicon).

**Heterologous expression of cDNAs**

cDNAs encoding rabbit α1A (X57689), rat β4 (L02315), and mouse α2δ2 (predominant brain splice variant that lacks exon 23 and 6 bp of exon 38, as described by Barclay and Rees, 2000) cDNAs, cloned into the pMT2 vector, were injected intranuclearly into \textit{Xenopus} oocytes as described previously (Canti et al., 1999), except that 4 ml of cDNA mixture was injected at 1 μg/μl. Recordings were made using two-electrode voltage clamp as described previously (Canti et al., 1999).

**Purkinje cell and granule cell preparation and I$_{Ba}$ measurement**

**Purkinje neurons.** Cells were dissociated from P4–P8 mice (Mintz et al., 1992) and plated onto concanavalin-A (2 μg/ml)-coated coverslips. Whole-cell $I_{Ba}$ was recorded 1–4 hr later with 5 mM Ba$^{2+}$ as described previously (Mintz et al., 1992). Purkinje cell (PC) identity was confirmed by labeling with an antibody to the PC-specific protein calbindin (Stratagene, La Jolla, CA). Results were expressed and analyzed as described previously.

**Cerebellar granule cells.** Granule cells (GCs) were isolated and cultured from P6–P8 mice, and whole-cell $I_{Ba}$ was recorded as described previously using 10 mM Ba$^{2+}$ (Pearson et al., 1995), except that the internal pipette solution contained (in mM): 100 HEPES, 50 EGTA, 0.57 CaCl$_2$, 2.25 MgCl$_2$, 3.68 ATP, and 0.1 GTP (Tris salt), pH 7.2 (320 mOsm).

Cells were used for analysis when the holding current at the holding potential was <20 pA for GCs and <50 pA for PCs. The holding current did not differ between genotypes. Leak current was subtracted using P8 protocol. Individual $V$–$I$ relationships were fitted with the modified Boltzmann equation $I = G_{max} \times (V - V_{rev})/(1 + \exp(-(V - V_{rev})/k))$, where $G_{max}$ is the maximum conductance, $V_{rev}$ is the reversal potential, $k$ is the slope factor, and $V_{0}$ is the voltage for 50% current activation.

**Single-channel recording**

All recordings were performed as described by Meir et al. (2000). Experiments were performed on cell-attached patches from PCs at room temperature (20–22°C). Recording pipettes were pulled from borosilicate tubes (World Precision Instruments, Sarasota, FL), coated with Sylgard (Sylgard 184; Dow Corning, Wiesbaden, Germany), and fire polished to form high-resistance pipettes (~10 MΩ with 100 mM BaCl$_2$). The bath solution was composed of (in mM): 135 K-aspartate, 1 MgCl$_2$, 5 EGTA, and 10 HEPES (titrated with KOH, pH 7.3). The patch pipettes were filled with a solution of the following composition (in mM): 100 BaCl$_2$, 10 tetraethylammonium (TEA)-Cl, 10 HEPES, and 200 mM TTX, titrated with TEA-OH to pH 7.4. Both solutions were adjusted to an osmolarity of 320 mOsm with sucrose. Data were sampled (Axopatch 200B and Digidata 1200 interface; Axon Instruments, Foster City, CA), at 5 kHz and filtered on-line at 1 kHz. Voltages were not corrected for liquid junction potential (Necher, 1995) measured to be ~15 mV in these solutions.

Leak subtraction was performed by averaging segments of traces with no activity from the same voltage protocol in the same experiment and subtracting this average from each episode using pCampl6 (Axon Instruments). Event detection was performed using the half-amplitude threshold method. Single-channel amplitude was determined by either a Gaussian fit to the binned amplitude distributions or the mean amplitude in two experiments at +10 mV when there was a small number of events.

All results are presented as mean ± SEM, and statistical differences were determined by the Student’s t test.

**GenBank accession numbers**

DNA and protein sequences described here have been deposited in GenBank under the following accession numbers: wild-type \textit{Cacna2d2}, AF247139; \textit{du} mutant transcript 1, AF247140; \textit{du} mutant transcript 2, AF247141; and \textit{du''} mutant transcript, AF247142.

