Novel Putative Nicotinic Acetylcholine Receptor Subunit Genes, $\alpha$5, $\alpha$6 and $\alpha$7, in Drosophila melanogaster Identify a New and Highly Conserved Target of Adenosine Deaminase Acting on RNA-Mediated A-to-I Pre-mRNA Editing

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ABSTRACT

Genome analysis of the fruit fly Drosophila melanogaster reveals three new ligand-gated ion channel subunits with the characteristic YXCC motif found only in &-nicotinic acetylcholine receptor subunits. The subunits are designated $\alpha$5, $\alpha$6, and $\alpha$7. Cloning of the $\alpha$5 embryonic cDNAs reveals an atypically large N terminus, part of which is without identifiable sequence motifs and is specified by two polymorphic alleles. Embryonic clones from $\alpha$6 contain multiple variant transcripts arising from alternative splicing as well as A-to-I pre-mRNA editing. Alternative splicing in $\alpha$6 involves exons encoding nAChR functional domains. The $\alpha$6 transcript is a target of the Drosophila adenosine deaminase acting on RNA (dADAR). This is the first case for any organism where a nAChR gene is the target of mRNA editing. Seven adenosines could be modified in the extracellular ligand-binding region of $\alpha$6, four of which are also edited in the $\alpha$6 ortholog in the tobacco budworm Heliothis virescens. The conservation of an editing site between the insect orders Diptera and Lepidoptera makes nAChR editing the most evolutionarily conserved invertebrate RNA editing site so far described. These findings add to our understanding of nAChR subunit diversity, which is increased and regulated by mechanisms acting at the genomic and mRNA levels.

NICOTINIC acetylcholine receptors (nAChR) mediate fast excitatory transmission at cholinergic synapses (Changeux and Edelstein 1998). They are pentameric membrane proteins that incorporate an integral ion channel (Unwin 1996). Typically, they become transiently permeable to cations ($\text{Na}^+$, $\text{K}^+$, $\text{Ca}^{2+}$) following the binding of the natural ligand, the neurotransmitter acetylcholine (ACh; Karlin and Arasas 1995). In vertebrates (McGehee and Role 1996) and in the nematode Caenorhabditis elegans (Fleming et al. 1997; Richmond and Jorgensen 1999), nAChRs are present both at the neuromuscular junction and in the nervous system. Large families of nAChR subunit genes have been identified: 17 in vertebrates (Le Novère and Changeux 1999; Elgooyen et al. 2001) and 27 in C. elegans (Mongan et al. 1998; Sattelle et al. 2002). Each nAChR subunit typically possesses an extracellular N-terminal domain containing residues responsible for agonist binding, four transmembrane regions (TM1–4), the second of which (TM2) lines the channel, and a long, poorly conserved intracellular loop between TM3

and TM4 that possesses multiple regulatory phosphorylation sites (Swope et al. 1999). In vertebrates, nAChR subunits are classified as either $\alpha$ or non-$\alpha$ types ($\beta$, $\gamma$, $\delta$, $\varepsilon$), the $\alpha$ subunits being characterized by the presence in the extracellular N-terminal domain of two adjacent cysteines in loop C of the ACh-binding site. Other specific residues in loops A and B of $\alpha$ subunits contribute to the ACh binding. Non-$\alpha$ subunits carry complementary ligand-binding domains (loops D–F) and they assemble with $\alpha$ subunits, typically with an $\alpha_{5-7}$, non-$\alpha_{5-7}$ stoichiometry (although other combinations are possible) in native neuronal heteropentameric receptors (Galzi and Changeux 1995; Arias 1997; Kuryatov et al. 2000). In the vertebrate central nervous system, $\alpha$ homopentameric receptors (e.g., $\alpha_{7-5}$) have also been detected (Drisdel and Green 2000).

In insects, cholinergic synaptic transmission is restricted to the nervous system (Breer and Sattelle 1987; Gundelfinger and Hess 1992; Lee and O’Dowd 1999). Both $\alpha$ and non-$\alpha$ nAChR subunit genes have been cloned from a variety of insect species and both homooligomeric and heterooligomeric receptors containing insect nAChR subunits have been (i) postulated to exist in situ (Schloss et al. 1991) and (ii) shown to assemble as functional recombinant receptors in Xenopus laevis oocytes (Marshall et al. 1990; Amar et al. 1994; Sgard et al. 1998). Insect nAChR $\alpha$ subunits more closely resemble in sequence vertebrate neuronal $\alpha$ subunits than vertebrate muscle $\alpha$ subunits. Nevertheless,
insect and vertebrate neuronal nAChRs show some distinct pharmacological properties; e.g., the insecticide imidacloprid appears to be more effective on insect nAChRs (Liu and Casida 1993; Matsuda et al. 1998, 2001; Lansdell and Millar 2000a).

The model organism Drosophila melanogaster is well suited to genetic, molecular, and physiological approaches to nAChR function. Patch-clamp electrophysiological recordings from native Drosophila neurons (Albert and Lingle 1993; Gundelfinger and Schulz 2000) point to a diversity of nAChR subtypes, as is the case in vertebrates (Cordero-Erausquin et al. 2000).

At present in D. melanogaster only six nAChR subunits have been cloned. Of these, ALS, SAD, Da3, and Da4 are α subunits (Bossy et al. 1988; Jonas et al. 1990; Sawruk et al. 1990a; Schulz et al. 1998; Lansdell and Millar 2000b), whereas ARD and SBD are referred to as non-α or β subunits (Hermans-Borgmeyer et al. 1986; Sawruk et al. 1990b).

Immunoprecipitation experiments show that ALS and SAD nAChR subunits may be part of the same receptor complex at synapses in the nervous system (Schulz et al. 2000). On the other hand, both immunoprecipitation and binding experiments suggest that Da3 and the β subunit ARD may coassemble in a receptor complex (Schloss et al. 1988; Chamaon et al. 2000). However, to date no fully functional recombinant nAChR has been generated in a heterologous system using only Drosophila nAChR subunits, although hybrid recombinant receptors containing a vertebrate neuronal β subunit have been employed to investigate, in vitro, the roles of the α subunits (Bertrand et al. 1994; Matsuda et al. 1998; Schulz et al. 2000).

