AuPS/ASB Meeting - Newcastle 2007

Free communications: Ion channels

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Chair: Joe Lynch
Relative counter-ion permeation in anion-selective glycine receptor channels did not vary between two different anions, supporting an ion pair mechanism

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The ligand-gated glycine receptor ion channel (GlyR) mediates fast inhibitory synaptic transmission in the central nervous system. The wild-type (WT) homomeric α1 GlyR, with a minimum pore diameter of 5.3 Å, is predominantly permeant to Cl− anions, while a proline deletion (P-2′Δ) creates a larger (6.9 Å) minimum pore diameter and a smaller anion-cation permeability (e.g., Keramidas et al., 2004).

In a series of whole cell dilution potential experiments to investigate the mechanism of counter-ion permeation in the above two channels expressed in HEK 293 cells, we have already measured relative permeabilities of counter-ion cations to anions and shown that the hydrated counter-ion size determines the anion-cation permeability (Sugiharto et al., 2006). The effective hydrated diameters of Cl−, NO3−, Cs+, Na+ and Li+ ions of 5.0, 5.2, 4.9, 6.5 and 7.4 Å, respectively, were calculated de novo, or re-calculated, from published data (Robinson & Stokes, 1965). We used the GHK (Goldman-Hodgkin-Katz) equation with activity corrections and PCl/Pcation values were determined in LiCl, NaCl and CsCl solutions from reversal potential shifts (corrected for liquid junction potentials) with −50% and −25% external salt dilutions. We showed that in the smaller WT GlyR channels, PCl/PCs = 5.1 ± 0.5, PCl/PNa = 12.4 ± 0.4 and PCl/PLi = 32 ± 5. However, in the larger mutant P-2′Δ GlyR channels, PCl/PCs = 2.0 ± 0.1, PCl/PNa = 3.5 ± 0.2 and PCl/PLi = 6.6 ± 0.5. For both channels, as the hydrated counter-ion cation size increased so did PCl/Pcation. In addition, the smaller channel displayed the greater range of relative permeabilities (Sugiharto et al., 2006). In anion-cation permeability measurements in a neuronal chloride channel, Franciolini & Nonner (1994) had previously shown that their counter-ion/co-ion permeability ratio was approximately constant for different anions.

We used whole-cell patch-clamp measurements to explore the counter-ion/co-ion permeability ratio for different anions to see if the same relationship held true for the WT GlyR channel, and if it could elicit further information about the mechanism of counter-ion permeation in that channel. We chose the nitrate anion, NO3−, which is similar in size to Cl− but has a different bi-ionic permeability (Lee et al., 2003). The solutions contained either 145 mM NaCl or NaNO3, together with 10 mM HEPES titrated to a pH of 7.4 with NaOH. In addition, each internal solution contained 2 mM CaCl2 and 5 mM EGTA, and the internal NaNO3 solution had 5 mM of its NaNO3 replaced by 5 mM NaCl to maintain a well-defined Ag/AgCl electrode potential. The diluted external solutions had the diluted Na-salt replaced by an osmotically equal concentration of sucrose. We initially measured the reversal potential in bi-ionic NaNO3 : NaCl solutions and determined a relative PNO3/PNa of 1.6 ± 0.1. We then did dilution potential measurements in NaNO3 solutions, determined their reversals potentials and, fitting the data to the GHK equation, showed that PNO3/PNa = 12.8 ± 0.6. This was very similar to the PCl/PNa value of 12.4 ± 0.4 and indicates that this counter-ion/co-ion permeability is not primarily determined by the anion permeability. It is supportive of counter-ion permeation being via neutral anion-cation pairs with PCl/Pcation determined by the larger hydrated size of the cation-counter-ions. It can readily be explained if (1) the ions permeate as neutral ion pairs, (2) the rate at which the ion pairs are formed is proportional to the rate at which the anions permeate, and (3) the hydrated sizes of the anions are smaller than those of the cations.

External divalent ions decrease counter-ion permeation in anion-selective glycine receptor channels without changing the minimum pore diameter of the channel

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The ligand-gated glycine receptor ion channel (GlyR) mediates fast inhibitory synaptic transmission in the central nervous system. Dilution potential measurements of \( P_{Cl}/P_{Na} \) in wild-type (WT) homomeric \( \alpha_1 \) GlyR have been found to give larger values, implying a greater anion selectivity, in the presence of external divalent ions than in their absence. In order to investigate monovalent anion-cation selectivity and particularly the mechanism underlying counter-ion permeation in ion channels (Sugiharto et al., 2006), it would be ideal to make such measurements in the absence of divalent ions. However, measurements in the literature are variously made with different concentrations of divalent cations. It is therefore particularly important to know whether the presence of divalent ions affects anion-cation permeability values.

