

Acknowledgements

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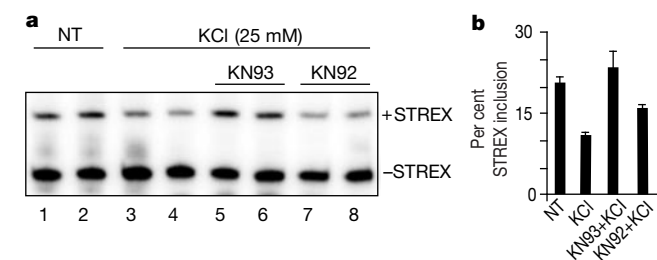
# A CaMK IV responsive RNA element mediates depolarization-induced alternative splicing of ion channels

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Calcium regulation of gene expression is critical for the long-lasting activity-dependent changes in cellular electrical properties that underlie important physiological functions such as learning and memory<sup>1</sup>. Cellular electrical properties are diversified through the extensive alternative splicing of ion channel pre-messenger RNAs<sup>2</sup>; however, the regulation of splicing by cell signalling pathways has not been well explored. Here we show that depolarization of GH<sub>3</sub> pituitary cells represses splicing of the STREX exon<sup>3</sup> in BK potassium channel transcripts through the action of Ca<sup>2+</sup>/calmodulin-dependent protein kinases (CaMKs). Overexpressing constitutively active CaMK IV, but not CaMK I or II, specifically decreases STREX inclusion in the mRNA. This decrease is prevented by mutations in particular RNA repressor sequences. Transferring 54 nucleotides from the 3' splice site upstream of STREX to a heterologous gene is sufficient to confer CaMK IV repression on an otherwise constitutive exon. These experiments define a CaMK IV-responsive RNA element (CaRRE), which mediates the alternative splicing of ion channel pre-mRNAs. The CaRRE presents a unique molecular target for inducing long-term adaptive changes in cellular electrical properties. It also provides a model system for dissecting the effect of signal transduction pathways on alternative splicing.

BK (Slo) channels participate in the repolarization and fast after-hyperpolarization of action potentials and are important in setting the firing properties of neurons<sup>4,5</sup>. Their diverse properties result largely from extensive alternative splicing of the *Slo* transcript<sup>6-11</sup>.



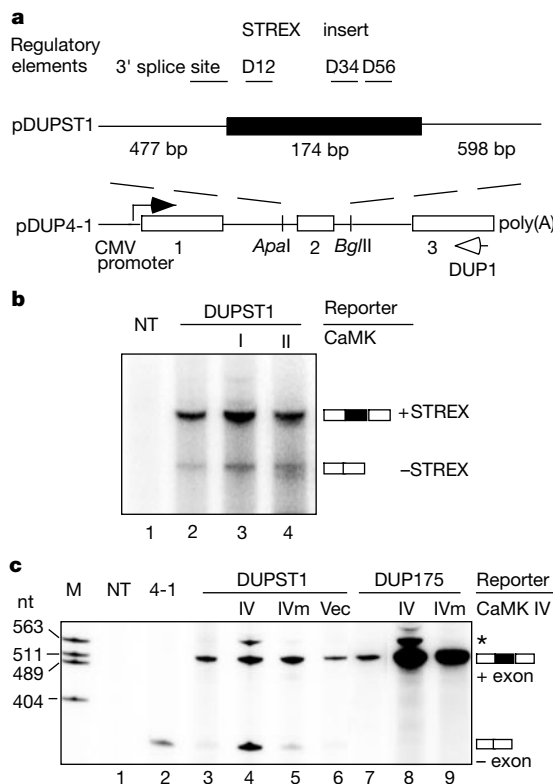
**Figure 1** Depolarization-induced changes in STREX splicing depend on CaMK. **a**, Denaturing gel electrophoresis of Slo RT-PCR products from GH<sub>3</sub> cells, including (+) or excluding (-) the STREX exon, 6 h after treatment with nothing (NT, lanes 1 and 2), KCl (lanes 3 and 4), KCl plus KN93 (lanes 5 and 6), or KCl plus KN92 (lanes 7 and 8). Two independent cell samples for each treatment are shown. **b**, Percentage of mRNA containing STREX after the treatments shown in **a**. Results are mean  $\pm$  s.d.,  $n = 3$ .

