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Control of alternative pre-mRNA splicing by Ca^{++} signals

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Abstract

Alternative pre-mRNA splicing is a common way of gene expression regulation in metazoans. The selective use of specific exons can be modulated in response to various manipulations that alter Ca^{++} signals, particularly in neurons. A number of splicing factors have also been found to be controlled by Ca^{++} signals. Moreover, pre-mRNA elements have been identified that are essential and sufficient to mediate Ca^{++}-regulated splicing, providing model systems for dissecting the involved molecular components. In neurons, this regulation likely contributes to the fine-tuning of neuronal properties.

Keywords

Alternative splicing; Ca^{++} signal; Pre-mRNA element; Splicing factor; Gene expression; Neuron; Brain; Ion channel; Neurodegenerative disease; Protein diversity; Fine-tuning

1. Introduction

Ca^{++} signalling is important for a variety of cellular processes such as muscle contraction, hormone secretion and gene transcription. In neurons, Ca^{++}-regulation of gene expression is known to be critical for the long-lasting changes associated with learning and memory [1]. Accumulating evidence supports that Ca^{++} signalling also controls the alternative splicing of precursor messenger RNA (pre-mRNA) transcripts (Fig. 1). Examples of this regulation have been identified in myotube cultures, endocrine cells, and mostly, in neuronal cells (Table 1).

The complex structure and function of the brain underlie the requirement for a high level of proteomic complexity. How can the human genome, with only the approximately 25,000 protein-coding genes [2], encode enough diverse proteins to achieve the high level of complexity to ensure, for example, that individual neurons are precisely “wired” with many others and that their functions are finely tuned? It is now clear that protein diversity can be greatly increased through alternative pre-mRNA splicing [3–5]. This is a particularly common way for the regulation of gene expression among neuronal genes and likely contributes to the fine-tuning of neuronal functions [6–8].

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The pre-mRNA of most eukaryotic genes contains both expressed regions (exons) that will be included in the mature mRNA and regions that will be excluded (called intragenic regions, i.e. introns) [9]. Removal of introns and joining of exons happen in the cell nucleus through pre-mRNA splicing [10–12]. After introns were discovered, Walter Gilbert predicted in his short essay "Why genes in pieces" in *Nature*: "…the splicing need not be a hundred percent efficient; changes in sequence can alter the process so that base pairing and splicing occurs only some of the time" [9]. Soon afterward, alternative transcripts from a common precursor RNA were discovered [13]. Since then, more and more eukaryotic genes have been found to undergo alternative splicing [6,14,15]. Currently, the estimated percentage of human genes with alternative splicing is more than 50% [16,17]. In some cases, a single pre-mRNA transcript has the potential to generate a large number of protein isoforms, for example, transcripts of the *Dscam*, *Neurexin* and *Slo* genes [4,18–38] (Fig. 2).

The consequences of alternative splicing include altered mRNA stability or subcellular localization and the addition or deletion of specific protein sequences. Functional differences among protein isoforms range from subtle modulations to on/off switches or antagonistic effects [39–41]. Defects in alternative splicing factors or aberrant inclusion of alternative exons can result in genetic diseases [42–47]. During the Cambrian "explosion" of metazoans, alternative splicing likely contributed to the expansion of proteomes [48]. Therefore, this is an important control in the transmission of genetic information from DNA to proteins. Currently with the huge amount of genomic information available from various genome sequencing projects, it is becoming imperative to understand how alternative splicing is controlled, particularly by cell signals, and to elucidate the role of this form of regulation in normal cell function and human genetic diseases.

Splicing happens in two transesterification steps within a large spliceosome complex containing more than 100 proteins assembled stepwise onto pre-mRNA introns [12,49]. During alternative splicing, the spliceosome assembly is altered so that a splice site (s) is optionally used depending on the cell type, developmental stage or sex, resulting in the inclusion or exclusion of alternative exon sequences in the mature mRNA (Fig. 1). Studies with tissue-or sex-specific exons identified both *cis*-acting pre-mRNA elements and *trans*-acting factors that control alternative splicing [15]. Depending on their location and effects on splicing, the RNA elements are called intronic or exonic splicing enhancers (ISE or ESE) or silencers (ISS or ESS). Many of these elements bind specific *trans*-acting factors (mostly proteins, but in some cases RNA) that enhance (enhancer) or inhibit (repressor) the assembly of the constitutive splicing factors. Well characterized examples of enhancer factors include most members of the arginine/serine-rich SR family that bind purine-rich ESE elements [50], and repressors include the heterogeneous ribonucleoprotein particle proteins hnRNP A1 and hnRNP I that bind purine-rich elements containing UAGG motifs and pyrimidine-rich elements containing UUCU, UCUU or CUCU motifs, respectively [51,52]. There are also elements/factors that function in a location-dependent way as either an enhancer or repressor, for example, the CA-repeat binding protein hnRNP L [53], the UGCAUG-binding proteins FOX-1 and -2 [54–61], and the YCAY-binding protein Nova-1 [62–66]. Binding of the *trans*-acting factors to the elements is thought to control mostly the early stages of spliceosome assembly before the first transesterification step [15]. For example, PTB prevents the binding of U2AF65, a factor of the E (early) complex, through competition or

looping out the target exons [67–69]. In mammalian systems, splicing regulation is generally dependent on the combinatorial effects of multiple pre-mRNA elements and *trans*-acting factors [70–72] (Fig. 1). The relative levels of positive and negative regulatory factors determine the generation of variant mRNA isoforms in specific cell types [73–75]. Brain-enriched splicing factors include Nova-1 and -2 [62–66], FOX-1 and -2 [54–61], PTBP2 (nPTB) and neuronal Hu proteins [76–83].

