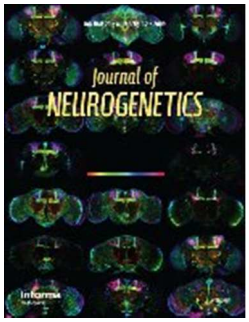

Ion channels as insecticide targets

Journal:	*Journal of Neurogenetics*
Manuscript ID	GNEG-2016-0041.R3
Manuscript Type:	Review
Date Submitted by the Author:	n/a
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Keywords:	ion channels, Drosophila, insecticides, insecticide resistance

Review

Ion channels as insecticide targets

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Keywords: insecticide resistance, ion channels, *Drosophila*

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Abstract

Ion channels remain the primary targets of most small molecule insecticides. This review examines how the subunit composition of heterologously expressed receptors determines their insecticide specific pharmacology and how the pharmacology of expressed receptors differs from those found in the insect nervous system. We find that the insecticide specific pharmacology of some receptors, like that containing subunits of the *Rdl* encoded GABA receptor, can be reconstituted with very few of the naturally occurring subunits expressed. In contrast, workers have struggled even to express functional insect nicotinic acetylcholine receptors, and work has therefore often relied upon expression of vertebrate receptor subunits in their place. We also examine the extent to which insecticide resistance associated mutations, such as those in the *para* encoded voltage gated sodium channel, can reveal details of insecticide binding sites and mode of action. In particular we examine whether mutations are present in the insecticide binding site and/or at sites that allosterically affect the drug preferred conformation of the receptor. We also discuss the ryanodine receptor as a target for the recently developed diamides. Finally, we examine the lethality of the genes encoding these receptor subunits and discuss how this might determine the degree of conservation of the resistance associated mutations found.

Introduction

The target sites and secondary effects of neuroactive insecticides have recently been reviewed (Casida & Durkin 2013). The current review therefore updates work on ion channels as targets (Bloomquist 1996) and examines the role of *Drosophila* genetics in the cloning of the genes that encode them. Five ion channels within the insect nervous system remain the primary targets for the development of small molecule insecticides. These are, first, the γ-aminobutyric acid (GABA) receptor containing subunits encoded by the *Resistance to dieldrin* gene or *Rdl* (the site of action of cyclodienes and fipronil), second, the glutamate gated chloride channel, third, the insect nicotinic acetylcholine receptor or nAChR (neonicotinoids and spinosyns), fourth, the voltage gated sodium channel encoded by homologs of the *Drosophila* gene *para* (DDT and pyrethroids) and fith the insect ryanodine receptor (ryanodine and the diamides). Here we will review the discovery and cloning of the first receptor subunits genes. We will examine the historical role that *Drosophila* genetics has played in the cloning of these important genes and compare their structure with those found in pest insects. We will then examine the extent to which the reconstitution of the full native pharmacology of these receptors is necessary in order to make valid inferences about insecticide specific aspects of their pharmacology. Finally, we will examine the extent to which resistance associated mutations in both *Drosophila* and pest insects have highlighted the binding sites and potential biophysical modes of action of the insecticides that target them. We will start our discussion with chloride ion channels gated either by GABA or glutamate.

The GABA gated chloride channel or GABA receptor

Lessons from Drosophila

γ-aminobutyric acid or GABA, is the major inhibitory neurotransmitter in both vertebrates and invertebrates and acts to open the pentameric transmembrane chloride channel within the GABA receptor (Bloomquist 1993). In vertebrates GABA

receptors are divided into $GABA_A$ and $GABA_B$ subtypes. The human brain $GABA_A$ receptor is composed of various combinations of α, β, or γ subunits, typically two α, two β and one γ subunit per pentamer, with each subunit type then conferring its own drug specific pharmacology on the final native receptor. However, until the cloning of insect GABA receptors from *Drosophila,* little was known about the subunit composition or pharmacology of the equivalent receptors in insects. Two different approaches were originally taken to the cloning of *Drosophila* GABA receptors. The first was positional cloning of the *Resistance to dieldrin* or *Rdl* locus which was isolated from field populations of *D. melanogaster* by screening with the cyclodiene insecticide dieldrin (Ffrench-Constant *et al.* 1993). Pharmacological work suggested that the cyclodiene insecticides (including dieldrin) interacted with insect GABA receptors and that picrotoxinin or 'PTX' had the same site of action and acts as a non-competitive antagonist (Bloomquist 1993). The second approach was to use conserved amino acid sequences within the ion channel lining of GABA gated chloride channels to design primers for use in the polymerase chain reaction or PCR (Henderson *et al.* 1994). Gratifyingly both approaches produced genes encoding GABA gated chloride ion channels. The *Rdl* locus encodes the Rdl GABA receptor subunit and the same *Rdl* containing sequence, termed LCCH1 (Henderson et al. 1994), was also recovered from the PCR based approach. The PCR based approach also recovered two other GABA receptor-like subunit encoding genes termed LCCH2 and LCCH3 (Henderson et al. 1994).

Heterologous expression and GABA receptor subunit composition in vivo

Vertebrate $GABA_A$ receptors are complex heteromultimers and co-expression of both α, β, or γ subunits together are necessary for heterologous expression in systems such as the *Xenopus* oocyte (Barnard *et al.* 1987). It is therefore highly surprising that transcripts from the *Rdl* gene could be readily expressed as Rdl homomultimers in both *Xenopus* oocytes and insect cells (ffrench-Constant et al. 1993, Shotkoski *et al.* 1994). These Rdl homomultimers not only form highly functional GABA gated chloride channels but currents from these channels can also be impeded by the application of either dieldrin (the insecticide used to recover the resistant mutant) or picrotoxinin (PTX) (ffrench-Constant et al. 1993). In this regard insect GABA receptors are very different from their vertebrate counterparts and should therefore probably be considered as corresponding to their own unique receptor subtypes (Buckingham *et al.* 2005).

Subsequent analysis of single ion channels in patch clamped *Drosophila* neurons confirmed that most GABA gated channels can be blocked by both dieldrin and PTX (Zhang *et al.* 1994). However measurements of the conductance of these native channels confirmed that they could not correspond to Rdl homomultimers and therefore that other unidentified subunits must also be present in the native receptor (Zhang *et al.* 1995). Moreover whilst the co-expression of LCCH3 with Rdl in heterologous systems adds bicuculine sensitivity to the pharmacology of the heterologously expressed receptor, again measurements of the single channel conductance of the Rdl-LCCH3 heteromultimer suggest that this is again not the native composition of most PTX sensitive GABA receptors in *Drosophila* (Zhang et al. 1995). In short the precise subunit composition of native GABA receptors containing Rdl subunits remains far from clear.

Finally, adding further complexity to likely insect GABA receptor subunit diversity, both alternative splicing and predicted RNA editing have been shown for the subunits encoded by *Rdl*. The *Rdl* transcript shows alternative splicing at two

locations where alternative exons of the same size are selected, termed 'a' or 'b' at position one and 'c' and 'd' at position two (ffrench-Constant & Rocheleau 1993). Similarly the *Rdl* transcript also shows several predicted sites for RNA editing and functional expression of transcripts altered by both alternative splicing and RNA editing shows that the combination of the two editing methods delivers an array of different subtypes with differing sensitivities to the agonist GABA (Jones *et al.* 2009). This combination of alternative splicing and RNA editing is a powerful method of obtaining numerous receptor subtypes from a single gene and is a theme we will be returning to as we examine other ion channel targets.

Resistance associated mutations in Rdl *and insecticide binding*

The Rdl containing GABA receptor is the site of action of cylcodiene insecticides like dieldrin, aldrin and endrin (Bloomquist 1993). Whilst resistance to cyclodienes has accounted for a staggering number of reported cases of insecticide resistance historically (ffrench-Constant 1994), as the cyclodienes have now been withdrawn, the Rdl containing receptor is now much more important as the target site for the phenylpyrazoles, such as the now widely used fipronils. To this end it was important to find the resistance associated mutation in *Rdl* both to examine its historical role in cyclodiene resistance and also its potential future role in conferring resistance to fipronil. Sequencing of several dieldrin resistant strains of *D. melanogaster* showed that a single alanine (alanine301 in *Drosophila*), within the predicted ion channel lining of the receptor (at the start of transmembrane segment M2, Figure 1a), could be replaced with either a serine or a glycine residue to make the *Drosophila* strains resistant to dieldrin. Replacement of this alanine within laboratory expressed Rdl homomultimers also confers dieldrin and PTX resistance on the associated GABA gated chloride fluxes. Importantly, a susceptible *Rdl* mini-gene also rescues resistance in transformed *D. melanogaster* (Stilwell *et al.* 1995) and correspondingly both A301S and A301G replacements (which are both semi-dominant) also confer resistance when expressed *in vivo* in transformed flies (Remnant *et al.* 2014).The observation that *replacements* of this alanine, either with a serine (addition of a hydroxyl group to the amino acid side chain) or a glycine (reduction of the side chain to a hydrogen group) can cause resistance, led to the hypothesis that it was the *presence* of this specific alanine at the base of the pore, close to the narrowest part of the ion channel (Figure 1b) that promotes drug *binding*. To test this hypothesis the detailed biophysics of the Rdl contraining receptor were examined and a model derived that suggests that dieldrin and PTX bind preferentially to the desensitized state of the insect GABA receptor. Further replacements of the key alanine residue not only directly alter the drug binding site but also allosterically destabilize the drug preferred (desensitized) state of the receptor (Zhang *et al*. 1994). This model is supported by the direct electrophysiological observation that mutants where the critical alanine is replaced with serine or glycine spend less time in the desensitized state (Zhang *et al*. 1994). Replacements of alanine301 therefore play a unique dual role in promoting insecticide resistance.

