CaV1.3 channels are unique among the high voltage-activated Ca\(^{2+}\) channel family because they activate at the most negative potentials and display very rapid calcium-dependent inactivation. Both properties are of crucial importance in neurons of the suprachiasmatic nucleus and substantia nigra, where the influx of Ca\(^{2+}\) ions at subthreshold membrane voltages supports pacemaking function. Previously, alternative splicing in the CaV1.3 C terminus gives rise to a long (CaV1.3\(_{42}\)) and a short form (CaV1.3\(_{42A}\)), resulting in a pronounced activation at more negative voltages and faster inactivation in the latter. It was further shown that the C-terminal modulator in the CaV1.3\(_{42}\) isoforms modulates calmodulin binding to the IQ domain. Using splice variant-specific antibodies, we determined that protein localization of both splice variants in different brain regions was similar. Using the transcript-scanning method, we further identified alternative splicing at four loci in the C terminus of CaV1.3 channels. Alternative splicing of exon 41 removes the IQ motif, resulting in a truncated CaV1.3 protein with diminished inactivation. Splicing of exon 41 causes a frameshift and exhibits a robust inactivation of similar intensity to CaV1.3\(_{42}\), and Alternative splicing of exons 44 and 48 are in-frame, altering interaction of the distal modulator with the IQ domain and tapering inactivation slightly. Thus, alternative splicing in the C terminus of CaV1.3 channels modulates its electrophysiological properties, which could in turn alter neuronal firing properties and functions.

Significance: Alternative splicing is an exquisite mechanism for customizing channel function within diverse biological niches.
dominant isoforms, we employed the transcript-scanning method (14, 15) to systematically identify novel and functional C terminus splice variants of Ca$_\alpha$1.3 that could be important in modulating gating properties of the channel. In addition to the Ca$_\alpha$1.3$_{42A}$ (12), we have identified and characterized the biophysical properties and subcellular localization of 4 novel splice isoforms: exon 41 (Ca$_\alpha$1.3$_{41A}$), exon 43 (Ca$_\alpha$1.3$_{43S,2}$), exon 44 (Ca$_\alpha$1.3$_{44A}$), and exon 48 (Ca$_\alpha$1.3$_{48R}$). Another splice isoform in exon 43 (Ca$_\alpha$1.3$_{43S}$) was described in our accompanying article (16). Alternative splicing in the C terminus causes hyperpolarized shifts in the activation and inactivation properties and modulates the degree of CDI, via changes in the IQ domain, or conserved proximal and distal domains (termed PRCD and DCRD), which could alter its C-terminal gating modulator (CTM) activity. All alternatively spliced Ca$_\alpha$1.3 channels examined in this study were functional and may contribute differentially to the overall firing property of neurons in specific nuclei, particularly in physiological and disease states.

**EXPERIMENTAL PROCEDURES**

**Generation of Polyclonal Antibodies against Ca$_\alpha$1.3$_{42}$ and Ca$_\alpha$1.3$_{42A}$**—The rat Ca$_\alpha$1.3$_{42}$ splice variant peptide (CCEDDSPTWSRQNYSYNRPSSMD) was subcloned in-frame at EcoRI and XhoI sites of expression plasmid pGEX-4T-1 (Amersham Biosciences). The resulting fusion protein was expressed in the host *Escherichia coli* BL21 (DES) cells. This GST-fused Ca$_\alpha$1.3$_{42}$ protein was purified and eluted with glutathione-agarose (Sigma, G4501). Purified Ca$_\alpha$1.3$_{42}$-GST protein was used to immunize female New Zealand White rabbits once a month. Complete Freund’s adjuvant (Sigma, F5881) was first mixed with GST fusion protein for immunization, and incomplete Freund’s adjuvant (Sigma, F5506) was used in subsequent injections once a month. Serum was pre-absorbed with a peptide antibody against Ca$_\alpha$1.342A channels in rabbits. The polyclonal peptide antibody was raised overnight at 4 °C with excess GST protein to remove contaminants. The resulting fusion protein was affinity purified from immobilized GST fusion protein with an affinity matrix (Calbiochem-Novabiochem) to retard fading. Preparations were analyzed using Carl Zeiss Laser Scanning System LSM 510. Anti-bassoon antibodies were used to identify the presence of Ca$_\alpha$1.3$\gamma$ subunit. The size of the PCR products ranged from 515 to 638 bp, spanning between 2 and 4 exons. We used 1 lot of rat brain cDNA libraries (Mar-

**Fluorescence Staining**—Brains of deeply anesthetized wild type C57 mice were dissected out without perfusion. Frozen brain coronal sections at 20 μm thickness from both wild type C57 and Ca$_\alpha$1.3$^{-/-}$ knock-out mice were prepared in a cryostat and mounted on glass slides. The brain sections were fixed with 4% paraformaldehyde in PBS. After washing and blocking with 4% normal goat serum in TBS (with 0.1% Triton X-100) for 2 h. Sections were incubated with primary antibody pAb_Ca$_\alpha$1.3$_{42}$ (used at 1:200) and primary antibody pAb_Ca$_\alpha$1.3$_{42A}$ (used at 1:100) overnight at 4 °C on the orbital shaker. After washing, sections were incubated with biotinylated goat anti-rabbit IgG (used at 1:500, Vector). After washing, sections were incubated with avidin-biotin complex (ABC) reagent (Vector) for 2 h, followed by DAB (0.12% H$_2$O$_2$ and 0.05% 3,3′-diaminobenzidine) (Sigma) for 20 min before mounting onto glass slides.