In addition to cell type, developmental stage and sex-specific regulation, alternative splicing can also be dynamically regulated in response to extracellular stimuli such as cytokines, hormones or neurotransmitters [24,84–88], adding a further dimension to the control of the flow of genetic information [86]. Examining the inducible alternative splicing events will help understand their roles in cell functions as well as provide systems where the dynamic changes of spliceosomal components can be tracked down to facilitate the mechanistic studies of splicing. Moreover, understanding the molecular basis of this process will help develop ways to reverse the aberrant splicing events in genetic diseases using extracellular factors. However, it is not clear in most cases how alternative splicing is controlled by external stimuli and intracellular signalling pathways. Ca^{++}-regulation of alternative splicing is among the regulatory events being intensively studied.

The following sections on the evidence for Ca^{++}-regulation of alternative splicing will be focused on the regulated alternative exons, the splicing factors and the pre-mRNA elements. It should be noted that both constitutive and alternative splicing are coupled with transcription and this topic has been covered in recent reviews [89–92]. Promoter-dependent regulation of alternative splicing by Ca^{++} stimuli has also been observed [84]. Readers are referred to these reviews and original papers for this aspect [84,89–92].

2. Alternative exons regulated by Ca^{++} signals

Table 1 lists some of the alternative exons whose inclusion in the mature mRNA can be changed upon stimulation by extra-cellular factors that activate Ca^{++} signalling pathways.

These factors include the ionophore ionomycin [93], the neurotransmitter glutamate or its analogue NMDA (*N*-methyl-$_D$-aspartate) [94–98], the muscarinic acetylcholine receptor agonist pilocarpine (inducing seizure) [99], the ionotropic glutamate receptor agonist kainate, the inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase thapsigargin [100–102], and depolarizing concentrations of KCl [84,98,101,103–106] (for a more complete list of the pharmacological agents and their descriptions, see Table 2). These stimuli can either promote or repress the inclusion of an exon (Table 1). Importantly, their effects on splicing can be inhibited by the L-type Ca^{++} channel blockers verapamil, nifedipine or nimodipine [95,96,102,103,107], by the NMDA receptor antagonist AP5 or MK-801 [84,95,96,103], by the Ca^{++}/calmodulin-dependent protein kinase inhibitors KN93 or KN62 [84,95,96,104,105], or by the cell permeable Ca^{++} chelator BAPTA-AM [101,108]. Together, these observations strongly support the regulation of alternative pre-mRNA splicing by variations in intracellular Ca^{++} levels.

Most of these regulated exons are in genes known to be important for neuronal functions. Examples include the neural cell adhesion molecule (NCAM) in axon guidance [102,109–113], the synapse-associated protein of 25 kDa (SNAP25), NMDA receptor type I (NMDAR1), BK and Ca^{++} channels and neurexin in synaptic function [8,96,108,114–123], the intracellular plasma-membrane calcium-pump isoforms 2 and 4 (PMCA2 and 4) and inositol triphosphate receptor type 1 (IP3R1) in Ca^{++} homeostasis [124–132], the caspase ICH-1 in neuronal survival/death [98,101,133], and the splicing factors Tra2 beta1 and Ania-6 in pre-mRNA splicing [99,106,134–136] (Table 1).

Inclusion of these alternative exons regulates the subcellular localization of proteins or their functions [25,26,119,120, 127–129,123,125,134,137–141], ranging from subtle changes such as current kinetics of ion channels to on or off switches in the sensitivity to protein kinases or hormones [26,114,139, 142–144] (Table 1). These observations suggest that the regulation of alternative splicing by Ca^{++} signals has effects on the expression of genes involved in a variety of cellular functions, from cell adhesion molecules at the synapse, ion channels in the cellular endoplasmic membrane, caspase in the cytosol as well as splicing factors inside the nucleus.

Of the various Ca^{++} signals that regulate splicing, those through the CaMK IV pathway have been the most intensively studied [104,105,107]. A prime example of the effect on splicing by this pathway is the BK channel STREX exon, one of the more than 10 alternative exons of the vertebrate *Slo* gene [19,21–35] (Fig. 2A). The BK channels contain both Ca^{++} and voltage-sensitive domains and are important in coupling Ca^{++} transients and electrical properties of excitable cells by participating in the repolarization and after hyperpolarization of action potentials [122] (Fig. 2B–D). These channels are important in neurotransmitter release [145], motor control [146], alcohol sensitivity [147–149], blood pressure and the fine-tuning of hearing frequencies [150,151]. The STREX-encoded peptide lies in the Ca^{++}-sensitivity domain at the COOH-terminal [30,152]. Inclusion of this exon confers higher voltage and Ca^{++} sensitivity on the channel [24,29–31,153], inhibition by PKA and oxidation [142,154], and sensitivity to glucocorticoids [143]. The exon is included in endocrine cells and neurons and enriched in the high frequency region of cochleae [22–24,84,107], contributing to the fine-tuning of hearing frequencies in birds and turtles [23,31]. Its inclusion is also regulated by steroid hormones in animals and by membrane depolarization in cultured cells [24,104,107,155–157]. Thus, the STREX exon inclusion is inducible by external stimuli and the regulation may modulate multiple properties of the BK channels.

CaMK IV regulation of the STREX exon inclusion has been examined in both cultured cells and in mice. In rat pituitary GH_3 cells and cerebellar neurons, addition of depolarizing concentrations of KCl leads to a decrease in transcripts containing the STREX exon [104,107] (Fig. 3). This effect can be specifically blocked by nifedipine [107], suggesting that L-type Ca^{++} channels mediate the depolarization-induced alternative splicing (Fig. 3). The splicing change is also completely blocked by KN93 [104], a CaM kinase inhibitor. Moreover, the repression of STREX exon inclusion is only seen with overexpressed constitutively active CaMK IV but not CaMK I or II [104]. Conversely, increased inclusion of the STREX exon was observed in the cerebella of CaMK $IV^{+/-}$ heterozygote mice

compared to that of the wild type [107]. These findings support the proposal that membrane depolarization activates the L-type Ca^{++} channels and CaMK IV to repress STREX exon inclusion.

Besides the *Slo* gene, similar regulation of the alternative splicing of NMDAR1 and other genes by membrane depolarization/CaMK IV has also been observed [84,104,105,107].

