Novel Splice Variants of Rat CaV2.1 That Lack Much of the Synaptic Protein Interaction Site Are Expressed in Neuroendocrine Cells*

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Voltage-gated Ca2+ channels are responsible for the activation of the Ca2+ influx that triggers exocytotic secretion. The synaptic protein interaction (synprint) site found in the II–III loop of CaV2.1 and CaV2.2 mediates a physical association with synaptic proteins that may be crucial for fast neurotransmission and axonal targeting. We report here the use of nested PCR to identify two novel splice variants of rat CaV2.1 that lack much of the synprint site. Furthermore, we compare immunofluorescence data derived from antibodies directed against sequences in the CaV2.1 synprint site and carboxyl terminus to show that channel variants lacking a portion of the synprint site are expressed in two types of neuroendocrine cells. Immunofluorescence data also suggest that such variants are properly targeted to neuroendocrine terminals. When expressed in a mammalian cell line, both splice variants yielded Ca2+ currents, but the variant containing the larger of the two deletions displayed a reduced current density and a marked shift in the voltage dependence of inactivation. These results have important implications for CaV2.1 function and for the mechanisms of CaV2.1 targeting in neurons and neuroendocrine cells.

Voltage-gated Ca2+ channels are a family of protein complexes that regulate the influx of Ca2+ into cells. At the center of this complex is a protein known as the α subunit, which forms the voltage-dependent, Ca2+ selective pore. Ca2+ channel properties and distribution are determined by the identity of the α subunit (1), by the association of accessory subunits (2, 3), and by alternative splicing of the α subunit (4, 5). The role of different Ca2+ channel types in exocytic secretion depends on the relationship between the channels and the exocytic apparatus (6). Toxins selective for different Ca2+ channel types have been used to show that central neurotransmission is predominantly evoked by the activation of CaV2.1 (P/Q-type channels) and CaV2.2 (N-type channels) (7, 8). The ability of these channels to evoke secretion may involve a synaptic protein interaction site (the “synprint” site) in the intracellular loop between domain II and domain III that interacts with the synaptic proteins syntaxin, SNAP-25 and synaptotagmin (9, 10). These interactions influence channel gating (11, 12) and may be important in determining the relationship between Ca2+ influx and exocytotic secretion. A peptide that mimics the interaction site has been shown to inhibit evoked exocytotic secretion from neurons, presumably by displacing syntaxin from its binding site on the Ca2+ channel II–III loop (13). The rba isoform of CaV2.1, which was isolated from rat brain (14), binds with SNAP-25 in vitro (15). Co-expression of SNAP-25 with the rba isoform causes a negative shift in steady-state inactivation that appears to be relieved through the formation of a complex with syntaxin and synaptotagmin (16). CaV2.1 channels exogenously expressed in superior cervical ganglion cells are able to evoke neurotransmission (17), but expression of channels in which the II–III loop had been deleted had reduced effectiveness (18). Furthermore, this loss of effectiveness was associated with a loss of presynaptic localization, suggesting that the synprint site is important in targeting or anchoring CaV2.1 to the presynaptic terminal (18).

Splice variants of human CaV2.2 have been identified that lack a large part of the II–III loop, including a large part of the synprint site (19). Because of the importance of this site, it is likely that these deletion variants have functions different from those of channels containing the interaction site. Although a deletion variant lacking 348 amino acids in the II–III loop was reported when CaV2.1 was cloned from rabbit brain (20), there have been no further characterizations of such deletion variants (4, 5). We have therefore conducted nested PCR experiments to detect alternative splicing within the II–III loop of rat CaV2.1. We report two novel splice variants that have large deletions within the II–III loop including large portions of the synprint site. PCR experiments on RNA extracted from various brains

