Unique cysteine-enriched, D2L5 and D4L6 extracellular loops in Ca\textsubscript{V}3 T-type channels alter the passage and block of monovalent and divalent ions

Wendy Guan\textsuperscript{1,2}, Robert F. Stephens\textsuperscript{1,2}, Omar Mourad\textsuperscript{1}, Amrit Mehta\textsuperscript{1}, Julia Fux\textsuperscript{1} & J. David Spafford\textsuperscript{*}

Invertebrate LCa\textsubscript{V}3 shares the quintessential features of vertebrate Ca\textsubscript{3} T-type channels, with a low threshold of channel activation, rapid activation and inactivation kinetics and slow deactivation kinetics compared to other known Ca\textsuperscript{2+} channels, the Ca\textsubscript{1} and Ca\textsubscript{2} channels. Unlike the vertebrates though, Ca\textsubscript{3} T-type channels in non-cnidarian invertebrates possess an alternative exon 12 spanning the D2L5 extracellular loop, which alters the invertebrate LCa\textsubscript{V}3 channel into a higher Na\textsuperscript{+} and lower Ca\textsuperscript{2+} current passing channel, more resembling a classical Na\textsubscript{1},Na\textsuperscript{+} channel. Cnidarian Ca\textsubscript{3} T-type channels can possess genes with alternative cysteine-rich, D4L6 extracellular loops in a manner reminiscent of the alternative cysteine-rich, D2L5 extracellular loops of non-cnidarian invertebrates. We illustrate here that the preferences for greater Na\textsuperscript{+} or Ca\textsuperscript{2+} ion current passing through Ca\textsubscript{3} T-type channels are contributed by paired cysteines within D2L5 and D4L6 extracellular loops looming above the pore selectivity filter. Swapping of invertebrate tri- and tetra-cysteine containing extracellular loops, generates higher Na\textsuperscript{+} current passing channels in human Ca\textsubscript{3.2} channels, while corresponding mono- and di-cysteine loop pairs in human Ca\textsubscript{3.2} generates greater Ca\textsuperscript{2+} current passing, invertebrate LCa\textsubscript{V}3 channels. Alanine substitutions of unique D2L5 loop cysteines of LCa\textsubscript{V}3 channels increases relative monovalent ion current sizes and increases the potency of Zn\textsuperscript{2+} and Ni\textsuperscript{2+} block by ~50× and ~10× in loop cysteine mutated channels respectively, acquiring characteristics of the high affinity block of Ca\textsubscript{3.2} channels, including the loss of the slowing of inactivation kinetics during Zn\textsuperscript{2+} block. Charge neutralization of a ubiquitous aspartate residue of calcium passing Ca\textsubscript{1}, Ca\textsubscript{2} and Ca\textsubscript{3} channels, in the outer pore of the selectivity filter residues in Domain II generates higher Na\textsuperscript{+} current passing channels in a manner that may resemble how the unique D2L5 extracellular loops of invertebrate Ca\textsubscript{3} channels may confer a relatively higher peak current size for Na\textsuperscript{+} ions over Ca\textsuperscript{2+} The extracellular loops of Ca\textsubscript{3} channels are not engaged with accessory subunit binding, as the other Na\textsuperscript{+} (Na\textsubscript{1}) and Ca\textsuperscript{2+} (Ca\textsubscript{1}/Ca\textsubscript{2}) channels, enabling diversity and expansion of cysteine-bonded extracellular loops, which appears to serve, amongst other possibilities, to alter to the preferences for passage of Ca\textsuperscript{2+} or Na\textsuperscript{+} ions through invertebrate Ca\textsubscript{3} channels.

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| HSF site     | High field strength site |
| D2L5         | Extracellular loop spanning the end of transmembrane segment 5 to the start of the pore selectivity filter (S5-P) in Domain II |
| D4L6         | Extracellular loop spanning the end of the pore selectivity filter to the start of transmembrane segment 6 (P-S6) in Domain IV |

\textsuperscript{1}B1-173, Department of Biology, University of Waterloo, Waterloo, ON N2L 3G1, Canada. \textsuperscript{2}These authors contributed equally: Wendy Guan and Robert F. Stephens. \textsuperscript{*}email: spafford@uwaterloo.ca
Ca\(_3\) T-type channels pass low voltage-activated, inward currents that contribute to pace-making in the mammalian cardiovascular system\(^1,2\), and to rhythmic spikes, such as low threshold Ca\(^{2+}\) potentials (LTCPs) in the thalamus\(^3\), a region of highest expression in the mammalian brain\(^4,5\). The most common T-type channel isoforms expressed both in the mammalian heart and brain are Ca\(_{3.1}\) and Ca\(_{3.2}\), which generate rapid, transient and mostly Ca\(^{2+}\)-selective ionic currents\(^6\).

We had previously expressed the first non-mammalian Ca\(_3\) T-type channel, LCaV3, which is a singleton gene derived from the pond snail, Lymnaea stagnalis\(^7,8\). The invertebrate Ca\(_{3}\) homolog shares in key biophysical features of mammalian counterparts, including a capacity to generate rapid and brief inward currents elicited by inhibitory input delivered by hyperpolarization which removes the channel refractoriness due to their inactivation at resting potentials\(^9\). The invertebrate LCaV3 homolog also possesses a "window current" of open channels from the significantly overlapping activation and inactivation curves at resting potentials\(^9\). Invertebrate LCaV3 also possesses the typical T-type channel's characteristically slow rate of deactivation which allows for a significant current influx, even when voltage changes are compelling LCaV3 channels to close\(^9\). A critical difference from vertebrate Ca\(_3\) T-type channels was encountered after in vitro expression of an unusual, alternative splice isoform spanning exon 12, which generated high Na\(^+\) current passing T-type channels\(^9\). This splice isoform expresses as the only isoform in the invertebrate heart\(^6\). High Na\(^+\) current passing T-type channels in invertebrates are generated by swapping of unique extracellular loops rising above the pore's signature, ion selectivity filter. We first evaluate the importance of the unique pattern of multiple cysteines in the variable extracellular loop in Domain II, by substitution of the cysteines in D2L5 extracellular loop with alanine residues. We observe that Δcys mutations in extracellular D2L5 loops possess a greater monovalent ion current size, and also alters the relative passage of divalent ion currents (Ba\(^{2+}\), Sr\(^{2+}\)) compared to Ca\(^{2+}\) and the relative block by Ca\(^{2+}\) and other divalent ions (Ni\(^{2+}\), Zn\(^{2+}\). We then swap extracellular loops between snail LCaV3 and mammalian Ca\(_{3}\) channels to illustrate that mostly Na\(^+\) or Ca\(^{2+}\) current passing T-type channel can be engendered with opposing pairs of extracellular loops containing specific patterning of cysteines that number 1 or 3 or 5 cysteines in Domains II, or 2 and 4 cysteines in Domain IV. What started as an examination of curious T-type Na\(^+\) currents within invertebrates has led to the discovery of the influences of cysteine-enriched extracellular loops contributing to a unique structure above the channel pore in the regulation of passage of altered Na\(^+\) or Ca\(^{2+}\) permeation and blockade through Ca\(_{3}\) T-type channels.

**Results**

**A variable sized Na\(^+\) current is a distinguishing feature of T-type channels.** The T-type Ca\(^{2+}\) channel homolog, LCaV3 from sample invertebrate species, pond snail Lymnaea stagnalis, shares the quintessential features of human Ca\(_{3.1}\), Ca\(_{3.2}\) and Ca\(_{3.3}\) channels, including a low threshold for channel activation below a typical resting membrane potential (~ 65 mV), rapid kinetics and slow de-activation kinetics compared to most other Ca\(_{3}\) channels (Fig. 1). Snail channels are notably different in possessing a 5–10 mV hyperpolarizing operating range of voltage-sensitivity and possess faster activation and inactivation kinetics compared to the human T-type channels (Fig. 1, Supplementary Table S1). A more remarkable difference in the invertebrate T-type channel homolog was first observed as a strikingly large size of outward currents carried by Ca\(_{3}\) channels (Fig. 1, Supplementary Table S1). We first evaluate the importance of the unique pattern of multiple cysteines in the variable extracellular loop in Domain II, by substitution of the cysteines in D2L5 extracellular loop with alanine residues. We observe that Δcys mutations in extracellular D2L5 loops possess a greater monovalent ion current size, and also alters the relative passage of divalent ion currents (Ba\(^{2+}\), Sr\(^{2+}\)) compared to Ca\(^{2+}\) and the relative block by Ca\(^{2+}\) and other divalent ions (Ni\(^{2+}\), Zn\(^{2+}\). We then swap extracellular loops between snail LCaV3 and mammalian Ca\(_{3}\) channels to illustrate that mostly Na\(^+\) or Ca\(^{2+}\) current passing T-type channel can be engendered with opposing pairs of extracellular loops containing specific patterning of cysteines that number 1 or 3 or 5 cysteines in Domains II, or 2 and 4 cysteines in Domain IV. What started as an examination of curious T-type Na\(^+\) currents within invertebrates has led to the discovery of the influences of cysteine-enriched extracellular loops contributing to a unique structure above the channel pore in the regulation of passage of altered Na\(^+\) or Ca\(^{2+}\) permeation and blockade through Ca\(_{3}\) T-type channels.

**A conserved framework of cysteine-containing D2L5 loops within Ca\(_{3}\) T-type channels of differing animal groups.** The dramatic increase in relative contribution of the Na\(^+\) current in the whole cell current of LCaV3 channels and T-type currents in snail heart cells is engendered by 17 and 28 amino acid changes, respectively between exon 12a and exon 12b, which represents less than 1% of the large ~322 kDa Ca\(_{3}\)
Figure 1. Expressed snail LCaV3 channels have more hyperpolarized voltage-sensitivities and faster kinetics than human Ca3 T-type Ca2+ channels. The more Na+ current passing isoform of snail LCaV3 with exon 12a does not vary in biophysical properties compared to more Ca2+ current passing isoform with exon 12b. (A) Representative Ca3 currents generated from −110 mV to voltage steps from near peak (−40 to −30 mV) in 10 mV steps beyond the reversal potential. (B) Current voltage relationships. (C) Activation and inactivation curves. (D) Comparison of biophysical parameters. Statistics comparison with LCaV3–12a using one-way ANOVA combined with a Student-Newman Keuls post hoc test with *p < 0.05, **p < 0.01. Data are represented as mean ± SEM. Grey shaded values represent statistically significant differences between snail LCaV3–12a channels. Table 1 provides more detailed statistical comparisons. Color coding of differing Ca3 channels: Ca3.1 (light blue), Ca3.2 (dark blue), Ca3.3 (green), LCaV3–12b (orange) and LCaV3–12a (red). Data contained in this figure were analyzed and illustrated using OriginPro 2018 (64-bit) SR1 b9.5.1.195.
Figure 2. All Ca3 T-type channels will pass Na+ in the presence of external Ca2+ at physiological concentrations to some degree, but the almost exclusive Na+ current passing through snail LCa3 channels requires a unique exon configuration and cysteine content spanning the D2L5 extracellular loop. (A) Amino acid alignment (including invertebrate Ca3 and human Ca3.x channels) illustrating the Domain II, L5 extracellular loop and descending helix sequences altered in snail LCa3 channels spanning exon 12a and exon 12b, and highlighting of 3 and 4 cysteine to alanine replacements for LCaV3–12a(Δcys) and LCaV3–12b(Δcys) mutants. (B) Representative traces and (C) graph illustrating the dramatic increase in peak current sizes in presence of external Na+ ions (Ca2+ and Na+ external solution) compared to when large weakly permeant monovalent ion, NMDG+ replaces Na+ ions (Ca2+ and NMDG+ external solution). Larger size of T-type currents in Δcys mutants suggests that disulphide bonds in extracellular loops is a contributor to Na+ or Ca2+ current passage through T-type channels. The graph contains mean ± SEM with data replicates (n) illustrated as grey diamonds. Data to generate graphs were compared in a parametric one-way ANOVA with Turkey post hoc test for statistical significance. Statistical significances are tabulated in Supplementary Tables S4 and S5. Data are significant (p < 0.05) unless stated, where n.s. = non-significant. Color coding of differing Ca3 channels: Ca3.1 (light blue), Ca3.2 (dark blue), Ca3.3 (green), LCa3–12b (orange), LCa3–12a (red), LCa3 Δcys mutants (striped orange or red bars, or dotted lines). Electrophysiology data contained in this figure were analyzed and illustrated using OriginPro 2018 (64-bit) SR1 b9.5.1.195. The alignment in Fig. 2a was created using MUltiple Sequence Comparison by Log- Expectation (MUSCLE) at website: https://www.ebi.ac.uk/Tools/msa/muscle/.
T-type channel protein (Fig. 2a). The presence of mutually-exclusive, alternative exons 12a and 12b includes most non-vertebrates Ca₃.3 T-type channels, with a pattern of conserved cysteine placement in the L5 (or S5-P) extracellular loop inDomain II (D2L5) that suggest a highly-organized structural framework of cysteine bridge pairs in invertebrate Ca₃.3 channels (Fig. 2a, Supplementary Figures S1, S2). Exon 12b has a penta-cysteine configuration C…CxC…CxC (most protostomes) or CxxC…CxC (some nematodes) and is always longer (range 48–55 aa long, average = 52 aa) than exon 12a (Fig. 2a, Supplementary Figures S1, S2). Exon 12a is shorter (38–46 aa long, average = 41 aa) with a nearly invariant tri-cysteine configuration of D2L5 extracellular loop: CxxC…C (Fig. 2a, Supplementary Figures S1, S2). Basal species like single cell choanoflagellates or cnidarians and vertebrate Ca₃.3 channels have short D2L5 loop (average ~ 39 aa) with no or a single cysteine residue, respectively (Fig. 2a, Supplementary Figure S1). Our goal in this research was to explore the importance of the framework of additional cysteines in the D2L5 loop of invertebrate Ca₃.3 channels in altering the relative peak sizes of the relative monovalent ion (Li⁺, Na⁺, K⁺, Ca⁺) currents, divalent ion currents (Ca²⁺, Ba²⁺, Sr²⁺), and current blockade by external Na⁺, Ca²⁺ and other divalent cations (Ni²⁺ and Zn²⁺).

