

## CHAPTER 3

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# Diversity of Insect Nicotinic Acetylcholine Receptor Subunits

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### Abstract

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels that mediate fast synaptic transmission in the insect nervous system and are targets of a major group of insecticides, the neonicotinoids. They consist of five subunits arranged around a central ion channel. Since the subunit composition determines the functional and pharmacological properties of the receptor the presence of nAChR families comprising several subunit-encoding genes provides a molecular basis for broad functional diversity. Analyses of genome sequences have shown that nAChR gene families remain compact in diverse insect species, when compared to their nematode and vertebrate counterparts. Thus, the fruit fly (*Drosophila melanogaster*), malaria mosquito (*Anopheles gambiae*), honey bee (*Apis mellifera*), silk worm (*Bombyx mori*) and the red flour beetle (*Tribolium castaneum*) possess 10–12 nAChR genes while human and the nematode *Caenorhabditis elegans* have 16 and 29 respectively. Although insect nAChR gene families are amongst the smallest known, receptor diversity can be considerably increased by the posttranscriptional processes alternative splicing and mRNA A-to-I editing which can potentially generate protein products which far outnumber the nAChR genes. These two processes can also generate species-specific subunit isoforms. In addition, each insect possesses at least one highly divergent nAChR subunit which may perform species-specific functions. Species-specific subunit diversification may offer promising targets for future rational design of insecticides that target specific pest insects while sparing beneficial species.

### Introduction

Since the groundbreaking sequencing of the first insect genome, that of the fruit fly *Drosophila melanogaster*, several other insect genomes have been sequenced allowing for detailed comparisons of gene families. In this chapter we explore the diversity of nicotinic acetylcholine receptor (nAChR) gene families in various insect species such as the fruit fly genetic model organism (*Drosophila melanogaster*), the malarial disease vector (*Anopheles gambiae*), the agriculturally beneficial honey bee (*Apis mellifera*), the commercially important silk worm (*Bombyx mori*) and the red flour beetle (*Tribolium castaneum*) which is a pest species of stored food. nAChRs are part of a ligand-gated ion channel superfamily found in species as diverse as bacteria and human and their best known role is molecular signalling in nervous systems and neuromuscular junctions as well as in nonneuronal cells. The central nervous system of insects is rich in nAChRs, more so than any other organism apart from the electroplax tissue of the electric fish. Insect

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nAChRs are therefore of interest for the study of nervous system signalling molecules and as targets for several classes of important insecticides.

### Nicotinic Acetylcholine Receptors (nAChRs)— Structure and Function

Nicotinic acetylcholine receptors (nAChRs) are prototypical members of the Cys-loop ligand-gated ion channel (cysLGIC) superfamily<sup>1,2</sup> which also includes ionotropic receptors for GABA, glycine and serotonin (5-hydroxytryptamine). nAChRs mediate the fast actions of acetylcholine (ACh) in the nervous system and at neuromuscular junctions and consist of five homologous subunits arranged around a central ion channel (Fig. 1). They act as molecular switches which change conformation upon binding to an agonist such as ACh to allow a net influx of ions into the cell.<sup>3</sup> Each subunit has four transmembrane domains (TM1-4) and possesses an N-terminal extracellular domain containing the characteristic Cys-loop motif consisting of two disulfide bond-forming cysteines separated by 13 amino acid residues. The Cys-loop plays a role in nAChR assembly<sup>4</sup> as well the kinetics of ion channel gating.<sup>5</sup> The ACh-binding site is located at the interface of two adjacent subunits and is formed by six distinct regions (loops A-F)<sup>6</sup> in the N-terminal extracellular domain with loops A, B and C being contributed by an  $\alpha$  subunit and loops D, E and F by either an  $\alpha$  or non- $\alpha$  subunit. Subunits possessing two adjacent cysteine residues in loop C which are important for ACh binding<sup>7</sup> are defined as  $\alpha$  subunits while subunits lacking these vicinal cysteines are classified as non- $\alpha$  ( $\beta$ ,  $\delta$ ,  $\epsilon$  or  $\gamma$ ).

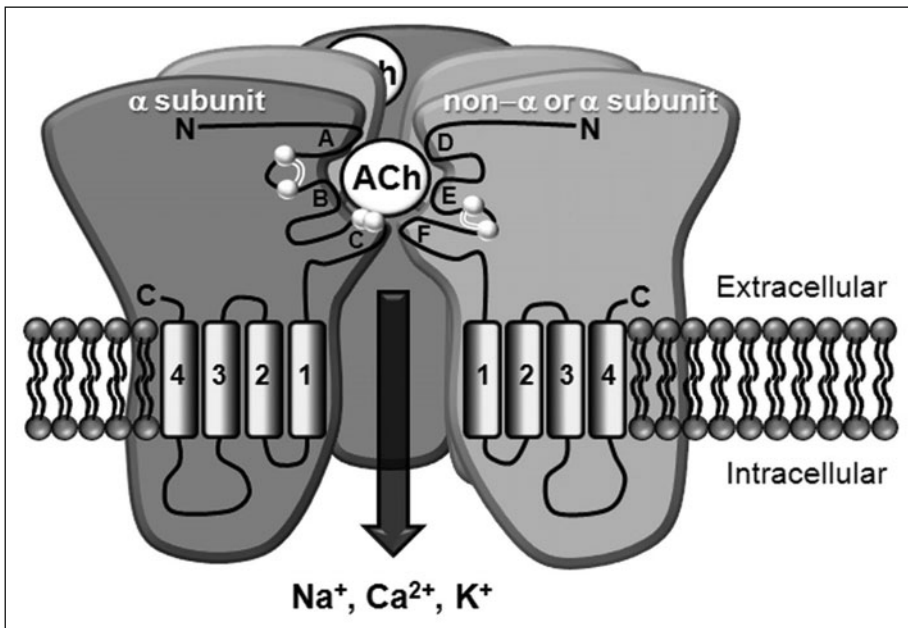


Figure 1. Structure of the nicotinic acetylcholine receptor. Schematic representation of a heteromeric receptor consisting of two  $\alpha$  (dark grey) and three non- $\alpha$  subunits (light grey). The polypeptide layout of two subunits are shown highlighting the Cys-loop (two white circles connected by a white double line), the two vicinal cysteines in loop C defining  $\alpha$  subunits and four transmembrane domains (TM1-4) with a large intracellular loop between TM3 and TM4. The six binding loops (A-F) that contribute to ligand binding are shown and two acetylcholine (ACh) molecules are bound to this particular nAChR. The five subunits that make up the receptor are arranged around a central cation-permeable channel.

nAChRs can exist as homomers of  $\alpha$  subunits or as heteromers of either two kinds of  $\alpha$  subunit, or, more commonly, of various combinations of  $\alpha$  and non- $\alpha$  subunits.<sup>8</sup> The subunit composition determines the functional and pharmacological properties of the nAChR, thus receptor diversity is generated by multiple-subunit-encoding genes in a given organism.

