T-type channels become highly permeable to sodium ions using an alternate extracellular turret region (S5-P) outside the selectivity filter

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Running title: Sodium-permeable T-type channels

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##Background:
Ion selectivity of voltage-gated channels is governed by selectivity filters

##Results:
Alternative turret region in Domain II promotes highly sodium-permeable T-type channels without major changes to gating and kinetic features

##Conclusion:
T-type channels can generate variable sodium or calcium-permeability by gene splicing

##Significance:
Ion selectivity in T-Type channels can be altered using extracellular domains outside the ion selectivity filter

##ABSTRACT
T-type (Ca\textsubscript{v}3) channels are categorized as calcium channels, but invertebrate ones can be highly sodium-selective channels. We illustrate that the snail LCa\textsubscript{v}3 T-type channel becomes highly sodium-permeable through exon splicing of an extracellular turret and descending helix in Domain II of the four domain Ca\textsubscript{v}3 channel. Highly sodium permeable T-type channels are generated without altering the invariant ring of charged residues in the selectivity filter that governs calcium selectivity in calcium channels. The highly sodium permeant T-type channel expresses in the brain and is the only splice isoform expressed in the snail heart. This unique splicing of turret residues offers T-type channels a capacity to serve as a pacemaking sodium current in the primitive heart and brain in lieu of Ca\textsubscript{v}1 type sodium channels, and substitute for voltage-gated sodium channels lacking in many invertebrates. T-type channels would also contribute substantially to sodium leak conductances at rest in invertebrates because of their large window currents.

Ca\textsubscript{v}3 T-type channels are members of the 4x6TM (4 Heterologous Domains of 6 Trans-Membrane segments) family of voltage-gated ion channels which includes calcium (Ca\textsubscript{v}1) and sodium (Na\textsubscript{v}) channels, and NALCN (1-3). The four heterologous domains of 4x6TM channels are considered to have evolved from two rounds of duplication of ancestral channels with one domain, such as the voltage-gated potassium channels (4). Key elements of each domain are a voltage sensor region (segments 1 to 4), and a re-entrant pore loop that extends from segments 5 and 6 and contains the selectivity filter thought to govern ion selectivity. The potassium channel pore mimics the hydration shell oxygen atoms that surround potassium ions in solution using conserved selectivity filter residues consisting of backbone carbonyl oxygens (5). This configuration makes it energetically feasible for surrogate oxygen groups to displace hydrating water molecules as potassium ions permeate (5). P-loops are contributed by four asymmetrical domains of calcium and sodium channels to a wider and shorter selectivity filter, which allows sodium ions to pass in a semi-hydrated (6) or hydrated state (7). Side chains of glutamate residues (EEEE) in the selectivity filter project carboxyl oxygens into the pore to create a high affinity pore locus for calcium ions in Ca\textsubscript{v}1 (L-type) and Ca\textsubscript{v}2 (non-L-type) calcium channels (8), where Na\textsuperscript{+} ions are prohibited from passing in the presence of low concentrations of external Ca\textsuperscript{2+} ions (\sim 1 \mu M) (9). T-type channels ubiquitously have a similar negatively-charged ring of residues as Ca\textsubscript{v}1 and Ca\textsubscript{v}2 channels in the selectivity filter, but, notably different, with aspartate residues replacing two of the glutamate residues in the 3\textsuperscript{rd} and 4\textsuperscript{th} positions (EEDD). Mammalian T-type channels (Ca\textsubscript{v}3.1, Ca\textsubscript{v}3.2 and Ca\textsubscript{v}3.3) have a \sim 10 fold lower calcium selectivity over sodium than Ca\textsubscript{v}1 and Ca\textsubscript{v}2 channels, but are still impermeant to sodium in the presence of low (10 \mu M) calcium (10,11). Here,
we show that the LCa,3 T-type channel from the pond snail *Lymnaea stagnalis* with a standard EEDD selectivity filter becomes highly permeant to monovalent cations through splicing of a novel extracellular turret, even in the presence of physiological (mM) concentrations of Ca^{2+} ions. These sodium-permeant T-type currents serve as the major pacemaker of primitive hearts, and likely proxy for sodium channels, when they are absent in many invertebrates. Our work confirms a capacity of snail channel currents to switch sodium and calcium permeabilities by an extracellular determinant, first postulated by Kostyuk, Mironov and Shuba, more than 30 years ago (12).

**Experimental Procedures**

**Cloning and expression of novel LCa,3 exon 12a splice variant** - Original cloning and expression work of LCa,3 T-type calcium channel contained exons +8b, 12b and -25c, was described in Senatore and Spafford (13) and the original gene sequence was deposited as GenBank Accession #: AF484084. Subsequently, we described LCa,3 T-Type calcium channel splicing of exon 8b deposited as GenBank Accession # JQ313138 and exon 25c deposited as GenBank Accession # JQ313139. LCa,3 -8b and +25C splice variants were described in Senatore and Spafford (14). Here, we describe novel exon 12a isoform (+8b, 12a, -25C), created by PCR using the original full length clone of LCa,3 in pIRE2 vector (+8b, 12b,-25c) as template. Novel splice variant (+8b, 12a, -25C) has been deposited as GenBank Accession #: JX292155. The inclusion of exons +8b (54%) and 12a (56%) and -25C (77%) is the most common configuration of these three exons in the adult brain while the adult heart does not commonly have a configuration of +8b (16%) or -25C (21%) (14). The adult heart configuration of exons most commonly found is -8b (84%), 12a (99%) and +25C (79%) (14). Novel exon 12a was confirmed in multiple PCR amplifications from snail brain cDNA (Fig. 1). All expressed plasmids were confirmed by sequencing (TCAG DNA Sequencing Facility, Sick Kids Hospital, Toronto).

**Culturing of snail heart cells** - Heart ventricle cells were cultured from anesthetized adult snails (25-35 mm shell length) using a modified protocol (15). Cut up ventricles were placed in 0.5 mM Ca^{2+} Leibowiz medium containing gentamycin (400 mg/ml) and 30 mM glucose and trypsinized (0.25% (w/v) trypsin, Sigma T9201) for 10 minutes, and then treated with 0.1% collagenase (Sigma Type II) in 0.5mM Ca^{2+} Leibowitz medium for 30 minutes. The digested hearts were then washed 3 times with 3.5 mM Ca^{2+} Leibowitz medium then plated on acid etched circular glass coverslips (16) and left for 24 hours to adhere at room temperature in 3.5 mM Ca^{2+} Leibowitz medium containing 400 mg/ml, gentamycin, 30 mM glucose and 2% fetal bovine serum, before patch clamp recording 24 hours later.

**Whole cell patch clamp recording** - We have detailed our optimized technique for the culture and maintenance of mammalian HEK-293T cells, calcium phosphate transfection and expression of ion channels, and their recording using whole-cell patch clamp in an online video journal (17). There are five sets of solutions for recording (Table 1, Table 2). For comparing ion permeabilities or drug block, a Valvelink8.2® gravity flow Teflon perfusion system (AutoMate Scientific, Berkeley CA) was used to toggle between differing external solutions during electrophysiological recording. Whole cell patch clamp recordings were carried out with an AxoPatch 200B amplifier, combined with a Digidata® 1440A Data Acquisition System and pCLAMP 10 Software. Patch pipettes for recording had pipette resistances of 2–5 MΩ (HEK-293T cells) or 5-10 MΩ (heart cells), and with typical access resistance maintained after breakthrough between 4 and 6 MΩ (HEK-293T cells) or 10 and 14 MΩ (heart cells). Only recordings with minimal leak (<10% of peak) and small current sizes (<500 pA) in HEK-293T 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 10.1 software (Molecular Devices). Protocols for measuring the voltage-sensitivity and kinetics, and curve fitting data are described in (13,14). The relative permeability of $P_x/P_x$, where $x$ is monovalent ion (Li^+, Na^+, K^+, Cs^+) was calculated by the following bi-ionic equation (18):
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\[ \frac{P_{Ca}}{P_x} = \frac{[x]_i}{4[Ca]_o} \exp(E_{rev}F/RT) \left( \exp(E_{rev}F/RT) + 1 \right) \]

Measurement of mRNA expression of exon 12a and exon 12b splice variants using qPCR - qPCR was carried out as described previously (14,19,20). RNA was extracted from entire juvenile and adult snails, where sexually immature juveniles have shell lengths of 1.0 to 1.5 cm, and reproduction-capable adults have shell lengths of 2.0 to 2.5 cm (21). qPCR primers sets (Table 3) were designed to selectively amplify universal and specific exon 12a and exon 12b splice variants of Lymnaea Ca3. The specificity of PCR primers pairs was initially assessed by comparing the size of PCR products amplified from a pooled cDNA library, with those amplified from cloned cDNAs. PCR primer efficiency for each primer set was then determined by generating relative standard curves using the 1:5 serial dilutions of pooled cDNA (1:5, 1:25, 1:125; and 1:625) as template for real time RT-PCR amplification. For each dilution, triplicate reactions were carried out in rigid 96-well PCR plates (Bio-Rad), with each well containing 0.5 µL of serially diluted cDNA, 5 µL of SsoFastTM EvaGreen® Supermix (Bio-Rad), 0.5 µL of each 10 µM primer from a set, and 3 µL of water. PCR amplification, fluorescence reading, and melt curve analyses were done using a Bio-Rad C1000TM Thermal Cycler equipped with a CFX96TM Real-Time System and run by CFX Manager Software (Bio-Rad). All cycle threshold values used for analysis were determined relative to the average cycle threshold value of the control gene, HPRT1 (hypoxanthine phosphoribosyltransferase 1).
RESULTS

