The Ducky Mutation in Ca\textit{cna2d2} Results in Altered Purkinje Cell Morphology and Is Associated with the Expression of a Truncated \(\alpha 2\delta-2\) Protein with Abnormal Function*

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The mouse mutant ducky, a model for absence epilepsy, is characterized by spike-wave seizures and cerebellar ataxia. A mutation in \textit{Ca}\textit{cna2d2}, the gene encoding the \(\alpha 2\delta-2\) voltage-dependent calcium channel accessory subunit, has been found to underlie the ducky phenotype. The \(\alpha 2\delta-2\) mRNA is strongly expressed in cerebellar Purkinje cells. We show that \(du/du\) mice have abnormalities in their Purkinje cell dendritic tree. The mutation in \(\alpha 2\delta-2\) results in the introduction of a premature stop codon and predicts the expression of a truncated protein encoded by the first three exons of \textit{Ca}\textit{cna2d2}, followed by 8 novel amino acids. We show that both mRNA and protein corresponding to this predicted transcript are expressed in \(du/du\) cerebellum and present in Purkinje cells. Whereas the \(\alpha 2\delta-2\) subunit increased the peak current density of the Ca\textit{v2.1}/\textit{\beta} channel combination when co-expressed \textit{in vitro}, co-expression with the truncated mutant \(\alpha 2\delta-2\) protein reduced current density, indicating that it may contribute to the \(du\) phenotype.

Voltage-gated Ca\textsuperscript{2+} (Ca\textit{v}) channels have been divided functionally into L-, N-, P/Q-, R-, and T-types (1). Each Ca\textit{v} channel is composed of a pore-forming \(\alpha\) subunit, associated at least in the case of the Ca\textit{v}1 and -2 subfamilies with an intracellular \(\beta\) subunit responsible for trafficking (2) and a membrane-anchored, but predominantly extracellular, \(\alpha 2\delta\) subunit, whose function is less well defined (2). Ca\textit{v}1.1 (\(\alpha 1\)S) is also associated with a \(\gamma\) subunit (\(\gamma\)1), and this may be true for other Ca\textit{v} channels (3), although the neuronal \(\gamma\) subunits may also subserve other functions (4). The \(\alpha\) subunit determines the main biophysical properties of the channel and is modulated by the other subunits (2). Mammalian genes encoding 10 \(\alpha\), 4 \(\beta\), 8 \(\gamma\), and 3 \(\alpha 2\delta\) subunits have been identified (1, 5, 6).

A number of spontaneous autosomal recessive mutant mouse strains have now been identified, involving mutations in each of the four different subunits that together compose a voltage-dependent calcium channel. They all have a similar phenotype that includes cerebellar ataxia and spike-wave seizures. Toting (\textit{Ca}\textit{cna1}a\textsuperscript{2h}) has a point mutation in \textit{Ca}\textit{cna1}a\textsuperscript{2} (\(\alpha 1\)A) (7), and a number of alleles of this mutant have now been identified, as summarized recently (8). Lethargic (\textit{Ca}\textit{cngb4}b\textsuperscript{2h}) represents a truncation mutation of the \(\beta\) subunit (9). Stargazer (\textit{Ca}\textit{cngb2}a\textsuperscript{2h}) has a truncation mutation in the \(\gamma 2\) subunit (3), although its role as a calcium channel subunit remains controversial (4, 10, 11). Finally, the two ducky alleles (\textit{Ca}\textit{cna2d2}a\textsuperscript{2h} and \textit{Ca}\textit{cna2d2}b\textsuperscript{2h}) both predict truncation mutations in the \(\alpha 2\delta-2\) subunit (12).

Homozygotes for the ducky (\(du\)) allele are characterized by ataxia and paroxysmal dyskinesia (13). The cerebellum is reduced in size (14), but we have previously found no loss of Purkinje cell (PC) bodies at postnatal day (P) 21 (12). In this study we observed a reduction in calcium channel current in P21 PCs isolated from \(du/du\) compared with +/- cerebella (12). The present study provides evidence that the \(du\) mutation results in the persistence of PCs with an immature and grossly abnormal morphology, including multiple primary dendrites and a reduction in the size of the PC dendritic tree. This is associated with loss of full-length \(\alpha 2\delta-2\) protein and expression of a truncated mutant \(\alpha 2\delta-2\) protein with aberrant function.

