The $\alpha_2\delta$-like Protein Cachd1 Increases N-type Calcium Currents and Cell Surface Expression and Competes with $\alpha_2\delta$-1

Highlights
- Cachd1 enhances $\text{Ca}_{\text{V}}2.2$ currents and increases $\text{Ca}_{\text{V}}2.2$ surface expression
- Effects of Cachd1 are not prevented by mutation in $\text{Ca}_{\text{V}}2.2$ VWA interaction site
- The effects of $\alpha_2\delta$-1 are prevented by the same mutation in $\text{Ca}_{\text{V}}2.2$
- Cachd1 competes with $\alpha_2\delta$-1 for its effects on $\text{Ca}_{\text{V}}2.2$

In Brief
Dahimene et al. examine the role of Cachd1, a protein with similarity to the auxiliary $\alpha_2\delta$ subunits of voltage-gated calcium channels. They find that Cachd1 increases N-type calcium currents substantially despite having a disrupted VWA interaction domain. Cachd1 also enhances channel trafficking and inhibits responses to $\alpha_2\delta$-1.
The \( \alpha_2\delta \)-like Protein Cachd1 Increases N-type Calcium Currents and Cell Surface Expression and Competes with \( \alpha_2\delta-1 \)

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SUMMARY

Voltage-gated calcium channel auxiliary \( \alpha_2\delta \) subunits are important for channel trafficking and function. Here, we compare the effects of \( \alpha_2\delta-1 \) and an \( \alpha_2\delta \)-like protein called Cachd1 on neuronal N-type (CaV2.2) channels, which are important in neurotransmission. Previous structural studies show the \( \alpha_2\delta-1 \) VWA domain interacting with the first loop in CaV1.1 domain-I via its metal ion-dependent adhesion site (MIDAS) motif and additional Cache domain interactions. Cachd1 has a disrupted MIDAS motif. However, Cachd1 increases CaV2.2 currents substantially (although less than \( \alpha_2\delta-1 \)) and increases CaV2.2 cell surface expression by reducing endocytosis. Although the effects of \( \alpha_2\delta-1 \) are abolished by mutation of Asp122 in CaV2.2 domain-I, which mediates interaction with its VWA domain, the Cachd1 responses are unaffected. Furthermore, Cachd1 co-immunoprecipitates with CaV2.2 and inhibits co-immunoprecipitation of \( \alpha_2\delta-1 \) by CaV2.2. Cachd1 also competes with \( \alpha_2\delta-1 \) for effects on trafficking. Thus, Cachd1 influences both CaV2.2 trafficking and function and can inhibit responses to \( \alpha_2\delta-1 \).

INTRODUCTION

Voltage-gated calcium (CaV) channels are key constituents of excitable cells, including muscles, neurons, and secretory cells, and are essential for their function (for a review, see Zamponi et al., 2015). The neuronal N-type (CaV2.2) and P/Q-type (CaV2.1) channels are critical for presynaptic release of neurotransmitters (for a review, see Nanou and Catterall, 2018), with N-type calcium channels playing a particularly important role in primary afferent neurotransmission involving pain pathways (for a review, see McGivern and McDonough, 2004). CaV2.1 subunits form the pore of the channels, determining their main biophysical and pharmacological properties (Zamponi et al., 2015), but the associated \( \beta \) and \( \alpha_2\delta \) proteins represent auxiliary subunits that are important contributors to the trafficking and biophysical properties of the channel complexes (Gurnett et al., 1996; Leung et al., 1987; Pragnell et al., 1994; Takahashi et al., 1987). The \( \beta \) subunits increase CaV currents by binding to the intracellular I-II linker (Pragnell et al., 1994), promoting folding (Van Petegem et al., 2004), hyperpolarizing current activation (Stea et al., 1993), preventing polyubiquitination (Page et al., 2016), and inhibiting proteasomal degradation (Attier et al., 2011; Waithe et al., 2011).

By contrast, the mechanism by which the \( \alpha_2\delta \) subunits increase trafficking and function of channel complexes is less well understood (Canti et al., 2005; Cassidy et al., 2014; Ferron et al., 2018; Kadurin et al., 2016; Savalli et al., 2016). The \( \alpha_2\delta-1 \) subunit, in combination with neuronal calcium channels, is the therapeutic target for gabapentinoid drugs, used for the alleviation of neuropathic pain conditions and as an add-on therapy in certain epilepsies (Field et al., 2006), and it is therefore important to understand its mechanism of action. The \( \alpha_2\delta \) proteins undergo several post-translational processing steps, including N-glycosylation, proteolytic cleavage into \( \alpha_2 \) and \( \delta \) (De Jongh et al., 1990; Ellis et al., 1988; Jay et al., 1991), and glycosyl-phosphatidylinositol (GPI) anchoring (Davies et al., 2010).

The recent structure of the skeletal muscle CaV1.1 complex (Wu et al., 2016) has revealed a complex interaction of \( \alpha_2\delta-1 \) with several extracellular loops in domains I-III of CaV1.1. In the present study, we have taken advantage of the insights provided by this structure to probe the role of the von Willebrand factor A (VWA) domain and investigate whether there is a role for other \( \alpha_2\delta \) domains in CaV channel function. In previous studies, by mutating the metal ion-dependent adhesion site (MIDAS) motif in the VWA domain of \( \alpha_2\delta \) subunits, we have shown that the VWA domains of both \( \alpha_2\delta-1 \) and \( \alpha_2\delta-2 \) are key to promoting calcium channel trafficking and function (Canti et al., 2005; Cassidy et al., 2014; Hoppa et al., 2012). The structure confirms the interaction of the MIDAS motif with the CaV1.1 α1 subunit (Wu et al., 2016). However, we also found that mutating the MIDAS motif reduced the trafficking of \( \alpha_2\delta-1 \) itself when it was expressed alone (Cassidy et al., 2014). In the present study, we have therefore taken the reciprocal step of mutating the residue in CaV2.2 with which \( \alpha_2\delta-1 \) is predicted to bind to examine whether other regions, such as their Cache domains, play a role in promoting
CaV2.2 trafficking and function. The Cache domains in z2δ-1, which have homology to domains in bacterial chemotaxis receptors (Anantharaman and Aravind, 2000), have also been shown to interact with the CaV1.1 z1 subunit (Wu et al., 2016). We have compared the effect of z2δ-1 with that of Cachd1, identified bioinformatically to be related to z2δ proteins (Whittaker and Hynes, 2002). Cachd1 has a VWA domain with a disrupted MIDAS motif but retains multiple predicted Cache domains. Surprisingly, we found that expression of Cachd1 increased both CaV2.2 currents and cell surface trafficking in both cell lines and neurons. By contrast, expression of Cachd1 did not increase the closely related CaV2.1 currents, indicating that this effect shows specificity for certain calcium channels. Furthermore, Cachd1 competed with z2δ-1 for binding to CaV2.2 and for its functional effects and can therefore inhibit responses to z2δ-1.