Loop cysteines regulate the Na⁺ ion dependent current through T-type channels. We replaced cysteine residues with alanine residues to convert the tri-cysteine loop of exon 12a and penta-cysteine loop of exon 12b in snail LCa₃,3 channels to superficially resemble the uni-cysteine arrangement in the Domain II L5 loops of vertebrate Ca₃.3 channels (Fig. 2a). Average increases of the peak current size in the presence of external Na⁺ replacing relatively non-permeant monovalent ion, NMDG⁺ was ~ 15 to ~ 20 fold for LCa₃,3–12a (Δcys) and ~ 2.3 to ~ 3.8 fold for LCa₃,3–12b (Δcys) (Fig. 2b,c). The relative permeability differences can be estimated in bi-ionic reversal experiments (Fig. 3), where high [Ca²⁺] in the external solution (4 mM) and a high concentration (100 mM) of monovalent ions (Li⁺, Na⁺, K⁺, Cs⁺) in internal solutions, generates a reversal potential (see inset, Fig. 3a) in a series of current-generating voltage steps that is considered to reflect the relative permeability of Ca²⁺ influx normalized to the permeability for the monovalent ion efflux (Fig. 3b). The calculated relative permeabilities using the bi-ionic method is provided on the bottom of page 34 of Fatt and Ginsborg based on measurement of reversal potential changes (Fig. 3b). We found the observed rank order from highest to lowest permeability to be Ca²⁺ > Na⁺ > K⁺ > Cs⁺ (Fig. 3a,b). A greater contributing Na⁺ current is reflected in a much weakened capacity of Ca²⁺ to block Na⁺ current (94–97% C₃,3 > LCa₃,3–12b > CaV3.3 > CaV3.2 = CaV3.1). An exception to this order is that the outward currents in loop cysteine mutated LCa₃,3–12b (Δcys) channels appear much more permeable to outward monovalent ions (Fig. 3) than expected based on their relative contribution to inward cation currents (Fig. 2b,c).

Loop cysteines regulate the relative contributions of Na⁺ and Ca²⁺ currents through T-type channels. We then measured the capacity of increasing extracellular Ca²⁺ doses ranging from 1 × 10⁻⁹ to 1 × 10⁻² to compete for passage through the T-type channel pore in the presence of extracellular Na⁺ ions at 60 mM. The voltage of expected peak sized currents shifts with changes in extracellular Ca²⁺ dose, so the peak sized current (Supplementary Figures S3, S4) was measured as the largest peak current resulting from a voltage step to − 65 mV, − 55 mV, − 45 mV and − 35 mV from a − 110 mV holding potential. Representative peak current traces are illustrated in Supplementary Figure S3 and illustrated graphically in Fig. 4. Human Ca₃,3 channels generate a typical U-shaped response curve, where the large Na⁺ passing current largely dissipates (94–97% in the presence of 10 µM of [Ca⁴⁺]ex, i.e. the bottom of the “U” in the U shaped curve) as increasing Ca²⁺ blocks the Na⁺ current from conducting through T-type channels (Figs. 4a, 6c). Increasing [Ca⁴⁺]ex further still, and the current sizes rise again, reflecting the greater ionic current sizes of human Ca₃,3 channels to Ca²⁺ ions than Na⁺ ions at physiological (mM) levels of [Ca⁴⁺]ex (Fig. 4a,d). A high Ca²⁺ current size through human Ca₃,3 channels appears to be a reflection of a high capacity of 10 µM Ca²⁺ to block the Na⁺ current (Fig. 4c) and the larger sized Ca²⁺ currents in response to physiological (mM) concentrations of external Ca²⁺ (Fig. 4d). Current sizes rise from ~ 5 to ~ 10-fold from Ca₃,3 to Ca₃,3–12a channels, respectively in response to increasing [Ca⁴⁺]ex from 10 µM to 10 mM levels (Fig. 4d) reflecting their rank order in observed Ca²⁺ current contributions (Fig. 2b,c) and relative Ca²⁺ to monovalent ion permeabilities to the whole cell current (Fig. 3a,b). The U-shaped response to increasing Ca²⁺ is considered an indicator that Na⁺ ions effectively compete for the limited cation binding sites as the cations funnel through the human Ca₃,3 pore at low micromolar [Ca⁴⁺]ex, and where Ca²⁺ will effectively outcompete Na⁺ ions based on their higher relative Ca²⁺ permeability at physiological (mM) levels of external Ca²⁺ (Fig. 4a). A greater contributing Na⁺ current is reflected in a much weakened capacity of Ca²⁺ to block Na⁺ from passing through snail LCa₃,3 channels, especially at the 10 µM level of [Ca⁴⁺]ex where the almost complete Ca²⁺ block of current through human channels (94–97%), falls to 81% and 44% for LCa₃,3–12b and LCa₃,3–12a channels, respectively, and falls even further to as low as 16% for LCa₃,3–12a Δcys (Fig. 4b). The reduced effectiveness of Ca²⁺ to block the snail Na⁺ current, especially in LCa₃,3–12a with cysteine loop mutations is evidence of an altered preference of the channel for passage of Ca²⁺ and Na⁺ ions. A monotonic and steady decline of current (Fig. 4b) instead of a U-shaped curve with increasing [Ca⁴⁺]ex doses, reflects the poor Ca²⁺ current passing capabilities through snail LCa₃,3 channels, regardless of which configuration (exon 12a or exon 12b) of D2L5 extracellular loop.

Relative sizes of ion currents generated by divalent ions (Ca²⁺, Ba²⁺, Sr²⁺) are altered in cysteine mutated Ca₃,3 channels. The uniqueness of Ca²⁺ as a permeant ion through T-type channels can be evaluated when other divalent ions replace Ca²⁺ such as barium (Ba²⁺) (Fig. 5a,b) or strontium (Sr²⁺) (Fig. 5c) as the charge carrier. Native LCa₃,3 channels generate larger peak Ba²⁺ and Sr²⁺ currents (~ 1.4 and ~ 1.3-fold increases, respectively) compared to equivalent Ca²⁺ currents (Fig. 5). Mutations of extracellular loop cysteines disrupts this ratio of relative peak sizes of divalent ion currents in a manner that is consistent with the dramatic current
Figure 3. Bi-ionic reversal potentials experiments quantifying the relative monovalent and divalent ion permeation through Ca3 T-type channels by means of measuring the monovalent ion efflux (with 100 mM internal Li+ or Na+ or K+ or Cs+ solutions) relative to Ca2+ influx (with 4 mM Ca2+ external solution). Comparisons are made for human Ca3 channels, snail LCaV3 with exons 12a or 12b and LCav3 Δcys mutants where cysteines replace alanines in exon 12. Graphs in (B) and (C) contain mean ± SEM, with replicates illustrated as grey diamonds. (A) Current voltage relationships, with highlights of the currents crossing near the reversal potential (inset). Note the scale of the Y-axis is extended for snail LCaV3 channels reflecting the greater monovalent ion permeation compared to human Ca3 channels. (B) Shifts in reversal potential reflect a relative permeability (P) change reflected in a PCa/Px ratio, where x is the monovalent ion, Li+ or Na+ or K+ or Cs+. Data to generate bar graphs were compared in a parametric one-way ANOVA with statistical significance evaluated in a Turkey post hoc test. Data contained in this figure were analyzed and illustrated using OriginPro 2018 (64-bit) SR1 b9.5.1.195. See Supplementary Tables S2 and S3 for table of mean ± SEM and results of ANOVA analyses, respectively. Data are significant (p < 0.05) unless stated, where n.s. = non-significant. Data for LCaV3–12b, LCav3–12a and Ca3.1 in this figure are reproduced integrally from Senatore et al.4. Color coding of differing Ca3 channels: Ca3.1 (light blue), Ca3.2 (dark blue), Ca3.3 (green), LCaV3–12b (orange), LCaV3–12a (red), LCav3 Δcys mutants (striped orange or red bars).

Loop cysteines contribute to the block of Ca3 channels by divalent cations (Ni2+ and Zn2+). T-type channels are mostly resistant to blockade from animal venoms, which contain toxins that specifically target particular ion channels and receptor subtypes, such as specific isoforms of vertebrate Ca2+ and Na+ channels.13 The closest subtype specific blocker for T-type channels are divalent ions such as Ni2+ and Zn2+ which highly discriminates Ca3.2 over Ca3.1 and Ca3.3 amongst vertebrate T-type channels. Snail LCaV3 channels have a 50% Zn2+ and Ni2+ blocking concentration (IC50 = 140 µM, 300 µM, respectively) that resembles the more weakly blocking Ca3.1 (197 µM, 250 µM) and Ca3.3 (159 µM, 216 µM) channels (Fig. 5).

We addressed whether cysteines in the extracellular loops will regulate the potency of Zn2+ and Ni2+ block. LCaV3 channels increased the 50% blocking concentration of Zn2+ and Ni2+, ~ 50-10-fold in cysteine loop mutated channels, to levels for Zn2+ and Ni2+ (~ 3 µM, 32 µM) that resemble the high blockade of Ca3.2 channels (0.8 µM, 12 µM, respectively) (Fig. 6). The similarities of LCaV3 Δcys channels in the blockade of Ca3.2 by Zn2+ extends beyond the similarly high potency, to unaltered inactivation kinetics in the presence of Zn2+, compared to the dramatic dose-dependent, slowing of inactivation kinetics for Ca3.1 and Ca3.3 channels in the presence of Zn2+ (Fig. 6a,b, insets).