Although no crystal structure of a full nAChR is currently available, the structure of the marine ray *Torpedo marmorata* electric organ nAChR has been resolved at 4.0 Å providing valuable insights into the three-dimensional structure of a cysLGIC.<sup>9</sup> More information has been added by further crystal structures such as that obtained at 1.94 Å for the N-terminal extracellular domain of the mouse  $\alpha 1$  muscle subunit.<sup>10</sup> The  $\alpha 1$  subunit is bound to  $\alpha$ -bungarotoxin, a snake toxin which was used in the first purification of a nAChR<sup>11</sup> and the crystal structure has provided important insights into protein-protein and protein-sugar interactions of the subunit-toxin complex.<sup>10</sup> In addition, the crystal structure of an ACh binding protein (AChBP) from the pond snail *Lymnaea stagnalis* that shares homology with the extracellular N-terminal region of the nicotinic acetylcholine receptor has been determined at 2.7 Å.<sup>12</sup> Also, the X-ray structure of a bacterial LGIC with a layout of five subunits similar to cysLGICs has been determined at 3.3 Å<sup>13</sup> accelerating the exciting prospect of a crystal structure for a complete eukaryotic nAChR. These structures have considerably enhanced our understanding of receptor function by permitting the construction of three-dimensional homology models of cysLGICs and the computational simulation of receptor dynamics as well as agonist docking in the ligand-binding site.

### Nicotinic Acetylcholine Receptors—Roles in Human Disease and as Drug Targets

The human nAChR family consists of 16 genes encoding 10  $\alpha$  and 6 non- $\alpha$  subunits.<sup>8</sup> There are separate families of muscle and neuronal nAChRs and the striking differences in pharmacological properties of nicotinic receptor subtypes found in different cells and tissues are mainly attributed to differences in their subunit composition. The importance of nAChRs is highlighted by their involvement in genetic and autoimmune disorders.<sup>14</sup> For instance, mutations in neuronal nAChR subunits  $\alpha 4$  and  $\beta 2$  are associated with autosomal dominant nocturnal frontal lobe epilepsy<sup>15</sup> while mutations in muscle nAChR subunits ( $\alpha$ ,  $\beta$ ,  $\delta$  and  $\epsilon$ ) underlie muscle weakening congenital myasthenia syndromes.<sup>16</sup> Examples of autoimmune diseases include myasthenia gravis where auto antibodies target muscle nAChRs<sup>17</sup> while auto antibodies to  $\alpha 7$  nAChRs, which function in the central nervous system, result in Rasmussen's encephalitis.<sup>18</sup> nAChRs play important roles in brain function and are the focus of research investigating them as targets for drugs designed to treat nicotine addiction, Alzheimer's disease, Parkinson's disease and Schizophrenia.<sup>19</sup>

### Insect Nicotinic Acetylcholine Receptors—Targets for Pest Control

Invertebrate nAChRs are of interest as they are effective targets for pest control. Nematode (worm) parasites infect a billion people and also cause many serious diseases in livestock as well as crop damage. Levamisole, pyrantel and morantel are anthelmintics (drugs used to control worm parasites) which target nAChRs functioning in body wall muscles of nematodes<sup>20</sup> and recently a novel class of drugs (the amino-acetonitrile derivatives) has been developed which target a different nAChR subtype of nematodes.<sup>21</sup>

With ACh being an abundant neurotransmitter in the nervous systems of many insect species, including the fruit fly, *Drosophila melanogaster*,<sup>22</sup> nAChRs are targeted by chemicals used for insect control, such as neonicotinoids<sup>23</sup> which have been the fastest-growing class of insecticides in modern crop protection. Imidacloprid [1-(6-chloro-3-pyridylmethyl)-2-nitroimino-imidazolidine] and other neonicotinoids now have worldwide annual sales of around \$US 1.56 billion, representing nearly 17% of the global insecticide market.<sup>24</sup> It has

been demonstrated that imidacloprid binds with high affinity to membrane preparations from diverse insects.<sup>25-27</sup> Radioligand binding and electrophysiological studies have demonstrated that imidacloprid shows agonist actions on nAChRs in cockroach neurons and displaces <sup>125</sup>I  $\alpha$ -bungarotoxin binding from central nervous system membranes.<sup>28</sup> Calcium imaging has demonstrated that imidacloprid acts on nAChRs in *D. melanogaster* cholinergic neurons<sup>29</sup> and whole-cell patch-clamp studies on the same neurons show imidacloprid to be a partial agonist.<sup>30</sup> The effectiveness of neonicotinoids as safe insecticides has been attributed, at least in part, to the selectivity for insect nicotinic receptors over mammalian nAChRs<sup>31</sup> and indeed the binding affinity of neonicotinoids to nAChRs correlates well with insecticidal efficacy.<sup>32</sup>

**The nAChR Gene Family in a Genetic Model Organism, the Fruitfly *Drosophila melanogaster***

***The First Complete Insect nAChR Gene Family To Be Described***

The first sequences of nAChR subunits, those of the electric rays *Torpedo californica* and *Torpedo marmorata*, were published in the early 1980s.<sup>33-37</sup> Subsequently, it has been shown that nAChR subunits from a variety of organisms are highly homologous sharing considerable amino acid identity.<sup>6</sup> This enabled development of Torpedo DNA probes to isolate *D. melanogaster* cDNA clones in a hybridisation screen which led to the determination of the first insect nAChR subunit sequence.<sup>38</sup> As summarised in Table 1, nine further *D. melanogaster* nAChR subunits were identified over the

**Table 1. Summary of all 10 *D. melanogaster* nAChR subunits which are listed in the order their sequences were published. The techniques used to determine their sequences are included**