Novel spliced turret alters monovalent ion permeability of snail T-type channels. T-type channels diversified into three vertebrate genes, but are rooted as a single T-type channel gene in extant relatives of animals spanning tissue and nervous system evolution (Trichoplax and cnidarians) and identified as LCa,3 gene in the pond snail, Lymnaea stagnalis (13,14). Snail LCa,3 resembles mammalian T-type channels in its rapid kinetics, and activity spanning low voltages for generating a depolarizing pacemaker current from rest to the threshold for eliciting action potential spikes (13), as well as conserved, developmentally-regulated exon splicing, such as with optional exons 8b and 25c (14). We have identified unique exon splicing for exon 12 in the invertebrate T-type channel which spans from the S5 membrane helix of Domain II, the extracellular loop, dubbed the “turret” to the descending pore helix that projects into the pore towards the selectivity filter (Fig. 2a). Remarkably, the 3’ splice sites for exons 12a and 12b occur five amino acids upstream but do not include the invariant selectivity filter (Fig. 2a), whose residues define the channel’s characteristic ion selectivity (22). Exon 12a is shorter (39 aa long) with a characteristic cysteine structure: CxexC...C, while exon 12b has a penta-cysteine structure C...CexC...C, and is longer (50 aa long) (Fig. 2a). Snail LCa,3-12a and LCa,3-12b were recorded with 2mM Ca2+ as the external charge carrier in transfected HEK-293T cells, using whole cell patch clamp as previously described (13,14), with cell culturing methods we describe in an online video publication (JoVE) (17). A notable difference for LCa,3-12a compared to LCa,3-12b in the ensemble of voltage-gated calcium currents, was a large outward current with voltage steps above 0 mV (Fig. 2b, d). We suspected that the large outward current with LCa,3-12a was carried by the outflow of 110 mM Cs+ from the intracellular patch pipette, a constituent normally present in the patch pipette to block K+ currents. Replacement of 110 mM internal Cs+ with equimolar impermeant monovalent cation N-methyl-D-glucamine (NMDG+), eliminated most of the outward current for LCa,3 isoforms, leaving a residual current, possibly carried by NMDG+, but also the 0.6 mM Li+ in the compound Li-GTP and other monovalent contaminants contained in the intracellular solution (Fig. 2c,d). Calcium channels have a high affinity for Ca2+ ions, and monovalent ions are normally excluded in the presence of physiological levels of extracellular calcium, [Ca2+]ex (10). Thus, it was surprising to observe that LCa,3-12a generated >15-fold greater peak inward currents in the presence of 2 mM Ca2+ with physiological levels of [Na+]ex at 135 mM, whereas LCa,3-12b was significantly less permeable with only a ~2.5-fold increase (Fig. 2e,f). With such a dramatic change in the pore’s ion permeability for LCa,3-12a, it is surprising that both snail LCa,3-12a and 12b isoforms have completely overlapping biophysical properties, such as voltage sensitivities and kinetics of activation and inactivation (Fig. 3a, 3b), as well as the size of the currents in divalent cations (Ba2+ currents being ~1.3 fold larger than Ca2+ currents) (Fig 3c) and similar sensitivity to block by Ni2+ ions (Fig. 3d). Outside of permeability changes, there is a notably faster recovery rate from inactivation (Fig. 3e) and slower deactivation rate for the LCa,3-12a isoform (Fig. 3f). Yet, taking it all together (see Table 4), it is surprising how an extracellular turret constituting ~1% of the channel’s length, has remarkably little effects to the T-type channel while dramatically altering monovalent cation permeability.

Snail T-type channel with exon 12a is more permeant to sodium ions than calcium ions

To quantify ion permeability differences, we measured peak sizes of T-type channel currents generated in bi-ionic conditions with intracellular solutions containing 100 mM of differing monovalent ions (Li+, Na+, K+ or Cs+) in the presence of external calcium ions (4 mM [Ca2+]ex). We compared the more sodium permeant snail LCa,3-12a and snail LCa,3-12b isoforms to the most sodium-impermeant human T-type channel, Ca,3.1. Sizes of outward currents were elicited from a -110 mV holding potential in voltage steps from -90 mV to +80 mV in 5 mV increments (Fig. 4a). The relative permeability of monovalent ions followed Eisenman’s ion selectivity model (23), where Li+ > Na+ > K+ > Cs+, and is consistent with the decreasing permeability with increasing crystal radii of monovalent ions from Li+ < Na+ < K+ < Cs+ (A+) = 0.60, 0.95, 1.33, 1.69 respectively (Fig. 4a,b). The contribution of each monovalent ion
calcium ions. The lack of calcium permeability of permeability of the human Ca
60 mM [Na
mM external calcium, in the presence of a constant through Ca
physiological (mM) levels (Fig. 5a). LCa,3 channels with exon 12a turret are significantly more permeable to monovalent ions than LCa,3 channels bearing the exon 12b turret. Both snail channel splice variants are much more sodium permeant than human Ca,3,1, which is considered the most sodium impermeant of the three mammalian T-type channels, Ca,3.1, Ca,3.2 and Ca,3.3 (10,11). L-type channels are in the ten to sixty-fold range less monovalent ion permeant than T-type channels with P
ratios of 424, 1170, 3000, 4200 (9,18). Another way to gauging the relative permeabilities is in the linear slope conductances between voltage steps from +70 to +80 mV where outward monovalent ion currents possess steeper conductance slopes in current voltage relations, reflecting the much greater observed outward monovalent current through snail LCa,3-12a isoform in the heart and other muscle (buccal mass), where exon 12b is completely absent (Fig. 6a). Whole animal Ca,3 transcript levels steeply decline during embryonic development, and LCa,3-12a continues to fall from juvenile to adults (Fig. 6b), likely largely attributable to the sharp decline of LCa,3 expression specifically in the heart (Fig. 6a). The sodium-impermeable isoform LCa,3-12b is of equal abundance as LCa,3-12a in the brain, but is almost exclusive in secretory reproductive tissue (prostate and alburnen gland), where LCa,3 expression rises from juvenile to adult animals (Fig. 6a). We examined the relative density of mRNA for all relevant voltage-gated cation channels comparing the heart and brain (Fig. 6c). Snail hearts only express two cation channel genes, the L-type channel, LCa,1 (24) and the highly sodium permeant T-type channel LCa,3-12a (Fig. 6c). Brain enriched genes such as the classical sodium channel serving to generate the action potential upstroke (LNa,1 (15)), is completely lacking in the snail heart. Nor does the heart express the brain

Sodium-permeable T-type channels is the only major sodium current in the snail heart

We evaluated mRNA density of the differing extracellular turrets (exon 12a and 12b) in snail tissues by qPCR to determine where we could expect to find sodium-permeable T-type channels. The highest density of T-type channels were found in the snail heart, and particularly, an almost exclusive expression of the sodium-permeant LCa,3-12a isoform in the heart and other muscle (buccal mass), where exon 12b is completely absent (Fig. 5c and see (10)). The consequence of the extracellular turret from exon 12a is to alter T-type channel ion selectivity, shifting a preference to passage of sodium ions over calcium ions especially at physiological concentrations, and reducing the effectiveness of calcium ions to prevent sodium ions from permeating the channel pore. A summary of the monovalent and calcium permeability results are shown in Table 5.

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enriched, synaptic non-L-type calcium channel, LCa,2 (25). We co-expressed and analysed LCa,1 (plus its required accessory β and αδ subunits) and LCa,3-12a channels in HEK-293T cells, which is a configuration expected to emulate the major cation channels in snail cardiomyocytes. Voltage ramps generated from -100 mV to +80 mV, separate low voltage-activated (LVA) T-type and high voltage-activated (HVA) L-type currents in 2 mM [Ba\(^{2+}\)]\(_{ex}\) and impermeant [NMDG\(^{+}\)]\(_{ex}\) at 100 mM (black curve, Fig. 6d). The high sodium permeation through LCa,3-12a channels is revealed after replacing the 100 mM NMDG\(^{+}\) with equimolar Na\(^{+}\) (red curve, Fig 6d). In the same recording, the HVA L-type current is unchanged when external NMDG\(^{+}\) is replaced with Na\(^{+}\), reflecting the channel’s barium-selective currents, and a lack of sodium permeability for L-type channels (Fig 6d). A voltage ramp recording of adult snail cardiomyocytes (Fig. 6f), generates a remarkably similar profile of the sodium permeant LVA T-type current and sodium-impermeant HVA L-type current. The ~11-fold increase in size of the combined sodium and barium LVA currents, upon addition of Na\(^{+}\) ions, is identical in cells transfected with LCa,3-12a channels and the endogenous LVA T-type current in cardiomyocytes (Fig. 6h). On the contrary, the ~two-fold current increase in HEK cells transfected with LCa,3-12b (Fig. 6e, 6h) or human Ca,3.1 (Fig. 6g, 6h) is not reflected in the T-type currents of cardiomyocytes, corroborating the qPCR data that LCa,3-12a is the exclusive T-type isoform in the snail heart. The similarity between cardiomyocytes and transfected LCa,3-12a / LCa,1 channels is also revealed in the makeup of current ensembles initiated from differing holding potentials (Fig. 7a). We used 2 mM Ba\(^{2+}\) and 100 mM Na\(^{+}\) external solution, to separate the kinetics of the slow barium L-type currents from the fast, T-type currents mostly carried by sodium ions. A holding potential of -60 mV will inactivate T-type sodium currents, leaving a residual L-type current (grey color), and a mostly pure T-type sodium current (red color) from the difference current generated from -110 mV and -60 mV holding potentials (Fig. 7a). We examined drug responses of currents in cardiomyocytes elicited by voltage ramp (Fig. 7b, 7c). Pharmacologically, we could separate the L-type from the T-type current using 1 μM isradipine, but both the L-type and T-type currents were similarly blocked by nickel and mibefradil (Fig. 7b, 7c). The sum of the electrophysiological data for cardiomyocytes (current kinetics, drug block, and sodium permeability) (Figs. 6 and 7), is consistent with the mRNA expression profiles in the heart, which indicate an exclusive expression of sodium permeant, LCa,3-12a and sodium-impermeant LCa,1 channels.

