J Mol Neurosci (2009) 39:40–48
DOI 10.1007/s12031-008-9159-2

Alternative Splicing in the Synaptic Protein Interaction Site of Rat $Ca_v2.2$ (α_{1B}) Calcium Channels: Changes Induced by Chronic Inflammatory Pain

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Received: 1 October 2008 / Accepted: 27 October 2008 / Published online: 6 January 2009

Abstract Voltage-gated N-type Ca^{2+} channels play a central role in regulation of neurotransmitter release. The II–III linker of N-type calcium channel α_{1B} subunit (exons 17–21 in rats) contains synaptic protein interaction (synprint) site, which plays a pivotal role in efficient neurotransmission. Using RT-PCR analysis of rat hippocampus and lumbar spinal cord transcripts, the expressions of $Ca_v2.2$ exon 18a and a novel $Ca_v2.2$ splice variant, which were detected in this study, have been investigated, and they showed tissue-specific pattern. The new variant contains a large deletion in II–III linker that produces a stop codon, which is predicted to produce a two-domain-truncated channel. To study the effect of inflammatory pain on the expression pattern of these variants, animals were treated with intraplantar formalin, and the amount of splice variants in lumbar spinal cord was measured. The results showed that chronic inflammatory pain increases the $Ca_v2.2$ mRNA levels lacking exon 18a and decreases the amount of full-length variants, which do not have any deletion. Determining different $Ca_v2.2$ splice variants in rat nervous system and the impact of inflammatory pain on the splicing pattern suggest a possible regulatory role for calcium channel alternative splicing.

Keywords Ca^{2+} channels · Alternative splicing · Pain · Spinal cord · Hippocampus · Rat

Funding: This research was funded by the Neuroscience Research Center, Shahid Beheshti University (M.C.), Tehran, Iran.

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Introduction

Voltage-gated calcium channels mediate rapid and voltage-dependent entry of calcium into many types of neural, muscle, and endocrine cells. Calcium entry contributes to a number of physiological functions, including neurotransmitter and hormone secretion, muscle contraction, and release of and modulation of other membrane ion channels (Reuter 1983; Tsien 1983).

Electrophysiological analysis categorizes native calcium currents into two major classes that differ in their voltage activation properties: high-voltage-activated calcium channels (L-, N-, P/Q- and R-types) and low-voltage-activated calcium channels (T-type; Hofmann et al. 1994). An individual channel is composed of multiple subunits (α_1, β, α_2/δ, γ). The α_1 subunit, known as the main pore-forming subunit, determines the major functional properties of the channel (Dunlap et al. 1995). The pore-forming α_1 subunit contains as a basic motif a tetrameric association of four domains each containing a series of six transmembrane α-helical segments, numbered S1–S6, which are connected by both intra- and extracellular loops (Dubel et al. 1992). Ten genes encoding Ca^{2+} channel α_1 subunits have been

cloned and classified systematically according to their characteristics (Ertel et al. 2000).

Splicing of α_{1B} mRNA also represents an important mechanism for optimization of neurotransmitter release in different regions of the nervous system (Lin et al. 1997, 1999; Pan and Lipscombe 2000). Alternative splicing occurs most often in cytoplasmic domains of Ca_v2 channels, where the effects on channel gating are hard to predict (Thaler et al. 2004).

N-type Ca^{2+} channels are essential for transmission of nociception. These channels are highly concentrated in presynaptic terminals of small-diameter myelinated and unmyelinated nociceptors that synapse in laminae I and II of the dorsal horn where they control neurotransmitter release (Holz et al. 1988; Maggi et al. 1990). Because of the importance of N-type calcium channels in the release of pronociceptive neurotransmitters and pain transmission in spinal cord, they are attractive targets for therapeutic intervention concerning chronic and neuropathic pain conditions (Snutch 2005).

There are several reports about the importance of exon 37a/ exon 37b splice site in the activity of N-type calcium channels in nociceptors (Bell et al. 2004; Castiglioni et al. 2006; Altier et al. 2007). Here, we focus on the alternative splicing in the linker of domains II and III of $Ca_v2.2$ and its role in pain transmission. An important part of this linker is called synaptic protein interaction site (synprint site), which associates with proteins of the presynaptic vesicle docking and release machinery (Sheng et al. 1994, 1996; Catterall 1999). An exon cassette called exon 18a in the $Ca_v2.2$ gene encoding 21 amino acids in the synprint site preferentially expresses in monoamine neurons in the rat brain (Ghasemzadeh et al. 1999). The insertion of this exon cassette induces a shift in the steady-state inactivation curve to more depolarized potentials without affecting the kinetics or voltage dependence of channel activation (Coppola et al. 1994).