Another Ca^{++} signalling component calcineurin also appears to have a role in the control of alternative splicing. The inclusion of the 175 nt CII exon of the PMCA4 gene is reduced by KCl and NMDA in rat granule neurons [97]. This reduction can be prevented by FK506, a calcineurin inhibitor [97]. These data suggest that Ca^{++}-dependent protein phosphatases and dephosphorylation events are required for depolarization-induced splicing in these cells.

For a single exon, its regulation by Ca^{++} signals can be different, depending on cell types or Ca^{++} signal stimuli. For example, inclusion of the STREX exon is repressed by depolarization in GH_3 cells and cerebellar neurons but enhanced in cortical neurons [84,104,107]. In the *Ania-6* gene, the retention of intron 6′ is promoted by glutamate but repressed by KCl depolarization [94], although both glutamate and KCl induce Ca^{++} signals and activate CaM kinases [158]. Thus, the control of alternative splicing by Ca^{++} signals is cell- and Ca^{++} signal-inducer dependent. Since the pre-mRNA elements are the same in a cell or among different cell types, except when RNA editing occurs [159], it is possible that diverse factors upstream of the RNA elements are involved [73–75], as is the case in the control of gene transcription [158].

These examples of Ca^{++} signal-regulated alternative splicing are found in several species including chick, rat, mouse and human, suggesting that this regulation is evolutionarily conserved.

Taken together, these observations from many independent studies strongly support that Ca^{++} signals control alternative splicing. This regulation controls the expression of a wide range of genes likely involving diverse factors, and is evolutionarily conserved.

However, most of these observations did not provide direct evidence for the involvement of the splicing regulatory components including the essential splicing factors and *cis*-acting pre-mRNA elements. To address this, in the following two sections, I will review literature on the splicing factors and pre-mRNA elements that are targeted by Ca^{++} signals.

3. Splicing factors regulated by Ca^{++} signals

Of the *cis*-acting elements in alternative splicing, most that have been studied to date are bound by protein factors, although, as in constitutive splicing, RNA secondary structures and *trans*acting small RNAs have also been described [12,160–162]. Protein splicing factors reportedly regulated by Ca^{++} signals at various levels are listed in Table 3.

The protein level of splicing factors can be controlled by Ca^{++} signals. In some cases, it takes several hours for the splice variant mRNA to change significantly, and it has been shown that *de novo* protein synthesis is required [97,163,164]. Interestingly, the nuclear

level of the hnRNP A1 protein is increased in depolarized neurons in regulating the exon 21 (or called C1 cassette exon) of NMDAR1 [72,84]. The increase is expected to contribute to the exclusion of exon 21 upon depolarization. Therefore, control of the protein level of splicing factors by membrane depolarization and the downstream Ca^{++} signalling is one way for Ca^{++} signals to regulate alternative splicing.

The alternative splicing of splicing factors themselves can also be regulated by Ca^{++} signals. For example, the inclusion of the alternative exons of hnRNP H3 and RNPS1 is specifically repressed by depolarization in cortical neurons [84], in contrast to the inclusion of the hnRNP A1 alternative exon 8, which is barely changed. The Ania-6 protein (also called cyclin L1) contains arginine/serine-rich (RS) domains that are characteristic of SR protein splicing factors and regulates alternative splicing [136,165]. The retention of the intron 6′ of Ania-6 is controlled by glutamate and depolarization in striatal neurons [94,95]. In another example, the exon III of the Tra2 beta1 is repressed in the brain during pilocarpine-induced seizure [99]. Since the alternative splicing of both Ania-6 and Tra2 beta1 is likely to alter their splicing activity [94,134,136,166], their own splicing regulation by Ca^{++} stimuli is expected to alter their target gene splicing as well.

Relocalization of splicing factors in response to Ca^{++} signals has also been observed, as in the cases of other cell signals [167–170]. In cortical neurons, thapsigargin or ischemia treament induces Tra2 beta1 relocalization from the nucleus to the cytoplasm [99]. This translocation is accompanied by an increased inclusion of a 61 nt cassette exon of the *ICH-1* gene.

Phosphorylation of splicing factors has been described in many other cases [85,87,171,172]. In Ca^{++}-regulated splicing, the CaM kinase inhibitor KN93 inhibits the splicing changes of the *Slo* gene [84,104]. Whether this is due to inhibition of the phosphorylation of a splicing factor remains unclear.

Phosphatases, including PP1 [173–180], PP2C-gamma [181,182], PP2A [178,183], and the pol II CTD phosphatase FCP1 [184], also interact with splicing factors or are known to control splicing. Of these, PP2A binds CaMK IV in a signalling complex [185,186]. Moreover, PP2A is also shown to inactivate CaMK IV by directly dephosphorylating the Thr196 of CaMK IV [187]. Since the CaMK IV activity is essential for its role in the control of splicing [104,105,107], it is possible that the PP2A dephosphorylation of CaMK IV at Thr196 has an effect on CaMK IV-regulated alternative splicing.

Protein methylation has also been shown to control splicing factors or splicing [188,189]. Currently there is no evidence available to support a role for methylation in Ca^{++}-regulated alternative splicing.

Can Ca^{++} or calmodulin (CaM) directly interact with the splicing machinery? In an experiment using calmodulin-affinity chromatography, several RNA binding proteins including hnRNP A2 and C were identified [190]. The binding of CaM to these splicing factors inhibits their phosphorylation by Casein kinase II [190]. SAP145 [191], a member of the spliceosome-associated proteins and a subunit of SF3 (b) [49], was also identified as binding to a CaM affinity column. In another report, polyadenylation specificity factor 30

(CPSF30) of *Arabidopsis thaliana* was found to bind calmodulin [192], which inhibits the RNA binding activity of CPSF30. In HeLa nuclear extracts, CPSF couples transcription with splicing [193]. Therefore, it will be interesting to know whether varying Ca^{++} concentrations and CaM can control any specific alternative splicing event through direct interaction with the factors that regulate splicing.

