

Ion channels as insecticide targets

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Review**Ion channels as insecticide targets****Richard H. ffrench-Constant¹, Martin S. Williamson², T. G. Emyr Davies² and Chris Bass¹**¹Biosciences, University of Exeter in Cornwall, Falmouth, UK²Rothamsted Research, Harpenden, Hertfordshire, UK**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 fifth 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

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3 receptors are divided into GABA_A and GABA_B subtypes. The human brain GABA_A
4 receptor is composed of various combinations of α , β , or γ subunits, typically two α ,
5 two β and one γ subunit per pentamer, with each subunit type then conferring its own
6 drug specific pharmacology on the final native receptor. However, until the cloning
7 of insect GABA receptors from *Drosophila*, little was known about the subunit
8 composition or pharmacology of the equivalent receptors in insects. Two different
9 approaches were originally taken to the cloning of *Drosophila* GABA receptors. The
10 first was positional cloning of the *Resistance to dieldrin* or *Rdl* locus which was
11 isolated from field populations of *D. melanogaster* by screening with the cyclodiene
12 insecticide dieldrin (Ffrench-Constant *et al.* 1993). Pharmacological work suggested
13 that the cyclodiene insecticides (including dieldrin) interacted with insect GABA
14 receptors and that picrotoxinin or 'PTX' had the same site of action and acts as a **non-**
15 **competitive antagonist** (Bloomquist 1993). The second approach was to use
16 conserved amino acid sequences within the ion channel lining of GABA gated
17 chloride channels to design primers for use in the polymerase chain reaction or PCR
18 (Henderson *et al.* 1994). Gratifyingly both approaches produced genes encoding
19 GABA gated chloride ion channels. The *Rdl* locus encodes the Rdl GABA receptor
20 subunit and the same *Rdl* containing sequence, termed LCCH1 (Henderson *et al.*
21 1994), was also recovered from the PCR based approach. The PCR based approach
22 also recovered two other GABA receptor-like subunit encoding genes termed LCCH2
23 and LCCH3 (Henderson *et al.* 1994).
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27 *Heterologous expression and GABA receptor subunit composition in vivo*

28 Vertebrate GABA_A receptors are complex heteromultimers and co-expression of both
29 α , β , or γ subunits together are necessary for **heterologous expression** in systems such
30 as the *Xenopus* oocyte (Barnard *et al.* 1987). It is therefore highly surprising that
31 transcripts from the *Rdl* gene could be readily expressed as Rdl homomultimers in
32 both *Xenopus* oocytes and insect cells (Ffrench-Constant *et al.* 1993, Shotkoski *et al.*
33 1994). These Rdl homomultimers not only form highly functional GABA gated
34 chloride channels but currents from these channels can also be impeded by the
35 application of either dieldrin (the insecticide used to recover the resistant mutant) or
36 picrotoxinin (PTX) (Ffrench-Constant *et al.* 1993). **In this regard** insect GABA
37 receptors are very different from their vertebrate counterparts and should therefore
38 probably be considered as corresponding to their own unique receptor subtypes
39 (Buckingham *et al.* 2005).
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42 Subsequent analysis of single ion channels in patch clamped *Drosophila*
43 neurons confirmed that most GABA gated channels can be blocked by both dieldrin
44 and PTX (Zhang *et al.* 1994). However measurements of the conductance of these
45 native channels confirmed that they could not correspond to Rdl homomultimers and
46 therefore that other unidentified subunits must also be present in the native receptor
47 (Zhang *et al.* 1995). Moreover whilst the co-expression of LCCH3 with Rdl in
48 heterologous systems adds bicuculine sensitivity to the pharmacology of the
49 heterologously expressed receptor, again measurements of the single channel
50 conductance of the Rdl-LCCH3 heteromultimer suggest that this is again not the
51 native composition of most PTX sensitive GABA receptors in *Drosophila* (Zhang *et*
52 *al.* 1995). In short the precise subunit composition of native GABA receptors
53 containing Rdl subunits remains far from clear.
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55 Finally, adding further complexity to likely insect GABA receptor subunit
56 diversity, both alternative splicing and predicted RNA editing have been shown for
57 the subunits encoded by *Rdl*. The *Rdl* transcript shows alternative splicing at two
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3 locations where alternative exons of the same size are selected, termed 'a' or 'b' at
4 position one and 'c' and 'd' at position two (ffrench-Constant & Rocheleau 1993).
5 Similarly the *Rdl* transcript also shows several predicted sites for RNA editing and
6 functional expression of transcripts altered by both alternative splicing and RNA
7 editing shows that the combination of the two editing methods delivers an array of
8 different subtypes with differing sensitivities to the agonist GABA (Jones *et al.* 2009).
9 This combination of alternative splicing and RNA editing is a powerful method of
10 obtaining numerous receptor subtypes from a single gene and is a theme we will be
11 returning to as we examine other ion channel targets.
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13 14 *Resistance associated mutations in Rdl and insecticide binding*

15 The *Rdl* containing GABA receptor is the site of action of cyclodiene insecticides like
16 dieldrin, aldrin and endrin (Bloomquist 1993). Whilst resistance to cyclodienes has
17 accounted for a staggering number of reported cases of insecticide resistance
18 historically (ffrench-Constant 1994), as the cyclodienes have now been withdrawn,
19 the *Rdl* containing receptor is now much more important as the target site for the
20 phenylpyrazoles, such as the now widely used fipronils. To this end it was important
21 to find the resistance associated mutation in *Rdl* both to examine its historical role in
22 cyclodiene resistance and also its potential future role in conferring resistance to
23 fipronil. Sequencing of several dieldrin resistant strains of *D. melanogaster* showed
24 that a single alanine (alanine301 in *Drosophila*), within the predicted ion channel
25 lining of the receptor (at the start of transmembrane segment M2, Figure 1a), could be
26 replaced with either a serine or a glycine residue to make the *Drosophila* strains
27 resistant to dieldrin. Replacement of this alanine within laboratory expressed *Rdl*
28 homomultimers also confers dieldrin and PTX resistance on the associated GABA
29 gated chloride fluxes. Importantly, a susceptible *Rdl* mini-gene also rescues
30 resistance in transformed *D. melanogaster* (Stilwell *et al.* 1995) and correspondingly
31 both A301S and A301G replacements (which are both semi-dominant) also confer
32 resistance when expressed *in vivo* in transformed flies (Remnant *et al.* 2014). The
33 observation that *replacements* of this alanine, either with a serine (addition of a
34 hydroxyl group to the amino acid side chain) or a glycine (reduction of the side chain
35 to a hydrogen group) can cause resistance, led to the hypothesis that it was the
36 *presence* of this specific alanine at the base of the pore, close to the narrowest part of
37 the ion channel (Figure 1b) that promotes drug *binding*. To test this hypothesis the
38 detailed biophysics of the *Rdl* containing receptor were examined and a model
39 derived that suggests that dieldrin and PTX bind preferentially to the desensitized
40 state of the insect GABA receptor. Further replacements of the key alanine residue not
41 only directly alter the drug binding site but also allosterically destabilize the drug
42 preferred (desensitized) state of the receptor (Zhang *et al.* 1994). This model is
43 supported by the direct electrophysiological observation that mutants where the
44 critical alanine is replaced with serine or glycine spend less time in the desensitized
45 state (Zhang *et al.* 1994). Replacements of alanine301 therefore play a unique dual
46 role in promoting insecticide resistance.
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51 52 *Further amino acid replacements associated with fipronil resistance*