RESULTS

Disruption of the Interaction Site between CaV2.2 and the z2δ-1 VWA Domain Prevents the Interaction between z2δ-1 and CaV2.2

In previous studies, we found that mutation of the MIDAS motif in z2δ-1 and z2δ-2 prevented the ability of these proteins to traffic CaV2 channels and abolished the increase in CaV1 and CaV2 currents, normally seen with wild-type (WT) z2δ-1 and z2δ-2 (Canti et al., 2005; Cassidy et al., 2014; Hoppa et al., 2012). However, trafficking of the z2δ-1 MIDAS mutant alone to the cell surface was also impaired (Cassidy et al., 2014), and our data indicate that z2δ-1 also interacts with the trafficking protein LR1P1 via its VWA domain (Kadurin et al., 2017). Therefore, in the present study, we took advantage of the recently described structure of the skeletal muscle calcium channel complex (Wu et al., 2016) and mutated the residue in CaV2.2 likely to coordinate the divalent cation together with the MIDAS interaction site of z2δ-1. The structure of z2δ-1 shows this to be residue D78, which is in the first extracellular loop of domain I; it corresponds by alignment to D122 in CaV2.2 (Figures 1A and 1B). This residue was mutated to uncharged alanine to disrupt the interaction with z2δ-1. D122A CaV2.2 was expressed at the same level as WT CaV2.2 in tsA-201 cells in the presence of β1b and z2δ-1 (Figure 1C). As we found previously (Kadurin et al., 2016), CaV2.2 showed robust co-immunoprecipitation with z2δ-1 (Figures 1C and 1D). In contrast, D122A CaV2.2 exhibited only weak co-immunoprecipitation (coIP) with z2δ-1 (Figures 1C and 1D), confirming a key role for D122 in this interaction.

The z2δ Homolog Cachd1 Is Exposed on the Cell Surface and Interacts with CaV2.2

The cryoelectron microscopy (cryo-EM) structure of CaV1.1 shows that z2δ-1 has four Cache domains (Anantharaman and Aravind, 2000; Wu et al., 2016), and there are interactions of the z1 subunit with these domains as well as with the VWA domain (Wu et al., 2016). The z2δ-like protein Cachd1 contains Cache domains, similar to the z2δ subunits, but its VWA domain has a highly disrupted MIDAS motif (Whittaker and Hynes, 2002). Indeed, in a preliminary report, Cachd1 was found to have no effect on CaV2.2 currents (Soubbrane et al., 2012). Because our experiments also suggest that the VWA domain has a dominant role in mediating the effects of z2δ-1, we decided to investigate whether Cachd1 showed any residual functional effect on CaV2.2 function.

We initially used a construct encoding zebrafish Cachd1 (zCachd1) that had been generated in a study to identify genes underlying particular nervous system development phenotypes (H. Stickney, A. Faro, G.T.P., and S.W.W., unpublished data). We subsequently confirmed our results with the rat construct rCachd1. There is very high sequence conservation, the two proteins being 85.6% identical at the amino acid level. Using a polyclonal antibody (Ab) raised against the predicted extracellular domain of zCachd1, which also recognizes human CACHD1 (G.T.P., G.J. Wright, and S.W.W., unpublished data), we observed a major band of the predicted molecular weight (MW) in whole-cell lysate (WCL) of tsA-201 cells transfected to express either zCachd1 or rCachd1 (Figure 1E). Cachd1 is predicted to be an N-glycosylated protein (Figure S1A). For rCachd1, the MW was ~168 kDa when glycosylated and ~148 kDa following deglycosylation with N-Glycosidase F (PNGase F), indicating that it has up to 7 N-glycosylation sites (Figure 1E), agreeing with the predicted number (Figure S1A). The glycosylation pattern is also compatible with the prediction that Cachd1 is a type I membrane protein (Figure S1A), in contrast to the GPI-anchored z2δ proteins (Davies et al., 2010). In addition to the major Cachd1 protein band, two lower MW minor bands were observed. For rCachd1, these were ~148 and ~137 kDa, reduced to ~133 and ~119 kDa following deglycosylation (Figure 1E). Similar results were found for zCachd1 (Figure 1E). Cell surface biotinylation indicated that the major band was the species on the plasma membrane (Figure 1E), suggesting that membrane-associated Cachd1 does not undergo post-translational proteolytic processing, unlike z2δ proteins.

To determine whether Cachd1 was co-localized on the cell surface with CaV2.2, we expressed the proteins in N2A or tsA-201 cells and imaged their localization. We found that both rCachd1 (Figure 1F) and zCachd1 (Figure S1B) were present on the cell surface, together with either WT CaV2.2 or D122A CaV2.2 and β1b. Partial colocalization of Cachd1 with CaV2.2-hemagglutinin (HA) on the cell surface was observed (Figure 1F, yellow regions). Even in permeabilized cells, most of the Cachd1 appeared to be associated with the cell surface (Figure S1B).

We then co-expressed CaV2.2 with a C-terminally GFP-tagged Cachd1 and found that immunoprecipitation (IP) of Cachd1_GFP with GFP Ab was able to coIP CaV2.2. As a control, there was no coIP of CaV2.2 using Cachd1 without a GFP tag (Figure 1G), expression of which was confirmed using Cachd1 Ab (Figure S1C). The interaction of Cachd1 with CaV2.2 was likely to be weaker than that observed for z2δ-1 because no coIP of Cachd1 with GFP_CaV2.2 was observed in experiments performed under conditions similar to those shown for z2δ-1 in Figure 1C (Figure S1D).

The D122A Mutation in CaV2.2 Prevents the Effect of z2δ-1 but Not Cachd1 on CaV2.2 Currents

In agreement with the coIP results, we found that expression of rCachd1 produced a consistent increase (4.5-fold) in WT CaV2.2 currents (in the additional presence of β1b) despite its disrupted MIDAS motif (Figures 2A–2C).
We then examined the effect of the D122A mutation on the ability of z2δ-1 and Cachd1 to increase CaV2.2 currents. We found that, although z2δ-1 increased the maximum conductance (Gmax) of WT CaV2.2 by 11.5-fold, it produced no increase in the case of D122A CaV2.2, for which the currents were of the same amplitude as WT CaV2.2 without z2δ-1 (Figures 2A–2C). Very similar results to those observed with z2δ-1 were obtained for z2δ-3 (Figure S2).

By contrast, we found that Cachd1 produced a similar increase (5.2-fold) in Gmax for D122A CaV2.2 to that observed for WT CaV2.2 (Figures 2A–2C). This result indicates that the effect of Cachd1 is unlikely to be dependent on co-ordination of a divalent cation between its disrupted MIDAS motif and loop I of the α1 subunit and, therefore, might involve other interactions with Cachd1. Like z2δ-1, Cachd1 induced a shift of current activation to more hyperpolarized potentials for both WT and D122A CaV2.2, as shown in the current-voltage (i-V) relationships (Figure 2B).

