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Letter

The nicotinic acetylcholine receptor gene family of the honey bee, *Apis mellifera*

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Nicotinic acetylcholine receptors (nAChRs) mediate fast cholinergic synaptic transmission and play roles in many cognitive processes. They are under intense research as potential targets of drugs used to treat neurodegenerative diseases and neurological disorders such as Alzheimer's disease and schizophrenia. Invertebrate nAChRs are targets of anthelmintics as well as a major group of insecticides, the neonicotinoids. The honey bee, *Apis mellifera*, is one of the most beneficial insects worldwide, playing an important role in crop pollination, and is also a valuable model system for studies on social interaction, sensory processing, learning, and memory. We have used the *A. mellifera* genome information to characterize the complete honey bee nAChR gene family. Comparison with the fruit fly *Drosophila melanogaster* and the malaria mosquito *Anopheles gambiae* shows that the honey bee possesses the largest family of insect nAChR subunits to date (11 members). As with *Drosophila* and *Anopheles*, alternative splicing of conserved exons increases receptor diversity. Also, we show that in one honey bee nAChR subunit, six adenosine residues are targeted for RNA A-to-I editing, two of which are evolutionarily conserved in *Drosophila melanogaster* and *Heliothis virescens* orthologs, and that the extent of editing increases as the honey bee lifecycle progresses, serving to maximize receptor diversity at the adult stage. These findings on *Apis mellifera* enhance our understanding of nAChR functional genomics and provide a useful basis for the development of improved insecticides that spare a major beneficial insect species.

[Supplemental material is available online at www.genome.org. Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. DQ026031–DQ026039.]

The honey bee, *Apis mellifera*, is an important beneficial insect in agriculture. In addition to producing honey and beeswax, the contribution of *A. mellifera* to crop pollination is valued at more than $14 billion dollars per year in the U.S. alone (United States Department of Agriculture http://www.ars.usda.gov/main/main.htm). Honey bees live in societies of considerable complexity and thus are studied as models for social behavior (Robinson et al. 1997).

The neonicotinoids are the newest major group of insecticides, which includes acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid, and thiamethoxam (Tomizawa and Casida 2005). The worldwide annual sales of neonicotinoids amounts to ~1 billion dollars, and they are used against piercing-sucking pests (aphids, leafhoppers, and whiteflies) of major crops. In France, the use of imidacloprid has been suspended over concerns that it may be having a drastic effect on bee populations (http://www.pan-uk.org/press/pr140604.htm), highlighting the importance that effective insecticides should also show selectivity within insects so that pollinators such as *A. mellifera* are spared. While the link between imidacloprid use and bee population decline has yet to be proven, studies have shown that imidacloprid is highly toxic to *A. mellifera* (Suchail et al. 2004) and at sublethal doses can alter honey bee foraging and learning (Guez et al. 2001; Lambin et al. 2001; Decourtye et al. 2004). Neonicotinoids act as agonists on their molecular targets, nicotinic acetylcholine receptors (nAChRs) (Matsuda et al. 2001), which are prototypical members of the cys-loop ligand-gated ion channel (LGIC) superfamily (Karlin 2002). The fast actions of acetylcholine (ACh) at synapses are mediated by nAChRs, which consist of five homologous subunits arranged around a central ion channel (Corringer et al. 2000; Unwin 2005). Analyses of completed genomes have revealed diverse nAChR gene families with mammals possessing 16 subunit genes, chicken, 17 (Millar 2003), *Fugu rubripes*, 28 (Jones et al. 2003), and *Caenorhabditis elegans*, at least 27 (Jones and Sattelle 2004). In contrast, *Drosophila melanogaster* and *Anopheles gambiae* have notably smaller nAChR gene families, each consisting of 10 subunits (Jones et al. 2005; Sattelle et al. 2005).

To date, four *A. mellifera* nAChR subunits (Apisα2, Apisα3, Apisα7-1, and Apisα7-2) have been identified (Thany et al. 2003, 2005), which are expressed in brain structures that play roles in learning and memory, olfactory signal processing, mechanosensory antennal input, and visual processing. These findings are consistent with ACh being a major excitatory neurotransmitter in the insect nervous system (Breer and Sattelle 1987; Lee and O'Dowd 1999). Patch clamp studies have demonstrated the existence of a distinct nAChR subtype in the honey bee nervous system that is blocked by the nAChR antagonists α-bungarotoxin (α-Btx), dihydroxy-β-erythroidine and methyllycaconitine, while nicotine and imidacloprid acted as partial agonists on this receptor (Goldberg et al. 1999; Déglise et al. 2002; Wustenberg and Grunewald 2004). Another study has shown the presence of two nAChR populations that differ in their responses to imidacloprid

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Figure 1. Protein sequence alignment of *A. mellifera* nAChR subunits. Dα1 of *D. melanogaster* is included for comparison. N-terminal signal leader peptides are underlined, and the loops implicated in ligand binding (LpA-F), as well as the four transmembrane regions (TM1–TM4) are indicated. The two cysteines forming the cys-loop are marked by asterisks and the vicinial cysteines characteristic of α subunits are highlighted in black shading. Putative N-glycosylation sites are boxed, potential cAMP, PKC, and CK2 phosphorylation sites are boxed with gray shading, and potential tyrosine kinase phosphorylation sites are enclosed in the gray-shaded ovals. Note that the Amelα3L, Amelα4 exon 4, and Amelα6 exon 8a splice variants are shown in the alignment.