A lowered Ca2+ passing preference through invertebrate Ca3 channels containing exon 12a may involve the cysteine-containing D2L5 extracellular loop neutralizing the universal aspartate residue located in the outer pore of the ion selectivity filter. So how do the 18 and 27 amino acid differences, respectively between exon 12a and exon 12b generate a starkly different relative Na+ and Ca2+ current contributions to the whole cell current observed through the ~ 322 kDa LCaV3 T-type channel protein? Both the starting amino acid residues (F891) and terminal amino acid (D902) spanning the unique 9 amino acid cysteine loop of invertebrate exon 12a, is within proximity (~ 1 to 2 amino acids) to D902 (above) and D923 of the ion selectivity filter of the high resolution structure of Ca3.118 (Fig. 7a,b). More than 20% of the non-cysteine residues spanning exon 12 are positively charged (arginine, lysine) or negatively charged amino acids (aspartate, glutamate) (Supplementary Figures S2, S5). The additional cysteine bridge contained in exon 12a may constrain the D2L5 extracellular loop within proximity to influence the pore selectivity filter by electrostatic and/or steric means on key amino acids that alter a preference for passage of Ca2+ or Na+ currents. The universally conserved aspartate residue of D924 in Ca3.1 is in a key position of the outer pore of the ion selectivity filter found in homologous position in all known Ca2+-selective (Ca1, Ca2, and Ca3) channels in the outer pore, and is absent in all known Na+ channels (Na1, Na2, and Na+ leakage conductance channels (NLCN)) (Supplementary Figure S4).14 We neutralized D975 in Ca3.2, the equivalent residue in position of D924 in Ca3.1 by replacing charged aspartate (D) residue with a polar asparagine (N) residue. Neutralizing the charged aspartate (D) residue of the D975N mutation dramatically increased the peak ionic current size observed through Ca3.2 channels in the presence of external Na+, in a manner that resembles how the additional D2L5 cysteine loop contained within exon 12a of invertebrate Ca3 channels may neutralize the universal aspartate residue in the outer pore of the ion selectivity filter of the more Ca2+ current passing channels (Fig. 7c,d). We did not observe expressible currents in Ca3.2 channels when this key aspartate residue in the outer pore was substituted with an alanine residue (D975A). The lack of expressible channels with the D975A mutation in Ca3.2 channels, appears to indicate that this key negatively-charged residue in the pore selectivity filter can be neutralized, but will not remain structurally viable if converted to a hydrophobic non-polar residue like alanine.

D2L5 extracellular loops are not the sole determinant for generating high Na+ current passing Ca3 T-type channels in invertebrates. Next, we addressed whether the extracellular D2L5 loop alone is
the sole determinant to engender a mostly $Ca^{2+}$ passing current through human $CaV3.1$ and $CaV3.2$ channels and a mostly $Na^+$ passing current of snail $LCaV3$ channels with exon 12a (Fig. 2b,c). We swapped snail D2L5 loops (spanning snail exons 12a and 12b) and the equivalent D2L5H loop region from human $CaV3.2$ channels onto human and snail $CaV3$ channel backgrounds. We found that swapped D2L5 loops in chimeric channels, in both backgrounds, that is $LCaV3$ D2L5H (Fig. 8a) and $CaV3.2$-12a (Fig. 8b), contribute to an intermediate preference between a mostly $Ca^{2+}$ passing current of human $CaV3.2$ and the more $Na^+$ passing current of snail $LCaV3$–12a channels. These findings suggest that regions other than D2L5 extracellular loops are likely to be contributing to the differences in relative contributions of $Na^+$ and $Ca^{2+}$ currents in $CaV3$ channels.

Evaluation of the opposing pairs of D2L5 and D4L6 extracellular loops to sizes of relative $Na^+$ and $Ca^{2+}$ currents through $CaV3$ channels. We evaluated the individual contributions of differing D4L6 extracellular loops that we discovered in the two different $CaV3$ genes containing 2 or 4 cysteines in many anthozoan and scyphozoan cnidarian species found in available genome and transcriptome databases, and the
alternative spliced D2L5 loops of other invertebrate CaV3 channels using different combinations of extracellular loop swaps in snail LCaV3 and human CaV3.2 channel backgrounds (see Fig. 8). The observed changes in relative contribution of Na+ and Ca2+ currents in these chimeric channels are dramatic, despite the small size of loop regions of D2L5 (24 and 35 amino acids) and D4L6 extracellular loops (16 and 19 amino acids) that were swapped compared to the total channel length of 2,353 and 2,685 amino acids for full-length CaV3.2 and LCaV3 channels, respectively.

Generating mostly Ca2+ current passing human CaV3.2-like channels in snail LCaV3 background. The relative Na+ ion contribution to the whole cell currents (illustrated in brackets below) was evaluated as the fold increase in current size in presence of 2 mM external Ca2+ when equimolar 135 mM external Na+ replaces 135 mM larger monovalent ion, NMDG+ (as illustrated in Fig. 2b,c). We attempted to transform snail LCaV3–12a which has the highest relative contribution of Na+ ions to the measured whole cell current (15.68 ± 0.34, n = 15) to resemble the high Ca2+ current passing channel, human CaV3.2 (1.31 ± 0.025, n = 16) (Fig. 8a).

Replacement of snail LCaV3 with either D2L5H or D4L6H loops of human CaV3.2 channels, cut the relative observed Na+-dependent, current size through snail LCaV3 channels by approximately half (7.42 ± 0.60, n = 12, 10).
A. Zinc block - LCₐv₃-12b

- 3 mM, 1 mM, 0.3 mM, 0.1 mM, 30 µM, 10 µM, 1 µM
- 300 pA, 100 µs
- 3 mM slows activation/inactivation kinetics (like Cav3.1 and Cav3.3)
- 0 to 3 mM

B. Zinc block - LCₐv₃-12b Δcys

- 200 pA, 100 µs
- With altered LCav3 extracellular loop, zinc now imparts no effect on inactivation kinetics (like Cav3.2)
- 0 to 100 µM

C. Zinc block – dose response

- LCₐv₃-12b, LCₐv₃-12a, Cav3.1, Cav3.3
- LCₐv₃-12b Δcys, LCₐv₃-12a Δcys
- Zinc block
- [zinc] μM: 0.3, 1, 3, 10, 30, 100, 300, 1000, 10000
- (n=7, 10, 7, 5, 6)
- LCₐv₃-12b: 137 ± 2.7 μM
- LCₐv₃-12a: 106 ± 3.3 μM

D. Nickel block – dose response

- LCₐv₃-12b Δcys = 32 μM
- LCₐv₃-12b = 300 μM
- Nickel block
- [nickel] μM: 3, 10, 30, 100, 300, 1000, 10000
- (n=5, 6)
- LCₐv₃-12b Δcys: 32 µM
- LCₐv₃-12b: 300 μM
The relative Ca²⁺ to Li⁺ or Na⁺ permeabilities in bi-ionic recording conditions correspond to the measured Na⁺ contribution to the whole cell current. To quantify the relative permeabilities of Ca²⁺ ion to monovalent ion X⁺ (PCa²⁺/PX⁺) in the extracellular loop chimeras, we evaluated the monovalent ion current as an outward current flux, relative to the inward Ca²⁺ influx, generated in bi-ionic conditions where the monovalent ion (Li⁺ or Na⁺) is held at intracellular concentrations at 100 mM in the presence of extracellular divalent cation (Ca²⁺) held at 4 mM (as previously illustrated for wild type and LCaV3 Δcys channels in Fig. 3).

The relative permeabilities of the Ca²⁺ influx to monovalent ion efflux (PCa²⁺/PLi⁺) (Fig. 9a) and (PCa²⁺/PNa⁺) (Fig. 9b) is a measure based on their influence on the reversal potential calculated in a bi-ionic Nernst potential comparison. Human Cav3.x channel response to Zn²⁺ and Ni²⁺ block of Cav3.1 and Cav3.3 channels, but are conferred the high potency of Zn²⁺ and Ni²⁺ block of Ca₃.2 channels as well as the characteristic Ca₃.2 behavior with a loss of the property where inactivation kinetics slows with increasing Zn²⁺ doses, after alanine replacement of cysteines in D2L5 extracellular loops. Generating mostly Na⁺ current passing (snail LCaV3–12a (15.68 ± 0.34, n = 17) in snail LCaV3-12b alone (2.36 ± 0.054, n = 17) bears a longer penta-cysteine loop of 37 amino acids and appears to be the invertebrate equivalent of the native human Ca₃.2 channel (Fig. 2a, Supplementary Figures S1, S2) in its passing of a high Ca²⁺ current of the total whole cell current. LCaV3–12b channels with the human D4L6H loop are no different statistically in the relative contribution of Ca²⁺ to the whole cell current (1.52 ± 0.060, n = 7) as the mostly Ca²⁺ current passing wild-type human Ca₃.2 channels (1.31 ± 0.025, n = 16) (Fig. 8a). Taken together, any of D2L5 loops from snail exon 12b or human (LCaV3–12b, ~ 15.7 fold) (Fig. 8b). Placement of snail exon 12b alone (1.96 ± 0.14, n = 6) or combination of exon 12b and the snail D4L6L loop (2.29 ± 0.11, n = 6) in human Ca₃.2 channels generates a high Ca²⁺ current passing channel (~ 1.52, ~ 2.27) in snail LCaV3, that is lost when the snail channel bears (lymmaea) snail exon 12a D2L5L loop (~ 7.9, ~ 15.7) or if a chimeric snail channel with D2L5H possesses its native D4L5L loop (~ 7.4).
A higher Na⁺ permeation through the loop chimeras correspond to a weaker Ca²⁺ block of the Na⁺ current at 10 μM of external Ca²⁺. The competition between Ca²⁺ and Na⁺ for passage through the CaV3 channel pore of the extracellular loop chimeras can be evaluated by increasing extracellular Ca²⁺ from

---

**Figure 7.** Neutralization of the negatively-charged “calcium beacon” residue in Cav3.2 channels generates high Na⁺ current passing T-type channels. (A) Side view and (B) top view of human Ca₃.1 channel (PDB: 6kzp, 3.1 Å resolution (Zhao et al.18), illustrating the opposing DI–DIII and DII–DIV pore loops alone (s5–P–s6), plus DI s1–s2 loop which contains a cysteine bonded to a cysteine in DII L5 (s5–P) loop in Ca₃.1. Unique extracellular loops (purple colored lines, sequence above) in DII L5 (s5–P) between F891–D902 and DIV L6 (P–s6) between D1791–Y1798 of Cav3.1 are positioned in invertebrate Cav3 channels. Regions F891–D902 and D1791–Y1798 in Cav3.1 are unresolved in the Cav3.1 structure, indicating that these extracellular loop regions are likely highly flexible in Cav3.1. F891 and D902 are within one amino acid of pore selectivity filter residues (E923 and D924) critical for calcium selectivity. It is modelled that the extracellular loop between cysteines (C1054–C1075) in exon 12a of LCav3 in position between F891–D902 in Ca₃.1, brings positively-charged amino acids in proximity of D924, a key aspartate residue omni-present in identified calcium (Ca₁, Ca₂ and Ca₃) channels to date. (C) Charge neutralization (D975N) of the “calcium beacon” in Cav3.2T-type channels generates high sodium current passing channels (D, sample currents; E, graph), revealed as the 6.48 ± 0.98, n = 11 fold increase in peak currents when 135 mM external Na⁺ replacing equimolar impermeant NMDG⁺ in the presence of 2 mM external Ca²⁺. Graph includes mean ± SEM with replicates (n) illustrated with grey diamonds. Large fold increases in sodium current passing channels can similarly be generated in Cav3.2 channels in replacement of D2L5 extracellular loops (Cav3.2-12a) or D2L5/D4L6 extracellular loop pairs (Cav3.2-12a/D4L6) (see Fig. 10). A potential mechanism for the greater Na in LCav3 channels is in the charge neutralization of the calcium beacon, by the juxta-positioning of positively-charged residues within the D2L5 extracellular loop contained within exon 12a. PDB files in Fig. 7a,b are illustrated using PyMOL Molecular Graphics System, Version 2.3, Schrödinger, LLC, [https://pymol.org/2/](https://pymol.org/2/). Data in Fig. 8c,d were analyzed and illustrated using OriginPro 2018 (64-bit) SR1 b9.5.1.195.
1 nM (1 × 10⁻⁹) to 10 mM (1 × 10⁻²) in the presence of a constant 60 mM external Na⁺ concentration as illustrated previously for wild type and LCaV3 Δcys channels in Fig. 4 and Supplementary Figure S3. The voltage of expected peak sized currents shift with changes in extracellular Ca²⁺ dose, so the peak sized current (Fig. 10, Supplementary Figure S7) was measured as the largest current size resulting from a voltage step to − 65 mV, − 55 mV, −

