The *Drosophila* nicotinic acetylcholine receptor subunits Da5 and Da7 form functional homomeric and heteromeric ion channels

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**Abstract**

**Background:** Nicotinic acetylcholine receptors (nAChRs) play an important role as excitatory neurotransmitters in vertebrate and invertebrate species. In insects, nAChRs are the site of action of commercially important insecticides and, as a consequence, there is considerable interest in examining their functional properties. However, problems have been encountered in the successful functional expression of insect nAChRs, although a number of strategies have been developed in an attempt to overcome such difficulties. Ten nAChR subunits have been identified in the model insect *Drosophila melanogaster* (Da₁-Da₇ and Dβ₁-Dβ₃) and a similar number have been identified in other insect species. The focus of the present study is the Da₅, Da₆ and Da₇ subunits, which are distinguished by their sequence similarity to one another and also by their close similarity to the vertebrate α₇ nAChR subunit.

**Results:** A full-length cDNA clone encoding the *Drosophila* nAChR Da₅ subunit has been isolated and the properties of Da₅-, Da₆- and Da₇-containing nAChRs examined in a variety of cell expression systems. We have demonstrated the functional expression, as homomeric nAChRs, of the Da₅ and Da₇ subunits in *Xenopus* oocytes by their co-expression with the molecular chaperone RIC-3. Also, using a similar approach, we have demonstrated the functional expression of a heteromeric ‘triplet’ nAChR (Da₅ + Da₆ + Da₇) with substantially higher apparent affinity for acetylcholine than is seen with other subunit combinations. In addition, specific cell-surface binding of [¹²⁵]α-bungarotoxin was detected in both *Drosophila* and mammalian cell lines when Da₅ was co-expressed with Da₆ and RIC-3. In contrast, co-expression of additional subunits (including Da₇) with Da₅ and Da₆ prevented specific binding of [¹²⁵]α-bungarotoxin in cell lines, suggesting that co-assembly with other nAChR subunits can block maturation of correctly folded nAChRs in some cellular environments.

**Conclusion:** Data are presented demonstrating the ability of the *Drosophila* Da₅ and Da₇ subunits to generate functional homomeric and also heteromeric nAChRs.

**Background**

Nicotinic acetylcholine receptors (nAChRs) are excitatory neurotransmitter receptors that are found in both vertebrate and invertebrate species. In insects, nAChRs are expressed throughout the nervous system and are the site of action for economically important insecticides such as spinosyns and neonicotinoids [1,2]. Detailed information is available concerning the structure of nAChRs, as a consequence of studies conducted with receptors purified from the electric organ of the marine ray *Torpedo* [3] and from X-ray crystallographic studies conducted with nAChR fragments [4] and also with the closely related acetylcholine binding protein [5]. Nicotinic receptors are assembled from five subunits arranged around a central cation-selective pore [6,7]. Conventional agonists, such as acetylcholine, activate the receptor by binding at an extracellular site located at the interface between two subunits [8], although recent evidence indicates that nAChRs can also be activated by ligands binding to an allosteric transmembrane site [9].

Ten nAChR subunits (Da₁-Da₇ and Dβ₁-Dβ₃) have been identified in the model insect *Drosophila melanogaster* and a similar number of subunits have been identified in other insect species [1,2]. Despite considerable
efforts, there has been only limited success in expressing insect nAChRs in artificial expressions systems [10,11] and, where functional expression has been achieved, ion channel currents have tended to be small or have been generated in response to relatively high agonist concentrations [12-14]. Experimental approaches that have had some success in overcoming problems associated with expression of insect nAChRs include the expression of subunit chimeras containing domains from other neurotransmitter receptors [15], co-expression of insect nAChRs with vertebrate subunits [16,17] or a combination of these approaches [18]. Co-expression with vertebrate nAChR subunits is an approach that has been used in the characterization of nAChR subunits cloned from insect pest species such as the aphid Myzus persicae [19,20] and the brown planthopper Nilapavarta lugens [21,22]. However, for most insect species for which nAChRs have been cloned, there have been no reports of successful heterologous expression. This includes nAChRs cloned from the honeybee Apis mellifera [23-25], diamondback moth Plutella xylostella [26,27], house fly Musca domestica [28-30], locust Locusta migratoria [31], mosquito Anopholes gambiae [25], red flour beetle Tribolium castaneum [25,32], silkworm Bombyx mori [25,33] and tobacco hornworm Manduca sexta [34].