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The abbreviations and trivial names used are: synprint site, synaptic protein interaction site; MNC, magnocellular neurosecretory cell; RT-PCR, reverse transcription-PCR.
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areas and cell types show that mRNA species coding for these variants are expressed in most brain areas. These mRNA species are also found in two types of neuroendocrine cell, PC12 cells (a rat pheochromocytoma cell line) and the magnocellular neurosecretory cells (MNCs) of the hypothalamus, acutely isolated from the supraoptic nucleus. To test whether the Ca\textsubscript{v}2.1 variants are expressed in these cell types, we compared the immunostaining of two antibodies directed against different portions of Ca\textsubscript{v}2.1, one that binds to a sequence in the II–III loop (which would therefore not recognize the deletion variants) and one that binds to the carboxyl terminus (which would therefore recognize channels with or without the deletions in the II–III loop). We found that both antibodies (which we will refer to as “selective” and “inclusive,” respectively) stained membranes in the somata and axon terminals of cultured hip- pocampal neurons, suggesting that these cells express channels containing an intact II–III loop. In both types of neuroendo- crine cells, however, the inclusive antibody showed robust staining of the plasma membrane, whereas the selective antibody failed to show clear plasma membrane staining. Expression in a mammalian cell line revealed that although both splice variants yielded Ca\textsuperscript{2+} currents, the variant containing the larger deletion resulted in current with a much lower density and with a marked depolarizing shift in the voltage dependence of inactivation. These data support the hypothesis that the pre- dominant form of Ca\textsubscript{v}2.1 expressed in these neuroendocrine cells lacks a portion of the II–III loop and have important impli- cations for Ca\textsuperscript{2+} channel function and targeting in neurons and neuroendocrine cells.

EXPERIMENTAL PROCEDURES

MNC Cultures—Male Long-Evans rats (200–300 g) were anesthetized with halothane and killed with a rodent guillotine following a protocol approved by the University of Saskatchewan Animal Care Committee. The supraoptic nucleus of the hypothalamus was dissected, and MNCs were isolated following treatment with trypsin (Sigma–Aldrich) as described previously (21–23). MNC axon terminals were isolated from neurohypophyses using a similar protocol, as described previously (24, 25).

Hippocampal Neuron Cultures—Hippocampi were isolated from Wistar rats on postnatal day 2 and dissociated using a published protocol (26) involving digestion with trypsin (Sigma–Aldrich). Neurons were plated on glass coverslips coated with poly-l-lysine (Sigma–Aldrich) and cultured in Neurobasal-A medium (Invitrogen) supplemented with B-27 (Invitrogen). Neurons were plated at a density of 50–60/mm\textsuperscript{2}. Immunocytochemistry was performed within 4 days.

PC12 Cultures—PC12 cells were obtained from American Type Culture Collection (ATCC) and cultured in Ham’s F12 medium with 2 mm l-glutamine and 1.5 g/liter sodium bicarbonate (82.5%; Sigma), horse serum (15%; Sigma), and fetal bovine serum (2.5%; Invitrogen) in a 5% CO\textsubscript{2} humidified atmosphere.

Primers and PCR—Rats were killed as described above. The brains were immediately removed, and various parts were dissected for total RNA extraction. RNA from these parts was isolated immediately or stored at −70 °C after being frozen in liquid nitrogen. RNA was extracted using TRIzol\textsuperscript{TM} reagent (Invitrogen). RNA was reverse-transcribed using SuperScript\textsuperscript{TM} first-strand synthesis system for RT-PCR (Invitrogen).

Variants with differences in the II–III loop were sought using nested RT-PCR. The sequence of the rat Ca\textsubscript{v}2.1 II–III loop (accession number NM_012918) was used to design two sets of primers (P1 forward, 5’-GGC ATG GTG TTC ATC TA-3’, 2065–2084, and P2 reverse, 5’-GAG CCC TGG CTC TCT TTG CT-3’, 2959–2978) that were then synthesized by the University Core DNA Services, University of Calgary. Initial amplification was carried out by 20 cycles of 95 °C for 35 s, 60 °C for 45 s, and 72 °C for 3 min after initial denaturation of 95 °C for 3 min and then the final elongation of 72 °C for 10 min using primers P1 and P2. The products were reamplified with nested primers (P3 forward, 5’-ACC CTC TTC GGG AAC TAC AC-3’, 2098–2117, and P4 reverse, 5’-CTC CCC ATC ATC GCC TTC TC-3’, 2878–2895) using 23 cycles of 95 °C for 35 s, 60 °C for 45 s, and 72 °C for 3 min after initial denaturation of 3 min at 95 °C and then a final elongation at 72 °C for 10 min. A negative control without cDNA was run with each reaction. Gels were visualized under UV light and photographed using Polaroid film, and the band intensities were measured using the NIH Image J software. The housekeeping gene gapdh was used as a positive control (P5 forward, 5’-CAT GAC AAC TTT GCC ATC GT-3’, 1336–1355, and P6 reverse 5’-ATG TAG GCC ATG AGG TTC AC-3’, 1816–1835; 31 cycle of 95 °C for 30 s, 60 °C for 40 s, and 72 °C for 1.5 min).