but not ACh (Nauen et al. 2001). The involvement of nAChRs in honey bee behavior has also been investigated. Injection of the nAChR agonist, nicotine, showed that potentiation of the cholinergic system improves short term memory (Thany and Gauthier 2005) and injection of the nAChR antagonist, mecamylamine, inhibited olfactory learning or memory recall depending upon the site of injection (Lozano et al. 1996, 2001). Recently it has been demonstrated that one distinct nAChR subtype, which is α-Btx sensitive, is involved in long-term memory, whereas a second subtype, which is α-Btx insensitive, but is affected by mecamylamine, plays a role in retrieval processes (Dacher et al. 2005). Interestingly, this mirrors to a certain extent the mammalian central nervous system, where there are two predominant nAChR subtypes, the α7 and α4β2 receptors, that are α-Btx sensitive and insensitive, respectively, and both receptor subtypes play a role in memory (for review, see Hogg et al. 2003). Since individual nAChR subunits can confer distinct pharmacological properties on a receptor (Romanelli and Gualtieri 2003), the multiple nAChR subtypes present in the honey bee nervous system are likely to be determined by their subunit composition. Identifying the full complement of honey bee nAChR subunits represents a critical step in understanding the variety of roles played by nAChRs in the honey bee nervous system and the exquisite repertoire of bee behavior, as well as in identifying particular targets of chemical compounds. Here we have used the *A. mellifera* genome to describe the complete honey bee nAChR gene family.

Results

Existence of 11 candidate nAChR subunit genes in the *A. mellifera* genome

Using TBLASTN, we identified 11 candidate nAChR subunits in the *A. mellifera* genome. The complete open-reading frames of each subunit were confirmed and completed by RT–PCR and RACE–PCR. An alignment of their protein sequences shows that the honey bee nAChR candidate subunits possess features common to members of the cys-loop LGIC superfamily (Fig. 1). These include an N-terminal signal peptide sequence, an extracellular N-terminal region with conserved residues in loops A–F that are involved in ACh binding, the dicysteine loop (cys-loop) consisting of two disulphide-bond forming cysteines separated by 13 amino-acid residues, four transmembrane regions (TM1–TM4), and a highly variable intracellular loop between TM3 and TM4. As with other LGIC subunits, the *Apis* nAChR subunits also possess potential N-glycosylation sites within the extracellular N-terminal domain and phosphorylation sites within the TM3–TM4 intracellular loop. In nine of the candidate subunits, two adjacent cysteine residues that are required for ACh binding (Kao and Karlin 1986) are present in loop C, defining them as α subunits. Due to the absence of the vicinial subunits, the remaining two candidates are designated β subunits.

The honey bee nAChR subunits show substantial sequence similarity with known nAChR subunits, in particular, those of other insects. As shown in Table 1, *Apis* and *Drosophila* nAChR subunits can share up to 84% amino-acid identity. With regard to vertebrate nAChR subunits, they show 25%–38% identity. A phylogenetic tree demonstrating the relationship between *Apis* nAChR subunits and those of *Drosophila* and *Anopheles* indicates orthologous relationships between the honey bee and fruit fly/mosquito subunits (Fig. 2). Previously characterized *Apis* nAChR subunits were named based on their closest human homolog (e.g., Apisα3 after the human α3 subunit [Thany et al. 2003]). However, several honey bee subunits do not have clear human homologs. Thus, to facilitate comparison of insect nAChR gene families and maintain consistency in insect subunit nomenclature, all honey bee nAChR subunits have been named after their *Drosophila* and *Anopheles* counterparts (any alternative nomenclature of *Apis* subunits is given in Table 1). As with *Anopheles* (Jones et al. 2005), the *Apis* counterpart of Dβ2 is of the α type (Amelα8). As is the case for *Drosophila* and *Anopheles* (Grauso et

Table 1. Percentage identity/similarity between *A. mellifera* and *D. melanogaster* nAChR subunit protein sequences

Subunit	Amelα1	Amelα2	Amelα3	Amelα4	Amelα5	Amelα6	Amelα7	Amelα8	Amelβ1	Amelβ2	Amelα9
		(Apisα2)			(Apisα7–2)		(Apisα7–1)	(Apisα3)			
	9.7	9.7	5.24	7.22	15.8	2.27	14.14	5.9	14.14	9.3	9.3
Amelα1	—	47/62	51/63	49/61	27/41	30/44	29/44	49/62	34/49	10/27	12/27
Dα1	**71/77**	51/66	53/66	50/63	28/43	31/46	31/48	52/65	36/52	10/27	13/29
Amelα2	47/62	—	47/62	45/61	27/46	31/47	31/47	48/63	36/52	11/30	11/29
Dα2	46/58	**74/82**	46/61	43/59	27/44	30/48	30/47	47/62	36/53	11/29	12/29
Amelα3	51/63	47/62	—	64/74	28/42	31/46	32/46	54/65	38/54	11/27	13/29
Dα3	48/59	44/60	**70/78**	58/68	26/41	30/45	32/47	50/63	36/51	10/26	12/28
Amelα4	49/61	45/61	64/74	—	26/43	31/46	31/47	50/64	38/54	11/28	13/29
Dα4	47/59	43/60	62/71	**78/84**	26/43	30/46	30/47	51/63	35/52	11/29	13/30
Amelα5	27/41	27/46	28/42	26/43	—	33/52	30/48	27/44	29/48	15/32	17/33
Dα5	32/46	32/48	32/47	31/48	**34/52**	58/69	65/74	32/47	31/49	13/29	13/29
Amelα6	30/44	31/47	31/46	31/46	33/52	—	53/62	33/48	31/48	14/32	14/32
Dα6	29/42	30/47	31/45	31/46	34/54	**73/82**	56/66	34/48	31/47	14/32	14/32
Amelα7	29/44	31/47	32/46	31/47	30/48	53/65	—	30/46	31/49	13/30	12/28
Dα7	29/44	31/46	31/46	31/47	30/47	56/69	**63/71**	31/47	30/47	13/29	13/30
Amelα8	49/62	48/63	54/65	50/64	27/44	33/48	30/46	—	39/56	12/31	14/32
Dβ2	46/60	47/64	51/64	49/63	26/45	30/47	29/45	**70/82**	37/55	13/31	14/33
Amelβ1	34/49	36/52	38/54	38/54	29/48	31/48	31/49	39/56	—	13/32	13/30
Dβ1	35/51	37/54	38/54	37/53	29/48	31/50	30/49	39/56	**84/88**	13/31	13/30
Amelβ2	10/27	11/30	11/27	11/28	15/32	14/32	13/30	12/31	13/32	—	32/55
Dβ3	14/29	16/32	15/32	16/33	18/31	17/36	14/29	16/34	16/33	17/34	17/35
Amelα9	12/27	11/29	13/29	13/29	17/33	14/32	12/28	14/32	13/30	32/55	—