Further amino acid replacements associated with fipronil resistance

The critical importance of alanine301 has been confirmed by the recent finding of yet another replacement of the equivalent residue, in this case to asparagine (A301N, but called A2'N, where 2' refers to the relative location of the residue within the second membrane spanning domain) in the fipronil resistant planthopper *Laodelphax striatellus* (Nakao *et al.* 2011). Further amino acid replacements within Rdl, beyond

replacements of alanine301 alone, have also been associated with resistance to fipronil. In a strain of *D. simulans* selected for fipronil resistance in the laboratory, both the original A301G replacement and a second replacement T350M in the third membrane spanning domain (M3) were found (Le Goff *et al.* 2005). Functional expression of these two replacements together confirmed that T350M enhances the effect of A301G (Le Goff et al. 2005). Recent structure modelling studies have shown that both A301 and T350 map closely to the proposed cyclodiene/fipronil (NCA-1A) site within the channel pore (Figure 1c) (Casida & Durkin 2015, Remnant et al. 2014) suggesting that the mutations impact directly on binding to this site. Here we therefore speculate that T350M may further destabilize the fipronil preferred desensitized state of Rdl and therefore increase resistance over and above that conferred by A301G alone. However, to our knowledge, this hypothesis currently remains to be tested. It is also interesting to note that the same amino acid replacement T345M also occurs at an equivalent position in the Rdl receptor subunit of *Anopheles gambiae* (Taylor-Wells *et al.* 2015). Finally, it is worth pointing out that the existing mutations do not appear to affect the binding and activity of two new classes of GABA receptor blockers, the isoxazolines and meta-diamides, which modelling studies suggest interact with different residues within the channel pore (Casida & Durkin 2015) (Figure 1c). These compounds are currently being developed for control of animal health pests (e.g. ticks and fleas) but may also find subsequent applications in crop protection.

Fitness phenotypes and Rdl *gene duplication*

Finally before leaving the insect GABA receptor it is worth discussing the likely negative fitness phenotypes associated with the *Rdl* gene itself. One often overlooked *Rdl* phenotype is that resistant mutants (R) in which alanine301 is replaced are also temperature sensitive paralytics (ffrench-Constant *et al.* 1993). That is to say that when raised to elevated temperatures that mutant adult flies take longer to recover (fly away from the substrate) than their susceptible (S) wild type counterparts. This temperature sensitive paralysis may be associated with a negative fitness phenotype for this resistance gene in the field. It is therefore extremely interesting to note the recent documentation of duplicated alleles of *Rdl* in *D. melanogaster* where both a resistant (R) and a susceptible (S) copy of the *Rdl* are found to be physically linked in tandem on the *same allele* (Remnant *et al.* 2013). This effectively 'compound' heterozygote (R+S), which would be expected to encode both susceptible and resistant subunits from the same allele, may therefore be an effective way of preserving the production of susceptible subunits even when the other allele was resistant (R+S/R).

The glutamate-gated chloride channel

Cloning and functional expression

Glutamate-gated chloride channels (GluCls) are members of the Cys-loop ligand-gated ion channel family that mediate inhibitory synaptic transmission in the nervous system of invertebrates (Wolstenholme 2012). As they are exclusively found in invertebrates they represent excellent highly selective nematicidal, acaricidal and insecticidal targets and are the primary target of the macrocyclic lactones (avermectins and milbemycins). They may also play a secondary role in the insecticidal activity of fipronil (Zhao *et al.* 2004). In contrast to nematodes and mites, the genomes of most insects contain a single GluCl gene with the subunit they encode assumed to assemble into homo-pentameric receptors (Dermauw *et al.* 2012, Jones &

Sattelle 2007, Knipple & Soderlund 2010). The subunit composition of GluCls in non-insect arthropods, such as mites is unknown (Wolstenholme 2012).
The first insect GluCl gene to be characterised was from *Drosophila* which when expressed in *Xenopus* oocytes produced a homomeric chloride channel gated by both glutamate and ivermectin (Cully *et al.* 1996).

Resistance associated mutations within GluCl
The first target-site mutation in an insect GluCl was also first described in *Drosophila* in a strain with resistance to nodulisporic acid, a natural insecticidal product isolated from an endophytic fungus, and weak (3-fold) cross-resistance to ivermectin (Kane *et al.* 2000). A combination of genetics and sequencing was used to identify a single mutation, P299S in the *Drosophila* GluCl channel of this strain that when expressed in *Xenopus* oocytes was found to reduce channel sensitivity to both compounds (Kane et al. 2000). The avermectin abamectin is also an effective miticide and two studies have reported mutations associated with resistance in the GluCl of *T. urticae* (Dermauw et al. 2012, Kwon *et al.* 2010), which in contrast to insects has six genes encoding GluCl (Dermauw et al. 2012). In the first study a G323D mutation in TuGluCl-1 was associated with ~20-fold resistance to abamectin in *T. urticae* and found to correlate with resistance in individual F2 progenies obtained by backcrossing (Kwon et al. 2010). A second study identified the same mutation in *TuGluCl-1* in a *T. urticae* strain with ~2000-fold resistance to abamectin, however, a novel substitution was also identified in the same strain, at an identical position (G326E), but in a different GluCl gene (*Tu_GluCl3*), suggesting these mutations may have an additive or synergistic effect in conferring resistance (Dermauw et al. 2012). Genetic association studies with two diagnostic doses of abamectin was used to link both mutations with resistance to lower abamectin concentrations, and the G326E substitution in Tu_GluCl3 with resistance to higher abamectin concentrations (Dermauw et al. 2012). Together these studies suggest that these two subunits might be the primary target-sites of abamectin in *T. urticae* and it would be interesting to explore if they coassemble to form a heteropentameric GluCl or form individual homopentameric receptors.

A point mutation associated with abamectin resistance has also been described in *P. xylostella* (Wang *et al.* 2016). In this case a A309V substitution at the N-terminus of the third transmembrane helix (M3) was associated with resistance in a field population introgressed into a susceptible strain (Wang et al. 2016). Homology modelling suggested that the A309V substitution modifies the abamectin-binding by an allosteric mechanism rather than eliminating a key binding contact (Wang et al. 2016). The modelling does however infer a more direct role for the G323/326 mutations identified in *T. urticae* since this residue within the M3 helix does show direct contact with ivermectin in the open state GluCl model developed by Wang and co-workers (Wang et al. 2016) (Figure 1c).

Alternative splicing and RNA editing of GluCl
Finally, perhaps to overcome the constraints of being encoded by a single gene, insect GluCls expand their coding capacity by mRNA splicing and editing (Jones & Sattelle 2007, Knipple & Soderlund 2010). A study on *P. xylostella* observed three naturally occurring transcript variants of PxGluCl in both abamectin resistant and susceptible strains (Liu *et al.* 2014). One of these, described as a '36-bp deletion', represents a transcript with alternative exon 9c. Transcripts with this exon were observed at higher frequency in abamectin resistant strains than susceptible strains and *in vitro*

expression of this isoform revealed that GluCl channels with exon 9c are less sensitive to both abamectin and glutamate than transcripts containing exon 9a, the primary variant observed in *P. xylostella* (Kwon et al. 2010, Liu et al. 2014). The finding that alternative exon usage in insect GluCls can influence insecticide binding/sensitivity was further supported by recent work characterising the transcriptional patterns of the GluCl of *Anopheles gambiae* (Meyers *et al.* 2015). A total of four isoforms were identified in this species that differ in their splicing of exons 3, 8 and 10 and result in differences in the N-terminal extracellular domain and intracellular loop region. Functional expression of two different isoforms, performed to examine their influence on modulating responses to the natural ligand and insecticides, revealed specific isoforms that were sensitive (AgGluCl-a1) and insensitive (AgGluCl-b) to ivemectin. These results suggest that residues encoded by alternative exons 3, 8 and 10, at least in part, define sensitivity to ivermectin and also suggest resistance to this insecticide in *An. gambiae* could arise simply through altered regulation of AgGluCl to favor ivermectin insensitive transcripts (Meyers et al. 2015).