Gel bands were cut and DNA was eluted using Quantum Prep\textsuperscript{TM} Freeze ‘N Squeeze DNA gel extraction spin columns (Bio-Rad Laboratories). DNA was precipitated with sodium acetate (pH 5.2) and ethanol and sequenced at the Plant Bio- technology Institute, National Research Council, Saskatoon, Canada. For the study of expression of these splice variants in MNCs, acutely isolated MNCs in culture (see above) were aspi- rated using a micropipette and subjected to single-cell RT-PCR (27).

Antibodies and Immunofluorescence—An affinity-purified goat polyclonal antibody directed against a sequence in domain IV of human Ca\textsubscript{v}2.1 (Santa Cruz Biotechnology, Santa Cruz, CA) and an affinity-purified rabbit polyclonal antibody directed against peptide residues 865–881 of rat Ca\textsubscript{v}2.1 (Alomone Labs, Jerusalem, Israel) were used for immunofluorescence experi- ments on cultured cells. Cells were fixed in phosphate-buffered saline with 2% paraformaldehyde, 0.05% sodium periodate, and 0.34% lysine (28) and permeabilized and blocked for 1 h with digitonin (100 μg/ml), bovine serum albumin (4%), normal donkey serum (4%), and sodium azide (0.02%) in phosphate-buffered saline. Primary antibodies (1:100) were incubated overnight at 4 °C followed by incubation for 1 h with secondary anti- bodies Alexa Fluor 488 donkey anti-goat (1:400, Invitrogen) or Alexa Fluor 555 donkey anti-rabbit goat (1:200, Invitrogen). Immunofluorescence was visualized using a Zeiss Axiovert 200 microscope with a 40\times objective and the appropriate filter sets. Images were captured using a cooled CCD camera. The immuno- blots were obtained using a standard protocol following solubi- lization of rat brain tissue.
Construction of Deletion Variants—Splice variants were created using overlap extension PCR. The full-length rat CaV2.1 channel (accession number NM_012918) was used to design primers, synthesized by the University Core DNA Services, University of Calgary. Both the large and the small II–III linker deletion variants used outside forward primer (U1BmgBl) 5′-CGA AAC AGA CGT GGA GCA GAG GCA CCC TTT TGA TGG-3′, 1227–1262, and inside reverse primers (L2D1) 5′-GCC GCT CTA GTG TCG CGG GGA CTG ACT TCT GCC ACC T-3′, 2240–2259, and (L2D2) 5′-GCC GGC CTA GTG TCG CCG CTA GCC AGC AGG TTC TGC T-3′, 2357–2376, respectively, to amplify the fragment flanking the upstream portion of the deletion and append a 20-base overlap homologous to the downstream flank. Similarly, the downstream flanking region for the large and small variants were amplified with forward primers (U3D1) 5′-AGG TGG CAG AAG TGA GTC CCC GCG ACG CCA CTA GGC CGG C-3′, 2842–2861, and (U3D2) 5′-AGC AGC ACC TGC TGG CTA GCC GCG ACG CCA CTA GCC CGG C-3′, 2842–2861, respectively, with a downstream reverse primer (L4SgrAI) 5′-CGC CGG TGT CTG CGC TCC CTG TCA TCG TG-3′, 2929–2957. These products similarly possessed a 20-base overlap homologous to the upstream flank. Thermocycling was conducted using Pfu DNA polymerase (Stratagene, La Jolla, CA) as per the manufacturer’s recommendations. The initial denaturation was carried out at 95 °C for 2 min followed by 35 cycles of denaturation (95 °C, 30 s), annealing (55 °C, 30 s), and extension (72 °C, 2 min) followed by a final extension at 72 °C for 10 min.

A second round of PCR using upstream and downstream PCR products plus outside forward (U1Xhol) and reverse (L4BmgBl) primers was conducted using TaqDNA polymerase (Invitrogen) as per the manufacturer’s recommendations. The initial denaturation was carried out at 95 °C for 2 min followed by 30 cycles of denaturation (95 °C, 45 s), annealing (65 °C, 45 s), and extension (72 °C, 4 min) followed by a final extension at 72 °C for 10 min.