The STREX exon of Slo confers higher Ca<sup>2+</sup> sensitivity on the channel<sup>3,11-14</sup>, and may contribute to the repetitive firing of adrenal chromaffin cells<sup>3,5</sup> and the tuning of hearing frequencies in cochlear hair cells<sup>11,13</sup>. The differential expression of STREX suggests that its splicing is modulated by many physiological factors<sup>3,11,15</sup>.

Polymerase chain reaction after reverse transcription (RT-PCR) of Slo mRNA indicated that GH<sub>3</sub> rat pituitary cells include the STREX exon in about 20% of their endogenous Slo transcripts (Fig. 1a, lanes 1, 2, and Fig. 1b). When these cells were depolarized by adding KCl (25 mM), STREX splicing was reduced by 50% in 6 h (lanes 3, 4). Adding the CaMK inhibitor KN93 to the media, before the KCl depolarization, completely blocked this decrease (30  $\mu$ M, lanes 5, 6). In contrast, the inactive analogue KN92 had only a slight effect (lanes 7, 8). Thus, depolarization of GH<sub>3</sub> cells represses STREX exon splicing, and this repression apparently occurs through a CaMK-dependent pathway.

CaMKs I, II and IV can be activated by cell depolarization and inhibited by KN93. CaMK IV, in particular, is important in depolarization-regulated gene expression<sup>16</sup>. Because all three enzymes are expressed in GH<sub>3</sub> cells (Supplementary Information Fig. 1a), any one of them might be involved in the repression of STREX splicing.

We wanted to determine whether the regulation was acting through the sequences surrounding the STREX exon itself and also was not an effect of transcriptional regulation or RNA degradation. The mouse STREX exon with partial flanking intron sequences



**Figure 2** CaMK IV overexpression represses STREX exon inclusion in HEK cells. **a**, The STREX splicing construct pDUPST1. The STREX insert replaces DUP4-1 sequences between the *Apa*I and *Bgl*II sites. The CMV promoter, primer extension primer DUP1 (arrows), exons (boxes), introns (lines) and regulatory elements are indicated. **b**, Primer extension assays of pDUPST1 co-transfected with CaMK I-dCT (I) or CaMK II-dCT (II). **c**, Primer extension assay as in **b** with CaMK IV. M, nucleotide size marker; NT, mock transfection (lane 1); 4-1, pDUP4-1 (lane 2); IV, CaMK IV-dCT (lanes 4 and 8); IVm, CaMK IV-dCTK75E (lanes 5 and 9); Vec, pcDNA3.1(+) (lane 6). Asterisk indicates apparently early terminated products from unspliced mRNA.



by multiple enhancer and repressor elements, which are found in flanking introns as well as the exon<sup>23,25,26</sup>. Exons controlled by pyrimidine-rich repressor elements often contain additional repressor elements in their upstream 3' splice site<sup>25,26</sup>. Further mutagenesis of the DUPST1 plasmid indicated that several intronic sequences, including the 3' splice site, were also affecting STREX splicing (Supplementary Information Fig. 2). However, the effect of point mutations in these elements was not as marked as that of the D56 mutation in pDUPST1, presumably because of redundancy in the regulatory sequences<sup>23</sup>. To avoid this redundancy, we transferred small segments from STREX to the CaMK IV non-responsive DUP175 exon, where the effect of each element could be measured in the absence of other STREX sequences. Although the D56 element is required for CaMK IV repression of the STREX exon in pDUPST1, it was not sufficient to repress splicing when transferred to the DUP175 exon (see Fig. 4b, lanes 1, 2; and data not shown). Other STREX elements were apparently needed for D56 to function.