The recently sequenced genome of D. melanogaster (Adams et al. 2000) has renewed interest in molecular and functional diversity in the Drosophila nAChR gene family. This has led to the cloning of three new, α7-like, putative nAChR subunit genes (Dα5, Dα6, and Dα7) and has provided evidence of genetic variation, alternative splicing, and site-specific mRNA editing events. Our results describe the first example of A-to-I pre-mRNA editing in a nAChR subunit.

MATERIALS AND METHODS

Animal strain and nucleic acids: For RACE experiments, cDNA cloning and developmental expression studies, D. melanogaster Canton-S strain poly(A)+ RNA and genomic DNA were provided by CLONTECH (Palo Alto, CA). D. melanogaster flies for Dα5 polymorphism analysis were of Canton-S strain (provided by Dr. J.-M. Dura, Institut de Génétique Humaine, Montpellier, France) and single-fly genomic DNA for PCR amplification was quickly extracted by incubating ground fly for 30 min at 37°C in 50 μl of 10 mM Tris pH 8, 1 mM EDTA, 25 mM NaCl, and 200 μg/ml Proteinase K. Drosophila lines for Dα6 editing studies were as follows: Drosophila adenosine deaminase acting on RNA (dADAR)− animals were females doubly heterozygous for the dADAR−/− and dADAR−/− alleles, both of which remove the dADAR locus (Palladino et al. 2000a). Hemizygous dADAR−/− males were also used. dADAR− controls were females doubly heterozygous for dADAR−/− and the balancer chromosome FM7a (dADAR−) or FM7a males. RNA was extracted by a modified LiCl/Urea method (Auffray and Rougeon 1980).

RACE and cloning of cDNAs: A double-strand cDNA library was prepared using the Marathon cDNA amplification kit (CLONTECH) on embryonic D. melanogaster poly(A)+ RNA following the manufacturer’s protocol. Rapid amplification of cDNA ends (RACE) was performed using the Advantage cDNA PCR kit (CLONTECH) with 5 μl of the Marathon products in a final volume of 50 μl. Gene-specific primers (Table 1) were used in combination with the Marathon adaptor primer API. For each gene, forward and reverse primers were chosen to give overlapping 5′ and 3′ RACE amplifications to confirm that they belong to the same gene. Thermal cycler parameters for Dα5 and Dα6 genes were as follows: five cycles at 94°C for 30 sec and at 72°C for 4 min, five cycles at 94°C for 30 sec and at 70°C for 4 min, and 20 cycles at 94°C for 30 sec and at 68°C for 4 min. For the Dα7 gene, we used 30 cycles at 94°C for 5 sec and at 68°C for 4 min. RACE products were checked by nested PCR or Southern blotting and then cloned in PCR-Script vector (Stratagene, La Jolla, CA). Automated sequencing was performed by the Biochemistry Department Sequencing Facility (University of Oxford, UK).

PCR amplifications of full-length cDNAs were performed with the proofreading Pfu Turbo DNA polymerase (Stratagene) on 5 μl of the Marathon cDNA products in a final reaction volume of 100 μl. Specific 5′ and 3′ end primers were deduced from RACE product sequences (Table 1). To amplify Dα5 full-length cDNA, we carried out 30 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 1 min, with extension at 72°C for 4 min. For Dα6 cDNA, thermal cycling conditions were as follows: 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 4 min. Blunt-end PCR products were submitted to A-tailing at 70°C and then cloned in pGEM-T vector. As described above, a large number of transforming colonies were screened by PCR using at least one gene-specific nested primer and positive clones were sequenced on both strands. The cDNA nucleotide differences with respect to the genomic sequence reported in databases have been confirmed with at least one independent amplification experiment to exclude the possibility of polymerase errors. Then, regions corresponding to nucleotide differences in cloned cDNAs were also amplified on genomic DNA using the proofreading polymerase, and products were sequenced to check for polymorphisms.

Dα6 RNA editing analysis: RNA editing was assayed using a bulk sequencing method as described in Palladino et al. (2000a). This method has been reported to be accurate to as low as 5% editing. Reverse transcriptase (RT)-PCR was performed on whole adult D. melanogaster RNA using the primer DART (see Table 1) to specifically prime the reverse transcription reaction and primers Dare-1 and Dare-2 were used to amplify the Dα6 transcript spanning all suspected editing sites (exons 4–9). Similar reactions were performed on whole RNA from dADAR−/− males, which lack dADAR, the only adenosine deaminase acting on RNA in Drosophila. Reaction products were subjected to automated sequence analysis.

Analysis of RNA editing in Heliothis virescens was performed using whole adult RNA as the template for RT-PCR. The primers AHVRT-1 and AHVRT-2 were used to specifically prime the reverse transcription reactions for Heliothis α7-1 and α7-2 transcripts, respectively. PCR was performed with the primer pairs Darv-1 and Dare-2 or Darv-2 and Dare-2 for the α7-1 or α7-2 genes, respectively. Amplification products were subjected to automated sequence analysis. Primers for the Dα6 ortholog exon 5 in Heliothis α7-2 (Hvex5.1 and Hvex5.2)
were used to amplify *H. virescens* genomic DNA from the same animals used for RNA isolation. The genomic PCR amplification product was subjected to direct sequencing to demonstrate that genomic products give a pure A signal at editing sites, ruling out a polymorphism.

**Quantitative RT-PCR:** Quantitative RT-PCR was performed using the Roche (Indianapolis) LightCycler apparatus and the Roche Expand reverse transcriptase for reverse transcription. *D. melanogaster* poly(A) + RNA (50 ng) from each of the three major developmental stages was incubated at 65°C for 10 min in the presence of 50 pmol random hexanucleotides. Then 1× RT buffer, 10 mM DTT, 1 mM of dNTP mix, 0.5 µl of Promega (Madison, WI) rRnasin, and 50 units of reverse transcriptase were added in a 20-µl final reaction volume. Reverse transcription conditions were as follows: 10 min at 30°C and 45 min at 42°C. A 2-µl sample of each RT product was used in the LightCycler-DNA Master SYBR Green I PCR reaction. The anti-Taq DNA polymerase antibody (GLONTECH) was used for hot-start reactions.