Taken together, the current evidence indicates that Ca^{++} signalling can control or interact with components of the splicing machinery. The regulation of hnRNP A1 by membrane depolarization to control the inclusion of exon 21 of the *NMDAR1* gene through the UAGG motifs is particularly well characterized. In most other cases, however, a direct molecular link between the regulation of the splicing factors by Ca^{++} signalling and a change in RNA splicing remains to be demonstrated.

4. Pre-mRNA elements responsive to Ca^{++} signals in the control of alternative splicing

Since most mammalian exons are controlled in a combinatorial way by multiple positive and negative regulatory elements (Fig. 1), the role of a single RNA element in splicing regulation is usually isolated from its endogeneous gene context and tested in a heterologous mini-gene, as has been done in studying the control of gene transcription [194,195]. This approach has also proved useful in studying the regulation of splicing by the CaM kinase pathway.

Two CaMK IV-responsive RNA elements (CaRREs) have been isolated [104,105,107] (Fig. 3), which, when transferred to a heterologous gene, confer CaMK IV regulation on an otherwise constitutive exon. Similarly, a UAGG motif is also shown to mediate depolarization-regulated alternative splicing [84], which is inhibited by the CaM kinase inhibitor KN93. These observations allow the coupling of depolarization/CaMK IV with short RNA sequence elements, providing regulatory systems much simpler than the endogeneous genes for characterizing the components in-between the stimuli and RNA elements.

From the example of the regulation of NMDAR1 exon 21, it appears that multiple elements, either the same (UAGG) or different (both CaRRE1 and 2), can function within the same exon to respond to Ca^{++} signals to control alternative splicing [84,104,105]. However, it is unclear whether this is a feature common to the regulation of other exons.

Actually, search in alternative splicing databases or genomes for these depolarization/CaMK IV-responsive RNA elements identified different groups of exons and target genes [84,105,107]. This raises questions whether the target genes of a particular element are involved in specific cellular functions and whether each element has its preferred Ca^{++} stimulus, since CaMK IV can be activated by different stimuli [196–199].

As mentioned earlier, a *trans*-acting factor has been identified in the case of the UAGG motifs of the NMDAR1 exon 21. This motif binds hnRNPA1 to repress the 5′ splice site and exon inclusion [72,84]. Interestingly, the nuclear level of the hnRNP A1 protein is

increased in depolarized neurons [84]. Further evidence with loss-of-function of hnRNP A1 will help verify its role in the inducible regulation of alternative splicing of endogenous genes in neurons.

Taken together, these studies couple Ca^{++} signals with specific pre-mRNA elements. This coupling isolates single RNA elements from the complex influences of their surrounding elements in the endogenous gene transcripts thus providing simple model systems for studying Ca^{++}-regulation of alternative splicing.

5. The impact of Ca^{++}-regulated alternative splicing on neuronal functions and diseases

As shown in Table 1 and references therein, alternative exons controlled by Ca^{++} signalling can affect a broad range of neuronal functions. Based on data from these Ca^{++} signal-regulated splicing events and work from others [7,8], I will speculate on the effect of Ca^{++}-regulated alternative splicing on neuronal functions and diseases.

5.1. Fine-tuning of neuronal functions

One fascinating property of neurons, for which the underlying molecular basis is not fully understood, is the fine-tuning of their functions. Interestingly, the functions of many neuronal genes are diversified and optimized through alternative splicing [6,8,18,200]. The large number of splice variants contributes greatly to the diversity of the protein functions, especially when functional proteins such as ion channels can be composed of heteromultimers of different splice variants. Take for example the production of 672 splice variants in all possible combinations of the mammalian BK channel subunits (Fig. 2). If any four variants are chosen to create a tetramer channel without considering their relative positions inside the channel, the total number of all possible combinations of every four variants can be calculated according to the algebra formula for “Combination” $C_{672}^{4}=672![4!\times(672-4)!]=(672\times671\times670\times669)/(4\times3\times2\times1)=8,421,345,240$. Thus, theoretically about eight billion heterotetramers of BK channels can be formed in total, all composed of four different variant subunits. Note that this calculation is still an underestimate since it has not considered the relative subunit positions of a channel, homotetramers and combinations of homo- and hetero-subunits. With this large number of ion channels, a high resolution spectrum of BK channel properties emerges (Fig. 4A). For example, for a Ca^{++} sensitivity range of 0–100 μM, the spectrum will be a much smoother gradient of Ca^{++} sensitivities formed by different channels when there are eight billion than when only one or ten channel variants are available. The fine-tuning of electrical properties is exemplified in turtle and bird cochlear hair cells where the BK channel kinetics is “tuned” along the tonotopic map [151]. Differential alternative splicing of the *Slo* gene along the map is also observed and proposed to contribute to the fine-tuning of hearing frequencies [151]. By analogy, the electrical and other properties of neurons could also be finely tuned this way.

Greater diversity of protein properties could be further obtained by varying the strength of the signals controlling alternative splicing, particularly for individual cells where only a

limited set of splice variants is often observed. For example, when only two variants are produced in a cell and their relative levels are dependent on the strength of a Ca^{++} stimulus (Fig. 4B), varying the stimulus strength will induce a gradient of the relative levels of the two variant proteins leading to the formation of heterotetrameric channels with varying ratios of variant subunits and the generation of electrical currents of higher diversity than with only two homotetramers. In fact, splicing changes can be KCl concentration-dependent [84,105], and different ratios of two BK channel subunits generate channel current kinetics in-between the two homotetramer channels [19,201]. This way, alternative splicing regulation by Ca^{++} signals will greatly contribute to the fine-tuning of electrical properties of individual neurons.

5.2. Autoregulation and adaptive changes

As shown in Table 1, the alternative splicing of several Ca^{++} channel genes is regulated by Ca^{++} signals and some exons of ion channel genes are regulated by their own channel ligands or by membrane depolarization. For example, the inclusion of the exons 5 and 21 of NMDAR1 is controlled by NMDA or glutamate, and the STREX exon of the BK channel by membrane depolarization [103,104]. Inclusion of these exons has known effects on the functions or localization of the specific channels [24,29,30,39,140,141,202]. Therefore, a given first round stimulus (for example, depolarization during electrical stimulation) could cause a change in the ion channel subunits via alternative splicing (Fig. 4C). This change will provide neurons with a different set of variant ion channels to respond to a second-round identical stimulus. The second-round stimulation will now have different effects on the neuronal electrical properties because of the different channels formed from the first round stimulation-induced alternative splicing. Such self-regulatory and adaptive changes are interesting when considering the most important functions of neurons and the brain: learning and memory, particularly electrophysiological memory that requires CaMK IV [203].

