Continuous Tunable Ca\textsuperscript{2+} Regulation of RNA-edited Ca\textsubscript{\textit{v}}1.3 Channels

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SUMMARY

Ca\textsubscript{\textit{v}}1.3 ion channels are dominant Ca\textsuperscript{2+} portals into pacemaking neurons, residing at the epicenter of brain rhythmicity and neurodegeneration. Negative Ca\textsuperscript{2+} feedback regulation of Ca\textsubscript{\textit{v}}1.3 channels (CDI) is therefore critical for Ca\textsuperscript{2+} homeostasis. Intriguingly, nearly half the Ca\textsubscript{\textit{v}}1.3 transcripts in brain are RNA edited to reduce CDI and influence oscillatory activity. It is then mechanistically remarkable that this editing occurs precisely within an IQ domain, whose interaction with Ca\textsuperscript{2+}-bound calmodulin (Ca\textsuperscript{2+}/CaM) is believed to induce CDI. Here we sought the mechanism underlying the altered CDI of edited channels. Unexpectedly, editing failed to attenuate Ca\textsuperscript{2+}/CaM binding. Instead, editing weakened the prebinding of Ca\textsuperscript{2+}-free CaM (apoCaM) to channels, which proves essential for CDI. Thus, editing might render CDI continuously tunable by fluctuations in ambient CaM, a prominent effect we substantiate in substantia nigral neurons. This adjustability of Ca\textsuperscript{2+} regulation by CaM now looms as a key element of CNS Ca\textsuperscript{2+} homeostasis.

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SUPPLEMENTAL INFORMATION
Supplemental Figures, Extended Discussion, and Supplemental References can be found with this article online at xxxxxx.

AUTHOR CONTRIBUTIONS
Hojjat Bazzazi performed extensive experiments and analysis. Manu Ben Johny pioneered and conducted the apoCaM/CI binding assays, performed molecular modeling, and undertook extensive analysis and software development. Paul Adams developed the SNc preparation and conducted all related experiments. David Yue conceived and supervised the project. All authors refined hypotheses, wrote the paper, and created figures.

COMPETING INTERESTS
None.
**INTRODUCTION**

Voltage-activated Cav1.3 channels constitute prominent Ca\(^{2+}\) entry portals into pacemaking neurons (Bean, 2007), owing to the more negative voltages required to open these ion channels (Xu and Lipscombe, 2001) (Figure 1A). Accordingly, these channels influence neurobiological functions ranging from circadian rhythms drawn from repetitive spiking in suprachiasmatic nucleus, to movement control modulated by pacemaking in substantia nigra (Chan et al., 2007; Obeso et al., 2008). Moreover, Cav1.3 channels often contribute the majority of Ca\(^{2+}\) entry in these settings, such as in substantia nigral neurons (Bean, 2007; Cardozo and Bean, 1995; Chan et al., 2007; Guzman et al.; Puopolo et al., 2007) whose loss is intimately connected with Ca\(^{2+}\) dysfunction in the setting of Parkinson’s disease (Bezproevanny, 2009; Surmeier and Sulzer, 2013).

Fitting with these Ca\(^{2+}\) entry actions, the opening of Cav1.3 channels is subject to extensive negative Ca\(^{2+}\) feedback regulation (CDI), critical for proper Ca\(^{2+}\) handling in these venues. CDI is triggered by the Ca\(^{2+}\)-sensing molecule calmodulin (CaM), which acts as a virtual subunit of channels (Erickson et al., 2001). The Ca\(^{2+}\)-free form (apoCaM) prebinds to the carboxy tail of channels (Erickson et al., 2001; Erickson et al., 2003a; Pitt et al., 2001), and subsequent Ca\(^{2+}\) binding to this CaM drives conformational changes that trigger CDI (DeMaria et al., 2001; Dunlap, 2007; Erickson et al., 2003a; Kim et al., 2004; Peterson et al., 1999; Zuhlke et al., 1999; Zuhlke et al., 2000). It has been widely proposed that a carboxy-terminal IQ domain (Figure 1A) serves as the Ca\(^{2+}\)/CaM effector site that induces CDI, and also as a potential tethering site for apoCaM (Figure 1B).

It is intriguing that nearly half the Cav1.3 transcripts in brain are RNA edited precisely and only within the IQ element (Figure 1A, blue circle), yielding variant channels whose reduced CDI tunes pacemaking in the brain (Huang et al., 2012). The mechanism underlying the editing effects on CDI is presently unknown, but we are now poised to achieve an atomic-level mechanistic understanding of these effects, in terms of single-residue downregulation of Ca\(^{2+}\)/CaM interaction with the IQ effector site. Indeed, several crystal structures of Ca\(^{2+}\)/CaM complexed with IQ-domain peptides of various Ca\(^{2+}\) channels have recently been resolved (Fallon et al., 2005; Mori et al., 2008; Van Petegem et al., 2005). Moreover, based on a structure for a Cav1.2 IQ domain with only a single glutamate-to-aspartate difference, a well-constrained homology model of the Cav1.3 complex is deduced in Figure 1C. Accordingly, we here sought to rigorously demonstrate that RNA-editing effects could indeed be attributed to precise reductions of Ca\(^{2+}\)/CaM binding to the IQ domain. Contrary to expectations, these natural variations within the IQ segment largely fail to attenuate Ca\(^{2+}\)/CaM binding, as do alanine mutations throughout. In a surprising turn, editing instead weakens prebinding of channels to Ca\(^{2+}\)-free CaM (apoCaM), which we substantiate as being essential for CDI (Ben Johny et al., 2013; Liu et al., 2010). This unanticipated outcome suggests that the actual effect of RNA editing is to reset downward the affinity of channels for apoCaM, so that fluctuations in ambient apoCaM can bias the fraction of channels lacking or endowed with resident CaM. In this manner, the strength of CDI of Cav1.3 channels could become a continuously tunable function of CaM levels, a prominent effect we establish directly in substantia nigral neurons. This newfound...
adjustability of Ca\(^{2+}\) feedback regulation by CaM now emerges as a key element of Ca\(^{2+}\) homeostasis across the brain.