Kaneko and colleagues in 2002 reported two novel human $Ca_v2.2$ calcium channel variants ($Ca_v2.2$ Δ1and $Ca_v2.2$ Δ2). These two splice variants contain large sequence deletions in the linker of domains II and III, including the synprint site. The voltage dependence of steady-state inactivation of these variants was shifted to more depolarized potentials, and recovery from inactivation occurred more rapidly than that of the full-length channel.

In the present study, the expression pattern of $Ca_v2.2$ synprint site splice variants in rat hippocampus and lumbar spinal cord is described. A novel splice variant, which contains a stop codon as a result of the deletion of large portions of $Ca_v2.2$ cytoplasmic II–III linker, was detected. Besides, the impact of chronic inflammatory pain on the alternative splicing of $Ca_v2.2$ II–III linker in lumbar spinal cord is reported.

Materials and Methods

Animals

In this study, male Wistar rats weighing 200–250 g were used. Animals were housed in groups of four and had free access to food and drinking water with a 12-h light/dark cycle. The injections were performed between 10:00 and 12:00 each day to reduce any variation between groups. Animal research protocols were approved by the Committee of Ethics in Research, Shahid Beheshti University (M.C.) and conformed to NIH guidelines for use of animals in research. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Animals were grouped in four groups of rats (four to six in each). To induce chronic inflammatory pain, 50 μl of 2% formalin was injected subcutaneously (s.c.) into the hind paws (Javan et al. 2005). The first group received single-dose formalin in the dorsal region of right paw. In the second group, injections were repeated for 4 days in different regions of the paws (first day: right/dorsal; second day: left/ventral; third day: right/ventral; fourth day: left/dorsal). For the other two control groups (single dose and repeated one), saline was injected to hind paw with the same protocol for formalin injections.

Gene Expression Study

One hour after the single-dose injections (formalin or saline) and 24 h after the last injections in the second and fourth groups (repeated ones), animals were anesthetized using CO_2 and then decapitated. Extracted samples of hippocampus and lumbar spinal cord were preserved in liquid nitrogen and then used for isolation of total cellular RNAs. The protocol was a modification of guanidine isothiocyanate–phenol–chloroform method using RNX^+ reagent (CinnaGen, Iran). Samples were used immediately in reverse transcription reaction using oligo-dT primer and M-MuLV reverse transcriptase (Fermentas) based on the manufacturer's protocol. The reactions were incubated at 42°C for 60 min and then inactivated at 70°C for 10 min.

To determine the possible position of Δ1 deletion (a 1,146-base-pair deletion between nucleotides 2412 and 3559 in humans, which corresponds to a 382-amino-acid deletion between Arg756 and Leu1139 in rats), two human (GeneBank accession number M94172) and rat (GeneBank accession number M92905) mRNAs were aligned, and the location of this deletion in the rats was guessed according to its site in humans. The $Ca_v2.2$ e18a and $Ca_v2.2$ Δ1 primers were designed flanking the regions of e18a insertion and Δ1 deletion, respectively. In addition, two other primers were used specifically for β-actin as an internal standard. The sequence of primers used was as

follows: $Ca_v2.2$ e18a forward: 5′-GTCAGCAAAGGCATG TTTTCATC-3′; $Ca_v2.2$ e18a reverse: 5′-GTGGTGATTTC GAGTTTCCTTATC-3′; $Ca_v2.2$ Δ1 forward: 5′-GCCAAT CAGAAG CTTGCTCTTC-3′; $Ca_v2.2$ Δ1 reverse: 5′-CGATGACCACAAGAATCACCATC-3′; β-actin forward: 5′-CCCAGAGCAAGAGAGGCATC-3′; β-actin reverse: 5′-CTCAGGAGGAGCAATGATCT-3′.

A semi-quantitative PCR method (Maron et al. 2002) was used for studying the expression of $Ca_v2.2$ splice variants in rat hippocampus and lumbar spinal cord. The reaction parameters and number of PCR cycles were adjusted to obtain a condition with linear relation between the number of PCR cycles and PCR products and with linear relation between the initial amount of cDNA template and PCR product. The PCR reactions were incubated at 95°C for 5 min, followed by 35 thermal cycles (60 s at 94°C, 60 s at 58°C, 60 s at 72°C) for $Ca_v2.2$ e18a and $Ca_v2.2$ Δ1 primers and 25 ones for β-actin primers. The final cycle was followed by a 10-min extension step at 72°C. Upon performing PCR reaction, PCR products were analyzed on 2% low melting point agarose (Roche, Germany) gel and bands were quantified by densitometry using Lab works analyzing software (UVP, UK).