Subunit Name	Technique Used to Identify Subunit	Year Published
D $\beta$ 1 or ARD (acetylcholine receptor <i>Drosophila</i> )	Hybridisation screen using Torpedo $\alpha$ and $\gamma$ nAChR subunit probes	1986 <sup>38</sup>
D $\alpha$ 1 or ALS (alpha-like subunit)	Hybridisation screen using chicken $\alpha$ 2 nAChR subunit probe	1988 <sup>39</sup>
D $\alpha$ 2 or SAD (second alpha-like subunit <i>Drosophila</i> )	Hybridisation screen using D $\alpha$ 1 nAChR subunit probe <sup>41</sup> or conserved 10 amino acid region preceding TM4 <sup>44</sup>	1990 <sup>41,44</sup>
D $\beta$ 2 or SBD (second beta-like subunit <i>Drosophila</i> )	Hybridisation screen using genomic clone probe isolated in ref. <sup>44</sup>	1990 <sup>45</sup>
D $\alpha$ 3	Hybridisation screen using D $\alpha$ 1 nAChR subunit probe and conserved 10 amino acid region preceding TM4	1998 <sup>46</sup>
D $\alpha$ 4	PCR using primers based on an EST clone	2000 <sup>42</sup>
D $\beta$ 3	Sequencing of an EST clone based on a gene predicted in the <i>D. melanogaster</i> genome	2002 <sup>43</sup>
D $\alpha$ 5	BLAST analysis <sup>124</sup> against <i>D. melanogaster</i> genome sequence	2002 <sup>40</sup>
D $\alpha$ 6	BLAST analysis <sup>124</sup> against <i>D. melanogaster</i> genome sequence	2002 <sup>40</sup>
D $\alpha$ 7	BLAST analysis <sup>124</sup> against <i>D. melanogaster</i> genome sequence	2002 <sup>40</sup>

next two decades.<sup>38–46</sup> During this time the *D. melanogaster* genome was sequenced<sup>47</sup> which greatly facilitated the identification of nAChR subunits, changing the strategy of isolating subunits from hybridisation screening to genome sequence analysis (Table 1). With the genome sequence available, it was shown that the complete fruit fly nAChR gene family consists of 10 subunits, seven of which are  $\alpha$  ( $\alpha 1$ – $\alpha 7$ ) and three are non- $\alpha$  ( $\beta 1$ – $\beta 3$ ).<sup>48</sup> Considering that humans possess 16 subunits<sup>8</sup> and the nematode worm *Caenorhabditis elegans* has at least 29,<sup>49</sup> the nAChR gene family of *D. melanogaster* is rather compact. However, as described in section 4, alternative splicing and RNA editing considerably increases the number of insect nAChR gene products.

### ***Distribution and Assembly of Drosophila AChRs***

The localisation of many gene products throughout the *Drosophila* body can be determined by immunohistochemistry or in situ hybridisation. These techniques have been used to show that several *Drosophila* nAChR subunits ( $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 7$ ,<sup>50</sup>  $\beta 1$  and  $\beta 2$ ) have overlapping distributions in various regions of the nervous system (for review see ref. 96 and refs. therein). A lot is known about the subunit composition of vertebrate nAChRs.<sup>8</sup> Unfortunately, this is not the case for *Drosophila*, largely due to the fact that expression of functional receptors in heterologous systems has so far been unsuccessful. Several studies, however, have provided clues regarding the assembly and functions of certain subunits (for review see ref. 96 and refs. therein). Thus, based on immunoprecipitation experiments, overlapping expression patterns and pharmacological properties of hybrid receptors consisting of *Drosophila*  $\alpha$  subunits and vertebrate non- $\alpha$  subunits, Chamaon et al proposed three possible receptor complexes.<sup>51</sup> One contains at least  $\beta 1$  and  $\beta 2$ , another includes  $\beta 1$  and  $\alpha 3$  whilst in the third at least  $\alpha 1$ ,  $\alpha 2$  and  $\beta 2$  are present. As noted by the authors, the genes encoding  $\alpha 1$ ,  $\alpha 2$  and  $\beta 2$  form a directly linked cluster in the *Drosophila* genome which may facilitate coordinated expression and regulation of coassembly of the three subunits. Another report, using radioligand binding and co-immunoprecipitation studies in transfected *Drosophila* S2 cells, has suggested that  $\beta 3$  can coassemble with  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$  or  $\beta 2$ .<sup>43</sup>

### ***Role for $\alpha 7$ in Drosophila Escape Behaviour***

The powerful genetic toolkit available in the model organism, *D. melanogaster*, can yield insights into behavioural roles for individual nAChR subunits. This was demonstrated by a study which used immunohistochemistry to show that  $\alpha 7$  protein is enriched in the dendrites of the giant fiber system which serves as a reflex circuit that triggers escape behaviours.<sup>50</sup> A fly strain with mutated  $\alpha 7$  showed no obvious abnormalities when compared with wild-type flies but mutant adult flies did fail to exhibit the giant fiber-mediated startle response to a sudden change in light levels, indicating that  $\alpha 7$  mediates the *Drosophila* escape response.

### ***Uncovering the Actions of Imidacloprid and Spinosad Using Drosophila nAChRs***

Work with *Drosophila* nAChRs has implicated certain subunits as targets of imidacloprid action. This has involved the use of heterologous expression systems such as *Xenopus laevis* (African clawed frog) oocytes<sup>52</sup> or a *D. melanogaster* cell line (Schneider S2 cells)<sup>53</sup> to study functional receptors. Unlike vertebrate nAChRs, reconstituting functional insect nAChRs in heterologous systems has proven elusive. Nevertheless, the fact that several *Drosophila* nAChR subunits can form functional nAChRs when co-expressed with a vertebrate  $\beta 2$  subunit in *Xenopus* oocytes has been exploited to identify  $\alpha 1$  and  $\alpha 2$  as candidate imidacloprid targets since  $\alpha 1/\beta 2$  and  $\alpha 2/\beta 2$  hybrid nAChRs were more neonicotinoid sensitive than the complete vertebrate  $\alpha 4/\beta 2$  receptor.<sup>54,55</sup> Also, the partial agonist actions of imidacloprid (and super-agonist actions of the second generation neonicotinoid clothianidin) reported for native *Drosophila* receptors are mimicked in the  $\alpha 2/\beta 2$  hybrid.<sup>30</sup> This approach has been extended to study whether vertebrate nAChRs (usually  $\alpha 4/\beta 2$  or  $\alpha 7$ ) can be rendered more sensitive to neonicotinoids when insect nAChR-specific amino acids or