**RESULTS**

**Genetic and physical mapping of the \textit{du} locus**

Two genetic crosses were used to refine the location of \textit{du} (Fig. 1a). Progeny representing 1460 meioses (564 backcross progeny and 448 intercross progeny) were typed with microsatellite markers 53.6–63.4 centimorgans (cM) from the centromere on mouse chromosome 9 (Dietrich et al., 1996). This region was assigned in overlapping yeast artificial chromosome (YAC) clones (Fig. 1b). Sequence tagged sites (STSs) to \textit{Dagl} and \textit{Lamb2} (Skytner
et al., 1995) localized both genes distal to the du critical region (Fig. 1b). The human orthologs of these genes map to chromosome 3p21 (Skytner et al., 1995). The STS sequences D31943, M13963, and X85990 demonstrated significant similarities with CISH (Uchida et al., 1997), GNAT1 (Blatt et al., 1988), and SEMA3B (Sekido et al., 1996), respectively. These genes map to human 3p21.3, indicating that the du gene is in a region of conserved linkage with this region.

**Cacna2d2 is a candidate gene for the du locus**

Cacna2d2 was identified as a candidate gene for du as a direct result of the conservation of linkage of human chromosome 3p21.3 with this region of mouse chromosome 9. Human chromosome 3p21.3 is frequently deleted in small cell lung carcinoma and has been the target of positional cloning efforts. One transcript (human gene CACNA2D2; GenBank accession number AF042792) isolated from this region showed 55.6% homology with the Cacna2d2 gene. Two mouse expressed sequence tags (GenBank accession numbers AA000341 and AA008996) with 91 and 82% nucleotide identity to CACNA2D2 were identified by Basic Local Alignment Search Tool analysis. This mouse sequence (gene Cacna2d2) was used to design a genomic PCR assay to test YACs for linkage to du.
Cacna2d2 is predominantly expressed in mouse brain in a restricted pattern

The predominant Cacna2d2 transcript is in brain, with lower levels in kidney and testis (Fig. 2ai), a pattern distinct from Cacna2d1 (Fig. 2aii) but similar to Cacna2d3 (Fig. 2a(ii)). By RT-PCR, no Cacna2d2 expression was detected in lung at any age studied (1, 2, 6, and 20 months), a result confirmed using two additional sets of PCR primers (data not shown). Detailed Cacna2d2 brain expression was studied in situ hybridization. A Cacna2d2 antisense RNA probe (exons 38–39) was hybridized to sections of P21 +/+ mouse brain. Analysis of whole-brain sagittal sections (Fig. 2bi) revealed the highest level of expression to be in the cerebellum, with moderate levels in medulla, pons, and striatum. Analysis of horizontal sections (Fig. 2bii) also shows expression in cortex, hippocampus, habenuela, and nuclei reticularis thalami (nRT). Figure 2c shows higher-resolution images of medulla (i), striatum (ii), cerebral cortex (iii), nRT (iv), habenuela (v), and hippocampus (vi). No signal was detected with a control sense probe (data not shown). Cerebellar expression is investigated further in Figure 5, demonstrating that the gene is highly expressed in PCs with only very low levels in the granule cell layer (GCL) (see Fig. 5e).

Cacna2d2 is mutated in du/du mice

No full-length Cacna2d2 transcript was detected by RT-PCR in du/du mice. A failure of amplification between exon 1 and 4–39 implied disruption of the gene (Fig. 3ai, top and middle panels). Additional analysis identified two distinct mutant transcripts. 3′ RACE of Cacna2d2 RNA in du/du identified a chimeric transcript (mutant transcript 1) composed of exons 1, 2, and 3 spliced to a novel sequence (X). RT-PCR using primers for Cacna2d2 exon 1 and region X gave a du-specific product (Fig. 3ai, bottom panel). Mutant transcript 1 encodes the first 414 nucleotides of Cacna2d2, followed by 24 novel nucleotides and a stop codon. Amplification between exons 1 and 3 (Fig. 3ai, top panel) reveals a low level of mutant transcript 1 in du/du mice. A low level of mutant transcript 2 (exons 2–39) is also detected by RT-PCR in du/du brain (Fig. 3a(ii)). Wild-type Cacna2d2 (5.5 kb) and these mutant transcripts sized ~1.5 and 5 kb can be detected by Northern analysis of +/+ and du/du cerebellar mRNA, respectively (Fig. 3b).