The nicotinic acetylcholine receptor

Subunit composition and structure of the nAChR

Nicotinic acetylcholine receptors (nAChRs) are cation permeable ion channels that mediate fast synaptic transmission in the central nervous system of both vertebrates and invertebrates, and at the neuromuscular junction of vertebrates. nAChRs are the primary target of two major insecticide classes, the neonicotinoids, which comprise seven major compounds with a market share of more than 25% of total global insecticide sales (Bass *et al.* 2015b), and the natural product-derived spinosyns (Sparks *et al.* 2001).

nAChRs are oligomers composed of five subunits arranged in combinations from a variety of different subunit subtypes. These subunits form multiple functional heteropentamers or homopentamers which have distinct functional and pharmacological profiles (Millar 2003). Like the ligand-gated chloride channels discussed in the previous section, nAChR subunits have a similar topology comprising an N-terminal extra cellular region containing the ligand binding site and four transmembrane segments (TM1 – TM4) that create the ion channel (Figure 2a,b). Acetylcholine (and neonicotinoids) bind at the interface of two subunits, each of which contribute 3 binding loops (A – C by an α subunit and D-F by an α or non-α neighbouring subunit) to the binding pocket (Corringer *et al.* 2000). The first insect nAChR subunit sequence was isolated from *Drosophila* using probes designed on sequences from electric rays (*Torpedo* spp.), a rich source of this receptor (Hermans-Borgmeyer *et al.* 1986). Later sequencing of the *Drosophila* genome revealed a relatively compact family of ten genes (Dα1- Dα 7 and Dβ1- Dβ3) encoding nAChR subunits (Adams *et al.* 2000), a number subsequently found to be representative of most other insects (Jones & Sattelle 2010). Despite the reduction in size of this gene family in insects when compared to vertebrates further research has revealed that the transcriptomic diversity generated by certain insect nAChR genes can be greatly expanded by posttranscriptional modifications such as alternative splicing and RNA editing (Grauso *et al.* 2002).

Functional expression of insect nAChR subunits and neonicotinoid binding

The subunit composition of human nAChR muscle-type receptors is well established ($\alpha1_2$, β, γ, δ, or $\alpha1_2$, β, ε, δ), and although neuronal subtypes are less well characterised various combinations of twelve different subunit subtypes (α2−α10 and β2−β4) have been defined (Millar 2003). In contrast, much less is known about the composition of insect nAChRs, to a large degree a reflection of significant difficulties encountered in expressing insect-only subunits in heterologous systems (Millar & Lansdell 2010). These issues have been somewhat circumvented by expressing insect nAChR subunits in combination with vertebrate subunits (such as vertebrate β2), or creating subunit chimeras containing ligand binding domains of insect subunits (Millar & Lansdell 2010). While these hybrid receptors are unlikely to fully reflect the native pharmacology of insect-only receptors they have proved useful to identify candidate neonicotinoid targets. For example Lansdell and Millar demonstrated that Dα1, Dα2 and Dα3 form a high-affinity imidacloprid binding site in *Drosophila* S2 cells when co-expressed with rat β2 subunits (Lansdell & Millar 2000). Significantly Dα3 (which showed highest affinity for imidacloprid) showed no specific binding of imidacloprid when co-expressed with alternative vertebrate non-α subunits such as rat neuronal β4, or muscle γ- or δ-subunits suggesting native *Drosophila* β-subunits also play an important role in defining the sensitivity of the nAChR to neonicotinoids.

Although the expression of hybrid receptors containing vertebrate β2 precludes the analysis of insect β receptors, mutagenesis of amino acid residues in important ligand binding loops of vertebrate β receptors to make them more 'insect-like' has been shown to result in significant shifts in sensitivity to imidacloprid, further predicting the importance of insect β subunits in neonicotinoid binding (Shimomura *et al.* 2001, Shimomura *et al.* 2006). To further circumvent issues with functional expression *in vitro*, researchers have resorted to alternative approaches, such as immunohistochemistry, to characterise the subunit composition of insect nAChRs and their potential involvement in neonicotinoid binding. An excellent example is the combination of co-immunoprecipitation studies using subunit selective antisera and radioligand binding to native nAChR preparations of the brown planthopper, *Nilaparvata lugens*. These approaches provided evidence that that the Nlα3, Nlα8 and Nlβ2 subunits of this species co-assemble in one complex to form a high affinity imidacloprid binding site while Nlα1, Nlα2 and Nlβ1 assemble to form a lower affinity site (Li *et al.* 2010). More recent work expressing certain *Drosophila* α subunits (Dα5, Dα6 and Dα7) in combination with the molecular chaperone RIC-3 has successfully achieved the generation of functional homomeric and heteromeric insect only nAChRs, providing greater optimism that the composition and sensitivity of insect nAChRs to neonicotinoids may yet be determined using heterologous systems (Lansdell *et al.* 2012, Watson *et al.* 2010).

The insect nAChR as a target for the spinosyns

In contrast to the neonicotinoids, the nAChR subtypes targeted by spinosyn insecticides are more clearly defined with this class of insecticides appearing to specifically target insect nAChR α6 subunits. Evidence for this, initially came from *Drosophila*, with a Dα6 knock-out mutant exhibiting >1000-fold resistance to spinosad (Perry *et al.* 2007). Subsequent studies on spinosad resistant insect pests have provided substantial additional evidence that α6 is the primary target of spinosyns (see below). Very recently the ease of genome engineering in *Drosophila* has been elegantly exploited in two studies to both confirm insect α6 nAChRs as the

target of spinosad and localise the binding site of this insecticide (Perry *et al.* 2015, Somers *et al.* 2015). In the first of these studies Perry et al. exploited two *Drosophila* mutants created by EMS mutagenesis, that are highly resistant to spinosad, to develop an *in vivo* rescue system (Perry et al. 2015). They then used this tool to demonstrate that while Dα1, Dα5 and Dα7 do not rescue the response to spinosad, four different isoforms of Dα6 and the α6 gene from three different pest insects are all able to rescue spinosad sensitivity. Insect α6 genes show a remarkable capacity to expand their transcriptional diversity through alternative splicing of two mutually exclusive exons (exon 3 and 8) and RNA A to I editing (Grauso et al. 2002). An important result of the study by Perry and co-workers was therefore the finding that expression of four Dα6 isoforms with different alternative exon 3 and exon 8 combinations resulted in significant difference in fly mortality to spinosad, despite the fact they were expressed in a uniform genetic background (Perry et al. 2015). This finding highlights the potential importance of alternative splicing in influencing the responsiveness of nAChRs to insecticides. In the second study, Somers et al. expressed two chimeric subunits, where the N-terminal half of Dα6 was fused to the C-terminal half of Dα7 (which exhibits >60% sequence similarity to Dα6) and vice versa, in a spinosad resistant, Dα6 null background to test their ability to rescue susceptibility to spinosad (Somers et al. 2015). Significantly only subunits containing the C-terminal region of Dα6 were able to respond to spinosad, suggesting, in contrast to neonicotinoid insecticides, that this region is critical for spinosad binding.

Resistance associated mutations and nAChR insecticide binding sites

The molecular characterisation of spinosad and neonicotinoid resistance has shed light on the binding, and selectivity of these insecticides and the nAChR subunits that are important in these interactions. In the case of neonicotinoids, resistance in several insect species was initially attributed to metabolic mechanisms, and, to date, modification of the receptor (i.e. target-site resistance) has only been described in a handful of insect species. Prior to the development of 'field-evolved' neonicotinoid resistance, work on *Drosophila* provided valuable information on the nAChR subunit subtypes that may be important in forming the binding site of this insecticide class. EMS mutagenesis followed by selection with the neonicotinoid nitenpyram was used to identify four highly resistant lines with mutations in Dα1 and Dβ2 that confer resistance to this neonicotinoid (Perry *et al.* 2008) and also other members of this class (Perry *et al.* 2012). This finding provided strong evidence that nAChRs containing either or both of these subunits form important sites of neonicotinoid action.