RESULTS

Functional effects of RNA editing in the IQ domain of Ca\(_{V1.3}\) channels

Figure 1D (far left subpanel) displays the electrophysiological signature of CDI for Ca\(_{V1.3}\) channels bearing a prototypic IQ domain, as directly coded by genomic DNA without editing. The central portion of the IQ element is comprised of the contiguous residues isoleucine-glutamine-aspartate-tyrosine (IQDY), and such channels correspond to ~60% of the transcripts across the brain (Huang et al., 2012). These channels are here expressed as a homogeneous population in HEK293 cells for optimal biophysical resolution. The resulting Ca\(^{2+}\) current decays rapidly (Ca, red trace), compared with the minimal decline of Ba\(^{2+}\) current (Ba, black trace). Because Ba\(^{2+}\) binds negligibly to CaM (Chao et al., 1984), the fractional decline of Ca\(^{2+}\) versus Ba\(^{2+}\) current after 300-ms depolarization quantifies the steady-state extent of CDI as mediated by CaM (right, CDI parameter).

With this baseline in mind, we can readily appreciate the effect of RNA editing to variably attenuate CDI (subpanels to right). The composition of the central IQ domain for each variant is displayed atop the corresponding set of exemplar currents, along with the prevalence of affiliated transcripts across the brain (Huang et al., 2012). The blunting of CDI is particularly intense for MQDY and MQDC variants, and MRDY and IRDY exhibit intermediate extents of attenuated CDI. Accordingly, adjusting the distribution of Ca\(_{V1.3}\) channels among these variants markedly influences the strength of CDI in the brain. The extent of CDI modulation reported here differs somewhat from that previously reported (Huang et al., 2012), owing to the use of more stringent intracellular Ca\(^{2+}\) buffering solutions used here (10 mM BAPTA versus 5 mM EGTA).

Given the presumed atomic-level understanding of the Ca\(^{2+}/\text{CaM}\) effector configuration (Figure 1B, C), we sought to achieve a high resolution understanding of these editing effects (Figure 1D), by corresponding CDI strength (CDI) with graded and well-understood decrements in the affinity of Ca\(^{2+}/\text{CaM}\) binding affinity to variant IQ modules. To determine binding properties, we used an extensively developed FRET two-hybrid approach (Chen et al., 2006; Dick et al., 2008; Erickson et al., 2001; Erickson et al., 2003a; Erickson et al., 2003b; Liu et al., 2010), where CFP-tagged CaM and YFP-tagged IQ domains were expressed in live HEK293 cells (Erickson et al., 2003a; Yang et al., 2006) (Figure 1E, left), allowing FRET efficiency (\(E_{\lambda}\)) within an individual cell to be plotted as a function of the free concentration of CFP–CaM in that cell. Given variable expression and concentrations in different cells, the ensemble of points yields the binding curve shown in Figure 1E (right). The specific curve here pertains to the prototypic IQ domain (IQDY), with a relative dissociation constant of \(K_{a,\text{EFF}}\) of 1700 microscope-specific \(D_{\text{free}}\) units. This corresponds to an absolute dissociation constant of \(K_d \approx 55 \text{ nM}\) (see Figure S1). That said, we expected that the effects of RNA editing could be rigorously understood in terms of a Langmuir function, shown as a hypothetical outcome in Figure 1F. Specifically, CDI would be plotted as a function of the association constant (\(K_{a,\text{EFF}}\)) for Ca\(^{2+}/\text{CaM}\) binding to the IQ element of a particular variant. The collection of such points, encompassing the IQDY module (green
symbol) and other variants (black symbols), should then decorate the black Langmuir curve defined by the relation \( \text{CDI} \propto \frac{K_{a,\text{EFF}}}{(K_{a,\text{EFF}} + \Lambda)} \) (Figure S2). For the prototypic IQDY module, the association constant \( K_{a,\text{EFF}} = 1/K_d,\text{EFF} = 1/1700 \) would be \( 5.88 \times 10^{-4} \) reciprocal \( D_{\text{free}} \) units, equivalent to an absolute association constant \( K_a = 1/K_d = 1/55 \text{nM} \) approximating \( 0.018 \text{nM}^{-1} \).

**Incongruencies of Ca\textsubscript{v1.3} IQ domain as Ca\textsuperscript{2+}/CaM effector site for CDI**

Thus positioned, we proceeded to correspond CDI with related Ca\textsuperscript{2+}/CaM association constants, not only for the RNA-editing variants, but also for point alanine mutations throughout the IQ domain. Naturally occurring alanines were substituted with threonine. Figure 2A summarizes the population effects on CDI in regard to both RNA editing (blue bars, far left) and alanine substitutions (gray and rose bars, right). The green symbol and dashed-horizontal line furnish the reference CDI for prototypic IQDY channels, and the precise amino-acid sequence of the IQ domain is aligned above for orientation. Substitutions at several positions produced strong suppression of CDI (rose bars), with manipulation at the position-zero isoleucine (I[0]A) yielding the largest sequela. Exemplar currents shown below (Figure 2B) illustrate more directly the effects of mutations producing the strongest CDI reductions.