The presence by RT-PCR of the two mutant transcripts in du/du mice suggested a duplication of exons 2 and 3, although additional bands were not detected by standard agarose gel electrophoresis and Southern blotting (data not shown). In contrast, PFGE and Southern blotting revealed a large genomic rearrangement (Fig. 3c). Exons 1 and 4–39 are present once per +/+ and du chromosome. This is demonstrated by the presence of single 190 kb NoI hybridizing fragments with the probe corresponding to these exons (Fig. 3ci, both genotypes. Exons 2–3 and region X are present once per +/+ chromosome and twice per du chromosome, as indicated by the single (190 kb) and double (190 and 600 kb) NoI fragments (Fig. 3ci), respectively. This supports a genomic duplication of exons 2–3 and region X. The large size (>150 kb) of this duplication precludes its identification by conventional PCR and sequencing or Southern blotting because internal primer sites and restriction sites have been duplicated without disruption, preventing any distinction between original and duplicated exons. The wild-type position of region X as 3′ to the Cacna2d2 gene was confirmed by PCR amplification of the PAC clones (Fig. 1b). In genomic DNA, the copy of region X common to +/+ and du/du contains two B2 repeat elements, and

from the du contig. Three positive clones (y203E7, y257D12, and y465F1) placed Cacna2d2 between D17914 and M13963, within the candidate interval (Fig. 1b). STS content mapping of four overlapping Cacna2d2-positive PAC1 artificial chromosomes (PACs) orientated the gene as 5′ to 3′ in a proximal to distal direction (Fig. 1b). An intragenic (CA)n repeat (a262–43.21) was nonrecombining with du in the backcross. Therefore, Cacna2d2 was a good positional and functional candidate for du.

The 5.5 kb Cacna2d2 cDNA (GenBank accession number AF247139) shared 91% nucleotide identity with CACNA2D2. The genomic structure of the Cacna2d2 gene has been determined (see Fig. 3d) (Barclay and Rees, 2000). Overall, mouse a262 shares 95% identity and 96.5% similarity with the human protein.
the du-specific copy contains a single B2 repeat (Fig. 3d). A plausible mutation mechanism, possibly mediated by the B2 repeats, is a head to tail duplication of Cacna2d2 exons 2–39 and region X, followed by a deletion including exons 4–39 of the original Cacna2d2.

**A second, distinct mutation of Cacna2d2 in du washer mice**

Recently, a spontaneous, autosomal recessive mouse mutant, with ataxia and paroxysmal dyskinesia, arose at The Jackson Laboratory. Breeding experiments established it as a novel ducky allele: du washer. Cortical EEG recordings from du washer/du washer revealed infrequent bilateral SWDs of high amplitude (500 μV) and 5–7 Hz (Fig. 4a).

These spontaneous discharges were accompanied by behavioral arrest. To determine whether these discharges were seizure related, an intraperitoneal injection of ethosuximide (100 mg/kg) was given, and the discharges were abolished.

Mutational analysis of Cacna2d2 in du washer/du washer mice by RT-PCR and genomic sequencing revealed a 2 bp deletion (TG) within exon 9 (Fig. 4b) predicted to cause premature truncation of the protein (GenBank accession number AF247142). Sequence analysis of 45 subclones of the du washer/du washer RT-PCR product failed to detect any wild-type transcript (data not shown). Northern analysis of mRNA from du washer/du washer brain showed no difference in

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**Figure 3**. The du mutation is a genomic rearrangement involving the Cacna2d2 gene. **a**, Two mutant transcripts can be identified by RT-PCR of total brain RNA from du/du mice. Two +/+ , two du/du samples, and a negative control (no RNA) are shown per gel. **i**, Top, Normal size amplification product of exons 1–3 is shown in +/+ and du/du RNA, with reduced levels in the latter. Middle, Amplification between exons 1 and 4 does not produce a product in du/du RNA, suggesting disruption of the Cacna2d2 gene in this region. 
Bottom, Amplification of the du-specific chimeric transcript of Cacna2d2 exons 1, 2, and 3 and a novel sequence X. **ii**, Overlapping PCR fragments spanning exons 2–39 of Cacna2d2 can be detected in +/+ and du/du RNA, with lower levels observed in du/du samples. **b**, Wild-type Cacna2d2 transcript (5.5 kb) is absent from du/du brain by Northern analysis using cerebellar mRNA and full-length Cacna2d2 as a probe. Low levels of two du-specific bands (~1.5 and 5 kb) are detected. The filter was rehybridized with β-actin as a control for RNA loading. **c**, PFGE shows duplication of Cacna2d2 exons 2 and 3 and region X in du/du genomic DNA. Southern analysis of NotI-digested genomic DNA separated by PFGE from +/+ , +/du, and du/du mice is shown. Blots were hybridized with Cacna2d2 probes: i, exon 1; ii, exons 2–3; iii, exons 4–39; iv, region X. Sizes are in kilobases. **d**, A scale representation of the genomic region containing Cacna2d2 (red) and region X (blue) in +/+ and du/du mice. N, NotI sites. The presence of one or two B2 repeats 5′ to region X is marked by a vertical line. The mutant transcripts 1 and 2 produced from each region in du/du are represented by colored boxes. The Cacna2d2 gene is arranged 5′ to 3′ in +/+ . In du/du, exons encoding mutant transcript 1 are shown in a 5′ to 3′ direction, and those encoding mutant transcript 2 are inverted and shown 3′ to 5′. The distance between exon 3 and region X is unknown but is >12 kb. The scale bar is in kilobases.