Alternative subunit nomenclature is given in parentheses and linkage groups are underlined. Proposed orthologs are shown in bold.

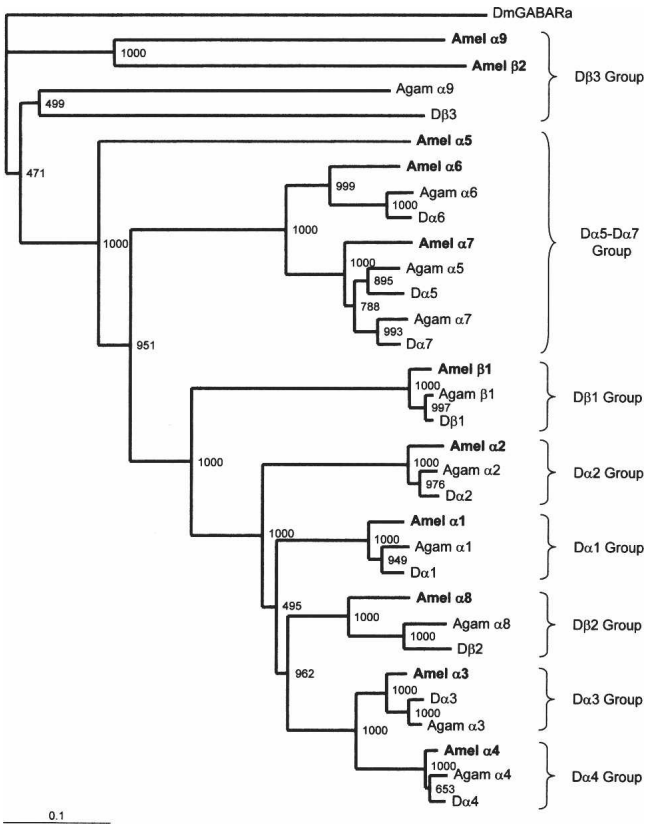

Figure 2. Tree showing relationships of *D. melanogaster*, *A. gambiae*, and *A. mellifera* nAChR subunit protein sequences. Numbers at each node signify bootstrap values with 1000 replicates and the scale bar represents substitutions per site. The *D. melanogaster* $GABA_A$ subunit (GenBank accession no. AAA28556) was used as an outgroup. The nAChR subunits shown in the tree (as well as GenBank accession nos.) are as follows: Dα1 (CAA30172), Dα2 (CAA36517), Dα3 (CAA75688), Dα4 (CAB77445), Dα5 (AAM13390), Dα6 (AAM13392), Dα7 (AAK67257), Dβ1 (CAA27641), Dβ2 (CAA39211), Dβ3 (CAC48166), Agamα1 (AY705394), Agamα2 (AY705395), Agamα3 (AY705396), Agamα4 (AY705397), Agamα5 (AY705399), Agamα6 (AY705400), Agamα7 (AY705402), Agamα8 (AY705403), Agamα9 (AY705404), and Agamβ1 (AY705405). *A. mellifera* nAChR subunits are shown in boldface type.

al. 2002; Jones et al. 2005; Thany et al. 2005), three *Apis* subunits (Amelα5, Amelα6, and Amelα7) show close homology with the vertebrate α7 subunit, sharing 34%, 44%, and 40% identity, respectively. Whereas Amelα6 is clearly orthologous to Dα6 (Fig. 2), the *Apis* subunits analogous to Dα5 and Dα7 were not so easy to determine. We designated Amelα7 as the Dα7 counterpart based on the fact that its N-terminal extracellular domain resembles that of Dα7 more closely than Dα5, showing 90% and 88% identity, respectively. The third α7-like subunit, Amelα5, is considerably distant from Dα5, as indicated by the long branch in the phylogenetic tree (Fig. 2).