To determine the Δ1 deletion position in rats, band was sequenced and compared with the NCBI genomic DNA reference sequence (NC-005120), mRNA reference sequence (NM-147141), and protein reference sequence (NP-671482) using Sequencher software (Gene Code Corporation, Ann Arbor, MI, USA).

Data Analysis

The values of $Ca_v2.2$ containing and lacking e18a along with $Ca_v2.2$ full length and deleted band densities and β-actin ones were obtained from gel analysis using Lab works software. Band densitometry was calculated and expressed as $Ca_v2.2$ 18a/β-actin and $Ca_v2.2$ Δ1/ β-actin, respectively. Data have been presented as mean ± SEM. The averages of different groups were compared using one-way ANOVA followed by Tukey's post test to check statistical significance. $P<0.05$ was considered as significant difference.

Results

Determination of Splice Variants in $Ca_v2.2$ Synprint Site

After performing PCR using specific primers for detection of e18a insertion and Δ1 deletion, two amplified fragments in each reaction were separated by gel electrophoresis.

As is shown in Fig. 1a, two 343 and 406 bp bands were observed in the PCR products of $Ca_v2.2$ e18a primers. In the PCR products of $Ca_v2.2$ Δ1 primers, two different bands were detected and showed a novel deletion in this site. The bands separated by gel electrophoresis were 1,232 and 278 bp in size (Fig. 1b).

The Δ1 deletion band detected in gel electrophoresis was different in size with what we expected according to the Δ1 deletion previously reported in human (Kaneko et al. 2002). Sequencing of Δ1 deletion band determined the deletion site as shown in Fig. 2.

The sequence of deleted variant showed a stop codon in the synprint site as a result of Δ1 deletion, which is predicted to produce a truncated protein with only 758 amino acids.

Tissue-Specific Expression Pattern of $Ca_v2.2$ Splice Variants

As shown in Fig. 3, there are significant differences between expression pattern of 18a exon cassette in hippocampus and lumbar spinal cord. In hippocampus, most of the α_{1B} subunits lack e18a, but in lumbar spinal cord, approximately half of the channels contain this exon encoding 21 amino acids. The expression pattern of Δ1 deletion is also different in these two regions. $Ca_v2.2$ full-

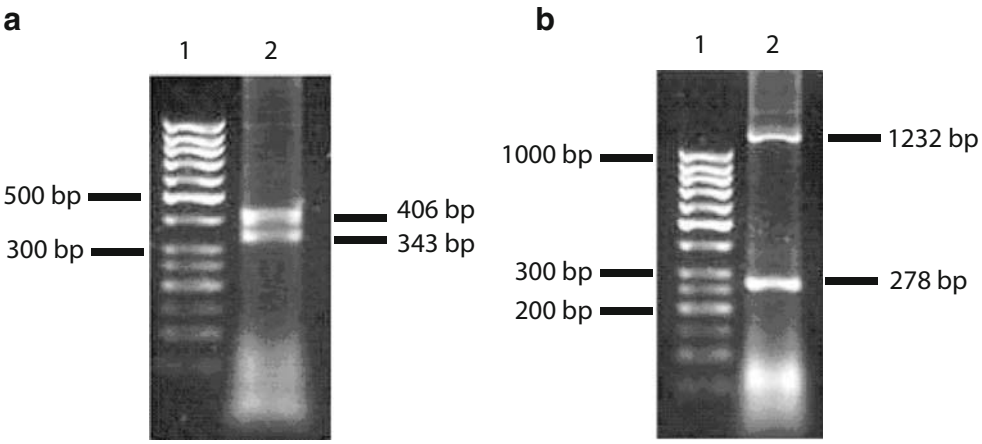

Figure 1 Gel electrophoresis pattern of PCR products for two different alternative splicing. **a** PCR products of $Ca_v2.2$ e18a primers. *Lane 1*= 100 bp DNA ladder, *lane 2*= $Ca_v2.2$ containing (406 bp) and lacking (343 bp) e18a variants in the intact rats. **b** PCR products of $Ca_v2.2$ Δ1 primers. *Lane 1*=100 bp DNA ladder, *lane 2*=$Ca_v2.2$ full length (1,232 bp) and Δ1 deleted (278 bp) variants in the intact rats