EXPERIMENTAL PROCEDURES

Construction of \(du\)-mut1 \(\alpha\).—The \(du\) mutant 1 construct (\(du\)-mut1 \(\alpha\)) was assembled by PCR (Platinum PfX polymerase; Invitrogen) of \(du/du\) total brain cDNA using primers corresponding to the cDNA sequence (GenBank\textsuperscript{TM} AF247140) containing engineered \(\textit{SmaI}\) and \(\textit{SpeI}\) restriction sites. The primer sequences are as follows: F, 5'- TTG/C- CCGGG/GAACATGCGGCGCCGGCCGCT-3', and R, 5'- TCT/CAGGT/CAGAATACCAGAAGACCAAA-3', with the recognition sites indicated in parentheses. The PCR product was digested with \(\textit{SmaI}\) and \(\textit{SpeI}\) and ligated into the corresponding sites of a modified pMT2 vector (Genetics Institute, Cambridge, MA). Insert sequence fidelity was determined by automated sequencing (PE Biosystems, Warrington, UK).

Genotyping—Mice were obtained from The Jackson Laboratory (Bar Harbor, ME), and a colony was established, as described previously (12). RNA was extracted from samples of mouse brain tissue using RNeasy Mini Kit and QiAmp DNA (Qiagen Ltd., Crawley, West Sussex, UK). RNA was reverse-transcribed using Moloney murine leukemia virus-reverse transcriptase (Promega, Southampton, UK) with 0.6 unit/ml \(\textit{RNasin}\) Ribonuclease Inhibitor (Promega) and 25 ng/\(\mu\)l Random Hexamers (Promega). PCRs were carried out with primers

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The abbreviations used are: Ca\textit{v}, voltage-gated Ca\textsuperscript{2+}; Ab, antibody; FL, first latency; ML, molecular layer; PBS, phosphate-buffered saline; PC, Purkinje cell; PCL, Purkinje cell layer; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; DAPI, 4,6-diamidino-2-phenylindole; P, postnatal day; GFP, green fluorescent protein.

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DNA was extracted by incubating 2 mm of tail-snip tissue in 75 μl of 25 mM NaOH, 0.2 mM Na2EDTA at 95 °C for 30 min followed by cooling to 4 °C and addition of 75 μl of 40 mM Tris-HCl, 5 μl of the resulting solution was amplified in the PCRs. Primers 49F and 50R, 5′-CTACAAATGGGACTCATGTA-3′, produce a 387-bp product from the wild-type allele only. Primers 29F, 5′-GCCGCATCTTGAACTGGA-3′, and 50R, 5′-CAGAGACCAATGAGACTGGA-3′ produce a 456-bp product from the du allele only.

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Truncated α2δ-2 in Ducky Mice

Heterologous Expression of cDNAs and Electrophysiology—GenBank™ accession numbers of cDNAs are given in parentheses. Calcium channel expression in COS-7 cells was investigated by whole cell patch clamp recording, essentially as described previously (19), by transfection of rat Cav 2.1 (M64373) E1868R (20), in conjunction with rat α1 (LG582761). Mouse α2-δ (AF247138) cDNAs were injected into Xenopus laevis oocytes, cDNAs encoding rabbit Cav 2.1 (M64373) E1686R (20), in conjunction with rat Cav 2.1 (M64373) E1868R (20), was composed of (in mm) 135 potassium aspartate, 1 MgCl2, 5 EGTA, and 10 HEPES (titrated with KOH, pH 7.3), and patch pipettes were filled with a solution of the following composition (in mm): 100 BaCl2, 1 tetraethylammonium-Cl, 10 HEPES, 200 mM tetrodotoxin, titrated with tetraethylammonium-OH to pH 7.4. Both solutions were adjusted to an osmolarity of 290 mosmol with sucrose. Data were sampled (Axonpatch 200B and Digidata 1200 interface, Axon Instruments, Foster City, CA) at 20 kHz and filtered online at 1 kHz. Voltages were not corrected for liquid junction potential (26). In this solution, the estimated liquid junction potential was +35 mV at +40 mV and at least 20 consecutive stimulations, the number of detectable multiple openings was considered to represent the number of channels active in these patches. The presence of a single channel or a multichannel opening was determined by performing a Gaussian fit to the binned amplitude distribution. A two-channel patch was considered to represent the number of channels active in these patches.