Target-site resistance to neonicotinoids was first described in an insect pest species in 2005 (Liu *et al.* 2005). Selection of a laboratory strain of the planthopper *N. lugens* with imidacloprid for 35 generations resulted in a strain with over 250-fold resistance compared to a susceptible reference. Radioligand binding experiments to whole body membrane preparations revealed a significantly lower level of [3H]imidacloprid-specific binding to preparations of the resistant strain and sequencing of *N. lugens* nAChR subunit genes identified a single point mutation at a conserved position (Y151S) in two nAChR subunits, Nlα1 and Nlα3. The causal effect of these mutations was examined by expression of hybrid nAChRs containing *N. lugens* α and rat β2 subunits, with the presence of Y151S associated with a substantial reduction in specific [3H]imidacloprid binding (Liu et al. 2005). The discovery of these mutations in the orthologs of *Drosophila* (Dα1 and Dα3), which have previously been shown to form a high-affinity binding site for imidacloprid (see above), provides

additional evidence of their importance in neonicotinoid binding. Finally, the identification of the same mutation at homologous residues in two distinct nAChR α subunits is intriguing and it is unclear if Nlα1 and Nlα3 subunits contribute to different populations of receptors, conferring resistance to each, or if they occur together in a heteromeric receptor. It should be noted that this mutation combination, although able to confer strong resistance to neonicotinoids, may also carry an unacceptable fitness penalty as these mutations have never been reported in resistant field populations of *N. lugens*.

In the Green Peach aphid *M. persicae*, significant resistance to neonicotinoids used as aphicides has also taken a surprisingly long time to evolve with control compromising levels of resistance only described in a clone of *M. persicae* collected in 2009, nearly twenty years after the introduction of imidacloprid (Bass *et al.* 2011). Binding assays using radiolabelled imidacloprid to native membrane preparations revealed that the very high affinity site present in aphids, and indeed other hemipterans, was completely lost in the resistant clone and the remaining lower affinity site was also altered compared to susceptible clones resulting in a significant overall reduction in binding affinity to the nAChR. Sequencing of the previously characterised nAChR subunit genes from resistant and susceptible *M. persicae* clones revealed a point mutation in the nAChR β1 subunit of the resistant clone resulting in an arginine to threonine substitution or R81T (Slater *et al.* 2012) (Figure 2). Remarkably the amino acid at this position had been previously identified as a key determinant of the selectivity of neonicotinoids for insects, over vertebrates (Shimomura et al. 2006). The high sensitivity of insect nAChRs to neonicotinoids is thought to result, in part, from interactions between the distinctive electronegative pharmacophore (nitro or cyano group) of these insecticides and conserved positively charged residues in loop D (such as R81) of β subunits (Shimomura et al. 2006, Tomizawa & Casida 2003, Tomizawa & Casida 2005). Indeed substituting the threonine residue in the chicken β2 subunit at this position with arginine or another basic residue greatly enhanced the affinity of heterologously expressed nAChRs (such as *Drosophila* Dα2/chicken β2 hybrids) for imidacloprid (Shimomura et al. 2006). The mutation identified in *M. persicae* therefore confers a 'vertebrate-like' quality to the β1 subunit of resistant aphids resulting in reduced sensitivity of the nAChR to neonicotinoids through the loss of direct electrostatic interactions of the electronegative pharmacophore with the basic arginine residue at this key position within loop D (Figure 2c). These results both provide further evidence of the importance of insect β1 subunits in forming the neonicotinoid binding site and provide additional validation of the current models of neonicotinoid binding and selectivity for insect nAChRs. Unlike Y151S in planthoppers, the R81T mutation in *M. persicae* does not appear to carry a strong fitness penalty and is seen at high frequency in resistant aphid populations from peach growing regions of Southern Europe (Slater et al. 2012).

More recent work has suggested that quantitative changes in nAChR subunits may represent an alternative form of target-site resistance to neonicotinoids with resistance to imidacloprid in a laboratory selected strain of the planthopper *N. lugens* associated with a reduction in both mRNA and protein levels of the nicotinic acetylcholine receptor (nAChR) subunit Nlα8 (Zhang *et al.* 2015). Functional validation of the observed down-regulation using RNA interference showed that knockdown of Nlα8 mRNA and protein levels causes a significant increase in imidacloprid resistance in *N. lugens* and demonstrated a decrease in Nlα8 expression is sufficient to confer resistance *in vivo*. As detailed above, previous work has

indicated that Nlα8 co-assembles with Nlβ2 to form a high affinity imidacloprid binding site. It is therefore significant that radioligand binding assay on native membrane preparations of the resistant strain revealed that the affinity of this binding site was reduced over 10-fold compared to an earlier (and hence less resistant) generation of the same strain (Zhang et al. 2015).

In the case of spinosad resistance, target-site resistance appears to be the primary route of resistance evolution, likely because, in contrast to other conserved insecticide receptors, insect α6 subunits are not essential for viability and so can tolerate a variety of mutations even if they result in loss of function. In this regard a wide range of genetic alterations have been described that result in truncated non-functional protein (Baxter *et al.* 2010, Hsu *et al.* 2012, Rinkevich *et al.* 2010). In most of these cases the mutations involved disrupt the normal pattern of alternative splicing of this gene. For example, an investigation of a spinosad resistant strain of Diamondback moth, *Plutella xylostella,* collected in Hawaii, identified a mutation within the ninth intron splice junction of *Pxα6* resulting in mis-splicing of transcripts and a predicted protein truncated between the third and fourth transmembrane domains (Baxter et al. 2010). More recently, spinosad resistance has been associated with two alternative point mutations that do not result in loss of function (Bao *et al.* 2014, Puinean *et al.* 2013, Silva *et al.* 2016, Somers et al. 2015). In the first case, the same non-synonymous point mutation resulting in a G275E substitution was identified in exon 9 of the α6 nAChR in three different pest insect species. An alternative amino acid replacement, P146S, was also recently associated with spinosad resistance in an EMS mutagenized line of *Drosophila*. As a result of recent advances in genome editing approaches the causality of this mutation and G275E were both demonstrated using the CRISPR/Cas9 system to edit the mutations into *Drosophila* and measure the effect of this on reduced sensitivity to spinosad (Somers et al. 2015, Zimmer *et al.* 2016). In contrast to mutations involving loss of the whole gene, specific amino acid replacements can also provide more specific information on the mode and site of insecticide binding. Homology modeling using the nematode glutamate-gated chloride channel structure predicts the G275E mutation to lie at the top of the third α-helical transmembrane domain of the nAChR α6 subunit, providing additional evidence that the transmembrane domain of α6 contains the spinosad binding site (Puinean et al., 2013). In contrast the P146S mutation is unlikely to reside in the spinosad binding site and its close proximity to the conserved Cys-loop indicates an alternative mode of action potentially by impairing the gating of the receptor (Somers et al. 2015).

The Para containing voltage gated sodium channel

Lessons from *Drosophila*

The screening of chemically induced *Drosophila* mutants for temperature sensitivity (Siddiqi & Benzer 1976, Suzuki 1970) has played an important role in isolating mutants of genes encoding important insecticide target sites. One such temperature sensitive paralytic mutant, termed *paralytic* (temperature sensitive) or *para*^{ts}, was isolated using a screen involving a shift of temperature from 22°C to 29°C upon which paralysis in *para*^{ts} flies is immediate but reversible (Suzuki *et al.* 1971). The structural gene encoding the voltage gated sodium channel was then cloned from the *para* locus via a chromosomal walk (Loughney *et al.* 1989). The polypeptide encoded by this locus will here be referred to as 'Para' and the gene itself as '*para*'. The *para* locus was shown to be both large and complex, spanning over 60 kb of genomic DNA and with a minimum of 26 predicted exons, encoding numerous transcripts via

alternative splicing (Loughney et al. 1989). Further studies in the Ganetzky laboratory showed that the *para* locus in fact has six different sites for alternative splicing and that at least 48 different splice forms could be generated (Thackeray & Ganetzky 1994). The variety of *para* splice forms found differed markedly between embryos and adults suggesting that the adult nervous system may contain a very different complement of sodium channels (Thackeray & Ganetzky 1994). This same pattern of alternative splicing is also conserved in *D. virilis* suggesting that this developmentally regulated variability does indeed have a functional role (Thackeray & Ganetzky 1995). To add to this bewildering array of alternative splice forms, the Ganetzky laboratory subsequently showed that *para* also contains three sites where RNA editing occurs (Hanrahan *et al.* 2000). In the RNA editing process, post-transcriptional editing of pre-mRNAs, through the action of dsRNA adenosine deaminases, modifies adenosine (A) residues to inosine (I), which then alters the coding potential of modified transcripts. These three RNA editing sites were again developmentally regulated and again conserved in *D. virilis*, supporting their functional role (Hanrahan et al. 2000). At each position a highly conserved region was found downstream of the RNA editing site in the following intron, and this region matched the exonic editing site (Hanrahan et al. 2000). This suggests a mechanism whereby the edited exon forms a base-paired secondary structure with the distant noncoding intronic sequence, in a similar manner to that seen in A-to-I RNA editing in vertebrate glutamate receptor subunits. More recently the functional significance of both editing and splice variants has been investigated via the heterologous expression of a range of different Para variants (here alternatively termed DmNa(V)) in *Xenopus* oocytes (Olson *et al.* 2008, Lin *et al.* 2009). These studies showed a wide range of voltage dependence in the gating properties of different DmNa(V) (Para) variants (Olson et al. 2008) and also differences in activation, inactivation and persistent current (Lin et al. 2009), proving that such variation does indeed have a meaningful biological role in generating insect sodium channel diversity. In summary, the *para* locus is large and complex and the variety of different mRNAs produced via a combination of both alternative splicing and RNA editing is large. Understanding the role of all of these different Para variants in the development and functioning of the insect nervous system therefore remains a major challenge.