RIC-3 is a nAChR-associated molecular chaperone that was originally characterised in the nematode Caenorhabditis elegans [35] but has also been identified in several other species, including mammals and insects [36]. It is a transmembrane protein that is able to enhance maturation (folding and assembly) of several nAChR subtypes [36]. For example, co-expression of RIC-3 with the vertebrate nAChR α7 subunit enhances levels of functional expression in Xenopus oocytes [35] and is able to facilitate the functional expression of α7 nAChRs in mammalian cell lines that are otherwise non-permissive for expression of α7 [37,38]. In some cell types it has been found that the α7 subunit can be expressed (subunit protein can be detected) but, in the absence of RIC-3, is unable to fold into a conformation that can be detected by radioligand binding or form functional nAChRs [37,38]. In addition, some success has been achieved in overcoming difficulties associated with expression of insect nAChRs by the co-expression with RIC-3 [39,40].

The Da5, Da6 and Da7 subunits of Drosophila show close sequence similarity to one another (53-63% amino acid identity [41]) and also have close similarity to the vertebrate nAChR α7 subunit (42-46% amino acid identity [42]). Of the three Drosophila subunits, Da5 and Da7 have the closest sequence similarity to one another and Da6 has the highest sequence similarity to the vertebrate α7 [43]. In the present study, we report the molecular cloning of the Da5 subunit, the only Drosophila nAChR subunit for which a full-length cDNA clone was not previously available in our laboratory. Heterologous expression studies with Da5, Da6 and Da7 are described in three host cell types: Drosophila S2 cells, human tsA201 cells and Xenopus oocytes. Functional expression of several subunit combinations has been achieved in Xenopus oocytes and has enabled the pharmacological properties of recombinant nAChRs to be examined. Evidence is provided that demonstrates the ability of subunits to form both homomeric and heteromeric nAChRs. Of particular note is evidence that Da5 can generate functional homomeric channels and that Da7 can form both homomeric and heteromeric channels. We are not aware of any previous studies demonstrating the ability of Da5 and Da7 subunits to generate such recombinant nAChRs, either with subunits cloned from Drosophila or with analogous nAChR subunits from other insect species.

Results

Molecular cloning of Da5

A full-length cDNA encoding the Drosophila nAChR Da5 subunit was isolated from a preparation of Drosophila embryo mRNA. The Da5 cDNA encodes an open reading frame of 807 amino acids corresponding to the previously described Da5 isoform B [41]. In agreement with previous studies [41], the Da5 cDNA isolated in this study contains an open reading frame encoding an unusually large N-terminal domain, extending some 300 amino acids upstream of the start methionine in most nAChRs subunits.

Heterologous expression of Da5 in Drosophila and human cell lines

The full-length coding sequence of the Da5 cDNA was sub-cloned into the Drosophila expression vector pRMHa3 (to facilitate expression in Drosophila S2 cells) and into pRK5 (to facilitate expression in human tsA201 cells). In cells transfected with pRMHa3-Da5 or pRK5-Da5 alone, no evidence of specific high-affinity binding of nicotinic radioligands ([125I]-α-bungarotoxin, [3H]-epibatidine or [3H]-methyllycaconitine) could be detected. The Da5 subunit was also co-expressed with an extensive series of Drosophila nAChR subunit combinations. Expression studies with more than 100 different subunit combinations (containing between 2 and 10 different Drosophila nAChR subunit subtypes) have been examined in our laboratory. However, no specific binding was detected with any these combinations (in the absence of any co-expressed chaperone proteins, see later). To illustrate the extent of these studies, details of all Drosophila nAChR subunit combinations containing the Da5 subunit are listed in Table 1. It is possible that the lack of radioligand binding is a consequence of the
Table 1 Radioligand binding to *Drosophila* nAChR subunit combinations