**Alternatively-spliced turrets for generating sodium permeable T-type channels are widespread in protostome invertebrates**

Analyses of Ca,3 genomic sequences reveal that alternative splicing of exon 12 that we discovered in the snail, Lymnaea stagnalis is widely present, but limited to protostomes (i.e. non-echinoderm invertebrates) (Fig. 8, Fig. 9, Table 6). Exon 12a is characteristically short (38 to 46 aa long, average = 40.7 aa) with a nearly invariant tri-cysteine structure: CxxC…C (Fig. 8). Exon 12b has a penta-cysteine structure C...CxC...CxC (most protostomes) or CxxC...C...CxC (some nematodes) and is always longer (range: 48 to 55 aa long, average = 52.1 aa) than exon 12a (Fig. 9). The three vertebrate T-type channels, Ca,3.1, Ca,3.2 and Ca,3.3, have an exon 12 with only one conserved cysteine (Fig. 8) compared to three and five cysteines in invertebrate exons 12a (Fig. 8) and 12b (Fig. 9), respectively. Paradoxically, the vertebrate exon 12 resembles the shorter exon 12a of invertebrates in size (38 to 39 aa long, average = 38.7 aa) which in snail LCa,3 we have shown imparts the properties of extreme sodium ion permeability for exon 12a-containing T-type channels.
DISCUSSION

Unique splicing of exon 12 in invertebrate T-type channels. We have functionally characterized the first non-vertebrate T-type calcium channel (13), which reveals a high conservation of quintessential features of vertebrate Ca$_3$1.1 and Ca$_3$3.2 T-type channels, including a similar low voltage range of activity with a peak current at -40 to -35 mV, with similar rapid activation and inactivation kinetics, and slow deactivation kinetics. Also conserved is developmentally-regulated splicing where T-type channels which lack exon 25c are highly down-regulated from embryo to adult animals, leading to a battery of biophysical changes amongst LCa$_3$, Ca$_3$.1 and Ca$_3$.2 (14). We also found that the presence of optional exon 8b in the I-II linker, specifically downregulates the level of protein expression of snail T-type channels in a manner consistent with Ca$_3$.1 (14). Here we describe unique exon splicing that generates highly sodium-permeant T-type channels utilizing an alternative exon 12a, which spans the extracellular turret of Domain II upstream of the highly conserved EEDD selectivity filter of T-type channels.

We show that invertebrate LCa$_3$ T-type channels will generate large outward currents in the presence of 100 mM intracellular monovalent ions accompanying the large driving force at high voltage steps much more so than human Ca$_3$.1, while also generating an inward calcium current in the presence of 4 mM extracellular calcium ions at voltage steps below the reversal potential (Fig. 2, Fig. 4). Permeability of monovalent ions decreases with increasing ion diameter size where Li$^+$ > Na$^+$ = K$^+$ > Cs$^+$ > NMDG$^+$. All T-type channels pass sodium ions when external calcium concentration is at exquisitely low levels (<10 μM external calcium ions). However the sodium current through human Ca$_3$.1 has less physiological relevance because it is almost completely blocked (96%) by low (10 μM) external calcium. With rising external calcium concentrations to physiological levels, nearly all of the current through human Ca$_3$.1 is carried by calcium (Fig. 5). The snail T-type channel isoforms, especially those harboring exon 12a, are not calcium-selective like human Ca$_3$.1. Calcium ions are not effective at blocking the sodium current at 10 μM external calcium, with snail T-type channels with exon 12a resisting the calcium block (44%), much more than isoforms with exon 12b (81%) or human Ca$_3$.1 (96%) (Fig. 5).

Increasing external Ca$^{2+}$ above 10 μM to physiological (mM) levels, does not generate the larger calcium currents for LCa$_3$ as is reflected in the “U” shape calcium dose response curve of human Ca$_3$.1 (10). Rather, the total current size dramatically falls monotonically (6.5-fold) for snail channels harboring exon 12a reflecting their reduced affinity for Ca$^{2+}$ in the pore that allows for greater permeability of Na$^+$ ions (Fig. 5). The extreme sodium permeability of LCa$_3$.3-12a is evident in voltage ramp-generated currents with amplitudes that are ~11 fold greater when external Na$^+$ replaces impermeant cation NMDG$^+$ in the presence of barium. The sodium permeability is not evident in the co-expressed snail L-type (LCa$_1$) channel in the same voltage ramp, and a much reduced (~2-fold instead of ~11-fold) sodium permeability for LCa$_3$ channels harboring exon 12b (Fig. 6). Snail heart cells only express T-type channels with exon 12a, and express the same sodium-permeable T-type current as the in vitro expressed gene, resembling its fast kinetics, low voltage of activation, nickel (mM) and mibebradil (mM) sensitivity and isradipine (μM) insensitivity (Fig. 6, Fig. 7).

Unique functions for highly sodium permeant (exon 12a) and less sodium permeant (exon 12b) T-type channels. The distinct tissue expression patterns of LCa$_3$ exons 12a (heart, muscle) and 12b (secretory glands), suggests specialized roles of different permeable T-type channels in different cell types. T-type channel transcript levels steeply decline during embryonic development through adulthood, reflected by a sharp decline in LCa$_3$.3-12a expression in juvenile to adult heart (Fig. 6). A downregulation of LCa$_3$.3-12a may relate to allometric scaling during development, where more prominent LCa$_3$ currents are found in smaller animals (non-adults) coinciding with faster heart rhythms (26). Expression of LCa$_3$.3-12a isoform indicates a significantly slower deactivation rate, and faster recovery rate from inactivation when compared to LCa$_3$.3-12b (Fig. 3). This is consistent with LCa$_3$.3-12a and adaptations in the snail heart, where a slower deactivation would serve to prolong the repolarization phase of the cardiac action.
potential, and a faster inactivation recovery prevents rundown between heart beats due to cumulative inactivation. Snails have only one (non-specialized) sodium channel gene (Na\textsubscript{v}1), compared to ten in vertebrates, and this gene is only expressed in the snail brain and is not expressed in the snail heart (Fig. 6) (15). Thus, LCa\textsubscript{v}3-12a is the only voltage-gated sodium channel generating pacemaker currents in ventricular cells of the primitive two-chambered snail heart, which lacks the appearance of specialized vertebrate sodium channels, like Na\textsubscript{v}1.5 and other neuronal Na\textsubscript{v} channels for fast conduction of heart rhythms.

The more calcium-selective isoform LCa\textsubscript{v}3-12b is of equal abundance to LCa\textsubscript{v}3-12a in the brain, and is the almost exclusive transcript associated with reproductive tissue of the hermaphroditic snail (prostate = male organ, albumen gland = female organ) (Fig. 6). The rise in transcripts of LCa\textsubscript{v}3-12b from juvenile to adults (Fig. 6) coincides with sexual maturation where the albumen gland takes up a role secreting factors that facilitate egg mass formation (27). Our observations of a much faster deactivation rate, and slower recovery rate from inactivation of LCa\textsubscript{v}3-12b channels promotes the typical pulsatile, burst firing of vertebrate T-type channels, where the rise in intracellular calcium may contribute to pacemaking, and possibly also to excitation-secretion coupling (14).

Snail LCa\textsubscript{v}3 (like its vertebrate counterparts) also has prominent “window” currents, where an estimated 1-2\% of available channels are open at rest (13). LCa\textsubscript{v}3-12a variants are highly abundant in the brain, and likely contribute to reported persistent sodium currents (28,29), serving as a significant sodium leak conductance (30). Even though highly sodium-permeant T-type channels are likely widespread in invertebrates, it would be hard to discern them with voltage-gated sodium channel currents if you weren’t looking for them. Drugs are not selective in most invertebrates, like snails which are insensitive to classical sodium blockers such as TTX (15), and neither are snail T-type channels easily discernible by Ni\textsuperscript{2+} block (13). Some species including nematodes like C. elegans, lack a sodium channel (Na\textsubscript{v}) gene in their genomes (31), which means that their sodium-permeant T-type channels with exon 12a likely serve as a critical source for generating sodium-dependent spikes in these organisms. Most invertebrates do contain gene homologs of hyperpolarization-activated and cyclic nucleotide-gated (HCN) channels, which presumably contributes to sodium currents in invertebrate pacemaker rhythms (32). An HCN channel homolog is notably absent in C. elegans too (31,32), which limits the possibilities of sodium current generating spikes in these animals from the conventional palette of ion channel genes. We suggest that T-type sodium channels have a role to play in lieu of sodium channels in C. elegans.