We have done a series of whole-cell patch-clamp dilution potential experiments on WT homomeric \( \alpha_1 \) GlyR channels expressed in HEK 293 cells in the presence and absence of different external concentrations of \( \text{Ca}^{2+} \) to carefully investigate the relationship between \([\text{Ca}^{2+}]_o\) and \( P_{Cl}/P_{Na} \). The solutions contained 145 mM NaCl, together with 10 mM HEPES titrated to a pH of 7.4 with NaOH. In addition, each internal solution contained 2 mM CaCl\(_2\) and 5 mM EGTA, and the external solution contained varying concentrations of \([\text{Ca}^{2+}]_o\). The diluted external solutions had the diluted Na-salt concentrations reduced to about half (75 mM) and one quarter (25 mM) and in each case were replaced by an osmotically equivalent concentration of sucrose. We have shown that dilution potential experiments performed in \([\text{Ca}^{2+}]_o\) ranging from 0 to 4 mM produced the following \( P_{Cl}/P_{Na} \) values: 12.4 ± 0.4 (32) [0 mM]; 13.4 ± 0.8 (6) [0.5 mM]; 15.5 ± 0.7 (6) [1 mM]; 19.3 ± 1.2 (6) [2.0]; 24.9 ± 1.8 (6) [4 mM], where the values are given as mean ± SEM, with the number of measurements in parentheses and [\text{Ca}^{2+}]_o in brackets. These results clearly show that the addition of at least 1.0 mM Ca\(^{2+}\) produced a significant increase in \( P_{Cl}/P_{Na} \) and at 4 mM Ca\(^{2+}\) the \( P_{Cl}/P_{Na} \) value was double the value obtained at zero calcium. It was suggested that this might be due to an effect of Ca\(^{2+}\) reducing the minimum pore diameter of the GlyR channel. To test this we measured the reversal potential in virtually symmetrical NaCl solutions (\( V_{rev} \)), the shift under bi-ionic NaCl:NaFormate conditions (\( \Delta V_{rev} \)) and the relative permeability to Cl\(^-\) of a large, but not impermeant, monovalent organic anion, formate, to give the results in the table below (the data are presented as the mean ± SEM, and number of measurements in parenthesis). All potentials were corrected for liquid junction potentials.

<table>
<thead>
<tr>
<th>( [\text{Ca}^{2+}]_o )</th>
<th>( V_{rev} ) (mV)</th>
<th>( \Delta V_{rev} ) (mV)</th>
<th>( P_{\text{Formate}}/P_{Cl} )</th>
<th>( V_{rev} ) (mV)</th>
<th>( \Delta V_{rev} ) (mV)</th>
<th>( P_{\text{Formate}}/P_{Cl} )</th>
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<tr>
<td>0 mM</td>
<td>NaCl:NaCl</td>
<td>NaCl:NaFormate</td>
<td>NaCl:NaCl</td>
<td>NaCl:NaFormate</td>
<td>NaCl:NaCl</td>
<td>NaCl:NaFormate</td>
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<tr>
<td>~1.6 ± 0.4</td>
<td>21.0 ± 0.7</td>
<td>0.38 ± 0.01 (7)</td>
<td>~2.4 ± 0.3</td>
<td>20.6 ± 0.8</td>
<td>0.38 ± 0.02 (7)</td>
<td></td>
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This clearly indicates that although formate has a much lower permeability than Cl\(^-\), 4 mM Ca\(^{2+}\) has no effect on the \( P_{\text{Formate}}/P_{Cl} \) and hence has not changed the minimum pore diameter of the channel. It hence seems likely that external Ca\(^{2+}\) ions are somehow directly reducing the permeation of the counter-ion Na\(^+\) maybe by making it energetically more difficult for Na\(^+\) ions to dissociate from an Na-Cl ion pair in the selectivity filter and more difficult for it to enter the external vestibule of the channel.