Features particular to certain *Drosophila* and *Anopheles* nAChR subunits are also evident in their *Apis* counterparts (Fig. 1). For instance, as with Dα1, Dα2, Dα3, Dα4, and Dβ2, the corresponding *Apis* subunits (Amelα1–Amelα4 and Amelα8) have an insertion in loop F, which, interestingly, may contribute to imidacloprid interactions (Shimomura et al. 2004). The Dα1, Dα2, and Dβ2 genes, as well as the *Anopheles* orthologs, Agamα1, Agamα2, and Agamα8, are similarly arranged and tightly clustered within 200 and 220 kb, respectively (Jones et al. 2005). In the *Apis* genome, only Amelα1 and Amelα2 are clustered, being situated within 120 kb of each other. Immunohistochemical and coimmunoprecipitation studies show that Dα1, Dα2, and Dβ2 are integral components of certain nAChR subtypes, leading to the suggestion that clustering may facilitate coordinate expression and coassembly of the nAChR subunits (Chamaon et al. 2002). The separation of the *Apis* Dβ2 ortholog Amelα8 from the cluster may thus result in diversification of receptor expression and coassembly. In line with this potential broadening of receptor complexity, studies indicate that Dβ2 may also be part of a receptor subtype that includes Dβ1 but not Dα1 and Dα2 (Chamaon et al. 2002). Two other subunits, Amelα7 and Amelβ1, lie in close proximity to each other in the *A. mellifera* genome, both located in linkage group 14.14. This is also the case for the *Anopheles* orthologs Agamα7 and Agamβ1, which are both on chromosome X at map positions 5D and 5C, respectively, whereas the *Drosophila* orthologs Dα7 and Dβ1 are located on different chromosomes, X and 3L, respectively (Jones et al. 2005).

Analysis of *Drosophila* and *Anopheles* nAChRs shows that each insect possesses a distantly related subunit sharing relatively low-sequence identity with other nAChR subunits. In the case of *Drosophila*, the subunit is of the non-α type (Dβ3) (Lansdell and Millar 2002), whereas in *Anopheles* it is an α subunit (Agamα9) (Jones et al. 2005). Interestingly, the honey bee has two distantly related subunits, one α (Amelα9) and the other non-α (Amelβ2), which are designated members of the "Dβ3 Group" (Fig. 2). It is interesting to speculate that duplication of a common ancestor gave rise to an α and a β subunit with the α subunit being lost in the *Drosophila* lineage, the β subunit disappearing in the *Anopheles* lineage, and both subunit types being retained in *Apis*. Indeed, the Amelα9 and Amelβ2 genes lie only within 10 kb of each other in the honey bee genome, suggesting that both subunits arose from an evolutionary recent duplication event from a common gene.

A comparison of *Apis* and *Drosophila* nAChR gene structures shows that only one ortholog pair (Dα6 and Amelα6) shares an identical set of exon–intron boundaries (Fig. 3). This conservation in gene structure is further highlighted by the *Anopheles* ortholog, Agamα6, also possessing the same exon composition (Jones et al. 2005). In other cases, *Apis* nAChR genes possess fewer introns than their *Drosophila* counterparts (e.g., Amelα1 and Amelβ1, which both possess two less than Dα1 and Dβ1, respectively), more introns (e.g., Amelα3, which has two more than Dα3), or the same number of introns (e.g., Amelα4). Both Amelα5 and Amelβ2 possess an uncommon exon–intron boundary within TM1. It is interesting to observe that in addition to having amino-acid sequences closely resembling the vertebrate α7 subunit, Amelα5, Amelα6, and Amelα7 possess exon–intron junctions found in mammalian, bird, and fish α7, as well as the closely related α8 subunits (Fig. 3) (Jones et al. 2003), indicating an ancient lineage for this receptor subtype.

Splice variants increase *Apis* nicotinic receptor diversity

Two *Apis* nAChR subunits, Amelα4 and Amelα6, have alternatively spliced exons most likely arising from tandem exon duplication (Kondrashov and Koonin 2001). As with Dα4 and Agamα4 (Lansdell and Millar 2000; Jones et al. 2005), Amelα4 possesses two alternatives for exon 4 (denoted exon4 and exon4′) (Fig. 4A). However, whereas Dα6 and Agamα6 have two alternatives for exon 3 (Grauso et al. 2002; Jones et al. 2005), Amelα6 has only a

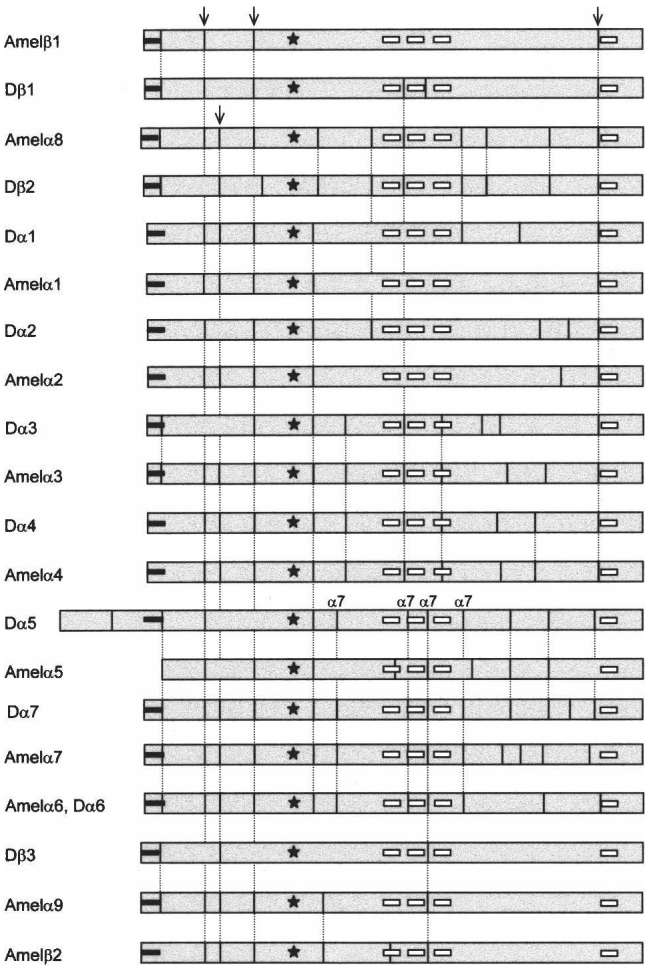

Figure 3. Exon composition of *D. melanogaster* and *A. mellifera* nAChR subunits. The N-terminal signal peptide is shown as a bar, the cys-loop is denoted by a star, and the four transmembrane regions are marked as white boxes. Conserved exon–intron boundaries are indicated by broken lines. Boundaries highly conserved in nAChR genes of invertebrates and vertebrates are highlighted by arrows, while boundaries particular to the α7 subunit are also indicated.