We next obtained binding relations for Ca\textsuperscript{2+}/CaM interacting with variant IQ segments relating to perturbations generating the greatest functional consequences (Figure 2A, rose bars), as well to those chosen at random (Figure 2A, dashed-gray bars). Figure 2C displays binding relations for exemplar whole-cell currents exhibiting the most pronounced CDI reductions. To facilitate comparison, the reference relation for the prototypic IQDY domain is shown in green (reproduced from Figure 1E). Notably, the decrease in affinity, if any, was modest at best. In the case of I[0]A for which CDI was nearly eliminated (Figure 2A, B), there is no discernible alteration in binding whatsoever (Figure 2C). Also unexpected was the outcome of binding-curve analysis for the RNA-editing variants, which revealed no decrement in Ca\textsuperscript{2+}/CaM interaction (Figure 2D). Accordingly, plots of the steady-state extent of CDI (CDI) versus the association constant of Ca\textsuperscript{2+}/CaM interaction with correlating IQ modules \( K_{a,\text{EFF}} \) altogether deviated from a Langmuir relation (Figure 2E; Figure S3). The symbol corresponding to the prototypic IQ is shown in green; symbols relating to RNA editing are plotted in blue; and those for alanine substitutions are graphed in red (large CDI reductions) or black (loci chosen at random). Notably, manipulations that left CDI unchanged featured altered binding affinity (black symbols tracking horizontal green line), and variations yielding marked CDI attenuation demonstrated unchanged binding (symbols hugging vertical green line). In all, it appears unlikely that RNA editing reduces CDI by diminishing Ca\textsuperscript{2+}/CaM binding to an IQ domain effector site; indeed, it seems that the IQ module alone does not represent a CDI effector site at all.

**RNA editing perturbs apoCaM interaction with an IQ domain preassociation locus**

Despite the incongruence of the IQ domain as Ca\textsuperscript{2+}/CaM effector, editing and alanine substitutions in this element nonetheless attenuated CDI (Figures 1, 2). To understand how this effect arises, we considered another potential role of the IQ domain, to furnish an important locus for channel preassociation with apoCaM (Figure 3A, configuration A). Since
channels devoid of a resident apoCaM fail to exhibit CDI (Ben Johny et al., 2013; Liu et al., 2010) (configuration E), it is plausible that IQ perturbations reduce CDI simply by diminishing channel/apoCaM affinity (Figure 3A, \(K_a\)) and promoting apoCaM-less channels. If so, then overexpressing recombinant CaM\(_{WT}\) should, by mass action, drive channels from configuration E to A (Figure 3A), where channels in the configuration A may undergo CDI by Ca\(^{2+}\)-driven transition to configuration I. Thus, overexpressing CaM\(_{WT}\) would be predicted to restore CDI in channels with altered IQ domains.

Remarkably, this resurgence of CDI is indeed observed (Figure 3B, C). For prototypic IQDY channels, CaM\(_{WT}\) overexpression hardly perturbs CDI (Figure 3B, green horizontal line; Figure 3C, leftmost subpanel) compared to control (Figure 1D, leftmost subpanel). This outcome would be expected if the high affinity of these channels for apoCaM always ensures the absence of channels in configuration E (Figure 3A), even with only endogenous CaM present. By contrast, for the RNA editing variants, elevating CaM\(_{WT}\) restored CDI essentially to prototypic levels (Figure 3B, blue bars). Exemplar traces visually corroborate this restoration for editing variants with the weakest CDI before augmenting CaM\(_{WT}\) (Figure 3C, MQDY and MQDC). Elevating CaM\(_{WT}\) also produced near-complete restoration of CDI for the majority of IQ mutations with initially deficient Ca\(^{2+}\) regulation (Figure 3B, rose bars). Alternatively, CDI remained at prototypic levels for mutations lacking initial CDI effects (Figure 3B, gray bars). These effects on CDI are visually substantiated by the exemplar traces below (Figure 3C, rightmost two subpanels), particularly for the I[0]A substitution that nearly eliminated CDI before CaM\(_{WT}\) supplementation. The modest residual deficit in CDI in I[0]A reflects perturbation of a different effector configuration (Ben Johny et al., 2013), as cartooned in configuration I of Figure 3A.

Still, the actions of overexpressing CaM\(_{WT}\) on CDI could be explained by alternative mechanisms, unless this functional restoration could be explicitly linked to decreased apoCaM interaction with channels. Thus, we performed FRET 2-hybrid binding assays for apoCaM paired with the entire CI region of channels (Figure 3D, leftmost subpanel). This arrangement supported robust binding for the prototypic IQDY pairing, as shown by the green data in the MQDY subpanel. Reassuringly, these assays indicated sharply reduced binding affinity for CI modules relating to editing variants and alanine substitutions with strong CDI effects (Figure 3D, black data).