**Evolutionary adaptations of the Domain II turret.** A pattern of evolution of the extracellular turret in Domain II of T-type channel is evident in their phylogeny (Fig. 10). The Ca\textsubscript{v}3 T-type channel class first appeared in the most primitive multicellular animals, as low voltage-activated calcium-selective counterpart to the Ca\textsubscript{v}1 and Ca\textsubscript{v}2 calcium channels. We have isolated and characterized the singleton T-type channel, TCa\textsubscript{v}3 cloned from the most primitive, extant relative to have a T-type channel, the placozoan, Trichoplax, which is multicellular, but lacking a tissue level organization. TCa\textsubscript{v}3 generates calcium currents consistent with other basal metazoans, such as the hydrozoan jellyfish where it serves as the only calcium current generated in muscle action potentials (33). All basal metazoans have short exon 12 turrets with a small number of cysteines (0 to 2). Insertion of an intron before exon 12 in primitive protostomes, the platyzoa (acoelomates), allowed a duplication of exon 12 to generate dual turrets, one highly sodium permeant tri-cysteine turret (exon 12a) and a more calcium-selective, penta-cysteine turret (exon 12b) in ecdysozan and lophotrochozoan protostomes. Associated with protostomes is the appearance of the coelom and internal organs such as the heart, which require fast sodium-dependent spikes to coordinate their body organs’ activities. Na\textsubscript{v}1 channels in vertebrates expanded to ten Na\textsubscript{v}1 channel genes fulfilling a universal spike generator role inside and outside the brain. Invertebrates, on the other hand, have a singleton Na\textsubscript{v}1 sodium channel gene, which is absent in many species (eg. C. elegans) and is not abundant outside the nervous system (eg. *Lymnaea* snail). Outfitting sodium permeant pores in T-type channels is a likely adaptation in
invertebrates in lieu of spikes generated by Na,1 channels.

**Importance of the high field strength site in the selectivity filter governing calcium and sodium permeation.** Ion channels have evolved unique ion pores that are selective for the two major external cations, calcium ions and sodium ions. Flexible side chains of carboxylate oxygens from negatively charged residues are proposed to bind cations with high affinity, and permeation is facilitated by incoming ions attracted to the negatively charged extracellular surface of the pore (34). These charged residues form a “high field strength site” at the outer end of the selectivity filter in equivalent position of the selectivity filter TLESWSM in the re-entrant pore contributed by equal domains of the homomultimeric prokaryotic sodium channel, NavAb (6). Backbone carbonyl residues of the preceding two residues TLESWSM form the two innermost ion coordination sites for the sodium channel’s selectivity filter (6). Replacement with three aspartates TLDWSD in the selectivity filter transforms the sodium-selective pore into a calcium-selective one in prokaryotic channels (35,36). Significantly, sodium and calcium ions enter, reside and exit through the selectivity filter in a semi- or fully hydrated form through a much broader pore than a potassium-selective pore, and where strategic positioning of a limited number of residues can tip the balance in favor of whether mostly sodium or calcium ions, or a mixture of both ions permeate (35). The sodium and calcium pore is a departure from the long and narrow, potassium selectivity filter (TVGYG), which is a Goldilocks’ “just right” fit for the dehydrated potassium ion, which underlies the potassium channels nearly exclusive selectivity for potassium ions (5).

Identity of the single amino acid base of the equivalent field strength glutamate residue in prokaryotic sodium channels serves as the basis for categorizing metazoan sodium and calcium channels. This position is contributed by four non-equivalent residues of Domains I, II, III and IV, in four domain containing channels which contains a lysine in the Domains III or II as DEKA (or DEEA) for Nav1 sodium selective channels, and lacking a lysine residue in the calcium-selective Ca,1 (EEEE), Ca,2 (EEEE) and primordial Na,1 (DEEA) or invertebrate Na,2 channels: (DEEA) (37). The importance of this residue position is evident in experiments where sodium channels take on the high calcium selectivity of calcium channels after replacement of the DEKA high field strength site position with EEEE, and vice-versa (8,38). Yet it is clear that the high field strength site in the selectivity filter isn’t the only critical feature to govern permeation through T-type channels. All T-type channels have a unique high field strength site residue of EEDD, but when mutated to more resemble calcium selective Ca,1 and Ca,2 channels (as EEED or EEDE), T-type channels become paradoxically more, not less permeable to sodium ions (22). And despite the consistency in the EEDD high field strength site, T-type channels highly vary in their sodium permeability that ranges from 20%, 25% and 40% of the current for Ca,3.1, Ca,3.2 and Ca,3.3 channels, respectively, for mammalian channels (1) and from 50% and >90% of the current through snail LCav3 channels with alternate novel extracellular SS-P turret domains in Domain II. T-type channels also widely vary in their relative permeation of calcium ions as the charge carrier versus barium ions, which is expected to reflect a variable calcium affinity for the pores of T-type channels (39). This is in contrast to other calcium channels. Macroscopic barium currents are consistently ~2 fold the size of calcium currents, regardless of whether these are invertebrate Cav1 or Cav2 channels or vertebrate Cav1 or Cav2 homologs (1).

So, how do invertebrate T-type channels become so highly sodium permeant using alternative splicing of extracellular regions outside the selectivity filter of the pore in Domain II? Snail exon 12a and exon 12b differ in the extracellular turret but there are also sequence differences that may be important in the descending helix just upstream of the selectivity filter in Domain II. We suggest that changes in the descending helix and the extracellular turret could contribute to the altered sodium permeation through T-type channels.

**Potential roles for changes in the descending helix.** The distal end of the descending helix away from the selectivity filter has three variable amino acid residues: AIV, ALI and SLV in Cav3.1, and snail LCav3-12a and LCav3-12b channels,
respectively. Negatively charged residues within the selectivity filter form transient binding partners outside the selectivity filter in structural intermediates of the calcium channel pore. Changes in the descending helix may be sufficient to reorient a selectivity filter residue, altering the relative permeation of sodium and calcium ions.

**Potential similarities of the T-type channel turret with the extracellular cap domain of K_{2P} channels.** There are possibilities of analogy between the extracellular turret of Domain II in T-type channels and the crystal structures of turret regions from two-pore potassium leak channels (K_{2P}) channels (40,41). The first of two pores of K_{2P} channels have a uniquely extended turret of 56 amino acids, which normally ranges between 5 to 20 amino acids in other potassium channels. The turret forms a highly ordered, extracellular cap, reminiscent to an A-frame, extending 35 angstroms above the lipid membrane with a cysteine residue at its apex which cross-links to the apex of the A-frame of a second K_{2P} subunit, in the two-fold symmetrical, K_{2P} channel (40,41). The dual A-frames perpendicular to each other form a space-filled “carafe plug” that restricts ion and drug access to the channel pore from above (40,41). K^+ ion passage is limited to two funnel-shaped side portals contained in the extracellular cap domain (40,41). Wall linings the side portals contain negatively charged residues, and variable sequences which could serve as a variable pre-filter for cations channeling to the selectivity filter below (40,41). The extracellular cap domain approaches to a close proximity of the selectivity filter where the negative C-terminal ends of the helical dipole from an extracellular helix lies just above and to the side of the selectivity filter, before sharply pivoting away from the selectivity filter at a conserved glycine residue before meeting with the descending pore helix that leads to the selectivity filter (40,41). The turret in Domain II of T-type channels is the shortest of the four extracellular turrets preceding pore helices that descends into the re-entrant pore, and thus is likely restricted to the lower quadrant of the outer vestibule where it may likely encounter the selectivity filter (1). The constraints on size and the conserved framework of uni-, tri-, and penta-cysteines exon 12 in vertebrates, and exons 12a and 12b of invertebrate T-type channels are consistent with these specialized turret variants changing the landscape in the external vestibule (analogously to K_{2P} channels) and forming regulatory structures that make specific contacts within the pore. Differing turrets are expected to bias the selectivity filter allowing the more favored passage of monovalent over divalent cations, in a pore that is expected to be similar to the wide bacterial sodium channel (eg. Na,Ab (6), Na,Rh (42)) which can accommodate more than one semi-hydrated ion at its more constrictive locus (the selectivity filter residues).

**Possible similarity of the extracellular turret domains in K_{2P}, K_{IR} and T-type channels as toxin defense shields.** Eukaryotic inward rectifying potassium channels (K_{IR}) also possess an extended turret region, albeit a minor external appendage compared to K_{2P} channels (43). Nonetheless, the turret of K_{IR} channels is substantial enough to alter the external surface landscape and contribute a unique resistance of these channels to classical invertebrate toxins (eg. from snake, spider and scorpion venoms), which typically block voltage-gated potassium channels when applied from the outside of the channels (44-46). Resistance to external toxins is a shared property with K_{2P} channels likely due to shielding with the elaborate extracellular cap domain of K_{2P} channels (47,48). Unique extracellular turret domains may also explain why there aren’t invertebrate pore-blocking toxins (from cone snail, spider and snake venom) for Ca_{1,3} T-type channels compared to other (Ca_{1,1} and Ca_{1,2}) calcium channels, where numbers of specific, high affinity toxins have been discovered (49). The extensive extracellular turret regions in K_{2P} (40,41), eukaryotic K_{IR} (43) and T-type channels are highly variable in sequence outside of key structural residues (eg. cysteines, glycine, prolines) or in the positioning of particular charged residues (eg. arginine, lysine). One can imagine that some sequence variation in extracellular regions may be adaptive as countermeasures against the arsenal of specific channel toxins from venoms of invertebrate predators.