Once complete, the two final PCR products were gel-purified and subcloned into pGEM T-easy (Promega) and transformed into DH5α competent cells. This amplified deletion region was then subcloned back into wild-type Cav2.1 channels at restriction endonuclease sites BmgBl and SgrAI. The correct splice variant identity was confirmed by sequencing by the University Core DNA Services, University of Calgary.

Electrophysiology—Culturing, maintenance, and transfection of tsA-201 cells via the calcium phosphate method was conducted as described by us previously (29). For electrophysiological characterization, full-length CaV2.1 channels or the two cloned CaV2.1 splice variants were co-transfected into tsA-201 cells with cDNAs encoding rat β1b, rat α2-δ1, and enhanced green fluorescent protein as described by us previously. Whole-cell recordings were conducted using external solution containing 5 mM BaCl2, 1 mM MgCl2, 10 mM HEPES, 40 mM tetraethylammonium chloride, 87.5 mM CsCl, 10 mM glucose (pH 7.7 with tetraethylammonium-OH), and internal solution containing 108 mM cesium-methanesulfonate, 4 mM MgCl2, 9 mM EGTA, and 9 mM HEPES (pH 7.2 with CsOH) using an Axopatch 200B amplifier and pCLAMP9 software. Steady-state inactivation curves were obtained by holding cells at different conditioning voltages (starting at −120 mV) for 10 s prior to stepping to a test potential of +10 mV. Data were sampled at 2 kHz and filtered at 1 kHz. Series resistance was compensated by 80%. Currents larger than 2 nA were eliminated from biophysical characterization but were included in the analysis of peak current densities. Steady-state inactivation curves were fitted using the Boltzmann equation. In Fig. 4, all error bars denote standard errors; statistical analysis was conducted using one-way analysis of variance.

RESULTS AND DISCUSSION

Identification of Splice Variants of CaV2.1—Nested PCR primers designed to encompass the RNA sequence coding for much of the CaV2.1 subunit II–III loop were used to probe RNA isolated from rat brain tissue. In addition to the full-length sequence, which generated a band close to the expected size of 797 bp, we identified three bands of smaller size. These three bands were isolated, and the DNA was sequenced. These sequences, which correspond to lengths of 332, 260, and 215 bp, were used to predict the amino acid sequences that would result from the deletions. Although the intermediate band would result in an mRNA containing a stop codon and would thus be unlikely to be physiologically relevant, the smallest and largest bands would result in unchanged reading frames with expected deletions of 194 and 155 amino acids. We will refer to these two deletion mutants as CaV2.1Δ1 and CaV2.1Δ2. The predicted structures and sequences of the two variants are shown in Fig. 1. The diagram in Fig. 1 shows the structure of the CaV2.1 α1 subunit. The II–III loop is the intracellular loop between domains II and III. The approximate location of the synprint site is shown by the thicker line, and the sites of the deletions in the identified mutants are shown by the arrows. The sequence coded for by CaV2.1Δ1 would correspond to a deletion beginning at amino acid 754 and ending at amino acid 947, whereas CaV2.1Δ2 would predict a deletion between amino acids 793 and 947. The amino acid sequences of the two deletion mutants are compared with the full-length sequence at the bottom of Fig. 1. The upper sequence is that of the full-length protein with the synprint site underlined, the second sequence is that of CaV2.1Δ1, and the bottom sequence is that of CaV2.1Δ2.

Tissue Distribution of the CaV2.1 Variants—PCR reactions were carried out on RNA isolated from a variety of brain areas, as well as in cultured neuroendocrine cells (see below). The results of such experiments are shown in the images of gels at the top of Fig. 2. The graph at the bottom of Fig. 2 displays the intensity of the bands for CaV2.1Δ1 (Δ1) and CaV2.1Δ2 (Δ2) in the listed brain regions, expressed as a fraction of the intensity of the band for gapdh. Both CaV2.1−Δ1 and CaV2.1−Δ2 are widely expressed in the brain. In all brain areas tested, with the exception of the hippocampus, the relative expression of CaV2.1−Δ1 is greater than that of CaV2.1−Δ2. Expression appears to be highest in the hypothalamus and cortex. Neither variant was seen in tissues from outside the brain (kidney, spleen, pancreas; data not shown).