The most rigorous test arises from the following realization regarding the fraction of channels bound to CaM with only endogenous CaM present—this fraction \(F_b\) is given by the ratio of the CDI measured before (Figure 2A, CDI) and after strong overexpression of CaM\(_{WT}\) (Figure 3B, CDI\(_{CaMhi}\)). Thus, if the mechanism in Figure 3A holds true, then the \(CDI/CDI_{CaMhi}(=F_b)\) should relate as a Langmuir function to the association constant for apoCaM binding to the relevant CI module (Figure S4). Indeed, plotting data in this manner strikingly resolves just such a relationship in Figure 3E. The green symbol corresponds to the prototypic IQDY configuration, blue symbols relate to RNA-editing variants, red symbols derive from point mutations yielding strongly weakened CDI with only endogenous CaM present, and black symbols report on mutations without appreciable CDI effects (Figure 3B, dashed-gray bars). Additional supporting data are summarized in Figure S5. Still
more reassuring are the results of homology modeling the C-lobe of apoCaM in complex with the CaV1.3 IQ domain (Figure 3F), based on an analogous NMR structure for NaV channels (Chagot and Chazin, 2011; Feldkamp et al., 2011). Notably, RNA editing would perturb deeply articulated anchor points within the homology model. In all, these outcomes argue well that RNA editing perturbs CDI primarily by diminishing channel affinity for apoCaM (Figure 3A).

**Continuously tunable Ca\(^{2+}\) regulation in substantia nigral neurons**

This new understanding of RNA editing (Figure 3A) opens the door to considerably expanded tunability of the Ca\(^{2+}\) regulation of CaV1.3 channels, beyond initial expectations. The prevailing original concept in Fig 1b—that editing modulates Ca\(^{2+}\)/CaM interaction with an IQ effector site—would portray RNA editing as adjusting CDI to a limited set of static strengths, much like the discretized settings on a rotary switch. By contrast, the alternative mechanism involving apoCaM (Figure 3A) predicts a more flexible configuration as diagrammed in Figure 4A. Viewed in this way, the CDI strength of each variant may be smoothly adjusted by fluctuating levels of naturally occurring ambient CaM (ΔapoCaM), which could variably redistribute channels between configurations that lack (configuration E) or manifest CDI (configuration A). Accordingly, CDI may be continuously adjusted in the manner of a rheostat, with each variant requiring a different level of CaM to achieve half-maximal CDI.

Among the settings where this connection would be most consequential are dopamine neurons of the substantia nigra pars compacta (SNc), whose loss is closely linked to Ca\(^{2+}\) dysfunction in Parkinson’s disease (Chan et al., 2007; Guzman et al., 2009). RNA editing of CaV1.3 channels is prevalent in these cells (Huang et al., 2012), so we tested for CaM-mediated upregulation of CDI in isolated murine SNc dopamine neurons, as exemplified in Figure 3B by GFP expression under the control of a tyrosine hydroxylase promoter. Whole-cell patch clamp recordings revealed, for the first time in these cells, the existence of modest CDI under control conditions, as illustrated by current waveforms averaged over multiple neurons (Figure 4C, left, with only endogenous CaM present (CaM\(_{\text{endo}}\))). Using a FRET-based genetically encoded sensor of CaM (BSCaM\(_{\text{IQ}}\)), we estimated the free concentration of CaM\(_{\text{endo}}\) to be 3.9 ± 1.0 μM (n = 7) based on measurements done in intrinsically non-fluorescent hippocampal neurons (Brody and Yue, 2000; Liu et al., 2010). Dihydropyridine antagonists verified that up to two thirds of the current was carried by CaV1.3 channels in SNc neurons (Figure S6). Moreover, the strength of CDI here was about half that of prototypic IQDY CaV1.3 channels (Figure 4D, CaM\(_{\text{endo}}\)), all consistent with significant CDI modulation by RNA editing. Importantly, upon internal perfusion with elevated CaM\(_{\text{WT}}\) protein (final concentration 100 μM), CDI was robustly enhanced (Figure 4C, right; Figure 4D, CaM\(_{\text{WT}}\)) to a level approaching that of prototypic IQDY constructs (Figure 4D, green). This exciting outcome is directly consistent with the tunability of CDI by CaM in SNc neurons, opening a new realm of research regarding Ca\(^{2+}\) homeostasis in this neurodegeneration-prone locus (Bean, 2007).
DISCUSSION

We have demonstrated that RNA editing of CaV1.3 channels downwardly modulates their Ca\(^{2+}\) regulation by an unexpected mechanism. Rather than attenuate Ca\(^{2+}/\)CaM binding to an effector site comprised by the channel IQ element alone (Figure 1B), editing variants reduce the affinity of channels for apoCaM (Figure 3E). This effect promotes the occurrence of channels uncharged by CaM and incapable of CDI. This alternative mechanism predicts that CDI of edited channels could become a smoothly continuous function of ambient CaM levels (Figure 4A), an outcome we corroborate in substantia nigral neurons (Figure 4, B–D).

These findings demonstrate that naturally occurring RNA editing of CaV1.3 channels acts to modulate CDI in ways that substantiate a recently emerging mechanism where apoCaM begins preassociated with the IQ and other channel elements (Figure 4A, configuration A), but the Ca\(^{2+}/\)CaM effector configuration (configuration I) involves substantial rearrangements and differs considerably from that originally proposed in Figures 1B, C. A surprising outcome here is that even single-residue changes may readily influence configurations outside the Ca\(^{2+}/\)CaM effector complex. For example, the impression from prior work has been that channels so avidly prebind to apoCaM that they would always possess a resident CaM (Findeisen et al., 2011; Yang et al., 2006), and that mutation of several IQ residues might be required to appreciably affect apoCaM interaction (Erickson et al., 2003a; Liang et al., 2003). This view need no longer be the norm. More broadly, the mechanism in Figure 4A adds to the growing awareness of direct biological actions by apoCaM, despite the historical focus on the functions of Ca\(^{2+}/\)CaM (Jurado et al., 1999).