**Other examples of alternative splicing that generates altered ion selectivity of ion channels.** Alternative splicing that generates permeation differences in ion channels is not common. We
discovered alternate exons in Domain II of NALCN channels, that alter the high field strength residue to generate calcium-like (EEEE) or sodium-like (EKKE) pores (of flatworms, mollusks, annelids, echinoderms and hemichordates) (19,50). Interestingly, some arthropods generate alternative pores in NALCN channels differently, using an alternate exon in Domain III to generate calcium-like (EEEE) or sodium-like (EEKE) pores in their NALCN channels (19,50). And last, a completely unique approach to altering ion selectivity is the use of alternative translational start sites to generate a truncated isoforms in K_2P2.1 channels that are sodium permeable, to produce offsetting depolarizing currents, in a channel type that typically only serves up hyperpolarizing potassium leak currents (51).

**Conclusions.** T-type channels have varying sodium permeability in the presence of an extracellular veil of novel turret residues in Domain II, which encode merely ~1% of the T-type channel protein. Each splice isoform is exclusively expressed in particular tissues, where they serve highly different functions conducting variable sodium and calcium currents. It is surprising that only a few amino-acids separate highly sodium- or more calcium-conducting channels, given that the ions have such different roles, where Na^+ ions are relatively inert and are much more abundant serving mostly an electrogenic role, while Ca^{2+} ions are maintained at very low levels in cells due to cytotoxicity, and serving as an exquisitely-sensitive signalling molecule. Extracellular concentrations of sodium ions are on the scale of ~50-100x that of Ca^{2+} ions or K^+ ions, so T-type channels that are permeant to monovalent ions are generating large currents, and steeper membrane depolarizations than typical calcium-selective channels. They also generate larger currents than high voltage-activated calcium channels because of the greater driving force for T-type channels operating at their much lower voltage range. It is remarkable that T-type channels evolved a mechanism outside the conventional selectivity filter to alter their ion selectivity. It is also intriguing to understand the physiological context (eg. for brain, heart and glands) in which T-type channels retain similar biophysical properties but possess dramatically different ion selectivities for sodium and calcium ions.

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**AUTHOR CONTRIBUTIONS**

Conceived and designed the experiments, analyzed the data, and contributed reagents/materials/analysis tools: AS WG ANB and JDS. Performed the experiments: AS WG and ANB. Wrote the paper: AS WG ANB and JDS.
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FIGURE LEGENDS:

Table 1. External and internal recording solutions (Part I)

Table 2. External and internal recording solutions (Part 2)

Table 3. Primers used for qPCR of control gene HPRT1, LCa.3, LNa.1 and LNa.2. LCa.3 primer sets include universal sequence primers, exon 12a isoform specific primers, and exon 12b isoform specific primers. PCR efficiency (E), goodness of fit R² values, and slopes for the standard curves used to characterize qPCR primer pairs are shown in red.

Table 4. Comparison of the biophysical parameters for snail LCa.3 channel currents recorded harboring either exon 12A or exon 12B in HEK-293T cells.

Table 5. Monovalent ion and calcium permeability parameters for snail LCa.3-12a or LCa.3-12ba channel or human Ca.3.1 channel currents expressed in HEK-293T cells

Table 6. Features of exons 11 and 12 of Cav3 channel homologs in sequenced metazoan genomes

Figure 1: Expression of exons 12a and 12b in *Lymnaea stagnalis* cDNA. A) PCR amplification of snail embryonic cDNA with nested primers flanking the mutually exclusive exons 12a and 12b produced two DNA fragments that could be distinguished in size on an agarose gel. B) Plasmid DNA inserts from (A) was excised from the pGEM-T Easy vector with EcoRI, and electrophoresed inserts were restricted to two sizes (451 bp for exon 12a, 484 bp for exon 12b (plus sizes additional polylinker sequence from the pGEM vector). Sequencing of multiple inserts of each size confirmed that the smaller plasmid insert encoded exon 12a and the larger clone encoded exon 12b.

Figure 2. Splicing of extracellular turret isoform (exon 12a) in the pore loop of Domain II in snail LCa.3 channels generates monovalent ion permeant T-type channels. (A) Aligned sequences illustrate mutually exclusive exons (12a and 12b) correspond to a portion of the domain II S5 helix, the turret and descending helix that descends into the pore vestibule. (B) Dramatic difference in outward Cs⁺ currents between LCa.3-12a and 12b in 2 mM [Ca²⁺]ₐₓ and 0.6 mM [Li⁺-GTP] in mostly eliminated by (C) replacement of 110 mM internal Cs⁺ with equimolar impermeant NMDG⁺. Sample traces (left) and current voltage curves (right) in B and C. (D) Ratio of maximum outward currents (at +40 mV) to peak inward current for B and C. n values in brackets. (E) Sample currents and (F) bar graph illustrating the 15 fold peak inward current size with addition of 135 mM [Na⁺]ₐₓ to 2 mM [Ca²⁺]ₐₓ with LCa.3-12a, compared to the ~2.5 fold change with LCa.3-12b.

Figure 3. Differing extracellular turrets in DII P-loop (exon 12a, 12b) do not alter major features outside of monovalent ion permeability. There were no major differences in most -biophysical properties between LCa.3-12a and LCa.3-12b in 2 mM [Ca²⁺]ₐₓ, including (A) current-voltage relationship for activation and steady-state inactivation, (B) inactivation kinetics and time to peak (inset), (C) current size differences in 2 mM [Ba²⁺]ₐₓ vs. [Ca²⁺]ₐₓ and (D) blockade by 1 mM Ni²⁺ (sample block, inset). There are measureable differences in (E) faster recovery rate from inactivation and (F) slower deactivation kinetics for LCa.3-12a. n values in brackets.

Figure 4. Monovalent ion (Li⁺, Na⁺, K⁺, Cs⁺) permeability through snail LCav3 T-type channels is especially dramatic with exon 12a compared to exon 12b extracellular turrets or human Cav3.1 channels. Inward calcium currents in 4 mM [Ca²⁺]ₐₓ and outward monovalent T-type channel currents were generated with intracellular solutions containing 100 mM of Li⁺, Na⁺, K⁺, or Cs⁺ and elicited from a -110 mV holding potential in voltage steps from -90 mV mV to +80 mV in 5 mV increments. (A)
Representative currents and their (B) current-voltage relationships. n values in brackets. (A or B, inset) Close-up of currents crossing the reversal potential, with reversal potentials labelled in B.

Figure 5. Snail LCa,3 channels with exon 12a are highly permeable to sodium ion and weakly permeant to calcium ions, compared to LCa,3 channels with exon 12b or human Cav3.1 channels. (A) Relative permeabilities of calcium to monovalent cations (PCa/Pv) generated using reversal potentials (shown in Figure 3b) inputted into a bi-ionic Nernst equation (materials and methods). (B) Slope conductance of outward currents (shown in Figure 3b) measured as the linear fit of currents generated from steps between +70 to +80 mV. (C) Increasing block of maximal ionic current (I/I\text{max}) in 60 mM Na\textsuperscript{+} external with increasing [Ca\textsuperscript{2+}]\text{ex} from 10\textsuperscript{-9} to 10\textsuperscript{-5} M reflects the competition between sodium and calcium ions to permeate the pore. (D) Bar graph illustrates the weak block of LCa,3-12a channel current (44%) compared to LCa,3-12b (81%) and human Ca,3.1 (96%) channels at 10 μM [Ca\textsuperscript{2+}]\text{ex}. (E) X-scale shown for calcium dose response curves (C) limited to rise in [Ca\textsuperscript{2+}]\text{ex} from 10 μM to 10 mM with corresponding (F) bar graph, reflecting the dramatic fold decrease (6.5x, 2.0x) in calcium permeability with snail LCa,3-12a and -12b channels, respectively compared to the dramatic fold increase (9.1x) in calcium current through the physiological range for external calcium ions. Statistics shown in Table S2.

Figure 6. Snail express two cardiac cation currents, the highly sodium-permeant T-type channel, LCa,3 and the calcium-selective, L-type channel, LCa,1. (A, B, C) quantitative RT-PCR standardized to Lymnaea HPRT1 control gene. (A) Relative mRNA expression levels of exon 12a and 12b in juvenile (juv) and adult (ad) snails, reflecting the almost exclusive expression of exon 12a in the snail heart and exon 12b in prostate and albumen glands. (B) The dramatic decline in LCa,3 channel expression in whole animals can mostly be attributed to the decline of LCa,3-12a expression in the developing heart. (C) Snail hearts lack mRNA expression of any sodium channel gene (LNa,1, LNa,2), nor LCa,2 channel, nor T-type channel gene with exon 12b. (D,E,F,G) Ramp protocols (-110 mV to +100 mV in 1 s) carried out in presence of external solutions: 2 mM Ba\textsuperscript{2+} and 100 mM Na\textsuperscript{+} ions or 2 mM Ba\textsuperscript{2+} and 100 mM NMDG\textsuperscript{+} reveal a covert, sodium-permeant, T-type current superimposed on the Ba\textsuperscript{2+} current in (F) cardiomyocytes in the presence of a sodium-impermeant, L-type current. The sodium-permeant, T-type currents in cardiomyocytes are indistinguishable from mammalian HEK-293T cells transfected with LCa,3 with exon 12a (D) with co-expression of LCa,1 and accessory subunits (α\text{δ}, β\text{δ}). (E) LCa,3 with exon 12b and (G) human Ca,3.1 has some Na\textsuperscript{+} permeability, but is not as Na\textsuperscript{+} permeant as the LCa,3-12a variant expressed in cardiomyocytes. (H) Bar graph +/- s.e.m. including sample data in D,E,F,G.