**C terminus Transcript Scanning by Nested PCR**—The transcript-scanning method has been described in detail by Mittman et al. (14) and Soong et al. (15) for the systematic identification of loci for alternative splicing of voltage-gated calcium channel genes. Here we briefly describe the method. Pairs of primers were used to amplify 5 amplicons covering overlapping regions of the C terminus of the Ca$_\alpha$1.3$\gamma$ subunit. The size of the PCR products ranged from 515 to 638 bp, spanning between 2 and 4 exons. We used 1 lot of rat brain cDNA libraries (Mar-
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Data were acquired using pClamp9 software (Molecular Devices), and analyzed and fitted using GraphPad Prism IV software and Microsoft (Seattle, WA) Excel. Data are expressed as mean ± S.E. Statistical analysis was performed using paired or unpaired Student’s t test. Current-voltage (I-V) curve relationships were obtained by step depolarization from a holding potential of −100 mV to various test potentials. I-V curves were fitted according to Equation 1: $I = G_{max}(V-F_{rev})(1 + exp[(V-V_{1/2act})/k_{1,act}])$, where $G_{max}$ is the maximum conductance of the cell, $E_{rev}$ is the reversal potential, $V_{1/2act}$ is the voltage for half-maximal activation, $k_{1,act}$ is the slope of Boltzmann function, and $n$ is the number of tested cells. Steady-state inactivation data and CDI were fitted to Equation 2: $amp1 = (1-amp2)/(1 + exp[(V-V_{1/2inact})/SF1]) + amp2/(1 + exp[(V-V_{1/2inact})/SF2])$, where $amp1$ is the initial current amplitude, $amp2$ is the final current amplitude, $V$ is the membrane potential of the conditioning pulse, $V_{1/2inact}$ is the potential for half-inactivation, and $SF$ is the slope factor. Activation data were fitted according to Equation 3: $G/G_{max} = F_{low}/(1 + exp[(V_{1/2,low} - V)/k_{low}]) + (1 - F_{low})/(1 + exp[(V_{1/2,high} - V)/k_{high}])$, where $G$ is the tail current, $G_{max}$ is the tail current evoked by a depolarization to +120 mV, $F_{low}$ is the fraction of the low threshold component, $V_{1/2}$ is the membrane potential of the test pulse, $V_{1/2,low}$, $V_{1/2,high}$, $k_{low}$, and $k_{high}$ are the half-activation potentials and slope factors for the low and high threshold components and $V_{1/2act}$ was calculated when $G = 0.5 G_{max}$.

RESULTS

Generation and Characterization of Ca$_V$1.3 Splice Variant-specific Antibodies—In the C terminus of Ca$_V$1.3, expression of longer isoform Ca$_V$1.3$_{42}$ is mediated by exon skipping of exon 42A in the pre-mRNA (8). The presence of exon 42A predicts a $\alpha_1$-subunit containing C termini 500 amino acids shorter than exon 42-containing subunits (GenBank accession number AF370010) (Fig. 1A). Although qualitative RT-PCR showed brain tissue expression of both Ca$_V$1.3 splice isoforms, with predominant exon 42 mRNA levels in both mouse and human (9), not much is known about differential protein expression due to lack of antibodies that differentiate between exons 42 and 42A. We therefore raised specific polyclonal antibodies against the Ca$_V$1.3$_{42}$ and Ca$_V$1.3$_{42A}$ channels. For Ca$_V$1.3$_{42A}$ channels, exons downstream of exon 42A are not encoded as such and, therefore, a GST fusion to a 27-amino acid sequence encoded by exons 45 and 46 (Fig. 1) generated against a GST fusion to a 27-amino acid sequence encoded by exons 45 and 46 (Fig. 1). The resulting antibody was denoted pAb_Ca$_V$1.3$_{42A}$. In both cases, antibodies were affinity purified before use.

To establish the specificity of pAb_Ca$_V$1.3$_{42}$ and pAb_Ca$_V$1.3$_{42A}$, we analyzed membrane proteins extracted from wild type and Ca$_V$1.3$^{-/-}$ knock-out mouse brain tissue via Western blotting. A single protein band with the predicted size of ~250 kDa was observed for Ca$_V$1.3$_{42}$ in the wild type (Fig. 1B, left) and ~180 kDa was observed for Ca$_V$1.3$_{42A}$ (Fig. 1B, middle), but was absent in Ca$_V$1.3$^{-/-}$ knock-out mouse. In addi-

The amplicons were subcloned into pGEMp$^\text{TM}$-T Easy vector and transformed into DH10B E. coli cells. A total of 237 colonies were picked as templates for nested PCR using the exon-specific primers listed in supplemental Table S1. The different splice combinations were differentiated based on their distinct migration patterns in 3% agarose gels (supplemental Fig. S2D). To verify the accuracy of gel analysis, plasmids extracted from representative colonies were sent for DNA sequencing.

To characterize their functional properties, the splice variations in the C terminus were substituted into the full-length wild type Ca$_V$1.3$_{42}$ and constructs Ca$_V$1.3$_{42A}$, Ca$_V$1.3$_{43S-2}$, Ca$_V$1.3$_{444}$, and Ca$_V$1.3$_{48S}$ were generated. The Ca$_V$1.3$_{444}$ (deleted exon 41) has exon 41 alternatively spliced out, thus generating a truncated protein that is different from Ca$_V$1.3$_{2}$ (contains exon 41). The Ca$_V$1.3$_{43S-2}$ (truncated exon 43) is subjected to alternative splicing at the region just after PCRD, thus generating a truncated protein with an intact PCRD. The Ca$_V$1.3$_{444}$ (deleted exon 44) has exon 44 alternatively spliced out, generating an in-frame protein that lacks exon 44. The Ca$_V$1.3$_{48S}$ construct (alternative 3’ splice acceptor site in exon 48) has a portion of exon 48 spliced out, generating an in-frame protein that lacks the first 45 amino acids of exon 48.