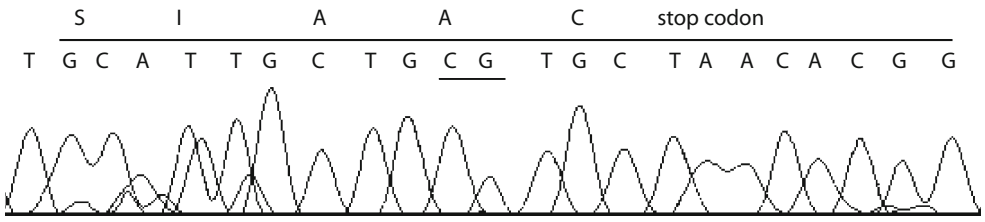

Figure 2 The sequence of Δ1 deletion in the rat synprint site. Δ1 deletion occurs after the $C_{c.2270}$ and $G_{c.3281}$, and a 21-kb intron is spliced out. As a result, a stop codon arises, which was predicted to produce a truncated protein with 758 amino acids

length variants without Δ1 deletion in the lumbar spinal cord are more abundant than hippocampus. In other words, deletion occurred in few $Ca_v2.2$ variants in the lumbar spinal cord, but it happened in hippocampus more frequently. As can be observed, the β-actin band densities did not show a significant difference in these two regions.

Effects of Inflammatory Pain on the Expression Pattern of $Ca_v2.2$ Splice Variants in Lumbar Spinal Cord

Following formalin-induced chronic inflammatory pain, some changes were detected in the expression patterns of $Ca_v2.2$ splice variants. Although β-actin band density did not change after saline or formalin treatment (Fig. 4a), the band density of e18a lacking variant increased after single-dose administration of formalin ($P<0.05$; Fig. 4b). The amount of $Ca_v2.2$ e18a containing variant was unchanged after the single-dose or chronic administration of formalin.

As shown in Fig. 4c, both single-dose and chronic administration of formalin reduced the amount of full-length variant without Δ1 deletion but did not have any effect on Δ1 deletion variant ($P<0.05$).

Discussion

The consequences of regulated alternative splicing among the genes that comprise voltage-gated calcium channels permits specialization of channel function, optimizing calcium signaling in different regions of the brain and in different cellular compartments (Lipscombe et al. 2002). Recent studies focus on N-type Ca^{2+} channels because of their importance in control of neurotransmitter release in different types of neurons.

In mammalian neurons, the synprint site, located within domain II–III linker of $Ca_v2.2$ subunits, associates with proteins of presynaptic vesicle docking and release machinery such as syntaxin1, SNAP25, Synaptotagmin 1, and CSP (Catterall 1999). The focus of this study was on exon cassette 18a encodes 21 aa located between exons 18 and 19 in $L_{II–III}$ of $Ca_v2.2$ genes, and the Δ1 deletion occurs at this site. Besides the expression pattern of these variants in hippocampus and lumbar spinal cord, the effects of chronic inflammatory pain on their expression levels have been investigated.

Novel $Ca_v2.2$ Splice Variant in Rats with a Stop Codon in Synprint Site

To study whether the synprint site deletion reported previously in humans (Kaneko et al. 2002) also exists in rats, human and rat cDNAs were aligned, and the possible region of Δ1 deletion was marked in the rat cDNA. The deleted band,