Heterologous expression of Para

The *Drosophila para* sodium channel alpha subunit expresses alone when injected into *Xenopus* oocytes (Warmke *et al.* 1997). However, co-expression with the gene product of the *temperature-induced paralysis*, locus *E* (*tipE*), a putative accessory subunit, results in both elevated sodium currents and accelerated current decay (Warmke et al. 1997, Feng *et al.* 1995). The resulting Para/TipE sodium channels have both biophysical and pharmacological properties that are similar to native insect channels. However they differ from the pharmacology of vertebrate sodium channels, most notably in their sensitivity to pyrethroid insecticides. Thus the insect Para channel is over 100-fold more sensitive to pyrethroids than rat brain type IIA sodium channels (Warmke et al. 1997).

Resistance associated mutations and insecticide binding

The voltage gated sodium channel is the primary site of action of pyrethroid insecticides and in pest insects, such as the house fly, *Musca domestica*, and the German cockroach, *Blattella germanica*, pyrethroid resistance is associated with a locus originally termed *knockdown resistance* or *kdr*. The first evidence that the *kdr*

locus might indeed encode a *para*-like channel came from observations of tight genetic linkage between *kdr* and molecular probes for the *para*-like gene in both house flies (Knipple *et al.* 1994, Williamson *et al.* 1993) and German cockroaches (Dong & Scott 1994). Subsequently, sequencing of both the *kdr* and *super-kdr* alleles of the house fly showed that both mutants are associated with point mutations in domain II of the Para-like sodium channel (Williamson *et al.* 1996). Specifically, a leucine to phenylalanine replacement (L1014F) in the hydrophobic IIS6 transmembrane section is found in both *kdr* and *super-kdr* alleles and a second methionine to threonine replacement (M918T) within the intracellular IIS4-S5 loop is unique to *super-kdr* (Williamson et al. 1996). Similarly, an equivalent mutation (L1014F) was shown to be associated with *kdr*-like mutants of the German cockroach (Miyazaki *et al.* 1996, Dong 1997). These equivalent mutations in two different resistant insects suggested a binding site for pyrethroids at the intracellular mouth of the channel pore in a region thought to be important for channel inactivation (Williamson *et al*. 1996).

Interestingly, *para*-associated (*kdr*-like) pyrethroid resistance has not, to our knowledge, been isolated from field strains of *D. melanogaster*. Despite the fact that *para* is X-linked and therefore *kdr*-like resistance should easy to indentify as it should be clearly sex linked. The absence of *kdr*-like (DDT and pyrethroid) resistance in field strains of *Drosophila* may be associated with the presence of the dominant *DDT-R* gene on chromosome II which confers resistance to DDT but ironically not to pyrethroids. However we can still examine the effects of different point mutations on the insecticide sensitivity of the *Drosophila* Para channel as although the original *para*^{ts} mutants were isolated on the basis of their paralysis at high temperature, the same point mutations conferring temperature sensitivity also overlap with those conferring resistance to DDT and pyrethroids (Pittendrigh *et al.* 1997). Specifically, six out of thirteen *para* mutants tested showed 10-30 fold resistance to DDT and the associated mutations were clustered into a few sites across the channel polypeptide (Figure 3a). First, within the intracellular loop between S4 and S5 in homology domains I and III, second, within the pore region of homology domain III and third, within S6 of homology domain III (Pittendrigh et al. 1997).

Numerous different pest species have now been shown to possess *kdr*-like mutations in their *para* homologous sodium channel genes (Dong *et al.* 2014, Davies *et al.* 2008). However the functionality of many of these mutations has not been tested via heterologous expression and mutagenesis. Current work on the mode of action of pyrethroids on the insect voltage gated sodium channel is, however, now divided into two camps. Those that favour a single binding site for pyrethroids (O'Reilly *et al.* 2006, Davies et al. 2008), as implicated by the original *kdr* and *super-kdr* mutations, and those that favour two different binding sites on the sodium channel polypeptide (Figure 3). A single binding site for fenvalerate and DDT has been predicted using a homology model of the house fly Para-like voltage gated sodium channel (O'Reilly et al. 2006). In this model, the sodium channel was modelled in the open conformation with the pyrethroid binding site as a hydrophobic cavity formed by domain II of the S4-S5 linker and the IIS5 and IIS6 helices (Figure 3b) (O'Reilly et al. 2006). Importantly, this binding site is predicted to be accessible to the lipid bilayer where the lipid-soluble insecticides are predicted to accumulate. Insecticide binding, and the consequent formation of contacts across different channel elements, is predicted to stabilize the open state of the channel. This stabilisation of the open state of the channel is consistent with the prolonged sodium tail currents induced by both pyrethroids and DDT (Vais *et al.* 2000). Conversely, in the closed state, repositioning

of the domain II S4-S5 linker would disrupt this binding site and help explain why pyrethroids have a higher affinity for the open state of the sodium channel (O'Reilly et al. 2006). Importantly, some of the residues on the helices that form the putative binding contacts are not conserved between vertebrates and invertebrates, which may help explain the 100-fold difference in pyrethroid potency discussed above. Similarly additional binding contacts on the II S4-S5 linker (in the *super-kdr* region) may also explain the higher affinity binding of pyrethroids over DDT (O'Reilly et al. 2006) and why the potency of DDT is relatively unaffected by *super-kdr* (Usherwood *et al.* 2007). It should be noted that in this model the *kdr* site does not have a direct interaction with the pyrethroid molecule and mutations at this site are postulated to affect pyrethroid binding via an indirect (allosteric) impact that alters the conformation of the adjacent pyrethroid binding site (Davies et al. 2008).

Evidence for a two binding site model comes from studies of *kdr*-like resistance in the Para-like sodium channel of the mosquitoes *Aedes aegypti* and *Anopheles gambiae*. In these mosquitoes, two resistance associated mutations map to the same binding site predicted from the house fly model, termed pyrethroid receptor site or 'PyR1', located in domain interface II/III. However, three other resistance associated mutations apparently map to a *second* binding site on the receptor, termed pyrethroid receptor site 2 or 'PyR2', which is located in domain interface I/II (Figure 3c) (Du *et al.* 2013). Further molecular modelling of the interaction between the mosquito sodium channel and deltamethrin suggests a possible additional binding site between the linker helix IL45 (domain I S4-S5 linker) and transmembrane helices IS5 and IIS6, with deltamethrin dibromoethenyl and diphenylether residues pointing in intra- and extra-cellular directions respectively (Du *et al.* 2015). Finally, recent evidence suggests that pairs of mutations can work in concert to synergise pyrethroid resistance. Thus a unique mutation, N1575Y in the cytoplasmic loop linking domains III and IV of the *Anopheles gambiae* Para channel, confers no resistance on its own but synergises the effect of the previously identified L1014F replacement in IIS6 (Wang *et al.* 2015). Molecular modelling supports a mechanism whereby this second mutation (N1575Y) allosterically alters the PyR2 binding site via a small shift in IIS6 (Wang et al. 2015). It therefore appears that secondary mutations can accumulate within the resistant receptor causing *allosteric* changes in the mutant receptor which accentuate the effect of the primary mutation. It will therefore be interesting to see if such effects can be proven for mutations in other receptors such as the secondary mutations seen around A301S in the Rdl containing GABA receptor (see discussion above).

