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$Ca_v\beta$ surface charged residues contribute to the regulation of neuronal calcium channels

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Abstract

Voltage-gated calcium channels are essential regulators of brain function where they support depolarization-induced calcium entry into neurons. They consist of a pore-forming subunit ($Ca_v\alpha_1$) that requires co-assembly with ancillary subunits to ensure proper functioning of the channel. Among these ancillary subunits, the $Ca_v\beta$ plays an essential role in regulating surface expression and gating of the channels. This regulation requires the direct binding of $Ca_v\beta$ onto $Ca_v\alpha_1$ and is mediated by the alpha interacting domain (AID) within the $Ca_v\alpha_1$ subunit and the α binding pocket (ABP) within the $Ca_v\beta$ subunit. However, additional interactions between $Ca_v\alpha_1$ and $Ca_v\beta$ have been proposed. In this study, we analyzed the importance of $Ca_v\beta_3$ surface charged residues in the regulation of $Ca_v2.1$ channels. Using alanine-scanning mutagenesis combined with electrophysiological recordings we identified several amino acids within the $Ca_v\beta_3$ subunit that contribute to the gating of the channel. These findings add to the notion that additional contacts besides the main AID/ABP interaction may occur to fine-tune the expression and properties of the channel.

Keywords: Ion channels, Calcium channels, Voltage-gated calcium channels, $Ca_v2.1$ channels, $Ca_v\beta$ subunit, Alanine-scanning mutagenesis

Main text

Neuronal high-voltage-activated (HVA) calcium channels are multisubunits complexes that support depolarization-induced calcium entry and downstream cellular functions [1]. They are composed of a pore-forming subunit ($Ca_v\alpha_1$) that consists of four homologous membrane domains, each composed of six transmembrane helices, connected via cytoplasmic linkers (I–II, II–III, and III–IV loops), and cytoplasmic amino- and carboxy termini. They require the co-assembly with ancillary subunits to ensure the proper functioning of the channel. Among these ancillary subunits, the cytoplasmic $Ca_v\beta$ regulates several aspects of HVA channels including their gating properties and expression at the cell surface (for review see [2]). $Ca_v\beta$ subunits are encoded by four different genes ($Ca_v\beta_{1-4}$) and belong to the family of membrane-associated guanylate kinase (MAGUK). They consist of a conserved core region formed by Src homology 3 (SH3) and guanylate kinase (GK) domains connected by a HOOK region, flanked by non-conserved amino- and carboxy-termini (Fig. 1a). The molecular assembly of the $Ca_v\alpha_1$/$Ca_v\beta$ complex relies on a conserved 18 residue sequence within the I–II loop of $Ca_v\alpha_1$ called α_1 interaction domain (AID) [3] that binds into a hydrophobic groove within the GK domain of $Ca_v\beta$ termed AID-binding pocket (ABP) [4–6] (Fig. 1a). This high-affinity interaction is critical for $Ca_v\beta$-mediated enhancement of $Ca_v\alpha_1$ surface expression and gating. Mutation of key residues within the ABP that weakens or abolishes AID-ABP interaction severely alters the functional influence of $Ca_v\beta$ [7]. Besides the AID/ABP interaction, additional low-affinity contacts between $Ca_v\alpha_1$ and $Ca_v\beta$ that do not involve the ABP have been proposed to confer essential $Ca_v\beta$ modulatory properties [8–10]. In this study, we aimed to assess the functional

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(See figure on next page.)