**Figure 8.** Swapping of snail and human D2L5 and D4L6 extracellular loops can generate high Na⁺ current passing human Ca₃.2 channels and high Ca²⁺ current passing snail LCa₃,3 channels. Representative traces (top panels) and graphs (bottom panels) illustrating the fold increases in peak currents when equimolar 135 mM [Na]ex replaces 135 mM [NMDG⁺]ex in presence of 2 mM [Ca²⁺] for D2L5 and D4L6 extracellular loop mutants in (A) snail LCa₃,3 (12a/12b) background, and (B) human Ca₃.2 background. Graphs illustrates mean ± SEM with replicates (n) illustrated by grey diamonds. Data to generate graphs were compared in a parametric one-way ANOVA with statistical significance evaluated in Tukey post hoc analyses. In graphs, data are not significant, unless stated, where **p < 0.01. Statistical significances for (A, B) are illustrated in Supplementary Tables S4 and S5. Color coding of differing Ca₃,3 channels: Ca₃.1 (light blue), Ca₃.2 (dark blue), Ca₃.3 (green), LCa₃,3–12b (orange), LCa₃,3–12a (red), LCa₃ Δcys mutants (striped orange or red bars), snail LCa₃ or human Ca₃.2 channels with chimeric extracellular loops (light purple). Data contained in this figure were analyzed and illustrated using OriginPro 2018 (64-bit) SR1 b9.5.1.195.
Figure 9. Normalized peak currents in response to voltage steps generated in conditions of extracellular 4 mM [Ca$^{2+}$] and 100 mM intracellular Li$^+$ (A), or 100 mM intracellular Na$^+$ (B), with reverse potentials and calculated relative permeabilities shown graphically. Note the differing reversal potentials and slope conductances of outward currents in the current–voltage relationships for (A, Li$^+$) and (B, Na$^+$), and a characteristic uniformity of voltage-dependent inward Ca$^{2+}$ currents peaking at more hyperpolarized voltages for wild type and extracellular loop chimeras of snail LCav3 T-type channels, compared to human Cav3.1, Cav3.2 and Cav3.3 channels (see Fig. 1 for biophysical description of wild type snail LCav3 and human Cav3.x channels). Graphs illustrate mean ± SEM with replicates (n) illustrated by grey diamonds. Data to generate graphs were compared in a parametric one-way ANOVA with statistical significance evaluated in Tukey post hoc analyses. Data displayed in graphs are not significant, unless stated, where **$p < 0.01$. Statistical significances for (A, B) are illustrated in Supplementary Tables S6 and S7, respectively. Color coding of differing Cav3 channels: Cav3.1 (light blue), Cav3.2 (dark blue), Cav3.3 (green), LCav3–12b (orange), LCav3–12a (red), LCav3 Δcys mutants (striped orange or red bars), snail LCav3 or human Cav3.2 channels with chimeric extracellular loops (light purple). Data contained in this figure were analyzed and illustrated using OriginPro 2018 (64-bit) SR1 b9.5.1.195.
45 mV and –35 mV from a –110 mV holding potential. We find that the degree to which CaV3 channels are Na+ current passing corresponds to CaV3 channels with a weaker Ca2+ block of the Na+ current (Fig. 10, Supplementary Figure S7). The chimeric snail Ca2+ channel LCaV3–12b with the human D4L6H loop, for example is equally Ca2+ current passing as human CaV3.2 and possesses an equivalently strong (90.63% ± 0.014, n = 7) Ca2+ block of the Ca2+ current compared to wild-type CaV3.2 (94.03% ± 0.015, n = 6) (Fig. 10, Supplementary Figure S7).

The more significantly Na+ current passing chimeras of LCaV3 correspondingly possess a weaker Ca2+ block of the Na+ current, such as LCaV3–12a/D4L6H (50.59% ± 0.036, n = 5) and LCaV3/D2L5H (65.71 ± 0.035, n = 5) (Fig. 10, Supplementary Figure S7).

The loop chimeras possessing a relatively high Na+ current diminishes rather than increase the size of whole cell currents when external Ca2+ concentrations rise through the physiological range. The high Na+ current passing LCaV3 channel with exon 12a exhibits a monotonic decline of current size as extracellular Ca2+ increases from 10 µM to 10 mM, as Ca2+ is less passing relative to blocking the more permeant Na+ ion from passage through the Ca3 channel pore (Fig. 10, Supplementary Figure S7). An equally steep monotonic decline in peak current size is observed with increasing Ca2+ concentration from 10 µM to 10 mM for the loop chimeric channels which possess high Na+ current passing capabilities such as LCaV3–12a/D4L6H and LCaV3–12b/D4L6H.

The more Ca2+ current generating loop chimeras, on the other hand, such as LCaV3–12b/D4L6H and LCaV3–D2L5H/D4L6H have a much reduced decline in current size with increasing external Ca2+ concentration rises suggesting that these channels are more readily passing Ca2+ rather than impeded by the competing Na+ (Fig. 10, Supplementary Figure S7).

Contributions of the differing single channel currents are inferred by evaluation of the recording of the composite whole cell currents. We have measured the relative contribution of Na+ and Ca2+ currents through differing Ca3 channels including chimeric and mutated channels by different approaches in this manuscript. These include: (a) measuring the relative Na+ and Ca2+ contribution to peak inward currents, or (b) as relative peak inward Ca2+ to peak outward monovalent (X+) ionic currents, and their influences on the reversal potential; (c) identifying individual current contributions by means of differing external and internal solutions lacking or containing variable concentrations of Na+ and Ca2+ ions; (d) comparing the relative contribution to peak currents or blockade with differing divalent ions: Ca2+, Ba2+, Sr2+, Ni2+, Zn2+; and differing monovalent ions: Cs+, K+, Na+, Li+. The differing contributions of single channel conductances for Na+ and Ca2+ ions would be gained in single channel recording. What we have observed at the whole cell level in this manuscript, nonetheless, is a consistency in the data set reflecting the greater Na+ or Ca2+ passing character through Ca3 T-type channels, gained in measurement of their observed relative contribution to peak Na+ and Ca2+ currents, the calculated permeabilities that were generated in bi-ionic solutions, the changing size of relative current con-

Figure 10. D2L5 and D4L6 extracellular loops regulate the degree of Ca2+ block of the Na+ current and the fold increase in relative peak Ca2+ current size when external Ca2+ rises from 10 µM [Ca2+]ex to the physiological (mM) range. (A) Normalized peak current sizes in response to increasing concentrations of [Ca2+]ex from 10–9 to 10–2 M in presence 60 mM [Na+]ex for wild type LCaV3–12a and LCaV3–12b channels, and chimeric snail LCaV3 channels with D2L5 and D4L6 extracellular loops from human CaV3.2. (B) Bar graphs of the normalized peak current blockade at 10 µM [Ca2+]ex, the maximally effective blocking Ca2+ concentration for human Ca3 channels (i.e. bottom of “U” shaped curve in A). (C) Bar graphs of the fold change in normalized peak currents from 10 µM to 10 mM [Ca2+]ex. Graphs illustrate mean ± SEM with replicates (n) illustrated as grey diamonds. Data to generate graphs were compared in a parametric one-way ANOVA with a Tukey post hoc analyses to test for statistical significances. Statistical significances are tabulated in Supplementary Tables S8 and S9 for (B,C). Data are significant (p < 0.01), unless stated, where n.s non-significant. Data for LCaV3–12b, LCaV3–12a and Ca3.1 in this figure are reproduced integrally from Senatore et al.9. Color coding of differing Ca3 channels: Ca3.2 (dark blue), LCaV3–12b (orange), LCaV3–12a (red), LCaV3 Δcys mutants (striped orange or red bars), snail LCaV3 or human Ca3.2 channels with chimeric extracellular loops (light purple). Data contained in this figure were analyzed and illustrated using OriginPro 2018 (64-bit) SR1 b9.5.1.195.
tributions with increasing external Ca\textsuperscript{2+} doses in the presence of Na\textsuperscript{+} ions, and the behaviors of differing monovalent or divalent ions other than Na\textsuperscript{+} and Ca\textsuperscript{2+} respectively.

**The differing current densities recorded from transfected HEK-293 T cells was not a significant factor in the assessment of the relative contributions of Na\textsuperscript{+} and Ca\textsuperscript{2+} currents through Ca\textsubscript{3} channels.** A possible caveat in working with in vitro expressed channels is the observed high variability in the current densities (pA/pF) in replicate recordings from the same or different batches of transfected HEK-293 T cells (Supplementary Figure S8), which can contribute to observable differences in channel properties. We observe no apparent influences of the highly variable current densities in individual whole cell recordings, such as the relative pattern of contributing Na\textsuperscript{+} and Ca\textsuperscript{2+} currents to the five wild type channels (Ca\textsubscript{3}.1, Ca\textsubscript{3}.2, Ca\textsubscript{3}.3, LCa\textsubscript{3}–12b, LCa\textsubscript{3}–12a) and eight chimeric LCa\textsubscript{3} or Ca\textsubscript{3} Δcys channels (Supplementary Figure S8). Expressed chimeric clones do generate a lower range of average current densities than their wild-type counterparts, for both the human Ca\textsubscript{3}.2 channels containing snail extracellular loops, and the snail LCa\textsubscript{3} channels containing mammalian extracellular loops (Supplementary Figure S9).

The generally lower current densities of chimeric channels could reflect a compromised membrane trafficking and/or expression because of the xeno-graphing of poorly-compatible extracellular loops to native channels. The highest average current densities (i.e. LCa\textsubscript{3}–12a, LCa\textsubscript{3}–12a Δcys) are enhanced for Na\textsuperscript{+} passing channels in our use of high 60 mM, 100 mM or 135 mM external [Na\textsuperscript{+}] compared to the much lower concentration of external Ca\textsuperscript{2+} of 2 mM used in our experiments (Supplementary Figure S9).

**Discussion**

**Why do invertebrate Ca\textsubscript{3} T-type channels have alternative spliced isoforms with a high preference for passage of Na\textsuperscript{+} currents?** We illustrate here that a major structural determinant for the greater relative passage of Na\textsuperscript{+} or Ca\textsuperscript{2+} ions through Ca\textsubscript{3} T-type channels involves cysteine-rich, extracellular D2L5 and D4L6 loops, which have alternative forms in non-vertebrates (see Supplementary Figure S1). Exon 12a spanning the D2L5 loop contributes to a more Na\textsuperscript{+} current passing isoform of invertebrate Ca\textsubscript{3} T-type channel that more resembles the classical Na\textsubscript{v}1.1 channels. We have recorded LCa\textsubscript{3} channels containing exon 12a in vitro and matched the expression phenotype to a corresponding Ni\textsuperscript{2+} and mibefradil sensitive, but 1,4-dihydropyridine insensitive, low voltage activated, Na\textsuperscript{+} current recorded in primary cultured, snail cardiomyocytes.