| Subunit combination | [125I]-α-BTX Binding | [3H]-epibatidine binding |
|---------------------|------------------------|--------------------------|
|                     | -RIC3      | +RIC3      | -RIC3      | +RIC3      |
| Da5                 | –          | –          | –          | –          |
| Da5/Da1             | –          | –          | –          | –          |
| Da5/Da2             | –          | –          | –          | –          |
| Da5/Da3             | –          | –          | –          | –          |
| Da5/Da4             | –          | –          | –          | –          |
| Da5/Da6             | –          | +          | –          | –          |
| Da5/Da7             | –          | –          | –          | –          |
| Da5/Dβ1             | –          | –          | –          | –          |
| Da5/Dβ2             | –          | –          | –          | –          |
| Da5/Dβ3             | –          | –          | –          | –          |
| Da5/Da1/Da2         | –          | –          | –          | –          |
| Da5/Da1/Dβ1         | –          | –          | –          | –          |
| Da5/Da1/Dβ2         | –          | –          | –          | –          |
| Da5/Da1/Dβ3         | –          | –          | –          | –          |
| Da5/Da2/Dβ1         | –          | –          | –          | –          |
| Da5/Da2/Dβ2         | –          | –          | –          | –          |
| Da5/Da2/Dβ3         | –          | –          | –          | –          |
| Da5/Da3/Dβ1         | –          | –          | –          | –          |
| Da5/Da3/Dβ2         | –          | –          | –          | –          |
| Da5/Da3/Dβ3         | –          | –          | –          | –          |
| Da5/Da4/Dβ1         | –          | –          | –          | –          |
| Da5/Da4/Dβ2         | –          | –          | –          | –          |
| Da5/Da4/Dβ3         | –          | –          | –          | –          |
| Da5/Da6/Da7         | –          | –          | –          | –          |
| Da5/Dβ1/Dβ2         | –          | –          | –          | –          |
| Da5/Dβ1/Dβ3         | –          | –          | –          | –          |
| Da5/Dβ2/Dβ3         | –          | –          | –          | –          |
| Da5/Da1/Da2/Dβ1     | –          | –          | –          | –          |
| Da5/Da1/Da2/Dβ2     | –          | –          | –          | –          |
| Da5/Da1/Da2/Dβ3     | –          | –          | –          | –          |
| Da5/Da1/Da3/Dβ1     | –          | –          | –          | –          |
| Da5/Da1/Da3/Dβ2     | –          | –          | –          | –          |
| Da5/Da1/Da3/Dβ3     | –          | –          | –          | –          |
| Da5/Da1/Da4/Dβ1     | –          | –          | –          | –          |
| Da5/Da1/Da4/Dβ2     | –          | –          | –          | –          |
| Da5/Da1/Da4/Dβ3     | –          | –          | –          | –          |
| Da5/Da2/Da3/Dβ1     | –          | –          | –          | –          |
| Da5/Da2/Da3/Dβ2     | –          | –          | –          | –          |
| Da5/Da2/Da3/Dβ3     | –          | –          | –          | –          |
| Da5/Da2/Da4/Dβ1     | –          | –          | –          | –          |
| Da5/Da2/Da4/Dβ2     | –          | –          | –          | –          |
| Da5/Da2/Da4/Dβ3     | –          | –          | –          | –          |

To illustrate the extent of radioligand binding studies undertaken, Table lists all subunit combinations containing Da5 that were examined in transfected *Drosophila* S2 cells. Binding studies were performed with [125I]-α-bungarotoxin (10 nM) and [3H]-epibatidine (30 nM). Combinations of *Drosophila* nAChR subunit cDNAs were transfected in the absence or presence of RIC-3 cDNA. Data indicating presence or absence of specific binding are derived from at least 3 independent experiments.

expressed subunit proteins failing to undergoing appropriate maturation (folding and assembly) due to a requirement for specific chaperone proteins, as has been reported for other nAChR subunits [37,38], or due to a requirement for additional nAChR subunits.
Previous studies have shown that, when co-expressed with a vertebrate β2 subunit, some Drosophila nAChR α-subunits can generate functional recombinant nAChRs and form high-affinity binding sites for nicotinic radioligands (see for example [16,44]). However, when Da5 was co-expressed with vertebrate β2 in Drosophila S2 cells or in human tsA201 cells, no specific radioligand binding could be detected. These findings with Da5 are similar to those conducted previously with the closely related Drosophila Da6 and Da7 subunits [15]. However, in control experiments conducted in parallel, high levels of specific radioligand binding were detected after co-expression of Drosophila Da2 and Da3 subunits with the rat β2 (Rβ2) subunit. This is in agreement with previous studies conducted with the Da2 + Rβ2 and Da3 + Rβ2 subunit combinations [17,45].