Gene-specific primers for PCR were designed in the low-homology intracellular TM5-TM4 loop of the nAChR subunits, to amplify a ~300-bp segment (Table 1). Specificity of PCR amplifications was checked by Southern blot. The *Drosophila* Rpl17A housekeeping gene (Noselli and Vincent 1992) was also amplified as a control for the amount of RNA used. A standard amplification curve for each gene was constructed using three or four serial dilutions of plasmids containing these genes and covering the expected range of their expression level. Thus, we used the following quantities to establish the reference curve: 3 × 10^{-5}, 3 × 10^{-4}, 3 × 10^{-3}, and 3 × 10^{-2} ng for *Dx5* and *Dx6* genes and 3, 3, 3, and 3 × 10^{-3} ng for the Rpl17A gene. The second derivative maximum method was used to estimate DNA concentration. Concentration values for each stage, expressed as ratio with the Rpl17A value, were calculated with regard to the embryonic concentration (that was taken as 100%).

**Computer analysis:** Sequence similarity searches were performed using the BLAST method (Altschul et al. 1990), the Genetics Computer Group (GCG) package was used for general DNA sequence analysis (Womble 1997), were deployed in the ClustalX program (Thompson et al. 1997), were deployed in a phylogenetic study of nAChR subunits. The phylogenetic tree was then drawn using the TreeView application (Page 1996). Analysis of RNA secondary structure was conducted using the M. Zucher mfold program (mfold server at http://bioinfo.math.rpi.edu/~mfold/rna/form1.cgi), setting a folding temperature of 25°C.

**Nomenclature:** During the preliminary stage of this work, four unknown nAChR subunit candidate genes had been annotated in the Genome Annotation Database (GadFly, http://flybase.bio.indiana.edu/annot/; annotation of March 2000): CG4498, CG4128, CG8109, and CG11822. The first three corresponded to the putative novel nAChR subunits we studied. The formal nomenclature for genes encoding *Drosophila*
nAChR subunits includes information derived from the gene’s chromosomal location but we formally named them *Dx5*, *Dx6*, and *Dx7*, respectively, for convenience (Table 2).

**nAChR subunit sequences:** GenBank accession numbers of the three new nAChR subunits genes are as follows: *Dx5* full-length cDNAs are AF272778 (from allele A) and AY036613 (from allele B); *Dx6* cDNA variants from type I to V are from AF321444 to AF321449; *Dx7* full-length cDNA (a misprocessed messenger) is AY036614. GenBank accession numbers and sequences of all the other nAChR subunits used in this work are available at the Ligand-Gated Ion Channel database (Le Novère and Changeux 1999, http://www.pasteur.fr/recherche/banques/LGIC/LGIC.html).

### RESULTS

**Identification of three new Drosophila genes with structural properties of nAChR subunits:** From a BLAST analysis against the *D. melanogaster* genome sequence at the Berkeley Drosophila Genome Project (BDGP, http://www.fruitfly.org), using previously cloned Drosophila nAChR subunit genes, high-score alignments were obtained for three different Drosophila genomic clones (see Table 2 for their accession numbers and the corresponding gene names). We recovered the three genomic sequences and reconstructed partial putative open reading frames (ORFs) using the BLAST alignments. Deduced partial ORFs revealed recognizable regions characteristic of a ligand-gated ion channel subunit: the “dicysteine loop” and the conserved ligand-binding loops (A-F), as well as the putative membrane-spanning (TM1-4) segments (Figure 1). The presence of a YXCC motif in loop C of the binding site confirmed that they represent nAChR subunits of the α type (Kao et al. 1984). As noted by Littleton and Ganetzky (2000), these subunits are closest to the vertebrate and *C. elegans* neuronal α7-like subunits (Figure 2). All three new genes are more similar to one another than to any previously described *D. melanogaster* nAChR subunit, displaying between 53 and 63% identity and between 63 and 71% similarity at the amino acid level to each other (Table 3). Moreover, they present the highest identity and closest phylogenetic relation with the *H. virescens* α7-like nAChR subunits, α7-1 and α7-2, identifying them as the budworm orthologs of *Dx5* and *Dx6*, respectively.

In the *Dx6* genomic sequence, two alternative versions of coding exon 3 (3a and 3b) and three repeats of coding exon 8 (8a, 8b, and 8c) were found (Figure 1 and Table 4). Exon 3 specifies 15 amino acids around loop D of the ligand-binding region and exons 3a and 3b differ in only four residues. Exon 8 encodes 29 amino acids that include the channel-lining region, TM2, as well as the extracellular region linking transmembrane regions TM2 and TM3. Exons 8a and 8b differ in six residues, which are located in the TM2-TM3 linker region, whereas 11 amino acid differences are encoded by 8c, some of which are part of TM2, and the others define a TM2-TM3 extracellular linker sequence completely different from that of exon 8a. This suggests the potential existence of six alternative transcripts encoding polypeptide variants of *Dx6* with potentially different physiological/pharmacological properties.

**Determination of 5’ and 3’ ends and genomic organization:** BLAST alignments did not allow the precise identification of the N-terminal exons and of the stop codon. Thus, 5’ and 3’ RACE experiments were performed in an attempt to clone full-length cDNAs for *Dx5*, *Dx6*, and *Dx7*. The deduced genomic structures and open reading frames are shown in Figure 1.

Using 5’ RACE experiments with two *Dx5* gene-specific primers we obtained and sequenced 12 different partial *Dx5* cDNA clones. They identified the same putative start codon and an N terminus containing two large coding exons, each almost 450 bp long, that precede the ligand-binding region and that share no similarity with any other known nAChR subunit domain. The RACE clones extended for a variable distance upstream of the ATG up to 290 bp. A 3’ RACE experiment resulted in a partial cDNA identifying a stop codon just downstream of the transmembrane segment TM4, a 181-bp 3’ untranslated region (UTR) portion and a polyadenylated tail. Thus, the *Dx5* genomic sequence spans a distance of nearly 38 kb and contains 12 coding exons (Figure 1A).

A 5’ RACE experiment for the *Dx6* gene yielded three partial cDNAs, defining an identical putative start codon and the first coding exon, but extending for a different distance up to 380 bp upstream of the ATG. On the genomic *Dx6* sequence, the first identified coding exon is separated from the second by a large intron of ~43.7 kb that does not seem to contain in its sequence any other predicted gene. For *Dx6*, 3’ RACE experiments were unsuccessful, so a putative stop codon and 139-bp...