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Cacna2d2 transcription levels compared with wild type (data not shown), suggesting stability of the mutated transcript. These observations suggest that Cacna2d2 mutations in du/du and du\(^2\)/du\(^2\) mice underlie the ducky phenotype of ataxia, SWDs, and paroxysmal dyskinesia.

Immunohistochemistry of du/du Purkinje and granule cells reveals no cell loss

In view of the cerebellar pathology in du/du mice, we wanted to identify whether there was any loss of PCs or GCs that might be responsible for this. However, immunohistochemical investigations using calbindin as a PC marker (Fig. 5a,b) and calretinin as a GC marker (Fig. 5c,d) did not identify loss of cell bodies in du/du cerebella at P21 (Fig. 5b,d). Similar observations were made for du\(^2\)/du\(^2\) (data not shown).

Absence of full-length a2\(\delta\)2 in du/du cerebellar Purkinje cells

In situ hybridization with a 3' Cacna2d2 anti-sense RNA probe (Fig. 5e,f) was used to demonstrate the presence of full-length Cacna2d2 message in +/+ PCs (Fig. 5e) and its absence in du/du PCs (Fig. 5f).

The possibility of compensatory upregulation of Cacna2d1 (Fig. 5g,h) and Cacna2d3 (Fig. 5i,j) transcript levels in du/du cerebella was investigated by in situ hybridization with antisense RNA probes. No major differences were observed in their distribution in du/du compared with +/+ cerebellum, and in particular there was no compensatory expression of a2\(\beta\)1 or a2\(\delta\)3 mRNA in du/du PCs.

Modulation of Ca\(^{2+}\) channel currents by a2\(\delta\)2

The physiological function of the a2\(\delta\)2 subunit encoded by the Cacna2d2 gene was investigated using in vitro expression and electrophysiology. To mimic the composition of the predominant calcium channels in cerebellar Purkinje cells, we examined the effect of a2\(\delta\)2 when coexpressed with a1A and b4 in Xenopus oocytes. Coexpression of a2\(\delta\)2 induced a large enhancement of a1A current amplitude (Fig. 6a,b). For example, at 0 mV, the increase was from \(-0.55 \pm 0.15\) (n = 14) to \(-1.8 \pm 0.27\) \(\mu\)A (n = 13), and there was a small hyperpolarization of current activation,
the voltage for 50% activation shifting from –6.4 ± 0.7 to –12.0 ± 1.4 mV (p < 0.01) (Fig. 6c). The maximum conductance was increased from 0.013 ± 0.003 to 0.036 ± 0.005 μS by coexpression of a2δ2. There was no effect of a2δ2 on steady-state inactivation of α1A/β4 currents (Fig. 6d).

Ca2+ channel currents in du/du Purkinje cells and granule cells

The effects of a2δ2 on the α1A/β4 current in vitro suggested that loss of wild-type a2δ2 may result in a reduction in Ca2+ current density. To test this, I\textsubscript{Ba} was examined in acutely dissociated cerebellar PCs from P4–P8 mice. I\textsubscript{Ba} density was clearly reduced in the PCs from du/du compared with +/+ and +/du mice (Fig. 7a,b). There was no effect on voltage dependence of activation (Fig. 7a) or on the kinetics of activation or inactivation (data not shown). Cell size, as determined by the capacitance, was not significantly different between the genotypes, being 14.9 ± 1.4, 15.1 ± 0.9, and 17.6 ± 1.7 pF in the +/+, +/du, and du/du PCs, respectively. To examine the basis for the reduction in the whole-cell I\textsubscript{Ba} in du/du PCs, single P-type Ca2+ channels were examined in the cell-attached mode from +/+ , +/du, and du/du PCs (Fig. 7c). There was no difference in the single-channel conductance or amplitude between the three genotypes (Fig. 7d). In cultured cerebellar GCs, taken from P6–P8 mice, there was no significant difference in I\textsubscript{Ba} density at any potential (Fig. 7e,f) or in cell capacitance between the genotypes.