Expression of the CaV2.1 Variants in Neuroendocrine Cells—Since the synprint site is associated with synaptic release, we sought to determine whether the variants are expressed in neu-
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The sequences of the II–III loop deletion splice variants of CaV2.1. The diagram shows the structure of the CaV2.1 α1 subunit with the synprint site of the II–III loop shown in bold and the location of the amino acids that mark the sequences deleted in the variants shown with arrows. The lower part of the figure compares the sequence of the full-length variant of rat CaV2.1 (FL) with those of CaV2.1-Δ1 (Δ1) and CaV2.1-Δ2 (Δ2). The sequence of the synprint site is underlined. The line above sequence 790–798 indicates a putative synaptic protein-binding site (see “Results and Discussion”). The numbers on the right refer to the amino acids at the end of each row in the full-length sequence of CaV2.1.

roendocrine cells. Patch clamp and PCR studies of cultured PC12 cells have shown that they express CaV2.1 channels (30). Fig. 2 shows the results of PCR experiments demonstrating that PC12 cultures express CaV2.1-Δ1. We also tested whether CaV2.1 variants are expressed in the MNCs. Single cell RT-PCR experiments have demonstrated that these cells express CaV2.1, as well as CaV1.2, CaV1.3, and CaV2.2 (27), and currents mediated by CaV2.1 have been characterized in both the MNC somata (22, 31) and axon terminals (24). Studies of evoked secretion from isolated MNC terminals suggest that currents mediated by CaV2.1 are important in mediating VP release (32). Since the MNC somata are highly enriched in the supraoptic nucleus of the hypothalamus, we first isolated RNA from this tissue to test for the presence of mRNA coding for the CaV2.1 splice variants. These experiments resulted in three bands close in size to those observed in whole brain (Fig. 2). We also probed for expression of the variants in acutely isolated MNCs using single cell RT-PCR. The result of one such experiment is illustrated in Fig. 2. The RNA corresponding to CaV2.1-Δ1 was observed in each of the five cells tested, and three also expressed CaV2.1-Δ2.

Immunofluorescence Evidence for the Expression of CaV2.1 II–III Loop Deletion Variants—We used immunocytochemical techniques to test whether cultured cells express channel variants lacking portions of the synprint site as proteins. Antibodies directed against a sequence within the synprint site (the selective antibody) and the carboxyl terminus (the inclusive antibody) of CaV2.1 were used to probe cultured neonatal hippocampal neurons, PC12 cells, and acutely isolated MNCs (Fig. 3). We reasoned that immunoreactivity to the inclusive antibody, in the absence of immunoreactivity to the selective antibody, would suggest the presence of II–III loop deletion variants of CaV2.1. Immunoreactivity to the two antibodies in hippocampal neurons is compared in Fig. 3A. The two antibodies clearly stain both the somatic membrane and the axon terminals of hippocampal neurons, suggesting that CaV2.1 channels including the synprint site are found in both areas. Our data do not, however, provide evidence for expression of CaV2.1 II–III loop deletion variants in hippocampal neurons. The patterns of staining for the two antibodies were clearly different in PC12 cells. Although there was robust membrane staining for the inclusive antibody, there was little or no staining observed on the membrane for the selective antibody. This suggests that a variant lacking a portion of the II–III loop is the predominant form in PC12 cells. Similar results were obtained for isolated MNCs. We compared the staining of the two antibodies both in the MNC somata isolated from the hypothalamus and in the large MNC axon terminals that may be isolated from the neurohypophysis (24, 25). The inclusive antibody showed clear staining in both locations, whereas the selective antibody showed little membrane staining. The lack of clear immunoreactivity on the MNC terminal membrane using the selective antibody is consistent with a previous report (25), and although punctuate staining near the plasma membrane of MNC somata has been observed in a tissue slice preparation, the authors of the report noted that they could not distinguish between membrane staining and staining of synaptic inputs onto the MNC somata (33). Our data support the conclusion that the dominant form of CaV2.1 in both neuroendocrine cell types is not immunoreactive to the selective antibody and therefore may
lack a portion of the II–III loop. Furthermore, these data suggest that an intact II–III loop is not necessary for the targeting of CaV2.1 to MNC axon terminals.