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**TABLE 2**

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Map position</th>
<th>Genomic clone</th>
<th>Gene name</th>
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<tbody>
<tr>
<td>CG4498</td>
<td>2L (34E4-34E5)</td>
<td>AE005342</td>
<td>nAcRalpha-34E (<em>Dx5</em>)</td>
</tr>
<tr>
<td>CG4128</td>
<td>2L (30D1-30E1)</td>
<td>AE005362</td>
<td>nAcRalpha-30D (<em>Dx6</em>)</td>
</tr>
<tr>
<td>CG8109</td>
<td>X (18C3-18C3)</td>
<td>AE005351</td>
<td>nAcRalpha-18C (<em>Dx7</em>)</td>
</tr>
</tbody>
</table>

Deduced from the *D. melanogaster* Genome Annotation Database (GadFly in FlyBase, http://flybase.bio.indiana.edu).

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...coding exon 8 (8a, 8b, and 8c) were found (Figure 1 and Table 4). Exon 3 specifies 15 amino acids around loop D of the ligand-binding region and exons 3a and 3b differ in only four residues. Exon 8 encodes 29 amino acids that include the channel-lining region, TM2, as well as the extracellular region linking transmembrane regions TM2 and TM3. Exons 8a and 8b differ in six residues, which are located in the TM2-TM3 linker region, whereas 11 amino acid differences are encoded by 8c, some of which are part of TM2, and the others define a TM2-TM3 extracellular linker sequence completely different from that of exon 8a. This suggests the potential existence of six alternative transcripts encoding polypeptide variants of *Dx6* with potentially different physiological/pharmacological properties.

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3' UTR were deduced by sequencing a *Dx6* expressed sequence tag (EST) clone (GH15518) provided by the BDGP resources. Its sequence carried the alternatively spliced exons 3b and 8a and confirmed the N-terminal exons we found in 5' RACE experiments. The complete *Dx6* genomic sequence covers a distance of almost 68 kb and contains 12 coding exons (Figure 1B).

For the *Dx7* gene, we obtained two 5' RACE clones covering an untranslated upstream region from putative ATG up to 305 bp. Nevertheless, these partial cDNAs carried in their sequence the small (~60 bp) unspliced intron 5, probably representing unprocessed messengers since its 5' and 3' splicing consensus are correct. In these clones, lack of splicing introduces a loss of the reading frame and a premature stop codon. A 3' RACE clone identified the stop codon and a 341-bp 3' UTR preceding a polyadenylated tail. The *Dx7* genomic sequence spans only ~14 kb (Figure 1C). Surprisingly, the 3' RACE cDNA did not include the putative, BLAST-identified exons 7 and 9 that specify sequences for the N-terminal moiety. Alternative coding exons 3 and 8 of the *Dx6* gene are red and green, respectively. In cDNAs, the gray regions correspond to the 5' and 3' untranslated regions. Plain lines indicate constitutive splicing whereas alternative splicing leading to different cDNA variants is shown by dotted lines. Lengths of cloned cDNAs and of the corresponding encoded polypeptides are also indicated. Both skipping of exon 5 (in *Dx5* cDNA) and unsplicing of intron 5 (in *Dx7* RACE clones) lead to truncated proteins (see text).  ■ Coding exons;  □ transmembrane domains.

**Figure 1.** — Genomic organization and structure of partial and full-length cDNAs of the new α7-like Drosophila nAChR subunit genes: (A) *Dx5*, (B) *Dx6*, and (C) *Dx7*. The genomic organization was deduced by BLAST alignment and RACE experiments (see text). Half arrows indicate the positions of primers used in RACE experiments. Exons are drawn to scale whereas the nucleotide intron lengths are just shown. Colored dots show ligand-binding domains of the extracellular N-terminal moiety. Alternative coding exons 3 and 8 of the *Dx6* gene are red and green, respectively. In cDNAs, the gray regions correspond to the 5' and 3' untranslated regions. Plain lines indicate constitutive splicing whereas alternative splicing leading to different cDNA variants is shown by dotted lines. Lengths of cloned cDNAs and of the corresponding encoded polypeptides are also indicated. Both skipping of exon 5 (in *Dx5* cDNA) and unsplicing of intron 5 (in *Dx7* RACE clones) lead to truncated proteins (see text).  ■ Coding exons;  □ transmembrane domains.

Cloning *Dx5* and *Dx6* full-length cDNAs from a *D. melanogaster* embryo: For the *Dx5* gene, a 2447-bp full-length cDNA, encoding a putative polypeptide of 807 amino acids, was cloned (Figure 1A). In its first two large N-terminal exons we found regions differing in the nucleotide sequence from the AE003642 genomic sequence. Nucleotide substitutions as well as an inser-
Amino acid differences between Da6 alternative exons are underlined. Functional domains formed by these exons are indicated on the top of the sequence. The highly conserved tryptophane residue of loop D is in boldface type.

The shorter cDNA from Da5 allele B was lacking the fifth exon and the incorrect splicing results in loss of the open reading frame with a premature stop codon being created in exon 6. This cDNA is predicted to encode a truncated 472-amino-acid polypeptide, consisting of only a soluble partial ligand-binding domain.

Two independent Da6 full-length cDNA amplification experiments yielded, as expected, several transcript variants, as exons 3 and 8 could occur in alternative combinations (Figure 1B). However, cDNA containing exon

<table>
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<tr>
<th>Exon 3a</th>
<th>DEKNQILTTLNWLNL</th>
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<tr>
<td>Exon 3b</td>
<td>DEKNQLLLTLNLWLSL</td>
</tr>
<tr>
<td>Exon 8a</td>
<td>GVTILSSLVFLVAESMPTTDWAPHL</td>
</tr>
<tr>
<td>Exon 8b</td>
<td>GVTILSSLVFLVAETLPQSDAPPL</td>
</tr>
<tr>
<td>Exon 8c</td>
<td>GVTILSSQTQVSLVNGVITYKTSIGAVPL</td>
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The percentages of identities/similarities between the D. melanogaster nAChR subunit, chicken α7 subunit (Ggα7), and H. virescens α7-1 and α7-2 subunits (Hvα7-1 and Hvα7-2) were calculated on protein sequences starting downstream of the putative peptide signal.
8c was found to be transcribed neither in the 25 full-length cDNAs we cloned nor in the 5′ RACE clones. We classified the \( \text{\textit{Dx6}} \) transcripts into five main variant categories, depending on the alternatively spliced exons 3 and 8 versions: type I (3a-8b), type II (3b-8a), type III (3b-8b), type IV (3ab-8a), and type V (3b-8ab). Types IV and V are unusual because they contain, without loss of the open reading frame, a duplicate of either exon 3 or exon 8, respectively, thereby including certain motifs twice in the encoded polypeptide subunit, notably the ligand-binding loop D region (exon 3), in the case of type IV, and the channel-lining transmembrane segment TM2 (exon 8) for type V. Because such structural organization for an ion channel subunit was never described before, the novel receptor properties introduced by exon repeating and their significance remain to be explored.