Fig. 1 $Ca_v\beta_3$ surface charged residues contribute to the modulation of $Ca_v2.1$ channels. **a** Cartoon representation of secondary structural elements of the rat $Ca_v\beta_3$ subunit in complex with the $Ca_v\beta_1$ interacting domain (AID) (PDB 1VYT). **b** Position of surface charged residues within the $Ca_v\beta_3$ subunit. Positively (H, histidine; R, arginine) and negatively (E, glutamic acid; D, aspartic acid) charged residues are shown in blue and red, respectively. **c** Mean current–voltage (*I/V*) relationship for $Ca_v2.1$ expressed alone (filled circles) and in combination with wild-type $Ca_v\beta_3$ (open circles). **d** Corresponding mean maximal macroscopic conductance (G_{max}) obtained from the fit of the *I/V* curves with the modified Boltzmann function (1) for $Ca_v2.1$ alone and in combination with WT and mutant $Ca_v\beta_3$ (ANOVA results: $F = 26.6$; $p < 0.0001$ and $F = 10.15$; $p < 0.0001$ for $Ca_v2.1$ expressed alone versus in the presence of $Ca_v\beta_3$ variants and $Ca_v2.1$ expressed with $Ca_v\beta_3$ wild-type versus with $Ca_v\beta_3$ variants, respectively. **e** Mean normalized voltage-dependence of activation for $Ca_v2.1$ expressed alone (filled circles) and in combination with WT $Ca_v\beta_3$ (open circles). **f** Corresponding mean half-activation potential values obtained from the fit of the activation curves with the modified Boltzmann function (1) for $Ca_v2.1$ alone and in combination with WT and mutant $Ca_v\beta_3$ (ANOVA results: $F = 34.29$; $p < 0.0001$ and $F = 9.965$; $p < 0.0001$ for $Ca_v2.1$ expressed alone versus in the presence of $Ca_v\beta_3$ variants and $Ca_v2.1$ expressed with $Ca_v\beta_3$ wild-type versus with $Ca_v\beta_3$ variants, respectively). **g** Mean normalized voltage-dependence of inactivation for $Ca_v2.1$ expressed alone (filled circles) and in combination with WT $Ca_v\beta_3$ (open circles). **h** Corresponding mean half-inactivation potential values obtained from the fit of the activation curves with the two-state Boltzmann function (3) for $Ca_v2.1$ alone and in combination with WT and mutant $Ca_v\beta_3$ (ANOVA results: $F = 47.9$; $p < 0.0001$ and $F = 27.84$; $p < 0.0001$ for $Ca_v2.1$ expressed alone versus in the presence of $Ca_v\beta_3$ variants and $Ca_v2.1$ expressed with $Ca_v\beta_3$ wild-type versus with $Ca_v\beta_3$ variants, respectively. Statistical analysis (ANOVA followed by Dunnett's post hoc multiple comparisons test) was performed for all $Ca_v\beta_3$ variants either against $Ca_v2.1$ expressed alone (black statistical symbols) or $Ca_v2.1$ expressed with WT $Ca_v\beta_3$ (red statistical symbols): * $p < 0.05$. The exact *p* values of the Dunnett's post hoc analysis are provided in Additional file 3: Table S2

importance of $Ca_v\beta$ surface charged residues in the regulation of $Ca_v2.1$ channels. To do so, we generated a number of $Ca_v\beta_3$ mutants where surface charged residues, most belonging to the GK domain, were replaced with an alanine (Fig. 1b), and recombinant $Ca_v\beta_3$ were expressed in *Xenopus* oocytes with $Ca_v2.1$ for electrophysiological analyses in the presence of 40 mM barium as charge carrier. $Ca_v\beta_3$ was chosen over $Ca_v\beta_4$ because it induces a more pronounced phenotype on $Ca_v2.1$ with faster inactivation kinetics, and also because according to our experience, the association of $Ca_v\beta_3$ with $Ca_v2.1$ in expression experiments is more complete than of $Ca_v\beta_4$ which would have made the interpretation of $Ca_v\beta_4$ variants slightly more difficult overall. As expected, the maximal macroscopic conductance (G_{max}) in cells expressing $Ca_v2.1$ was increased by 3.3-fold ($p = 0.0001$) in the presence of wild-type (WT) $Ca_v\beta_3$ compared to cells expressing $Ca_v2.1$ alone (Fig. 1c, d, Additional file 1: Fig. S1, Additional file 2: Table S1). Similarly, all $Ca_v\beta_3$ variants, except the E347A mutant, produced a significant increase of $Ca_v2.1$ conductance indicative of the proper expression of $Ca_v\beta_3$ mutants (Fig. 1d, Additional file 1: Fig. S1, Additional file 2: Table S1). However, $Ca_v\beta_3$-dependent potentiation of $Ca_v2.1$ currents was significantly reduced when residues D343, D344, E347, E354 (located in the GK domain), and R358 (located in the N-terminus) were mutated (ranging from 1.4-fold reduction for $Ca_v\beta_3$ R358A to 2.0-fold reduction for $Ca_v\beta_3$ E347A compared to WT $Ca_v\beta_3$) (Fig. 1d, Additional file 1: Fig. S1, Additional file 2: Table S1). While the exact underlying mechanisms have not been further investigated in this study, this alteration is likely to have resulted from either a decreased surface expression of the channel, or from a decreased $Ca_v\beta$-dependent potentiation of the single channel gating (channel open probability and latency to first channel opening). Consistent with the latest, we observed that while co-expression of WT $Ca_v\beta_3$ produced a 10.7 mV hyperpolarizing shift ($p = 0.0001$) of the mean-half activation potential of $Ca_v2.1$, this effect was significantly reduced when the channel was co-expressed with $Ca_v\beta_3$ D343A, D344A, and E347A (Fig. 1e and f, Additional file 1: Fig. S2, Additional file 2: Table S1). In contrast, mutation of residues E354 and R358 did not alter $Ca_v\beta_3$-mediated hyperpolarization of the voltage-dependence of activation of $Ca_v2.1$ suggesting that the effect of $Ca_v\beta_3$ mutants on $Ca_v2.1$ conductance may have resulted from distinct gating alteration. In that respect, we note that while $Ca_v\beta_3$ H206A did not alter the maximal macroscopic conductance of $Ca_v2.1$-expressing cells (Fig. 1d, Additional file 1: Fig. S1, Additional file 2: Table S1), it reduced the hyperpolarizing shift of the voltage-dependence of activation produced by WT $Ca_v\beta_3$ (Fig. 1f, Additional file 1: Fig. S2, Additional file 2: Table S1). Finally, we assessed the effect of $Ca_v\beta_3$ surface charged residues on the voltage-dependence of inactivation of the channel. Co-expression of WT $Ca_v\beta_3$ produced a 16.7 mV hyperpolarizing shift ($p = 0.0001$) of the mean-half inactivation potential of $Ca_v2.1$ (Fig. 1g and h**,** Additional file 1: Fig. S3, Additional file 2: Table S1). Although this effect was significantly altered upon mutation of $Ca_v\beta_3$ surface charged residues, the magnitude of this alteration remained modest and all $Ca_v\beta_3$ mutants retained their ability to significantly enhance the voltage-dependence of inactivation of the channel (Fig. 1h, Additional file 1: Fig. S3, Additional file 2: Table S1). Indeed, the weakest enhancement was observed with $Ca_v\beta_3$ E339A and H348A which still produced a 9.1 mV 9.4 mV hyperpolarized shift, respectively, suggesting