The images at the bottom of Fig. 3 show the results of immunoblots of rat brain tissues using the selective (left) and inclusive (right) antibodies. The selective antibody is directed against the same sequence as an antibody developed by the Catterall laboratory (34) and appears to recognize a similar pattern of polypeptides, including those with apparent masses of about 210, 190, and 160 kDa. The image at the right shows the pattern of bands recognized by the inclusive antibody, which displays a similar pattern of bands with an additional band just above 175 kDa, which could correspond to one of the splice variants.

**Biophysical Properties of the Deletion Variants**—To determine the functional characteristic of the deletion variants, we used PCR to create the deletions in the rat brain CaV2.1 cDNA (rbA isoform (14)) and co-expressed these variants with rat β1β2, rat α1-δ, and enhanced green fluorescent protein in tsA-201 cells followed by whole cell patch clamp recordings. As shown in Fig. 4A, both the full-length CaV2.1 channel, as well as the CaV2.1-Δ1 and CaV2.1-Δ2 constructs, yielded detectable whole cell currents, which were, however, significantly smaller for the CaV2.1-Δ1 isoform (Fig. 4B). The position of the current voltage relation was similar among the three channel subtypes (Fig. 4C). In contrast, the steady-state inactivation curve of the CaV2.1-Δ1 isoform was shifted toward more depolarized voltages (Fig. 4D) in a manner reminiscent of what was observed with synprint deletions in the human CaV2.2 channels (19). The CaV2.1-Δ2 isoform, on the other hand, showed steady-state inactivation properties that were similar to those observed with the full-length channel. These data indicate that there is a functional consequence to alternate splicing of the CaV2.1 synprint region.

**The Significance of II–III Loop Deletion Variants in CaV2.1**—Although several functional splice variants of CaV2.1 have been identified (35–39), including the optional insertion of a tripep-
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FIGURE 4. Functional characterization of CaV2.1 deletion variants in tsA-201 cells. A, representative whole cell currents obtained from full-length CaV2.1 (filled circles), CaV2.1-Δ1 (upright triangles), and CaV2.1-Δ2 (inverted triangles) channels co-expressed with rat β1 and α2-δ, at a test potential of +40 mV. FL, full-length. B, a bar graph showing peak current densities of the three constructs. Δ1, CaV2.1-Δ1; Δ2, CaV2.1-Δ2. The numbers in parentheses reflect the numbers of experiments. C, families of current-voltage (IV) relations obtained from CaV2.1, CaV2.1-Δ1, and CaV2.1-Δ2. The IV curves were normalized to the same peak value to facilitate comparison of the position of the shapes of the ensemble data. The bar graph shows the half-activation potentials obtained from Boltzmann fits to the IV data. The numbers in parentheses reflect the numbers of experiments. D, steady-state inactivation curves obtained from the three channel variants. The data were fitted with the Boltzmann equation. The bar graph illustrates half-inactivation potentials determined from fits to individual steady-state inactivation curves. The numbers in parentheses reflect the numbers of experiments.

tide sequence (VEA) in the II–III loop (38), our observations represent the first identification of splice variants of rat CaV2.1 that lack large portions of the II–III loop (4, 5). These variants appear to be analogous to two alternatively spliced variants that have deletions within the synprint site in human CaV2.2 (19). Both deletions in CaV2.2 resulted in a rightward shift in the voltage dependence of inactivation. Although the smaller deletion in CaV2.1 (CaV2.1-Δ2) resulted in currents that were similar in amplitude and biophysical properties to the full-length channel, the variant containing the larger of the two deletions, CaV2.1-Δ1, yielded currents that were of much smaller amplitude, displayed a slight rightward shift (−6 mV) in the voltage at which the current peaked and a large rightward shift (−40 mV) in the voltage dependence of inactivation. Although syntaxin causes a hyperpolarizing shift in the half-inactivation voltage of rat (11, 12) and human (19) CaV2.2 and does interact with the rabbit isoform of CaV2.1 (15), syntaxin does not bind to the rat CaV2.1 and does not cause a shift in its voltage dependence of inactivation (16). Consistent with these findings, we did not observe a hyperpolarizing shift in the voltage dependence of inactivation of either the full-length CaV2.1 or the two deletion variants upon co-expression with syntaxin (data not shown). Although there is evidence for the binding of a complex of synaptic proteins to CaV2.1 (which includes syntaxin, SNAP-25, and synaptotagmin (16)), the loci and large size of the regions deleted in CaV2.1-Δ1 and -Δ2 make binding of such a complex appear unlikely. SNAP-25 has been shown to bind to a motif in CaV2.2 found between residues 781 and 789 (LRASCEALY (40)), and a highly homologous site is found in CaV2.1 at residues 790–798 (LLASREALY; Fig. 1). This sequence is completely absent in CaV2.1-Δ1 and is severely truncated in CaV2.1-Δ2. These observations, together with our imaging and electrophysiological data, suggest that the deletion of the synprint region of CaV2.1 may have multiple consequences including altered coupling to the synaptic release machinery, altered subcellular targeting, and altered channel function. The predominance of these deletion variants in neuroendocrine cells suggests that they may have a role in triggering non-synaptic exocytotic release. The lower levels of steady-state inactivation in CaV2.1-Δ1 may indicate that the inactivation of CaV2.1 plays a role in synaptic release that is less important for neuroendocrine release. The observation that a deletion mutant is present in the MNC terminals suggests that the II–III loop is not necessary for axonal targeting of CaV2.1, despite the observation that selective excision of the II–III loop of CaV2.1 appears to inhibit axonal targeting or anchoring in superior cervical ganglion neurons (18). Targeting in MNCs may therefore depend on sequences in a different portion of the channel.