On the other hand, we were unable to obtain a full-length \( \text{\textit{Dx7}} \) cDNA clone, and considering the exon skipping features of the RACE clones, we suspect \( \text{\textit{Dx7}} \) could not give a correctly processed nAChR subunit but, as in the case of the short version of \( \text{\textit{Dx5}} \) from allele B, a putative truncated soluble polypeptide consisting of only part of the ligand-binding domain.

For comparison purposes, a putative \( \text{\textit{Dx7}} \) full-length cDNA, based on both the high similarity with \( \text{\textit{Dx5}} \) and the overlapping 5′ and 3′ RACE sequences, is nevertheless described. Such a putative cDNA would be 2338 bp long and specify a 563-amino-acid polypeptide (Figure 1C).

**\( \text{\textit{Dx6}} \) gene RNA editing:** Interestingly, many \( \text{\textit{Dx6}} \) variant cDNAs showed some nucleotide differences compared to the genomic clone AE003626. Some of these consist of site-specific adenosine-to-guanosine A-to-I pre-mRNA editing-like transitions that lead to amino acid changes in the ligand-binding domain of the encoded protein (Table 6). In fact, A-to-I conversions in pre-mRNA result in the appearance of a guanosine in double-stranded cDNA (Emeson and Singh 2001). Sequence analysis of 25 embryonic transcripts, derived from two independent amplification experiments, showed that three amino acids could be changed by four possible adenosine-to-guanosine transitions in exon 5 (designated as sites 3–6) and one amino acid in exon 6 (site 7), thereby defining five specific candidate-editing positions. The frequency of such editing is shown in Table 7. We also checked for the existence of genetic variation in these \( \text{\textit{Dx6}} \) regions. PCR amplification on \( \text{\textit{D. melanogaster}} \) genomic DNA, using primers surrounding exons 5 and 6 and direct sequencing, revealed that the nucleotide in genomic DNA was adenosine at all these five positions, confirming that post-transcriptional modifications, such as editing, occur in this case.

Thus, addition of A-to-I RNA editing modifications to alternative splicing reveals the existence of at least nine \( \text{\textit{Dx6}} \) isoforms in the embryo (Table 6; type I is an

### Table 5

<table>
<thead>
<tr>
<th>Exon 1</th>
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<tr>
<td>F</td>
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Nucleotide and amino acid differences in exons 1 and 2 of the two \( \text{\textit{Dx5}} \) polymorphic alleles are in boldface type. Nucleotide positions, in parentheses, are indicated for the \( \text{\textit{Dx5}} \) allele A (GenBank accession no. AF272778). Putative N-linked glycosylation sites are underlined.

### Table 6

| \( \text{\textit{A-to-I Dx6 editing sites}} \) |
|------------------|------|
| Exon 5           | Exon 6 |
| 1 2 3 4 5 6 7    |       |
| ↓                | ↓      |
| (775) aacattggtgcaaaca taac aute(846) aat(939) |
| ↓                | ↓      |
| ↓                | ↓      |
| ↓                | ↓      |
| ↓                | ↓      |
| SV               | R G S/D |
|                  | M S     |

Numbers in parentheses correspond to the nucleotide sequence of the \( \text{\textit{Dx6}} \) type I cDNA (GenBank accession no. AF921145) and the amino acid position is indicated in superscript. Nucleotides and amino acids changed by mRNA editing are set in boldface type.

1. \( \text{\textit{Dx6}} \) cDNA types detected in Drosophila embryo (see text for full description).
2. \( \text{\textit{Dx6}} \) adult EST cDNA.
TABLE 7

<table>
<thead>
<tr>
<th>Site</th>
<th>%³</th>
<th>%⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>4</td>
<td>&lt;5</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>5</td>
<td>68</td>
<td>78</td>
</tr>
<tr>
<td>6</td>
<td>80</td>
<td>94</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

³ Calculated from 25 Dø6 embryonic cDNA clones.
⁴ Calculated from the ratio of A/G chromatogram peaks from direct sequencing of embryo RT-PCR.

unmodified sequence with respect to the genomic one). Among these isoforms, type II is similar to type I but is missing the first three nucleotides of exon 11 (CAG₁₉₀₅₋₁₇). Because noncoding regions can also be targets of editing (Rütter et al. 1999), skipping of this triplet could be explained if we suppose the AG 3’ splicing consensus of intron 10 is subject to editing, becoming GG. In this case, the splicing machinery will recognize the next AG, in the downstream CAG₁₉₀₅₋₁₇, as splicing consensus, resulting in the elimination of the amino acid Ser410 in the translated protein. Interestingly, the Dø6 EST (GH15518) has edited sites 4-7, but also revealed two additional adenosine-to-guanosine transitions and amino acid changes in exon 5: site 1-A₁₇₇G (Asn133 → Ser) and site 2-A₁₇₈G (Ile134 → Val). Thus, we have evidence for seven A-to-I editing positions in the Dø6 gene, five of which could be present in a small region of 19 bp around loop E of the ACh-binding site. Editing could result in eight potential nontemplated amino acid changes and, amazingly, >30,000 Dø6 nAChR isoforms are theoretically possible through RNA editing and alternative splicing, without considering any linkage between these events.