It is noteworthy that, for both WT CaV2.2 and D122A CaV2.2, we observed that the barium current (IBa) in the presence of rCachd1 had an apparent reversal potential that was ~11.6 mV more negative compared with WT CaV2.2 currents in the
presence of $\alpha_{2,\delta}^d$, suggesting a possible effect of Cachd1 on ion selectivity (Figure 2D). Under the same recording conditions, no effect was observed of rCachd1, expressed alone, on endogenous conductances in tsA-201 cells, which might independently account for this effect on the reversal potential. Furthermore, the $\omega$-conotoxin GVIA (GVIA) completely abolished CaV2.2 currents when coexpressed with $\beta_1b$ and Cachd1, as it did when $\beta_1b$ and $\alpha_{2,\delta}^d$ (Figures 2E and 2F). Note that the negative shift in reversal potential induced by Cachd1, relative to $\alpha_{2,\delta}^d$, remains present in this dataset prior to $\omega$-conotoxin GVIA application (Figure 2F).

Surprisingly, rCachd1 did not increase currents through the related CaV2.1 channel under the same conditions, although $\alpha_{2,\delta}^d$ produced the expected effect (Figure 2G; Figures S3A and S3B), indicating that there is selectivity in the effect of Cachd1 for specific calcium channel isoforms.

The D122A Mutation in CaV2.2 Reduces the Effect of $\alpha_{2,\delta}^d$ but Not Cachd1 on CaV2.2 at the Plasma Membrane

We then compared the cell surface expression of WT and D122A CaV2.2-HA, either in the presence or absence of $\alpha_{2,\delta}^d$ or Cachd1, using N2A cells. All conditions included the $\beta$ subunit $\beta_1b$, and

Figure 2. D122A Mutation of CaV2.2 Abolishes CaV2.2 Current Enhancement by $\alpha_{2,\delta}^d$ but Not Cachd1

(A) Example families of CaV2.2 currents for WT CaV2.2-HA (top row) and D122A CaV2.2-HA (bottom row), co-expressed with $\beta_1b$ and either no $\alpha_{2,\delta}^d$ (left), $\alpha_{2,\delta}^d$ (center), or rCachd1 (right). Holding potential = −80 mV, steps between −50 and +60 mV for 50 ms (top, applies to all traces).

(B) Mean (± SEM) current-voltage relationships for the conditions shown in (A). WT CaV2.2-HA (solid circles; n = 9, 18, and 27 for no $\alpha_{2,\delta}^d$, $\alpha_{2,\delta}^d$, and Cachd1, respectively) were co-expressed with $\beta_1b$ and either no $\alpha_{2,\delta}^d$, $\alpha_{2,\delta}^d$, or Cachd1 (green). The individual and mean data were fit with a modified Boltzmann equation (STAR Methods).

(C) $G_{\text{max}}$ (nanosiemens [nS]/picofarad [pF]) from the current-voltage relationships shown in (B). Individual data (same symbols as in B) and mean ± SEM are plotted.

(D) Bar charts of mean $V_{\text{rev}}$ (millivolt) for the conditions shown in (B). WT CaV2.2-HA (solid bars) and D122A CaV2.2-HA (open bars) were co-expressed with $\beta_1b$ and either no $\alpha_{2,\delta}^d$, $\alpha_{2,\delta}^d$, or Cachd1 (green). ns, not significant; ***p < 0.001, ****p < 0.0001 (1-way ANOVA and Sidak’s post hoc test correcting for multiple comparisons).
CaV2.2-HA was N-terminally GFP-tagged to identify all transfected cells. WT CaV2.2-HA was well expressed at the cell surface when co-expressed with α2δ-1, which resulted in a 7.3-fold increase compared with its cell surface expression in the absence of α2δ-1 (Figures 3A and 3C). In contrast, D122A CaV2.2-HA exhibited a very low expression level at the plasma membrane, which was similar in the presence and absence of α2δ-1 (Figures 3B and 3C). In contrast, intracellular expression of WT or D122A CaV2.2-HA (Figures 3A and 3B) was not significantly different with and without α2δ-1 (Figure 3D).

In the same experiment, we also investigated the effect of rCachd1 on cell surface expression of CaV2.2-HA. We found that it produced an increase of 2.9-fold in cell surface expression of WT CaV2.2-HA (Figures 3A and 3C). Very similar results were obtained for zCachd1 in tsA-201 cells (Figures S4A and S4B). Of great interest, and similar to its effect on calcium currents, is that rCachd1 increased cell surface expression of D122A CaV2.2-HA by an extent similar to its effect on WT CaV2.2-HA (a 2.7-fold increase compared with D122A CaV2.2-HA alone; Figures 3B and 3C). Intracellular expression of WT or D122A CaV2.2-HA (Figures 3A and 3B) was not significantly different with or without rCachd1 (Figure 3D).

To understand the mechanism of action of Cachd1, we compared the endocytosis rates of CaV2.2 in the presence of...
The D122A Mutation Abolishes the Effect of z2δ-1 on the Trafficking of Cav2.2 into Cultured Hippocampal Neurites

Because we have found the presence of z2δ to be a key regulator of trafficking of Cav2.2 into neuronal processes (Kadurin et al., 2016), we investigated whether the D122A mutation would influence this. Cultured hippocampal neurons were transfected after 7 days in culture, by which time there was already extensive neurite outgrowth. All conditions included j1b and either without z2δ or plus either z2δ-1 or Cachd1 (Figures 3E–3G), using a method described previously (Cassidy et al., 2014). We found that Cachd1 reduced the endocytosis rate of Cav2.2 (Figures 3E–3G). The mean endocytosis time constant was increased from 8.5 min for Cav2.2 + j1b to 15.4 min in the additional presence of Cachd1 (Figure 3F). This is unlike z2δ-1, which has no effect on Cav2.2 endocytosis (Cassidy et al., 2014), a result confirmed here. This effect of Cachd1 on endocytosis may therefore contribute to the increased cell surface expression of Cav2.2.

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**The D122A Mutation Abolishes the Effect of z2δ-1 on the Trafficking of Cav2.2 into Cultured Hippocampal Neurites**

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In the absence of α2δ-1, there was almost no trafficking of WT CaV2.2-HA into hippocampal neurites (Figures 4A and 4C). The same was true for D122A CaV2.2-HA, its level being similar in the presence and absence of α2δ-1 (Figures 4B and 4C).

We also analyzed cell surface expression of CaV2.2 in the cell bodies of these hippocampal neurons and found essentially the same result; the increase in cell surface expression resulting from α2δ-1 was abrogated by the D122A mutation (Figures 4D and 4E), although an intracellular signal was present for both WT and D122A CaV2.2-HA (Figure 4D).