Figure 7. Separation of snail cardiac LCa,3-12a (T-type) and LCa,1 (L-type) currents using holding potentials (HP) and drugs (isradipine, mibefradil, nickel) in whole-cell patch clamp recording. (A) Fast T-type currents (red color) isolated by difference current in 2 mM [Ba\textsuperscript{2+}]\text{ex} and 100 mM [Na\textsuperscript{+}]\text{ex} with a holding potential (HP) of -110 mV versus -60 mV in whole cell recording of native LCa3/LCa1 channel currents in snail cardiomyocytes. (B) Ramp protocols (-110 mV to +100 mV in 800 ms) carried out in presence of external solutions 2 mM [Ba\textsuperscript{2+}]\text{ex} and 100 mM [Na\textsuperscript{+}]\text{ex} and differing doses of drug perfused on cardiomyocytes. Isradipine at 1 μM is a specific blocker of L-type currents while mibefradil and nickel are non-specific blockers of L-type and T-type currents. C) Dose response curves +/- s.e.m. for isradipine (n=6), mibefradil (n=8), nickel (n=8); sample data in (B).

Figure 8: Aligned amino acid sequences of exon 12A in metazoan Ca,3 channel homologues. Exon 12a codes for novel extracellular “turret” residues from the middle of Domain II, segment five through the descending pore helix to five amino acids upstream of selectivity filter glutamate (purple) residue, and invariant aspartate residue after it, conserved in all calcium channels (orange residue). Exon 12a is flanked by conserved charged residues (red), surrounding a set of variable amino acids, outside of very conserved cysteine (yellow) residues. Exon 12a is ~40 aa with a tri-cysteine motif (CxxC...C).

Vertebrate T-Type channels resemble the size of exon 12A in length (~39 aa) but are missing the most downstream two cysteines of invertebrate exon 12a. Animals bearing T-type channels below the
protostome invertebrates (placozoan and cnidarians) have zero or two cysteines in exon 12a and lack an exon 12b. Most protostome invertebrates possess an alternative exon 12b (see Figure 9) turret in addition to exon 12a, and these are highlighted and outlined.

**Figure 9: Aligned amino acid sequences of exon 12B found in protostome Ca,3 channel homologues.** Exons 12B code for novel extracellular “turret” residues from the middle of Domain II, segment five through the descending pore helix to five amino acids upstream of selectivity filter glutamate (purple) residue, and invariant aspartate residue after it, conserved in all calcium channels (orange residue). 12b exons are flanked by conserved charged residues (red), surrounding a set of variable amino acids, outside of very conserved cysteine (yellow) residues. Exon 12b is ~52 aa with a penta-cysteine motif (C...CxC...CxC) or more rarely (CxxC...C...CxC), in some nematodes. Most protostome invertebrates possess a shorter alternative exon 12a turret (see Figure 8) in addition to longer exon 12b, and these are highlighted and outlined.

**Figure 10. Phylogeny and proposed evolution for exon 12 which governs monovalent ion permeability in Ca,3 T-type channels.** (A) Gene tree illustrates the conservation of exon 12a, (smaller, blue exon) and exon 12b (larger, green exon) with vertical black lines indicating location of cysteine residues. Introns indicated by triangles. (B) Proposed evolutionary progression: an intron separating exons 11 and 12; appearance of a tri-cysteine motif characteristic of exon 12a highly sodium-permeant isoforms; an alternative exon 12b with a penta-cysteine motif characteristic of less sodium permeant channels in protostomes. Evolution of a uni-cysteine motif in exon 12 of Ca,3 channels which supports a more calcium-selective channel in vertebrates.
Table 1. External and internal recording solutions (Part I)

[Ca^{2+}]_{ex} or [Ba^{2+}]_{ex} solution for Figure 2 and 2 (minus Figure 2e, 2f)

| External | CaCl$_2$ | BaCl$_2$ | TEA-Cl | HEPES |
|----------|----------|----------|--------|-------|
| pH 7.4 with TEA-OH |
| Internals | # | CsCl | NMDG+ | EGTA | Mg-ATP | Li-GTP | HEPES |
| 1 | 110 | 0 | 10 | 3 | 0.6 | 10 |
| 2 | 0 | 110 | 10 | 3 | 0.6 | 10 |

1 pH 7.2 with CsOH

[Ca^{2+}]_{ex} +/- [Na^{+}]_{ex} solutions for Figure 2e, 2f, 10b, 10c

| External | CaCl$_2$ | NaCl | NMDG+ | TEA | HEPES |
|----------|----------|------|-------|-----|-------|
| pH 7.4 with TEA-OH |
| Internals | # | CsCl | NaCl | KCl | LiCl | EGTA# | TEA-Cl | HEPES |
| 1 | 110 | 0 | 0 | 0 | 0 | 10 | 10 | 10 |
| 2 | 0 | 100 | 0 | 0 | 0 | 10 | 10 | 10 |
| 3 | 0 | 0 | 100 | 0 | 0 | 10 | 10 | 10 |
| 4 | 0 | 0 | 0 | 100 | 0 | 10 | 10 | 10 |

1 pH 7.2 with CsOH

Bi-ionic solutions for reversal potential experiments, Figures 4, 5a, 5b

| External | CaCl$_2$ | TEA-Cl | HEPES |
|----------|----------|--------|-------|
| pH 7.4 with TEA-OH |
| Internals | # | CsCl | NaCl | KCl | LiCl | EGTA# | TEA-Cl | HEPES |
| 1 | 110 | 0 | 0 | 0 | 0 | 10 | 10 | 10 |
| 2 | 0 | 100 | 0 | 0 | 0 | 10 | 10 | 10 |
| 3 | 0 | 0 | 100 | 0 | 0 | 10 | 10 | 10 |
| 4 | 0 | 0 | 0 | 100 | 0 | 10 | 10 | 10 |

1 pH 7.2 with XOH where X= Cs, Na, K, or Li.

# pH 8.0 with XOH
Table 2. External and internal recording solutions (Part 2)

Dose response to $[Ca^{2+}]_{ex}$ in presence of 60 mM $[Na^+]_{ex}$, Figure 5c-5f

| External | in mM | $Ca^{2+}$ free | CaCl$_2$ | NaCl | EGTA | HEPES | Glucose |
|----------|-------|----------------|---------|------|------|-------|---------|
| 1        | 1x10^-9 | 0.01 | 60 | 1.246 | 10 | 26.3 |
| 2        | 1x10^-7 | 1.00 | 60 | 2.236 | 10 | 20.4 |
| 3        | 1x10^-5 | 1.00 | 60 | 1.002 | 10 | 24.1 |
| 4        | 1x10^-4 | 0.10 | 60 | 0    | 10 | 29.7 |
| 5        | 3x10^-4 | 0.30 | 60 | 0    | 10 | 29.1 |
| 6        | 1x10^-3 | 1.00 | 60 | 0    | 10 | 27.0 |
| 7        | 3x10^-3 | 3.00 | 60 | 0    | 10 | 21.0 |
| 8        | 1x10^-2 | 10.00 | 60 | 0    | 10 | 0.0  |

pH 7.4 with TEA-OH

| Internal | CsCl | EGTA | Mg-ATP | Li-GTP | HEPES |
|----------|------|------|--------|--------|-------|
|          | 110  | 10   | 3      | 0.6    | 10    |

pH 7.4 with CsOH

Solutions for Figure 7

| External | BaCl$_2$ | NaCl | NMDG+ | TEA-Cl | MgCl$_2$ | HEPES |
|----------|---------|------|-------|--------|----------|-------|
| 1        | 2       | 100  | 0     | 50     | 1        | 10    |
| 2        | 2       | 100  | 0     | 50     | 1        | 10    |

1 pH 7.9 with CsOH

| Internal | CsCl | 4-AP | EGTA | Mg-ATP | HEPES |
|----------|------|------|------|--------|-------|
| #        | 135  | 5    | 10   | 2      | 10    |

1 pH 7.7 with CsOH
Table 3. Primers used for qPCR of control gene HPRT1, LCa,3, LNa,1 and LNa,2. LCa,3 primer sets include universal sequence primers, exon 12a isoform specific primers, and exon 12b isoform specific primers. PCR efficiency (E), goodness of fit R² values, and slopes for the standard curves used to characterize qPCR primer pairs are shown in red.