Given the numerous indications of strong variations of CaM under differing physiological and disease-related contexts (Bezprovanny, 2009; Black et al., 2004; Chafouleas et al., 1982; Ikeda et al., 2009; Lesnick et al., 2007; Yacoubian et al., 2008; Zhang et al., 2005), the rheostat-like connection between CaM levels and Ca\(^{2+}\) feedback gain on Ca\(^{2+}\) influx now looms as a potentially important dimension of Ca\(^{2+}\) homeostasis and dysfunction (Figure 4A). In this regard, it is worth considering the CaM dependence of aggregate CDI exhibited by a channel population comprised of prototypic IQDY and editing variants. Figure 4E shows the projected CDI response relations for individual CaV1.3 species, based on our apoCaM binding data (Figure 3D, black curves). Layered atop RNA editing, roughly one third of CaV1.3 channel transcripts in substantia nigra exhibit a long splice variant featuring a competitive ICDI inhibitor of apoCaM binding to channels (Bock et al., 2011; Liu et al., 2010). This splicing would multiplex the RNA editing effects to a parallel set of CDI response relations (Huang et al., 2012; Liu et al., 2010), shown by the set of blue relations in Figure 4E (Extended Discussion). Each curve represents CDI sensitivity to apoCaM variations over a limited concentration range, as constrained by the 1:1 stoichiometry of apoCaM binding to channels. However, when the aggregate response of a population of variants is considered, by averaging the individual curves with weighting factors specified by transcript and splice prevalence in substantia nigra (Bock et al., 2011; Huang et al., 2012), the far more extended relationship shown in Figure 4F results (black curve; Extended Discussion). Reassuringly, our experimental estimates of CDI responsiveness and estimated CaM (black symbols from Figure 4C–4D) fit well with this projected aggregate response relation (black curve). This agreement between data and prediction should be taken as
approximate, because our estimate of free endogenous CaM concentration was obtained using a FRET-based CaM sensor expressed in readily transfectable hippocampal neurons, rather than SNc neurons \textit{per se}. This outcome then reflects an elegant mechanism to render CDI tunable over a large dynamic range of CaM levels, unachievable by a single Ca\textsubscript{V}1.3 variant. Interestingly, SNc neurons at baseline populate a ‘setpoint’ right in the middle of this response relationship (Figure 4F, dashed line labeled CaM\textsubscript{endo}), as if to optimally exploit the full dynamics of this system. CDI can thereby adapt smoothly and continuously over a maximal range of CaM levels. Altogether, this system of adjustable interdependence (Figure 4G), rooted in the role of the IQ domain as an apoCaM preassociation locus, now merits exploration in a vast array of neurophysiological and pathophysiological contexts.

**EXPERIMENTAL PROCEDURES**

**Molecular biology**

Our baseline Ca\textsubscript{V}1.3 construct (α\textsubscript{1DΔ1626}; or Ca\textsubscript{V}1.3\textsubscript{short} in Figure 5B) was closely similar to a naturally occurring rat brain variant (α\textsubscript{1D}; AF3070009 (Xu and Lipscombe, 2001), encoding 1643 amino acids) that terminates 18 residues after the IQ domain in the carboxy terminus. To facilitate mutagenesis, α\textsubscript{1DΔ1626} was engineered with a silent and unique \textit{Kpn} I restriction site at a position encoding amino acids G\textsubscript{1538}T\textsubscript{1539}, ~50 residues upstream of the IQ domain. Additionally, α\textsubscript{1DΔ1626} contains a unique \textit{Xba} I restriction site followed by a stop codon, both of which reside immediately after the IQ domain ending in V\textsubscript{1624}G\textsubscript{1625}. The engineered construct α\textsubscript{1DΔ1626} as cloned within mammalian expression vector pCDNA6 (Invitrogen), thereby permitted rapid substitution of mutated segments of ~260 bp between \textit{Kpn} I and \textit{Xba} I restriction sites. Actual point mutations were introduced via QuikChange\textsuperscript{®} mutagenesis (Agilent), where the template was a short stretch of α\textsubscript{1DΔ1626} (~1500 bp) encompassing the \textit{Kpn} I to \textit{Xba} I segment, all as cloned within a ~3.5 kb pCR-Blunt II-TOPO vector (Invitrogen). After complete sequence verification between \textit{Kpn} I and \textit{Xba} I restriction sites, mutated segments were cloned into α\textsubscript{1DΔ1626} via these same sites, yielding full-length channel constructs with point mutations. For FRET 2-hybrid constructs, fluorophore-tagged CaM constructs were made as described (Erickson et al., 2003a). Other FRET constructs were made by replacing CaM in these constructs with appropriate PCR amplified and mutated IQ segments, via unique \textit{Not} I and \textit{Xba} I sites flanking CaM (Erickson et al., 2003a). Throughout, all segments subject to PCR or QuikChange\textsuperscript{®} (Agilent) were verified in their entirety by sequencing.