One possibility is an interaction site found in the carboxyl terminus of both CaV2.1 and CaV2.2 that interacts with the modular adaptor proteins CASK and Mint1 (41). Experiments in cultured hippocampal neurons demonstrated that this sequence is necessary and sufficient for axonal targeting of CaV2.2 in hippocampal neurons (41, 42). The homolog of CaV2.1 and CaV2.2 in snail neurons lacks the synprint site entirely, and axonal targeting in these neurons was found to require the interaction of the channels with CASK and Mint1.
When Ca\textsubscript{v}2.2 variants lacking the II–III loop were expressed in cultured hippocampal neurons, they were transported to the axon terminals, suggesting that the synprint site is not necessary for axonal targeting (44). These variants were, however, deficient in their ability to form presynaptic clusters, suggesting that the synprint site has a role in the formation of presynaptic structures. The observation that deletion variants of both Ca\textsubscript{v}2.1 and Ca\textsubscript{v}2.2 are found in axon terminals suggests that axonal targeting may be mediated by mechanisms other than those requiring an interaction with synaptic proteins. One possibility is that an interaction with CASK and Mint1 is also important in the axonal targeting of Ca\textsubscript{v}2.1. Expression studies in mice hippocampal cultures, however, suggest that the carboxyl terminus of Ca\textsubscript{v}2.1 is not indispensable for axonal targeting (45). Ca\textsuperscript{2+} channel targeting experiments in a polarized epithelial cell suggest that association with different \(\beta\) subunits can be an important determinant of axonal targeting for Ca\textsubscript{v}2.1 but not Ca\textsubscript{v}2.2 (46). More work will be required to elucidate the mechanisms of Ca\textsubscript{v}2.1 targeting in neurons and neuroendocrine cells.

We have identified two novel splice variants of rat Ca\textsubscript{v}2.1 that lack much of the II–III loop, including the synaptic protein interaction site. Such deletion variants appear to be expressed in two types of neuroendocrine cells, including in the axon terminals of the MNCs. Furthermore, we have shown that one of the variants displays a marked difference in voltage-dependent inactivation. These data have important implications about the function of both Ca\textsubscript{v}2.1 and Ca\textsubscript{v}2.2 are found in axon terminals suggests that axonal targeting may be mediated by mechanisms other than those requiring an interaction with synaptic proteins. One possibility is that an interaction with CASK and Mint1 is also important in the axonal targeting of Ca\textsubscript{v}2.1. Expression studies in mice hippocampal cultures, however, suggest that the carboxyl terminus of Ca\textsubscript{v}2.1 is not indispensable for axonal targeting (45). Ca\textsuperscript{2+} channel targeting experiments in a polarized epithelial cell suggest that association with different \(\beta\) subunits can be an important determinant of axonal targeting for Ca\textsubscript{v}2.1 but not Ca\textsubscript{v}2.2 (46). More work will be required to elucidate the mechanisms of Ca\textsubscript{v}2.1 targeting in neurons and neuroendocrine cells.