The calcium activated calcium channel or ryanodine receptor

Structure of the ryanodine receptor

Ryanodine receptors (RyRs) are homotetrameric calcium release channels and are the largest known ion channels. RyRs mediate the controlled release of Ca^{2+} from intracellular stores to initiate a wide variety of cellular processes, not least excitation-contraction coupling in muscle tissues (Lanner *et al.* 2010). Mammals possess three RyR isoforms (RyR1-3) each derived from a separate gene that are localized in different tissues. In contrast insect RyRs are encoded by a single gene with an ORF of >15,000 bp (Sattelle *et al.* 2008). RyRs are the target of ryanodine, a plant-derived alkaloid and natural insecticide, and the synthetic diamide insecticides, currently comprising the phthalic diamide flubendiamide and anthranilic diamides chlorantraniliprole and cyantraniliprole. The first insect RyR gene to be sequence characterised was from *Drosophila*, and was identified using a cDNA probe of the

rabbit skeletal muscle RyR, with the receptor showing less than 50% sequence identity to mammalian isoforms (Takeshima *et al.* 1994). Early attempts at functional expression of the *Drosophila* receptor in Chinese hamster ovary cells were only partially successful (Xu *et al.* 2000), however, subsequent expression of this receptor in insect Sf9 cells was much more robust, and sufficient to characterize the action of anthranilic diamides and to generate a stable cell line (Cordova *et al.* 2006). This system was further exploited to identify regions of the RyR that are critical for diamide binding (Tao *et al.* 2013). Replacement of a 46 amino acid segment of the *Drosophila* C-terminus with that from the plant parasitic nematode, *Meloidogyne incognita*, which is insensitive to anthranilic diamides, resulted in a functional RyR which lacked sensitivity to these compounds suggesting this region is critical to diamide sensitivity in insect ryanodine receptors (Tao et al. 2013). Beyond *Drosophila*, functional expression of the RyR of the model lepidopteran, *Bombyx mori*, has identified additional putative sites of diamide binding (Kato *et al.* 2009). HEK cells expressing modified *B. mori* RyRs with an N-terminal deletion (residues183–290) or where the 20% C-terminus (amino acids 4111–5084) was replaced with the rabbit RyR2 counterpart sequence, failed to produce Ca^{2+} mobilization in the presence of flubendiamide suggesting these two regions are essential for diamide sensitivity (Kato et al. 2009).

Insecticide resistance and insecticide binding sites

Use of radiolabelled diamides and ryanodine on native muscle membrane preparations of the house fly suggested that the major anthranilic and phthalic diamide insecticides bind to insect RyRs at different allosterically coupled sites (Isaacs *et al.* 2012). This finding was subsequently called into question by the result of work elucidating the molecular basis of diamide resistance in the diamondback moth, *P. xylostella* (Troczka *et al.* 2012). This species is notorious both as a pest of cruciferous crops and also for its ability to rapidly evolve resistance to insecticides used for control. Remarkably, reports of resistance in *P. xylostella* to diamides emerged just a few years after their introduction (Troczka et al. 2012, Wang & Wu 2012). Sequencing of the RyR from strains collected from the Philippines and Thailand with over 200-fold resistance to both chlorantraniliprole and flubendiamide revealed different non-synonymous mutations in each of the resistant strains that in both cases lead to the same glycine to glutamic acid substitution (G4946E) in the transmembrane spanning region of the RyR (Figure 4a) (Troczka et al. 2012). Subsequent radioligand binding studies with *P. xyostella* thoracic microsomal membrane preparations provided direct evidence of the role of the G4946E mutation on diamide specific binding with the binding of the tritiated flubendiamide derivative [^{3}H]PAD1 profoundly reduced in preparations of the resistant strain (Steinbach *et al.* 2015a). Finally, expression of the *P. xylostella* RyR in *Sf*9 cells demonstrated that the sensitivity of heterologously expressed receptors to flubendiamide and chlorantraniliprole is dramatically reduced in cells stably expressing the G4946E modified RyR, providing final unequivocal validation that the G4946E RyR mutation impairs diamide insecticide binding (Troczka *et al.* 2015).

Sequencing of a second chlorantraniliprole resistant field population of *P. xylostella*, this time from China, identified three novel amino acid substitutions, E1338D, Q4594L and I4790M at high frequency in addition to the G4946E mutation, which was observed at lower frequency (20%) (Guo *et al.* 2014). Crossing the resistant strain with a susceptible strain, and subsequent analysis of the frequency of these mutations in a subset of the F2 generation that had survived a diagnostic dose of

chlorantraniliprole, showed an increase in frequency of all three novel mutations compared to an untreated subset of the F2 population. Less convincing were the results of fluorescent probe based assays which largely failed to provide compelling evidence of the functional significance for the novel mutations due to the low specificity of the diamide-like probe employed.

More recent homology modelling of the *P. xylostella* RyR based on a cryo-EM structure of rabbit RyR1 has been used to unambiguously locate two of the four mutations (G4946E, I4790M) in close proximity to each other in the voltage sensor domain (Figure 4b,c) (Steinbach et al. 2015a). The observation that G4946E is located in trans-membrane helix S4 close to the S4–S5 linker domain thought to be involved in the modulation of the voltage sensor suggests that the wild-type residue in this position may act as a glycine hinge at the interface between the S4 helix and the S4-S5 linker. The mutation to glutamic acid observed in resistant strains is therefore likely to have a significant impact on movement of the S5 and S6 helices that control channel gating, and hence a direct knock on effect on binding of diamide insecticides to the receptor. Interestingly the I4790M occurs in helix S2 opposite and in close proximity to G4946E and together these two residues may define the diamide binding site on the receptor (Figure 4c) (Steinbach et al. 2015a, Troczka et al. 2015). Interestingly I4790 is conserved in Lepidoptera, with all other insects and arachnids exhibiting alternative amino acids at this position suggesting this residue may in part underlie the species-specific differences exhibited in flubendiamide and chlorantraniliprole binding at the RyR (see below).

Contrary to conclusions drawn from the work on the house fly (Isaacs et al. 2012), the observed cross-resistance of resistant strains of *P. xylostella* to anthranilic and phthalic diamides, and the demonstration that the G4946E mutation results in insensitivity to both classes suggests that they share a common binding site. Further comparison of diamide binding to thoracic muscle preparations of *Musca domestica* and *Heliothis virescens* using [^{3}H] ryanodine [^{3}H]chlorantraniliprole and [^{3}H]flubendiamide reconciled these two studies by providing evidence that there may be more than one type of diamide site in insects and species-specific differences in diamide binding at the receptor (Qi *et al.* 2014). In the housefly, *Musca domestica*, binding data suggest [^{3}H]Chlo, [^{3}H]Ry, and [^{3}H]Flu bind at three distinct sites, however, in the caterpillar pest, *Heliothis*, anthranilic and phthalic diamide insecticides displace each other suggesting they bind at the same or closely coupled sites (Qi et al. 2014).

Conclusions and future directions

This brief over-view of the five major ion channel targets for current small molecule insecticides has attempted to emphasize how our knowledge of these important receptors in pest insects has been enriched by studies in the genetic model *Drosophila*. Whilst studies of insecticide resistance associated mutations have identified not only the receptor subunits to which the insecticides bind but also their likely binding sites on those subunits. Importantly, however, studies of insecticide binding have also reinforced important lessons about receptor biophysics. Thus it is clear that many insecticides (such as the cyclodienes and pyrethroids) bind to specific biophysical forms of their receptors (the cyclodienes binding preferentially to the desensitized state of the insect GABA receptor for example). Critically therefore, insecticide resistance associated mutations can either be directly within the insecticide binding site and/or they can alter the stability of the drug preferred conformation of the receptor. This begins to explain how mutations within the drug binding site can be

synergized by distant mutations that act allosterically on the drug preferred conformation of the receptor. It will therefore be important in the future to examine the growing number of target site mutations in the light of this dual role hypothesis.

Acknowledgements

This article is a review for a special issue of the Journal of Neurogenetics to honour the lifetime achievements of Barry Ganetzky from the University of Wisconsin-Madison.

Figure legends:

Figure 1: a) GABA and glutamate gated chloride channels share similar structures with individual subunits comprising a large extracellular N-terminal domain (for ligand binding) and four transmembrane domains (M1-M4). The mature receptor (b) is a pentamer and gates a chloride-selective channel following GABA/Glu binding. The positions of mutations implicated in cyclodiene/fipronil resistance at the GABA-R (red circles) and avermectin resistance at the GluCl (yellow circles) are shown in a) and the corresponding regions of the mature receptors highlighted in b). Structural models of these binding sites (AVE, avermectin and FIP, fipronil) are shown in c) (adapted from Casida and Durkin (Casida & Durkin 2015).