In contrast to D56, the STREX 3' splice site had a large effect on DUP175 splicing (Fig. 3). The constitutive DUP175 exon was not responsive to CaMK IV (Fig. 3d, lanes 1–3). When the 3' splice site upstream of DUP175 was replaced with the corresponding sequence from STREX, this DUP175ST exon was strongly repressed by CaMK IV (lanes 4–6). Mutations M1 and M2 in the STREX splice site

almost eliminated CaMK IV repression, yielding a nearly constitutive exon (Fig. 3c, d, lanes 7–9 and 10–12, respectively). Another constitutive CaMK IV, activated by point mutations rather than by truncation, also repressed DUP175ST exon inclusion (Supplementary Information Fig. 3). Thus, the upstream 3' splice site of STREX is sufficient to confer CaMK IV-mediated repression on a heterologous exon, and the precise sequence in its polypyrimidine tract is critical to its function. We call this sequence a CaMK IV-responsive RNA element (CaRRE).

For the STREX exon, both D56 and the CaRRE were needed for CaMK IV repression of splicing. In contrast, with DUP175 only the CaRRE was needed. When most of the Slo intron sequences were deleted from DUPST1, splicing of STREX still responded to the CaRRE and D56 but was lower overall (see Fig. 5c, DUPST2; and Supplementary Information Fig. 2b, lanes 1, 3). This indicates that additional elements contribute to STREX regulation. Similar differences in the requirements of native and reporter exons for regulatory elements are seen in other systems<sup>23,25</sup>. It will require extensive mutagenesis to identify all the elements affecting STREX splicing; however, from our results it is clear that particular RNA elements confer CaMK responsiveness on an exon.

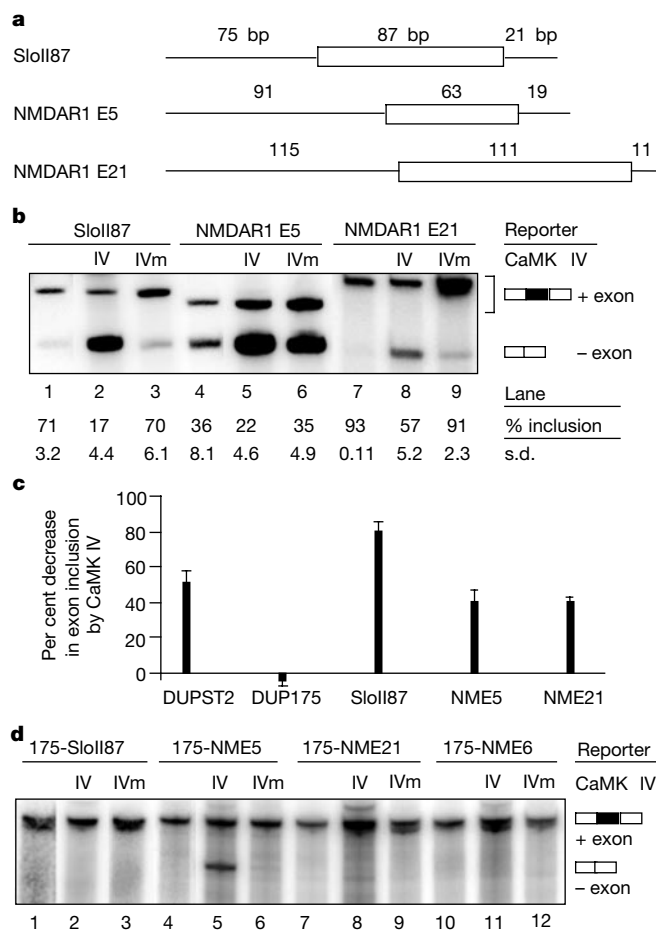
We next determined whether the CaRRE would respond to depolarization in GH<sub>3</sub> cells as it did to CaMK IV. Tests with several constructs indicated that GH<sub>3</sub> cells show even stronger STREX or DUP exon inclusion than HEK cells, making the exons less sensitive to repression by the 3' splice site. To make a more repressible CaRRE-dependent exon, we modified the DUP175 exon to contain both the 3' splice site and the D56 repressor element from STREX (Fig. 4). The DUP175-D56 exon, containing just the D56 element, was completely included in GH<sub>3</sub> cells and was not repressed by depolarization (Fig. 4b, lanes 1, 2). When the CaRRE was added to this exon, exon inclusion was reduced to about 60% in normal media (CaRRE-D56; lane 3). Most significantly, depolarization now further reduced splicing to about 30% (lane 4). Again, the M1 and M2 mutations abolished this repression (CaRRE-M1-D56 and CaRRE-M2-D56; lanes 5–8). Thus, the CaRRE confers repression by depolarization, supporting the idea that depolarization and overexpressed CaMK IV repress splicing through the same pathway.