single exon. For α6 exon 8, both *Apis* and *Anopheles* have two alternatives, while *Drosophila* has three, although the mosquito possesses exons analogous to Dα6 8b and 8c (Jones et al. 2005), while the honey bee clearly possesses 8a and 8b-like exons (Fig. 4A).

As previously observed for *Drosophila* nAChRs, alternative splicing introduces amino-acid changes in functionally significant regions (Lansdell and Millar 2000; Grauso et al. 2002). For the two versions of Amelα6 exon 8, residues in the region linking TM2 with TM3 are altered (Fig. 4A). Since studies using chimeric vertebrate α7/α3 receptors as well as site-directed mutagenesis in α7 have shown that this region is involved in coupling agonist binding to ion channel gating (Campos-Caro et al. 1996), alternative splicing of Amelα6 exon 8 may alter the response of ion channel function upon agonist binding.

Both Dα3 and Agamα3 possess extraordinarily long intracellular domains between TM3 and TM4 (Schulz et al. 1998; Jones et al. 2005). However, the *Apis* ortholog, Amelα3, does not have such an extended region (Fig. 1), although use of different splice sites gives rise to two variants, Amelα3L (long variant) and Amelα3S (short variant), which have intracellular domains differing in size by 13 amino-acid residues (Fig. 4C). It is worth noting that Amelα3L has two extra phosphorylation sites. Since phosphorylation of the intracellular loop is involved in regulating several aspects of receptor function such as desensitization and aggregation (Hopfield et al. 1988; Borges and Ferns 2001), the two splice variants have the potential to alter several receptor properties (Schulz et al. 1998; Jones et al. 2005).

Truncated transcripts for several *Drosophila* nAChR subunits have also been described. For instance, Dα4 cDNAs lacking exon 2 ($D\alpha4^{\Delta exon2}$) have been identified (Lansdell and Millar 2000), while in other cases, omission of exon 4 from Dα4 ($D\alpha4^{\Delta exon4}$) and exon 5 from Dα5 ($D\alpha5^{\Delta exon5}$) result in frameshifts and the introduction of premature stop codons (Lansdell and Millar 2000; Grauso et al. 2002). For Dα7, a premature stop codon is introduced by lack of splicing intron 5 (Grauso et al. 2002). RT–PCR was performed to determine whether similar truncated transcripts could be detected for the corresponding *Apis* nAChR subunits. As with *Anopheles* (Jones et al. 2005), truncated honey bee cDNAs analogous to $D\alpha4^{\Delta exon2}$ and $D\alpha5^{\Delta exon5}$ were not detected, whereas $Amel4^{\Delta exon4}$ and truncated Amelα7 transcripts were identified, both having premature stop codons (Fig. 4B). In addition, RT–PCR analysis revealed a novel truncated variant, where lack of splicing intron 9 in Amelα3 results in the introduction of a premature stop codon (Fig. 4C). It remains to be determined whether these truncated transcripts are removed by a process such as nonsense-mediated decay, which rapidly degrades mRNAs with premature stop codons (Hillman et al. 2004). Otherwise, if the truncated transcripts are translated, it would be of interest to determine the functional role of the resulting proteins. Perhaps they regulate receptor expression in a similar manner to a truncated variant of the mouse α7 subunit, which acts as a dominant negative when cotransfected with full-length α7 in HEK 293 cells (Saragoza et al. 2003). Alternatively, the truncated receptors may be modulating cholinergic synaptic transmission by acting as an ACh "sponge" in a manner similar to that of the molluscan ACh-binding protein (Smit et al. 2003). However, the truncated receptors lack some of the loops important for ligand binding, most notably loop C, which is crucial for ACh interaction; thus, their ability to bind ACh is questionable and remains to be determined. The novel truncated Amelα3 transcript (Fig. 4C), however, is the first abbreviated insect variant reported to possess all ligand-binding loops as well as the first three transmembrane domains. With the complete N-terminal extracellular domain as well as TM2, which lines the ion channel, the truncated transcript may well assemble with other subunits to form a functional receptor. Since analysis of subunit mutants suggests a role for TM4 in channel gating (Mitra et al. 2004), it is likely that the Amelα3 variant would have a profound effect on ion channel properties. The Amelα3 truncation also possesses four putative phosphorylation sites (Fig. 4C); thus, it may serve to diversify several characteristics of receptor function.