Electrophysiological Recordings and Data Analysis—Whole cell patch clamp recordings were used to characterize the wild type long form Ca$_V$1.3$_{42}$ and short form Ca$_V$1.3$_{42A}$, as well as the splice variants: Ca$_V$1.3$_{444}$, Ca$_V$1.3$_{43S-2}$, Ca$_V$1.3$_{444}$, and Ca$_V$1.3$_{48S}$. The Ca$^{2+}$ currents were recorded at room temperature using the whole cell patch clamp electrophysiology technique from transiently transfected mammalian human embryonic kidney 293 (HEK 293) cells according to methods described previously (17, 18). Outward K$^+$ currents were blocked by Cs$^+$ in the internal and external solutions. Cells were transiently transfected with wild type long form Ca$_V$1.3$_{42}$, short form Ca$_V$1.3$_{42A}$, or the splice variants, Ca$_V$1.3$_{444}$, Ca$_V$1.3$_{43S-2}$, Ca$_V$1.3$_{444}$ or Ca$_V$1.3$_{48S}$, rat $\beta_2a$ and $\alpha_\delta$ subunits using the standard calcium phosphate transfection method. $I_{Ba}$ or $I_{Ca}$ were recorded at room temperature using the whole cell patch clamp technique 48–72 h after transfection. For whole cell patch clamp recording, the internal solution (patch-pipette solution) contained the following (in mM): 138 Cs-MeSO$_4$, 5 CsCl, 0.5 EGTA, 10 HEPES, 1 MgCl$_2$, 2 mg/ml of Mg-ATP, pH 7.3 (adjusted with CsOH), 290 mosmol with glucose. The external solution contained the following (in mM): 100 HEPES, 140 tetraethylammonium methanesulfonate, 5 BaCl$_2$, or 5 CaCl$_2$ (pH adjusted to 7.4 with CsOH and osmolality to 290–310 with glucose). Pipettes, of 1.5–2.5 megohm resistance, were used. Whole cell currents, obtained under voltage clamp with an Axopatch 200B amplifier (Molecular Devices, Union City, CA), were filtered at ~5 kHz and sampled at ~50 kHz, and the series resistance was typically <5 megohm after >70% compensation. A P/4 protocol was used to subtract on-line the leak and capacitive transients.
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Multiple bands were detected with commercial pAb_Ca_\(\alpha_{1.3}\) antibody (Fig. 1B, right) in the 250 to 150 kDa range, indicating the expression of different Ca_\(\alpha_{1.3}\) channel splice isoforms of different sizes. Visual inspection revealed that Ca_\(\alpha_{1.3}\) channels with a longer C terminus appear to be the dominant species.

In the CA3 region of the hippocampus, a similar immunostaining pattern was observed for both pAb_Ca_\(\alpha_{1.3}\) and pAb_Ca_\(\alpha_{1.3,42A}\) antibodies (Fig. 1C). Strong labeling of both Ca_\(\alpha_{1.3}\) and Ca_\(\alpha_{1.3,42A}\) proteins was restricted mostly to the pyramidal cell soma in the stratum pyramidale (SP) (Fig. 1C, left panels). Some interneurons in the CA3 region are also immunostained for Ca_\(\alpha_{1.3}\) and Ca_\(\alpha_{1.3,42A}\) proteins. The most dense labeling was observed in the cell soma and proximal dendrites for both cell types, with diminishing intensity in the distal dendritic regions. Bassoon, a presynaptic vesicle marker, stained the stratum lucidum, a mossy fiber recipient layer of the CA3 subfield.

Similar Localization of Both Ca_\(\alpha_{1.3}\) and Ca_\(\alpha_{1.3,42A}\) Proteins in Brain and Spinal Cord Slices—Using polyclonal antibodies pAb_Ca_\(\alpha_{1.3}\) and pAb_Ca_\(\alpha_{1.3,42A}\), we mapped the expression patterns of Ca_\(\alpha_{1.3}\) splice variants in the mouse brain and spinal cord tissues (Fig. 2). Stronger staining was observed for both splice variants in the septum region, the cerebellar Purkinje cells, the paraventricular thalamic nucleus, the paraventricular hypothalamic nucleus, the ventral medial hypothalamus, the dorsal lateral geniculate nucleus, and some nuclei from the brain stem including hypoglossal nucleus, dorsal motor nucleus of vagus, and locus coeruleus. A weaker staining was observed in the bed nucleus of stria terminalis, the central amygdaloid nucleus, and the lateral preoptical area. In the spinal cord, immunostaining with both pAb_Ca_\(\alpha_{1.3}\) and pAb_Ca_\(\alpha_{1.3,42A}\) antibodies were confined to neurons in the gray matter, and similar staining patterns were observed in all animals examined. The staining was present at all spinal levels of the gray matter, as shown in a representative coronal section.
from the lumbar segment (Fig. 2). The staining was most intense at the ventral horn motor neurons, with some small neurons stained in the intermediate zone and in the dorsal horn as well. In the ventral horn neuron, both soma and the proximal dendrites were strongly stained.