DISCUSSION

Our data provide strong evidence that the ducky phenotype is associated with mutations in the Cacna2d2 gene. This is discussed together with a consideration of the normal expression pattern and function of the VDCC a2δ2 accessory subunit it encodes and how disruption of this function leads to the phenotypic features of ataxia and spike-wave seizures.

Cacna2d2 is disrupted in du and du <sup>2d</sup> mice

These studies demonstrate that wild-type Cacna2d2 transcript is absent from the brain of du/du and du<sup>2d</sup>/du<sup>2d</sup> mice. In du/du mice, a genomic rearrangement disrupts Cacna2d2 and duplicates a nonfunctional open reading frame, region X, although the exact mechanism remains unclear. Mutant transcripts 1 and 2 are present at very low levels in du/du mice and, if translated, would encode proteins that are unlikely to function normally. The product of mutant transcript 1 would lack most of the α2 subunit that includes the transmembrane domain, whereas that of mutant transcript 2 is unlikely to be trafficked correctly without a signal sequence. In du2J/du2J mice, a 2 bp deletion in exon 9 of Cacna2d2 would result in a truncated protein lacking >800 amino acids, including the transmembrane domain. This is the first mammalian phenotype associated with disruption of an α2 subunit gene and should allow the physiological roles of a2δ2 and the other a2δ subunits to be characterized further.

Cacna2d2 is predominantly expressed in mouse brain

Northern analysis of CACNA2D2 in human tissue showed highest expression in heart, pancreas, and skeletal muscle and lower levels in kidney, liver, placenta, and brain (Klugbauer et al., 1999). A separate study reported highest expression in lung and testis and significant levels in brain, heart, and pancreas (Gao et al., 2000) and suggested that the pattern in the former study may reflect cross-hybridization of the probe with CACNA2D1. The expression pattern presented here corresponds more closely with the latter study, and the differences observed (particularly in lung) may be attributed to species differences and/or developmental differences. This is not unprecedented (Fougerousse et al., 2000).

In brain, Cacna2d2 expression was highest in cerebellar PCs but was also detected in cerebral cortex, hippocampus, cerebellar GCs, nRT, habenula, pons, and medulla. The genes encoding the α2δ1, α2δ2, and α2δ3 subunits show generally distinct patterns of expression within the cerebellum. Cacna2d1 is predominantly expressed in the GCL, Cacna2d2 is predominant in the Purkinje cell layer (PCL), and Cacna2d3 expression is detected in the molecular layer (ML) (Klugbauer et al., 1999; Hobom et al., 2000, present study). Most of the α1, β, and γ subunit genes share at least one region of expression with Cacna2d2, making it difficult to predict in vivo interactions based on expression profiles. However, the similarity of the ducky phenotype to that observed in mice with mutations in genes encoding the α1A and β4 subunits (Fletcher et al., 1996; Burgess et al., 1997) and their predominant
PC expression pattern suggests that α2β2 contributes to the P-type current.

α2β2 interacts in vitro with the α1A/β4 combination

In vitro studies have shown that α2β1 and α2β3 subunits increase peak current amplitude and alter the kinetics of inactivation for a number of different α1 subunits (Walker and De Waard, 1998; Dolphin et al., 1999; Klugbauer et al., 1999). In vitro studies with human α2β2 also demonstrate increased peak current amplitude for several α1 subunits (Gao et al., 2000). Our results show that mouse α2β2 causes a 2.8-fold increase in maximum conductance for the α1A/β4 subunit combination when coexpressed in Xenopus oocytes.