Da5/5HT3A subunit chimera
As has been described previously for the Da6 and Da7 subunits [15], a chimera was constructed containing the N-terminal ligand-binding domain of the Da5 subunit fused to the transmembrane and C-terminal regions of the mouse 5-HT3A subunit (5HT3A). Despite the inability of the intact Da5 subunit to be detected by [125I]-α-bungarotoxin binding when expressed in Drosophila S2, expression of the Da5/5HT3A chimera resulted in high levels of cell-surface [125I]-α-bungarotoxin binding (Figure 1). These data from recombinant subunit chimeras is consistent with evidence derived from native Drosophila nAChRs that Da5 forms part of an α-bungarotoxin binding nAChR [46]. Expression studies with the intact and chimeric Da5 subunit indicate that, in common with the Da6 and Da7 subunits, inefficient folding and assembly can be attributed to domains present in the C-terminal subunit domain. Similar conclusions have also been made concerning the closely related vertebrate α7 subunit [47].

The influence of co-expressing combinations of subunit chimeras was also examined. In comparisons to the level of [125I]-α-bungarotoxin binding detected with Da5/5HT3A alone, higher levels of specific cell-surface binding were detected when the Da5 chimera was co-expressed with other subunit chimeras (Da6/5HT3A and Da7/5HT3A; Figure 1). However, the levels of specific binding detected were not significantly higher than would have been expected from a possible additive effect of co-expressing these chimeras. Consequently, this data cannot be used as evidence to support the possibility of heteromeric co-assembly, as was the case previously for studies conducted with the Da6 and Da7 subunit chimeras [15].

Heterologous expression with RIC-3
Previous studies have demonstrated that the molecular chaperone protein RIC-3 can enhance maturation of several nAChRs [36]. This finding has prompted us to examine the influence of co-expressing Da5 with RIC-3 in cultured cell lines, as we have done previously for other Drosophila nAChR subunits [39]. Various combinations (see Table 1 and Figure 2 for details) of Da5, Da6 and Da7 were expressed with either CeRIC-3 or DmRIC-3 in both Drosophila S2 cells and human tsA201 cells. No specific cell-surface [125I]-α-bungarotoxin binding was detected when any of these subunits were expressed individually with RIC-3. However, specific binding was detected when Da5 was co-expressed with Da6 (Figure 2), albeit at a lower level than seen with the subunit chimeras (Figure 1). Interestingly, no specific binding was detected when these two subunits were also co-expressed with Da7 (or other subunits; see Table 1), suggesting that Da7 may co-assemble with Da5 or Da6 and, in doing so, impair receptor assembly or maturation. Similar results were obtained in both cell types examined (Figure 2), although specific binding was detected in mammalian cells only when they were cultured at a temperature lower than 37°C. As has been reported previously [15,17], lowering the temperature of transfected mammalian cells from 37°C to 25°C for 24
hours (the temperature at which \textit{Drosophila} S2 cells are maintained) facilitates receptor assembly and cell-surface expression. As has been discussed previously with respect to insect nAChR subunits [15,17], the detection of specific radioligand binding only in mammalian cells cultured at 25°C is likely to be a consequence of more efficient subunit folding and assembly at lower temperatures.

\textbf{Expression in \textit{Xenopus} oocytes}

\textit{Xenopus} oocytes were injected with cRNA encoding various combinations of the \textit{Drosophila} nAChR subunits Da5, Da6 and Da7. No evidence of functional expression was detected for any subunit combination in the absence of co-expressed RIC-3. However, when co-expressed with CeRIC-3, functional responses to acetylcholine were detected in oocytes injected with either the Da5 or the Da7 subunit, indicating the presence of functional homomeric Da5 and Da7 nAChRs (Figure 3). However, even when co-expressed with RIC-3, functional expression was somewhat inconsistent, being observed in some but not all batches of oocytes tested (responses greater than 5 nA were observed in only about a third of the oocyte batches tested). Dose–response curves indicate that acetylcholine has a similar EC\textsubscript{50} for these two homomeric receptor subtypes (8.8 ± 2.5 μM 6.7 ± 1.7 μM, respectively). In contrast, no functional expression was detected when Da6 was co-expressed with CeRIC-3.