Moreover, in some of the 25 Dø6 cloned cDNAs, we found that other silent, not A-to-I editing-like nucleotide modifications could also be present at three other positions. The existence of these modifications in the embryonic transcripts was confirmed by independent RT-PCR experiments. Referring to the Dø6 type I cDNA numbering, nucleotide modifications are C₁₁₁₁_A in exon 9 encoding the TM3 segment and C₁₅₆₀_T in exon 10 and C₁₆₈₃_T in exon 11, both belonging to the long intracellular TM3-TM4 loop. Direct sequencing of a PCR amplification product covering these regions on genomic DNA did not reveal mixed signal at the three positions, suggesting an absence of polymorphism in these regions.

Dø6 gene A-to-I pre-mRNA editing via dADAR: It was previously shown that an adenosine deaminase acting on RNA (ADAR) is responsible for A-to-I pre-mRNA editing (reviewed by Rütter and Emeson 1998). Drosophila possesses one adenosine deaminase enzyme acting on RNA, dADAR. Mutants in dADAR have been generated and shown to lack A-to-I editing of known target ion channels and receptors (Palladino et al. 2000a,b). We made use of dADAR-deficient animals to prove that the Dø6 gene underwent editing and that the editing proceeded through hydrolytic deamination of A-to-I via dADAR. Dø6 transcripts were amplified by RT-PCR from wild-type, whole adult RNA. The resulting product, which was a mixture of the population of expressed/modified transcripts, was subjected to direct sequencing analysis. All sites, with the exception of site 3, were detected as a mixed sequence signal (Figure 3A). Sites 1 and 2 were detected at a low level that disappeared in dADAR mutants (data not shown). The ratios of A/G signal were reproducible from site to site among different RT-PCR reactions and when using different sequencing primers. Thus, we believe that the levels of editing observed using this technique are a reasonable measure of the levels of conversion at a given site. We repeated the experiment on embryonic RNA and confirmed that the frequency of editing in each site seen in sequencing cDNA products was consistent with the A/G signal levels seen by this method (Table 7). Then, we analyzed Dø6 transcripts from a dADAR-deficient mutant background. As illustrated in Figure 3A, all detectable mixed sequence signals were reduced to pure A signals at all sites of editing in Dø6. Thus, dADAR, the identified RNA editase of several other ion channels and receptors, is also the enzyme performing A-to-I pre-mRNA editing of Dø6 transcripts in Drosophila.

Evolutionary conservation of nAChR editing: The new Drosophila nAChRα subunit nucleotide sequences are homologous to nAChR genes in the Lepidoptera, H. virescens, named α7-1 and α7-2. We decided to determine whether transcripts of both genes were targets for dADAR. Using RT-PCR and PCR on genomic DNA, followed by direct sequencing, we demonstrated that the α7-2 site 2 was a genomically encoded G (Figure 4). All other homologous editing site positions in Heliothis α7-2 contained A in the genomic DNA (Figures 3B and 4). The RT-PCR data revealed that editing was not detectable at sites 1 and 7 (not shown). This might have been expected for site 1, which is edited at a low level in Dø6 transcripts. However, site 7, which is edited to a high extent in Dø6, is apparently not edited in Heliothis α7-2 transcripts. Surprisingly, sites 3-6, all of which are in exon 5 of Dø6, are edited in H. virescens to a great extent. Site 3, which is barely detectable in Dø6 (occurrence is <5%), is edited to a high degree in α7-2. Sites 4-6 are highly edited, with sites 5 and 6 in α7-2 being almost completely edited. This pattern of editing of the Dø6 homolog in Heliothis is quite different from that seen in Drosophila Dø6 (Figure 3C). Nevertheless, RNA editing of exon 5 of this nAChRα subunit has been conserved between the orders Diptera and Lepidoptera. This is the most highly conserved RNA editing event yet reported in invertebrates.

Using this same approach, we examined whether the other Heliothis α7-like gene, α7-1, exhibits editing cor-
responding to that in Da6/Heliothis α7-2 exon 5. We found no evidence of editing in α7-1 or its homolog, the Drosophila Da5 gene (data not shown).

**Primary sequence analysis:** Figure 5 shows an alignment of the proteins encoded by Da5 (allele A), Da6 (type I), and Da7 (putative full-length cDNA) genes with the *C. elegans* ACR-16 subunit (Ballivet et al. 1996), the chicken neuronal α7 nAChR subunit (Couturier et al. 1990), the *Torpedo californica* muscular α1 subunit (Noda et al. 1982), and the two *H. virescens* α7-like nAChR subunits.

The Da5-encoded protein exhibits an unusually large
N terminus of 304 amino acids specified by the first two coding exons. Analysis of Da5 primary sequence for the presence of a signal peptide consensus and other protein domains did not show any homology with known proteins or domains, other than arginine-rich (amino acids 129–144) and serine-rich (amino acids 266–280) regions. The SignalP prediction server detected an internal signal peptide (underlined in Figure 5). We can speculate that a post-translational maturation of Da5 polypeptide could occur by protease cleavage to yield a mature nAChR subunit able to insert into the plasma membrane in the correct topology. The putative protein encoded by Da7 also seems to have a candidate internal signal peptide whereas the Da6 deduced protein sequence shows a putative signal peptide in the N terminus of the protein (both underlined in Figure 5).

The three new D. melanogaster subunits possess the two 13 residue-spaced cysteines involved in the “cys-loop” disulphide bridge that determines the correct folded status of the ligand-binding, extracellular domain. All the T. californica nAChR α subunit structural canonical residues of the main ligand-binding loops are present in the novel Drosophila subunits (Arias et al. 1997; Corringer et al. 2000): W90 and Y90 of loop A; W150 and Y151 of loop B; and Y190, Cys192, and Y198 of loop C are conserved. Moreover, Da5, Da6, and Da7 show some vertebrate non-α muscle nAChR subunit residues that belong to the complementary ligand-binding region loops D (D85) and F (D106; T. californica δ subunit numbering), which are present in the homooligomeric-forming chicken α7 subunit. The more variable loop E is also present in Da5, Da7 (δR115; T. californica δ subunit numbering), and Da6 (γK124; T. californica γ subunit numbering). The N-terminal extracellular region of the novel Drosophila subunits shows conservation of the three putative chicken α7 N-linked glycosylation sites (N23, N67, and N110). Additional N-linked glycosylation sites are present in the large N terminus of Da5 protein and some reside in the region of the insertion/deletion polymorphism (Table 5). Multiple putative serine/threonine kinase phosphorylation sites were identified in the large cytoplasmic loop between TM3 and TM4, where phosphorylation is known to act, regulating the desensitization rate (Swope et al. 1999) and/or receptor expression and localization (Williams et al. 1998).