**Cachd1 Increases the Trafficking of CaV2.2 into Hippocampal Neurites**

We therefore also investigated the effect of Cachd1 on trafficking of CaV2.2-HA into hippocampal neurites (Figure 5A). We found that it produced a consistent increase of WT CaV2.2-HA by 3.3-fold (Figures 5A and 5B), although this was less than the 6.8-fold increase produced by α2δ-1 in the same experiment. However, in this experimental context, Cachd1 was much less able to traffic D122A CaV2.2-HA into neurites than WT CaV2.2-HA (Figures 5A and 5B), unlike the result observed in the N2A cell line. This result is in agreement with our previous finding that trafficking of CaV2.2 is more stringently controlled in neurons than in cell lines (Kadurin et al., 2016).

**Figure 5. Cachd1 Promotes CaV2.2-HA Distribution in Hippocampal Neurites**

(A) Representative confocal images showing neurites of hippocampal neurons expressing CaV2.2-HA WT (left) or CaV2.2-HA D122A (right) together with β1b and mCherry in the absence (top row) or presence (bottom row) of Cachd1. Expression of mCherry is shown in red. Scale bars, 20 μm.

(B) Bar chart (mean ± SEM) showing neurite expression of WT and D122A CaV2.2-HA, determined by HA staining of intact cells prior to permeabilization. Shown are data for 137 (WT, blue solid), 144 (D122A, blue open), 200 (WT + α2δ-1, red solid), 111 (D122A + α2δ-1, red open), 175 (WT + Cachd1, green solid), and 152 (D122A + Cachd1, green open) neurites from 3 experiments. Data were normalized to the WT CaV2.2-HA + α2δ-1 condition in each experiment. ****p < 0.0001, *p = 0.0473 between WT CaV2.2-HA + α2δ-1 and D122A CaV2.2-HA + Cachd1, and #p = 0.5563 between D122A CaV2.2-HA and D122A CaV2.2-HA + Cachd1 (1-way ANOVA and Sidak’s post hoc analysis correcting for multiple comparisons).

**Cachd1 Competes with α2δ-1 for Interaction with CaV2.2**

The preceding experiments indicate that Cachd1 does not utilize the domain I D122 interaction site on CaV2.2, which is required by α2δ-1 for interaction via its MIDAS motif. Because Cachd1 still has a VWA domain, albeit with a disrupted MIDAS motif, we wondered whether Cachd1 might potentially be an antagonist at this site, interfering with the effect of α2δ-1. We found that Cachd1 concentration-dependently reduced the coloP of α2δ-1 with GFP_CaV2.2, by 71% when both cDNAs were transfected in equal amounts (Figures 6A and 6B), indicating that Cachd1 can obstruct the interaction site on CaV2.2 utilized by α2δ-1.

Furthermore, in experiments measuring CaV2.2 cell surface expression (Figures 6C and 6E), the additional presence of Cachd1 significantly reduced the effect of α2δ-1 on cell surface expression of CaV2.2-HA by 28.4% (Figure 6E, purple bar) but had no effect on its intracellular expression (Figure 6F). In this experiment, the increase in cell surface expression of CaV2.2-HA in the presence of Cachd1 alone was 54.4% of the CaV2.2-HA + α2δ-1 level (Figure 6E, green bar), and this increase with Cachd1 was still observed for D122A CaV2.2 (47.9% of the CaV2.2 + α2δ-1 level; Figure 6E, open green bar). The additional presence of α2δ-1 had no effect on the increase of D122A CaV2.2 cell surface expression in the presence of Cachd1 (Figure 6E, open purple bar).

In a direct parallel with these results, we observed that Cachd1 co-expression significantly reduced α2δ-2 currents in the presence of α2δ-1, almost to the level of CaV2.2 currents in the presence of Cachd1 alone (Figures 7A and 7B), but α2δ-1 had no effect on the ability of Cachd1 to increase D122A CaV2.2...
currents (Figures 7C and 7D). Interestingly, when α2δ-1 and Cachd1 were co-expressed, the reversal potential for WT CaV2.2 currents was identical to that observed with α2δ-1 alone (Figure 7E), pointing to preferential α2δ-1 interaction on the cell surface. By contrast, for D122A CaV2.2 currents, the reversal potential in the presence of both α2δ-1 and Cachd1 was similar to that for Cachd1 alone (Figure 7F), reinforcing the evidence for a lack of interaction of this mutant with α2δ-1.

**DISCUSSION**

In this study, we uncovered a mechanism for influencing CaV2.2 channel trafficking and function mediated by the α2δ-like protein Cachd1, despite its VWA domain having a disrupted MIDAS motif.

We first established the importance of interaction of CaV2.2 with the α2δ-1 VWA domain for its cell surface expression and function by mutating the predicted α2δ interaction site in CaV2.2 (D122), which is in the first extracellular loop of domain I. This mutation completely abolished the ability of α2δ-1 to increase the trafficking of CaV2.2 and to increase CaV2.2 currents, indicating that it is the main interaction site between the channel and α2δ. This was confirmed by our colP results.

Surprisingly, Cachd1 consistently produced a 4.5-fold increase in CaV2.2 currents and also increased the cell surface expression of CaV2.2 by 2.9-fold. However, in contrast to α2δ-1...
subunits, neither the trafficking effects of Cachd1 in N2A cells nor its effect on calcium channel currents were affected by the presence of the D122A mutation in CaV2.2. Therefore, these effects of Cachd1 are likely not to be mediated via its disrupted MIDAS motif but, rather, due to interactions of the Cache or other domains in the protein. Interestingly, our results indicate that the effect of Cachd1 on cell surface expression of CaV2.2 involves a reduction in CaV2.2 endocytosis. It is highly unlikely that this is a non-specific effect because we have previously provided many examples of protein constructs that do not increase CaV2.2 currents or cell surface expression (Ferron et al., 2008; Kadurin et al., 2016, 2017; Macabuag and Dolphin, 2015).

In α2δ-1 and α2δ-2, the key MIDAS motif in the VWA domain contains three polar or negatively charged residues and has the sequence DVSGS. It is these three residues (D259, S261, and S263 in rat α2δ-1 used here), plus two others (T331 and D363) in separate loops of the VWA domain that, together with the VWA protein ligand (CaV2.2 in this study), coordinate a divalent cation. In α2δ-3 and α2δ-4, one of these other coordinating residues is non-polar, but the MIDAS motif is intact. We confirm here that the increase in CaV2.2 currents caused by α2δ-3 is also abolished by the D122A mutation in CaV2.2. A similar result was found for the interaction of CaV1.2 and S263 in rat α2δ-1 used here), plus two others (T331 and D363) in separate loops of the VWA domain that, together with the VWA protein ligand (CaV2.2 in this study), coordinate a divalent cation.

By contrast, Cachd1 contains a VWA domain that has a disrupted MIDAS motif (DHGAS), a sequence that is conserved in the human, rat, mouse, and zebrafish Cachd1 proteins. This conservation across species supports the possibility that it may retain some function. Indeed, the ability of Cachd1 to increase the trafficking of CaV2.2 into hippocampal neurites was significantly reduced for D122A CaV2.2, suggesting that the disrupted MIDAS motif in Cachd1 may play some role in the interaction required for CaV2.2 trafficking into neurites. This result also indicates that there may be more stringent trafficking requirements for this channel in neurons. We drew a similar conclusion in a previous study, in which we showed that immature pro-α2δ-1 could traffic CaV2.2 to the cell surface in non-neuronal cells but not into hippocampal neurites, where
mature proteolytically processed \( \alpha_2\delta-1 \) was required (Kadurin et al., 2016).