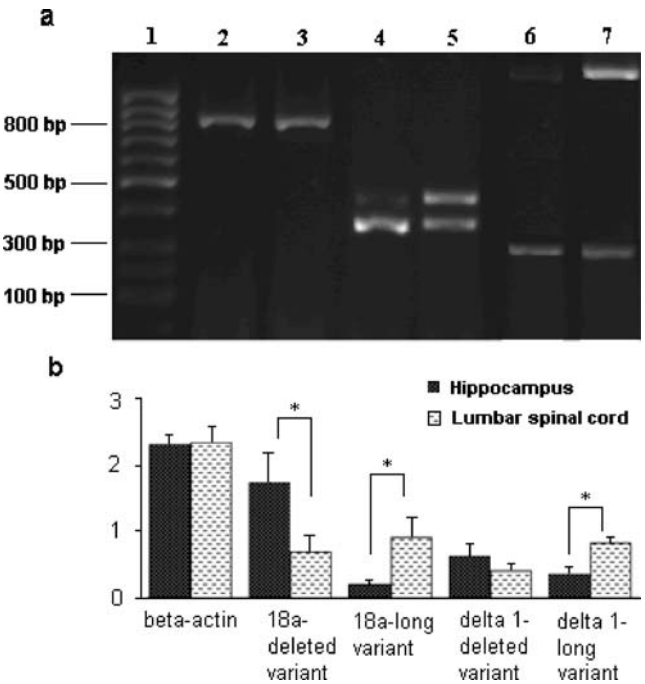

Figure 3 Comparative expression pattern of different $Ca_v2.2$ splice variants in rat hippocampus and lumbar spinal cord. **a** Gel electrophoresis of β-actin, $Ca_v2.2$ e18a, and $Ca_v2.2$ Δ1 PCR products. *Lane 1*=100 bp DNA ladder, *lane 2*=β-actin in hippocampus, *lane 3*=β-actin in lumbar spinal cord, *lane 4*=$Ca_v2.2$ e18a containing (406 bp) and lacking (343 bp) variants in hippocampus, *lane 5*=$Ca_v2.2$ e18a containing and lacking variants in lumbar spinal cord, *lane 6*=$Ca_v2.2$ full length (1,232 bp) and Δ1 deletion (278 bp) variants in hippocampus, *lane 7*=$Ca_v2.2$ full length and Δ1 deletion variants in lumbar spinal cord. **b** Quantitative comparison of β-actin, $Ca_v2.2$ e18a, and $Ca_v2.2$ Δ1 PCR products' band densities in hippocampus and lumbar spinal cord. Data have been shown as mean ± SEM; *$P<0.05$