**Transfection of HEK293 cells**

For electrophysiology experiments, HEK293 cells were cultured in 10-cm plates, and channels were transiently transfected by a calcium phosphate protocol (Brody et al., 1997). We applied 8 µg of cDNA encoding the desired channel α\textsubscript{i} subunit, along with 8 µg of rat brain β\textsubscript{2a} (M80545) and 8 µg of rat brain α\textsubscript{2δ} (NM012919,2) subunits. β\textsubscript{2a} minimized voltage inactivation, enhancing resolution of CDI. Additional cDNAs were added as required in co-transfections. All of the above cDNA constructs were driven by a cytomegalovirus promoter. To enhance expression, cDNA for simian virus 40 T antigen (1–2 µg) was co-transfected. For fluorescence resonance energy transfer (FRET) 2-hybrid experiments, transfections and experiments were performed as described (Erickson et al., 2003a).
Electrophysiology and FRET experiments were performed at room temperature 1–2 d following transfection.

**Neuron culture**

SNc neurons were isolated from C57BL/6 mice and FVB/N mice expressing GFP under the tyrosine hydroxylase promoter (TH-GFP) (GENSAT (Gong et al., 2003); Rockefeller University). Mouse brains were rapidly removed after decapitation and placed in ice-cold solution containing the following (in mM): NaCl, 59.4; NaHCO₃, 25; Glucose, 25; Sucrose, 75; KCl, 2.5; NaH₂PO₄, 2.3; CaCl₂, 0.9; MgCl₂, 14.9. Brains were sectioned into 400 μm coronal slices, and SNc dissected using a 22 gauge needle. SNc pieces were enzymatically digested with 3 mg/mL of proteinase XXIII (Sigma) for 1 hr at 37°C in dissociation solution containing (in mM): NaSO₄, 82; K₂SO₄, 30; HEPES (pH 7.4), 10; Glucose, 10; MgCl₂, 5; at 305–310 mOsm adjusted with glucose. All solutions were continuously bubbled with 95% O₂, 5% CO₂. Following digestion, pieces were washed in Tyrode’s solution containing (in mM): NaCl, 150; KCl, 4; CaCl₂, 2; Glucose, 10; and HEPES (pH 7.4), 10; at 305–310 mOsm adjusted with glucose. Tyrode’s solution was supplemented with 1 mg/mL of bovine serum albumin and trypsin inhibitor (Invitrogen). After wash, SNc pieces were moved to DMEM/F12 GlutaMAX (Gibco) supplemented with 3% FBS, N2 supplement, B27 supplement, NEAA, and glia-derived neurotrophic factor (GDNF) (25 ng/mL). The pieces were triturated into isolated neurons with a series of glass pipettes and plated on glass coverslips coated with poly-L-lysine, and incubated at 37°C, 95% O₂, and 5% CO₂ until experiments performed between 1 and 5 days later. SNc neurons were identified by characteristic size and morphology, as exemplified by GFP expressing neurons from TH-GFP mice.

**Whole-cell recording**

For both recombinant channels in HEK cells, and endogenous channels in SNc neurons (C57BL/6 mice), whole-cell recordings were obtained at room temperature using Axopatch 200A or 200B amplifiers (Axon Instruments). Electrodes were pulled with borosilicate glass capillaries (World Precision Instruments, MTW 150-F4), resulting in 1–3 MΩ resistances, before series resistance compensation of 80%. The internal solutions contained, (in mM): CsMeSO₃, 135; CsCl₂, 5; MgCl₂, 1; MgATP, 4; HEPES (pH 7.3), 5; and BAPTA, 10; at 290 mOsm adjusted with glucose. The bath solution contained (in mM): TEA-MeSO₃, 140; HEPES, 10, pH 7.3; CaCl₂ or BaCl₂, 40; 300 mOsm, adjusted with glucose.

**FRET optical imaging**

FRET 2-hybrid experiments were carried out in HEK293 cells and analyzed, largely as described (Erickson et al., 2003a). During imaging, the bath solution was a Tyrode’s buffer containing either 2 mM Ca²⁺ alone for apoCaM interaction experiments, or 2 mM Ca²⁺ and 10 μM ionomycin to elevate intracellular Ca²⁺ for Ca²⁺/CaM interaction experiments. In parallel experiments with cells expressing the genetically encoded Ca²⁺ indicator TN-XL (Mank et al., 2006; Tay et al., 2012), we confirmed that our ionomycin treatment achieved saturating concentrations of Ca²⁺ with respect to CaM binding. Concentration-dependent spurious FRET was subtracted from raw data prior to binding-curve analysis (Dick et al., 2008; Erickson et al., 2003b). Acceptor-centric measures of FRET were obtained with the
3-FRET algorithm as described (Erickson et al., 2003a). Complementary donor-centric measures of FRET were obtained with the E-FRET method (Ben Johny et al., 2013; Chen et al., 2006). In- vitro binding assays were performed as described previously (Erickson et al., 2003a). Standard-deviation error bounds on $K_{\text{EFF}}$ estimates were determined by Jacobian error matrix analysis (Johnson, 1980). BSCaM$_{\text{IQ}}$ sensor measurements of free CaM concentration at rest were determined by previously established protocols (Liu et al., 2010) on mouse hippocampal neurons, cultured (Brody and Yue, 2000) and transiently transfected with plasmids encoding the sensor using polyethyleneimine PEI reagent (Polysciences, Warrington, PA). Sensor measurements in this setting were favored because of the readily transfectable nature of hippocampal versus SNc neurons.