53 The critical importance of alanine301 has been confirmed by the recent finding of yet
54 another replacement of the equivalent residue, in this case to asparagine (A301N, but
55 called A2^N, where 2^o refers to the relative location of the residue within the second
56 membrane spanning domain) in the fipronil resistant planthopper *Laodelphax*
57 *striatellus* (Nakao *et al.* 2011). Further amino acid replacements within *Rdl*, beyond
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3 replacements of alanine301 alone, have also been associated with resistance to
4 fipronil. In a strain of *D. simulans* selected for fipronil resistance in the laboratory,
5 both the original A301G replacement and a second replacement T350M in the third
6 membrane spanning domain (M3) were found (Le Goff *et al.* 2005). Functional
7 expression of these two replacements together confirmed that T350M enhances the
8 effect of A301G (Le Goff *et al.* 2005). Recent structure modelling studies have shown
9 that both A301 and T350 map closely to the proposed cyclodiene/fipronil (NCA-1A)
10 site within the channel pore (Figure 1c) (Casida & Durkin 2015, Remnant *et al.* 2014)
11 suggesting that the mutations impact directly on binding to this site. Here we therefore
12 speculate that T350M may further destabilize the fipronil preferred desensitized state
13 of Rdl and therefore increase resistance over and above that conferred by A301G
14 alone. However, to our knowledge, this hypothesis currently remains to be tested. It
15 is also interesting to note that the same amino acid replacement T345M also occurs at
16 an equivalent position in the Rdl receptor subunit of *Anopheles gambiae* (Taylor-
17 Wells *et al.* 2015). Finally, it is worth pointing out that the existing mutations do not
18 appear to affect the binding and activity of two new classes of GABA receptor
19 blockers, the isoxazolines and meta-diamides, which modelling studies suggest
20 interact with different residues within the channel pore (Casida & Durkin 2015)
21 (Figure 1c). These compounds are currently being developed for control of animal
22 health pests (e.g. ticks and fleas) but may also find subsequent applications in crop
23 protection.
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27 *Fitness phenotypes and Rdl gene duplication*

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

47 *Cloning and functional expression*

48 Glutamate-gated chloride channels (GluCl) are members of the Cys-loop ligand-
49 gated ion channel family that mediate inhibitory synaptic transmission in the nervous
50 system of invertebrates (Wolstenholme 2012). As they are exclusively found in
51 invertebrates they represent excellent highly selective nematicidal, acaricidal and
52 insecticidal targets and are the primary target of the macrocyclic lactones
53 (avermectins and milbemycins). They may also play a secondary role in the
54 insecticidal activity of fipronil (Zhao *et al.* 2004). In contrast to nematodes and mites,
55 the genomes of most insects contain a single GluCl gene with the subunit they encode
56 assumed to assemble into homo-pentameric receptors (Dermauw *et al.* 2012, Jones &
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3 Sattelle 2007, Knipple & Soderlund 2010). The subunit composition of GluCl_s in
4 non-insect arthropods, such as mites is unknown (Wolstenholme 2012).
5 The first insect GluCl gene to be characterised was from *Drosophila* which when
6 expressed in *Xenopus* oocytes produced a homomeric chloride channel gated by both
7 glutamate and ivermectin (Cully *et al.* 1996).
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9 10 *Resistance associated mutations within GluCl*

11 The first target-site mutation in an insect GluCl was also first described in *Drosophila*
12 in a strain with resistance to nodulisporic acid, a natural insecticidal product isolated
13 from an endophytic fungus, and weak (3-fold) cross-resistance to ivermectin (Kane *et*
14 *al.* 2000). A combination of genetics and sequencing was used to identify a single
15 mutation, P299S in the *Drosophila* GluCl channel of this strain that when expressed
16 in *Xenopus* oocytes was found to reduce channel sensitivity to both compounds
17 (Kane *et al.* 2000). The avermectin abamectin is also an effective miticide and two
18 studies have reported mutations associated with resistance in the GluCl of *T. urticae*
19 (Dermauw *et al.* 2012, Kwon *et al.* 2010), which in contrast to insects has six genes
20 encoding GluCl (Dermauw *et al.* 2012). In the first study a G323D mutation in
21 TuGluCl-1 was associated with ~20-fold resistance to abamectin in *T. urticae* and
22 found to correlate with resistance in individual F2 progenies obtained by backcrossing
23 (Kwon *et al.* 2010). A second study identified the same mutation in *TuGluCl-1* in a *T.*
24 *urticae* strain with ~2000-fold resistance to abamectin, however, a novel substitution
25 was also identified in the same strain, at an identical position (G326E), but in a
26 different GluCl gene (*Tu_GluCl3*), suggesting these mutations may have an additive
27 or synergistic effect in conferring resistance (Dermauw *et al.* 2012). Genetic
28 association studies with two diagnostic doses of abamectin was used to link both
29 mutations with resistance to lower abamectin concentrations, and the G326E
30 substitution in *Tu_GluCl3* with resistance to higher abamectin concentrations
31 (Dermauw *et al.* 2012). Together these studies suggest that these two subunits might
32 be the primary target-sites of abamectin in *T. urticae* and it would be interesting to
33 explore if they coassemble to form a heteropentameric GluCl or form individual
34 homopentameric receptors.
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37 A point mutation associated with abamectin resistance has also been described
38 in *P. xylostella* (Wang *et al.* 2016). In this case a A309V substitution at the N-
39 terminus of the third transmembrane helix (M3) was associated with resistance in a
40 field population introgressed into a susceptible strain (Wang *et al.* 2016). Homology
41 modelling suggested that the A309V substitution modifies the abamectin-binding by
42 an allosteric mechanism rather than eliminating a key binding contact (Wang *et al.*
43 2016). The modelling does however infer a more direct role for the G323/326
44 mutations identified in *T. urticae* since this residue within the M3 helix does show
45 direct contact with ivermectin in the open state GluCl model developed by Wang and
46 co-workers (Wang *et al.* 2016) (Figure 1c).
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49 50 *Alternative splicing and RNA editing of GluCl*

51 Finally, perhaps to overcome the constraints of being encoded by a single gene, insect
52 GluCl_s expand their coding capacity by mRNA splicing and editing (Jones & Sattelle
53 2007, Knipple & Soderlund 2010). A study on *P. xylostella* observed three naturally
54 occurring transcript variants of PxGluCl in both abamectin resistant and susceptible
55 strains (Liu *et al.* 2014). One of these, described as a '36-bp deletion', represents a
56 transcript with alternative exon 9c. Transcripts with this exon were observed at higher
57 frequency in abamectin resistant strains than susceptible strains and *in vitro*
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3 expression of this isoform revealed that GluCl channels with exon 9c are less
4 sensitive to both abamectin and glutamate than transcripts containing exon 9a, the
5 primary variant observed in *P. xylostella* (Kwon et al. 2010, Liu et al. 2014). The
6 finding that alternative exon usage in insect GluCls can influence insecticide
7 binding/sensitivity was further supported by recent work characterising the
8 transcriptional patterns of the GluCl of *Anopheles gambiae* (Meyers et al. 2015). A
9 total of four isoforms were identified in this species that differ in their splicing of
10 exons 3, 8 and 10 and result in differences in the N-terminal extracellular domain and
11 intracellular loop region. Functional expression of two different isoforms, performed
12 to examine their influence on modulating responses to the natural ligand and
13 insecticides, revealed specific isoforms that were sensitive (AgGluCl-a1) and
14 insensitive (AgGluCl-b) to ivermectin. These results suggest that residues encoded by
15 alternative exons 3, 8 and 10, at least in part, define sensitivity to ivermectin and also
16 suggest resistance to this insecticide in *An. gambiae* could arise simply through
17 altered regulation of AgGluCl to favor ivermectin insensitive transcripts (Meyers et al.
18 2015).
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21 22 **The nicotinic acetylcholine receptor**