RESULTS

Comparison of Morphology of du/du and +/+ PCs—The du/du cerebellum exhibits normal foliation and laminar structure. There are no gaps in the PC layer, and their perikarya form a single row. The thickness of each layer is reduced in du/du compared with wild-type littermates as described previously (12). Two techniques were used to compare the morphology of cerebellar PCs between the different genotypes in more detail as follows: first, classical Golgi impregnation, and second, microinjection of PC somata with Lucifer Yellow and neurobiotin. Both methods revealed a changed PC cytoarchitecture in homozygous du/du cerebellum at P21–26, which was the latest period in which morphology could be studied, given that the du/du mice die by P35. In the Golgi-impregnated cerebella, PCs were examined in detail in multiple cerebellar sections from one +/+ and six du/du mice. Atypical initial lateral extensions of the primary dendrite were seen in du/du but not in +/+ PCs (compare +/+ in Fig. 1A to du/du in Fig. 1, B, C, and F). The primary dendrites then bend apically in a delayed targeting of the pial surface, which they frequently fail to reach (e.g. Fig. 1, B and C). Thickened tertiary branchlets were found to bend downwards in some du/du PCs, giving a “weeping willow” appearance (Fig. 1B, closed arrowhead). Additionally, PC somata are frequently multipolar, exhibiting up to three primary dendrites (Fig. 1, D–F), which, in the cells shown in Fig. 1, E and F, extend laterally rather than targeting the pial surface. Similar results were obtained with the cell-filling technique, for which 4+ or 4 du/du PCs were examined in detail. The du/du PCs displayed a dendritic arbor that was significantly less complex, reduced in size, and frequently did not reach the border of the molecular layer (compare the typical +/+ PC in Fig. 2A with three du/du PCs shown in Fig. 2B). Additionally, the shafts of the main and secondary dendritic branches of du/du PCs were often thickened (Fig. 2B). In one du/du PC, the dendrites drooped downward into the granule cell layer giving a weeping willow appearance (Fig. 2B, top right panel), similar to that seen in some of the Golgi-impregnated du/du PCs (Fig. 1B). Following formation of the skeleton of the dendritic trees (Fig. 2C), the numbers of dendrites were found to be reduced in du/du PCs (Fig. 2D, left panel), and the total length of the dendritic tree was also reduced (Fig. 2D, center panel). How-
ever, the number of branch points on the longest dendrites was unchanged (Fig. 2D, right panel).

The du 5' Mutant Transcript Is Present and Translated in du/du Cerebellar PCs—We have shown previously, by in situ hybridization using a 3' antisense probe, that no full-length transcript for Cacna2d2 is present in du/du cerebellum, whereas a strong signal was obtained in +/- cerebellar PCs (12). In the present study we performed in situ hybridization with a 5' Cacna2d2 antisense RNA probe to examine whether a truncated message was present in du/du PCs. This confirmed the presence of full-length transcript for Cacna2d2 in +/- PCs (Fig. 3A). The data would also not be inconsistent with the additional presence of transcript in small Bergmann glial cell bodies (Fig. 3A, arrows, see also Fig. 8). The results also demonstrated a low level of message hybridizing to the 5' probe in du/du PCs (Fig. 3B, compared with Fig. 3A). This, together with the absence of full-length transcript in du/du PCs shown in our previous study (12), provides evidence for a low level of transcription of du mutant transcript 1 (12) in these cells.