5.3. Neuronal death and neurodegenerative diseases

Aberrant alternative splicing of several genes or deficiencies in alternative splicing factors have been implicated in neuronal diseases [43]. Ca^{++} is also involved in neuronal diseases [204–206]. In normal neurons, Ca^{++} levels are under homeostatic control. However, when the balance is tilted, deficient neuronal functions or diseases develop [204]. For example, when excess Ca^{++} ions are present, neuronal toxicity occurs leading to neuron death or contributing to neurodegenerative diseases [204,206]. Inhibition of CaMK IV also leads to neuron death in cell cultures [207], and CaMK IV deficiency in mice results in loss of learning or memory or in less drug tolerance [208–211]. The ion channels involved in Ca^{++} toxicity including the AMPA and NMDA receptors undergo alternative splicing [202,204], and inclusion of the NMDAR1 exons 5 and 21 is controlled by membrane depolarization and CaMK IV [84,104,105,107,212]. In addition, the ICH-1S variant induced by thapsigargin or ischemia prevents cell death [213,214]. Therefore, it will be interesting to determine whether the regulation by Ca^{++} signals of these and other ion channels and apoptosis-related genes is important for Ca^{++} homeostasis in normal cells or imbalance during neuronal death and in the development of neurodegenerative diseases.

6. Perspectives

Even with the progress described above, much remains to be learned about Ca^{++} control of alternative splicing and its role in cellular, particularly neuronal, functions and diseases. For example, what is the nature of the molecular link between CaMK IV and the *trans*-acting splicing factors that function through the CaRRE elements? What is the physiological impact of a specific stimulus through the regulation of a group of alternative exons? How important is this inducible alternative splicing in neuronal and whole brain functions such as learning and memory? Understanding the molecular basis of this regulation will help us to address these questions.

In addition to the traditional molecular, cellular, biochemical and genetic approaches for the examination of individual genes or proteins, several recent developments could be helpful in answering these questions. Firstly, large-scale proteomic approaches may help identify splicing factors and their posttranslational modifications induced by Ca^{++} signals. Secondly, whole genome RNA interference libraries have been developed recently and applied to whole genome loss-of-function screening of genes [215–222]. Screening for alternative splicing factors by RNAi has already been carried out in fly cells [215,216]. Hopefully, the now available mammalian RNAi libraries could also be applied for functional screening of genes essential in inducible alternative splicing. Thirdly, splicing-sensitive microarrays and whole genome exon arrays [64,78,82,83,223–225], combined with loss-of-functions of splicing factors (such as knockout or RNAi), will allow the identification of a group of exons/genes controlled by a particular factor during Ca^{++}-regulation of splicing. Lastly, bioinformatics approaches with highly predictive RNA elements could also help identify a group of exons regulated by Ca^{++} signals, as has been demonstrated for the alternative splicing factor Nova-1 [62]. These recent developments will facilitate the study of Ca^{++}-regulated splicing and will allow a more accurate assessment of the physiological impact of a particular splicing factor/RNA element through regulating the alternative splicing of a specific functional group of genes.

In summary, Ca^{++} signal-regulated alternative splicing modulates the function of a variety of genes that have important roles in cellular, particularly neuronal, functions and diseases. Recent studies have identified downstream splicing factors and coupled Ca^{++} signals with pre-mRNA elements. Further detailed analyses of individual elements/exons using model systems shall help to bring the molecular basis of this regulation into focus. Future studies with genomewide coverage of alternative exons and splicing factors shall help in our understanding of the impact of splicing regulation on cellular functions and its role in the development of diseases.

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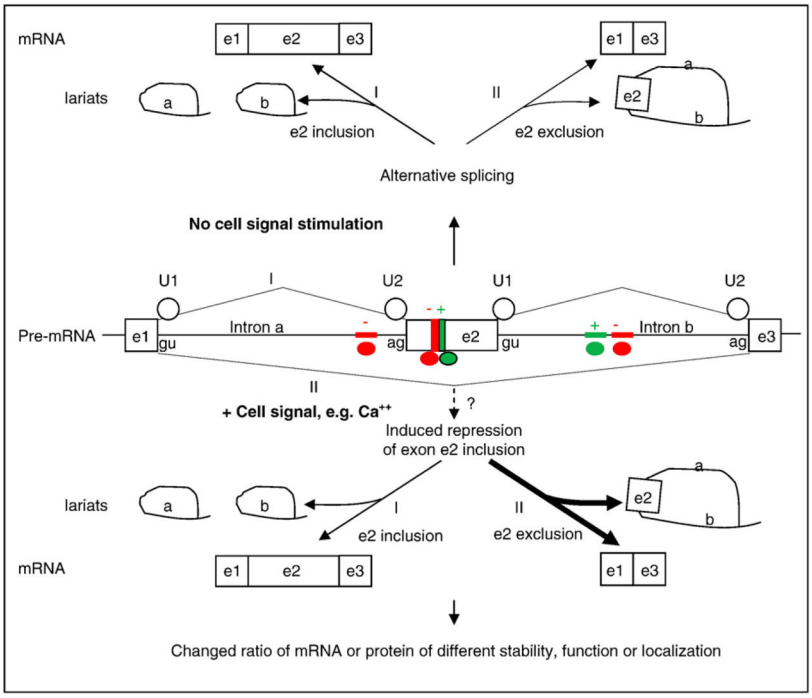

Fig. 1.