The two Amelα4 splice variants are differentially expressed

RT–PCR was performed to determine which of the 11 *Apis* nAChR subunits as well as all splice variants are transcribed at different stages of honey bee development, including four larval stages (L_0–L_3), three pupal stages (P_1, P_3, and P_4), and the following tissues from adults: mushroom bodies, optic lobes, brains, head,

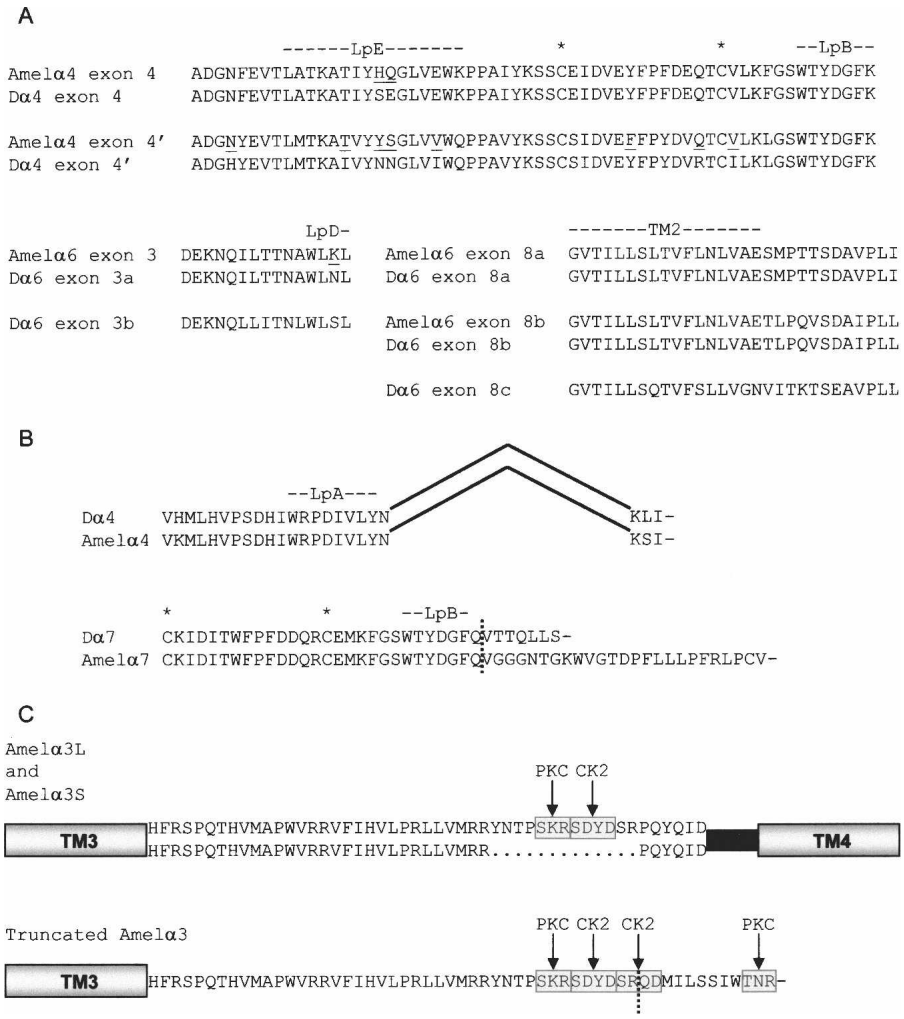

Figure 4. Splice variants of *A. mellifera* nAChR subunits. (*A*) Comparison of alternative exons of *D. melanogaster* and *A. mellifera*. *Apis* residues that differ from those of the orthologous *Drosophila* exon are underlined. (*B*) Conservation of truncated nAChR transcripts between *D. melanogaster* and *A. mellifera*. Omission of exon 4 of Dα4 and Amelα4 results in a frameshift and premature termination of translation, while nonsplicing of intron 5 of Dα7 and the equivalent intron of Amelα7 introduces a loss of the reading frame and a premature stop codon. (*C*) Novel splice variants in *Apis* nAChRs. The TM3 and TM4 domains are represented schematically, while the sequence of a portion of the intervening intracellular loop is shown highlighting the three splice variants, and the remainder of the loop is represented by a black bar. Loops involved in ligand binding and transmembrane regions are indicated, while the cys-loop is marked by asterisks. Potential phosphorylation sites are highlighted by gray shading and broken lines mark the start of altered reading frames resulting from unspliced introns.

and whole bodies. All 11 subunits, as well as all splice variants, are transcribed in each developmental stage and tissue tested (see Supplemental material) with one exception. Amelα4 exon4 transcripts were detected in all stages and tissues, whereas transcripts of Amelα4 exon4′ splice variants were not observed in larvae and were particularly more abundant in the mushroom bodies, optic lobes, and brain (see Supplemental material). Since alternative splicing of Amelα4 exon4 substitute residues in the vicinity of the cys-loop, which has been shown to be important for complete receptor assembly (Green and Wanamaker 1997) and radioligand-binding assays, indicate that Dα4 with exon 4′ assembles less efficiently than with exon4 (Lansdell and Millar 2000), Amelα4 exon 4′ subunits may serve to modulate receptor assembly during the later stages of honey bee development and in tissues rich in neural activity such as the mushroom bodies and optic lobes.

Amelα6 undergoes A-to-I pre-mRNA editing

Pre-mRNA A-to-I editing modifies select adenosine (A) residues to inosine (I) in transcripts, which is interpreted as guanosine (G), thereby generating mRNA with a nucleotide composition that differs from the corresponding genomic DNA (Seeburg 2002). RNA editing has been observed in several *Drosophila* nAChR subunits, including two sites in loop D of Dβ1, one site in TM2 of Dβ2, one site in TM3, three sites in TM4 of Dα5, and seven sites in loops E to F in Dα6 (Grauso et al. 2002; Hoopengardner et al. 2003; Sattelle et al. 2005). To determine whether orthologous *Apis* nAChR subunits are also RNA edited, the equivalent regions of Amelβ1, Amelα8, Amelα5, and Amelα6 were amplified with high-fidelity proofreading DNA polymerase. For Amelβ1, Amelα8, and Amelα5, the sequences of the resulting amplification products were identical to those of genomic DNA with no indication of A-to-G changes (data not shown), showing that these regions of the three subunits are not RNA edited.