We noted the chicken α7C167 homologous position in the Da6 subunit is an aspartic acid residue. The mutation G189D, reported as G189D in Matsuda et al. (2000), was previously generated in chicken α7 subunit and was shown to reduce receptor sensitivity to the neonicotinoid insecticides. We note that an aspartic acid in the homologous position is also present in the H. virescens H. virescens 7-2 subunits as well as in the insect Locusta migratoria Loc3 subunit (Hermesen et al. 1998).

Due to alternative splicing and RNA editing, the Dα6 proteins display variability in their amino acid composition (Table 6). For example, editing at site 1 in adult EST cDNA disrupts an asparagine residue (N→S) that is highly conserved in the other homologous subunits. On the other hand, the I→V transition, in edited site

Figure 5.—Novel D. melanogaster nAChR α7-like subunit sequences. Protein alignment was performed/displayed with the Genedoc program. The conserved residues are highlighted in gray. The Da5 protein sequence is from allele A. Da6 is from the unedited type I variant cDNA carrying alternative exons 5a and 8b (sequences of the other alternative exons are shown on the top of the Da6 sequence and dots indicate conserved residues with type I version). Da6 residues, which can be changed by pre-mRNA editing, are boxed. The putative Da7 protein sequence is deduced by BLAST alignment with Da5 protein (italic type) and from the 5’ and 3’ RACE clones. Hva7-1 and Hva7-2 are the H. virescens α7-like subunits. CeACR-16 is the C. elegans ACR-16 subunit. Gga7 is the chicken α7 subunit. Tcρ1 is the T. californica α1 subunit. Ligand-binding consensus residues are in boldface type. Thick solid lines indicate the TM1–4 putative membrane-spanning segments. Putative peptide signals are underlined. The amino acid numbering starts after the peptide signal for Hva7-1, Hva7-2, CeACR-16, Gga7, and Tcρ1. Solid diamonds indicate conserved N-glycosylation sites with the chicken α7 subunit. Additional putative N-glycosylation sites in the large N terminus of Da5 are doubly underlined. Putative phosphorylation sites in the TM3-TM4 loop are underlined.
2, could reintroduce a conserved valine residue. In the embryonic \textit{Da6} type IV variant, editing at site 3 introduces the transition H → R in loop E, thus adding another positively charged residue. The coding triplet corresponding to the conserved N-linked glycosylation site chicken α7N110 belongs to editing sites 4 and 5. In some variant transcripts this glycosylation site can be eliminated by the N → S/G transition.

The alternative splicing of exon 8a or 8b versions will introduce in the \textit{Da6} subunit the asparagine residue at the chicken α7L234 homologous position in TM2 (N is also present in the homologous position of \textit{Da5} and \textit{Da7} subunits). This Leucine residue has been shown to be involved in the cation selectivity of the chicken α7 homomeric receptor since the transition L254Q decreases the Ca\textsuperscript{2+} affinity and permeability of this receptor (\textit{Bertrand et al.} 1993). Due to the similar polarity of Q and N amino acids, we predict that subunits encoded by \textit{Da5}, \textit{Da7}, and \textit{Da6} containing either exon 8a or 8b should have a different permeability to Ca\textsuperscript{2+} than the nontemplated subunits carrying exon 8c. Thus, alternative splicing of exon 8 could have important consequences on cation permeability of \textit{Da6}-containing nAChRs. More interestingly, this position corresponds to the so-called Q/R site in glutamate receptors, which in the GluR-B AMPA receptor is a target of A-to-I mRNA editing with the consequent Q → R amino acid transition, resulting also in cation permeability modification (\textit{Sommer et al.} 1991). It is noteworthy that for both receptor molecules two different post-transcriptional mechanisms have been used to modify one key amino acid position.

\textbf{\textit{Da5} and \textit{Da6} developmental expression:} A developmental study of the expression of \textit{Da5} and \textit{Da6} genes was conducted by quantitative RT-PCR, using staged RNA samples, with reference to the expression of the housekeeping \textit{Rpl17A} gene. On the basis of the plasmid dilutions used to construct the standard curve, we deduce that \textit{Da5} and \textit{Da6} are expressed at very low levels during development, on the order of 1000-fold lower than the level of ribosomal protein. However, even when MgCl\textsubscript{2} concentration and annealing temperature were optimized for each gene PCR amplification, it was difficult to obtain comparable amplification efficiencies, thereby precluding any comparison of the expression between these genes at the same developmental stage. Consequently, the results were derived from RT-PCR replicates and \textit{Da5} and \textit{Da6} transcript levels were expressed as a percentage of the levels in the embryonic stage (Table 8). The findings indicate a major expression of \textit{Da5} and \textit{Da6} in the embryo that declines, severely in the case of \textit{Da5}, in larvae and adults.

\textbf{DISCUSSION}

Three novel α7-like nAChR subunit genes have been identified in \textit{D. melanogaster}, \textit{Da5}, \textit{Da6}, and \textit{Da7}. All three genes are transcribed and quantitative RT-PCR indicates that \textit{Da5} and \textit{Da6} are developmentally regulated (the \textit{Da7} expression level was not studied). The high degree of identity among the \textit{Da5}, \textit{Da6}, and \textit{Da7} genes and the conservation of seven intron positions (data not shown) lead to the hypothesis that they are the product of duplication events.

We obtained from embryonic RNA full-length cDNAs for \textit{Da5} and \textit{Da6} genes, showing the existence of several expressed variants of \textit{Da6} resulting from alternative splicing of coding exon 3 and exon 8 repeats and multisite pre-mRNA editing. Moreover, we have evidence for the existence of two polymorphic alleles of the \textit{Da5} gene leading to transcripts with amino acid changes in an atypically large N terminus. The cDNA derived from allele \textit{B} could encode for a soluble protein whereas a classical nAChR subunit from allele \textit{A} cDNA could be generated only if it is subject to post-translational maturation, which remains to be demonstrated. In fact, it has been recently shown that a soluble ACh-binding protein (AChBP) secreted by glial cells is involved in cholinergic synapse modulation in an invertebrate (\textit{Smit et al.} 2001). AChBP may decrease or even terminate the postsynaptic ACh response. One may imagine that \textit{Da5} (allele \textit{B}) and \textit{Da7} truncated polypeptides could take part in analogous mechanisms in \textit{Drosophila}.