Alternative isoforms of exon 12 are present in non-cnidarian invertebrate Ca\textsubscript{3} T-type channel genomes to generate alternative mostly Na\textsuperscript{+} current (exon 12a) or more Ca\textsuperscript{2+} current (exon 12b) passing channels. The alternative extracellular loop in Domain II of Ca\textsubscript{3} T-type channels make a first appearance in extant relatives of the Platyhelminthes (Supplementary Figures S1, S5). Platyhelminthes (which include the flatworms) are basal multicellular invertebrates with a rudimentary body cavity (pseudo-coelom). While possession of a Na\textsubscript{v}1 channel gene is optional outside of the vertebrates, Ca\textsubscript{3} T-type channel genes are ubiquitously found in genomes of every multicellular animal to date outside of basal sponge and ctenophores. We propose that Ca\textsubscript{3} T-type channels with exon 12a as an important contributor to Na\textsuperscript{+} influx in lieu of Na\textsubscript{v}1 channels which are lacking often outside the central nervous systems of non-vertebrates.

**Anthozoan and scyphozoan contain alternative D4L6 extracellular loops in their Ca\textsubscript{3} channels.** We combed through available genome and transcriptome databases to identify alternative extracellular loops varying in sequence, size and patterning of cysteines, resembling the D2L5 loops spanning exon 12a and exon 12b of invertebrate Ca\textsubscript{3} channels. The difference is that this evolutionary pathway involves cnidarians and D4L6 loops instead, and a sequence diversity created through gene duplication rather than alternative splicing. Cnidarians are the only non-vertebrates outside of the most basal representatives in single cell choanoflagellates (Salpingoeca) and placozoans (Trichoplax), without tri-cysteine D2L5 loops (exon 12a) and/or penta-cysteine D2L5 (exon 12b) extracellular loops (Supplementary Figures S1, S2). Instead anthozoan and scyphozoan classes of cnidarians are uniquely endowed amongst non-vertebrates, (besides flatworms), in containing the D4L6 cysteine pair so far remains unresolved in the high-resolution structure of Ca\textsubscript{3}.1 (Supplementary Figure S4). Intra-domain cysteine bridges within extracellular loops
are expected to provide structural stability in support of binding the large extracellular domain of the α2 subunit of the CaVα2δ gene, spanning contacts that encompass D1L5, D2L5 and D3L5 extracellular loops of CaV1.1. PDB files are illustrated using PyMOL Molecular Graphics System, Version 2.3, Schrödinger, LLC, https://pymol.org/2/. Left panels: side view of nanoparticle channel complex with voltage-sensor domains (magenta color) and pore loop domains (light pink color) with bound accessory subunits: Cavβ1, Cavα2δ (light blue color); Navβ1, Navβ2 (light green color). Extracellular loop regions of ion channels bound to accessory subunits are highlighted darker blue (for CaV1.1) and highlighted darker green (for NaV1.1). Cysteine bonded pairs in the extracellular loops are labelled (mustard yellow color), with key negatively-charged (red color) and positively-charged (blue color) residues of the pore selectivity filter, indicated. Middle panels (side view) and right panel (top down view) of the opposing DI–DIII and DII–DIV pore loops alone (s5-s6), plus DI s1–s2 loop which contains a cysteine bonded to a cysteine in DII L5 (s5-s) loop in Cav3.1. The positioning of the unique cysteine-rich extracellular loops contained in non-vertebrate Cav3 channels in DII s5-P (between F891–D902) and DIV P-s6 (between D1791–Y1798) are labelled, by darker-purple dotted lines.

Figure 11. High resolution structures of (A) rabbit CaV1.1 channel (PDB: 6jpa, 2.6 Å resolution, (Zhao20), (B) human NaV1.7 channel (PDB: 6j8g), 3.2 Å resolution (Shen21) and (C) human Cav3.1 channel (PDB: 6kzp, 3.1 Å resolution (Zhao et al.18). PDB files are illustrated using PyMOL Molecular Graphics System, Version 2.3, Schrödinger, LLC, https://pymol.org/2/.
der Waals diameter of 2 Å, which forces $\text{Ca}^{2+}$ ions to pass through T-type channels as fully dehydrated $\text{Ca}^{2+}$ very distant to influence the pore selectivity filter residues directly in $\text{CaV}3.1$, unlike the cysteine-spanning loop that the D2L5 extracellular region is likely a highly flexible structure in vertebrate $\text{CaV}3.1$ channel.

relative passage of $\text{Ca}^{2+}$ and $\text{Na}^{+}$ ions through the pore selectivity filter. Also noteworthy is that the extracellular loops of opposing D2 and D4 domains are dramatically lower in profile above the membrane, and lying in much closer proximity to the pore selectivity filter, compared to the D1 and D3 where the extracellular loops loom much higher above the pore selectivity filter (Fig. 11c). The unique cysteine loop containing 9 amino acids of exon 12a of invertebrate $\text{CaV}3$ channels spans the unresolved D2L5 extracellular loop in $\text{CaV}3.1$ between F891 and D902. Both F891 and D902 are within 1 and 2 amino acids from the negatively-charged aspartate residue (D924) in a key position to attract incoming $\text{Ca}^{2+}$ ions. The position of D924 in $\text{CaV}3.1$ channel is universally-featured in all calcium-selective channels, located just above the pore selectivity residue E923 in $\text{CaV}3.1$. We propose that the uniquely structured 9 amino acid cysteine loop in D2L5 of invertebrate $\text{CaV}3$ channels may bring its positive residues (arginine or lysine residues) in proximity to neutralize the aspartate residue (D924) in a manner that damps the $\text{Ca}^{2+}$ current passing capability and promotes a higher $\text{Na}^{+}$ current passing capability, as we observed in the D975N $\text{CaV}3.2$ mutant (Fig. 7). We speculate a neutralizing role of the equivalent position of D975 in $\text{CaV}3.2$ channels with positively-charged residues in the D2L5 extracellular loop of invertebrate $\text{CaV}3.3$–12a channels, although we caution that we did not directly test this possibility within this manuscript.

Why do all invertebrate T-type channels possessing the major $\text{Na}^{+}$ current passing channel engendered with exon 12a always also possess an extra cysteine pair of cysteines in the D4L6 loop compared to vertebrate T-type channels? We found that exon 12a from invertebrates only generates a high $\text{Na}^{+}$ current passing channel in the normally high $\text{Ca}^{2+}$ passing $\text{CaV}3.2$ background, when the D4L6 extracellular loop of invertebrate $\text{LaCaV}3$ is also swapped into vertebrate $\text{CaV}3.3$ channels. The extra pair of cysteines in D4L6 extracellular loop of invertebrate $\text{LaCaV}3$ channels is located within the unresolved sequence region of $\text{CaV}3$ spanning D1791 and Y1798 (Fig. 7a,b). Residues spanning between D1791 and Y1798 are very distant to influence the pore selectivity filter residues directly in $\text{CaV}3.1$, unlike the cysteine-spanning loop within D2L5 of invertebrate $\text{LaCaV}3$ channels. How the D4L6 extracellular loop working in concert with the D2L5 extracellular loop in opposing domains to influence the relatively high $\text{Na}^{+}$ current passing capabilities may be resolved in the cryo-electron microscopy of invertebrate T-type channels.

The extra cysteine pair (of exon 12a) and two cysteine pairs (of exon 12b) contained in the D2L5 extracellular loops of invertebrate $\text{LaCaV}3$ channels influence the relative Na⁺ and Ca²⁺ current sizes, and the relative divalent ion current sizes (Ca²⁺, Ba²⁺, Sr²⁺) and blockade (Ca²⁺, Ni²⁺, Zn²⁺). We observe that alanine substitution of cysteines in Δcys mutants of the tri-cysteine and penta-cysteine D2L5 loops, spanning exon 12a and exon 12b generates strikingly similar phenotypes, even though these two D2L5 loops contribute to very different phenotypes in native conditions, involving a high Na⁺ current (exon 12a) or more high Ca²⁺ current (exon 12b) passing capacity respectively onto snail $\text{LaCaV}3$ channels. The Δcys mutants possess a common phenotype include a high permeability of outward monovalent ion currents (Li⁺, Na⁺, K⁺) compared to inward Ca²⁺ currents. With increasing external Ca²⁺ ions to 10 µM, we observed a severely weakened block of the Ca²⁺ ion of the Na⁺ current. Further evidence of altered preferences for ion passage through Δcys mutants is the differing peak sizes of ionic currents with divalent cations as the charge carrier, where Ca²⁺ are larger-sized currents in the Δcys mutants and Ba²⁺ and Sr²⁺ are larger-sized currents in wild type $\text{LaCaV}3$ channels (Fig. 5).
We observed not only differences in the relative current sizes generated in response to differing extracellular divalent ions (Ca\(^{2+}\), Ba\(^{2+}\), Sr\(^{2+}\)) in Δcys mutants but the potency and drug characteristics of the blockade of the Ba\(^{2+}\) currents by divalent ions (Ni\(^{2+}\) and Zn\(^{2+}\)) (Fig. 6). Δcys mutants possess an increase in potency of Ni\(^{2+}\) and Zn\(^{2+}\) blockade. The exons 12a LCaV3 channels possess a weak blocking capacity for Ni\(^{2+}\) and Zn\(^{2+}\) resembling mammalian CaV3.1 and CaV3.3 channels, and then increase to the ~50 and ~10-fold higher Ni\(^{2+}\) and Zn\(^{2+}\) potency of native mammalian CaV3.2 T-type channels, in the Δcys mutants. With higher concentrations of Zn\(^{2+}\), we observed a correspondingly greater drug-induced slowing of inactivation kinetics shared between native LCaV3, CaV3.1 and CaV3.3 channels. Both the Δcys mutant channels of LCaV3 and native CaV3.2 channels possess a higher Ni\(^{2+}\) and Zn\(^{2+}\) potency, and lacking the slowing of inactivation kinetics associated with Zn\(^{2+}\) blockade.

**Zn\(^{2+}\) and Ni\(^{2+}\) blockade, the redox sensitivity of CaV3.2 and consequences of cysteine mutations of loop cysteines in LCaV3 may be linked.** Our discovery of a similar potency and characteristics of the blockade with divalent cations (Ni\(^{2+}\), Zn\(^{2+}\)) between LCaV3–Δcys and CaV3.2 channels is relevant in context of the published findings of the unique sensitivity of CaV3.2 channels amongst mammalian CaV3 channels to oxidation of its disulphide bonds within extracellular loops. Oxidizing agents like s-nitrosothiols or lipoic acid or dithionitrobenzoic acid (DTNB) reduce the size of T-type channel currents when applied, and this inhibition of currents can be relieved by mutation of unique, extracellular cysteines contributed from the Domain I s1–s2 linker\(^{15,22}\), the D1L5 loop\(^{23}\), and the D2L5 loop\(^{15,24}\). Thiol group reduction with dithiothreitol (DTT) or l-cysteine has the opposite effect, enhancing T-type channel currents, by a means that is specific to CaV3.2 channels, and is prevented by mutations that alter the high affinity Zn\(^{2+}\) and Ni\(^{2+}\) block of CaV3.2 channels, at a particular histidine residue (H191) within an Asp–Gly–His motif in the Domain I, s3–s4 extracellular loop of mammalian CaV3.2\(^{2,24}\). Both cysteine mutations of the extracellular D2L5 in snail LCaV3 and the H191 mutation in Domain I, s3–s4 loops of CaV3.2 appear to be generating a strikingly similar phenotype. Substitution of D2L5 extracellular loop cysteines, increases Zn\(^{2+}\) and Ni\(^{2+}\) binding affinity of snail LCaV3 channels, whereas, mutation of H191 in the D1, S3–S3 extracellular loop of CaV3.2 decreases Zn\(^{2+}\) and Ni\(^{2+}\) binding affinity with consequences of a lowered sensitivity of their disulphide bonds in its cysteine-enriched extracellular loops to oxidation.