For Amelα6, however, six RNA-editing sites were observed, two of which are conserved in the *Drosophila* and *Heliothis virescens* orthologs, Dα6 and Hvα7-2, respectively (Grauso et al. 2002) (Fig. 5). The genomic DNA and adult cDNA sequence traces shown in Figure 5 were taken from the same individual bee, indicating that sequence variation likely arose at the RNA level, thereby eliminating the possibility that they are polymorphisms. Editing at five of the six sites alters amino acid residues, all of which are situated in functionally significant regions. For instance, an N-glycosylation site in loop E is eliminated by one case of editing. Since loop E contributes to ligand binding and N-glycosylation has also been linked to ligand binding as well as channel desensitization and conductance (Corringer et al. 2000; Nishizaki 2003), editing at this site has considerable potential to alter receptor function. In the remaining cases, editing alters residues near or within the cys-loop, which, like alternative splicing of Amelα4 exon4, may affect receptor assembly. Analysis of Amelα6 editing at different stages of honey bee development shows that in larvae, five of the six sites undergo editing, the extent of which increases throughout development so that in adults, four sites are predominantly edited. In pupae, from P_3 onward, editing was observed at the sixth site, which increases considerably the potential diversity of subunit isoforms, as a lysine residue can be converted to either arginine, glutamic acid, or glycine. Interest-

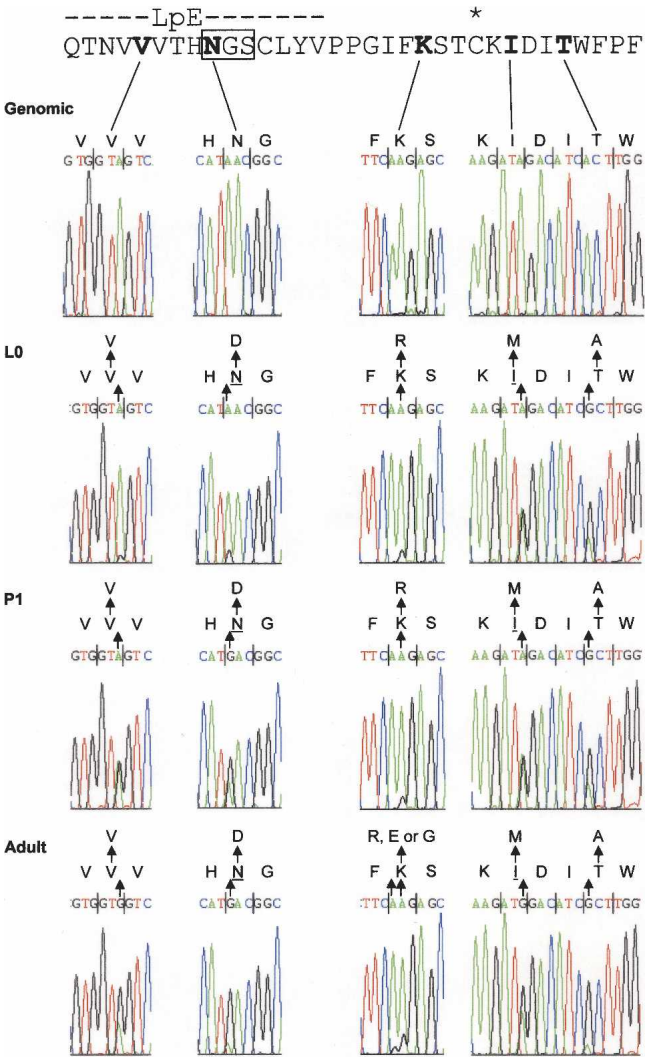

Figure 5. RNA editing of Amelα6. Sequencing traces of RT-PCR products from larval (L_0), pupal (P_1), and adult stages are compared with amplified genomic DNA. Editing is shown by mixed A and G signals. Amino acids also affected by editing in *D. melanogaster* and *H. virescens* are underlined. The edited region of Amelα6 is included with loop E indicated, N-glycosylation sites boxed and a cysteine, which is part of the cys-loop, marked by an asterisk. Amino acids targeted by editing are highlighted in bold.

ingly, the elevated editing in the later stages of development is consistent with findings that RNA editing is particularly important in the nervous system function of *Drosophila* adults (Palladino et al. 2000) and that the highest levels of RNA editing are seen in adult flies (Keegan et al. 2005).

Discussion

We have used the available *A. mellifera* genome information to complete the characterization of the honey bee nAChR gene family, thus describing the first complete set of Hymenoptera nAChR subunits and the third insect nAChR gene family following those of the two Diptera, *A. gambiae* (Jones et al. 2005) and *D. melanogaster* (Sattelle et al. 2005). The three insect species represent ~280 million years of evolution (Carpenter and Burnham 1985; De Gregorio and Lemaitre 2002) where the nAChR gene family has remained compact with *A. mellifera* having 11 genes encoding nAChR subunits, whereas both *D. melanogaster* and *A. gambiae* possess 10 genes (Jones et al. 2005; Sattelle et al. 2005). The nAChR subunit composition of *Apis* most closely resembles that of *Anopheles* in that both possess nine α and one β subunit, while *Drosophila* has seven α and three β. The extra honey bee subunit is a β subunit (Amelβ2) making *A. mellifera* only the second insect known to possess more than one non-α type subunit.