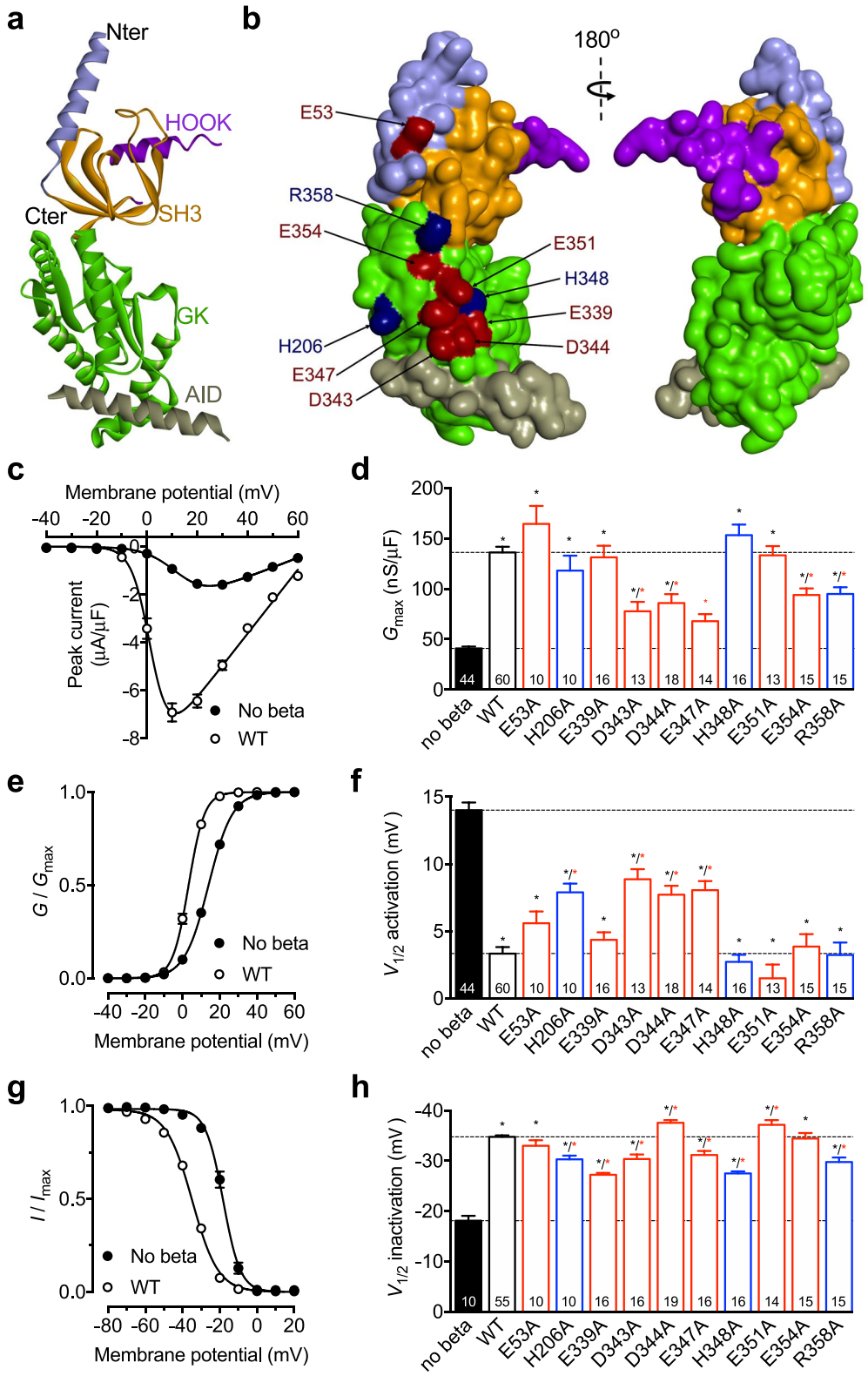

Fig. 1 (See legend on previous page.)

that $Ca_v\beta_3$ surface charged residues have minimal influence on the voltage-dependence of inactivation of $Ca_v2.1$ channels. These data however allow us to conclude that for the $Ca_v\beta_3$ mutations for which there is a reduced G_{max} (Fig. 1d), the channels under study remain in the $Ca_v2.1$ / $Ca_v\beta_3$ complex form.

While AID-ABP interaction is a prerequisite for $Ca_v\beta$-dependent modulation of HVA channels, additional interactions are expected to contribute to $Ca_v\beta$ modulatory properties [8, 9]. Here, we reported that $Ca_v\beta$ surface charged residues located outside of the ABP play a significant role in $Ca_v\beta_3$-dependent modulation of $Ca_v2.1$ channels. In particular, residues D343, D344, and E347 appear to form a hot-spot at the surface of the GK domain to influence activation of the channel, with limited effect on its inactivation. It is of interest that this cluster of residues is in close proximity to the AID sequence itself (Fig. 1B). These data are consistent with previous studies showing that the effect of $Ca_v\beta$ on the voltage-dependence of $Ca_v2.1$ channel activation is largely reconstituted by the core region of $Ca_v\beta$ [11]. The question then arises as to how surface charged residues regulate channel gating. One possibility is via enabling additional low affinity interactions between $Ca_v\beta$ and other parts of $Ca_v\alpha_1$. For instance, the amino- and carboxy-termini as well as the III–IV loop of $Ca_v\alpha_1$ have been shown to interact directly with $Ca_v\beta$ [8, 9, 12, 13]. In addition, it was reported that the orientation of $Ca_v\beta$ relative to $Ca_v\beta_1$ is essential for $Ca_v\beta$-mediated regulation of the channel activation [14, 15]. Therefore, it is a possibility that surface charged residues, by supporting low affinity interactions, may contribute to the proper positioning of $Ca_v\beta$. Inherent to our study are a number of limitations that will need to be addressed in future studies. First, in addition to $Ca_v\beta$, $Ca_v2.1$ associated with $Ca_v\alpha_2\delta$ that on the one hand mediates its own effects on the channel, and on the other hand influences the modulatory input of $Ca_v\alpha_2\delta$. For that reason, $Ca_v\alpha_2\delta$ was purposely left out of our experiments to simplify the mechanistic analysis of $Ca_v\beta_3$ variants. However, given the important role of $Ca_v\alpha_2\delta$ in the modulation of $Ca_v2.1$, the present findings will need to be confirmed in the presence of $Ca_v\alpha_2\delta$ where it can be expected that allosteric modulations will add another level of complexity to the regulation described in the present study. Second, in this study we used $Ca_v\beta_3$ because it produces a more pronounced phenotype on $Ca_v2.1$ evidenced by faster inactivation kinetics compared for instance to $Ca_v\beta_4$, and also because according to our experience the associated of $Ca_v\beta_3$ with $Ca_v2.1$ in expression experiments is more complete than of $Ca_v\beta_4$ which would have made the interpretation of the data more complicated. However, and although $Ca_v\beta_3$ represents a legitimate subunit that does associate with $Ca_v2.1$ in native condition, $Ca_v\beta_4$ remains the major isoform found co-associated with $Ca_v2.1$ in the brain and therefore it will be important to confirm our findings in the presence of $Ca_v\beta_4$. And third, another potential limitation inherent to our experimental settings is the use of *Xenopus* oocytes where trace levels of endogenous $Ca_v\beta$ have been reported. While such an endogenous $Ca_v\beta$ is unlikely to have played a major role in the regulation of recombinant $Ca_v2.1$ since otherwise we would not have observed any effect of the co-expression of $Ca_v\beta_3$, it would nevertheless be important to reproduce these findings in a mammalian cell line.