MA-stretch residues are critical for ion conduction of 5-HT$_{3A}$ receptors


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5-Hydroxytryptamine type 3 (5-HT$_3$) receptors are members of the Cys-loop ligand-gated ion channel receptor superfamily. Members of this family, that include nicotinic acetylcholine (nACh), glycine and GABA$_A$ receptors, convert the chemical messages conveyed by neurotransmitters into electrical impulses through the selective conduction of ions. Electron microscopic images of the Torpedo marmorata nACh receptor suggest that residues within a helical stretch (the MA-stretch) of the M3-M4 cytoplasmic loop line intracellular portals that are an obligate pathway for ion flux (Unwin, 2005). It has recently been demonstrated that three arginine residues (R432, R436, R440) within the human 5-HT$_{3A}$ MA-stretch are critical determinants of single channel conductance ($\gamma$) (Kelley et al., 2003). The collective replacement of these residues by their 5-HT$_{3B}$ counterparts (producing the 5-HT$_{3A}$QDA receptor) increased $\gamma$ 29-fold, with R436D exerting the greatest influence.

In the current study, the influence of all 5-HT$_{3A}$ receptor MA-stretch residues (positions 426 to 442) on $\gamma$ was investigated. A combination of alanine and arginine scanning and the substituted cysteine accessibility method (SCAM) was employed. Site-directed mutagenesis was used to introduce alanine, arginine and cysteine residues along the MA-stretch, one at a time. Mutations were introduced into a 5-HT$_{3A}$QDA receptor (Kelley et al., 2003) to allow detection of single channel events evoked by 5-HT in outside-out membrane patches. All mutant subunits produced functional receptors when expressed in tsA-201 cells, with the exception of the 5-HT$_{3A}$QDA(W442R) and 5-HT$_{3A}$QDA(W442C) subunits.

The introduction of alanine residues along the MA-stretch typically resulted in a decrease in $\gamma$ compared to 5-HT$_{3A}$QDA receptors. In particular, with the exception of the R426A mutation, removal of a charged residue resulted in a significant change in $\gamma$ ($p < 0.01$). The greatest change in $\gamma$ was a 2-fold decrease observed with the D436A mutation. Alanine mutant receptors were treated as controls for the remaining experiments.

The mutation of MA-stretch residues to the positively charged arginine residue typically resulted in a decrease in $\gamma$ compared to alanine controls. This decrease was significant with the introduction of arginine at positions 427 and 431 to 440 ($p < 0.05$, $n = 3-7$). The greatest reduction in $\gamma$ was observed with arginine present at the 436 and 440 positions. In contrast, $\gamma$ was not reduced when arginine occupied positions 427, 428 and 431. Unexpectedly, the L429R mutation significantly increased $\gamma$ ($p < 0.01$, $n = 12$).

Typically, the introduction of cysteine residues had little effect on receptor $\gamma$ compared to control alanine mutants. Of the cysteine mutant subunits analyzed, addition of the positively charged methanethiosulfonate (MTS) reagent, MTSEA (200 $\mu$M), to the electrode solution reduced receptor $\gamma$ compared to controls. The changes in $\gamma$ mimicked the effects observed with the introduction of the positively charged arginine residues at all but three positions, suggesting successful reaction of MTSEA with introduced cysteine residues. This decrease in $\gamma$ was significant at receptors containing the E434C to D436C and V438C to A440C mutations ($p < 0.001$, $n = 4-5$), the greatest change being seen with the modification of cysteine residues at positions 435, 436 and 440.

This work establishes that a substantial portion of the 5-HT$_{3A}$ MA-stretch influences ion conduction. In particular, residues at the 436 and 440 positions are the major critical determinants of $\gamma$.