We have shown previously that, when the three polar or charged residues of the \( \alpha_2\delta-1 \) MIDAS motif are mutated to alanine, \( \alpha_2\delta-1 \) still associates with CaV2.2, as judged by its ability to occlude antigenic epitopes within the Cache domains of CaV2.2 (Cassidy et al., 2014), although it fails to promote CaV2.2 trafficking. This indicates that there are certainly additional interaction sites as well as the MIDAS site interaction between \( \alpha_2\delta-1 \) and CaV2.2. The cryo-EM structure of the skeletal muscle calcium channel complex indicates clearly that \( \alpha_2\delta-1 \) interacts with CaV1.1 via multiple sites in addition to the divergent cation-mediated VWA domain interaction, including an interaction of a Cache domain with the turret of pore loop 5 in domain III (Wu et al., 2016). Such an interaction with the pore domain of CaV2.2 could also potentially explain the effect of Cachd1 on the apparent reversal potential. Because Cachd1 was able to co-IP CaV2.2, partially co-localized with CaV2.2 on the cell surface of transfected cells, and also affected the reversal potential of these channels, it is clear that Cachd1 is not solely a trafficking protein but influences functional channels in the plasma membrane. The influence of another protein on the reversal potential of a channel, interpreted as an effect on its selectivity filter, has been observed previously (Stephan et al., 2018). The lack of effect of Cachd1 on CaV2.1 currents may relate to a particular splice variant or be common to all isoforms of CaV2.1 and should allow us to localize the site of selective interaction of Cachd1 with CaV2.2 in the future.

The finding that Cachd1 was able to inhibit the co-IP between CaV2.2 and \( \alpha_2\delta-1 \) and reduce the effect of \( \alpha_2\delta-1 \) on CaV2.2 cell surface expression and CaV2.2 currents indicates that, in vivo, it could play either a positive or an inhibitory role on CaV2.2 currents, depending on the degree of association of the CaV2.2 channels with \( \alpha_2\delta-1 \). From mRNA expression screens, Cachd1 is widely expressed in many tissues, including the brain, lungs, and small intestine. Of particular interest here is that Cachd1 mRNA expression was highest in dorsal root ganglia of all mouse tissues examined (see the transcriptome database described in Ray et al., 2018), raising the intriguing possibility that Cachd1 may modulate the efficacy of \( \alpha_2\delta-1 \) following neuropathic injury, a hypothesis that we will investigate in future studies.

Within the brain, there is strong expression of \( \alpha_2\delta-1 \) mRNA in the mouse hippocampus (Schlick et al., 2010). It is expressed strongly in CA1 and also present in dentate granule neurons. Cachd1 mRNA is also expressed in the mouse hippocampus, and, within the pyramidal cell layer, it is particularly prominent in CA3 but also in CA1 (Allen Mouse Brain Atlas; mouse.brain-map.org/api/index.html). Thus, Cachd1 and \( \alpha_2\delta-1 \) are likely to be expressed in overlapping cell types in the hippocampus. Within the rat hippocampus, there is a robust signal for \( \alpha_2\delta-1 \) protein in synaptic regions, including the dentate gyrus molecular layer, the \textit{stratum lucidum} of CA3, and the CA1 \textit{stratum oriens} and \textit{stratum radiatum} (Nieto-Rostro et al., 2014; Taylor and Garrido, 2008); however, there are no equivalent data available for Cachd1 because of the paucity of antibodies and lack of knockout control tissue. Furthermore, in a large-scale proteomic study of non-neuronal cell lines, several proteins interacting with Cachd1 have been described recently (Huttlin et al., 2017); this suggests other potential roles for this protein in non-excitable cells (Rutledge et al., 2017).

In the future, it will be of great interest to determine the effect of Cachd1 on native calcium channels and whether its expression is altered in conditions such as neuropathic injury of primary afferent neurons to further elucidate its physiological role and to understand whether it competes endogenously with \( \alpha_2\delta-1 \) or other \( \alpha_2\delta \) subunits.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental Information includes four figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.10.033.

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**AUTHOR CONTRIBUTIONS**

D.Y.H. and W.S.P. made cDNA constructs. G.T.P. made the Cachd1 Ab, and Gavin J. Wright (Wellcome Trust Sanger Institute) for facilities for protein production and Ab purification. S.W.W. and G.T.P. thank Ana Faro and other lab members for insightful discussions regarding Cachd1.

**DECLARATION OF INTERESTS**

The authors declare no competing financial interests.
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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**       |        |            |
| αδ-1 Ab             | Sigma-Aldrich | Cat #: C5105; RRID:AB_258885 |
| Anti-CaV2.2 II-III loop Ab (rabbit polyclonal) | (Raghib et al., 2001) | n/a |
| Anti-PA Ab rat monoclonal | Sigma-Aldrich | Cat #: 11815016001; RRID:AB_390914 |
| Anti-HA Ab rabbit   | Sigma-Aldrich | Cat #: H6908; RRID:AB_260070 |
| Anti-GAPDH Ab       | Ambion | Cat #: AM4300; RRID:AB_2536381 |
| Anti-GFP Ab (Living Colors, rabbit polyclonal) | Takara Bio Clontech | Cat #: 632375 |
| Anti-rabbit Alexa fluor 594 | Thermo Fisher | Cat #: R37117; RRID:AB_2556545 |
| Anti-rat Alexa fluor 488 | Therma Fisher | Cat #: A-11006; RRID:AB_2534074 |
| Anti-mouse Alexa fluor 647 | Thermo Fisher | Cat #: A32728; RRID:AB_2633277 |
| Anti-rat fluorescein isothiocyanate | Sigma-Aldrich | Cat #: F1763; RRID:AB_259443 |
| Goat anti-rabbit HRP | Biorad | Cat #: 1706515; RRID:AB_11125142 |
| Goat anti-rat HRP   | Biorad | Cat #: 5204-2504; RRID:AB_619913 |
| Goat anti-mouse HRP | Biorad | Cat #: 1721011; RRID:AB_11125936 |
| Affinity-purified Cachd1 rabbit polyclonal Ab | G. T. Powell and S.W Wilson, UCL | n/a |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| α-conotoxin GVIA | Alomone | Cat #: C-300 |
| Penicillin-Streptomycin (10,000 U/mL) | Invitrogen | Cat #: 15140-122 |
| Poly-L-lysine | Sigma-Aldrich | Cat #: P.6282 |
| Dulbecco’s modified Eagle’s medium | Thermo Fisher | Cat #: 10012-029 |
| GlutaMAX | Invitrogen | Cat #: 35050-038 |
| Fugene | Promega | Cat #: E2311 |
| Polyjet | Tebu-bio Ltd | Cat #: 189-SL100688-1 |
| Opti-MEM | Thermo Fisher | Cat #: 41965-039 |
| Neurobasal Medium | Invitrogen | Cat #: 10888-022 |
| B27 | Thermo Fisher | Cat #: 17504-044 |
| HEPES | Sigma-Aldrich | Cat #: H3735 |
| Horse serum | Invitrogen | Cat #: 26050-088 |
| Lipofectamine 2000 | Invitrogen | Cat #: L3000-008 |
| Premium Grade EZ-link Sulfo-NHS-LC-Biotin | Thermo Fisher | Cat #: 21335 |
| Glycine | Sigma-Aldrich | Cat #: G8898 |
| SDS | VWR | Cat #: 444062F |
| Protease Inhibitors | Roche | Cat #: 11697498001 |
| DTT | Melford | Cat #: M81015 |
| SDS-polyacrylamide gel electrophoresis | Invitrogen | Cat #: EA0375BOX |
| polyvinylidene fluoride (PVDF) membrane | Biorad | Cat #: 1620177 |
| streptavidin-agarose beads | Thermo Fisher | Cat #: 20347 |
| Igepal | Sigma-Aldrich | Cat #: 13021 |
| PNGase-F | Roche Applied Science | Cat #: 11365177001 |
| Digitonin | Millipore | Cat #: 300410 |
| A/G PLUS Agarose slurry Santa Cruz | Santa Cruz | Cat #: Sc-2003 |
| Paraformaldehyde | Sigma-Aldrich | Cat #: P6148 |
| Goat serum | Invitrogen | Cat #: 6210-072 |
| Triton X-100 | Thermo Fisher | Cat #: 28314 |