Mechanism of the altered Ca2+ channel current in du/du PCs

The in vitro expression data suggested that disruption of α2β2 expression in ducky mice may result in a decrease of the Ca2+ channel current in cells that express Cacna2d2. Electrophysiological recordings from isolated du/du PCs confirmed this hypothesis, with a 35% decrease in the peak P-type Ca2+ current density in du/du compared with +/+ PCs. This result has been confirmed recently by Ca2+ imaging experiments (J. Brodbeck and A. C. Dolphin, unpublished results). Furthermore, the comparable single P-type Ca2+ channel conductance in the two genotypes indicates that the reduction in Iba density reflects either a change in the number of functional channels or their open probability. The ducky mouse represents the first example of an accessory VDCC subunit mutant with a measurable effect in PCs. Recordings from α1A mutant mice PCs also revealed changes in the P-type Ca2+ current compared with that in wild-type PCs (Dove et al., 1998; Lorenzon et al., 1998; Wakamori et al., 1998; Jun et al., 1999). Similar studies performed on lethargic mice, however, showed no differences from wild type, potentially as a result of compensation by other β subunits (Burgess et al., 1999). The low levels of Cacna2d1 and Cacna2d3 transcripts in the PCs may preclude such compensation in PCs of du/du mice, and indeed no upregulation of these mRNAs was seen in du/du PCs. In vivo recordings from cultured du/du and +/+ GCs demonstrated no significant difference in the Ca2+ channel current, consistent with the lower expression levels of Cacna2d2 in the GCL.

Calcium channel dysfunction and the ducky phenotype

It is likely that several features of the ducky phenotype, including the SWDs, ataxia, and paroxysmal dyskinesia, are attributable to loss of full-length functional α2β2 in neurons of ducky mice. The occurrence of these traits in other mice with mutations in genes encoding VDCC subunits supports this hypothesis. Homozygous Cacna1dα, Cacnb4β, Cacng2α, du, and duβ mice...
all exhibit generalized bilaterally symmetrical SWDs with a frequency of 5–7Hz (Noebels and Sidman, 1979; Noebels et al., 1990; Hosford et al., 1992; present study). Evidence from animal models suggests that SWDs are generated by aberrant thalamocortical oscillations involving neocortical pyramidal neurons, thalamic relay neurons, and GABAergic neurons of the nRT (Snead, 1995). T-type Ca$^{2+}$ currents underlie thalamic oscillations, and VDCCs have an essential role in presynaptic release of neurotransmitters, providing two potential mechanisms linking Ca$^{2+}$ currents and thalamocortical circuits (Coulter, 1997). Reduction of excitatory but not inhibitory synaptic transmission in the thalamicus of lethargic and tottering mice has been documented previously (Caddick et al., 1999), and it was proposed that a net enhanced GABAergic input in thalamocortical neurons may synchronize them into a burst firing mode. In contrast, hippocampal neurotransmitter release appears to be stabilized by a Ca$^{2+}$ current compensatory mechanism in the same mice (Qian and Noebels, 2000). Additional work is required to elucidate the mechanism of SWD generation in ducky mice. However, the expression of Cacna2d2 within the nRT and cortical pyramidal neurons suggests a similar mechanism may be involved.

An ataxic gait is first detectable between P10 and P21 in tottering, lethargic, stargazer, and ducky homozygotes (Snell, 1955; Green and Sidman, 1962; Dickie, 1964; Noebels et al., 1990). The high levels of expression of all the corresponding VDCC subunit genes in cerebellar neurons, particularly PCs, provides an obvious anatomical correlate with the presumed cerebellar dysfunction underlying the ataxia. PC loss has been documented in some, but not all, of these mutant strains; for example, it is observed in $g^b$ (Heckroth and Abbott, 1994). In du/du mice, no loss of PC somata was seen, but preliminary findings indicate major PC dendritic abnormalities (Brobeck and Dolphin, unpublished results). The du/du PCs in which a reduced Ca$^{2+}$ channel current was documented were obtained, for technical reasons, from ducky mice too young and developmentally immature to manifest an ataxic gait. However, it is reasonable to assume that the functional deficit is also present in older mice, and its presence before the overt phenotype is seen demonstrates that it is not merely a secondary effect. It is noteworthy that mutations in the human ortholog of the tottering gene $CACNA1A$ is associated with ataxia and seizures in the lethargic ($lh$) mouse. Cell 88:385–392.


Caddick SJ, Wang C, Fletcher CF, Jenkins NA, Copeland NG, Hosford DA (1999) Excitatory but not inhibitory synaptic transmission if reduced in lethargic ($Cacnb4\alpha$) and tottering ($Cacna1A\alpha$) mouse thalamus. J Neurophysiol 81:2066–2074.


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