23 *Subunit composition and structure of the nAChR*

24 Nicotinic acetylcholine receptors (nAChRs) are cation permeable ion channels that
25 mediate fast synaptic transmission in the central nervous system of both vertebrates
26 and invertebrates, and at the neuromuscular junction of vertebrates. nAChRs are the
27 primary target of two major insecticide classes, the neonicotinoids, which comprise
28 seven major compounds with a market share of more than 25% of total global
29 insecticide sales (Bass et al. 2015b), and the natural product-derived spinosyns
30 (Sparks et al. 2001).
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32 nAChRs are oligomers composed of five subunits arranged in combinations
33 from a variety of different subunit subtypes. These subunits form multiple functional
34 heteropentamers or homopentamers which have distinct functional and
35 pharmacological profiles (Millar 2003). Like the ligand-gated chloride channels
36 discussed in the previous section, nAChR subunits have a similar topology
37 comprising an N-terminal extra cellular region containing the ligand binding site and
38 four transmembrane segments (TM1 – TM4) that create the ion channel (Figure 2a,b).
39 Acetylcholine (and neonicotinoids) bind at the interface of two subunits, each of
40 which contribute 3 binding loops (A – C by an α subunit and D-F by an α or non- α
41 neighbouring subunit) to the binding pocket (Corringer et al. 2000). The first insect
42 nAChR subunit sequence was isolated from *Drosophila* using probes designed on
43 sequences from electric rays (*Torpedo* spp.), a rich source of this receptor (Hermans-
44 Borgmeyer et al. 1986). Later sequencing of the *Drosophila* genome revealed a
45 relatively compact family of ten genes ($D\alpha 1$ - $D\alpha 7$ and $D\beta 1$ - $D\beta 3$) encoding nAChR
46 subunits (Adams et al. 2000), a number subsequently found to be representative of
47 most other insects (Jones & Sattelle 2010). Despite the reduction in size of this gene
48 family in insects when compared to vertebrates further research has revealed that the
49 transcriptomic diversity generated by certain insect nAChR genes can be greatly
50 expanded by posttranscriptional modifications such as alternative splicing and RNA
51 editing (Grauso et al. 2002).
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Functional expression of insect nAChR subunits and neonicotinoid binding

The subunit composition of human nAChR muscle-type receptors is well established ($\alpha 1_2$, β , γ , δ , or $\alpha 1_2$, β , ϵ , δ), and although neuronal subtypes are less well characterised various combinations of twelve different subunit subtypes ($\alpha 2$ – $\alpha 10$ and $\beta 2$ – $\beta 4$) 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 $\beta 2$), 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 $D\alpha 1$, $D\alpha 2$ and $D\alpha 3$ form a high-affinity imidacloprid binding site in *Drosophila* S2 cells when co-expressed with rat $\beta 2$ subunits (Lansdell & Millar 2000). Significantly $D\alpha 3$ (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 $\beta 4$, 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 $\beta 2$ 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 $N\alpha 3$, $N\alpha 8$ and $N\beta 2$ subunits of this species co-assemble in one complex to form a high affinity imidacloprid binding site while $N\alpha 1$, $N\alpha 2$ and $N\beta 1$ assemble to form a lower affinity site (Li *et al.* 2010). More recent work expressing certain *Drosophila* α subunits ($D\alpha 5$, $D\alpha 6$ and $D\alpha 7$) 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 $\alpha 6$ subunits. Evidence for this, initially came from *Drosophila*, with a $D\alpha 6$ 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 $\alpha 6$ 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 $\alpha 6$ nAChRs as the

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3 target of spinosad and localise the binding site of this insecticide (Perry *et al.* 2015,
4 Somers *et al.* 2015). In the first of these studies Perry *et al.* exploited two *Drosophila*
5 mutants created by EMS mutagenesis, that are highly resistant to spinosad, to develop
6 an *in vivo* rescue system (Perry *et al.* 2015). They then used this tool to demonstrate
7 that while D α 1, D α 5 and D α 7 do not rescue the response to spinosad, four different
8 isoforms of D α 6 and the α 6 gene from three different pest insects are all able to rescue
9 spinosad sensitivity. Insect α 6 genes show a remarkable capacity to expand their
10 transcriptional diversity through alternative splicing of two mutually exclusive exons
11 (exon 3 and 8) and RNA A to I editing (Grauso *et al.* 2002). An important result of the
12 study by Perry and co-workers was therefore the finding that expression of four D α 6
13 isoforms with different alternative exon 3 and exon 8 combinations resulted in
14 significant difference in fly mortality to spinosad, despite the fact they were expressed
15 in a uniform genetic background (Perry *et al.* 2015). This finding highlights the
16 potential importance of alternative splicing in influencing the responsiveness of
17 nAChRs to insecticides. In the second study, Somers *et al.* expressed two chimeric
18 subunits, where the N-terminal half of D α 6 was fused to the C-terminal half of D α 7
19 (which exhibits >60% sequence similarity to D α 6) and vice versa, in a spinosad
20 resistant, D α 6 null background to test their ability to rescue susceptibility to spinosad
21 (Somers *et al.* 2015). Significantly only subunits containing the C-terminal region of
22 D α 6 were able to respond to spinosad, suggesting, in contrast to neonicotinoid
23 insecticides, that this region is critical for spinosad binding.
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27 *Resistance associated mutations and nAChR insecticide binding sites*

28 The molecular characterisation of spinosad and neonicotinoid resistance has shed light
29 on the binding, and selectivity of these insecticides and the nAChR subunits that are
30 important in these interactions. In the case of neonicotinoids, resistance in several
31 insect species was initially attributed to metabolic mechanisms, and, to date,
32 modification of the receptor (i.e. target-site resistance) has only been described in a
33 handful of insect species. Prior to the development of 'field-evolved' neonicotinoid
34 resistance, work on *Drosophila* provided valuable information on the nAChR subunit
35 subtypes that may be important in forming the binding site of this insecticide class.
36 EMS mutagenesis followed by selection with the neonicotinoid nitenpyram was used
37 to identify four highly resistant lines with mutations in D α 1 and D β 2 that confer
38 resistance to this neonicotinoid (Perry *et al.* 2008) and also other members of this
39 class (Perry *et al.* 2012). This finding provided strong evidence that nAChRs
40 containing either or both of these subunits form important sites of neonicotinoid
41 action.
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44 Target-site resistance to neonicotinoids was first described in an insect pest
45 species in 2005 (Liu *et al.* 2005). Selection of a laboratory strain of the planthopper *N.*
46 *lugens* with imidacloprid for 35 generations resulted in a strain with over 250-fold
47 resistance compared to a susceptible reference. Radioligand binding experiments to
48 whole body membrane preparations revealed a significantly lower level of
49 [3H]imidacloprid-specific binding to preparations of the resistant strain and
50 sequencing of *N. lugens* nAChR subunit genes identified a single point mutation at a
51 conserved position (Y151S) in two nAChR subunits, N1 α 1 and N1 α 3. The causal
52 effect of these mutations was examined by expression of hybrid nAChRs containing *N.*
53 *lugens* α and rat β 2 subunits, with the presence of Y151S associated with a substantial
54 reduction in specific [3H]imidacloprid binding (Liu *et al.* 2005). The discovery of
55 these mutations in the orthologs of *Drosophila* (D α 1 and D α 3), which have previously
56 been shown to form a high-affinity binding site for imidacloprid (see above), provides
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3 additional evidence of their importance in neonicotinoid binding. Finally, the
4 identification of the same mutation at homologous residues in two distinct nAChR α
5 subunits is intriguing and it is unclear if N1 α 1 and N1 α 3 subunits contribute to
6 different populations of receptors, conferring resistance to each, or if they occur
7 together in a heteromeric receptor. It should be noted that this mutation combination,
8 although able to confer strong resistance to neonicotinoids, may also carry an
9 unacceptable fitness penalty as these mutations have never been reported in resistant
10 field populations of *N. lugens*.