Abbreviations

ABP: AID-binding pocket; AID: Alpha interaction domain; GK: Guanylate kinase; HVA: High-voltage-activated channels; MAGUK: Membrane-associated guanylate kinase; SH3: Src homology 3.

Supplementary Information

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Additional file 1: Fig. S1. Effect of $Ca_v\beta_3$ mutants on $Ca_v2.1$ current density. a Mean current–voltage (*I/V*) relationship for $Ca_v2.1$ channels expressed alone (filled circles) and in the presence of wild-type (WT) $Ca_v\beta_3$ ancillary subunit (open circles). b–k Legend same as in (a) but for $Ca_v2.1$ channels expressed with $Ca_v\beta_3$ mutants (open red circles). The smooth lines correspond to the fit of the *I/V* curve with the modified Boltzmann function (1). The dotted line shows the position of the *I/V* curve for $Ca_v2.1$ expressed with WT $Ca_v\beta_3$ for comparison. **Fig. S2.** Effect of $Ca_v\beta_3$ mutants on the voltage-dependence of activation of $Ca_v2.1$ channels. a Mean normalized voltage-dependence of activation for $Ca_v2.1$ channels expressed alone (filled circles) and in the presence of wild-type (WT) $Ca_v\beta_3$ ancillary subunit (open circles). b–k Legend same as in (a) but for $Ca_v2.1$ channels expressed with $Ca_v\beta_3$ mutants (open red circles). The smooth lines correspond to the fit of the activation curve with the modified Boltzmann function (2). The dotted line shows the voltage-dependence of activation for $Ca_v2.1$ expressed with WT $Ca_v\beta_3$ for comparison. **Fig. S3.** Effect of $Ca_v\beta_3$ mutants on the voltage-dependence of inactivation of $Ca_v2.1$ channels. a Mean normalized voltage-dependence of inactivation for $Ca_v2.1$ channels expressed alone (filled circles) and in the presence of wild-type (WT) $Ca_v\beta_3$ ancillary subunit (open circles). b–k Legend same as in (a) but for $Ca_v2.1$ channels expressed with $Ca_v\beta_3$ mutants (open red circles). The smooth lines correspond to the fit of the inactivation curve with the two-state Boltzmann function (3). The dotted line shows the voltage-dependence of inactivation for $Ca_v2.1$ expressed with WT $Ca_v\beta_3$ for comparison.

Additional file 2: Table S1. Electrophysiological properties of $Ca_v2.1$ channel expressed in *Xenopus* oocytes in the presence of $Ca_v\beta_3$ mutants. Statistical analysis (one-way ANOVA followed by Dunnett's post hoc multiple comparisons test) was performed for all $Ca_v\beta_3$ variants against $Ca_v\beta_3$ wild-type (WT): $*p < 0.05$. β decreased conductance; β depolarized shift of voltage-dependence; β hyperpolarized shift of voltage-dependence.

Additional file 3: Table S2. Statistical summary. One-way analysis of variance (ANOVA) followed by Dunnett's post hoc multiple comparisons test was used to determine statistical significance between $Ca_v\beta_3$ variants against channel expressed alone (top table) and against channel expressed with wild-type (WT) $Ca_v\beta_3$ (bottom table). Adjusted *p* values from Dunnett's multiple comparisons test are presented.

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Authors' contributions
N.W. and M.D.W. designed and supervised the study. A.T.V.M. and N.W. performed the experiments. N.W. analyzed the data. N.W. and M.D.W. wrote the manuscript. All authors critically revised the manuscript and contributed significantly to this work. All authors read and approved the final manuscript.

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Availability of data and materials
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Declarations

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Not applicable.

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Competing interests
The authors declare that they have no competing interests.

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