Expression of pairwise subunit combinations (with CeRIC-3) gave dose–response curves that were not significantly different (P > 0.05) to that of homomeric Da5 or Da7 nAChRs (Table 2 and Figure 3). Consequently, it was not possible to conclude whether pairwise heteromeric receptors were expressed. One pairwise combination (Da6 + Da7) failed to generate consistent responses, an indication that co-assembly of Da6 with Da7 blocks formation of functional nAChRs in oocytes. However, when all three subunits (Da5, Da6 and Da7) were co-expressed with CeRIC-3, dose–response data indicated a single population of receptors with a significantly higher (ANOVA, P < 0.05; Student’s t-test P < 0.01) apparent affinity for acetylcholine (13.5 ± 1.7 nM; Figure 3) than that of either of the two homomeric nAChRs (Da5 or Da7) or any of the putative pairwise subunit combinations (Table 2). For all subunit combinations examined (see Table 2), functional responses to acetylcholine were completely blocked by a 10 min pre-incubation with 100nM α-bungarotoxin. This block was completely reversible but occurred on a slow timescale, recovery taking, typically, about 15 minutes (Figure 4).

No significant differences were observed in pharmacological properties when nAChRs were co-expressed with DmRIC-3 [39], rather than CeRIC-3 (data not shown).

\textbf{Discussion}

The Da5, Da6 and Da7 subunits differ from other \textit{Drosophila} nAChR subunits in their close sequence similarity to the vertebrate a7 nAChR subunit [41,48], a subunit that is notable for its ability to form both homomeric
and heteromeric nAChRs [49-52]. In addition to being one of the best characterised homomeric nAChRs, the vertebrate \( \alpha_7 \) subunit can co-assemble into heteromeric nAChRs by co-assembly with the \( \alpha_8 \) subunit (in avian species) [50]. Although an \( \alpha_8 \) subunit is not present in mammals, recent evidence indicates that the mammalian \( \alpha_7 \) subunit can also form functional heteromeric nAChRs by co-assembly with \( \beta_2 \) [51,52].

Relatively limited information is available about the physiological roles of the D\( \alpha_5 \), D\( \alpha_6 \) and D\( \alpha_7 \) subunits in Drosophila, or about the role of analogous subunits in other insect species. There is, however, evidence from studies of native nAChRs in Drosophila that D\( \alpha_5 \) forms part of a nAChR that is sensitive to \( \alpha \)-bungarotoxin [46], D\( \alpha_6 \) forms part of the spinosad-sensitive nAChR [53] and that D\( \alpha_7 \) is required for the visually-mediated cholinergic escape response [54].

As has been discussed elsewhere [10,11], difficulties have been encountered in the efficient functional expression of insect nAChRs. Here we report the cloning of a

![Figure 3](image)

**Figure 3** Functional expression of Drosophila nAChR subunit combinations in Xenopus oocytes. 

A) Dose–response curves for acetylcholine are shown for homomeric D\( \alpha_5 \) nAChRs (open circles) homomeric D\( \alpha_7 \) nAChRs (open squares) and for triplet D\( \alpha_5 + \alpha_6 + \alpha_7 \) nAChRs (closed circles). B) Dose–response curves for acetylcholine are shown for heteromeric D\( \alpha_5 + \alpha_6 \) nAChRs (open circles) and D\( \alpha_5 + \alpha_7 \) nAChRs (closed circles) In all cases, nAChR subunits were co-expressed with CeRIC-3. Data are means ± SEM of 3–8 independent experiments.