A possible evolution of the configuration of unique cysteine bridges amongst different CaV3 channels. The simplest organism with a CaV3 channel are the single-cell choanoflagellates, and they lack two sets of cysteine pairs in their extracellular loop. Choanoflagellate CaV3 channels are lacking one universal cysteine pair in the DIL5 extracellular loop found in other CaV3 channels (**Supplementary Figure 4), and lacking the unique cysteine bridge pair between the voltage sensor domain (C104, D1s1–s2) and the pore domain (C889, D2L5), present in CaV3.1 and other vertebrate CaV3 channels (Supplementary Figures S1, S2, S5). The unique C104–C889 bridge pair between the voltage-sensor and pore domain is also lacking amongst the cnidarians. Instead, cnidarians possess two different CaV3 genes, unlike most other invertebrates which contain only one CaV3 gene in their genome. One cnidarian gene isoform resembles the vertebrate conditions with one cysteine pair in the D4L6 extracellular loop and one gene isoform resembling the condition within all other non-vertebrates, including the single-cell choanoflagellates with two cysteine pairs located in the D4L6 extracellular loop (Supplementary Figures S1, S4, S6). The protostome invertebrates, including the basal Platyhelminthes (eg. free living flatworm, Macrostomum lignano) to the basal chordates (eg. tunicate, Ciona intestinalis) contain one or both of alternative exon 12a and exon 12b (Supplementary Figures S1, S5), after an intron splice junction separating exons 11 and 12 appeared in common ancestors of the Platyhelminthes. Alternative exons 12a provides the equivalent D2L5 extracellular cysteine (C889) that bridges to C104 of the voltage-sensor domain found in CaV3.1 channels (C209–C0151 in LCaV3–12a, Supplementary Figure S4, red color), and also provide its own 9 amino acid sequence flanked by cysteines spanning F891 and D902 in D2L5 (C889, D2L5) present in CaV3.1 and other vertebrate CaV3 channels (Supplementary Figures S1, S2, S5). The unique C104–C889 bridge pair between the voltage-sensor and pore domain is also lacking amongst the cnidarians. Instead, cnidarians possess two different CaV3 genes, unlike most other invertebrates which contain only one CaV3 gene in their genome. One cnidarian gene isoform resembles the vertebrate conditions with one cysteine pair in the D4L6 extracellular loop and one gene isoform resembling the condition within all other non-vertebrates, including the single-cell choanoflagellates with two cysteine pairs located in the D4L6 extracellular loop (Supplementary Figures S1, S4, S6). The protostome invertebrates, including the basal Platyhelminthes (eg. free living flatworm, Macrostomum lignano) to the basal chordates (eg. tunicate, Ciona intestinalis) contain one or both of alternative exon 12a and exon 12b (Supplementary Figures S1, S5), after an intron splice junction separating exons 11 and 12 appeared in common ancestors of the Platyhelminthes. Alternative exons 12a provides the equivalent D2L5 extracellular cysteine (C889) that bridges to C104 of the voltage-sensor domain found in CaV3.1 channels (C209–C0151 in LCaV3–12a, Supplementary Figure S4, red color), and also provide its own 9 amino acid sequence flanked by cysteines spanning F891 and D902 in D2L5 (C889, D2L5) present in CaV3.1 and other vertebrate CaV3 channels (Supplementary Figures S1, S2, S5). The unique C104–C889 bridge pair between the voltage-sensor and pore domain is also lacking amongst the cnidarians. Instead, cnidarians possess two different CaV3 genes, unlike most other invertebrates which contain only one CaV3 gene in their genome. One cnidarian gene isoform resembles the vertebrate conditions with one cysteine pair in the D4L6 extracellular loop and one gene isoform resembling the condition within all other non-vertebrates, including the single-cell choanoflagellates with two cysteine pairs located in the D4L6 extracellular loop (Supplementary Figures S1, S4, S6). The protostome invertebrates, including the basal Platyhelminthes (eg. free living flatworm, Macrostomum lignano) to the basal chordates (eg. tunicate, Ciona intestinalis) contain one or both of alternative exon 12a and exon 12b (Supplementary Figures S1, S5), after an intron splice junction separating exons 11 and 12 appeared in common ancestors of the Platyhelminthes. Alternative exons 12a provides the equivalent D2L5 extracellular cysteine (C889) that bridges to C104 of the voltage-sensor domain found in CaV3.1 channels (C209–C0151 in LCaV3–12a, Supplementary Figure S4, red color), and also provide its own 9 amino acid sequence flanked by cysteines spanning F891 and D902 in D2L5 (C889, D2L5) present in CaV3.1 and other vertebrate CaV3 channels (Supplementary Figures S1, S2, S5). The unique C104–C889 bridge pair between the voltage-sensor and pore domain is also lacking amongst the cnidarians. Instead, cnidarians possess two different CaV3 genes, unlike most other invertebrates which contain only one CaV3 gene in their genome. One cnidarian gene isoform resembles the vertebrate conditions with one cysteine pair in the D4L6 extracellular loop and one gene isoform resembling the condition within all other non-vertebrates, including the single-cell choanoflagellates with two cysteine pairs located in the D4L6 extracellular loop (Supplementary Figures S1, S4, S6). The protostome invertebrates, including the basal Platyhelminthes (eg. free living flatworm, Macrostomum lignano) to the basal chordates (eg. tunicate, Ciona intestinalis) contain one or both of alternative exon 12a and exon 12b (Supplementary Figures S1, S5), after an intron splice junction separating exons 11 and 12 appeared in common ancestors of the Platyhelminthes. Alternative exons 12a provides the equivalent D2L5 extracellular cysteine (C889) that bridges to C104 of the voltage-sensor domain found in CaV3.1 channels (C209–C0151 in LCaV3–12a, Supplementary Figure S4, red color), and also provide its own 9 amino acid sequence flanked by cysteines spanning F891 and D902 in D2L5 (C889, D2L5) present in CaV3.1 and other vertebrate CaV3 channels (Supplementary Figures S1, S2, S5).
Novel cysteine bridge pairs in extracellular loop spanning D1s1–s2, D2L5 and D4L6 in CaV3 channels are structurally independent. We demonstrate from our D2L5 and D4L6 loop swaps on mammalian CaV3.2 and invertebrate LCaV3 channels that the extracellular loop pairs, operate in a complementary and additive manner, contributing to a change in the relative passage of Ca2+ or Na+, depending on whether the extracellular loop was derived from a highly Ca2+ current passing or high Na+ current passing CaV3 T-type channel. If there was an inter-dependence of cysteines amongst domains, we may have expected that the xeno-grafting of individual or dual extracellular loops would uniformly disable the proper folding of extracellular loops and the functional expression of CaV3 channels. Our observed functional independence of D2L5 and D4L6 loops is consistent with the likely independent evolution of D2L5 loops within common ancestors of non-cnidarian invertebrates, and D4L6 loops in cnidarians, without consequences to the integrity of the CaV3 channel structure as a whole.

The extra cysteine pairs in extracellular loops of CaV3 channels, compared to other Ca2+ channels (CaV1 and CaV2) and to Na+ channels (NaV2 and NaV1) is a likely reflection of the constraints on the flexibility of extracellular loops loom above the pore domain of CaV3 channels, in spite of the absence of required auxiliary subunit binding that occupies most of the extracellular loop domains of Na+ (NaV1) and Ca2+ (CaV1 and CaV2) channels. Notably, the enrichment of cysteines in extracellular loop of invertebrate CaV3 channels are restricted to D2L5 and D4L6 extracellular loops, in opposing domains, D2 and D4, which have a closer proximity to the pore selectivity filter than the other opposing domains, D1 and D3 (Fig. 11c).

As we have observed here, the dramatic enrichment and diversity of cysteine pairs in extracellular loops loom above the pore of invertebrate CaV3 channel contribute to a dramatic change in the contribution of the relative current sizes of monovalent ion currents (Li+, Na+, K+, Cs+) and divalent ion (Ca2+, Ba2+, Sr2+) currents through CaV3 channel pores, and also changes the potency and characteristic of the block by divalent ions (Ca2+, Ni2+, Zn2+). The large number of cysteine pairs in extracellular domains likely contributes to an observed unique regulation of CaV3 channel activity by oxidation of its disulphide bonds. Future high-resolution structures will clarify the unique structural diversity of cysteine bridges in extracellular loops of non-mammalian CaV3 channels. Further exploration will help to explain fully why CaV3 channels, especially in invertebrates, invested in such a large and diverse set of cysteine pairs in extracellular loops, given that they are not necessary in CaV3 channels for accommodating large auxiliary subunits of the Na+ (NaV1) and Ca2+ (CaV1 and CaV2) channels.

Methods
Cloning and expression of snail LCaV3 channels. The original, mostly Ca2+ current passing isoform of the invertebrate LCaV3 T-type channel (GenBank Accession #: AF484084), isolated from pond snail, Lymnaea stagnalis, was expressed and characterized in a configuration contained exon 12b, as well as optional exon 18b spanning the I–II linker, but lacking exon 25c of the III–IV linker. LCaV3 T-Type Ca2+ channels containing exon 12b, but lacking in exon 8b (GenBank Accession #: JQ313138) and containing exon 25c (GenBank Accession #: JQ313139) were subsequently described in Senatore and Spafford. Novel exon 12a isoform (+ 8b, − 25C) deposited as GenBank Accession #: JX292155, is compared with exon 12b isoform (+ 8b, − 25C) which is the configuration of the three exons that is more commonly expressed in the snail brain than in the snail heart, where there is exclusive expression of the mostly Ca2+ current passing CaV3 T-type channel with exon 12a.

mRNA isolation and quantitation of mRNA expression from juvenile and adult snail tissue has been described previously. Chimeras channels were generated by swapping synthetic gene fragments (ordered from BioBasic) at uniquely engineered restriction enzyme sites, as explained below:

Construction of D2L5 loops lacking cysteine residues in snail LCaV3 channels. LCaV3–12b (9031 bp mRNA transcript) was subcloned into pGEMT vector with unique BglII and Sall restriction sites (positions: 1,391–4,521) and novel silent restriction sites AvrII and Eco47III (AfeI) were created by Quikchange mutagenesis (Stratagene, Agilent Technologies) that cut at (positions: 3,338–3,567) spanning the coding sequence for exon 12a (39 amino acids) and exon 12b (50 amino acids). Synthetic DNA (ordered from BioBasic International) spanning the I–II linker, but lacking exon 25c of the III–IV linker was subsequently described in Senatore and Spafford. Novel exon 12a isoform (+ 8b, − 25C) deposited as GenBank Accession #: JX292155, is compared with exon 12b isoform (+ 8b, − 25C) which is the configuration of the three exons that is more commonly expressed in the snail brain than in the snail heart, where there is exclusive expression of the mostly Ca2+ current passing CaV3 T-type channel with exon 12a.

mRNA isolation and quantitation of mRNA expression from juvenile and adult snail tissue has been described previously. Chimeras channels were generated by swapping synthetic gene fragments (ordered from BioBasic) at uniquely engineered restriction enzyme sites, as explained below:

Creation of D975N mutant in human CaV3.2 channels. Human CaV3.2, coded by CACNA1H gene (7,762 bp mRNA transcript) of Genbank Accession #: AF051946, was subcloned (from positions: 342–3,921) into pBluescript II vector from restriction sites spanning NotI and AgeI restriction sites in the Bluescript vector polylinker generated by synthetic oligo insertion. Novel silent restriction sites AvrII was created into human CaV3.2 subclone by Quikchange mutagenesis (Stratagene, Agilent Technologies), and combined with native BsrGI restriction site downstream of the AvrII site, cut out the region (positions: 2,873–3,018) spanning the homologous exon 12a (39 amino acids) and exon 12b (50 amino acids) of human CaV3.2. Synthetic DNA (ordered from BioBasic International) spanning the AvrII and BsrGI restriction sites were inserted into the human CaV3.2 subclone, which included D2L5 loop changes for LCaV3–12a Δcys, LCaV3–12b Δcys each of which contained a silent NruI and MluI restriction sites, respectively, for rapid validation of individual cloned plasmid stocks for their unique D2L5 loop identity in LCaV3.