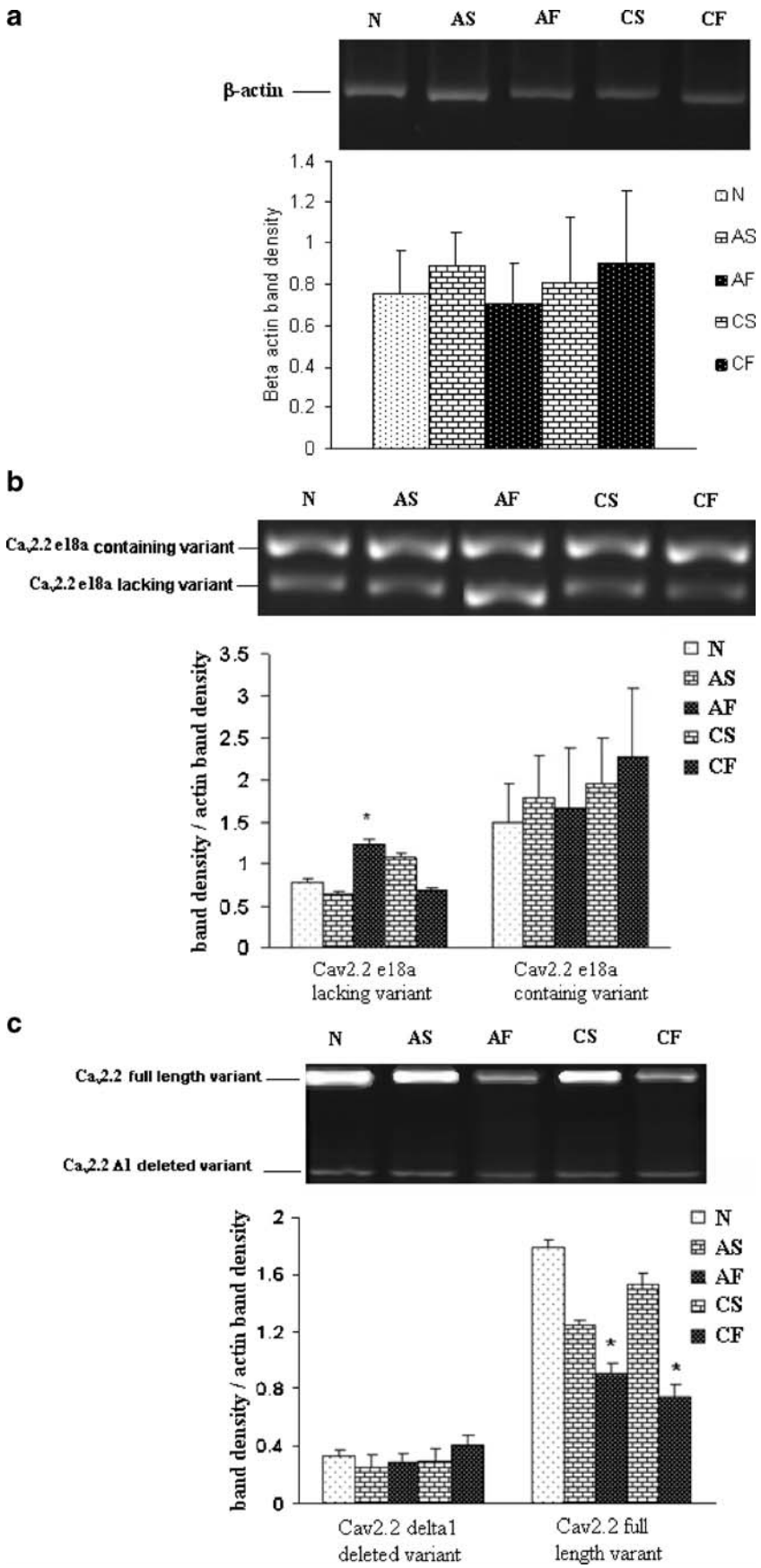

Figure 4 Effects of chronic inflammatory pain on $Ca_v2.2$ alternative splicing in rat lumbar spinal cord. **a** Effects of chronic inflammatory pain on β-actin expression. As it can be observed, no significant changes occur after saline or formalin treatment. **b** Effects of chronic inflammatory pain on the $Ca_v2.2$ e18a alternative splicing. The $Ca_v2.2$ e18a lacking variants levels increase after single-dose injection of formalin. No changes can be detected in the other situations. **c** Effects of chronic inflammatory pain on the $Ca_v2.2$ Δ1 deletion alternative splicing. The $Ca_v2.2$ full-length variants levels decrease both after single-dose and chronic administration of formalin but not after saline injections. *N* normal, *AS* acute saline, *AF* acute formalin, *CS* chronic saline, *CF* chronic formalin. Data have been shown as mean ± SEM; **P*<0.05 when compared with normal rats

which was amplified by the PCR, was sequenced and showed a novel deletion in rat synprint site (Fig. 2).

As is shown in Fig. 5a, as a result of Δ1 deletion, a large portion of $Ca_v2.2$ II–III linker is deleted, which is predicted to produce a truncated protein in rats. This deletion is similar to one previously reported in human by Kaneko et al. (2002). Full-length II–III linker region of $Ca_v2.2$ variants is encoded by six exon cassettes in humans (17 to 22) and five in rats (17 to 21; Fig. 5b). In the case of Δ1 deletion in rats, an intron with GT-AG sites (nucleotides between $C_{c.2270}$ and $G_{c.3281}$) is spliced out and deletes a large part of synprint site (exon 19 and 20). Interestingly a stop codon arises as a result of this deletion, which is predicted to produce a truncated protein with 758 amino acids containing two domain (the complete α_{1B} subunit contains 2,336 amino acids in rats). To the best of our knowledge, there is just one report (published in abstract form) of N-type calcium channel splice variant, which has a stop codon in the middle of the structure (Mittman and Agnew 2000). So, it will be very interesting to detect the protein that resulted from Δ1 deletion variants in different region of CNS and investigate whether such a structure, which is similar to half part of the complete α_{1B} subunit, is functional or not.

There are some reports about recombinant truncated voltage-gated Ca^{2+} channels. Ahern et al. (2001) cut the four-domain DHPR α_{1S} in the cytoplasmic linker between domain II and III and expressed fragments separately. Although excitation–contraction was not coupled, charge movement was detected in the I–II domain that is expressed alone. A two-domain I–II construct of neuronal α_{1A} Ca^{2+} channels also recovered charge movements like $\alpha_{1S\ I–II}$ domain. Moreover, they characterized the recombinant two-domain form ($\alpha_{1A\ I–II}$) and showed that co-expression of the $\alpha_{1A\ I–II}$ significantly reduces the current amplitude of $\alpha_{1A}/\beta_{1a}/\alpha_2\delta$ channel via competition for β subunit (Arikkath et al. 2002). Similar results about α_{1A} were reported by Raike et al. (2007). Interestingly, it has been demonstrated that a truncated form of α_{1B} protein, similar in structure to the $\alpha_{1A\ I–II}$, inhibits the current generated by the full-length α_{1B} protein (Raghib et al. 2001). Suppression requires interaction between a truncated construct and a related full-length channel, which activates a component of the unfolded protein response to suppress translation (Page et al. 2004). However, little is known about the tissue specificity and expression regulation of these truncated variants.