Many other proteins involved in cellular excitation show complex splicing variation. To examine whether CaMK IV affects other exons, we cloned several variant exons into the DUP4-1 reporter and tested their splicing in the presence or absence of CaMK IV (Fig. 5). We found that another exon of Slo (SloII87; see Methods), as well as exons 5 and 21 of NMDAR1 (*N*-methyl-D-aspartate receptor 1; ref. 27) were also repressed by CaMK IV (Fig. 5b). NMDAR1 exons 5 and 21 show a splicing decrease similar to the STREX exon in DUPST2 (lanes 5, 8). SloII87 was more strongly repressed (lane 2).

To examine whether the 3' splice site is a common target of CaMK IV repression, we transferred only the 3' splice sites of these exons to DUP175. We found that the splice site of NMDAR1 exon 5 also conferred CaMK IV responsiveness (Fig. 5d, lane 5), whereas the sites from SloII87, NMDAR1 exon 21 and the constitutive NMDAR1 exon 6 had no effect (lanes 2, 8, 11). Thus, CaMK IV apparently instigates a coordinate change in the splicing of a group of variant exons. It will be interesting to test additional transcripts that affect cell excitation, and the physiological effect of these splicing changes by CaMK IV.

Pyrimidine-rich elements are often binding sites for the polypyrimidine-tract binding protein (PTB)<sup>27,28</sup>, which is implicated in the repression of a number of exons. Indeed, PTB is known to bind the 3' splice site of NMDAR1 exon 5 (ref. 27). PTB is hence an appealing target for CaMK IV; however, we have not observed any changes in PTB on GH<sub>3</sub> cell depolarization, nor have we seen the direct phosphorylation of PTB by purified CaMK IV. Thus, the identity of the CaMK IV targets requires further investigation.

In summary, we have shown that both cell depolarization and



**Figure 5** CaMK IV repression and a CaRRE in other ion channel exons. **a**, Exons SloII87, and 5 (E5) and (E21) of NMDAR1 inserted into pDUP4-1. **b**, Primer extension assay of the plasmids shown in **a** transfected alone (lanes 1, 4, 7), or with CaMK IV-dCT (IV, lanes 2, 5, 8) or -dCT75E (IVm, lanes 3, 6, 9). **c**, Per cent reduction in exon inclusion by CaMK IV-dCT for DUPST2, DUP175, and the plasmids shown in **b** (mean  $\pm$  s.d.,  $n \geq 3$ ). **d**, Primer extension assay of DUP175-derived plasmids (containing 3' splice sites as in Fig. 3c) transfected alone (lanes 1, 4, 7, 10), or with IV (lanes 2, 5, 8, 11) or IVm (lanes 3, 6, 9, 12).

CaMK IV signalling regulate the alternative splicing of ion channels through a defined RNA regulatory element. Through the CaRRE, chronic input by action potentials is likely to decrease STREX inclusion, and thus decrease the Ca<sup>2+</sup> sensitivity and opening probability of BK channels. This, consequently, will alter the repolarization, after-hyperpolarization, and firing properties of later waves of action potentials. Thus, the CaRRE provides a molecular target for controlling activity-driven long-lasting changes in cellular electrical properties. In cerebellar neurons, where STREX is expressed<sup>15</sup>, the late phase of long-term depression requires both transcription and CaMK IV (ref. 29). It will be interesting to determine whether the regulated splicing of Slo is also involved.