Figure 2: a) nAChRs share a similar overall structure to the ligand-gated chloride channels with individual subunits containing four trans-membrane domains (M1-4) and a large extracellular N-terminal domain. The mature receptor (b) is a pentamer of either identical or non-identical subunits and gates a cation-selective channel following acetylcholine binding to the extracellular domain. Neonicotinoids such as imidacloprid activate the channel by binding to the same site. The only field-confirmed case of target site resistance to these compounds involves mutation (red circle) of arginine 81 to threonine (R81T) in *Myzus persicae* β1 subunit that causes repulsion of imidacloprid binding at the α/β subunit interface as shown in (c) (taken from Bass *et al*. (Bass *et al.* 2015a)).

Figure 3: The voltage gated sodium channel as a target for pyrethroids. a) Diagram of the voltage gated sodium channel showing the four repeat domains (I-IV), each comprising six membrane spanning helices (S1-S6). The positions of resistance mutations that identify pyrethroid binding sites PyR1 (red circles) and PyR2 (blue circles) are highlighted. The proposed PyR1 (b) and PyR2 (c) sites are shown in more detail, taken from O'Reilly and co-workers (O'Reilly et al. 2006) and Du and co-workers (Du et al. 2013). Positions of the mutations originally identified in pyrethroid and DDT resistant *para*^{ts} *Drosophila* strains are also shown (yellow circles).

Figure 4: Structure of the insect ryanodine receptor and the location of resistance associated mutations. a) Topology of the C-terminal domain of the insect RyR showing the six membrane spanning helices (TM1-6) with the positions of the two resistance mutations (I4790M & G4946E) that map to this region of the channel highlighted. b) Shows a full model of the insect RyR based on the recently reported structure of a rabbit RyR, with the membrane domain (boxed) further magnified in c) to show the close proximity of the resistance associated mutations (adapted from (Steinbach *et al.* 2015b)).