The characterization of the full complement of honey bee nAChR subunits presents an important basis for associating particular nAChR subtypes with key aspects of behavior, identifying receptor subtypes targeted by neonicotinoids as well as developing insecticides with improved selectivity. Indeed, comparison of complete insect nAChR gene families has identified a highly divergent subunit group (the Dβ3 group) as well as species-specific proteome diversification arising from alternative splicing and RNA editing, all of which represent promising subunit differences to target for future rational insecticide design. While studies using heterologous expression systems such as *Xenopus laevis* oocytes have proven instructive in characterizing vertebrate nAChRs (Corringer et al. 2000) and low levels of functional expression of an insect α subunit, αL1, have been observed in *Xenopus* oocytes (Marshall et al. 1990), expression of functional insect nAChRs has so far proven elusive (Sattelle et al. 2005). Nevertheless, *Drosophila* nAChR α subunits can form robust functional channels when coexpressed with a vertebrate β2 subunit (Bertrand et al. 1994) and studies on such hybrid receptors have provided insights into the selectivity of neonicotinoids for insect nAChRs over those of vertebrates (Matsuda et al. 1998; Ihara et al. 2003), regions of subunit proteins involved in neonicotinoid interactions (Shimomura et al. 2002, 2003, 2004), and the actions of different neonicotinoids (Ihara et al. 2004). Also, computer models of insect nAChRs have been recently generated, which permit docking experiments to assess interactions with compounds of interest (Sattelle et al. 2005). Similar studies combining functional expression with molecular modeling of *Apis* nAChRs are likely to prove useful in screening for novel compounds that show low selectivity for honey bee receptors and in dissecting the mechanisms of insecticide actions and selectivity on nAChRs.

Methods

Identification of nAChR subunits in the *A. mellifera* genome

To identify putative nAChR subunits, we screened the *A. mellifera* genome (database version 34.2b available at http://www.ensembl.org/Apis_mellifera/index.html and assembly version 3.0 available at http://www.ensembl.org/Apis_mellifera/) with each of the 10 *D. melanogaster* nAChR subunit cDNA sequences using the TBLASTN algorithm (Altschul et al. 1990). Candidate honey bee nAChR subunits were identified based on their considerable sequence homology with previously characterized nAChR subunits (sequences with lowest similarity had *E*-value 1e-21), particularly at the N-terminal ligand-binding domain and the four transmembrane regions. RT–PCRs were performed to verify the open-reading frame sequences of each subunit. Since BLAST was unable to identify the highly variable N-terminal signal peptides, 5′-RACE, using the Roche 5′/3′ RACE kit, was performed to complete the nAChR subunit sequences.

The multiple protein-sequence alignment was constructed with CLUSTALX (Thompson et al. 1997) using the slow-accurate mode with a gap-opening penalty of 10 and a gap-extension penalty of 0.1 as well as applying the Gonnet 250 protein weight matrix (Benner et al. 1994). The protein alignment was viewed using GeneDoc (http://www.psc.edu/biomed/genedoc). The neighbor-joining method (Saitou and Nei 1987) and bootstrap resampling (Felsenstein 1985), available with the CLUSTALX program, were used to construct a phylogenetic tree, which was then displayed using the TreeView application (Page 1996). Signal peptide cleavage sites were predicted using the SignalP 3.0 server (Dyrlov Bendtsen et al. 2004) and membrane-spanning regions were predicted using the TMpred program (available at http://www.ch.embnet.org/software/TMPRED_form.html). The PROSITE database (Falquet et al. 2002) was used to identify potential cyclic AMP (cAMP), protein kinase C (PKC), CK2, and potential kinase phosphorylation sites.

Dissection of *A. mellifera* tissues

Honey bee pupae and larvae were taken from the hive. Their developmental stage was determined using pigmentations of eyes, joints, and legs as described by Winston (1987). Adult honey bees were collected at the entrance of the hive. Bees were anaesthetized on ice and dissection of *A. mellifera* tissues were performed under a stereomicroscope in sterile 1X PBS. The brain was removed from the capsule head free of cuticle and trachea. When necessary, brain parts were separated manually. The tissues were then frozen in liquid nitrogen before RNA and genomic DNA extraction.

Reverse transcription and polymerase chain reaction

Genomic DNA was extracted from adult bees using the DNeasy Tissue Kit (Qiagen) and total RNA was extracted from various developmental stages and tissues using the RNeasy Mini Kit (Qiagen). First-strand cDNA was synthesized from 1 µg of total RNA using Superscript III First-Strand Synthesis Super Mix (Invitrogen). Nested RT–PCR reactions were performed to detect transcript of all honey bee nAChR subunits and variants. Primer pairs that recognize different exons were used to allow identification of cDNA-specific products (see Supplemental material for PCR primer sequences). The PCR reactions were performed in a total volume of 50 µL composed of Taq polymerase and 1X PCR buffer (Sigma), 0.2 mM dNTP mix (Roche), 0.4 µM each primer, and 2 µL first-strand cDNA template. The nested PCR approach involved two reactions each with 30 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec/500 bp amplified. The first PCR was used at a final dilution of one in 5000 as template for the second nested PCR reaction. For RNA-editing analysis, nested PCR using the proofreading Pfu Turbo DNA polymerase (Stratagene) in 2 × 30-cycle reactions was performed on at least two independently made first-strand cDNAs. PCR products were analyzed by electrophoresis in a TAE gel and then purified using the QIAquick Gel Extraction Kit (Qiagen) before being sequenced by the dye termination method at the Biochemistry Sequencing Facility, University of Oxford.

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