Identification and Correction of Cloning Error in Rat CaV1.342 Clone—Alternative splicing in human CaV1.3 results in CaV1.342A channels with more pronounced activation of calcium current at negative voltages and faster inactivation due to enhanced CDI (9). However, this diminished CDI of the CaV1.342 isoform was not replicated in rat clones, and was attributed to a single valine to alanine switch in its distal carboxyl tail (Fig. 3A, highlighted purple) (19). Sequencing analysis of the PCR fragment amplified with primers flanking exons 48–49 revealed GTC (coding for valine) in both rat brain (Fig. 3B) and rat heart cDNA (data not shown), instead of GCC (coding for alanine) at position 2123 in rat CaV1.342 clone (Fig. 3B).

The amplified fragment was cloned into cloning vector pGEM®-T Easy, and sequencing analysis for the correct clone was performed against rat CaV1.3 mRNA (GenBank accession number NM017298) before subcloning into rat CaV1.342 clone. We systematically investigated the biophysical properties of both $I_{Ba}$ and $I_{Ca}$ for the corrected clone CaV1.3A2123V and CaV1.342A as illustrated in Fig. 3, C–E, and Tables 1–3. The
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**TABLE 1**

Comparison of $I_{Ca}$ electrophysiological properties of CaV1.3 channels containing long form (CaV1.3A2123V), short form (CaV1.342A), and splice variants ΔA41, 43S-2, ΔA44, and 48S

| Construct        | $V_{rev}$ (mV) | $V_{1/2 inact}$ (mV) | $k_{inact}$ | $V_{1/2 act}$ (mV) | $k_{act}$ | $G_{max}$ (mV) | $k_{act}$ | $V_{1/2 inact}$ (mV) | $k_{inact}$ |
|------------------|----------------|----------------------|-------------|-------------------|-----------|----------------|-----------|-------------------|-------------|
| CaV1.3A2123V     | 49.0 ± 0.61    | -23.3 ± 0.49         | -8.03 ± 0.49| -10.50 ± 0.95     | 15.13 ± 0.91| 16.3 ± 0.49   | 7.39 ± 0.50| -44.24 ± 0.47     | 5.90 ± 0.40 |
| CaV1.342A        | 41.59 ± 0.70   | -32.1 ± 0.49         | -8.03 ± 0.49| -10.50 ± 0.95     | 15.13 ± 0.91| 16.3 ± 0.49   | 7.39 ± 0.50| -44.24 ± 0.47     | 5.90 ± 0.40 |
| CaV1.341         | 41.59 ± 0.80   | -32.1 ± 0.49         | -8.03 ± 0.49| -10.50 ± 0.95     | 15.13 ± 0.91| 16.3 ± 0.49   | 7.39 ± 0.50| -44.24 ± 0.47     | 5.90 ± 0.40 |
| CaV1.34S-2       | 42.04 ± 0.74   | -34.3 ± 0.49         | -8.03 ± 0.49| -10.50 ± 0.95     | 15.13 ± 0.91| 16.3 ± 0.49   | 7.39 ± 0.50| -44.24 ± 0.47     | 5.90 ± 0.40 |
| CaV1.34G        | 42.04 ± 0.74   | -34.3 ± 0.49         | -8.03 ± 0.49| -10.50 ± 0.95     | 15.13 ± 0.91| 16.3 ± 0.49   | 7.39 ± 0.50| -44.24 ± 0.47     | 5.90 ± 0.40 |

*p < 0.001, compared to CaV1.3A2123V (unpaired t test). Values shown are mean ± S.E.

*p < 0.01, compared to CaV1.3A2123V (unpaired t test). Values shown are mean ± S.E.

Current trace profile for CaV1.3_42A displayed a slower inactivating $I_{Ba}$ compared with the corrected clone CaV1.3A2123V (Fig. 3C, gray traces), but showed an early and much pronounced inactivation of $I_{Ca}$ (Fig. 3C, black traces). In the I-V curves, CaV1.3_42A showed a pronounced shift in the hyperpolarized direction, indicating a more negatively activating channel in
both Ba\(^{2+}\) and Ca\(^{2+}\) (Fig. 3D). This was also reflected in a pronounced hyperpolarized shift in voltage for half-maximal activation, \(V_{1/2 \text{act}}\), by 11.22 mV in Ba\(^{2+}\) (see Table 1) and 10.87 mV in Ca\(^{2+}\) (see Table 2) with respect to CaV1.3A2123V (both \(p < 0.001\); unpaired \(t\) test). This negative shift was because of the significant decrease in slope of activation, \(k_{\text{act}}\), by 2.43 mV in Ba\(^{2+}\) (see Table 1) and 2.57 mV in Ca\(^{2+}\) (see Table 2). From the profiles of the exemplary traces in Fig. 3C, it is clear that the corrected CaV1.3A2123V clone is not deprived of CDI, it is 2-fold smaller than the short variant CaV1.3a22 (\(f_{-10} = 300\) ms, CaV1.3A2123V 0.35 ± 0.03, \(n = 14\); CaV1.3a22 0.75 ± 0.04, \(n = 13\)). Hence, correction of the valine to alanine in position 2123 in the rat CaV1.3a22 clone was sufficient to replicate the diminished CDI observed in human CaV1.3a22. This confirms previous experiments by Liu et al. (19) that the valine residue is crucial for interaction of the CTD with the calcium-sensing apparatus in the proximal C terminus and could greatly repress the CDI of CaV1.3 channels.