**Table 2. Orders and key roles of insect species that have their complete nAChR gene family described**

Species	Order	Importance	Genome Size (Mega Bases)	nAChR Subunit Gene Number
<i>A. gambiae</i>	Diptera	malaria vector	278	10
<i>A. mellifera</i>	Hymenoptera	pollination, honey production, social and behavioural model	262	11
<i>B. mori</i>	Lepidoptera	silk production, Lepidopteran model	429	12
<i>D. melanogaster</i>	Diptera	genetic model organism	118	10
<i>T. castaneum</i>	Coleoptera	pest of stored food, Coleopteran model	204	12

subunit regions have been introduced. In this way, residues in loops C,<sup>56</sup> D,<sup>57-59</sup> E<sup>60</sup> and F<sup>58</sup> as well as an insertion in loop F<sup>56</sup> have been shown to contribute to imidacloprid sensitivity. Also, Dβ1<sup>57</sup> and Dβ2<sup>59</sup> have been highlighted as additional subunits targeted by neonicotinoids. These studies using amino acid substitutions have led to the postulation that the formation of hydrogen bond networks plays a key role in neonicotinoid interactions.<sup>61</sup> Support for this view is also derived from structural studies in which snail AChBP bound with neonicotinoids has been crystallised.<sup>62,63</sup>

Spinosad is an insecticide which is derived from fermentation products of the soil dwelling bacterium *Saccharopolyspora spinosa*.<sup>64</sup> It acts on nAChRs but not at the same site as imidacloprid,<sup>65</sup> indicating they may act on separate nAChR types.<sup>66</sup> Indeed this is likely to be the case as a Dα6 knockout mutant strain of *D. melanogaster* was shown to be 1181-fold more resistant to spinosad than the control strain, identifying this subunit as a major spinosad target.<sup>67</sup>

**Characterisation of Complete nAChR Gene Families from Five Insect Species Spanning Over 300 Million Years of Evolution**

***A Core Group of nAChR Subunits Is Highly Conserved in Different Insect Species***

Since the publication of the *D. melanogaster* genome in 2000,<sup>47</sup> the genomes of several other insect species have since been sequenced. This information has so far been used to characterise the complete nAChR gene families from *Anopheles gambiae* (malaria mosquito),<sup>68,69</sup> *Apis mellifera* (honey bee),<sup>70,71</sup> *Tribolium castaneum* (red flour beetle)<sup>72,73</sup> and *Bombyx mori* (silk worm).<sup>74,75</sup> These species represent diverse orders which span over 300 million years of evolution (Table 2)<sup>73</sup> during which the nAChR gene families of these five insect species have remained compact consisting of 10 (*D. melanogaster* and *A. gambiae*), 11 (*A. mellifera*) or 12 (*B. mori* and *T. castaneum*) subunits.

Each of the five insect nAChR gene families has seven core groups of subunits that are highly conserved between species (Fig. 2).<sup>76</sup> Thus, Anopheles, Apis, Bombyx and Tribolium have subunit equivalents of Dα1-7, Dβ1 and Dβ2. The different insect species have the same number of core group subunits with the exception of *T. castaneum* which has an extra Dβ2-like subunit arising most likely through a gene duplication event (Fig. 2).<sup>72</sup> Dα5, Dα6 and Dα7 have been placed into a single group (Fig. 2) due to their considerable sequence homology with vertebrate α7 subunits (Table 3).<sup>76,77</sup> The presence of α7-like subunits also in nematodes<sup>78,79</sup> and trematodes<sup>80</sup> indicates an ancient lineage for this receptor subtype. The remaining insect



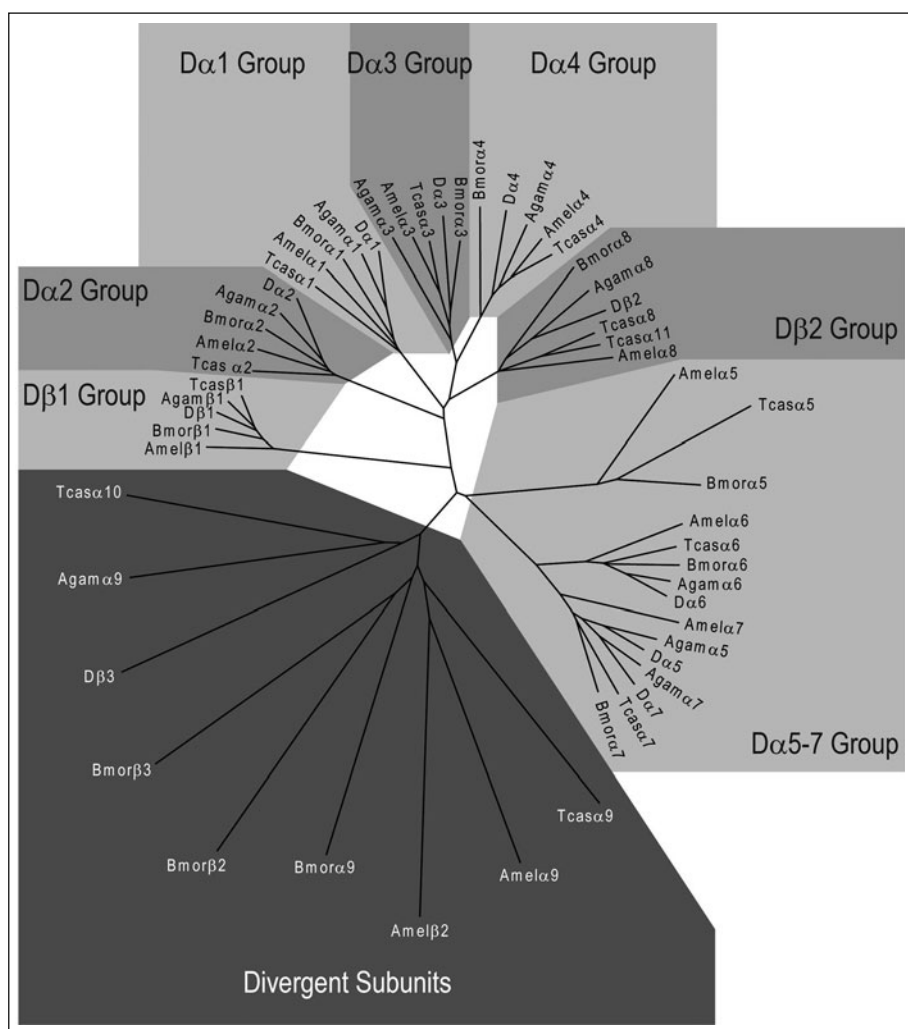


Figure 2. Tree showing the nAChR gene families *A. gambiae*, *A. mellifera*, *B. mori*, *D. melanogaster* and *T. castaneum*. Based on their high amino acid sequence homology, several insect nAChR subunits cluster into groups. Each insect possesses at least one divergent subunit that does not fall into any of these groups.