**Homology modeling of Ca$^{2+}$/CaM complexed with Cav1.3 IQ domain**

We used the python based homology modeling software MODELLER (Eswar et al., 2006) to build models of the Cav1.3 IQ domain (comprising positions -12 through +11 in Figure 2A) bound to Ca$^{2+}$/CaM. Briefly, to model Ca$^{2+}$/CaM bound IQ-domain, we used Cav1.2 structure (2BE6 (Van Petegem et al., 2005)) as a template. The IQ domains of Cav1.2 and Cav1.3 are only different by a single residue (E[+2] versus D[+2]). Molecular models and atomic structures were visualized and rendered using PyMOL v1.2r1 (DeLano Scientific, LLC).

**Homology modeling for C-lobe of apoCaM complexed with IQ domain**

We used the python based homology modeling software MODELLER (Eswar et al., 2006) to build models of Cav1.3 IQ domain bound to the C-lobe of apoCaM. Our starting templates were the NMR structures of Nav1.5 and Nav1.2 IQ domains {Chagot, 2011 #610; Feldkamp, 2011 #611} with templates aligned as follows:

| Cav1.3  | KFYATFLIQDYFRKFRKRV |
|--------|---------------------|
| Nav1.5 | EEVSAMVIQRAFRRHLQR  |
| Nav1.2 | EEVSAIVQRAYRRYLLKQ  |

Molecular models and atomic structures were visualized and rendered using PyMOL v1.2r1 (DeLano Scientific, LLC).

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Highlights

• Unexpected mechanism explains how RNA editing tunes Ca\(^{2+}\) channel regulation by CaM
• Substantiates major role of Ca\(^{2+}\) channel IQ domain to tether Ca\(^{2+}\)-free calmodulin
• Fluctuations in ambient calmodulin predicted to tune Ca\(^{2+}\) homeostasis across brain
• Elevated calmodulin enhances Ca\(^{2+}\) regulation of Ca\(^{2+}\) channels in substantia nigra
Figure 1. Functional effects of RNA editing of CaV1.3 channels, hypothesized to occur as perturbation of Ca\(^{2+}\)/CaM complexed alone with channel IQ domain

(A) Schematic of main pore-forming \(\alpha_{1D}\) subunit of CaV1.3 channel. Shown are cytoplasmic amino (N) and carboxy (C) termini, containing main elements implicated in CDI. CI, \(\text{Ca}^{2+}\) inactivation region spanning proximal channel carboxy tail (~160 aa). CI contains elements involved in CaM regulation. IQ domain (IQ), terminal CI segment (~30 aa) believed preeminent in binding CaM. Dual vestigial EF-hand region (EF) spanning proximal ~100 aa of CI. NSCaTE element on channel N terminus of CaV1.2 and CaV1.3 channels, proposed as N-lobe Ca\(^{2+}\)/CaM effector site (Dick et al., 2008; Tadross et al., 2008).

(B) Popular hypothesis about how CDI arises from CaM interactions with elements described in panel A. In this view, Ca\(^{2+}\)/CaM binding to the IQ element alone (right) triggers CDI. ApoCaM may prebind to the IQ element in a different way (left), positioning CaM as a ‘resident’ Ca\(^{2+}\) sensor. (C) Homology model of Ca\(^{2+}\)/CaM complexed with CaV1.3 IQ domain (dark blue helix, carboxy-terminal end to right). Ca\(^{2+}\) ions, yellow balls.

(D) Exemplar recombinant CaV1.3 whole-cell currents expressed in HEK293 cells. Leftmost subpanel pertains to prototypic channels with IQDY version of IQ domain. 0.2 nA scale bar pertains to Ca\(^{2+}\) current (red) throughout. Black Ba\(^{2+}\) current scaled down ~3× to facilitate...
comparison of decay kinetics, here and throughout. CDI metric, defined at right. Other subpanels pertain to various RNA edited variants, demonstrating a spectrum of reduced CDI strengths. Parentheses contain percent of corresponding transcripts across mouse brain.

(E) FRET 2-hybrid assay for interaction of CaV1.3 IQ domain and Ca\(^{2+}\)/CaM. Left, cartoon of relevant FRET pair. Right, 3\(^3\)-FRET binding curve plots FRET efficiency (\(E_A\) from YFP standpoint) versus free concentration of CFP-CaM\(_{WT}\) (\(D_{\text{free}}\)). Each symbol, mean ± SEM of ~19 cells. Smooth curve, \(E_A = E_{A,\text{max}} \cdot D_{\text{free}}/(K_{d,\text{EFF}} + D_{\text{free}})\), where \(E_{A,\text{max}}\) equals plateau value and \(K_{d,\text{EFF}}\) is given by x-intercept of vertical dotted line. Green bar calibrates \(D_{\text{free}}\) units to nM.

(F) Proposed strategy for quantitative confirmation of RNA editing mechanism involving Ca\(^{2+}\)/CaM complex with IQ element, as portrayed in panel C. CDI metric of particular construct is to be plotted versus corresponding effective association constant \(K_{a,\text{EFF}}\), deduced from data as in panel E. Symbols and fit portray hypothetical outcome: green symbol, prototypic IQ species; black symbols, RNA edited variants; smooth fit, Langmuir function as described in main text.
Figure 2. $Ca^{2+}$/CaM effector role of IQ domain to explain functional effects of RNA editing?