Region-Specific Expression Pattern of $Ca_v2.2$ Splice Variants

Alternative splicing in cytoplasmic II–III loop of the α_{1B} mRNAs is differentially regulated in hippocampus and lumbar spinal cord. As it has been shown in Fig. 3, β-actin expresses in these two regions equally, but there are some differences in the $Ca_v2.2$ splice variants levels. In the hippocampus, most of the channels lack e18a, and exon skipping is a frequent event in this region, while approximately half of the channels contain this exon in lumbar spinal cord. Because of the physiological, biophysical, and pharmacological differences of e18a containing and e18a lacking variants (Pan and Lipscombe 2000; Rott and Horn 2003; Thaler et al. 2004), these variations can cause tissue specificity through the nervous system.

Δ1 deletion variants levels are similar in hippocampus and lumbar spinal cord, but full-length variants are more abundant in lumbar spinal cord in comparison with hippocampus. If the expected Δ1 deletion variant produces a truncated protein, which has negative effect on full-length channel synthesis (Raghib et al. 2001; Page et al. 2004), more full-length variants in lumbar spinal cord means more functional channels in this region. These data suggest that regional regulation of $Ca_v2.2$ alternative splicing can cause structural and functional diversity in CNS, and it will be a good target for designing new drugs, which affect specific region of CNS with attenuated side effects on the other parts.

Alternative Splicing Modulation by Chronic Inflammatory Pain

Generally speaking, little is known about the mechanisms which regulate expression of alternative spliced exons of α_{1B} in different regions of nervous system. To study the impact of chronic inflammatory pain on $Ca_v2.2$ alternative splicing, formalin pain was selected as a commonly used model of persistent pain (Dubuisson and Dennis 1977; Abbott et al. 1995; Damas and Liegeois 1999; Fu et al. 2001), and lumbar spinal cord was extracted from treated animals as an important site in pain transmission to analyze the changes in $Ca_v2.2$ mRNA levels. Besides, lumbar spinal cord contains N-type channels in its dorsal horn terminals associated with medium-sized Aδ fibers and small-diameter C-type fibers, both of which are responsible for pain perception (Gribkoff 2006). There were several reports about behavioral and histological consequences of formalin as pain inducer (Matthias and Franklin 1992; Damas and Liegeois 1999; Fu et al. 2001) and its mechanism of function (Lee et al. 2008), so we just focused on molecular events that occurred after chronic inflammatory pain induction.

As is shown in Fig. 4, several changes were observed in the expression pattern of e18a variants and Δ1 deletion variants in formalin-treated animals, while β-actin band densities, which were used as an internal standard, did not change following formalin administration (Fig. 4a).

Single-dose formalin injection caused an increase in $Ca_v2.2$ mRNA levels lacking exon 18a but no changes in