subunits do not have such close sequence relationships with those of vertebrates. Subunit homologs have also been found in other species such as *Ctenocephalides felis* (cat flea),<sup>81</sup> *Locusta migratoria* (migratory locust),<sup>82</sup> *Musca domestica* (house fly),<sup>83-85</sup> *Myzus persicae* (green peach aphid),<sup>86,87</sup> *Nilaparvata lugens* (brown plant hopper)<sup>88</sup> and *Schistocerca gregaria* (desert locust),<sup>89,90</sup> suggesting that the core groups are common to insects. Generally, equivalent nAChR subunits from different insect species have greater than 60% identity in their amino acid sequences. In addition to amino acid identity, distinct features are also conserved in core group nAChR subunits as summarised in Table 3. Interestingly, insect orthologs of Dβ2 are α subunits (e.g., Agamα8 and Amelα8 in Fig. 2), suggesting a change in functional role of the subunit in the *Drosophila* lineage.

**Table 3. Amino acid sequence features particular to insect nAChR subunits when comparing *Anopheles*, *Apis*, *Bombyx*, *Drosophila* and *Tribolium***

Group	% Sequence Identity to Closest Human Homolog	Notable Features
Dα1	38-40% to human α2	Polypeptide insert in loop F which is involved in ligand binding
Dα2	36-38% to human α2	Polypeptide insert in loop F which is involved in ligand binding
Dα3	28-40% to human α2	Polypeptide insert in loop F which is involved in ligand binding. Agamα3 and Dα3 have unusually long intracellular domains between TM3 and TM4
Dα4	38-39% to human α2	Polypeptide insert in loop F which is involved in ligand binding. Alternative splicing of exon 4
Dα5-7	42-46% to human α7 <i>Apis</i> , <i>Bombyx</i> and <i>Tribolium</i> α5 have lower identity	Insect α6 subunits have alternative splicing of exons 3 and 8. Insect α6 subunits have conserved and distinct RNA A-to-I editing except for Agamα6
Dβ1	39-40% to human α2	
Dβ2	38-40% to human α2	Polypeptide insert in loop F which is involved in ligand binding. Is an α subunit in non- <i>Drosophila</i> species
Divergent	12-22% to human α2	Short intracellular domain between TM3 and TM4. Several divergent subunits lack the GEK amino acid motif preceding TM2 which is important for cation selectivity

***Insect Species Possess a Distinct Complement of Divergent nAChR Subunits***

Analysis of the five complete nAChR gene families has shown that insects possess at least one divergent subunit (Fig. 2) that shows low sequence homology to all other known nAChR subunits (less than 29% identity). Unlike core group subunits, analogous divergent subunits in different insects are difficult to assign. In addition to low sequence homology, divergent subunits possess extremely small intracellular domains between TM3 and TM4 and several examples, particularly those of *B. mori*, lack the highly conserved GEK amino acid motif preceding TM2<sup>74</sup> which is important for cation selectivity.<sup>91</sup> These subunits do not possess amino acid residues known to confer anion selectivity but they may form nAChRs with distinct ion channel characteristics. Currently, little is known about divergent nAChR function although it has been shown that Dβ3 can co-assemble with other nAChR subunits and influence ligand binding.<sup>43</sup> Each of the five insect species possesses a different set of divergent nAChR subunits. For example, *T. castaneum* has two divergent subunits which are both α,<sup>72</sup> *A. mellifera* also possesses two divergent subunits but one is α and the other β<sup>70</sup> whilst there are three divergent subunits (one α and two β) in *B. mori*.<sup>74</sup> Thus, the divergent subunits may perform species-specific roles and therefore be of interest as targets to control insect pests while sparing beneficial species.



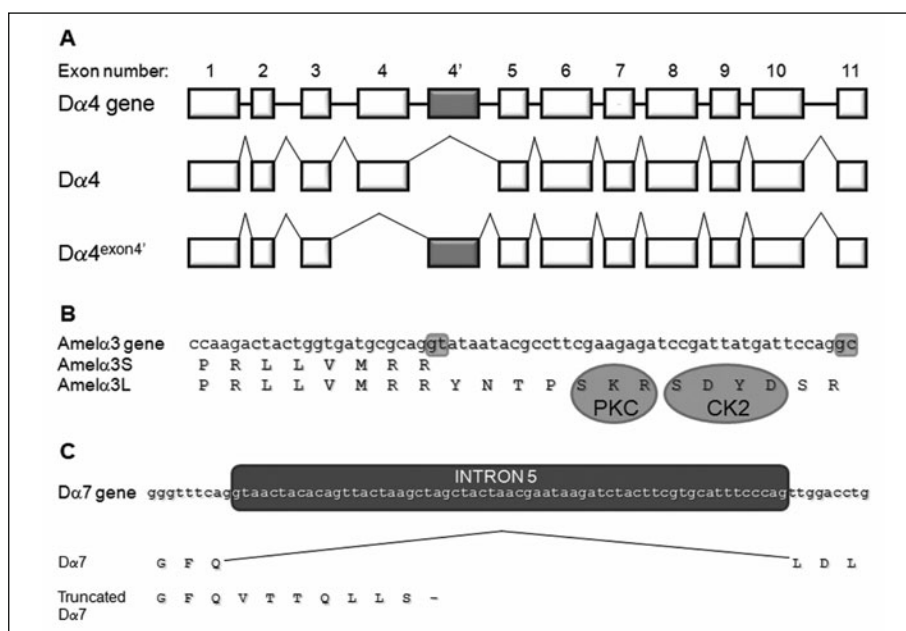


Figure 3. Examples of different forms of alternative splicing in insect nAChR subunits. A) Alternative splicing of exons. Dα4 possesses two alternatives for exon 4 (denoted exon 4 and exon 4')<sup>42</sup> which most likely arose through tandem exon duplication.<sup>125</sup> B) Use of different splice donor sites (highlighted in grey boxes) in the Amelα3 gene generates two intracellular domains differing in size by 13 amino acids.<sup>70</sup> The long variant (Amelα3L) has extra protein kinase C (PKC) and casein kinase 2 (CK2) phosphorylation sites which may regulate various aspects of receptor function.<sup>94,95</sup> C) A truncated variant of Dα7 is generated by the failure to excise intron 5 which introduces a premature stop codon (shown as a dash).<sup>40</sup>