Diagram of Ca^{++} signal-regulation of alternative splicing. Shown in the middle is a cassette exon (e2) with two flanking introns (a and b) and constitutive exons (e1 and e3), with exonic and intronic *cis*-acting pre-mRNA elements as splicing enhancers (green, +) or silencers (red, −) and *trans*-acting splicing factors (ovals) indicated. Without signal stimulation (above), the pre-mRNA is spliced in two pathways (I and II) with a given ratio leading to the inclusion (I) or exclusion (II) of the alternative exon e2 and the respective lariats. With signal induction, like Ca^{++} (below), one pathway (here for example, II, heavier arrows) over the other pathway (I) is favored in a particular cell to promote the production of one variant mRNA. Through this regulation, Ca^{++} signals will change the relative ratio of the variant mRNA or protein isoforms of different properties. (?: mostly unknown intermediate steps).

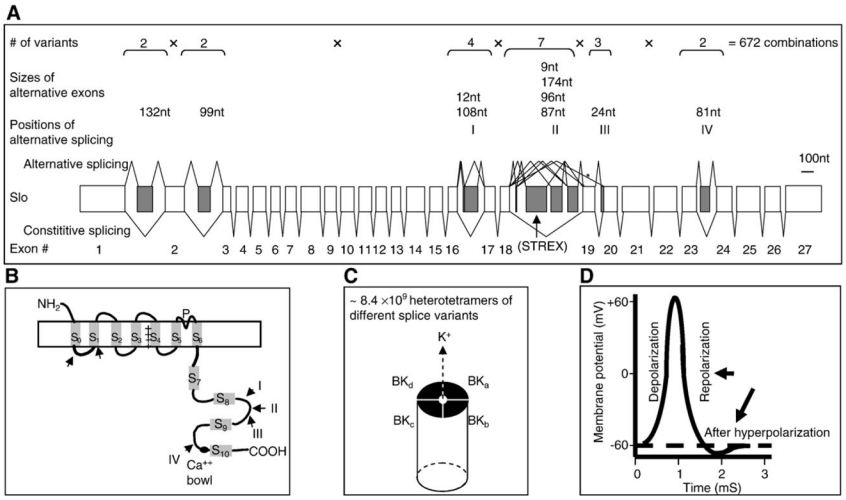

Fig. 2.

An example of the generation of diverse protein functions through alternative splicing of genes important in neuronal functions: complex alternative splicing patterns of the middle exons of the mammalian BK potassium channel gene *Slo* (GenBank accession #: U23767). In A, the exon/intron organization of the *Slo* gene and alternative splicing positions, exon (boxes) sizes and possible number of variant transcripts produced from each position are indicated above the gene, with the exon numbers below it. The total number of channel subunits produced from all possible combinations of alternative splicing is 2×2×4×7×3×2=672, as indicated. Position of the STREX exon is indicated with an arrow. The length (nt) bar is for exon sizes. In B, positions where the alternative exons are inserted relative to the protein domains are indicated with arrows. S: transmembrane (or putative) domains. P: channel pore. Black oval: Ca^{++} bowl. The S4 voltage-sensitive domain is labelled with "+"s. (C) A channel heterotetramer of four different variant subunits a, b, c and d, with the total number of such heterotetramers indicated above the channel (see text for calculations). (D) Diagram of an action potential with depolarization, repolarization and afterhyperpolarization phases indicated. BK channels participate in the last two phases (arrows).

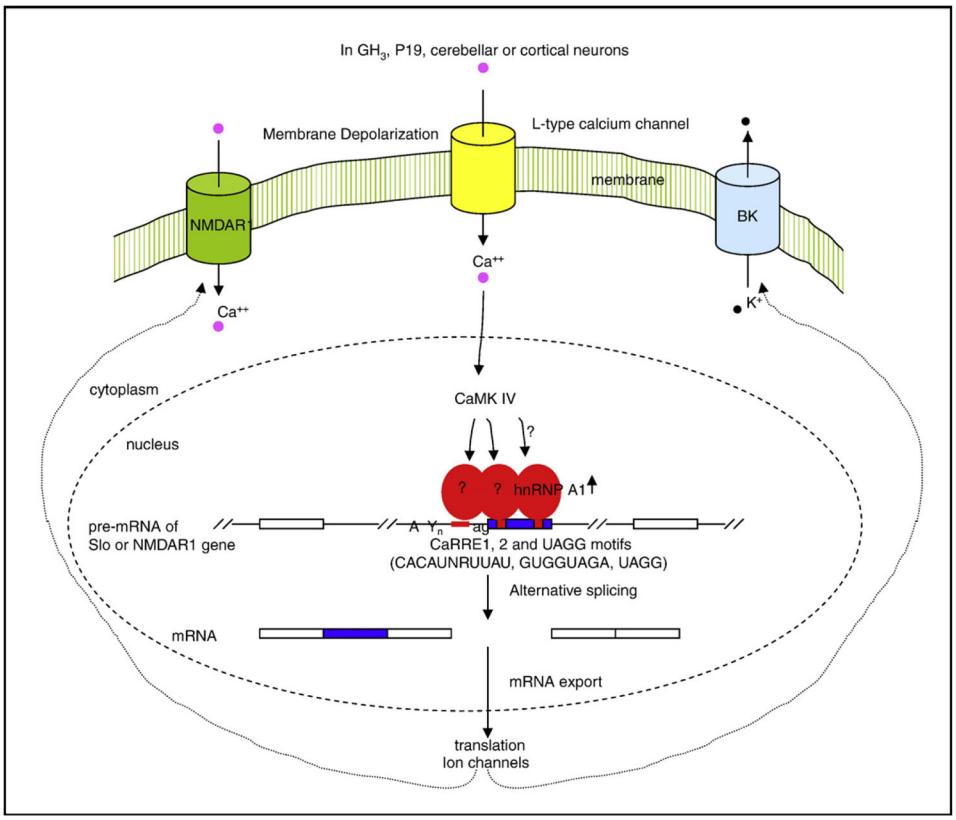

Fig. 3.