11 In the Green Peach aphid *M. persicae*, significant resistance to neonicotinoids
12 used as aphicides has also taken a surprisingly long time to evolve with control
13 compromising levels of resistance only described in a clone of *M. persicae* collected
14 in 2009, nearly twenty years after the introduction of imidacloprid (Bass *et al.* 2011).
15 Binding assays using radiolabelled imidacloprid to native membrane preparations
16 revealed that the very high affinity site present in aphids, and indeed other
17 hemipterans, was completely lost in the resistant clone and the remaining lower
18 affinity site was also altered compared to susceptible clones resulting in a significant
19 overall reduction in binding affinity to the nAChR. Sequencing of the previously
20 characterised nAChR subunit genes from resistant and susceptible *M. persicae* clones
21 revealed a point mutation in the nAChR β 1 subunit of the resistant clone resulting in
22 an arginine to threonine substitution or R81T (Slater *et al.* 2012) (Figure 2).
23 Remarkably the amino acid at this position had been previously identified as a key
24 determinant of the selectivity of neonicotinoids for insects, over vertebrates
25 (Shimomura *et al.* 2006). The high sensitivity of insect nAChRs to neonicotinoids is
26 thought to result, in part, from interactions between the distinctive electronegative
27 pharmacophore (nitro or cyano group) of these insecticides and conserved positively
28 charged residues in loop D (such as R81) of β subunits (Shimomura *et al.* 2006,
29 Tomizawa & Casida 2003, Tomizawa & Casida 2005). Indeed substituting the
30 threonine residue in the chicken β 2 subunit at this position with arginine or another
31 basic residue greatly enhanced the affinity of heterologously expressed nAChRs (such
32 as *Drosophila* Da2/chicken β 2 hybrids) for imidacloprid (Shimomura *et al.* 2006).
33 The mutation identified in *M. persicae* therefore confers a 'vertebrate-like' quality to
34 the β 1 subunit of resistant aphids resulting in reduced sensitivity of the nAChR to
35 neonicotinoids through the loss of direct electrostatic interactions of the
36 electronegative pharmacophore with the basic arginine residue at this key position
37 within loop D (Figure 2c). These results both provide further evidence of the
38 importance of insect β 1 subunits in forming the neonicotinoid binding site and
39 provide additional validation of the current models of neonicotinoid binding and
40 selectivity for insect nAChRs. Unlike Y151S in planthoppers, the R81T mutation in
41 *M. persicae* does not appear to carry a strong fitness penalty and is seen at high
42 frequency in resistant aphid populations from peach growing regions of Southern
43 Europe (Slater *et al.* 2012).

44 More recent work has suggested that quantitative changes in nAChR subunits
45 may represent an alternative form of target-site resistance to neonicotinoids with
46 resistance to imidacloprid in a laboratory selected strain of the planthopper *N. lugens*
47 associated with a reduction in both mRNA and protein levels of the nicotinic
48 acetylcholine receptor (nAChR) subunit N1 α 8 (Zhang *et al.* 2015). Functional
49 validation of the observed down-regulation using RNA interference showed that
50 knockdown of N1 α 8 mRNA and protein levels causes a significant increase in
51 imidacloprid resistance in *N. lugens* and demonstrated a decrease in N1 α 8 expression
52 is sufficient to confer resistance *in vivo*. As detailed above, previous work has
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3 indicated that Nl α 8 co-assembles with Nl β 2 to form a high affinity imidacloprid
4 binding site. It is therefore significant that radioligand binding assay on native
5 membrane preparations of the resistant strain revealed that the affinity of this binding
6 site was reduced over 10-fold compared to an earlier (and hence less resistant)
7 generation of the same strain (Zhang et al. 2015).
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9 In the case of spinosad resistance, target-site resistance appears to be the
10 primary route of resistance evolution, likely because, in contrast to other conserved
11 insecticide receptors, insect α 6 subunits are not essential for viability and so can
12 tolerate a variety of mutations even if they result in loss of function. In this regard a
13 wide range of genetic alterations have been described that result in truncated non-
14 functional protein (Baxter et al. 2010, Hsu et al. 2012, Rinkevich et al. 2010). In most
15 of these cases the mutations involved disrupt the normal pattern of alternative splicing
16 of this gene. For example, an investigation of a spinosad resistant strain of
17 Diamondback moth, *Plutella xylostella*, collected in Hawaii, identified a mutation
18 within the ninth intron splice junction of *Pxa6* resulting in mis-splicing of transcripts
19 and a predicted protein truncated between the third and fourth transmembrane
20 domains (Baxter et al. 2010). More recently, spinosad resistance has been associated
21 with two alternative point mutations that do not result in loss of function (Bao et al.
22 2014, Puinean et al. 2013, Silva et al. 2016, Somers et al. 2015). In the first case, the
23 same non-synonymous point mutation resulting in a G275E substitution was
24 identified in exon 9 of the α 6 nAChR in three different pest insect species. An
25 alternative amino acid replacement, P146S, was also recently associated with
26 spinosad resistance in an EMS mutagenized line of *Drosophila*. As a result of recent
27 advances in genome editing approaches the causality of this mutation and G275E
28 were both demonstrated using the CRISPR/Cas9 system to edit the mutations into
29 *Drosophila* and measure the effect of this on reduced sensitivity to spinosad (Somers
30 et al. 2015, Zimmer et al. 2016). **In contrast to mutations involving loss of the whole
31 gene, specific amino acid replacements can also provide more specific** information on
32 the mode and site of insecticide binding. Homology modeling using the nematode
33 glutamate-gated chloride channel structure predicts the G275E mutation to lie at the
34 top of the third α -helical transmembrane domain of the nAChR α 6 subunit, providing
35 additional evidence that the transmembrane domain of α 6 contains the spinosad
36 binding site (Puinean et al., 2013). In contrast the P146S mutation is unlikely to reside
37 in the spinosad binding site and its close proximity to the conserved Cys-loop
38 indicates an alternative mode of action potentially by impairing the gating of the
39 receptor (Somers et al. 2015).
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44 **The Para containing voltage gated sodium channel**

45 Lessons from *Drosophila*

46 The screening of chemically induced *Drosophila* mutants for temperature sensitivity
47 (Siddiqi & Benzer 1976, Suzuki 1970) has played an important role in isolating
48 mutants of genes encoding important insecticide target sites. One such temperature
49 sensitive paralytic mutant, termed *paralytic* (temperature sensitive) or *para^{ts}*, was
50 isolated using a screen involving a shift of temperature from 22°C to 29°C upon
51 which paralysis in *para^{ts}* flies is immediate but reversible (Suzuki et al. 1971). The
52 structural gene encoding the voltage gated sodium channel was then cloned from the
53 *para* locus via a chromosomal walk (Loughney et al. 1989). **The polypeptide encoded
54 by this locus will here be referred to as 'Para' and the gene itself as 'para'**. The *para*
55 locus was shown to be both large and complex, spanning over 60 kb of genomic
56 DNA and with a minimum of 26 predicted exons, encoding numerous transcripts via
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3 alternative splicing (Loughney et al. 1989). Further studies in the Ganetzky
4 laboratory showed that the *para* locus in fact has six different sites for alternative
5 splicing and that at least 48 different splice forms could be generated (Thackeray &
6 Ganetzky 1994). The variety of *para* splice forms found differed markedly between
7 embryos and adults suggesting that the adult nervous system may contain a very
8 different complement of sodium channels (Thackeray & Ganetzky 1994). This same
9 pattern of alternative splicing is also conserved in *D. virilis* suggesting that this
10 developmentally regulated variability does indeed have a functional role (Thackeray
11 & Ganetzky 1995). To add to this bewildering array of alternative splice forms, the
12 Ganetzky laboratory subsequently showed that *para* also contains three sites where
13 RNA editing occurs (Hanrahan et al. 2000). In the RNA editing process, post-
14 transcriptional editing of pre-mRNAs, through the action of dsRNA adenosine
15 deaminases, modifies adenosine (A) residues to inosine (I), which then alters the
16 coding potential of modified transcripts. These three RNA editing sites were again
17 developmentally regulated and again conserved in *D. virilis*, supporting their
18 functional role (Hanrahan et al. 2000). At each position a highly conserved region was
19 found downstream of the RNA editing site in the following intron, and this region
20 matched the exonic editing site (Hanrahan et al. 2000). This suggests a mechanism
21 whereby the edited exon forms a base-paired secondary structure with the distant
22 noncoding intronic sequence, in a similar manner to that seen in A-to-I RNA editing
23 in vertebrate glutamate receptor subunits. **More recently the functional significance of
24 both editing and splice variants has been investigated via the heterologous expression
25 of a range of different Para variants (here alternatively termed DmNa(V)) in *Xenopus*
26 oocytes (Olson et al. 2008, Lin et al. 2009). These studies showed a wide range of
27 voltage dependence in the gating properties of different DmNa(V) (Para) variants
28 (Olson et al. 2008) and also differences in activation, inactivation and persistent
29 current (Lin et al. 2009), proving that such variation does indeed have a meaningful
30 biological role in generating insect sodium channel diversity.** In summary, the *para*
31 locus is large and complex and the variety of different mRNAs produced via a
32 combination of both alternative splicing and RNA editing is large. Understanding the
33 role of all of these different Para variants in the development and functioning of the
34 insect nervous system therefore remains a major challenge.