## RNA Editing and Alternative Splicing Broadens the Insect Nicotinic “Receptorome”

### Alternative Splicing

Two *Drosophila* nAChR subunits (Dα4 and Dα6)<sup>40,42</sup> and their orthologs in other species have exons that are alternatively spliced, which effectively substitutes amino acids in regions important for receptor function and assembly.<sup>69,70,72,74</sup> For example, insect α4 subunits have two alternatives for exon 4 (denoted exon 4 and exon 4')<sup>42</sup> with different residues within, or in the vicinity of, the Cys-loop, which has been shown to be important for complete receptor assembly (Fig. 3).<sup>4</sup> Consistent with this, radioligand-binding assays indicate that Dα4 containing exon 4' assembles less efficiently than subunits with exon 4.<sup>42</sup> Interestingly, RT-PCR analysis revealed that the two Amelα4 splice variants are differentially expressed throughout the honey bee life cycle with exon 4 variants present at each developmental stage whereas exon 4' variants were detected only in pupae and adults.<sup>70</sup> This suggests that exon 4' subunits may serve to modulate receptor assembly in the later stages of honeybee development. Conservation of alternative splicing can also be seen in insect α6 subunits for exons 3 and 8<sup>40,69,70,72,74</sup> although the number of alternative exons can vary between species. For example, Agamα6 and Bmorα6 have two alternatives for exon 8<sup>69,92</sup> while Amelα6 and Dα6 have three.<sup>40,92</sup> Different residues introduced in functionally significant regions through alternative splicing can also vary between species. In one case, alternative splicing of Agamα6 exon 8 substitutes a valine for a leucine in the TM2

domain which lines the ion channel, whereas the leucine residue is conserved in all splice variants of Amel $\alpha$ 6, Bmor $\alpha$ 6, D $\alpha$ 6 and Tcas $\alpha$ 6.<sup>69,92</sup> A mutation of the equivalent leucine in chick  $\alpha$ 7 to valine resulted in nAChRs with a reduced rate of desensitisation and an enhanced sensitivity to ACh.<sup>93</sup> Thus, alternative splicing may generate nAChR subunit isoforms with functional properties particular to certain insect species. Alternative splicing of exons can also be species specific. For example, Bmor $\alpha$ 8 is the only known member of the D $\beta$ 2 group to have alternative splicing of exon 7, which introduces variation in TM2 and TM3 thereby potentially giving rise to variants with distinct ion channel properties.<sup>74</sup>

Species-specific nAChR subunit isoforms can also be generated through the differential use of splice sites, as in Amel $\alpha$ 3 where two variants (long and short forms) have TM3-TM4 intracellular loops that differ in length by 13 amino acid residues (Fig. 3).<sup>70</sup> The long form has two extra putative phosphorylation sites which may have an effect on receptor properties since phosphorylation of the TM3-TM4 intracellular loop regulates several aspects of receptor function such as desensitisation and aggregation and could affect the action of insecticides.<sup>77,94,95</sup>

For several insect nAChR subunits, truncated transcripts have been detected where an exon is missing or where premature stop codons have been introduced either by omission of an exon which results in a frame shift or lack of splicing an intron (Fig. 3).<sup>40,42,69,70,74,96</sup> It remains to be determined whether the truncated transcripts are translated into proteins *in vivo* and if so it will be of interest to determine their role. It has been suggested that they may act as an 'ACh sponge' serving to terminate cholinergic transmission in a manner similar to that of the molluscan ACh-binding protein<sup>40,96,97</sup> although their ability to interact with ACh is questionable since all truncated subunits, with the exception of truncated Amel $\alpha$ 3,<sup>70</sup> lack at least one loop involved in ligand binding. Another possible role is to regulate receptor expression similar to a truncated variant of the mouse  $\alpha$ 7 nAChR subunit which acts as a dominant negative when cotransfected with full length  $\alpha$ 7 in HEK 293 cells.<sup>98</sup>

### RNA Editing

RNA A-to-I editing involves the modification of select adenosine (A) residues to inosine (I) in pre-mRNA transcripts by adenosine deaminases acting on RNA (ADARs).<sup>99</sup> Since inosine is interpreted by cellular machineries as guanosine (G), A-to-I editing generates transcripts with a nucleotide composition different from that of the corresponding genomic DNA (Fig. 4). This has the potential to alter amino acid residues thus generating multiple protein isoforms. RNA editing occurs particularly in gene products which are involved in neuronal signaling,<sup>100</sup> consistent with neurological phenotypes observed for ADAR-deficient *Caenorhabditis elegans*,<sup>101</sup> *Drosophila melanogaster*<sup>102</sup> and mice.<sup>103</sup>

RNA A-to-I editing has been observed in five *D. melanogaster* nAChR subunits which alter amino acid residues in functionally significant regions.<sup>40,50,100</sup> For example, editing of D $\alpha$ 5, D $\alpha$ 7 and D $\beta$ 2 alters residues in the TM2, 3 and 4 domains, thereby potentially affecting ion channel characteristics.<sup>6,104,105</sup> RNA editing may also affect the ligand binding properties of two subunits (D $\alpha$ 6 and D $\beta$ 1) since residues are altered in the extracellular N-terminal region. RNA editing is less widespread in nAChR subunits of other insect species. For instance, two *T. castaneum* nAChR subunits (Tcas $\alpha$ 6 and Tcas $\beta$ 1) are edited<sup>72</sup> whilst in *A. mellifera* RNA editing was only seen in Amel $\alpha$ 6.<sup>70</sup> RNA editing of Amel $\alpha$ 6 alters nine amino acid residues in a confined area located in the vicinity of loop E.<sup>70,92</sup> Up to five of these residues are also altered through editing in other insects such as *B. mori*, *D. melanogaster*, *H. viriscens*, *M. domestica* and *T. castaneum* (Fig. 4).<sup>85,92</sup> Interestingly, conserved editing in  $\alpha$ 6 of different species removes an N-glycosylation site in loop E which may affect receptor maturation, channel desensitisation and conductance.<sup>106,107</sup> The reverse appears to be the case for *M. domestica* where the equivalent N-glycosylation site is created through editing of asparagine to serine (Fig. 4).<sup>85</sup> Several editing sites in Amel $\alpha$ 6, however, are not conserved in other insects and no RNA editing at all was