Coupling of Ca^{++} signals with pre-mRNA elements that are sufficient to mediate Ca^{++}-regulated alternative splicing. Shown is a diagram of the pathway from membrane depolarization to the CaRRE and the UAGG motifs. In several neuronal or endocrine cells as indicated, membrane depolarization induces Ca^{++} signals through L-type Ca^{++} channels, likely activating CaMK IV, which targets pre-mRNA elements CaRRE1 and 2, probably also the UAGG motif, to control the inclusion of the exons, for example, STREX exon of Slo or exons 5 and 21 of NMDAR1. The changed exon compositions will later translate into proteins with different electrical properties (STREX, exon 5) or subcellular localizations (exon 21) to incorporate into the ion channels in cell membranes, to impact cellular electrical properties. In the pre-mRNA, the branch point (A), polypyrimidine tract (Yn) and 3′ AG (ag), and the exons (boxes) and introns (lines) are indicated. The CaRREs or the UAGG motif is shown as red bars. CaRRE1 was found in the upstream intron of the STREX exon and the NMDAR1 exon 5, and CaRRE1, 2 and UAGG motifs in the NMDAR1 exon 21. hnRNPA1 and the unknown factors are as red ovals. The “?” next to the hnRNP A1 pathway is to reflect the fact that this regulation requires CaM kinases but an effect by CaMK IV is not demonstrated yet.

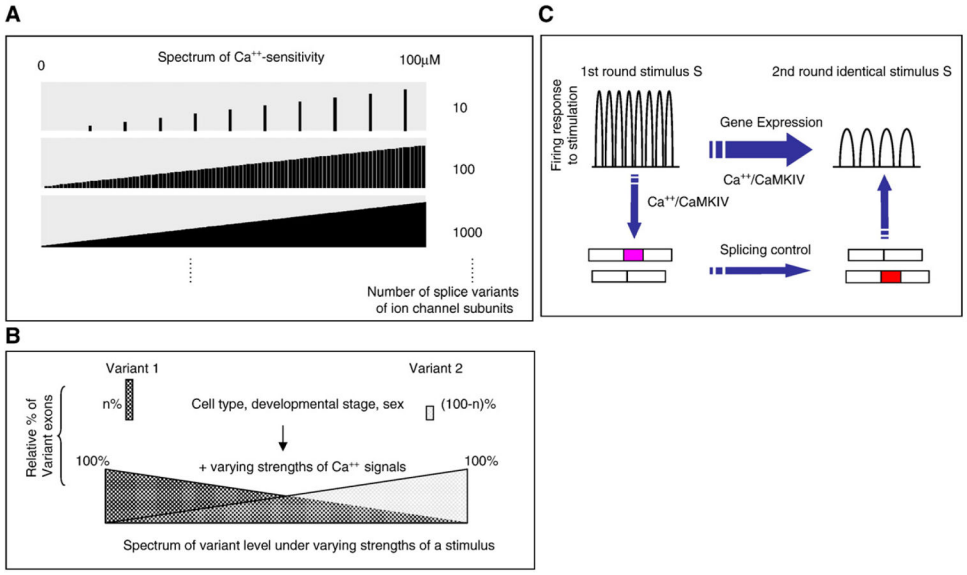

Fig. 4.

Speculations on the impact of Ca^{++}-regulated splicing on protein and neuronal functions. (A) On the fine-tuning of the Ca^{++} sensitivity of ion channels (for example, the BK channels). With Ca^{++} sensitivities between 0 to 100 μM (top), and increasing number of different ion channels (right) available through alternative splicing, the spectrum of the Ca^{++} sensitivity is becoming increasingly refined with narrower intervals (suppose the Ca^{++} concentration intervals are even). For simplicity, only the effect of up to 1000 variant channels is shown, with more indicated by "… …". (B) Modulation of splice variant ratios beyond cell-specific expression of a limited number of variants by cell signals such as Ca^{++}. Shown are two splice variants expressed in a cell with n% for variant 1 and (100-n)% for variant 2 when there is no signal. This relative percentage can be shifted smoothly if varying strengths of Ca^{++} stimuli are applied to modulate the splicing resulting in a spectrum of variant percentages ranging from 0% to 100% for each variant. (C) Diagram of the possible contribution of Ca^{++}/CaMK IV-regulated alternative splicing to changes in neuronal electrical properties during electrophysiological memory. Changed electrical firing during the 1st and 2nd round stimulations is indicated with a different amplitude and frequency. This change requires gene expression through CaMK IV. The control of alternative splicing of ion channels by CaMK IV to either promote or repress the inclusion of certain exons is expected to contribute to the changes in ion channel subunit compositions and therefore the firing properties.