We therefore generated an α2β-2 antipeptide antibody utilizing an immunizing peptide corresponding to amino acids 102-117, with the intent of examining whether a mutant protein was expressed in du/du cerebellum. This sequence is near the N terminus of α2β-2 and is also present in the predicted protein product of the du mutant transcript 1, termed du-mut1 α2 (Fig. 4A). This antibody, called Ab(102-117), was first characterized against heterologously expressed α2β-2. On Western blots of gels run under reducing conditions, α2 is separated from the δ moiety to which it is disulfide-bonded under native conditions. Ab(102-117) specifically recognized the α2 moiety of α2β-2 (as a broad band at about 150 kDa) and not the α2 moiety of α2-1 when both α2-1 and α2β-2 were overexpressed in COS-7 cells (Fig. 4B). It also recognized an ~10-kDa protein product of the du-mut1 α2 cDNA expressed in COS-7 cells (Fig. 4B). When Ab(102-117) was used to examine the immunochemical localization of α2β-2 in these cells, the epitope was accessible in non-permeabilized cells, orienting it exofacially (Fig. 4C, 1st and 2nd columns, upper panel). Additional intracellular staining was observed when the cells were permeabilized (Fig. 4C, 1st and 2nd columns, lower panel). When du-mut1 α2 was expressed, very little immunostaining was observed in non-permeabilized cells (Fig. 4C, 3rd column, upper panel), although a large number of cells were present in the field (Fig. 4C, 4th column, upper panel), whereas intense intracellular immunostaining, localized to intracellular organelles, was observed when the cells were permeabilized (Fig. 4C, 3rd and 4th columns, lower panel). The lack of staining with the anti-GFP antibody in non-permeabilized GFP-positive cells was observed when the cells were permeabilized (Fig. 4C, 3rd and 4th columns, lower panel). The predicted protein molecular mass of the entire du-mut1 α2-truncated protein is 16 kDa, which is larger than the ~10-kDa band observed here. We therefore utilized another antipeptide antibody, generated against the epitope represented by amino acids 16-29, which is within the predicted signal sequence of α2β-2, to further examine the processing of the du-mut1 α2 protein. When du-mut1 α2 was expressed in COS-7 cells, Ab(16-29) recognized a 16-kDa band in lysates of these cells (Fig. 5A, lane 1). No smaller molecular weight bands were
observed on this gel (but see Fig. 7). This suggested that the signal sequence of du-mut1 α2 is at least partly uncleaved when it is expressed in COS-7 cells. Ab(16–29) also recognized a well defined 120-kDa protein when full-length α2-2 was expressed (Fig. 5A, lane 2) and no bands when α2-1 was expressed as a control (Fig. 5A, lane 3). This 120-kDa protein is likely to represent an immature form of α2-2 before cleavage of the signal sequence, which normally precedes glycosylation. The protein molecular mass of the α2 moiety including the 6-kDa signal sequence is calculated to be 113 kDa. Unlike Ab(102–117), Ab(16–29) did not recognize a band of 150 kDa, indicating that, as expected, the signal sequence is cleaved from the mature glycosylated form of α2-2.

We then examined the immunolocalization of du-mut1 α2 in COS-7 cells, using Ab(16–29). Immunostaining for this epitope was not observed at the plasma membrane in non-permeabilized cells expressing α2-2, indicating that the signal sequence is cleaved before insertion of α2-2 into the plasma membrane. Furthermore, this epitope was also generally not observed on the exofacial side of the plasma membrane when du-mut1 α2 was expressed (Fig. 5B, non-permeabilized cells). Diffuse intracellular staining was observed when the cells were permeabilized, for both α2-2- and du-mut1 α2-expressing cells (Fig. 5B).

**Immunopurification of du-mut1 α2 from du/du Cerebellum—**

The use of an Ab(102–117) immunoaffinity column allowed the isolation of a low abundance protein of ~10 kDa from du/du cerebellum (Fig. 6A, lane 1), which was detected using the same antibody. This protein is very similar in molecular weight to the du-mut1 α2 protein isolated in the same way from lysates of COS-7 cells expressing du-mut1 α2 (Fig. 6A, lane 2). If a protein product were produced from du mutant transcript 2 (GenBank™ accession number AF247141) in du/du cerebellum (12), its predicted molecular mass would be ~100 kDa. This would also be recognized by Ab(102–117), but no higher molecular weight immunoreactive bands were observed from 4 to 20% gradient gels of proteins isolated from du/du cerebellum (data not shown, n = 2). A broad band of protein of ~150 kDa, representing the α2 moiety of α2-2, was isolated from +/- cerebellum using the same immunoaffinity column and detected using the same antibody (Fig. 6B, lane 1). This protein was the same molecular weight as that isolated by the same antibody from COS-7 cells transfected with α2-2, with the broad band probably representing different glycosylation states (Fig. 6B, lane 2).