(Continued on next page)
Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) | Molecular probes | Cat # n5995050 |
| VectaShield | Vector Laboratories | Cat H1000 |
| papain | Sigma-Aldrich | Cat # P4762 |
| L-cysteine | Sigma-Aldrich | Cat # c7755 |
| bovine serum albumin | First Link UK ltd | Cat # 41-00-410 |
| DNase | Sigma-Aldrich | Cat # D5025 |
| Hank's basal salt solution | Thermo Fisher | Cat # 14175-053 |
| α-bungarotoxin Alexa Fluor® 488 conjugate (BTX488) | Thermo Fisher | Cat # B13422 |
| fetal bovine serum | Invitrogen | Cat # 10270 |

Critical Commercial Assays

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Bradford Assay | BioRad | Cat # 500-0006 |
| ECL 2 | Thermo Fisher | Cat # 32132 |

Experimental Models: Cell Lines

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| tsA-201 cells | ECACC | Cat # 96121229 |
| Neuro2A cells | ATCC | CCL-131 |

Experimental Models: Organisms/Strains

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Rat Sprague Dawley male | UCL bred in house | n/a |

Oligonucleotides

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Primer for introducing the D122A mutation, reverse 5'-GACATAGGCGTCTTGGCCCCGTCAG-3' | this paper | n/a |
| Primer for introducing the D122A mutation forward 5'-CTGACGGGGCCAAGACGCCTATGTC-3' | this paper | n/a |

Recombinant DNA

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Rabbit Ca<sub>2.2</sub> HA | (Cassidy et al., 2014) | n/a |
| Rat β1b (X61394) | (Pragnell et al., 1991) | n/a |
| Rat α<sub>2</sub>β-1 (M86621) | (Kim et al., 1993) | n/a |
| HA tagged α<sub>2</sub>β-1 | (Kadurin et al., 2012) | n/a |
| Rat Ca<sub>2.1</sub> | (Brodbeck et al., 2002) | n/a |
| Human TASK3 (KCNK9) (NM_001282534) | obtained from Prof. A Mathie | n/a |
| Zebrafish zCachd1 | G. T. Powell and S.W Wilson, UCL. | n/a |
| Rat rCachd1 | OriGene | Cat # RN217577 |
| GFP_Ca<sub>2.2</sub>-HA | (Macabuag and Dolphin, 2015) | n/a |
| Ca<sub>2.2</sub>-BBS | (Cassidy et al., 2014) | n/a |
| Ca<sub>2.2</sub>-HA D122A | This paper | n/a |
| GFP_Ca<sub>2.2</sub>-HA D122A | This paper | n/a |
| rCachd1_GFP | This paper | n/a |
| zCachd1_GFP | G. T. Powell and S.W Wilson, UCL. | n/a |
| Mouse α<sub>2</sub>δ-3 (AJ010949) | (Klugbauer et al., 1999) | n/a |
| mcherry (AY678264) | (Shaner et al., 2004) | n/a |
| CD8 | (Shy et al., 2014) | n/a |

Software and Algorithms

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| ImageJ | National Institutes of Health | RRID:SCR_003070 |
| GraphPad Prism 5 or 7 | [Website](https://www.graphpad.com) | n/a |
| Origin-Pro 2015 | Microcal Origin, Northampton, MA | n/a |
| pCLAMP 9 | Molecular Devices | n/a |
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled where possible by the Lead Contact, Annette Dolphin (a.dolphin@ucl.ac.uk).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines

Cell lines were plated onto cell culture flasks, coverslips coated with poly-L-lysine, and cultured in a 5% CO2 incubator at 37°C. tsA-201 cells (ECACC, female sex) were cultured in Dulbecco’s modified Eagle’s medium in the presence of 10% fetal bovine serum, penicillin, streptomycin and 2% GlutaMAX (Invitrogen). N2A cells (ATCC, male sex) used for immunocytochemistry experiments, were cultured in DMEM and OPTI-MEM (1:1), supplemented with FBS (5%), penicillin (1 unit/ml), streptomycin (1 µg/ml), and GlutaMAX (1%).

Primary Hippocampal cultures

Hippocampal neurons were obtained from P0 rat pups (Sprague-Dawley, male), as previously described (Morales et al., 2000). All experiments were performed in accordance with the UK Home Office Animals (Scientific procedures) Act 1986, using a Schedule 1 method, with UCL ethical approval. Briefly, hippocampi were dissected and treated for 40 min at 37°C with a papain solution containing: 70 units/ml of papain, 0.2 mg/ml L-cysteine, 0.2 mg/ml bovine serum albumin (BSA), 1 mg/ml DNase and 5 mg/ml glucose (all from Sigma Aldrich) in Hank’s basal salt solution (HBSS) medium (Invitrogen). Hippocampi were then washed twice with plating solution (Neurobasal medium supplemented with B27 (Thermo Fisher Scientific; 2%), HEPES (10 mM), horse serum (5%), glutamine (0.5 mM) and 1 unit/ml penicillin, 1 µg/ml streptomycin), and the neurons were mechanically dissociated using fire-polished glass Pasteur pipettes with decreasing diameter. Approximately 75 x 10³ cells in 100 µl of plating solution were seeded onto sterile poly-lysine-coated glass coverslips. After 2 h, the plating solution was replaced with 1 ml of growth medium (serum-free Neurobasal medium supplemented with B27 (4%), 2-mercaptoethanol (25 µM), glutamine (0.5 mM), and 1 unit/ml penicillin, 1 µg/ml streptomycin), half of which was replaced every 3-4 days. At 7 days in vitro and 2 h before transfection, half of the medium was removed, and kept as ‘conditioned’ medium, and 500 µl of fresh medium was added.