Other adaptive changes that might result from regulated BK channel splicing include neurotransmitter release and hormone secretion. In addition to the exons tested here, the large number of splice variants of ion channels, and the widespread alternative splicing in neuronal and endocrine cells in general, provide many likely targets for Ca<sup>2+</sup> signalling. Thus, this regulation is an attractive molecular mechanism for the fine-tuning of cellular electrical properties<sup>30</sup>. The STREX exon also provides a working system to study the effects of cell signalling on splicing and to identify the molecules involved. □

## Methods

### Cloning genomic DNA encoding regulated exons

Genomic DNA containing the mouse STREX exon with partial flanking introns was subcloned from the mSlo bacterial artificial chromosome (BAC) clone 75D24, identified from the mouse BAC library (Genome Systems) using the *Hind*III–*Xho*I fragment of Slo cDNA as a probe<sup>3</sup>. The partial intronic sequences surrounding the 87-bp exon at alternative splicing site II of Slo (thus named SloII87, encoding a 29-amino-acid insert<sup>8</sup>) were obtained with the mouse DNA Genomic Walker kit (Clontech). We amplified NMDAR1 (*N*-methyl-D-aspartate receptor NR1) exons 5 and 21 by PCR from human genomic DNA using Pfu DNA polymerase, on the basis of GenBank genomic sequences Z32773 and Z32774.

### Plasmid construction

Exons with flanking intron sequences were cloned between the *Apa*I and *Bgl*II sites of pDUP4-1 (ref. 17). Mutants were made by PCR using Pfu DNA polymerase. CaMK IV-dCT and CaMK IV-dCTK75E inserts with Flag tags at the amino termini<sup>20,21</sup> were cloned into pcDNA3.1(+) at the *Bam*HI site. CaMK II-dCT (residues 1–29)<sup>19</sup> was cloned into pcDNA3.1(+) with a Flag tag at the N terminus. pDUP175 is the same as pDUP171 (ref. 22), except that the middle exon and the partial flanking introns were recombined into pDUP4-1 between the *Apa*I and the *Bgl*II site<sup>17</sup>, to give it the same restriction sites as the other splicing constructs. We confirmed all constructs by sequencing. The translation stop codons for pDUPST1, pDUPST1-D34 and pDUPST1-D56 mRNA are the same; thus, their differences are unlikely to be caused by nonsense-mediated RNA decay.

### Cell culture, treatments and transfections

We cultured GH<sub>3</sub> cells in HAM F10 with 10% horse serum plus 5% fetal bovine serum and HEK cells in DMEM with 15% calf serum. For the depolarization of GH<sub>3</sub> cells, overnight cultures were treated by adding KCl to 25 mM (plus 3.8 mM in the F10 media), with or without CaMK inhibitor KN93 (30 μM) or the inactive analogue KN92 (30 μM). Transfections were done with SuperFect (Qiagen) using overnight cultures at about 60% confluency. For HEK cells, 2 μg of splicing plasmid and/or 6 μg of CaMK plasmid, its mutant, or a vector control were used per well of a six-well plate. For transfections in GH<sub>3</sub> cells, 12 μg of splicing reporter plasmid was used for each 90-mm plate. KCl was added 3 h after transfection and maintained thereafter. After transfection, RNA was extracted at 16–20 h for HEK cells, or 2 days for GH<sub>3</sub> cells.

### RT-PCR and primer extension

We performed RT-PCR as described<sup>3</sup>, except that the RT product was amplified for only 25 cycles, a <sup>32</sup>P-labelled downstream primer was included, and the PCR products were quantified by phosphorimager. The ratios of the bands were constant as monitored from 22 to 26 cycles, suggesting that the amplification was in linear phase. We found that cell lines LA-N-5, PC12, AtT20 and GH<sub>3</sub> had partial inclusion of STREX. Primer extensions were done as described<sup>17</sup>, except that the primer was DUP1 (sequence: 5'-GCAGTCACTCAGTGTGGCA-3') and the annealing temperature was 55 °C. This primer gives rise to a 342 bp primer extension product when the middle exon from pDUP4-1 is not included in HEK cells. The length of the test exon-included product should be 341 plus the test exon length.

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