35 36 37 38 39 *Heterologous expression of Para*

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

50 51 52 53 *Resistance associated mutations and insecticide binding*

54 The voltage gated sodium channel is the primary site of action of pyrethroid
55 insecticides and in pest insects, such as the house fly, *Musca domestica*, and the
56 German cockroach, *Blattella germanica*, pyrethroid resistance is associated with a
57 locus originally termed *knockdown resistance* or *kdr*. The first evidence that the *kdr*
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3 locus might indeed encode a *para*-like channel came from observations of tight
4 genetic linkage between *kdr* and molecular probes for the *para*-like gene in both
5 house flies (Knipple *et al.* 1994, Williamson *et al.* 1993) and German cockroaches
6 (Dong & Scott 1994). Subsequently, sequencing of both the *kdr* and *super-kdr* alleles
7 of the house fly showed that both mutants are associated with point mutations in
8 domain II of the Para-like sodium channel (Williamson *et al.* 1996). Specifically, a
9 leucine to phenylalanine replacement (L1014F) in the hydrophobic IIS6
10 transmembrane section is found in both *kdr* and *super-kdr* alleles and a second
11 methionine to threonine replacement (M918T) within the intracellular IIS4-S5 loop is
12 unique to *super-kdr* (Williamson *et al.* 1996). Similarly, an equivalent mutation
13 (L1014F) was shown to be associated with *kdr*-like mutants of the German cockroach
14 (Miyazaki *et al.* 1996, Dong 1997). These equivalent mutations in two different
15 resistant insects suggested a binding site for pyrethroids at the intracellular mouth of
16 the channel pore in a region thought to be important for channel inactivation
17 (Williamson *et al.* 1996).
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20 Interestingly, *para*-associated (*kdr*-like) pyrethroid resistance has not, to our
21 knowledge, been isolated from field strains of *D. melanogaster*. Despite the fact that
22 *para* is X-linked and therefore *kdr*-like resistance should be easy to identify as it should
23 be clearly sex linked. The absence of *kdr*-like (DDT and pyrethroid) resistance in field
24 strains of *Drosophila* may be associated with the presence of the dominant *DDT-R*
25 gene on chromosome II which confers resistance to DDT but ironically not to
26 pyrethroids. However we can still examine the effects of different point mutations on
27 the insecticide sensitivity of the *Drosophila Para* channel as although the original
28 *para*^{ts} mutants were isolated on the basis of their paralysis at high temperature, the
29 same point mutations conferring temperature sensitivity also overlap with those
30 conferring resistance to DDT and pyrethroids (Pittendrigh *et al.* 1997). Specifically,
31 six out of thirteen *para* mutants tested showed 10-30 fold resistance to DDT and the
32 associated mutations were clustered into a few sites across the channel polypeptide
33 (Figure 3a). First, within the intracellular loop between S4 and S5 in homology
34 domains I and III, second, within the pore region of homology domain III and third,
35 within S6 of homology domain III (Pittendrigh *et al.* 1997).
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37 Numerous different pest species have now been shown to possess *kdr*-like
38 mutations in their *para* homologous sodium channel genes (Dong *et al.* 2014, Davies
39 *et al.* 2008). However the functionality of many of these mutations has not been
40 tested via heterologous expression and mutagenesis. Current work on the mode of
41 action of pyrethroids on the insect voltage gated sodium channel is, however, now
42 divided into two camps. Those that favour a single binding site for pyrethroids
43 (O'Reilly *et al.* 2006, Davies *et al.* 2008), as implicated by the original *kdr* and *super-*
44 *kdr* mutations, and those that favour two different binding sites on the sodium channel
45 polypeptide (Figure 3). A single binding site for fenvalerate and DDT has been
46 predicted using a homology model of the house fly *Para*-like voltage gated sodium
47 channel (O'Reilly *et al.* 2006). In this model, the sodium channel was modelled in the
48 open conformation with the pyrethroid binding site as a hydrophobic cavity formed by
49 domain II of the S4-S5 linker and the IIS5 and IIS6 helices (Figure 3b) (O'Reilly *et al.*
50 2006). Importantly, this binding site is predicted to be accessible to the lipid bilayer
51 where the lipid-soluble insecticides are predicted to accumulate. Insecticide binding,
52 and the consequent formation of contacts across different channel elements, is
53 predicted to stabilize the open state of the channel. This stabilisation of the open state
54 of the channel is consistent with the prolonged sodium tail currents induced by both
55 pyrethroids and DDT (Vais *et al.* 2000). Conversely, in the closed state, repositioning
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3 of the domain II S4-S5 linker would disrupt this binding site and help explain why
4 pyrethroids have a higher affinity for the open state of the sodium channel (O'Reilly et
5 al. 2006). Importantly, some of the residues on the helices that form the putative
6 binding contacts are not conserved between vertebrates and invertebrates, which may
7 help explain the 100-fold difference in pyrethroid potency discussed above. Similarly
8 additional binding contacts on the II S4-S5 linker (in the *super-kdr* region) may also
9 explain the higher affinity binding of pyrethroids over DDT (O'Reilly et al. 2006) and
10 why the potency of DDT is relatively unaffected by *super-kdr* (Usherwood *et al.*
11 2007). It should be noted that in this model the *kdr* site does not have a direct
12 interaction with the pyrethroid molecule and mutations at this site are postulated to
13 affect pyrethroid binding via an indirect (allosteric) impact that alters the
14 conformation of the adjacent pyrethroid binding site (Davies *et al.* 2008).

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16 Evidence for a two binding site model comes from studies of *kdr*-like
17 resistance in the **Para**-like sodium channel of the mosquitoes *Aedes aegypti* and
18 *Anopheles gambiae*. In these mosquitoes, two resistance associated mutations map to
19 the same binding site predicted from the house fly model, termed pyrethroid receptor
20 site or 'PyR1', located in domain interface II/III. However, three other resistance
21 associated mutations apparently map to a *second* binding site on the receptor, termed
22 pyrethroid receptor site 2 or 'PyR2', which is located in domain interface I/II (Figure
23 3c) (Du *et al.* 2013). Further molecular modelling of the interaction between the
24 mosquito sodium channel and deltamethrin suggests a possible additional binding site
25 between the linker helix IL45 (domain I S4-S5 linker) and transmembrane helices IS5
26 and IIS6, with deltamethrin dibromoethenyl and diphenylether residues pointing in
27 intra- and extra-cellular directions respectively (Du *et al.* 2015). Finally, recent
28 evidence suggests that pairs of mutations can work in concert to synergise pyrethroid
29 resistance. Thus a unique mutation, N1575Y in the cytoplasmic loop linking domains
30 III and IV of the *Anopheles gambiae* **Para** channel, confers no resistance on its own
31 but synergises the effect of the previously identified L1014F replacement in IIS6
32 (Wang *et al.* 2015). Molecular modelling supports a mechanism whereby this second
33 mutation (N1575Y) allosterically alters the PyR2 binding site via a small shift in IIS6
34 (Wang *et al.* 2015). It therefore appears that secondary mutations can accumulate
35 within the resistant receptor causing *allosteric* changes in the mutant receptor which
36 accentuate the effect of the primary mutation. It will therefore be interesting to see if
37 such effects can be proven for mutations in other receptors such as the secondary
38 mutations seen around A301S in the **Rdl** containing GABA receptor (see discussion
39 above).
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44 **The calcium activated calcium channel or ryanodine receptor**