D2L5 mutagenesis in snail LCaV3–12a/12b backgrounds. LCaV3–12b (9031 bp mRNA transcript) was subcloned into pGEMT vector with unique BglII and Sall restriction sites (positions: 1,391–4,521) and novel silent restriction sites AvrII and Eco47III (AfeI) were created by Quikchange mutagenesis (Stratagene, Agilent Technologies) that cut at (positions: 3,338–3,567) spanning the coding sequence for exon 12a (39 amino acids)
and exon 12b (50 amino acids). Synthetic DNA (ordered from BioBasic International) spanning the AvrII and Eco47III restriction sites were inserted into the LCaV3 subclone, which included D2L5 loop changes for human CaV3.2 Genbank Accession # AF051946 (positions: 2,873–3,069), each of which contained a silent NruI, MluI, AgeI restriction sites, respectively, for rapid validation of individual cloned plasmid stocks for their unique D2L5 loop identity in LCaV3.

D2L5 mutagenesis in human CaV3.2 backgrounds. Human CaV3.2, coded by CACNA1H gene (7,762 bp mRNA transcript) of Genbank Accession # AF051946, was subcloned (from positions: 342–3,921) into pBluescript II vector from restriction sites spanning NotI and AgeI restriction sites in the Bluescript vector polylinker generated by synthetic oligo insertion. Novel silent restriction sites AvrII was created into human CaV3.2 subclone by Quikchange mutagenesis (Stratagene, Agilent Technologies), and combined with native BsrGI restriction site downstream of the AvrII site, cut out the region (positions: 2,873–3,018) spanning the homologous exon 12a (39 amino acids) and exon 12b (50 amino acids) in snail LCaV3 channels. Synthetic DNA (ordered from BioBasic International) spanning the AvrII and BsrGI restriction sites were inserted into the human CaV3.2 subclone, which included D2L5 loop swaps for snail LCaV3 exon 12a and LCaV3 exon 12b and a D975N substitution, each of which contained a silent DpnI, MluI and AgeI restriction sites, respectively, for rapid validation of individual cloned plasmid stocks for their unique D2L5 loop identity in human CaV3.2.

D4L6 mutagenesis in snail LCaV3–12a/12b backgrounds. Snail LCaV3–12b was subcloned into pGEMT vector (at positions: 4,522–6,858) with unique SalI restriction site and a silent MluI restriction site created in the original concatenation of cDNA fragments to generate full length LCaV3–12b. Novel silent restriction sites BbsI and XhoI were created by Quikchange mutagenesis (Stratagene, Agilent Technologies) that cut at (positions: 3,338–3,567) spanning the coding sequence for the LCaV3 D4L6 loop. Synthetic DNA (ordered from Biobasic International) spanning the BbsI and XhoI restriction sites were inserted into the LCaV3 subclone, which included D4L6 loop changes coding for 16 amino acids (positions: 5,540–5,587) for human CaV3.2 to generate LCaV3 D4L6H which contained a silent MfeI restriction sites, for rapid validation of individual cloned plasmid stocks for the unique identity of LCaV3 D4L6H.

D4L6 mutagenesis in human CaV3.2 backgrounds. The complete C terminus of human CaV3.2 (at positions: 3,922–7,138) was subcloned into pBluescript vector in a modified polylinker utilizing a unique AgeI restriction site in the coding region and a downstream XbaI site only present in the pcDNA 3.1 mammalian expression vector. Silent restriction sites for XhoI were created in the CaV3.2 subclone by synthetic DNA insertion of a SphI and AvrII cDNA fragment (ordered from Biobasic International) into the CaV3.2 subclone that allowed for insertion of another DNA synthetic construct (ordered from Biobasic International) between SphI and XhoI (positions: 5,285–5,666 in CaV3.2) spanning the 20 amino acids coding sequence for the LCaV3 D4L6 loop. Synthetic DNA (ordered from BioBasic International) spanning the SphI and XhoI restriction sites were inserted into the LCaV3 subclone, which included D4L6 loop changes coding for 20 amino acids (positions: 5,540–5,587) for human CaV3.2 to generate LCaV3 D4L6L which contained silent DraII and DpnI restriction sites, for rapid validation of individual cloned plasmid stocks for the unique identity of LCaV3 D4L6L.

Phylogenetic analyses. CaV3 channel genes were derived from transcriptome or genome sequences available by BLAST searching of publicly available websites: GenBank (NIH genetic sequence database), DOE JGI (U.S. Department of Energy Joint Genome Institute) and EnsembleGenomes (EBI and Wellcome Trust Sanger Institute). Multiple alignments were carried out in using MUltiple Sequence Comparison by Log-Expectation (MUSCLE) at website: https://www.ebi.ac.uk/Tools/msa/muscle/ and gene trees were created with Phylogeny.fr at website: https://www.phylogeny.fr.

Statistical analyses. Numerical values in electrophysiological experiments were compared using a parametric one-way ANOVA within OriginPro 2018 (64-bit) SR1 b9.5.1.195. (OriginLabs). Statistical significance by means comparison was conducted using a Tukey post hoc test, unless otherwise stated.

PDB file illustrations. PDB files are illustrated using PyMOL Molecular Graphics System, Version 2.3, Schrödinger, LLC, https://pymol.org/2/.
In vitro expression of Cav3 channels in HEK-293T cells. We have detailed our optimized technique for the culture and maintenance of mammalian HEK-293T cells, Ca\textsuperscript{2+} phosphate transfection and expression of ion channels, and their recording using whole-cell patch clamp in an online video journal\textsuperscript{27}.

Snail LCaV3 clones including chimeric and mutated derivatives were expressed within bi-cistronic pIRES2-EGFP vector, which generates green fluorescence upon mercury lamp excitation of positively transfected cells. Human CaV3.1, CaV3.2 and CaV3.3 channels contained in pcDNA3.1 vector, were co-expressed with a pTracer plasmid, which provided green fluorescence for identification of positively transfected cells. Expressed human CaV3 channels were originally a gift from Dr. Edward Perez-Reyes (U. of Virginia, Charlottesville) and Dr. Gerald Zamponi (U. of Calgary, Calgary).

All transient transfection procedures, external and internal solutions, voltage-protocols for electrophysiology, post-recording and phylogenetic analyses were carried out as stated previously, including the primary cell culture

| CaCl\textsubscript{2} | BaCl\textsubscript{2} | NaCl | NMDG+ | TEA | HEPES |
|----------------------|-----------------|------|-------|-----|-------|
| **External\textsuperscript{a}** | | | | | |
| 0                    | 2               | 0    | 135   | 25  | 10    |
| 0                    | 2               | 135  | 0     | 25  | 10    |
| 2                    | 0               | 0    | 135   | 25  | 10    |
| 2                    | 0               | 135  | 0     | 25  | 10    |
| #                    | CaCl\textsubscript{2} | NMDG+ | EGTA\textsuperscript{a} | Mg-ATP | Li-GTP | HEPES |
| **Internals\textsuperscript{b}** | | | | | |
| 1                    | 110             | 0    | 10    | 3   | 0.6   | 10    |

Table 2. Recording solutions to measure relative inward sodium and/or calcium conductances using NMDG+ (Figs. 2, 7, 8). \textsuperscript{a}pH 7.4 with TEA-OH. \textsuperscript{b}pH 7.2 with CsOH.

| CaCl\textsubscript{2} | TEA-Cl | HEPES |
|----------------------|--------|-------|
| **External\textsuperscript{a}** | | | |
| 4                    | 155    | 10    |
| #                    | CaCl\textsubscript{2} | NaCl | KCl | LiCl | EGTA\textsuperscript{a} | TEA-Cl | HEPES |
| **Internals\textsuperscript{b}** | | | | | | | |
| 1                    | 100    | 0     | 0    | 0    | 10    | 10    | 10    |
| 2                    | 0      | 100   | 0    | 0    | 10    | 10    | 10    |
| 3                    | 0      | 0     | 100  | 0    | 10    | 10    | 10    |
| 4                    | 0      | 0     | 0    | 100  | 10    | 10    | 10    |

Table 3. Bi-ionic solutions for determination of relative PCa / Px permeabilities (Figs. 3, 9). \textsuperscript{a}pH 7.4 with TEA-OH. \textsuperscript{b}pH 7.2 with XOH where X = Cs, Na, K, or Li. \textsuperscript{c}pH 8.0 with XOH.

| In mM | Ca\textsuperscript{2+} free | CaCl\textsubscript{2} | NaCl | EGTA | HEPES | Glucose |
|-------|-----------------------------|----------------------|------|------|-------|---------|
| **External\textsuperscript{a}** | | | | | | |
| 1     | 1 × 10\textsuperscript{-9}  | 0.01                 | 60   | 1.246| 10    | 10      | 26.3    |
| 2     | 1 × 10\textsuperscript{-7}  | 1.00                 | 60   | 2.236| 10    | 10      | 20.4    |
| 3     | 1 × 10\textsuperscript{-5}  | 1.00                 | 60   | 1.002| 10    | 10      | 24.1    |
| 4     | 1 × 10\textsuperscript{-4}  | 0.10                 | 60   | 0    | 10    | 10      | 29.7    |
| 5     | 3 × 10\textsuperscript{-4}  | 0.30                 | 60   | 0    | 10    | 10      | 29.1    |
| 6     | 1 × 10\textsuperscript{-3}  | 1.00                 | 60   | 0    | 10    | 10      | 27.6    |
| 7     | 3 × 10\textsuperscript{-3}  | 3.00                 | 60   | 0    | 10    | 10      | 21.0    |
| 8     | 1 × 10\textsuperscript{-2}  | 10.00                | 60   | 0    | 10    | 0       | 0.0     |
| **CaCl\textsubscript{2}** | **EGTA** | **Mg-ATP** | **Li-GTP** | **HEPES** |
| **Internals\textsuperscript{b}** | | | | | | |
| 110   | 10                           | 3                   | 0.6   | 10    |       |

Table 4. Ca\textsuperscript{2+} ex dose response with 60 mM [Na\textsuperscript{+}] ex (Figs. 4, 10; Supplementary Figures S31, S7). \textsuperscript{a}pH 7.4 with TEA-OH. \textsuperscript{b}pH 7.4 with CsOH.

In vitro expression of Cav3 channels in HEK-293T cells. We have detailed our optimized technique for the culture and maintenance of mammalian HEK-293T cells, Ca\textsuperscript{2+} phosphate transfection and expression of ion channels, and their recording using whole-cell patch clamp in an online video journal\textsuperscript{27}.

Snail LCaV3 clones including chimeric and mutated derivatives were expressed within bi-cistronic pRES2-EGFP vector, which generates green fluorescence upon mercury lamp excitation of positively transfected cells. Human CaV3.1, CaV3.2 and CaV3.3 channels contained in pcDNA3.1 vector, were co-expressed with a pTracer plasmid, which provided green fluorescence for identification of positively transfected cells. Expressed human CaV3 channels were originally a gift from Dr. Edward Perez-Reyes (U. of Virginia, Charlottesville) and Dr. Gerald Zamponi (U. of Calgary, Calgary).

All transient transfection procedures, external and internal solutions, voltage-protocols for electrophysiology, post-recording and phylogenetic analyses were carried out as stated previously, including the primary cell...
culture of snail heart cells. Ca\textsuperscript{2+} channels were expressed in human embryonic kidney (HEK293T) cells via Ca\textsuperscript{2+} phosphate transfections.