| Real time RT primers: | Amplification |
|-----------------------|---------------|
|                       | Length | length | Tm (NN) | GC% | qPCR E | R²  | Slope |
| Lymnaea HPRT1 5'      | 145 | 24 | 53.86 | 42 | 90.4 | 0.985 | -3.576 |
| Lymnaea HPRT1 3'      | 23 | 53.06 | 43 | 90.4 | 0.985 | -3.576 |
| LCav3 Univ 5'         | 139 | 24 | 57.34 | 50 | 104.7 | 0.986 | -3.215 |
| LCav3 Univ 3'         | 24 | 54.14 | 42 | 104.7 | 0.986 | -3.215 |
| LCav3 12B 5'          | 145 | 23 | 57.05 | 52 | 90.4 | 0.984 | -3.576 |
| LCav3 12B 3'          | 23 | 57.82 | 52 | 90.4 | 0.984 | -3.576 |
| LCav3 12A 5'          | 134 | 23 | 57.05 | 52 | 92.3 | 0.977 | -3.523 |
| LCav3 12A 3'          | 26 | 57.29 | 46 | 92.3 | 0.977 | -3.523 |
| LNav1 5'              | 124 | 20 | 54.3  | 50 | 111.9 | 0.987 | -3.066 |
| LNav1 3'              | 23 | 52.85 | 43 | 111.9 | 0.987 | -3.066 |
| LNav2 5'              | 113 | 24 | 51.95 | 38 | 107.9 | 0.959 | -3.147 |
| LNav2 3'              | 24 | 54.43 | 42 | 107.9 | 0.959 | -3.147 |
Table 4. Comparison of the biophysical parameters for snail LCa,3 channel currents recorded harboring either exon 12A or exon 12B in HEK-293T cells.

|                      | LCa,3 12A                   | LCa,3 12B                   | Significance | p value |
|----------------------|----------------------------|----------------------------|--------------|---------|
| **Activation**       |                            |                            |              |         |
| \(V_{0.5}\) (mV)    | -53.63 ±0.35 (n24)         | -53.48 ±0.34 (n13)         | n.s.         | 0.768   |
| Slope (mv)           | 5.60 ±0.09 (n29)           | 5.46 ±0.14 (n13)           | n.s.         | 0.348   |
| **Activation NMDG**  |                            |                            |              |         |
| \(V_{0.5}\) (mV)    | -63.50 ±0.60 (n4)          | -67.40 ±0.78 (n5)          | **           | 0.004   |
| Slope (mv)           | 6.00 ±0.01 (n4)            | 5.57 ±0.43 (n5)            | n.s.         | 0.348   |
| **Inactivation**     |                            |                            |              |         |
| \(V_{0.5}\) (mV)    | -70.21 ±0.38 (n20)         | -70.89 ±0.49 (n16)         | n.s.         | 0.262   |
| Slope (mV)           | 2.73 ±0.04 (n20)           | 2.93 ±0.08 (n16)           | *            | 0.019   |
| **Activation kinetics** |                          |                            |              |         |
| TTP -55 mV (ms)      | 14.68 ±0.63 (n24)          | 12.62 ±0.70 (n16)          | *            | 0.035   |
| TTP -10 mV (ms)      | 3.19 ±0.39 (n24)           | 2.35 ±0.12 (n16)           | n.s.         | 0.088   |
| **Inactivation kinetics** |                          |                            |              |         |
| \(\tau\) -55 mV (ms) | 38.40 ±1.67 (n24)          | 40.07 ±2.56 (n16)          | n.s.         | 0.561   |
| \(\tau\) -10 mV (ms) | 17.20 ±0.48 (n24)          | 15.67 ±0.56 (n16)          | *            | 0.041   |
| **Deactivation kinetics** |                        |                            |              |         |
| \(\tau\) -100 mV (ms)| 1.97 ±0.06 (n12)           | 1.37 ±0.05 (n19)           | ***          | 0.000   |
| \(\tau\) -60 mV (ms) | 6.05 ±0.30 (n12)           | 5.45 ±0.52 (n19)           | n.s.         | 0.386   |
| **Inactivation Recovery** |                          |                            |              |         |
| % recovery at 0.25 s  | 33.14 ±0.96 (n13)          | 26.19 ±0.86 (n13)          | ***          | 0.000   |
| % recovery at 4 s     | 93.69 ±0.69 (n13)          | 93.42 ±0.58 (n13)          | n.s.         | 0.759   |
| \(T_{0.5}\) (ms)     | 921.49 ±22.45 (n13)        | 767.76 ±26.09 (n13)        | ***          | 0.000   |

Statistical comparisons were done using a one-way ANOVA combined with a Student-Newman-Keuls post hoc test with * P<0.05, ** P<0.01 and *** P< 0.001; n.s., not significant.
Table 5. Monovalent ion and calcium permeability parameters for snail \( \text{LCa}_v\text{-}12\text{a} \) or \( \text{LCa}_v\text{-}12\text{ba} \) channel or human \( \text{Ca}_v\text{.3.1} \) channel currents expressed in HEK-293T cells

| reversal potentials : | Li   | s.e.m. | n  | Na   | s.e.m. | n  | K    | s.e.m. | n  | Cs   | s.e.m. | n  |
|----------------------|------|--------|----|------|--------|----|------|--------|----|------|--------|----|
| Erev \( \text{LCav}_3\text{-}12\text{a} \) | 9.91 | 0.27   | 8  | 15.54| 0.31   | 6  | 22.19| 0.44   | 12 | 34.06| 1.02   | 9  |
| Erev \( \text{LCav}_3\text{-}12\text{b} \) | 11.99| 0.33   | 5  | 19.31| 0.27   | 5  | 28.74| 0.65   | 9  | 34.48| 1.10   | 10 |
| Erev \( \text{Cav}_3\text{.1} \)     | 20.62| 0.72   | 6  | 29.21| 1.84   | 7  | 38.47| 1.82   | 6  | 39.93| 1.58   | 6  |

| relative permeabilities : | \( P_{\text{Ca}}/P_{\text{Li}} \) | s.e.m. | n  | \( P_{\text{Ca}}/P_{\text{Na}} \) | s.e.m. | n  | \( P_{\text{Ca}}/P_{\text{K}} \) | s.e.m. | n  | \( P_{\text{Ca}}/P_{\text{Cs}} \) | s.e.m. | n  |
|--------------------------|----------------------------------|--------|----|----------------------------------|--------|----|----------------------------------|--------|----|----------------------------------|--------|----|
| \( P_{\text{Ca}}/P_{\text{Li}} \) \( \text{LCav}_3\text{-}12\text{a} \) | 22.74                           | 0.41   | 8  | 32.01                           | 0.71   | 6  | 50.25                           | 1.56   | 12 | 114.51                          | 8.85   | 9  |
| \( P_{\text{Ca}}/P_{\text{Na}} \) \( \text{LCav}_3\text{-}12\text{b} \) | 25.89                           | 0.59   | 5  | 41.32                           | 0.75   | 6  | 78.04                           | 2.81   | 9  | 113.84                          | 6.97   | 10 |
| \( P_{\text{Ca}}/P_{\text{K}} \) \( \text{Cav}_3\text{.1} \)     | 45.28                           | 2.11   | 6  | 89.56                           | 8.21   | 6  | 140.16                          | 12.02  | 5  | 154.65                          | 7.99   | 5  |

| slope conductance : | Li       | s.e.m. | n  | Na       | s.e.m. | n  | K       | s.e.m. | n  | Cs       | s.e.m. | n  |
|---------------------|----------|--------|----|----------|--------|----|----------|--------|----|----------|--------|----|
| outward \( g_x \) \( \text{LCav}_3\text{-}12\text{a} \) | 0.100    | 0.0057 | 8  | 0.079    | 0.0042 | 5  | 0.051    | 0.0037 | 12 | 0.027    | 0.0017 | 8  |
| outward \( g_x \) \( \text{LCav}_3\text{-}2\text{b} \)     | 0.075    | 0.0046 | 3  | 0.060    | 0.0047 | 8  | 0.042    | 0.0032 | 5  | 0.014    | 0.0008 | 11 |
| outward \( g_x \) \( \text{Cav}_3\text{.1} \)     | 0.026    | 0.0018 | 8  | 0.033    | 0.0046 | 7  | 0.014    | 0.0014 | 8  | 0.0033   | 0.0012 | 8  |

| anomalous mole fraction: | \( \text{LCav}_3\text{-}12\text{a} \) | \( \text{LCav}_3\text{-}12\text{b} \) | \( \text{hCav}_3\text{.1} \) |
|--------------------------|----------------------------------|----------------------------------|----------------------------------|
| % current \( \log [\text{Ca}]_{\text{ex}} \) | % current \( \log [\text{Ca}]_{\text{ex}} \) | % current \( \log [\text{Ca}]_{\text{ex}} \) |
| block | s.e.m. | n  | block | s.e.m. | n  | block | s.e.m. | n  |
| -9    | 5.19%  | 1.61% | 7    | 3.47%  | 1.60% | 6    | 0.00%  | 0.00% | 4  |
| -7    | 0.00%  | 0.00% | 7    | 0.21%  | 0.16% | 6    | 34.10% | 4.78% | 4  |
| -5    | 44.46% | 4.03% | 7    | 81.22% | 1.58% | 6    | 96.06% | 0.49% | 4  |
| -4    | 48.80% | 4.02% | 7    | 89.31% | 0.95% | 6    | 92.27% | 0.50% | 4  |
| -3.52 | 67.08% | 3.99% | 7    | 94.72% | 0.52% | 6    | 85.15% | 0.88% | 4  |
| -3    | 86.40% | 2.18% | 7    | 95.55% | 0.83% | 6    | 79.24% | 1.02% | 4  |
| -2.52 | 92.94% | 0.95% | 7    | 93.68% | 1.32% | 6    | 73.11% | 1.80% | 4  |
| -2    | 91.49% | 1.03% | 7    | 90.65% | 1.99% | 6    | 64.10% | 3.17% | 4  |