45 *Structure of the ryanodine receptor*

46 Ryanodine receptors (RyRs) are homotetrameric calcium release channels and are the
47 largest known ion channels. RyRs mediate the controlled release of Ca²⁺ from
48 intracellular stores to initiate a wide variety of cellular processes, not least excitation-
49 contraction coupling in muscle tissues (Lanner *et al.* 2010). Mammals possess three
50 RyR isoforms (RyR1-3) each derived from a separate gene that are localized in
51 different tissues. In contrast insect RyRs are encoded by a single gene with an ORF of
52 >15,000 bp (Sattelle *et al.* 2008). RyRs are the target of ryanodine, a plant-derived
53 alkaloid and natural insecticide, and the synthetic diamide insecticides, currently
54 comprising the phthalic diamide flubendiamide and anthranilic diamides
55 chlorantraniliprole and cyantraniliprole. The first insect RyR gene to be sequence
56 characterised was from *Drosophila*, and was identified using a cDNA probe of the
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3 rabbit skeletal muscle RyR, with the receptor showing less than 50% sequence
4 identity to mammalian isoforms (Takeshima *et al.* 1994). Early attempts at functional
5 expression of the *Drosophila* receptor in Chinese hamster ovary cells were only
6 partially successful (Xu *et al.* 2000), however, subsequent expression of this receptor
7 in insect Sf9 cells was much more robust, and sufficient to characterize the action of
8 anthranilic diamides and to generate a stable cell line (Cordova *et al.* 2006). This
9 system was further exploited to identify regions of the RyR that are critical for
10 diamide binding (Tao *et al.* 2013). Replacement of a 46 amino acid segment of the
11 *Drosophila* C-terminus with that from the plant parasitic nematode, *Meloidogyne*
12 *incognita*, which is insensitive to anthranilic diamides, resulted in a functional RyR
13 which lacked sensitivity to these compounds suggesting this region is critical to
14 diamide sensitivity in insect ryanodine receptors (Tao *et al.* 2013). Beyond
15 *Drosophila*, functional expression of the RyR of the model lepidopteran, *Bombyx*
16 *mori*, has identified additional putative sites of diamide binding (Kato *et al.* 2009).
17 HEK cells expressing modified *B. mori* RyRs with an N-terminal deletion
18 (residues 183–290) or where the 20% C-terminus (amino acids 4111–5084) was
19 replaced with the rabbit RyR2 counterpart sequence, failed to produce Ca²⁺
20 mobilization in the presence of flubendiamide suggesting these two regions are
21 essential for diamide sensitivity (Kato *et al.* 2009).
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25 *Insecticide resistance and insecticide binding sites*

26 Use of radiolabelled diamides and ryanodine on native muscle membrane preparations
27 of the house fly suggested that the major anthranilic and phthalic diamide insecticides
28 bind to insect RyRs at different allosterically coupled sites (Isaacs *et al.* 2012). This
29 finding was subsequently called into question by the result of work elucidating the
30 molecular basis of diamide resistance in the diamondback moth, *P. xylostella*
31 (Trocza *et al.* 2012). This species is notorious both as a pest of cruciferous crops and
32 also for its ability to rapidly evolve resistance to insecticides used for control.
33 Remarkably, reports of resistance in *P. xylostella* to diamides emerged just a few
34 years after their introduction (Trocza *et al.* 2012, Wang & Wu 2012). Sequencing of
35 the RyR from strains collected from the Philippines and Thailand with over 200-fold
36 resistance to both chlorantraniliprole and flubendiamide revealed different non-
37 synonymous mutations in each of the resistant strains that in both cases lead to the
38 same glycine to glutamic acid substitution (G4946E) in the transmembrane spanning
39 region of the RyR (Figure 4a) (Trocza *et al.* 2012). Subsequent radioligand binding
40 studies with *P. xylostella* thoracic microsomal membrane preparations provided direct
41 evidence of the role of the G4946E mutation on diamide specific binding with the
42 binding of the tritiated flubendiamide derivative [³H]PAD1 profoundly reduced in
43 preparations of the resistant strain (Steinbach *et al.* 2015a). Finally, expression of the
44 *P. xylostella* RyR in Sf9 cells demonstrated that **the sensitivity of heterologously**
45 **expressed receptors to flubendiamide and chlorantraniliprole** is dramatically reduced
46 in cells stably expressing the G4946E modified RyR, providing final unequivocal
47 validation that the G4946E RyR mutation impairs diamide insecticide binding
48 (Trocza *et al.* 2015).
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52 Sequencing of a second chlorantraniliprole resistant field population of *P.*
53 *xylostella*, this time from China, identified three novel amino acid substitutions,
54 E1338D, Q4594L and I4790M at high frequency in addition to the G4946E mutation,
55 which was observed at lower frequency (20%) (Guo *et al.* 2014). Crossing the
56 resistant strain with a susceptible strain, and subsequent analysis of the frequency of
57 these mutations in a subset of the F2 generation that had survived a diagnostic dose of
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3 chlorantraniliprole, showed an increase in frequency of all three novel mutations
4 compared to an untreated subset of the F2 population. Less convincing were the
5 results of fluorescent probe based assays which largely failed to provide compelling
6 evidence of the functional significance for the novel mutations due to the low
7 specificity of the diamide-like probe employed.
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9 More recent homology modelling of the *P. xylostella* RyR based on a cryo-
10 EM structure of rabbit RyR1 has been used to unambiguously locate two of the four
11 mutations (G4946E, I4790M) in close proximity to each other in the voltage sensor
12 domain (Figure 4b,c) (Steinbach et al. 2015a). The observation that G4946E is located
13 in trans-membrane helix S4 close to the S4–S5 linker domain thought to be involved
14 in the modulation of the voltage sensor suggests that the wild-type residue in this
15 position may act as a glycine hinge at the interface between the S4 helix and the S4-
16 S5 linker. The mutation to glutamic acid observed in resistant strains is therefore
17 likely to have a significant impact on movement of the S5 and S6 helices that control
18 channel gating, and hence a direct knock on effect on binding of diamide insecticides
19 to the receptor. Interestingly the I4790M occurs in helix S2 opposite and in close
20 proximity to G4946E and together these two residues may define the diamide binding
21 site on the receptor (Figure 4c) (Steinbach et al. 2015a, Troczka et al. 2015).
22 Interestingly I4790 is conserved in Lepidoptera, with all other insects and arachnids
23 exhibiting alternative amino acids at this position suggesting this residue may in part
24 underlie the species-specific differences exhibited in flubendiamide and
25 chlorantraniliprole binding at the RyR (see below).
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27 Contrary to conclusions drawn from the work on the house fly (Isaacs et al.
28 2012), the observed cross-resistance of resistant strains of *P. xylostella* to anthranilic
29 and phthalic diamides, and the demonstration that the G4946E mutation results in
30 insensitivity to both classes suggests that they share a common binding site. Further
31 comparison of diamide binding to thoracic muscle preparations of *Musca domestica*
32 and *Heliothis virescens* using [³H] ryanodine [³H]chlorantraniliprole and
33 [³H]flubendiamide reconciled these two studies by providing evidence that there may
34 be more than one type of diamide site in insects and species-specific differences in
35 diamide binding at the receptor (Qi et al. 2014). In the housefly, *Musca domestica*,
36 binding data suggest [³H]Chlo, [³H]Ry, and [³H]Flu bind at three distinct sites,
37 however, in the caterpillar pest, *Heliothis*, anthranilic and phthalic diamide
38 insecticides displace each other suggesting they bind at the same or closely coupled
39 sites (Qi et al. 2014).
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43 **Conclusions and future directions**