By using an Ab(16–29) immunoaffinity column, a protein of ~16 kDa was isolated from du/du cerebellum (Fig. 6C, lane 1). This protein is very similar in molecular weight to the du-mut1 α2 protein isolated in the same way from COS-7 cells expressing du-mut1 α2 (Fig. 6C, lane 2). Furthermore, a protein of about 120 kDa was isolated from +/- cerebellum using the same immunoaffinity column (Fig. 6D, lane 1). This protein was the same molecular weight as that isolated by the same antibody from COS-7 cells transfected with α2-2 (Fig. 6D, lane 2).

The basis for the difference in molecular weight between the du-mut1 α2 species recognized by the two antibodies was fur-
Fig. 6. Immunopurification of du-mut1 α2 from dudu cerebellum and the α2 moiety of full-length a2δ-2 from +/+ cerebellum. Immunoblot analysis of a2δ-2 proteins were immunocaptured from detergent-solubilized dudu or +/+ cerebellar membranes using column-immobilized peptide antibodies. Samples were separated on either 4–20% gradient gels (A and B), a 20% gel (C), or on a 7.5% gel (D). Arrows indicate the major protein bands detected. A and C, proteins from dudu cerebella (lane 1) or from COS-7 cells expressing du-mut1 α2 (lane 2) isolated using Ab(102–117) and Ab(16–29), respectively. The respective apparent molecular mass values are ~10 and 16 kDa. B and D, proteins from +/+ cerebella (lane 1) or from COS-7 cells expressing full-length a2δ-2 (lane 2) isolated with Ab(102–117) and Ab(16–29), respectively. The respective apparent molecular mass values are 150 and 120 kDa.

Fig. 7. Processing of du-mut1 α2. A, immunoblot analysis of du-mut1 α2 and fragments derived from cleavage of the signal peptide. Lysate (80 μg of protein) of COS-7 cells expressing du-mut1 α2 was separated on a 10–20% gradient gel and probed with Ab(16–29) (lane 1) or Ab(102–117) (lane 2). A 16-kDa band is detected with both antibodies. Ab(16–29) also recognizes a band of 6 kDa, and Ab(102–117) recognizes a major band of ~10 kDa. B, amino acid sequence of du-mut1 α2. The respective binding sites for Ab(16–29) and Ab(102–117) are shown in bold. The entire signal leader sequence is underlined with its predicted cleavage site marked by an arrow (40). C shows the calculated molecular mass values for du-mut1 α2 (16 kDa) and the two proteolytic fragments as follows: du-mut1 α2 following cleavage of the signal sequence (solid line), ~10 kDa, and the cleaved signal sequence (dotted line, ~6 kDa).

Further evidence was obtained here for staining of Bergmann glia (Fig. 8B, left panel, *). In dudu cerebellum, we observed that immunostaining with this antibody was concentrated largely in the cell bodies of PCs (Fig. 8B, right panel). This is likely to represent the 16-kDa uncleaved du-mut1 α2 species. The immunostaining was lost when the antibody was preincubated with the immobilizing peptide (Fig. 8B). No differences were observed between +/+ and dudu PCs when Bergmann glia were visualized using an anti-glial fibrillary acidic protein antibody (results not shown).

Modulation of CaV2.1 Ca2+ Channel Currents by a2δ-2 and du-mut1 α2—The possible pathological function of the du-mut1 α2 protein encoded by the Cacna2d2du gene was investigated using in vitro expression and electrophysiology. To mimic the PC complement of calcium channel subunits, the cDNAs corresponding to rat CaV2.1 and β2 were transfected into COS-7 cells, with or without a2δ-2 or du-mut1 α2 cDNA, and the resulting CaV currents (I Ca) recorded. Co-expression of a2δ-2 increased CaV2.1/β2 I Ca currents, inducing a 2.9-fold enhancement of amplitude at 0 mV (Fig. 9A, and I-V relationships in Fig. 9B), with no significant shift in the voltage dependence of current activation (V 0.5) for activation was ~0.7 ± 0.7 mV (n = 28) for CaV2.1/β2 and ~10.7 ± 0.8 mV (n = 42) for CaV2.1/β2/α2δ-2. There was no significant effect of a2δ-2 on the activation or inactivation of the CaV2.1/β2 combination (Fig. 9A and results not shown).