METHOD DETAILS

Molecular biology and constructs

cDNAs encoding the following proteins were used: calcium channel Ca₂⁺.2 (rabbit, GenBank: D14157), containing an extracellular HA tag (Cassidy et al., 2014), p11 (rat, GenBank: X61394), z₂δ-1 (rat, GenBank: M86621), HA-tagged z₂δ-1 (Kadurin et al., 2012), rat Ca₂⁺.2.1 (GenBank: M64373), human TASK3 (KCNK9) cDNA (GenBank: NM_001282534) and mCherry. Zebrafish zCachd1 was cloned from a zebrafish cDNA library. Rat rCachd1 cDNA (GenBank: NM_001191758) was purchased from OriGene. Note that the Cachd1 gene was misnamed Cacna2d4 in the original bioinformatics paper in which it was identified as z₂δ-like (Whittaker and Hynes, 2002). All cDNAs were subcloned into the expression vectors pMT2, pcDNA3 and pCAGGS. In some experiments, Ca₂⁺.2-HA also had the green fluorescent protein, mut3bGFP (GFP), fused to the N terminus (Macabuag and Dolphin, 2015). The D122A mutation was introduced into Ca₂⁺.2.2 by mutating aspartate at position 122 of rabbit Ca₂⁺.2.2 to alanine by PCR. C-terminal GFP fusion proteins of both zCachd1 and rCachd1 were made by standard techniques, and used where stated. The sequences of all constructs were confirmed by DNA sequencing.

Antibodies and other materials

Ca channel Abs used were: z₂δ-1 Ab (mouse monoclonal against z₂δ-1 moiety, Sigma-Aldrich, epitope identified in Cassidy et al., 2014)), anti-Ca₂⁺.2.2 II-III loop Ab (rabbit polyclonal) (Raghib et al., 2001). A bespoke, affinity-purified Cachd1 rabbit polyclonal Ab was raised by Cambridge Research Biochemicals (Billingham, UK) against the predicted extracellular domain of zCachd1 protein, produced by transient transfection of mammalian cells (Durocher et al., 2002) (G.T.P., S.W.W., and Gavin J. Wright, unpublished data). Purified Ab activity was confirmed by enzyme-linked immunosorbent assay. Other Abs used were anti-HA (rat monoclonal, Roche), anti-HA (rabbit polyclonal, Sigma), anti-GAPDH Ab (mouse monoclonal, Ambion), and GFP Ab (Living Colors, rabbit polyclonal; BD Biosciences). For immunocytochemistry, secondary Abs (1:500) used were anti-rabbit-Alexa Fluor 594, anti-rat-Alexa Fluor 488, anti-mouse-Alexa Fluor 647 (Life Technologies) or anti-rat fluorescein isothiocyanate (Sigma-Aldrich). The secondary Abs used for Western Blotting were goat anti-rabbit, goat anti-rat, and goat-anti-mouse Abs coupled to horseradish peroxidase (HRP) (Biorad). u-conotoxin GVIA was purchased from Alomone, and applied by local perfusion.

Cell line transfection

For co-IPs and electrophysiological studies, tsA-201 cells were transfected using Fugene6 (Promega, Fitchburg, WI) according to the manufacturer’s protocol. For immuno-cytochemistry, tsA-201 cells were transfected using PolyJet (SignaGen) according to the manufacturer’s protocol. N2A cells were re-plated onto poly-lysine coated coverslips and transfections were carried out using PolyJet.
The protocol described below was adapted from a procedure described previously (Gurnett et al., 1997). A tsA-201 cell pellet derived from transfected Cachd1 or empty vector where appropriate. When these experiments involved both CaV2.2 and Cachd1, CaV2.2, β1b, α2δ-1 and Cachd1 were added in a ratio of 3:2:2:2, with empty vector replacing α2δ-1 or Cachd1 where appropriate. For co-IP experiments CaV2.2 (with or without GFP and HA tags, as stated), β1b and α2δ-1 were transfected in a ratio of 2:1:2. For co-IP competition experiments the transfection mix contained CaV2.2, β1b, α2δ-1 (TASK3, Cachd1 or a 1:1 mix of both) in a ratio of 3:2:2:0.5. α2δ-1 was replaced by empty vector or rCachd1 when appropriate.

**Neuronal transfection**

The hippocampal cultures were then transfected using Lipofectamine 2000, at a ratio of 1:2 to DNA mix (1 μg/μl). After 2 h, the transfection mixes were replaced with growth medium consisting of 50% conditioned and 50% fresh medium. The DNA mix consisted of cDNAs in pCAGGS encoding WT CaV2.2 or D122A CaV2.2, mCherry, at a ratio of 3:2:2:0.5. α2δ-1 was replaced by empty vector or rCachd1 when appropriate.

**Cell surface biotinylation, cell lysis, deglycosylation and immunoblotting**

The procedures were modified from those described in more detail previously (Kadurin et al., 2012; Kadurin et al., 2016). Briefly, 72 h after transfection, tsA-201 cells were incubated for 30 min at room temperature with 0.5 mg/ml Premium Grade EZ-link Sulfo-NHS-LC-Biotin (Thermo Scientific) in PBS and the reaction was quenched with 200 mM glycine. The cells were resuspended in PBS, pH 7.4 at 4 °C containing 1% Igepal; 0.1% SDS and protease inhibitors (PI, Complete, Roche), to allow cell lysis, cleared by centrifugation at 18,000 × g and assayed for total protein (Bradford assay, Biorad). Cleared WCL corresponding to 20–40 μg total protein was mixed with Laemmli sample buffer (Davies et al., 2010) supplemented with 100 mM dithiothreitol (DTT), resolved by SDS-polyacrylamide gel electrophoresis (PAGE) on 3%–8% Tris-Acetate (Invitrogen) and transferred to polyvinylidene fluoride (PVDF) membrane (Biorad). The proteins were revealed by immunoblotting performed with the corresponding Abs essentially as described previously (Kadurin et al., 2012). The signal was obtained by HRP reaction with fluorescent product (ECL 2; Thermo Scientific) and membranes were scanned on a Typhoon 9410 phosphorimager (GE Healthcare). Biotinylated lysates (equalized to between 0.5 and 1 mg/ml total protein concentration) were applied to 40 μl prewashed streptavidin-agarose beads (Thermo Scientific) and rotated overnight at 4 °C. The beads were then washed 3 times with PBS containing 0.1% Igepal and, when required, the streptavidin beads were deglycosylated for 3 h at 37 °C with 1 unit of PNGase F (Roche Applied Science). The samples containing precipitated cell surface protein fractions were then analyzed by immunoblotting with the indicated Abs as described previously (Kadurin et al., 2012).