44 This brief over-view of the **five** major ion channel targets for current small molecule
45 insecticides has attempted to emphasize how our knowledge of these important
46 receptors in pest insects has been enriched by studies in the genetic model *Drosophila*.
47 Whilst studies of insecticide resistance associated mutations have identified not only
48 the receptor subunits to which the insecticides bind but also their likely binding sites
49 on those subunits. Importantly, however, studies of insecticide binding have also
50 reinforced important lessons about receptor biophysics. Thus it is clear that many
51 insecticides (such as the cyclodienes and pyrethroids) bind to specific biophysical
52 forms of their receptors (the cyclodienes binding preferentially to the desensitized
53 state of the insect GABA receptor for example). Critically therefore, insecticide
54 resistance associated mutations can either be directly within the insecticide binding
55 site and/or they can alter the stability of the drug preferred conformation of the
56 receptor. This begins to explain how mutations within the drug binding site can be
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3 synergized by distant mutations that act allosterically on the drug preferred
4 conformation of the receptor. It will therefore be important in the future to examine
5 the growing number of target site mutations in the light of this dual role hypothesis.
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8 **Acknowledgements**

9 This article is a review for a special issue of the Journal of Neurogenetics to honour
10 the lifetime achievements of Barry Ganetzky from the University of Wisconsin-
11 Madison.
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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 resistance to these compounds involves mutation (red circle) of arginine 81 to threonine (R81T) in *Myzus persicae* β 1 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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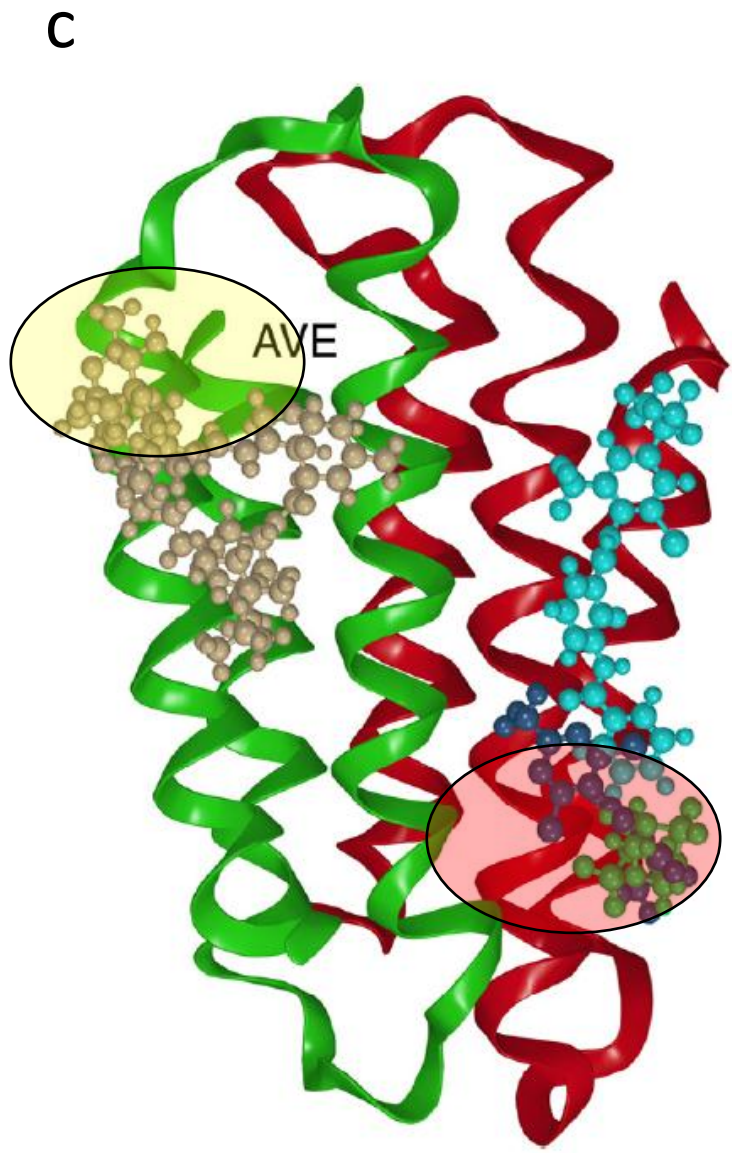
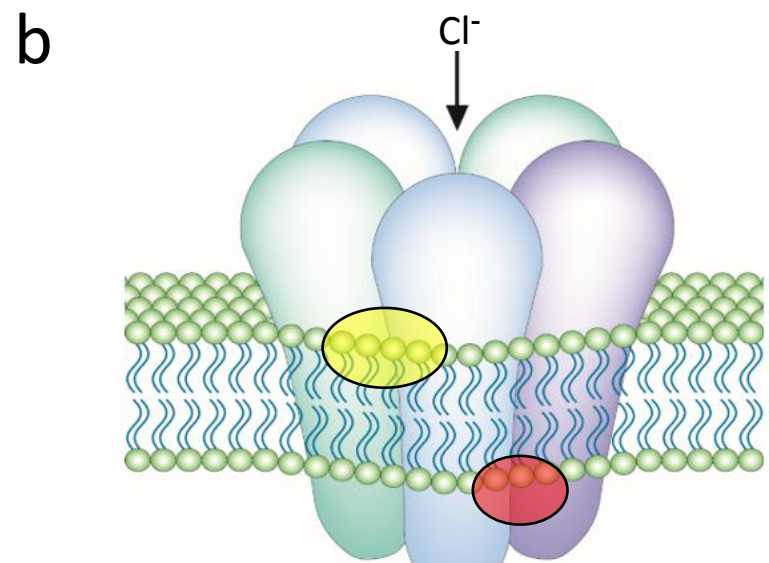
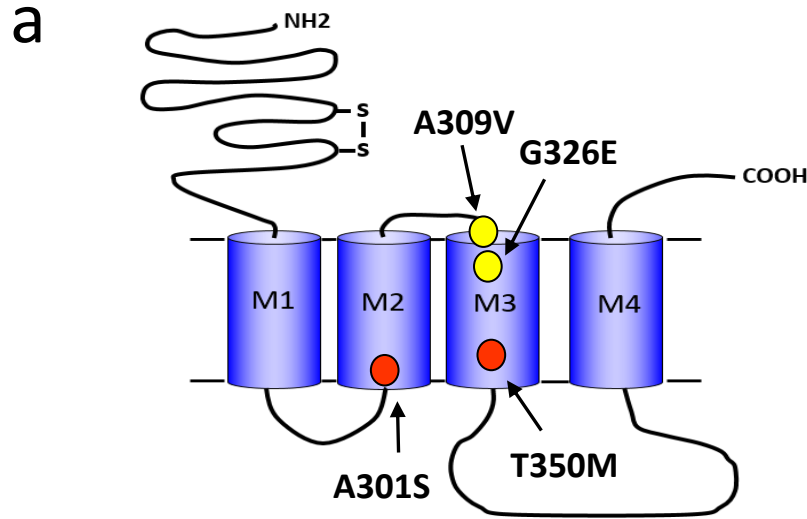
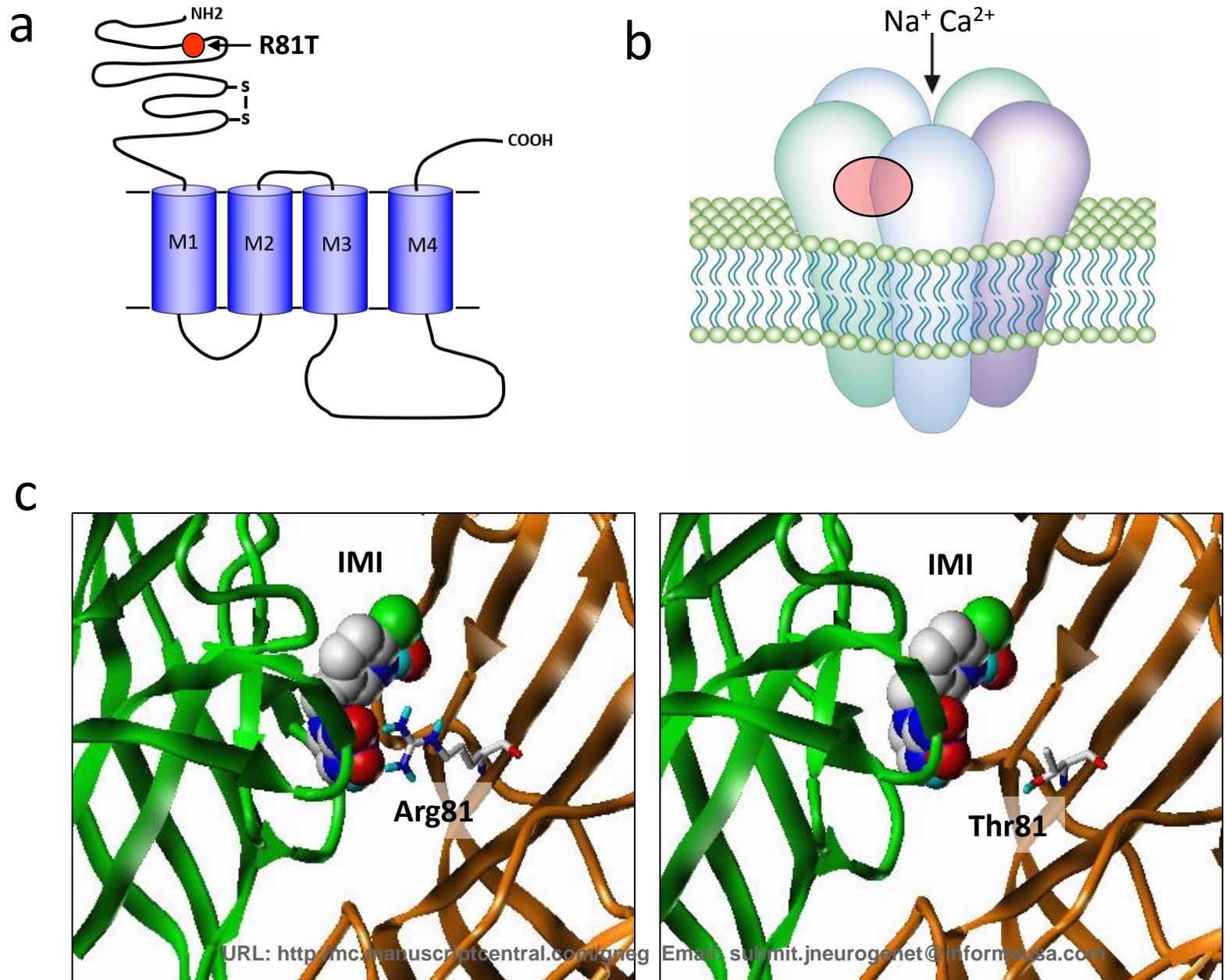