(A) Population data for $CDI$ metric of different RNA editing variants (left cluster in blue), and of various point-alanine substitutions (right cluster, rose and gray). Metric for prototypic IQDY species shown in green. Rose bars, strongest CDI reduction by mutations. Dashed-gray bars, mutations without appreciable CDI effects that were nonetheless chosen at random for subsequent $Ca^{2+}$/CaM binding analysis. Bars show mean ± SEM, derived from 4–6 cells each for editing, and ~6 cells each for the alanine scan.
(B) Exemplar current traces corresponding to indicated point-alanine substitutions. Format as in Figure 1D.

(C) FRET 2-hybrid interaction curves for Ca\(^{2+}\)/CaM versus IQ domain of point-alanine substitutions. As reference, green curve reproduces fit for prototypic IQDY species (from Figure 1E). Black data and fits correspond to whole-cell currents directly above in panel B. Each symbol bins data from ~3, 4, 7, and 8 cells (left to right).

(D) FRET 2-hybrid interaction curves for Ca\(^{2+}\)/CaM versus IQ domain of RNA editing variants. Format as in panel C. Each symbol bins data from ~7, 6, 9, and 6 cells (left to right).

(E) CDI plotted as a function of \(K_{a,\text{EFF}}\) deviates from Langmuir function (Figure 1F), arguing against CDI reduction arising from diminished Ca\(^{2+}\)/CaM with solitary IQ element acting as effector site. Horizontal bars, standard deviation of \(K_{a,\text{EFF}}\) deduced from Jacobian error analysis (Johnson, 1980).
Figure 3. ApoCaM prebinding role of IQ domain explains effects of RNA editing
(A) Channels require preassociation with apoCaM (configuration A) to undergo CDI. Channels without apoCaM (configuration E) cannot undergo CDI. RNA editing and alanine scanning might perturb association constant $K_a$ for apoCaM preassociation, thereby reducing CDI by populating configuration E.

(B) Population data for CDI metric under strong overexpression of CaM WT ($CDI_{CaM_{WT}}$). Different RNA editing variants (left cluster in blue). Various point-alanine substitutions (right cluster, rose and gray). Metric for prototypic IQDY species shown in green. Rose bars, strongest CDI reduction by mutations in Figure 2A. Gray bars, mutations without appreciable CDI effects in Figure 2A that were nonetheless chosen for CaM overexpression studies. Dashed-gray bars, subset of gray-bar constructs chosen at random for additional FRET analysis of apoCaM binding below. Bars show mean ± SEM derived from ~5 cells each.

(C) Exemplar current traces for selected constructs during CaM overexpression. Leftmost subpanel, prototypic IQDY species. Other subpanels, RNA-editing variants and point-alanine-substitution constructs, as labeled. Format as in Figure 1D.

(D) FRET assays of apoCaM binding to entire CaV1.3 CI region (cartoon at left). FRET 2-hybrid interaction curves. Green data and fit within MQDY subpanel display properties for IQDY construct (symbols bin ~10 cells each). Black data, interaction data and fit corresponding to RNA editing variants and alanine-substituted constructs, as labeled. Symbols average ~4, 4, 6, and 5 cells each (left to right).

(E) Plotting the ratio of $CDI_{CaM_{hi}}/CDI_{CaM_{WT}}$ (from Figures 2A, 3B) as function of $K_{a,EFF}$ decorates Langmuir curve (black line), arguing for apoCaM preassociation function of the IQ domain. Wild-type data in green.
(F) Homology model of C-lobe of apoCaM (cyan) complexed with Cav1.3 IQ domain (blue). Left, complex with prototypic IQ domain showing hotspots in red. Right, model with MQDY variant, showing potential steric clash of M[0] with apoCaM.
Figure 4. Tuning of CDI by CaM in substantia nigral neurons (SNc)
(A) Refocusing new mechanistic scheme to emphasize how changes in ambient apoCaM levels (ΔapoCaM) may continuously tune strength of CDI.
(B) Confocal image of SNc neuron expressing GFP under tyrosine hydroxylase promoter.
(C) Whole-cell currents from SNc neurons, averaged from $n = 7$ (left) and $n = 6$ (right) cells. Format as in Figure 1D. Left, with endogenous levels of CaM. Right, after strongly increasing CaM levels via pipet dialysis of recombinant CaMWT.
(D) Bar graph summary of data at left.
(E) CDI-CaM response curves for various RNA editing species, deduced from FRET 2-hybrid data in Figure 3D. Black curves, RNA editing species in splice variants lacking a competitive inhibitor ICDI module. Blue curves, parallel behavior of RNA editing species in splice variants containing an ICDI module (Extended Discussion).

(F) Aggregate CDI-CaM response curve (black), averaged over the curves of the entire population of RNA-edited species. Weighting factors specified by transcript and splice prevalence in substantia nigra. Gray and blue zones and curves reproduce response characteristics of individual RNA editing variants. Dashed lines, CDI response and CaM estimates for SNc neurons before and after CaM supplementation, taken from panel D.

(G) Conceptual scheme of Ca$^{2+}$ homeostasis, incorporating continuous CaM tuning of CDI strength as projected in panel F. Gray outline, generic pacemaking neuron with depolarizing Cav1.3 and hyperpolarizing SK channels. Ca$^{2+}$ negative feedback gain on Cav1.3 opening is continuously adjustable by CaM (as in panel F), in the manner of a rheostat-controlled gain element.