| Increase (fold) | s.e.m. | n  |
|----------------|--------|----|
| \( \text{LCav}_3\text{-}12\text{a} \) | 15.40  | 0.83 | 5  |
| \( \text{LCav}_3\text{-}12\text{b} \) | 2.53   | 0.10 | 7  |
| \( \text{hCav}_3\text{.1} \)     | 1.27   | 0.09 | 4  |
Table 6. Features of exons 11 and 12 of Cav3 channel homologs in sequenced metazoan genomes

| Phylum                  | Subphylum/Class/Order | Species            | Common Name                      | 11/12A Intron | Size 12A | Size 12B | #Cys 12A | #Cys 12B |
|------------------------|-----------------------|--------------------|----------------------------------|---------------|-----------|-----------|----------|----------|
| Vertebrates            |                       |                    |                                  |               |           |           |          |          |
| Phylum Chordata        | Subphylum Vertebrata  | Homo sapiens       | human                            | ✓             | 38        | 1         |          |          |
| Phylum Chordata        | Subphylum Vertebrata  | Xenopus tropicalis | Western Clawed Frog              | ✓             | 38        | 1         |          |          |
| Phylum Chordata        | Subphylum Vertebrata  | Oikopleura dioica  | marine tunace                     | ✓             | 39        | 1         |          |          |
| Phylum Chordata        | Subphylum Vertebrata  | Branchiostoma forsteri | lancelet or Amphioxus             | ✓             | 41        | 2         |          |          |
| Phylum Hemichordata    | Class Enteropneusta   | Saccoglossus auerevskii | acorn worm                      | ✓             | 39        | 3         |          |          |
| Phylum Echinoidea      | Class Echinoidea      | Strongylocentrotus purpuratus | California Purple Sea Urchin    | ✓             | 38        | 3         |          |          |
| Phylum Annelida        | Class Polychaeta      | Capitella teleta   | polychaete worm                  | ✓             | 50        | 5         |          |          |
| Phylum Mollusca         | Class Gastropoda      | Lymnaea stagnalis  | giant pond, freshwater snail     | ✓             | 39        | 50        | 3        | 5        |
| Phylum Mollusca         | Class Gastropoda      | Biotrichia glabrata | Biorhaphus Pianorb, freshwater snail | ✓             | 39        | 50        | 3        | 5        |
| Phylum Mollusca         | Class Gastropoda      | Aplysia californica | California Sea Hare              | ✓             | 46        | 51        | 3        | 5        |
| Phylum Mollusca         | Class Gastropoda      | Latia gigantea     | Och Limpet                       | ✓             | 38        | 51        | 3        | 5        |
| Phylum Arthropoda       | Class Insecta         | Tribolium castaneum | red flour beetle                 | ✓             | 38        | 55        | 3        | 5        |
| Phylum Arthropoda       | Class Insecta         | Drosophila melanogaster | fruit fly                       | ✓             | 38        | 55        | 3        | 5        |
| Phylum Arthropoda       | Class Insecta         | Drosophila virilis  | fruit fly                        | ✓             | 38        | 55        | 3        | 5        |
| Phylum Arthropoda       | Class Insecta         | Anopheles gambiae   | african malarial mosquito        | ✓             | 40        | 55        | 3        | 5        |
| Phylum Arthropoda       | Class Insecta         | Aedes aegypti      | yellow fever mosquito            | ✓             | 42        | 55        | 3        | 5        |
| Phylum Arthropoda       | Class Insecta         | Calanus finmarchicus | southern house mosquito         | ✓             | 41        | 55        | 3        | 5        |
| Phylum Arthropoda       | Class Insecta         | Daphnia pulex      | jewel worm                       | ✓             | 40        | 52        | 3        | 5        |
| Phylum Arthropoda       | Class Insecta         | Mesembryon pteroporum | common water flea               | ✓             | 39        | 52        | 3        | 5        |
| Phylum Arthropoda       | Class Echiacea        | Daphnia pulex      | common water flea               | ✓             | 39        | 52        | 3        | 5        |
| Phylum Arthropoda       | Class Mysidaea        | Strigea montium    | littoral centipede              | ✓             | 38        | 52        | 3        | 5        |
| Phylum Arthropoda       | Class Arachnida       | Loxosceles scutulatus | blacklegged Tick               | ✓             | 39        | 52        | 3        | 5        |
| Phylum Nematoda         | Order Rhabditida      | Trichosphaeralia   | parasitic roundworm             | ✓             | 43        | 48        | 3        | 5        |
| Phylum Nematoda         | Order Rhabditida      | Nematodera pacifica | free-living diplogastrid nematode | ✓             | 46        | 52        | 3        | 6        |
| Phylum Nematoda         | Order Rhabditida      | Heterorhabditia australis | entomophaseric nematode         | ✓             | 43        | 50        | 3        | 6        |
| Phylum Nematoda         | Order Rhabditida      | Strongyloides rotti | gastrointestinal parasitic nematode | ✓             | 43        | 52        | 3        | 5        |
| Phylum Nematoda         | Order Rhabditida      | Caenorhabditis elegans | free-living roundworm           | ✓             | 43        | 50        | 3        | 5        |
| Phylum Nematoda         | Order Rhabditida      | Caenorhabditis briggsi | free-living roundworm           | ✓             | 43        | 50        | 3        | 5        |
| Phylum Nematoda         | Order Rhabditida      | Meloidogyne javanica | Northern Root-Knot Nematode     | ✓             | 43        | 50        | 3        | 5        |
| Phylum Nematoda         | Order Rhabditida      | Ditylenchus rhabditis | free-living nematode            | ✓             | 43        | 55        | 3        | 5        |
| Phylum Nematoda         | Order Rhabditida      | Acrocerus suum     | pacific parasitic roundworm      | ✓             | 43        | 53        | 3        | 5        |
| Phylum Nematoda         | Order Rhabditida      | Acrocerus nematode  | Pacific parasitic roundworm      | ✓             | 43        | 53        | 3        | 5        |
| Phylum Nematoda         | Order Nematodina      | Loa loa            | African Eye Worm                 | ✓             | 42        | 3         |          |          |
| Phylum Nematoda         | Order Nematodina      | Wuchereria bancrofti | filarial nematode               | ✓             | 42        | 3         |          |          |
| Phylum Nematoda         | Order Nematodina      | Brugia malayi      | filarial nematode               | ✓             | 42        | 3         |          |          |
| Phylum Platyhelminthes  | Class Turbellaria     | Schistosoma mansoni | Freshwater Planarian             | ✓             | 38        | 3         |          |          |

| Non-vertebrate Bilateria |                       |                    |                                  |               |           |           |          |          |
| Phylum Cnidaria         | Class Anthozoa        | Nematostoma vectensis | Starlet Sea Anemone             | ✓             | 39        | 0         |          |          |
| Phylum Cnidaria         | Class Hydrozoa        | Acrozoa digitifera  | Table Coral                     | ✓             | 39        | 0         |          |          |
| Phylum Cnidaria         | Class Hydrozoa        | Hydromedusa tetraptera | Freshwater Hydrozoan polyp      | ✓             | 34        | 0         |          |          |
| Phylum Mollusca         | Class Polychaeta      | Trichoplax adhaerens | polychaete worm                 | ✓             | 40        | 2         |          |          |

| Basal metazoan          |                       |                    |                                  |               |           |           |          |          |
| Phylum Platyhelminthes  | Class Trichoplaxia    | Trichoplax adhaerens | polychaete worm                 | ✓             | 38        | 3         |          |          |
Figure 1. PCR amplified cDNA template coding for exons 12a and 12b in *Lymnaea stagnalis* cDNA.
Figure 2: Splicing of extracellular turret isoform (exon 12a) in the pore loop of Domain II in snail LCav3 channels generates monovalent ion permeant T-type channels.
Figure 3: Differing extracellular turrets in Domain II pore loop (exons 12a, 12b) do not alter major biophysical features outside of monovalent ion permeability.
Figure 4. Monovalent ion (Li+, Na+, K+, Cs+) permeability through snail LCav3 T-type channels is especially dramatic with exon 12a compared to exon 12b extracellular turrets or human Cav3.1 channels.
Figure 5. Snail LCav3 channels with exon 12a are highly permeable to sodium ions and weakly permeant to calcium ions, compared to LCav3 channels with exon 12b or human Cav3.1 channels.
Figure 6. Snail hearts contain two cation currents, the highly sodium permeant T-type channel (LCav3) and the calcium-selective, L-type channel (LCav1).
Figure 7. Separation of snail LCav3-12a (T-type) and LCav1 (L-type) calcium currents in the heart using holding potentials (HP) and drugs (isradipine, mibefradil, nickel) in whole-cell patch clamp recording.
Figure 8. Aligned amino acid sequences of exon 12A in metazoan Ca,3 channel homologues.
**Figure 9** Aligned amino acid sequences of exon 12B found in protostome Ca,3 channel homologues.
Figure 10. Phylogeny and proposed evolution for exon 12 which governs monovalent ion permeability in Cav3 T-type channels.
T-type Channels Become Highly Permeable to Sodium Ions Using an Alternate Extracellular Turret Region (S5-P) Outside the Selectivity Filter
Adriano Senatore, Wendy Guan, Adrienne N. Boone and J. David Spafford

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