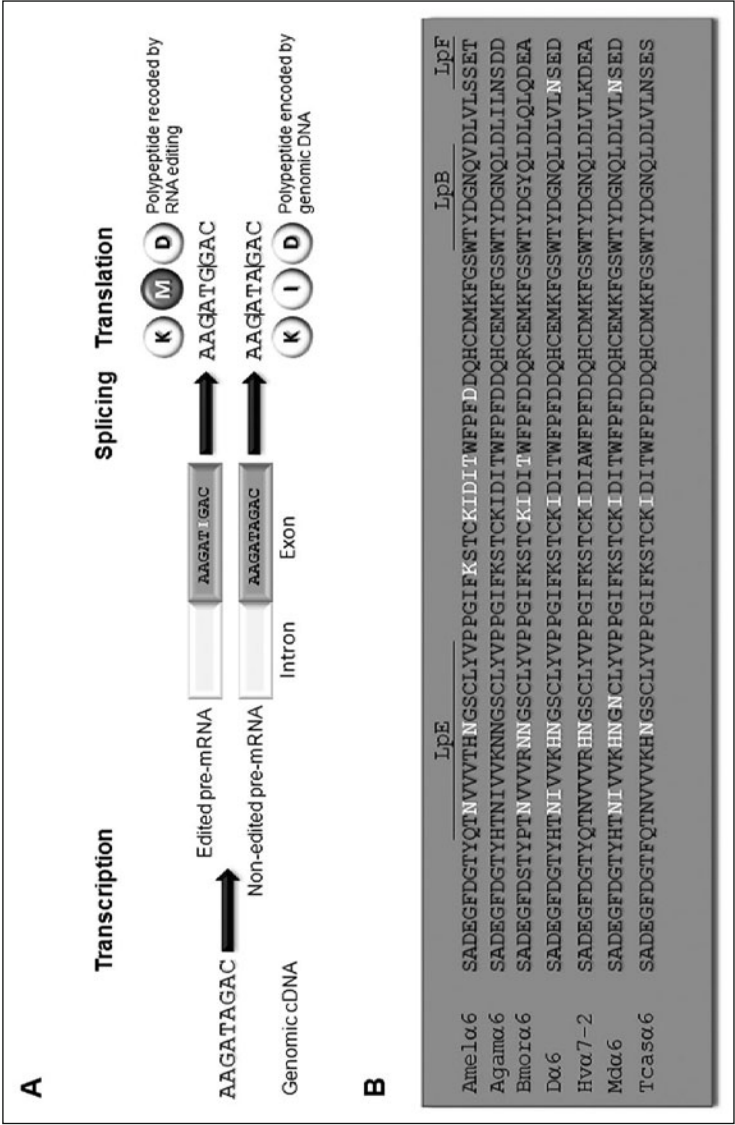


Figure 4. RNA A-to-I editing recodes the genome. A) Schematic of the RNA editing process. Select adenosine (A) residues in pre-mRNA are modified to inosine (I) by adenosine deaminases acting on RNA (ADARs). Since inosine is interpreted by cellular machineries as guanosine (G), A-to-I editing generates mRNA transcripts with a nucleotide composition that differs from the corresponding genomic DNA. This has the potential to alter amino acid residues thus generating multiple protein isoforms. B) A comparison of RNA A-to-I editing in Dα6 with orthologs in *A. gambiae*, *A. mellifera*, *B. mori*, *H. virescens*, *M. domestica* and *T. castaneum*. Ligand binding domains LpB, LpE and LpF are indicated and amino acids altered by RNA editing are highlighted in white text.

detected in Agam $\alpha$ 6 of *A. gambiae*.<sup>69</sup> Thus, RNA editing can generate species-specific nAChR subunit isoforms. It has been observed that genomically-encoded guanosines in certain insect  $\alpha$ 6 subunits including Agam $\alpha$ 6 are in fact A-to-I editing sites in other species leading to the suggestion that RNA editing maintains phylogenetic conservation while broadening protein diversity possibly as part of an evolutionary mechanism.<sup>92,108</sup> Studies on *Drosophila* have shown that RNA editing is particularly important in the nervous system function of adults.<sup>102</sup> This may hold true for other insect species since the greatest extent of nAChR RNA editing was observed in adults of *A. mellifera*.<sup>70</sup>

## Conclusion and Prospects

### *Small Gene Families with Large-Scale Proteome Diversity*

Characterisation of the first two complete insect nAChR gene families, those from the diptera *D. melanogaster* and *A. gambiae*, revealed a small complement of subunits numbering 10 in both species.<sup>48,69,96</sup> It was surprising that the third insect nAChR gene family to be described, that of the hymenoptera *A. mellifera*, possessed a similar number of subunits (11)<sup>70</sup> since the honey bee displays a far more complex behavioural repertoire than either the fruit fly or malaria mosquito. With the characterisation of *B. mori*<sup>74</sup> and *T. castaneum*<sup>72</sup> nAChR subunits, a consensus emerged that insect nAChR gene families remained compact over 300 million years of evolution. However, while the gene numbers are relatively small compared to other organisms, the number of insect nAChR gene products can be much larger due to alternative splicing and RNA editing which have the potential to generate a receptor proteome with diversity far greater than that suggested by the number of genes alone. In addition to broadening the nAChR proteome in a given insect species, alternative splicing and RNA editing generates species-specific subunit variants with potentially distinct functional characteristics. Since subunit composition determines nAChR pharmacological and functional characteristics, a major goal in determining proteome diversity would be to elucidate the stoichiometry of subunits and their isoforms in insect nAChRs in vivo.