In contrast, co-expression of du-mut1 α2 induced a consistent reduction in CaV2.1/β2 I Ca throughout the voltage range (Fig. 9, C and D). This amounted to a 51% inhibition at 0 mV (Fig. 9E) and also resulted in a ~+5 mV shift in V 0.5 for activation to ~3.5 ± 1.1 mV (n = 12, p < 0.01 compared with CaV2.1/β2). The mean current densities at 0 mV under the two
different conditions are compared in Fig. 9E. Similar results were obtained when these calcium channel subunits, in this case including rabbit CaV 2.1, were expressed in Xenopus oocytes (see Ref. 12 and data not shown).

Effect of α2β-2 and du-mut1 α2 on Single Ca²⁺ Channel Currents Formed by CaV 2.1—We compared single channel parameters between cell-attached patches of COS-7 cells transfected with CaV 2.1/β3 cDNA, either without α2β-2 (Fig. 10A) or co-expressed with either full-length α2β-2 (Fig. 10B) or the du-mut1 α2 (Fig. 10C). These experiments were performed in order to differentiate between a mechanism that involves changing the biophysical properties of CaV 2.1 channels and a mechanism that involves changing the trafficking or membrane expression levels of the CaV 2.1 channels, imposed by either α2β-2 or du-mut1 α2.

Once opened, CaV 2.1 channels showed an average single channel conductance of 9.9 ± 0.4 pS (n = 8) for CaV 2.1/β3, which was not significantly affected by co-expression of α2β-2 (10.2 ± 0.6 pS, n = 8) or du-mut1 α2 (8.8 ± 1.0 pS, n = 6) (Fig. 10D, left). This conductance is similar to that of P-type channels recorded from wild-type and du/du PCs under the same conditions (12). More detailed analysis demonstrated openings to three distinct amplitude levels, as has also been shown in native Purkinje cells (28), level 2 being the most prominent in our recordings (Fig. 10D, middle and right, see legend for conductance and amplitude values). Neither the conductance nor the amplitude of the three current levels was significantly affected by expression of α2β-2 or du-mut1 α2 (data not shown).

Neither α2β-2 nor du-mut1 α2 caused any significant change in mean open or closed times or in the pattern of voltage dependence of CaV 2.1 channels (Fig. 10E). We also examined the activation kinetics by measuring the latency to first opening of the channels in response to a square voltage pulse (Fig. 10F). CaV 2.1 channel activation was not influenced by the subunits examined (Fig. 10F, left), at any voltage (Fig. 10F, right). In addition, the voltage dependence of inactivation (Fig. 10G) was not influenced by either α2β-2 or du-mut1 α2.

Although the presence of α2β-2 caused an ~3-fold increase in whole cell current amplitude, all the single channel parameters were indistinguishable between the three conditions. This implies that the basic active unit in the whole cell current (an individual channel) remains unchanged, and the modulation by α2β-2 must involve an alteration in the number of active channels in the membrane.

DISCUSSION

PCs from du/du Mice Have a Reduced Dendritic Arbor—PC somata form a monolayer by 10 days postnatally in the mouse, and their dendrites reach the pial surface at day 20, coinciding with the completion of granule cell migration and concomitant parallel fiber production (29). The PC soma typically exhibits one primary dendrite, which emerges apically, and one axonal
process projecting in the opposite direction. The PC dendritic trees develop most dramatically between postnatal day 9 and 20, reaching 80% of their adult dimension in this period (30). PCs from du/du mice appear immature, reduced both in size and complexity, with multiple primary dendrites and small arbors that often terminate well below the pial surface. Thickened secondary and tertiary dendritic trunks are also present. The multipolar appearance of some of the du/du PCs may be a remnant of their immature stage (in which the normal resorption of all somatic filopodia fails to occur), with some of these processes continuing to develop into dendrites as found in weaver and staggerer mouse mutants (31).