**Identification and Expression of Novel C-terminal Spliced CaV1.3 Channels**—Transcript scanning of CaV1.3 from rat brain cDNA revealed alternative splicing in five loci on the C terminus, resulting in at least eight splice variants, namely CaV1.3a22, CaV1.3a22a, CaV1.3IQ4, CaV1.3a41, CaV1.3a45, CaV1.3a45s, CaV1.3a44 and CaV1.3n85. Altogether there are three alternative splice acceptor sites, two exon skipings, one cassette exon, and one intron retention. In all cases, use of the canonical "gt...ag" splice junctions in the alternative forms was preserved (supplemental Fig. S3). To quantify the frequency of various C-terminal splice isoforms, a first round RT-PCR using primers situated on flanking e37 and 3’ UTR was performed. This was followed by colony PCR of bacterial colonies containing plasmids subcloned with PCR products of various splice variants. Agarose gel electrophoresis of PCR products isolated from each colony will help identify and enumerate the frequency of occurrence of each splice variant. Of 237 bacterial colonies screened, CaV1.3a45s appeared to be the most abundant (44%), followed by CaV1.3a42 (16.1%), CaV1.3IQ4 (13.5%), CaV1.3a44 (9.7%), CaV1.3a45s (6.3%), CaV1.3a43s-2 (5.1%), and CaV1.3a44i (4.6%) (supplemental Table S1). We next characterized the four novel splice variants, which alters the C terminus of CaV1.3 (Fig. 4A). CaV1.3a41 deletes exon 41 and truncates the C terminus beyond the EF-hand, whereas CaV1.3a45s deletes the constitutive "intron" in exon 43 and removes the DCRD of CaV1.3a22. Length altering CaV1.3a44 and CaV1.3a45s splice variants shortened the length between PCRD and DCDR, and may hence remove the secondary structures critical for CTM interaction with the proximal C terminus. All four splice variants may modulate CTM function and affect inactivation properties of the channel. To characterize these splice variants, the sequence alterations in these exons were genetically engineered into the C terminus sequence of CaV1.3a22 constructs, and compared against this predominant reference isoform. One other novel splice variant that alters the C terminus of CaV1.3, CaV1.3a45i was further characterized in the accompanying article (16).

**Alternative Splicing in CaV1.3 C-terminal Modulates CDI**—The current trace profile for CaV1.3a45s exhibited a slower inactivating \(I_{\text{rev}}\) compared with CaV1.3a2123V, or the other three splice variants (Fig. 4, B–E, gray traces), but showed an early and much pronounced inactivation of \(I_{\text{act}}\) (Fig. 4, B–E, black traces). All splice variants except for CaV1.3a44i activated at more negative potentials as observed by the hyperpolarized shift in \(V_{1/2 \text{act}}\) in both Ba\(^{2+}\) (CaV1.3a45s-2 by 8.01 mV; CaV1.3a44 by 9.45 mV; CaV1.3a45s by 11.22 mV; see Fig. 4, F–I, and Table 1) and Ca\(^{2+}\) (CaV1.3a43s-2 by 7.07 mV; CaV1.3a44 by 12.29 mV; CaV1.3a45s by 10.32 mV; see Fig. 4, J–M, and Table 2) with respect to CaV1.3a2123V (\(p < 0.001\); unpaired \(t\) test). This shift is predominately caused by a decrease in the slope of activation \(k_{\text{act}}\) (see Tables 1 and 2). The hyperpolarized shift observed in CaV1.3a44s was not observed in the equivalent human clone (9), possibly due to unidentified amino acid differences between the rat and human clones at the regions governing gating. CaV1.3a44i showed a slight, although statistically significant (\(p < 0.001\); unpaired \(t\) test), depolarized shift in \(V_{1/2 \text{act}}\) by 3.96 mV in Ba\(^{2+}\) and 0.91 mV in Ca\(^{2+}\), with a slight increase in the slope of activation (\(k_{\text{act}}\) increased by 0.66 in Ba\(^{2+}\)). As expected, deletion of exon 41 in CaV1.3a41i, which removed the entire IQ motif, resulted in a very small degree of inactivation by Ca\(^{2+}\) even at 300 ms (\(f_{-10} = 0.15 ± 0.02\); see Fig. 4N). Alternative splicing of exon 43 in CaV1.3a45s-2, which caused a frameshift and deletion of CTM, exhibited a very pronounced CDI, comparable with that of CaV1.3a42 (\(f_{-10} = 0.74 ± 0.03\); see Fig. 4O). Skipping of exon 44 and the use of the alternative acceptor site on exon 48 results in two splice variants, CaV1.3a44s and CaV1.3n85 which retained CTM and exhibit smaller \(f_{-10}\) than CaV1.3a42 (\(f_{-10} = 0.64 ± 0.02\); CaV1.3n85 0.68 ± 0.02; see Fig. 4, P and Q). It is plausible that the secondary structure changes brought about by these splice variants may affect the efficacy of interaction between the DCDR and the calcium-sensing apparatus.

**Hyperpolarized Shifts in Activation and Inactivation Properties of CaV1.3 Splice Variants**—To enable a more accurate assessment of the voltage activation of these channels, we analyzed the tail currents (\(G\)) obtained at the end of a short depo-

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**Table 2**

Comparison of \(I_{\text{rev}}\) electrophysiological properties of CaV1.3 channels

| Construct | \(V_{\text{rev}}\) \(\text{mV}\) | \(I-V\) | \(k_{\text{act}}\) |
|-----------|-----------------|--------|----------------|
| CaV1.3A2123V | 62.12 ± 0.72 | –12.28 ± 0.53 | –9.39 ± 0.30 |
| CaV1.3a22 | 58.61 ± 0.73 | –23.15 ± 0.39 | –6.82 ± 0.27 |
| CaV1.3a22a | 62.21 ± 0.90 | –11.37 ± 0.71 | –9.61 ± 0.40 |
| CaV1.3a45s | 57.89 ± 1.18 | –22.60 ± 0.74 | –8.1 ± 0.48 |
| CaV1.3a45i | 62.12 ± 0.72 | –12.28 ± 0.53 | –9.39 ± 0.30 |
| CaV1.3n85 | 58.61 ± 0.73 | –23.15 ± 0.39 | –8.10 ± 0.48 |

\(p < 0.001\), compared to CaV1.3A2123V (unpaired \(t\) test). Values shown are mean ± S.E.