### *Upcoming New nAChR Gene Families of Interest*

Genome projects have either been completed or are in progress enabling the characterisation of nAChR gene families from other insect species. For example, the genome sequences of 12 *Drosophila* species were published in 2007<sup>109</sup> providing a far greater scope for comparative genome data analysis and studying with fine resolution nAChR diversity in a single phyla. The yellow and dengue fever mosquito *Aedes aegypti* genome has also been published<sup>110</sup> allowing for comparative studies with the malaria mosquito as well as with other species. Genome projects currently underway include those of the West Nile virus mosquito *Culex pipiens* ([http://www.broad.mit.edu/annotation/genome/culex\\_pipiens.4/Info.html](http://www.broad.mit.edu/annotation/genome/culex_pipiens.4/Info.html)), the human body louse *Pediculus humanus humanus*,<sup>111</sup> the pea aphid *Acyrthosiphon pisum* (<http://www.hgsc.bcm.tmc.edu/projects/aphid/>) which is an agricultural pest and the parasitoid wasp *Nasonia vitripennis* (<http://www.hgsc.bcm.tmc.edu/projects/nasonia/>) which is an important organism in the biological control of insect pests. Sequence information produced from such projects will provide further insights into the diversity of insect nAChR gene families. Cross hybridisation approaches still nevertheless have an important role to play in determining nAChR sequences of insects for which no genome information is currently available. For example, the cockroach *Periplaneta americana* played an important role as an early insect neurobiology model providing access to an identified cholinergic synapse and thereby facilitating combined biochemical and electrophysiological studies.<sup>112-114</sup> This orthopteran species is now being explored by Lapiéd and colleagues to determine the members of the nAChR family and their functional roles.

### ***Behavioural Studies, Forward and Reverse Genetics in Dissecting Functional Roles of nAChRs***

Genome sequence information and well-characterised nAChR gene families provide an invaluable basis for the further study of nAChR functional diversity. As we have illustrated the study of *D. melanogaster* mutants has the potential to pinpoint single nAChR subunits either in particular behavioural roles or as insecticide targets. These are examples of forward genetics which aim to find the genetic basis of a phenotype or trait. *B. mori* is the second most widely used genetic model insect after *D. melanogaster* due to the ease of their rearing and the availability of mutants from genetically homogenous inbred lines serve as a potentially useful tool for forward genetic studies.<sup>75</sup>

Reverse genetics, as the name implies, proceeds in the opposite direction of forward genetics by seeking to determine possible phenotypes arising from a specific DNA sequence. This is usually achieved by knocking down the function of a gene of interest. An example involves creating *D. melanogaster* mutants for D $\alpha$ 7 by using P-elements to assess the role of the subunit in vivo.<sup>50</sup> RNA interference (RNAi) is a powerful reverse genetics approach, first characterised in *C. elegans*, involving the introduction of double-stranded RNA which results in silencing of the corresponding gene.<sup>115</sup> In 2003, a genome-wide RNAi screen was published using *C. elegans* which involved silencing 16,757 genes (corresponding to approximately 86% of the genome) in a general survey of gene function.<sup>116</sup> More recently, genome-wide screens covering over 90% of the *D. melanogaster* genome have been applied to *Drosophila* cells to identify genes playing roles in specific processes, one example being neural outgrowth.<sup>117</sup> No nAChR subunits were implicated in this study but a similar screen specifically addressing cholinergic signalling may reveal the importance of various nAChR subunits as well as identify novel genes involved in nAChR signalling. Parental RNAi, where RNA interference arising from double-stranded RNA introduced into the mother also spreads to the offspring, is highly efficient in *T. castaneum*.<sup>73</sup> Thus, the beetle provides a powerful tool for studying nAChR gene function in an insect pest species.

*A. mellifera* is a key model for social behaviour as well as learning and these features have been exploited in studies of the involvement of nAChRs in honey bee behaviour. Injection of the nAChR agonist, nicotine, showed that potentiation of the cholinergic system improves short-term memory<sup>118</sup> and injection of the nAChR antagonist, mecamylamine, inhibited olfactory learning or memory recall depending on the site of injection.<sup>119,120</sup> It has also been demonstrated that one distinct nAChR subtype, which is sensitive to the antagonist  $\alpha$ -bungarotoxin, is involved in long-term memory, whereas a second subtype, which is insensitive to  $\alpha$ -bungarotoxin but is affected by mecamylamine, plays a role in retrieving information stored during single-trial learning.<sup>121</sup> Interestingly, this mirrors to a certain extent the mammalian central nervous system where there are two predominant nAChR subtypes,  $\alpha$ 7 and  $\alpha$ 4/ $\beta$ 2 receptors, that are  $\alpha$ -bungarotoxin sensitive and insensitive, respectively, both of which play a role in memory.<sup>122</sup> The development of compounds known to target specific honey bee nAChRs will allow these behavioural studies to be performed with finer resolution to elucidate the role of particular subunits in various aspects of behaviour.

### ***Towards a New Era of Improved, Safer Pesticide Design***

The characterisation of complete insect nAChR gene families has shown that while it is evident that most nAChR subunits are highly conserved between diverse insect species, alternative splicing and RNA editing as well as the presence of divergent subunits present species-specific isoforms which can perhaps be exploited for the development of compounds that target particular insects pests such as *A. gambiae* and *T. castaneum* while sparing beneficial insects such as *A. mellifera* and *B. mori*. Computer three-dimensional models of insect nAChRs have been

generated based on the snail AChBP which permit docking experiments to assess interactions with compounds of interest.<sup>96</sup> Also, the *T. marmorata* nAChR X-ray structure was used to build models of five theoretical subtypes of *A. mellifera* nAChRs ( $\alpha 1/\beta 1$ ,  $\alpha 3/\beta 2$ ,  $\alpha 4/\beta 2$ ,  $\alpha 6/\beta 2$  and  $\alpha 9$ ).<sup>123</sup> Docking simulations showed that both imidacloprid and the insecticide fipronil, which blocks GABA-gated chloride channels, bind to the honey bee nAChRs with the involvement of numerous hydrogen bonds and hydrophobic interactions, the number of which varied depending on receptor subtype. Now that crystal structures are available for AChBP with imidacloprid and other neonicotinoids docked,<sup>62,63</sup> further improvements of such models can be anticipated.

A major goal yet to be achieved which would greatly facilitate the search for improved/novel insecticides is the successful expression of functional insect nAChRs in heterologous systems such as *Xenopus laevis* oocytes or cell lines. This would enable testing of numerous compounds on nAChRs of known subunit composition. Together with molecular modelling, this would likely prove invaluable in screening for compounds that show selectivity for specific nAChR subtypes, thereby enhancing safety and providing guidelines for minimising adverse effects on beneficial species, as well as facilitating an improved understanding of insecticide-receptor interactions.

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