**Co–Immunoprecipitation**

The protocol described below was adapted from a procedure described previously (Gurnett et al., 1997). A tsA-201 cell pellet derived from one confluent 75 cm² flask was resuspended in co-IP buffer (20 mM HEPES (pH 7.4), 300 mM NaCl, 1% Digitonin and PI), sonicated for 8 s at 20 kHz and rotated for 1 h at 4 °C. The samples were then diluted with an equal volume of 20 mM HEPES (pH 7.4), 300 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, with PI (to 0.5% final concentration of Digitonin), mixed by pipetting and centrifuged at 20,000 × g for 20 min. The supernatants were collected and assayed for total protein (Bradford assay; Biorad). 1 mg of total protein was adjusted to 2 mg/ml with co-IP buffer and incubated overnight at 4 °C with anti-GFP polyclonal Ab (1:200; BD Biosciences). 30 μl A/G PLUS Agarose slurry (Santa Cruz) was added to each tube and further rotated for 2 h at 4 °C. The beads were then washed three times with co-IP buffer containing 0.2% Digitonin. The beads were then resuspended in 2 x Laemmli buffer with 100 mM DTT and analyzed alongside equalized aliquots of the initial lysate prior to co-IP by SDS-PAGE and western blotting as described above. The reverse co-IP experiments between Cachd1_GFP and CaV2.2-HA were performed under identical conditions except that the NaCl concentration in the co-IP buffer was 150 mM, and the beads were washed twice in co-IP buffer containing 0.1% Digitonin.

**Immunocytochemistry**

Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 5 min, incubated with blocking buffer (20% goat serum, 4% BSA in PBS) for 1 h at room temperature before being incubated with rat anti-HA (Roche) diluted 1:200 in 0.5x blocking buffer at 1 h at room temperature. When permeabilization was included, cells were permeabilized with 0.2% Triton X-100 for 5 min before being incubated with the second primary Ab, rabbit anti-CaV2.2 II-II loop (1:250), for 1 h at room temperature. For hippocampal neurons, primary Ab incubation was carried out at 4 °C overnight. After washing, samples were incubated with secondary Abs, anti-rat Alexa Fluor 488, anti-rat Alexa Fluor 594 and anti-rabbit Alexa Fluor 488, at a dilution of 1:500 for 1 h at room temperature. 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) was used to visualize the nuclei. Coverslips were washed and mounted in VectaShield (Vector Laboratories).

**Endocytosis assay**

N2A cells were transfected with a CaV2.2 construct tagged with a double bungarotoxin binding site epitope (CaV2.2-BBS) (Cassidy et al., 2014), β1b and either empty vector, α2δ-1 or rCachd1. After 40 h expression, cells were washed twice with Krebs-Ringer solution with HEPES (KRH) (in mM: 125 NaCl, 5 KCl, 1.1 MgCl₂, 1.2 KH₂PO₄, 2 CaCl₂, 6 Glucose, 25 HEPES, 1 NaHCO₃) and incubated with 10 μg/ml α-bungarotoxin Alexa Fluor® 488 conjugate (BTX-488) (Thermo Fisher Scientific) at 17 °C for 30 min. The

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unbound BTX-488 was removed by washing with KRH, and the labeled cells were returned to 37°C for the kinetic assay. Endocytosis was terminated by fixing the cells with cold 4% PFA-sucrose in PBS at the specified time. The cells were then permeabilized and intracellular CaV2.2 was labeled using the rabbit anti-CaV2.2 II-III loop Ab as described above.

**Image Analysis**

N2A and tsA-201 cell samples were viewed on an LSM 780 confocal microscope (Zeiss) using either 63x/1.4 or 40x/1.3 numerical aperture oil-immersion objective in 16-bit mode. The tile function (3x3 tiles, each tile consisting of 1024x1024 pixels) was used and every transfected cell within the image was analyzed to remove collection bias. Hippocampal neurons were viewed using a 20x objective (neuronal processes) or 63x objective (soma); individual neurons were selected on the basis of mCherry expression. Acquisition settings, chosen to ensure that images were not saturated, were kept constant for each experiment. Images are individual optical sections, unless otherwise stated.

Images were analyzed using ImageJ (National Institutes of Health). For N2A cells, the freehand line tool (5 pixels) was used to manually trace the plasma membrane to measure the mean intensity of cell-surface staining. Intracellular staining was measured using the freehand selection tool, excluding the nucleus and the plasma membrane. For hippocampal neurons, two concentric circles (100 and 150 μm diameter) were centered on the soma and the freehand line tool (3 pixels) was used to trace the neuronal processes between the circles, using the mCherry image as the template. The background fluorescence was measured in an area with no transfected cells and subtracted from the mean intensity.

**Electrophysiology**

Calcium channel currents in transfected tsA-201 cells were investigated by whole cell patch-clamp recording. The patch pipette solution contained in mM: Cs-aspartate, 140; EGTA, 5; MgCl₂, 2; CaCl₂, 0.1; K₂ATP, 2; HEPES, 10; pH 7.2, 310 mOsm with sucrose. The external solution for recording Ba²⁺ currents contained in mM: tetraethylammonium (TEA) Br, 160; KCl, 3; NaHCO₃, 1.0; MgCl₂, 1.0; HEPES, 10; glucose, 4; BaCl₂, 1.0; pH 7.4, 320 mOsm with sucrose. 1 mM extracellular Ba²⁺ was the charge carrier. Pipettes of resistance 2-4 MΩ were used. An Axopatch 1D or Axon 200B amplifier was used, and whole cell voltage-clamp recordings were sampled at 10 kHz frequency, filtered at 2 kHz and digitized at 1 kHz. 70%-80% series resistance compensation was applied, and all recorded currents were leak subtracted using P/8 protocol. Membrane potential was held at –80 mV. Analysis was performed using pCLAMP 9 (Molecular Devices) and Origin 7 (Microcal Origin, Northampton, MA). IV relationships were fit by a modified Boltzmann equation as follows: \( I = G_{\max} \frac{(V-V_{\text{rev}})}{[1+\exp(-(V-V_{\text{50, act}})/k)]} \) where \( I \) is the current density (in pA/pF), \( G_{\max} \) is the maximum conductance (in nS/pF), \( V_{\text{rev}} \) is the apparent reversal potential, \( V_{50, \text{ act}} \) is the midpoint voltage for current activation, and \( k \) is the slope factor.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Data were analyzed with GraphPad Prism 7 (GraphPad software, San Diego, CA) or Origin-Pro 2015 (OriginLab Corporation, Northampton, MA, USA). All data are shown as mean ± SEM; “n” refers to number of cells or neurites, unless indicated otherwise, and is given in the figure legends, together with details of statistical tests used. Experiments where representative data are shown were repeated at least 3 times, unless otherwise stated. Graphpad Prism 7 was used for statistical analysis. Statistical significance between two groups was assessed by Student’s t test, as stated. One-way ANOVA and the stated post hoc analysis was used for comparison of means between three or more groups.