Genomic variation and alternative splicing have been reported also in human nAChR subunits (\textit{Villarroel} 1999; \textit{Weiland et al.} 2000). For some vertebrate ligand-gated and voltage-gated ion channels, it has been shown that alternative splicing or pre-mRNA editing of subunit transcripts can greatly increase structural and functional diversity (\textit{Sommer et al.} 1991; \textit{Kohler et al.} 1993; \textit{Niswender et al.} 1999). In the insect \textit{D. melanogaster}, both alternative splicing and mRNA editing contribute to chloride channel subunit diversity (\textit{French-Constant} and \textit{Roccheleau} 1993; \textit{Semenov} and \textit{Pak} 1999). The \textit{Drosophila} nAChR subunit \textit{D} has been reported to be subject to alternative splicing of an encoding exon, generating subunits with different assembly efficiency (\textit{Lansdell} and \textit{Millar} 2000b). Editing has been shown in sodium channel and calcium channel genes (\textit{Smith et al.} 1998; \textit{Hanrahan et al.} 2000). Our work provides

\begin{table}
\centering
\caption{\textit{Da5} and \textit{Da6} expression level}
\begin{tabular}{lccc}
\hline
 & Embryo & Larvae & Adult \\
\hline
\textit{Da5} (\(n = 3\)) & 100 & 16 ± 2 & 17 ± 2 \\
\textit{Da6} (\(n = 3\)) & 100 & 39 ± 6 & 40 ± 5 \\
\hline
\end{tabular}
\end{table}

Results from three independent experiments were expressed as ratio to the \textit{Rpl17A} value. Larvae and adult values were expressed relative to the value obtained on the embryo, which was taken as 100% (see MATERIALS AND METHODS). Means (±SEM) were finally calculated.

\begin{align*}
\text{TABLE 8}
\end{align*}
additional evidence for an increased diversity in nAChR subunits arising by alternative splicing of exons encoding functional domains and, moreover, the first evidence for pre-mRNA editing in any nAChR subunit. In fact, A-to-I conversions occur in several specific positions in Δ66 embryonic transcripts that are restricted to the ligand-binding regions.

**Δ66 editing is dADAR dependent:** In the mutant Drosophila dADAR, that completely lacks ADAR activity, site-specific A-to-I editing of all known pre-mRNA targets in Drosophila is abolished. RT-PCR on dADAR mutant RNA for the Δ66 gene showed only adenosine in all the seven editing sites we identified, thus demonstrating that Δ66 editing is dADAR dependent and is abolished in the ADAR mutant fly. In mammals editing by ADAR has been shown to occur within the context of predicted RNA secondary structure formed through interactions between exon and intron sequences (Higuchi et al. 1993). We searched for RNA secondary structure leading to base pairing between the main group of editing sites in Δ66 exon 5 and its downstream or upstream intron. In both cases, the edited region seems to form base pairing within exon 5 itself (data not shown). A similar result was obtained for the Fsp site in the para channel (Hanrahan et al. 2000).

The presence of two additional putative sites of editing, found only in the adult Δ66 EST clone (sites 1 and 2), suggests that some sites could be edited in a stage-specific manner. The existence of such developmentally regulated editing has been also demonstrated at two of the four editing sites in the D. melanogaster para channel transcript, the Sp and Sf sites (Hanrahan et al. 2000). We speculate that Δ66 alternative splicing of multiple exons could also be developmentally regulated, as recently found for the exon 4 region of the Drosophila Discam pre-mRNA (Celotto and Graveley 2001).

**Functional significance of Δ66 variants:** Most of the Δ66 residues changed by both alternative splicing and pre-mRNA editing are localized to key functional domains like the ligand-binding loops and TM2. We know that the dADAR mutant fly, where editing is missing in a number of ligand and voltage-gated ion channels, exhibits various age-dependent behavioral deficits accompanied by neurodegeneration (Palladino et al. 2000b). The dADAR mutant has also been independently discovered in a genetic screen for mutants sensitive to hypoxia conditions (Ma et al. 2001). Electrophysiological recordings on primary culture of dADAR mutant neurons show that the para channel conductivity is altered in the mutant fly especially in oxygen deprivation conditions (Ma et al. 2001). This result clearly indicates that misediting of channels could result in neuronal activity defects. Thus the not-edited/edited Δ66 subunit-containing receptors could play a critical role in nervous system function and integrity. Because editing at some Δ66-specific sites occurs also in the homologous positions of the α7-2 H. virescens gene (sites 3–6), we demonstrate for the first time a high degree of evolutionary conservation of the pre-mRNA editing between two distantly related insect groups (moths and flies). This implies modifications introduced by editing are of functional relevance.

Residue changes introduced by such post-transcriptional modifications suggest novel target residues for further investigation, which could be initially studied by site-directed mutagenesis of homologous positions in the well-characterized homomeric-forming chicken α7 subunit. Furthermore, as nAChRs are known targets of insect control chemicals, such as imidacloprid, a detailed study on subunit variants in a simple model such as the fruit fly could contribute to new approaches for controlling the pest species H. virescens.

With the three novel nAChR subunits, the Drosophila nAChR family now stands at nine members. A tenth gene (CG11822) is designated as nicotinic-like in the annotated Drosophila genome. On the basis of the 5′ and 3′ ends of the corresponding EST clone (SD09326) we cloned and sequenced a putative full-length cDNA (GenBank accession no. AY005148) and compared its protein sequence to the other nAChR genes. We found that it is only weakly related to any other existing group, but may be of interest (Figure 2).

Finally, our findings provide an important additional example that post-transcriptional modifications play a role in expanding protein diversity in an organism with a small genome such as Drosophila and that the gene number does not correspond to animal complexity. The 10 members of the D. melanogaster nAChR subunit family identified so far and the diversity resulting from their expression may help account for the physiological and pharmacological diversity observed in native Drosophila nAChRs.

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