Thus, although we have shown that PCs are not lost in duidu cerebella at P21 (12), we now find that the PC dendritic tree is reduced in size and shows other abnormalities, such as weeping willow dendrites and dendritic thickening. Similar abnormalities have been found in a number of the spontaneously occurring CaV 2.1 mouse mutants (8), and some of these (in particular tglia) also show PC loss in older mice (32). The mechanism of the altered PC morphology in du/du mice may result

![Diagram of CaV2.1 currents](http://www.jbc.org/.../2018)
either from the reduced PC calcium channel currents, which we observed in P5-P9 PCs, before the extensive growth of the dendritic arbor (12) or more directly from the loss of α2δ-2, with the possible additional consequences of expression of a truncated mutant α2 protein. A number of the human genetic diseases involving CaV2.1, for example familial hemiplegic migraine (33), cerebellar ataxia, and PC degeneration are associated with mutations that have been shown to produce a reduction in CaV2.1 calcium currents in vitro (34). However, the mechanism whereby such molecular changes are translated into morphological and functional abnormalities remains to be determined.

A Truncated Mutant Protein Derived from the 5′ Mutant Transcript of Cacona2d2 Is Expressed in du Mice—The in situ hybridization studies demonstrated that although wild-type Cacona2d2 transcript is absent from the brain of du/du mice, because of the genomic rearrangement that disrupts Cacona2d2 (12), a 5′ mutant transcript (du mutant transcript 1) is present in du/du PCs. This transcript is predicted to encode a protein (du-mut1 α2) that lacks most of the α2 subunit and the whole of the δ subunit, including its transmembrane domain. It is frequently the case that mRNA encoding mutant transcripts, where a frameshift or point mutation introduces one or more premature stop or nonsense codons, is unstable and subject to nonsense-mediated mRNA decay (35). Indeed, although a second mutant transcript 2, predicted to be formed from exons 2–39, was identified by reverse transcriptase-PCR and Northern blot in du/du mouse brain, it was not observed by in situ hybridization in du/du PCs (12). Furthermore, the 5′ du mutant transcript 1 appeared to be present at a low level in du/du brain (12). To determine whether this mutant transcript was translated, we used two α2δ-2 anti-peptide antibodies, which were raised against peptides within the du-mut1 α2 sequence, Ab(16-29) and Ab(102–117).

It has been established, from studies with site-directed antipeptide antibodies, that the topology of the α2δ-1 subunit is such that the α2 subunit, which has an N-terminal leader signal sequence, is entirely extracellular (36–38). The α2 subunit is disulfide-bonded to a transmembrane δ subunit, and both subunits have been found to be involved in the interaction with the CaV1.2 subunit (38, 39). Now that two other α2δ subunit genes have been cloned, it is assumed that they have the same topology, and indeed, high homology is present between the N termini of α2δ-1 and α2δ-3, with the clear prediction of a cleaved signal peptide in both sequences. In contrast, although a putative signal peptide is found in α2δ-2, it is much longer. By using prediction analysis, it is found to have a potential cleavage site after position 64 (40) (Fig. 7B), whereas only 2% of eukaryotic signal peptides are longer than 35 residues (40). In particular, it has a longer sequence N-terminal to the putative hydrophobic signal sequence (−42 amino acids) than α2δ-1 or α2δ-3 (which are −3 and 11 amino acids, respectively). Such “n regions” are found to be less than 25 amino acids in 80% of secreted or transmembrane proteins where they occur (41). Therefore, it remains unclear whether this signal sequence is cleaved efficiently, as cleavage is often delayed when the signal sequence is long (42). This results in extended transit times through the endoplasmic reticulum-Golgi apparatus, which may be required for highly glycosylated proteins (42). Such an explanation is likely to be the reason for our observation using Ab(16–29), of a 120-kDa immunolabeled protein when α2δ-2 was expressed in COS-7 cells. This is likely to represent the α2 subunit of full-length α2δ-2 (predicted protein molecular mass of 113 kDa), which is immature in that it has an uncleaved signal peptide and, judging by the molecular weight, no added carbohydrate.