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Figure 1

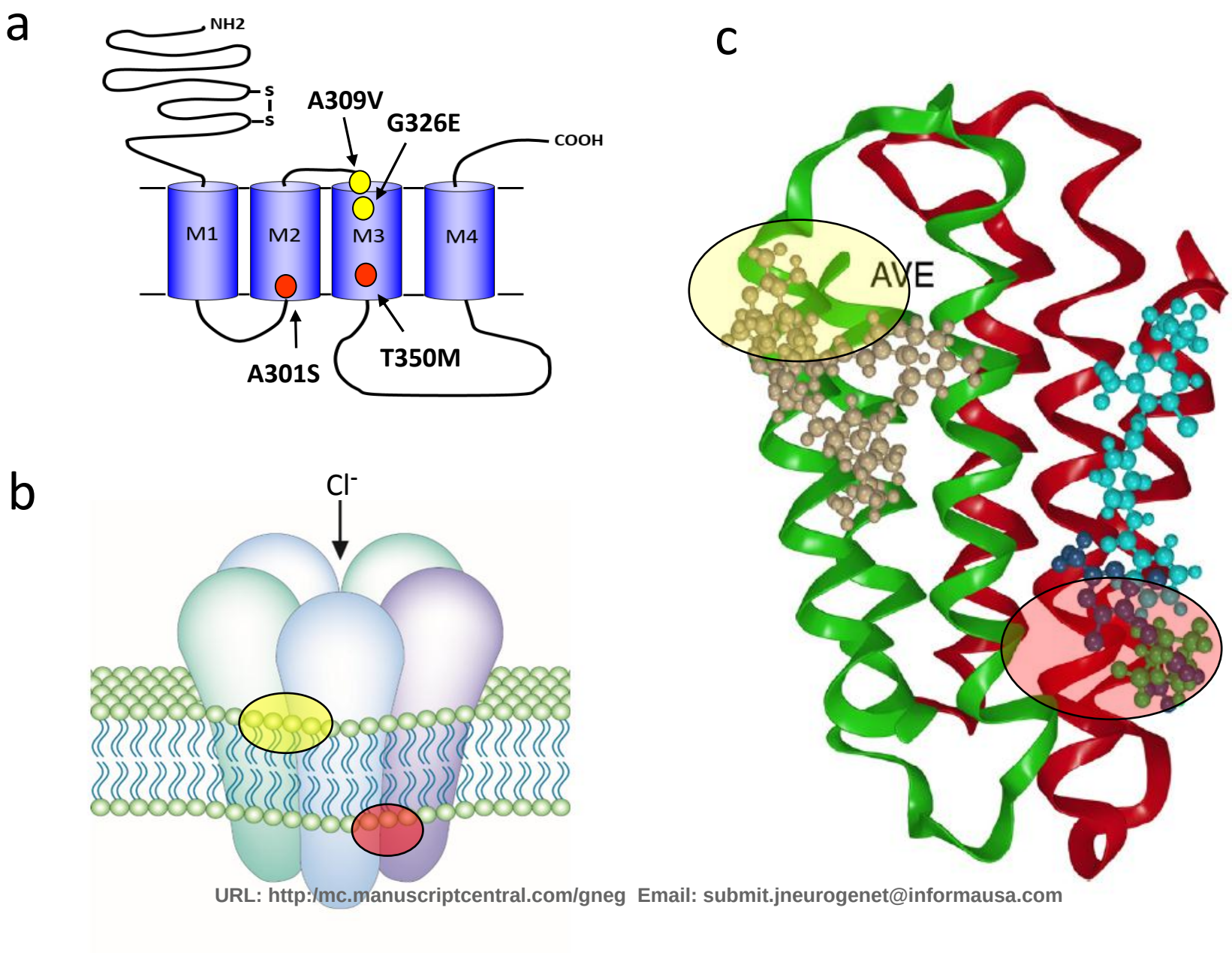

Figure 2

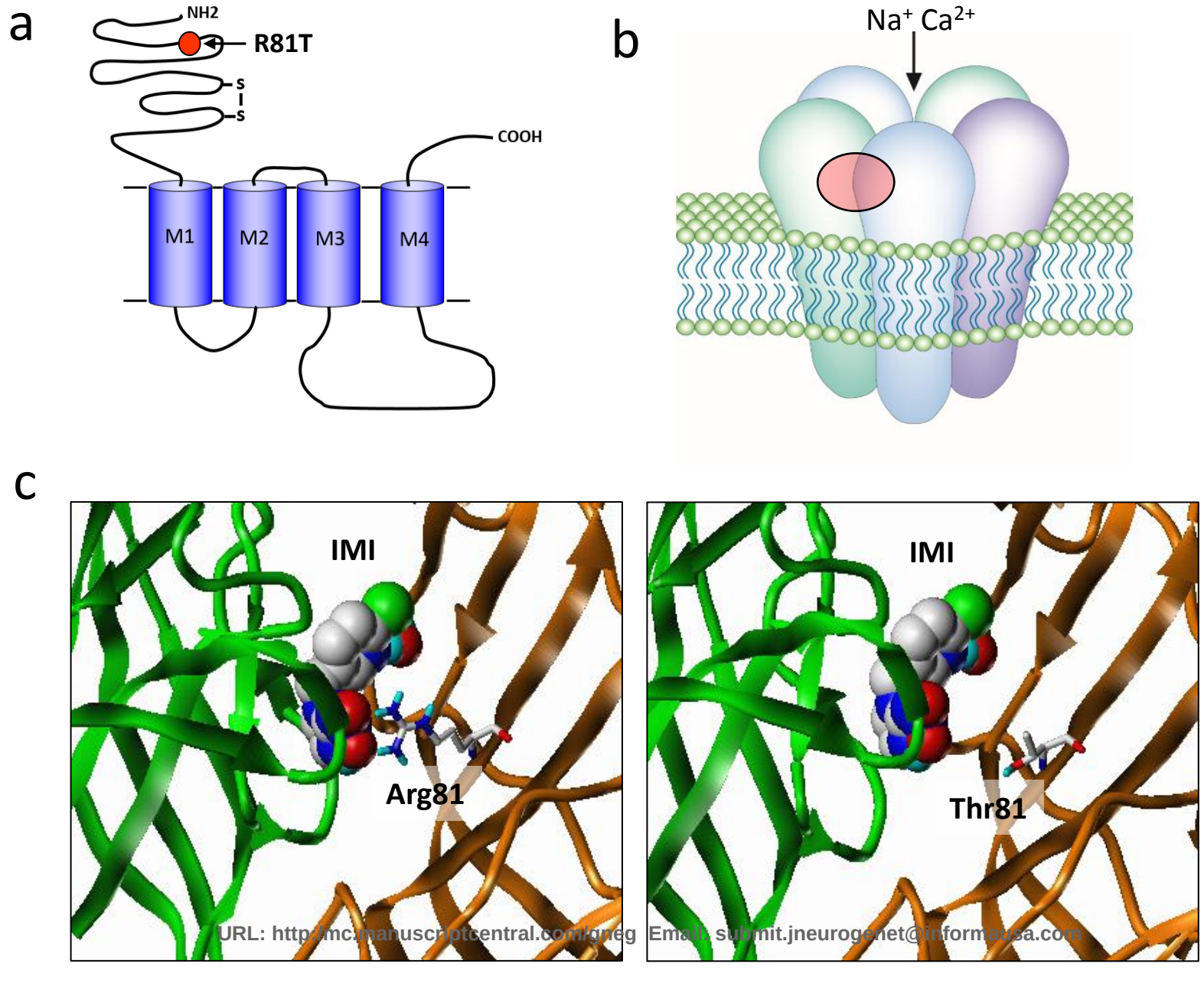

Figure 3

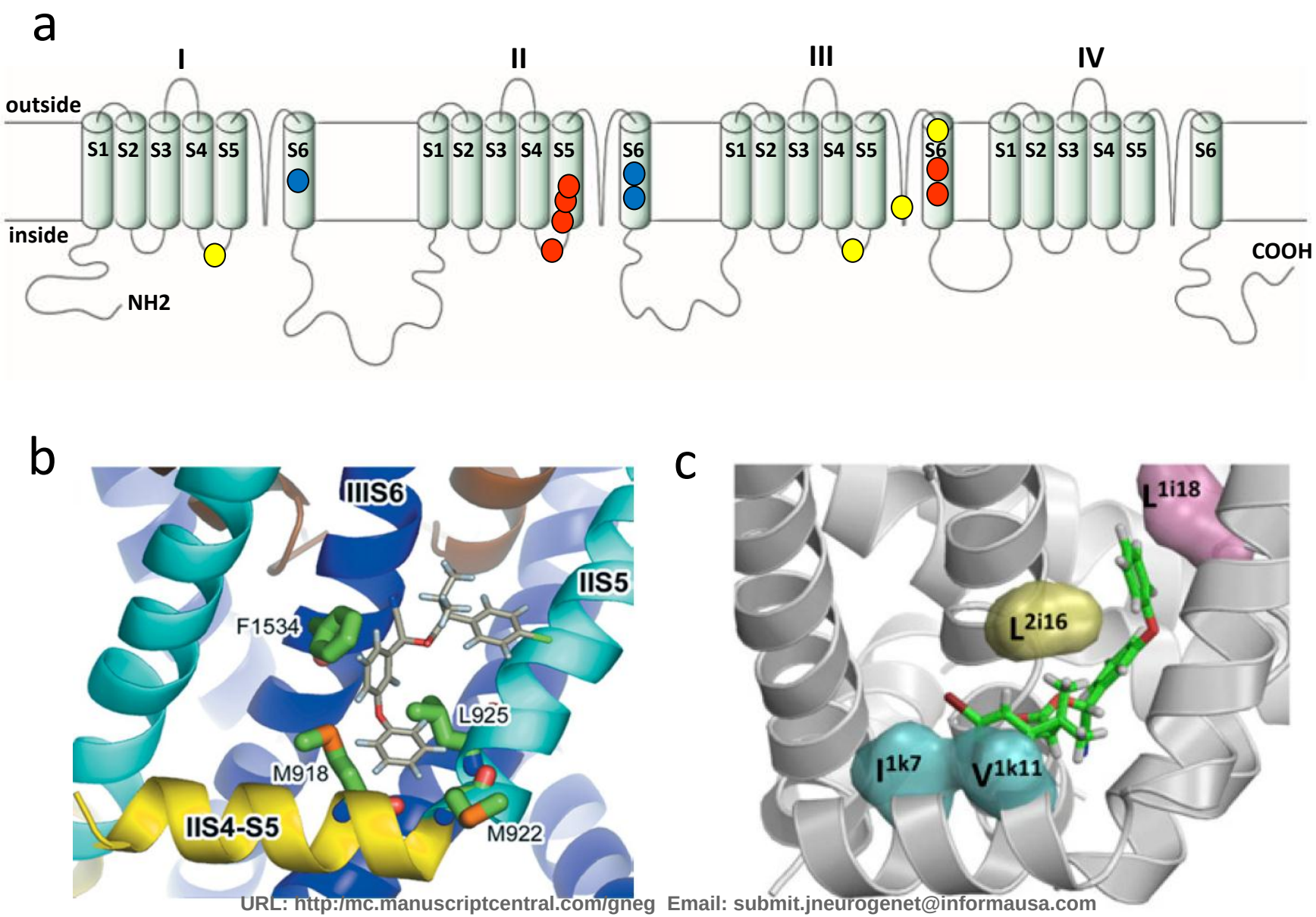
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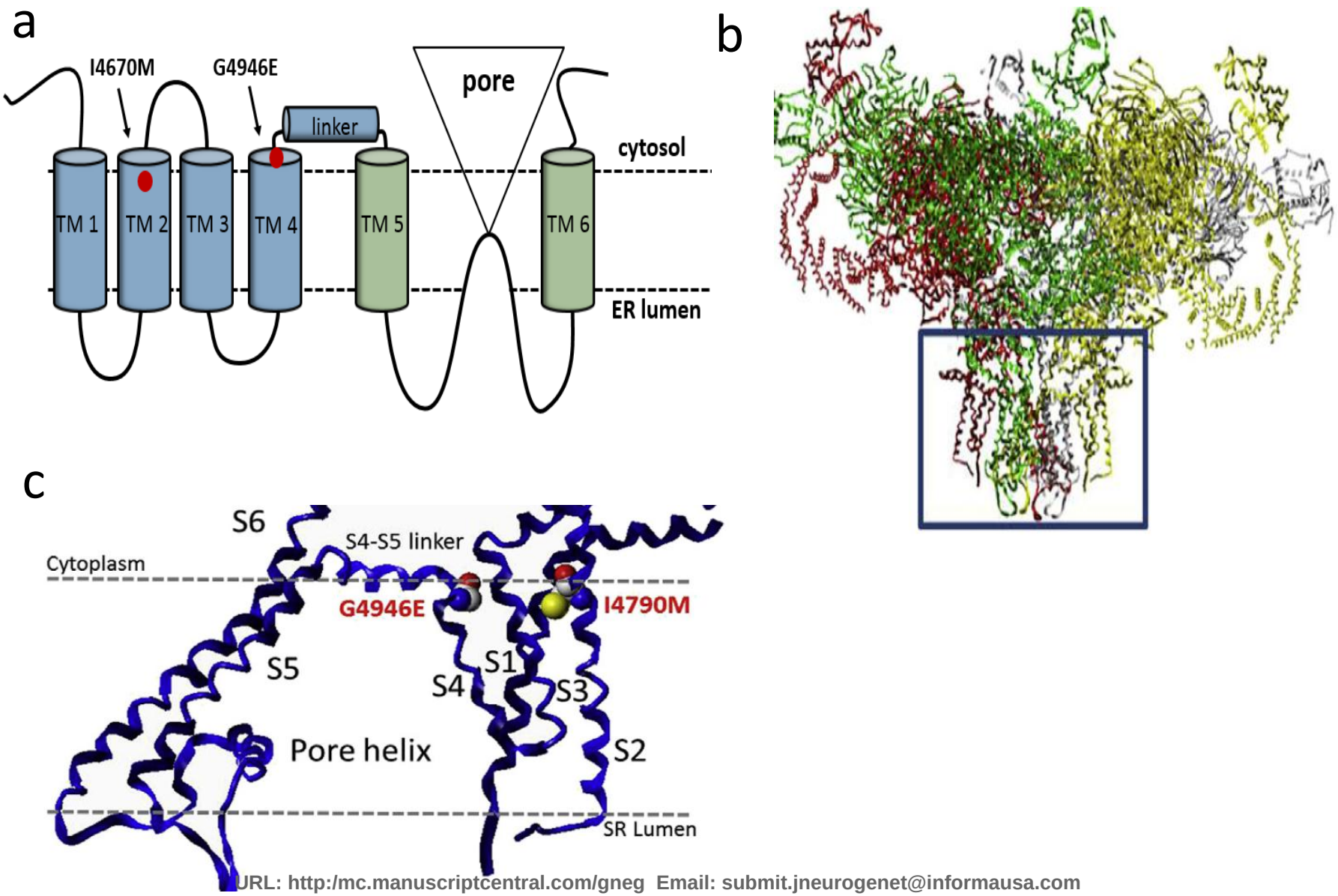
Figure 4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