Figure 2



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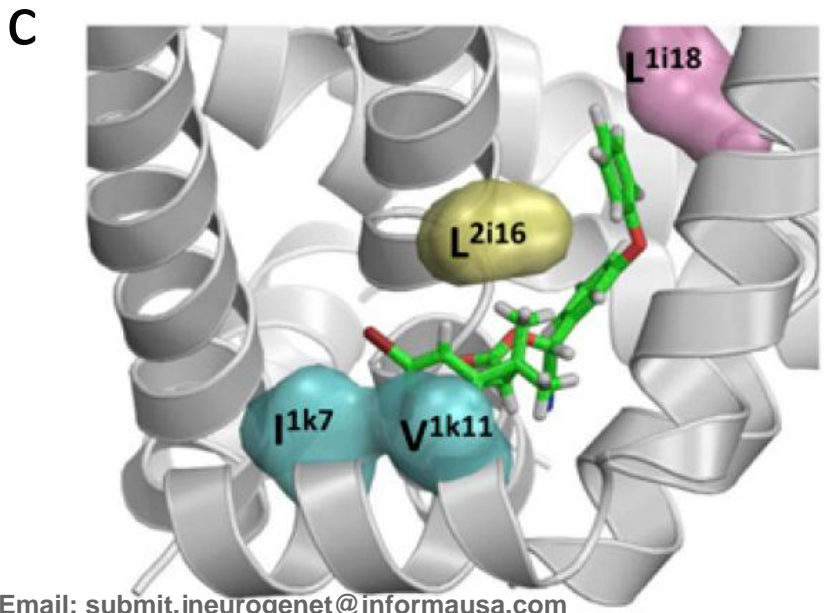
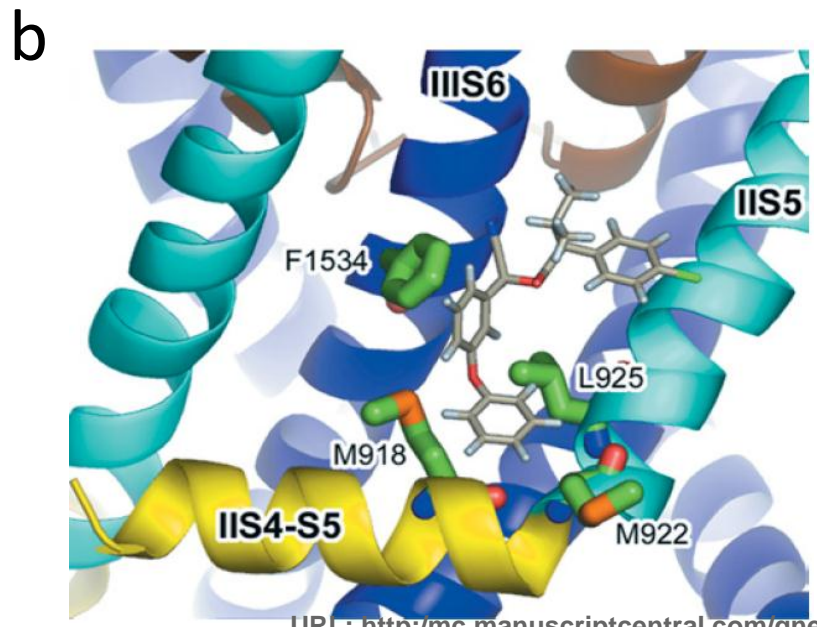
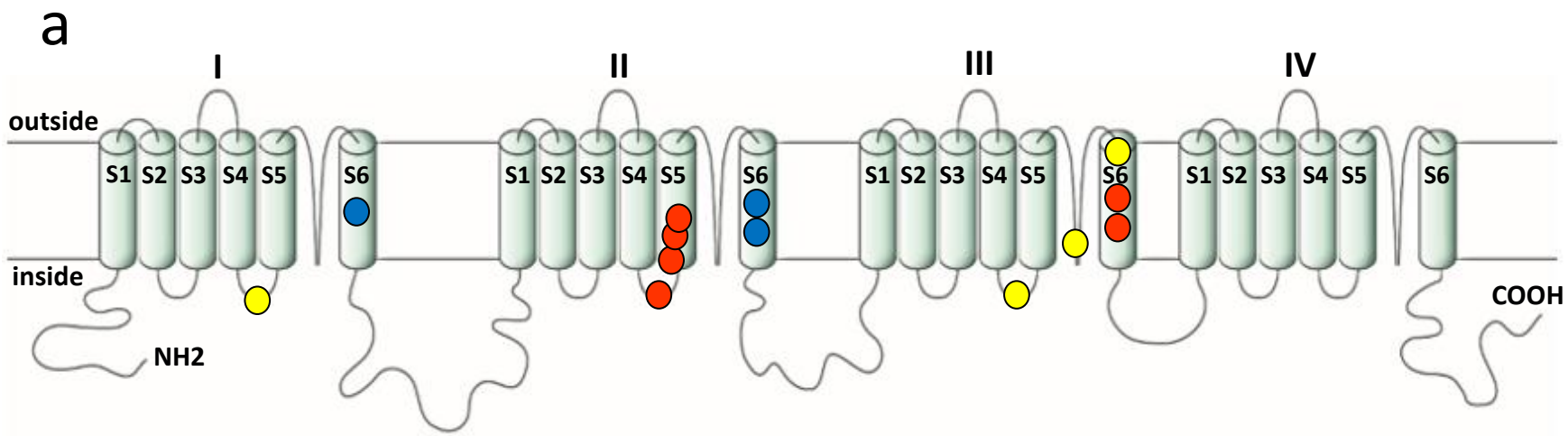


Figure 4