It appears that in the case of du-mut1 α2, expressed in COS-7 cells, the truncated protein is processed such that the signal sequence remains at least in part uncleaved, because both Ab(16–29) and Ab(102–117) recognized a band of ~16 kDa, the predicted size for the uncleaved du-mut1 α2, and Ab(16–29) also recognized a fainter band of about 6 kDa, which would represent the cleaved signal peptide. However, the predominant band recognized by Ab(102–117) but not Ab(16–29) was a ~10-kDa protein, which is therefore likely to represent du-mut1 α2 with its signal peptide cleaved. This result corresponded exactly with the molecular weight of the native du-mut1 α2 immunocaptured from du/du cerebellum by the same antibody, indicating that it is a stable in vivo species in these mice. This study also confirmed the previous indication (12) that du mutant transcript 2, which would be recognized by Ab(102–117), is not translated. A 16-kDa protein was immunocaptured by Ab(16–29) from du/du cerebellum, indicating that the signal sequence remains, in part, uncleaved from du-mut1 α2. The reason that this species was not also immunocaptured by Ab(102–117) may indicate that Ab(102–117) is of lower affinity, as also suggested by the data in Fig. 8.

Immunolocalization of α2δ-2 and du-mut1 α2 in Cerebellum—In cerebellar sections, we found, using Ab(102–117), that α2δ-2 is expressed in wild-type PC somata and also in the ML of the cerebellum, suggesting localization in PCs. It is also possible that some of the immunostaining arises from cerebellar afferents or from Bergmann glia, and this will be investigated in the future. In du/du cerebellum, a low level of immunostaining was observed with the same antibody. These results support the finding that du-mut1 α2 is expressed in du/du cerebellum. Immunoreactivity in du/du cerebellar sections was also observed using Ab(16–29), where staining, presumably representing the uncleaved du-mut1 α2, was concentrated in PC somata. In agreement with the expression study in COS-7 cells and the immunopurification data from cerebellum, this suggests that du-mut1 α2 retains, in part, the putative signal sequence at its N terminus and does not appear to be secreted. When du-mut1 α2 was expressed in COS-7 cells both Ab(16–29) and Ab(102–117) recognized an epitope that was only expressed intracellularly, indicating that du-mut1 α2 is unlikely to be secreted or inserted into the plasma membrane as a transmembrane protein.

The Functional Interaction of the CaV2.1/β3 Combination with α2δ-2—The similarity of the ducky phenotype to that observed in mice with mutations in genes encoding the CaV2.1 (7) and β3 (9) subunits and their predominant PC expression pattern suggests that α2δ-2 contributes to the P-type current. This is reinforced by our finding that the currents formed by both rat and rabbit CaV2.1 co-expressed with β3, which is the main PC β subunit, were strongly enhanced by α2δ-2, in two expression systems (COS-7 cells and Xenopus oocytes).

Previous in vitro studies have shown that α2δ-1, α2δ-2, and α2δ-3 subunits act to increase the maximum conductance of a number of expressed calcium channel α1/β subunit combinations at the whole cell level (2, 43–46). However, this may be dependent to some extent on the specific combination of α1 and β subunits expressed. Furthermore, the effects of α2δ subunits on kinetics and voltage dependence of activation are more minor (2, 44). We have also investigated this for the calcium channel subunit combinations used in the present study, and we show that α2δ-2 had no influence on voltage-dependent properties and had no effect on single channel conductance or other biophysical parameters of the CaV2.1/β3 channels themselves. This implies that α2δ-2 probably has its main effect on the lifetime of the channel complex in the plasma membrane, either by enhancing trafficking or reducing turnover. In agree-
ment with this proposed mechanism, it has previously been found that α2δ-1 increased the amount of CaV1.2 protein expressed in Xenopus oocytes (47).

In contrast, the protein product of du mutant transcript 1, du-mut1 αδ, produced a consistent reduction in CaV2.1/β3 currents in COS-7 cells. Thus, whereas loss of full-length α2δ-2 is likely to be the most important contributing factor, the expression of the truncated du-mut1 αδ may also contribute to the du/du phenotype, via an additional suppressive effect, possibly by interfering with the correct trafficking of α1 subunits.

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The Ducky Mutation in Cacna2d2 Results in Altered Purkinje Cell Morphology and Is Associated with the Expression of a Truncated α2δ-2 Protein with Abnormal Function

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