**Table 3**

Comparison of the kinetics of recovery from inactivation in Ba\(^{2+}\)

| Construct | \(t_{1/2} \text{rev}\) | \(t_{1/2} \text{act}\) |
|-----------|-----------------|---------------|
| CaV1.3A2123V | 0.61 ± 0.12 | 7.74 ± 2.97 |
| CaV1.3a22 | 0.76 ± 0.22 | 20.84 ± 7.45 |
| CaV1.3a22a | 0.63 ± 0.11 | 7.65 ± 2.42 |
| CaV1.3a44 | 0.44 ± 0.09 | 7.08 ± 2.86 |
| CaV1.3a44s | 0.40 ± 0.09 | 24.07 ± 10.05 |
| CaV1.3n85 | 0.53 ± 0.12 | 8.51 ± 3.69 |

\(p < 0.001\), compared to CaV1.3A2123V (unpaired \(t\) test). Values shown are mean ± S.E.
larizing pulse to various potentials. In addition, to determine the inactivation properties of the channels under steady-state conditions, transfected cells were held at various potentials for 15-s and currents evoked before and after the inactivating pulse were compared. The data obtained are displayed in Fig. 5, A–D, and Table 1.

**FIGURE 4.** Current-voltage relationships of CaV1.3 alternatively spliced variants. A, schematic representation of alternatively spliced CaV1.3 channel constructs. The channel backbone consists of CaV1.3 (GenBank accession number: D38101, white box), whereas the cytosolic tail consists of CaV1.3 long form (CaV1.3long) or alternatively spliced variants IQΔ1, 43S, 43S-2, and 48S (black). The stop codons for IQΔ1, 43S, and 43S-2 are indicated by black and white filled circles. Numbering follows the CaV1.3 amino acid sequence. B–E, representative I\(_{\text{Ba}}\) (gray) and I\(_{\text{Ca}}\) (black) traces during depolarization to 10 mV for alternatively spliced constructs Δ41, 43S-2, Δ44, and 48S. The I\(_{\text{Ba}}\) and I\(_{\text{Ca}}\) traces were scaled to enable comparison between the two profiles. Current scales were drawn for both I\(_{\text{Ba}}\) (gray) and I\(_{\text{Ca}}\) (black). The time scales for each I\(_{\text{Ba}}\) and I\(_{\text{Ca}}\) pair are the same. F–I, normalized I-V plots for I\(_{\text{Ba}}\) of alternatively spliced constructs Δ41, 43S-2, Δ44, and 48S. The curves were fitted with the equation described under “Experimental Procedures.” In parentheses are the number of cells recorded. J–M, same as F–I, but for I\(_{\text{Ca}}\). N–Q, calcium-dependent inactivation of current through alternatively spliced variants Δ41, 43S-2, Δ44, and 48S. The fraction of peak current, I\(_{\text{peak}}\), remained at time intervals of 300 ms upon depolarization for I\(_{\text{Ba}}\) and I\(_{\text{Ca}}\). f value indicates the strength of the CDI. The curves are visual fits of the values plotted to facilitate comparison. The number of cells recorded are given in parentheses.
Alternative Splicing in Ca$_V$1.3 C Terminus

Just as in the I-V analyses, the novel splice variants produced a hyperpolarized shift in $V_{1/2}$act of the tail current analyses compared with Ca$_V$1.3$^{4123V}$. The changes observed here were also significant ($V_{1/2}$act, Ca$_V$1.3$^{341V}$, $\bar{\Delta}12.68 \pm 0.57$, $n = 7$; Ca$_V$1.3$^{43S-2V}$, $\bar{\Delta}32.24 \pm 0.57$, $n = 9$; Ca$_V$1.3$^{43S+44V}$, $28.75 \pm 0.88$, $n = 8$; Ca$_V$1.3$^{48SV}$, $28.72 \pm 0.65$, $n = 10$; $p < 0.001$ unpaired t test). The activation slope factors for the splice variants were significantly smaller than Ca$_V$1.3$^{4123V}$. In addition, all four splice variants had small shifts in $V_{1/2}$act, recovery at the early part of the slow phase, where $\sim 81\%$ of channels were recovered by 2 s. Ca$_V$1.3$^{44V}$ started with rapid recovery near the early part of the slow phase, reaching $\sim 60\%$ recovery of channels by 20 ms, respectively (Fig. 5G). It has a steeper slope than Ca$_V$1.3$^{4123V}$ in the fast phase ($\tau_p$ Ca$_V$1.3$^{4123V}$, 7.74 $\pm$ 2.91 ms; Ca$_V$1.3$^{43S-2V}$, 24.07 $\pm$ 10.05 ms; $p = 0.06$, unpaired t test) and a gentler slope in the slow phase ($\tau_p$ Ca$_V$1.3$^{43S-2V}$, 0.40 $\pm$ 0.09 ms; $p = 0.10$, unpaired t test). Recovery of Ca$_V$1.3$^{44V}$ channels reached $\sim 87\%$ by 2 s in the slow phase, slightly greater than the $\sim 82\%$ recovery of Ca$_V$1.3$^{4123V}$ channels. Ca$_V$1.3$^{48SV}$, like Ca$_V$1.3$^{4123SV}$, began with rapid recovery at the early part of the slow phase, where $\sim 63\%$ of the channels had recovered by 100 ms (Fig. 5H). Despite the slightly more gentle slope than Ca$_V$1.3$^{4123SV}$ at the late slow phase, $\sim 86\%$ of channels were recovered by 2 s.