Table 1

List of alternative exons regulated by Ca^{++} signals

Gene name	Regulated exon/alternative splice site	Exon size (nt)	Type of alternative splicing	Stimulus	Effect on exon inclusion	Inhibitor	Kinase/PPase involved	Exon inclusion effect	Protein function	Cell/tissue	Species	Reference
Ania-6	Intron 6′	1689	Intron retention	50 mM KCl	Repression (of intron retention)	KN93, nifidepine		Truncated protein, protein localization	pol II associated, pre-mRNA splicing	Striatal neuron	r	Sgambato'03 [95], Berke'01 [94]
Ania-6	Intron 6′	1689	Intron retention	100 μM glutamate	Promotion	AP5		Truncated protein, protein localization		Striatal neuron	r	Sgambato'03 [95], Berke'01 [94]
Calcium channelα1A	1A-b EF hand, exon 37b	97	Mutually exclusive	Kainate	Decrease			Controls Ca^{++}-dependent facilitation	Ca channel	Hippocampus	r	Vigues'99 [100], Soong'02 [139], Jurkat-Rott'04 [123], Chaudhuri'04 [114]
CD45	Exons 4, 5, 6	129, 147, 141	Cassette	Ionomycin	Repression				Cell adhesion	B cells	m	Ogimoto'93 [93]
hPMCA2	A	33, 60, 42	Cassette	56 mM KCl, thapsigargin	Repression (on 33, 60 nt exons)	BAPTA-AM		Membrane targeting	Ca^{++} pump, Ca^{++} homeostasis	IMR32 human neuroblastoma	h	Strehler lab'96–'06 [101,125,127,128]
ICH-1	Exon 9 (AF136232)	61	Cassette	Thapsigargin, ischemia	Promotion			Early translation termination, prevent cell death	Caspase, cell growth/death	Cortical neurons	r	Daoud'02 [106], Jiang'98,'99 [213,138]
IP3R1	S1, S2 (A,B,C) and S3	45, (A,B,C), 27	Cassette	25 mM KCl, NMDA	Promotion	Nimodipine, MK-801, KN62		Disrupt ATP binding motif for PKA phosphorylation (S2)	Ca^{++} release from intracellular stores	Granule neurons	r	Nakagawa'91 [132], Nucifora'95 [131], Choi'04 [96]
NCAM	Exons 16, 17	208, 117	Cassette	Thapsigargin	Repression	Verapamil			Cell adhesion, interaction, axon growth	Myotube cultures	c	Rafuse'96 [102], Owens'87 [112]
Neurexin IIalpha	Exon 11/ site 3	27	Cassette	50 mM KCl	Repression	BAPTA-AM, CdCl2		In ligand binding domain	Neuronal synaptic adhesion	Cortical neuron	r	Rozic-Kotliroff'07 [108]
NMDAR1	E5, E21(C1)	63, 111	Cassette	25–50 mM KCl	Repression	KN93, nifidepine, AP5	CaMK IV	Current amplitude, channel localization on cell membrane	Neurotransmission, Ca^{++} signal	P19, hippocampal neurons	r, m	Mu'03 [212], Lee'07 [105], An'07 [84]
PMCA4	CII	175	Cassette	25 mM KCl, NMDA	Repression	FK506	Calcineurin	Lower affinity for calmodulin	Ca^{++} pump	Granule neurons	r	Guerini'00,'99 [97,98]
Slo	STREX	174	Cassette	25–50 mM KCl	Repression (promotion in cortical neurons)	KN93, nifidepine	CaMK IV	Ca^{++}, voltage, PKA and glucocorticoid sensitivity	Electrical firing, neuro-transmission	Granule neurons, GH3, cortical neurons	r	Xie'01,'05 [104,107], An'07 [84]
SNAP25	a, b	118, 118	Mutually exclusive	Decreased CaMK IV	Promotion of exon a		CaMK IV	Variable synaptic vesicle release	Synaptic vesicle release	Cerebellar	m, r	Xie'05 [107], Hepp'01 [115]
Tra2 beta1	Exon III	163	Cassette	Pilocarpine-induced seizure	Repression				Pre-mRNA splicing	Hippocampus, cortex	r	Daoud'99 [99], Stoilov'04 [134]

The list is according to gene name/description in alphabetical order, followed by other properties including exon, stimuli, inhibitor, protein function, reference, etc. PPase: protein phosphatase. NA: not available. For species, c: chick, h: human, r: rat, m: mouse. For more exons, see Xie et al., *RNA*'05 [107], Lee et al., *Plos Biol.*'07 [105] and An et al., *Plos Biol.*'07 [84].

Table 2

An alphabetical list of pharmacological agents/treatments referred to in this paper

Chemical/treatment	Effect on Ca^{++} signalling
Acetylcholine	Agonist of acetylcholine receptor, increase Ca^{++} influx
AP5	Antagonist of NMDA receptor, reduce Ca^{++} influx
BAPTA-AM	A cell permeable Ca^{++} chelator, reduce cytosolic Ca^{++}
$CdCl_2$	A voltage-sensitive Ca^{++} channel blocker
FK506	A calcineurin inhibitor
Glutamate	Agonist of glutamate receptor, increase Ca^{++} influx through ionotropic glutamate receptors
Ionomycin	Ionophore, increase intracellular Ca^{++}
Ischemia	increase intracellular Ca^{++}
Kainate	Ionotropic glutamate receptor agonist, increase Ca^{++} influx
KCl (25–50 mM)	Induce Ca^{++} influx through Ca^{++} channels
KN62	CaM kinase inhibitor
KN93	CaM kinase inhibitor
MK-801	Antagonist of NMDA receptor, reduce Ca^{++} influx
Nifedipine	L-type calcium channel blocker
Nimodipine	L-type calcium channel blocker
NMDA	Agonist of NMDA receptor, increase Ca^{++} influx
Pilocarpine	Agonist of muscarinic acetylcholine receptor, induce seizure, increase Ca^{++}
Thapsigargin	Inhibitor of the sarco/endoplasmic reticulum Ca^{++} ATPase, elevate cytosolic Ca^{++}
Verapamil	L-type calcium channel blocker

Table 3

List of RNA binding proteins/splicing factors regulated by Ca^{++} signals

Splicing factor	Ca^{++} signal-inducer/inhibitor/partner	Splicing factor change/interaction	Earliest time splicing change detected	Cell/extract	Reference
CPSF30	CaM	Inhibited RNA binding activity	NA	*A. thaliana*	Delaney et al. [192]
hnRNP A1	Depolarization	Increased nuclear protein level	12 h	Cortical culture	An and Grabowski [84]
hnRNP A2	CaM	Binds CaM column, inhibited phosphorylation by Casein kinase 2, colocalization with CaM in nucleus	NA	Rat liver	Bosser et al. [190]
hnRNP C	CaM	Binds CaM column, inhibited phosphorylation by Casein kinase 2, colocalization with CaM in nucleus	NA	Rat liver	Bosser et al. [190]
hnRNP H3	Depolarization	Repressed exon inclusion	24 h	Cortical culture	An and Grabowski [84]
PP2A	CaMK IV	Dephosphorylated Thr196 of CaMK IV and inhibited gene transcription	NA	In vitro	Tokumitsu et al. [185], Anderson et al. [187]
RNPS1	Depolarization	Repressed exon inclusion	6 h	Cortical culture	An and Grabowski [84]
SAP145	CaM	Binds CaM column	NA	NA	Agell et al. [191]
Tra2 beta1	Thapsigargin	Cytoplasmic accumulation	1 h	Cortical culture	Daoud et al. [106]

The list is according to the protein name/description in alphabetical order. NA: not available.