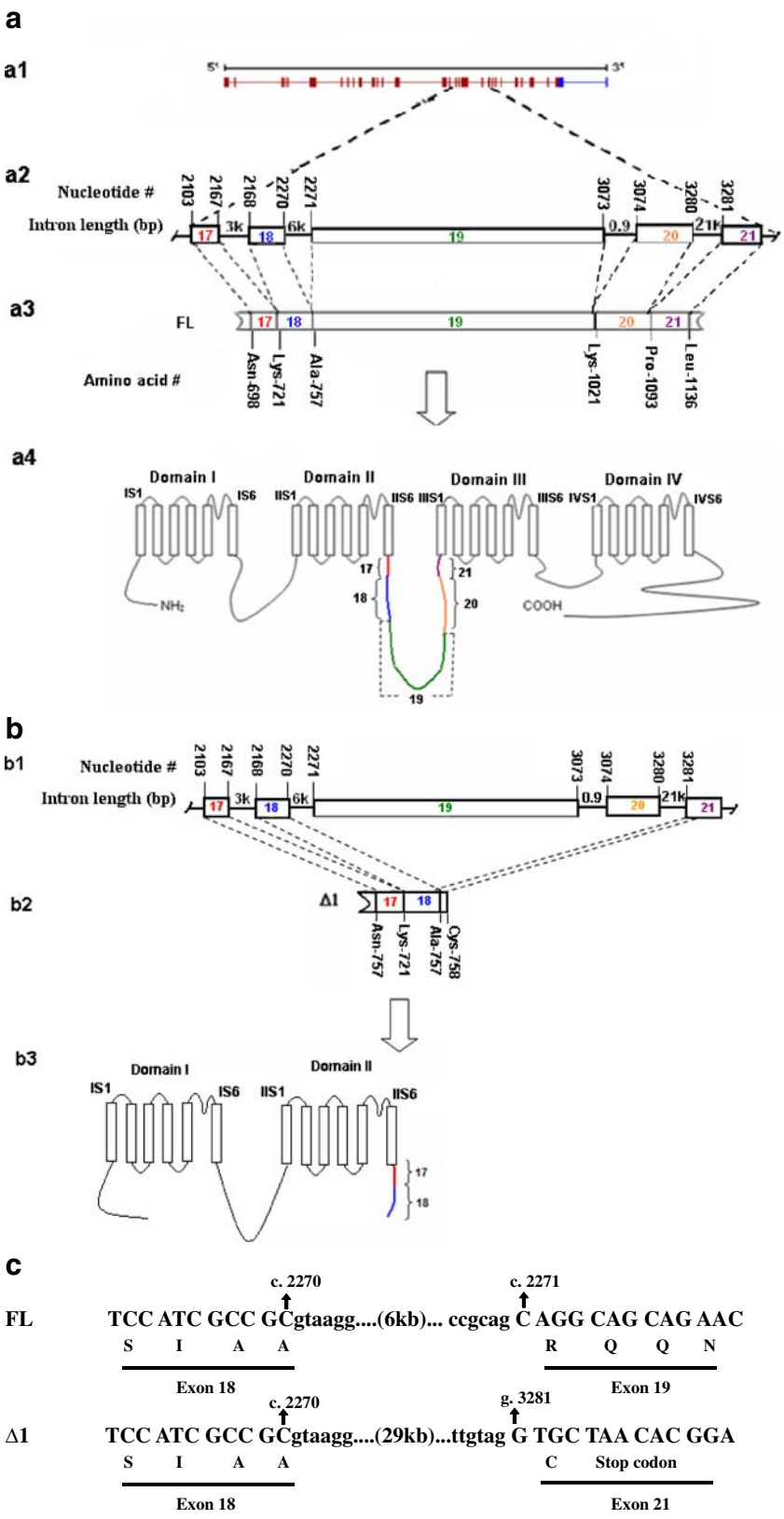

Figure 5 Alternative splicing in the cytoplasmic linker of domains II and III of rat $Ca_v2.2$. **a** An alternative splicing in II–III linker of $Ca_v2.2$, which produces a full-length variant; *a1* N-type calcium channel alpha 1B subunit ($Ca_v2.2$) gene structure; *a2* schematic diagram of the gene region encoding for II–III linker. Full-length II–III linker is encoded by exons 17 to 21; *a3* mRNA sequence corresponding to full-length II–III linker of $Ca_v2.2$; *a4* schematic structure of rat N-type calcium channel with full-length II–III linker showing the parts encoded by exons 17 to 21. **b** Alternative splicing in II–III linker of $Ca_v2.2$ that deletes a large part of mRNA encoding for II–III linker and leads to an expected two-domain calcium channel; *b1* schematic diagram of the gene region encoding for II–III linker; *b2* mRNA sequence corresponding to II–III linker, which is produced as a result of Δ1 deletion. Exons 19 and 20 with the flanking introns are spliced out, and a stop codon arises; *b3* two-domain calcium channel structure, which is expected to be produced as a result of Δ1 deletion in rats. **c** Genomic DNA sequence at boundary sites of $Ca_v2.2$ full length and deleted variants. In the case of Δ1 deletion, a large intron (29 kb) with consensus gt-ag terminals is spliced out. As a result, a stop codon arises, which is expected to produce a truncated protein with 758 amino acids. *IS1* First α-helical segment of domain I, *IS6* sixth α-helical segment of domain I, *IIS1* first α-helical segment of domain II, *IIS6* sixth α-helical segment of domain II, *IIIS1* first α-helical segment of domain III, *IIIS6* sixth α-helical segment of domain III, *IVS1* first α-helical segment of domain IV, *IVS6* sixth α-helical segment of domain IV

the level of $Ca_v2.2$ e18a containing variants. According to the electrophysiological differences between these two variants (Pan and Lipscombe 2000; Thaler et al. 2004), this alteration may lead to channels with attenuated excitability and fewer exocytosis events. The other alterations that have been investigated were reduction in the full-length variant levels, both after single and repeated injections of formalin (Fig. 4c). These alterations may be the result of change in splicing or overall decrease in $Ca_v2.2$ mRNA levels, but in both cases, they cause reduction in the amount of functional channels necessary for neurotransmitter release. It will be useful to investigate changes in other important sites for pain transmission and perception such as dorsal root ganglia to determine the exact impact of chronic inflammatory pain on N-type calcium channels. Expanding our knowledge about the molecular effects of chronic inflammatory pain in nervous system can help us to design new drugs to control the pain.

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