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Ligand-gated ion channels respond to specific neurotransmitters by transiently opening an integral membrane ion-selective pore, allowing ions to move down their electrochemical gradient. A distinguishing feature of all members of the ligand-gated ion channel superfamily is the presence of a 13 amino acid disulfide loop (Cys-loop) in the extracellular ligand-binding domain. Structural data derived from the acetylcholine receptor place this loop at the interface between the ligand binding domain and the transmembrane pore-forming domain where it is ideally located to participate in coupling ligand binding to channel opening. We have introduced specific mutations into a conserved motif at the mid-point of the Cys-loop of the GABA<sub>A</sub> receptor subunits α<sub>1</sub>, β<sub>2</sub> and γ<sub>2S</sub> where the sequence reads aromatic, proline, aliphatic (ArPAl motif). Receptors carrying a mutation in the Cys-loop of one of their subunits were expressed in L929 cells and responses to both GABA and drugs were assessed using the whole-cell patch clamp technique. Drug potentiation and direct activation were significantly enhanced by mutations in this Cys-loop but these effects were subunit-dependent. Currents in response to agonists were larger when mutations were carried in the α and β subunits but not in the γ subunit. In contrast, potentiation of current responses by diazepam, etomidate and pentobarbital were all enhanced when mutations were carried in the α and γ subunits, but not the β subunit. Since the disruption of interactions mediated through the ArPAl motif enhances the mutant receptor’s response to both agonist and drugs we suggest that this motif in the Cys-loop of the wild-type receptor participates in interactions that create activation barriers to conformational changes during channel gating. The participation of subunits in channel gating is however, asymmetrical.
Functional characterisation of ten myotonia-associated mutations of the hClC-1 chloride channel
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Myotonia congenita (MC) is a rare muscle stiffness disease of humans, and some other animals, associated with mutations in the gene, CLCN1. When correctly expressed and trafficked, its protein product, the wild type (WT) skeletal muscle chloride channel, ClC-1, passes chloride currents that buffer the depolarising influence of t-tubular potassium accumulation on membrane potential. This prevents involuntary after-discharges following tetanic muscle contractions. At least 120 different human ClC-1 (hClC-1) mutations are now known, although details of less than 80 have so far been published (Colding-Jørgensen, 2005) and less than half of these have been analysed to determine the specific reason for their failure to function or to be expressed (Pusch, 2002; Grunnet et al., 2003; Simpson et al., 2004). We have begun to investigate the remainder by heterologous expression and whole-cell patch-clamping in cultured HEK293 cells. Plasmid hClC-1 cDNA constructs were prepared by site-directed mutagenesis to mimic the naturally-occurring mutants and we used these to transfect our HEK cells. We also co-expressed some severely truncated mutants with complimentary C-tail peptide fragments to obtain functional reconstitution and unambiguous evidence that expression of the mutant had been achieved. Typically, after 24-48 hours had elapsed, cells were tested for evidence of voltage-gated chloride currents and/or hClC-1 protein expression. Using these methods, ten previously uncharacterised, myotonia-associated, hClC-1 mutations (S70L, G190R, C271R, G276D, W303R, G305E, E717X, S728L, Q807X and fs872X) were studied. No chloride currents could be obtained from cells transfected with G276D, G305E, E717X or Q807X. Robust functional rescue of truncations E717X and Q807X could, however, be achieved when either was co-expressed with C-tail fragment, G721-L988, indicating strong expression, correct trafficking to the plasma membrane and a potential for future therapy of these and similar truncations caused by premature stop codons. Interestingly, the open probabilities of both the fast gate (P_{o}^{f}) and common gate (P_{o}^{c}) of W303R and fs872X were shifted by tens of mV in the depolarising direction, sufficient to keep these channels almost closed within the activation range of WT channels. Minimum values of P_{o}^{c} for fs872X and C271R were reduced by ∼70%, or more, at negative potentials and V_{1/2} for C271R was shifted in excess of +150mV. These characteristics are typical of hClC-1 mutants that have been associated with the dominant form of myotonia congenita as these mutant subunits have a dominant negative effect on WT subunits when paired in ClC-1 channel dimers. From one compound heterozygous patient (S70L/S728L), neither mutant was significantly different from WT. Some of these myotonia-associated genetic variations may prove to be benign hClC-1 polymorphisms, the actual cause of the myotonia lying, for example, in the SCN4A sodium channel. Unexpectedly, however, chloride currents in cells transfected with the G190R mutant (associated with dominant MC) were also similar to WT, despite G190 being central to the chloride-binding motif, GSGIPE, and almost totally conserved across the CLC family of proteins from prokaryotes to humans. It is apparent that an absence of chloride currents in the case of some missense mutants and truncations could explain their association with recessive MC and that the association of other mutants with dominant MC could be accounted for by their strikingly altered voltage dependence of gating. More evidence will be required, however, to reveal any solid linkage between several of the mutants and either form of MC.