**Whole cell patch clamp recordings.** Whole cell electrophysiology recordings were obtained using an Axopatch 200B or Multicllamp 700B amplifiers (Molecular Devices), sampled through a Digidata 1440A A/D converter (Molecular Devices) to a PC computer. Patch pipettes for recording had pipette resistances of 2–5 M\(\Omega\) (HEK-293 T cells) or 5–10 M\(\Omega\) (heart cells), and with typical access resistance maintained after breakthrough between 2.5 M\(\Omega\) and 6 M\(\Omega\) (HEK-293 T cells) or 10 and 14 M\(\Omega\) (heart cells). Only recordings with minimal leak (<10% of peak) and small current sizes (<500 pA) in HEK-293 T cells were used due to loss of voltage clamp above 500 pA. Series resistance was compensated to 70% (prediction and correction; 10-µs time lag). Offline leak subtraction was carried out using the Clampfit 10.2 software (Molecular Devices). For all recordings, offline leak subtraction was carried out and data was filtered using a 500 Hz Gaussian filter in Clampfit 10.2. Reversal potentials were calculated in bi-ionic conditions using a calculation of relative permeabilities, as described previously\(^9\). Protocols for measuring the voltage-sensitivity and kinetics, and curve fitting data are described previously\(^9\). A Valvelink 8.2 gravity flow Teflon perfusion system (AutoMate Scientific) was utilized to compare ionic current sizes generated by differing external monovalent or divalent ions.

**Ionic solutions used in electrophysiology experiments.** Divalent cations for Ca\textsubscript{3} channel blockade were purchased from Sigma-Aldrich: Nickel (II) chloride hexahydrate (Cat #: 203,866) and zinc chloride (Cat #: 229,997). The standard recording solutions to measure ionic conductances with external calcium or barium or strontium ions (in Figs. 1, 5, 6, 7) is shown in Table 1.

The relative inward monovalent ion conductance involving sodium compared to calcium ions using NMDG\(^+\) (measured in Figs. 2, 7, 8) is shown in Table 2.

The relative permeability of PCa/Px, where x is monovalent ion (Li\(^+\), Na\(^+\), K\(^+\), Cs\(^+\)) was calculated by the following bi-ionic equation provided on the bottom of page 34 of Fatt and Ginsborg (1958)\(^10\):

\[
\frac{P_{Ca}}{P_x} = \frac{[x]}{4[Ca]_0} \exp(\frac{E_{rev,F}}{RT}) \left( \exp(\frac{E_{rev,F}}{RT}) + 1 \right)
\]

The bio Bi-ionic solutions for determining the relative PCa / Px permeabilities (measured in Figs. 3, 9) is shown in Table 3.

The ionic solutions used in Fig. 4 and Fig. 10 to measure the relative change in peak current sizes in response to differing external calcium ion concentrations in the presence of 60 mM external sodium ion is provided in Table 4.

**Contribution to the field.** Variable, and asymmetrical extracellular loops in Ca\textsubscript{3} channels possess strategically-positioned charged amino acid residues and intra-loop disulphides, contributing to structures that regulate the relative passage and block of Na\(^+\) and Ca\textsuperscript{2+} currents. The weak conservation amongst extracellular loop sequences overall, even amongst closely related homologs of cation channel class of Na\textsubscript{v}, Ca\textsubscript{v} and NALCN channels, suggest that each eukaryotic cation channel gene has a composition of extracellular loops from different domains that may operate quite uniquely and independently from each other, possibly contributing to different properties such as ion passage, cation drug block and sensitivity to venom toxins. A changeable external scaffold provides for potential adaptation to changing cellular environments within different organisms, without changing the fundamental high field strength (HFS) site of the pore’s selectivity filter, which is a highly conserved and characteristic sequence within different classes of voltage-gated Na\(^+\) (Na\textsubscript{v}) and Ca\textsuperscript{2+} (Ca\textsubscript{v}) channels, and NALCN. Determination of the functional consequences to the structural differences in nature’s alternative extracellular loops will aid in design of human medicines, and in pest control (yeast, protest, algal and invertebrate) involving the variable external scaffolds of voltage-gated Na\textsubscript{v}, Ca\textsubscript{v} and NALCN channels as targets.

Received: 31 March 2020; Accepted: 6 July 2020
Published online: 24 July 2020

**References**

1. Mangoni, M. E. et al. Bradycardia and slowing of the atrioventricular conduction in mice lacking CaV3.1/alpha1G T-type calcium channels. *Circ. Res.* **98**, 1422–1430. [https://doi.org/10.1161/01.RES.0000225862.14314.49](https://doi.org/10.1161/01.RES.0000225862.14314.49) (2006).
2. Chen, C. C. et al. Abnormal coronary function in mice deficient in alpha1H T-type Ca\textsuperscript{2+} channels. *Science* **302**, 1416–1418. [https://doi.org/10.1126/science.1098268](https://doi.org/10.1126/science.1098268) (2003).
3. Llinas, R. & Jahnson, H. Electrophysiology of mammalian thalamic neurones in vitro. *Nature* **297**, 406–408 (1982).
4. McKay, B. E. et al. Ca(V)3 T-type calcium channel isoforms differentially distribute to somatic and dendritic compartments in rat central neurons. *Eur. J. Neurosci.* **24**, 2581–2594. [https://doi.org/10.1111/j.1460-9568.2006.05136.x](https://doi.org/10.1111/j.1460-9568.2006.05136.x) (2006).
5. Talley, E. M. et al. Differential distribution of three members of a gene family encoding low voltage-activated (T-type) calcium channels. *J. Neurosci.* **19**, 1895–1911 (1999).
6. Shcheglovitov, A., Kostyuk, P. & Shuba, Y. Selectivity signatures of three isoforms of recombinant T-type Ca\textsuperscript{2+} channels. *Biochim. Biophys. Acta* **1768**, 1406–1419. [https://doi.org/10.1016/j.bbamem.2007.02.017](https://doi.org/10.1016/j.bbamem.2007.02.017) (2007).
7. Senatore, A. & Spafford, J. D. Transient and big are key features of an invertebrate T-type channel (LCav3) from the central nervous system of *Lymnaea stagnalis*. *J. Biol. Chem.* **285**, 7447–7458. [https://doi.org/10.1074/jbc.M109.090753](https://doi.org/10.1074/jbc.M109.090753) (2010).
8. Senatore, A. & Spafford, J. D. Gene transcription and splicing of T-type channels are evolutionarily-conserved strategies for regulating channel expression and gating. *PLoS One* **7**, e37409. [https://doi.org/10.1371/journal.pone.0037409](https://doi.org/10.1371/journal.pone.0037409) (2012).
9. Senatore, A., Guan, W., Boone, A. N. & Spafford, J. D. T-type channels become highly permeable to sodium ions using an alternative extracellular turret region (S5-P) outside the selectivity filter. *J. Biol. Chem.* 289, 11952–11969. https://doi.org/10.1074/jbc.M114.551473 (2014).

10. Fatt, P. & Ginsborg, B. L. The ionic requirements for the production of action potentials in crustacean muscle fibres. *J. Physiol.* 142, 516–543 (1958).

11. Pauling, L. *Nature of the Chemical Bond and Structure of Molecules and Crystals* 3rd edn, 644 (Cornell University Press, Cornell, 1960).

12. McRory, J. E. et al. Molecular and functional characterization of a family of rat brain T-type calcium channels. *J. Biol. Chem.* 276, 3999–4011. https://doi.org/10.1074/jbc.M008215200 (2001).

13. Lewis, R. J. & Garcia, M. L. Therapeutic potential of venom peptides. *Nat. Rev. Drug Discov.* 2, 790–802. https://doi.org/10.1038/nrd1197 (2003).

14. Kang, H. W., Moon, H. J., Joo, S. H. & Lee, J. H. Histidine residues in the IS3–IS4 calcium channel. *FEBS Lett.* 581, 5774–5780. https://doi.org/10.1016/j.febslet.2007.11.045 (2007).

15. Lee, J. H., Gomora, J. C., Cribs, L. L. & Perez-Reyes, E. Nickel block of three cloned T-type calcium channels: Low concentrations selectively block alpha1H. *Biophys. J.* 77, 3034–3042. https://doi.org/10.1016/S0006-3495(99)77134-1 (1999).

16. Traboulsee, A. et al. Subunit-specific modulation of T-type calcium channels by zinc. *J. Physiol.* 578, 159–171. https://doi.org/10.1113/jphysiol.2006.114496 (2007).

17. Sun, H. S., Hui, K., Lee, D. W. & Feng, Z. P. Zn2+ sensitivity of high- and low-voltage activated calcium channels. *Biophys. J.* 93, 1175–1183. https://doi.org/10.1529/biophysj.106.103333 (2007).

18. Zhao, Y. et al. Cryo-EM structures of apo and antagonist-bound human Cav3.1. *Nature* 576, 492–497. https://doi.org/10.1038/s41586-019-1801-3 (2019).

19. Stephens, R. F., Guan, W., Zhorov, B. S. & Spafford, J. D. Selectivity filters and cysteine-rich extracellular loops in voltage-gated sodium, calcium, and NALCN channels. *Front. Physiol.* 6, 153. https://doi.org/10.3389/fphys.2015.00153 (2015).

20. Zhao, Y. et al. Molecular basis for ligand modulation of a mammalian voltage-gated Ca(2+) channel. *Cell* 177, 1495 e1412-1506 e1412. https://doi.org/10.1016/j.cell.2019.04.043 (2019).

21. Shen, H., Liu, D., Wu, K., Lei, J. & Yan, N. Structures of human Nav1.7 channel in complex with auxiliary subunits and animal toxins. *Science* 363, 1303–1308. https://doi.org/10.1126/science.aaw2493 (2019).

22. Lee, W. Y. et al. Molecular mechanisms of lipooic acid modulation of T-type calcium channels in pain pathway. *J. Neurosci.* 29, 9500–9509. https://doi.org/10.1523/JNEUROSCI.5803-08.2009 (2009).

23. Karmazinova, M. et al. Cysteines in the loop between IS3 and the pore helix of Ca(V)3.1 are essential for channel gating. *Pflugers. Arch.* 460, 1015–1028. https://doi.org/10.1007/s00424-010-0874-5 (2010).

24. Nelson, M. T. et al. Reducing agents sensitive C-type nociceptors by relieving high-affinity zinc inhibition of T-type calcium channels. *J. Neurosci.* 27, 8280–8286. https://doi.org/10.1523/JNEUROSCI.1800-07.2007 (2007).

25. Madeira, F. et al. The EMBL-EBI search and sequence analysis tools APIs in 2019. *Nucleic Acids Res.* 47, W636–W641. https://doi.org/10.1093/nar/gkz268 (2019).

26. Dereeper, A. et al. Phylogeny.fr: Robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res.* 36, W465–W469. https://doi.org/10.1093/nar/gknz180 (2008).

27. Senatore, A., Boone, A. N. & Spafford, J. D. Optimized transfection strategy for expression and electrophysiological recording of recombinant voltage-gated ion channels in HEK-293T cells. *J. Vis. Exp.* https://doi.org/10.3791/2314 (2011).

Acknowledgements
This work was funded through a Heart and Stroke Foundation of Canada Grant-In-Aid and NSERC Discovery Grant to J.D.S., and an NSERC Canada Graduate Scholarship to RFS. We thank Dr. Boris Zhorov for helpful discussion surrounding the molecular modeling of D2L5 and D4L6 extracellular loops.

Author contributions
Conceived and designed the experiments, analyzed the data, and contributed reagents/materials/analysis tools: R.F.S., J.F. and J.D.S. Performed the experiments: W.G., R.F.S., O.M., A.M., J.F. and J.D.S. Wrote the paper: J.D.S.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-69197-3.

Correspondence and requests for materials should be addressed to J.D.S.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2020