Deletion of Exon 41 in Ca$_V$1.3 Decreases Current Density—Alternative splicing of Ca$_V$1.3 in the C terminus results in short Ca$_V$1.3$^{42\underline{A}A}$ with a gross increase in current density (9). We hence measured the current density from the novel Ca$_V$1.3 C terminus splice variants and compared the density against Ca$_V$1.3$^{4123V}$. The current density of Ca$_V$1.3$^{42\underline{A}A}$ was $3.3$ times smaller than Ca$_V$1.3$^{4123V}$ (see Fig. 5F, Ca$_V$1.3$^{42\underline{A}A}$: 88.39 $\pm$ 12.48 pA/pF, Ca$_V$1.3$^{4123V}$: 26.53 $\pm$ 4.22 pA/pF; $p < 0.001$, respectively (Fig. 5A).
Alternative Splicing in Ca$_{\text{v}}$1.3 C Terminus

Krussel-Wallis test, followed by Dunn’s multiple comparison post test). Even when depolarized to −40 mV, the current density was also significantly smaller (Ca$_{\text{v}}$1.3A2123V: 8.15 ± 1.29 pA/pF, Ca$_{\text{v}}$1.3A411: 2.06 ± 0.30 pA/pF; p < 0.001, Krussel-Wallis test, followed by Dunn’s multiple comparison post test). Ca$_{\text{v}}$1.3A411 also sustained a small, but not statistically significant decrease in current density compared with Ca$_{\text{v}}$1.3A2123V (see Fig. 5K). In contrast, however, Ca$_{\text{v}}$1.3A43S,2 and Ca$_{\text{v}}$1.3A48S channels displayed small increases in current density to Ca$_{\text{v}}$1.3A2123V (see Fig. 5, J and L), but these were not statistically significant.

DISCUSSION

Here we present systematic identification and functional characterization of the biophysical properties and subcellular localization of alternative splice variants in the C terminus of Ca$_{\text{v}}$1.3. Such study has not been described for Ca$_{\text{v}}$1.3 channels before. Alternative splicing of the C terminus affects the modulatory effects of CTM in Ca$_{\text{v}}$1.3A2, providing functional diversity to Ca$_{\text{v}}$1.3 channels in rat brain tissues. Activation at negative voltages is one hallmark of Ca$_{\text{v}}$1.3 channels crucial for adequate neurotransmitter release in inner hair cells and pacemaker function in the sinoatrial node (20) and in neuronal excitability (6, 21). Deletion of CTM and shortening the intervals between PCRD and DCRD causes a hyperpolarized shift of $V_{\text{r}}$ by about 10 mV by decreasing the slope factor of the activation curves.

The functional properties of Ca$_{\text{v}}$1.3A2 and Ca$_{\text{v}}$1.3A2A channels have been studied in previous reports. Activation of calcium current through Ca$_{\text{v}}$1.3A2A channels was more pronounced at negative voltages, and inactivation was faster because of enhanced CDI. It was determined by Ca$_{\text{v}}$1.3 channel truncations that the modulatory activity was restricted to the last 116 amino acids of the C terminus and involves interaction of a DCRD with a PCRD as shown by mutation analysis (9). Gating properties of these Ca$_{\text{v}}$1.3C116 channels, which lack the DCRD, were reversed by co-expression of corresponding C-terminal peptide C116. Intramolecular protein interaction in the C-terminal peptide C116. Intramolecular protein interaction in the C-terminal domain of a DCRD with a PCRD as shown by mutation analysis (9). Two studies using Ca$_{\text{v}}$1.3A2 and Ca$_{\text{v}}$1.3A2A have shown that CaBP family members (CaBP1 through CaBP5) are restricted to retinal rod and cone cells (28). Hence, it might be interesting to examine the modulation of these CaM-like proteins in the brain on electrophysiological properties of Ca$_{\text{v}}$1.3 C-terminal splice isoforms.

Stronger expression of both splice isoforms was selectively detected in specific brain regions, namely the paraventricular hypothalamic nucleus and locus coeruleus of the brain stem (Fig. 2), where Ca$_{\text{v}}$1.3 were shown to be selectively stimulated by Bay K8644 in Ca$_{\text{v}}$1.2DHP$^{-/-}$ mice (23). Surprisingly, weaker staining of both splice isoforms were observed in the central amygdala, the bed nucleus of the stria terminalis, and the lateral preoptical area, where significant Bay K8644-induced Fos expression was observed (23). It was speculated that selective stimulation of Ca$_{\text{v}}$1.3 channels restricts neuronal activation to a specific set of mainly limbic, hypothalamic and brainstem areas, which are associated with brain functions concerning integration of emotion/depression-related behavior (24). It would be interesting to characterize the expression profile of Ca$_{\text{v}}$1.3 splice variants in these specific regions, especially because quantitative PCR using the TaqMan gene expression assay in our accompanying article (16) has shown ~60% Ca$_{\text{v}}$1.3A2 and ~5% Ca$_{\text{v}}$1.3A2A mRNA expression in amygdala, leaving 35% unaccounted C-terminal splice variants. Although quantitative differences at the mRNA levels may not necessarily correlate with protein expression differences, it allows us to speculate on the channel isoform primarily responsible for the observed activity-dependent responses.