The effect of mutation E232Q and clofibric acid derivatives on gating in the human skeletal muscle chloride ion channel, ClC-1

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The chloride ion channel, ClC-1, is predominantly found in skeletal muscles. A decreased chloride conductance due to mutations in the gene, CLCN1, induces hyperexcitability in the muscle and is the cause of the disease myotonia congenita. The ClC-1 protein is a homodimer with each monomer containing 18 α-helices (labeled A-R), which are variable in length, tilted in relation to the vertical transmembrane axis (Dutzler et al., 2002) and wrapped around a common centre. This orientation allows each monomer to form its own protopore with multiple chloride binding sites, its own selectivity filter and its own independent fast gate by bringing together appropriate amino acids at the ends of several different α-helices. Both fast gates are then simultaneously regulated by the slower, common gating, with unknown structural basis. When the X-ray crystal of a prokaryotic CLC became available, it was proposed that the side chain of E148 (equivalent to E232 in ClC-1) works as the fast gate (Dutzler et al., 2002; Dutzler et al., 2003), by swinging in and out of the pore and thereby controlling the chloride conductance. Studies on ClC-0 support this view (Dutzler et al., 2003; Traverso et al., 2003), but also show that fast gating might involve global conformational changes (Accardi & Pusch, 2003; Traverso et al., 2003). Dominant negative effects on common gating suggest intersubunit allosteroy and it is possible that an interaction pathway between fast gates could constitute this "common gate". We sought to investigate whether common gating would be affected by mutation E232Q of the putative fast gate. Neither of the two gating processes has been studied before in this mutant of ClC-1. Using site-directed mutagenesis the glutamate at position 232 was substituted by a glutamine and the mutated protein was then expressed in HEK293 cells. In ClC-0, the equivalent mutation (E166Q) has been shown to abolish fast gating. As expected, whole-cell patch-clamping of mutant E232Q revealed that in ClC-1 fast gating was also eliminated. Furthermore, by using a method of patch-clamp analysis that allows us to separate fast from common gating, we observed that common gating was simultaneously absent. Application of the divalent cation, Zn2+, which has been shown to stabilise a closed state of common gating and thereby block the channels (Duffield et al., 2005), was no longer effective at blocking chloride currents which confirmed the absence of common gating. In a further effort to elucidate the nature of common gating and its relation to the fast gate, we used additional known CLC channel inhibitors, 2-(4-chlorophenoxy) propionic acid (CPP) and trichlorophenoxy acetic acid (TCPAA), to test whether they could restore gating, as previously shown for the E166A mutant of ClC-0 (Traverso et al., 2003). We expected a block of E232Q currents that could be fitted by a single exponential (i.e., recovery of the fast gate), as seen in the E166A mutant (Traverso et al., 2003). However, E232Q currents subjected to either inhibitor revealed a two exponential block. To test if this second, slower, exponential corresponded to the common gate, mutant channels were then exposed to Zn2+. If, indeed, common gating had been recovered, we expected Zn2+ to bind to the channel and block its currents, but the results we obtained were both complex and inconclusive. We observed that large currents were 40-50% blocked, while smaller currents only showed a 10-15% block. Furthermore, block of currents was voltage independent. Therefore, the second, slow, exponential observed in the mutant in the presence of either inhibitor can not, at this stage, be definitely ascribed to the common gate. Further experiments are needed to explain the presence of the second exponential.

Novel modulation of Na\textsubscript{v} channels in dorsal root ganglion neurons by veratridine

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The expression and activity of voltage-gated Na\textsuperscript{+} channels (Na\textsubscript{v}s) is a major determinant of neuronal excitability. While the modulation of Na\textsubscript{v}s on the transcriptional level is relatively well studied, the mechanisms regulating Na\textsubscript{v} cell surface levels remain largely unknown. One mechanism for the regulation of the epithelial Na\textsuperscript{+} channels (ENaC) is by ubiquitination followed by endocytosis and proteasomal degradation. Internalization of ENaC is triggered by an elevation of intracellular [Na\textsuperscript{+}] which via a feedback mechanism leads to the activation of Nedd4 ubiquitin ligases and subsequent ubiquitination of ENaC (Dinudom et al., 1998). A rapid Na\textsuperscript{+}-induced Na\textsubscript{v} internalization has also been reported to regulate Na\textsubscript{v} cell surface levels in embryonic cortical neurons (Paillart et al., 1996), however, it is also not known whether this mechanism is relevant in other neuronal cell types. This activation-induced internalization of Na\textsubscript{v} channels is likely to reflect a protection mechanism by which neurons can respond to increased external stimuli by reducing their ability to generate action potentials. Such a mechanism may be of particular importance in dorsal root ganglion (DRG) neurons as significant alterations of the Na\textsuperscript{+} current density and Na\textsuperscript{+} subtype profile in the DRG contribute to the pathogenesis of pain states. In the current study, we investigated whether activation of Na\textsubscript{v}s and elevation of intracellular [Na\textsuperscript{+}] in DRG neurons lead to an alteration of the Na\textsuperscript{+} current density.