Our work has shown that besides exons 42 and 42A, alternative splicing in other exons of the C-terminal generates three classes of functionally distinct Ca$_{\text{v}}$1.3 channel variants. Alternative splicing of exon 41 causes either deletion of only the IQ domain or the entire exon 41, causing a premature stop codon and Ca$_{\text{v}}$1.3 channel without the IQ domain pertinent to CaM interaction and hence CDI. Downstream of exon 42, alternative splicing occurring in exons 43, 44, and 48 could either delete the CTM or shorten the distance between PCRD and DCRD. The implications of retaining just the PCRD when alternative splicing occurs at exon 43 (Ca$_{\text{v}}$1.3A43S) were examined in our accompanying article (16). A similar degree of hyperpolarized shift in activation and inactivation properties was observed in splice variant Ca$_{\text{v}}$1.3A43S-2 with robust CDI that closely resembles that of Ca$_{\text{v}}$1.3A22A. Alternative splicing in exons 44 and 48 are in-frame. The resultant secondary protein structure (i.e. α-helices) between PCRD and DCRD and hence, efficacy of interaction between these two domains may be altered, causing a less robust CDI. Alternative splicing in the C terminus generates Ca$_{\text{v}}$1.3 channel isoforms with a spectrum of electrophysiological properties. Differences in the inactivation pattern of Ca$_{\text{v}}$1.3 C-terminal splice isoforms could underlie different shapes or firing rates of action potentials, as observed in different types of neurons (25). Interaction of Ca$_{\text{v}}$1.3 with the dihydropyridine (DHP) antagonist is state-dependent, and alternative splicing of the Ca$_{\text{v}}$1.3 C-terminal should also have a strong impact on the efficiency of DHP inhibition as this correlates with the amount of Ca$_{\text{v}}$1.3 channels inactivated (4, 5).

Two studies using Ca$_{\text{v}}$1.3A2 and Ca$_{\text{v}}$1.3A2A have shown that CaM-like Ca$^{2+}$-binding proteins (CaBPs) are integral subunits and modulators of Ca$_{\text{v}}$1.3 channels, suppressing its inactivation (26, 27). In both studies, CaBP1 strongly inhibits inactivation of $I_{\text{Ca}}$ and $I_{\text{Ba}}$ through interactions with the IQ domain and N-terminal of Ca$_{\text{v}}$1.3, whereas CaBP4 appears to modulate CDI differentially depending on the Ca$_{\text{v}}$1.3 isoform used. CaBP family members (CaBP1 through CaBP5) have varying expression patterns, and only CaBP1 and CaBP2 can be expressed as multiple, alternatively spliced variants in the brain and retina, whereas CaBP5 through CaBP5 are restricted to retinal and cone cells (28). Hence, it might be interesting to examine the modulation of these CaM-like proteins in the brain on electrophysiological properties of Ca$_{\text{v}}$1.3 C-terminal splice variants, especially given the differential modulation on CDI and current densities observed in Ca$_{\text{v}}$1.3A41, Ca$_{\text{v}}$1.3A44, and Ca$_{\text{v}}$1.3A48, which could potentially expand the baseline CaM regulatory profile of the various Ca$^{2+}$ signaling proteins in the nervous system. Besides CaBPs, Ca$_{\text{v}}$β subunits were found to increase membrane expression of Ca$_{\text{v}}$1.2 channels via ubiquitination and stability of the calcium channel complex (29). Two endoplasmic reticulum retention motifs were identified in the prox-
imal C-terminal region of these CaV1.2 channels, which are highly conserved in CaV1.3 channels. Although co-expression of the Ca\(\alpha\)\(\beta\) subunit did not alter retention of the CaV1.2 C terminus in the endoplasmic reticulum, it mediated a marked increase in cell surface and total protein expression of the full-length channel (29). In CaV1.3\(_{4A4}\), both endoplasmic reticulum retention motifs are removed via alternative splicing and could alter the cell surface expression and hence decrease the current density. For exploring the mechanism by which splicing alters calcium currents, we further characterized ON-gating currents (\(Q_{ON}\)) via depolarizing cells to positive potentials at which no ionic inward and outward currents were observed (see supplemental Fig. S4). \(Q_{ON}\) reflects the capacitative voltage-sensor movements upon depolarization during channel gating. Alternative splicing of exon 42A removes the CTM and results in a \(~2\)-fold greater \(Q_{ON}\) compared with CaV1.3\(_{42}\), whereas removal of exon 41 causes a \(~4\)-fold decline in \(Q_{ON}\) compared with CaV1.3\(_{42}\). This suggests that alternative splicing of exon 41 may decrease current density via down-regulation of functional surface expression of the channels. However, although the current densities of CaV1.3\(_{42S}\) and CaV1.3\(_{44}\) are not significantly altered as compared with CaV1.3\(_{42}\), their \(Q_{ON}\) values were significantly decreased as well.

In the brain, CaV1.3 channels couple neuronal activity to transcriptional events, mediating long term potentiation in the amygdala and participating in the consolidation of fear memory (30). The CTM in the long CaV1.3\(_{42}\), variant may be suitable for longer lasting Ca\(^{2+}\) signals triggered by stronger depolarization inducing CREB phosphorylation and synaptic plasticity, and the ITTL motif in CTM is crucial for interaction with the macromolecular signaling complex formed by Shank (31). It would be interesting to determine whether alternative splicing in the C terminus of CaV1.3 alters its predominantly soma-dendritic localization or its synaptic clustering, and whether substitution of the ITTL motif may alter its coupling with Shank and other adaptor proteins at the postsynaptic density to influence pCREB signaling.

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