Treatment of cortical neurons with the neurotoxin veratridine activates Na\textsubscript{v} channels by inhibiting channel inactivation and inducing persistent opening of the channels. This in turn leads to an increase in intracellular Na\textsuperscript{+} that results in a reduction in the whole-cell Na\textsuperscript{+} current density. To determine if veratridine had a similar effect on DRG neurons, we incubated primary cultures of DRG neurons with veratridine at 37°C for different periods of time after which Na\textsuperscript{+} current density was measured using whole-cell patch clamping. The bath solution consisted of (in mM) NaCl (70), KCl (5), CaCl\textsubscript{2} (2), MgCl\textsubscript{2} (1.5), NMDG-Cl (50), Hepes (10), TEA-Cl (10) and CdCl\textsubscript{2} (10), whereas the pipette solution contained (in mM) CsF (130), CsCl (15), NaCl (5), Hepes (10) and EGTA (10). Veratridine reduced the Na\textsuperscript{+} current density with a half-life of \textasciitilde15 min and steady-state was reached after \textasciitilde45 min, at which time only approximately 20% of the original Na\textsuperscript{+} current density remained. Next we investigated whether this effect was due to Na\textsuperscript{+}. The experiments were repeated in bath solutions where Na\textsuperscript{+} was replaced with equimolar NMDG\textsuperscript{+}. Under these conditions, veratridine failed to reduce the Na\textsuperscript{+} current density. Furthermore, the veratridine-mediated down-regulation in Na\textsuperscript{+} current density was mimicked by elevating the Na\textsuperscript{+} concentration in the pipette solution from 5 to 70 mM. This resulted in a significant reduction in Na\textsuperscript{+} current density during whole-cell recording over a period of 20 min. To determine if the down-regulation of Na\textsuperscript{+} current density in DRG neurons by veratridine was due to alterations in Na\textsubscript{v} trafficking, DRGs were incubated with veratridine first for 3 min at 37°C to activate Na\textsubscript{v} and then at 4°C for 1 hour to inhibit trafficking. Under these conditions, veratridine had no effect on Na\textsubscript{v} current density in the neurons, suggesting that the Na\textsuperscript{+}-induced reduction in Na\textsuperscript{+} current density may involve trafficking of Na\textsubscript{v} channels.

Taken together, these data suggest that elevation of intracellular [Na\textsuperscript{+}] leads to a decrease in Na\textsuperscript{+} current density of DRG neurons, which may be due to increased Na\textsubscript{v} endocytosis as reported for Na\textsubscript{v}s in the embryonic brain and ENaC in the kidney. The cellular mechanisms behind this process remain to be elucidated and future studies will investigate the potential involvement of Nedd4 ubiquitin ligases.

The lolitrem family of fungal alkaloids originate in planta as a result of grass-endophyte symbiosis. These indole diterpene compounds are secondary metabolites that are produced when perennial ryegrass is infected with the endophytic fungus Neotyphodium lolii. The most abundant compound is lolitrem B which is the main compound responsible for a neurological condition called ‘ryegrass staggers’ which impairs motor function in grazing animals. We recently showed that motor function deficits induced by lolitrem B in mice are mediated by BK channels. It is not known where lolitrem B binds to the BK channel, nor which structural features of the inhibitor confer its high potency (IC50 = 4 nM). The aim of this study was to determine which components of the lolitrem structure are important for BK channel inhibition. We used a suite of eight lolitrem compounds, four of which were isolated from endophyte-infected ryegrass seed, from which the other four were obtained by chemical modification. Compounds with subtle structural differences were chosen, so that data on their ability to inhibit BK channels would provide insights into the key structural features required for inhibition. Using inside-out membrane patches from hSlo HEK cells expressing human BK channels, we examined the lolitrem concentration-response relationship and the conductance-voltage relationship. The presence of an isoprene unit or an acetate group conferred an increase in apparent affinity for BK channels, whereas stereoisomer conformation had less influence.