Table 2 Functional properties of recombinant nAChRs expressed in Xenopus oocytes

| Subunits | \( EC_{50} \) (\( \mu \)M or nM)* | Hill slope | \( n \)** | \( I_{\text{max}} \) [\( I_{\text{mean}} \)] † (nA) |
|----------|-----------------|-------------|-----|------------------|
| D\( \alpha_5 \) | 8.8 ± 2.5 \( \mu \)M | 1.1 ± 0.3 | 6 | 200 [141 ± 25] |
| D\( \alpha_6 \) | – | – | † | – |
| D\( \alpha_7 \) | 6.7 ± 1.7 \( \mu \)M | 1.0 ± 0.3 | 4 | 86 [45 ± 13] |
| D\( \alpha_5 + \alpha_6 \) | 8.6 ± 2.4 \( \mu \)M | 1.0 ± 0.1 | 5 | 47 [20 ± 8] |
| D\( \alpha_5 + \alpha_7 \) | 1.6 ± 0.3 \( \mu \)M | 1.0 ± 0.1 | 3 | 53 [39 ± 8] |
| D\( \alpha_6 + \alpha_7 \) | – | – | † | – |
| D\( \alpha_5 + \alpha_6 + \alpha_7 \) | 13.5 ± 1.7 nM* | 1.2 ± 0.3 | 6 | 150 [107 ± 12] |

* Note, \( EC_{50} \) value for D\( \alpha_5 + \alpha_6 + \alpha_7 \) is expressed as nM, rather than \( \mu \)M.

** \( EC_{50} \) and Hill slopes are means ± SEM of separate fits to dose–response curves derived from independent experiments (\( n = 3–6 \)).

† Relatively small currents were detected with all subunit combinations, as indicated by the size of the maximum current that was detected with each subunit combination (\( I_{\text{max}} \)) and the mean maximum current (\( I_{\text{mean}} \)) from between 6–20 different oocytes.

‡ Subunit combinations that failed to generate functional responses are indicated by a dash. This is based on studies conducted with at least 5 batches of oocytes that generated functional nAChRs with other subunit combinations and at least 10 oocytes from each batch (\( n > 50 \)).
full-length cDNA of the Drosophila D\(\alpha\)5 subunit corresponding to a previously described isoform B [41]. Other isoforms of D\(\alpha\)5 described previously (isoforms A and C) [41] are a consequence of alternative splicing and have fewer exons than isoform B. Isoform A lacks exon 7, which codes for part of the second transmembrane domain, whilst isoform C lacks exon 5, which codes for the region containing the extracellular Cys-loop. The cloning of the D\(\alpha\)5 subunit was first reported in 2002 [41] but no expression studies were described at that time. More recently, it has been reported that D\(\alpha\)5 does not generate functional homomeric nAChRs when expressed in Xenopus oocytes, even when co-expressed with RIC-3 [40]. Functional expression was, however, reported in the same study when D\(\alpha\)5 was co-expressed with D\(\alpha\)6 and RIC-3 [40]. In the present study, we have detected functional responses when D\(\alpha\)5 is co-expressed with D\(\alpha\)6 but, in contrast to the previous study [40], we have also obtained evidence for the functional expression of homomeric D\(\alpha\)5 nAChRs. Similarly, we have demonstrated that D\(\alpha\)7 can form both homomeric and heteromeric nAChRs. As far as we are aware, there have been no previous reports of the successful functional expression of D\(\alpha\)7, as either a homomeric or a heteromeric nAChR. Given the difficulties encountered in obtaining reproducible functional expression of insect recombinant nAChRs, it is not surprising that there may be some apparent differences in subunit combinations found to generate functional receptors in this and previous studies, particularly since the focus of the most detailed previous study was the identification of a spinosyn-sensitive nAChRs [40].

Our studies conducted in cell lines provided evidence that the pairwise combination D\(\alpha\)5 + D\(\alpha\)6 generates a high affinity radioligand binding site, a finding that agrees with previous studies demonstrating functional expression of D\(\alpha\)5 + D\(\alpha\)6 nAChRs in oocytes [40]. Interestingly, we have found no evidence of specific binding when D\(\alpha\)7 was co-expressed with D\(\alpha\)5 and D\(\alpha\)6 in the same cell lines. This lack of specific binding would seem to suggest that, in the two cell lines examined, co-assembly of D\(\alpha\)7 with the D\(\alpha\)5 and D\(\alpha\)6 subunit interferes with the formation of correctly assembled complexes. We observed a somewhat similar situation in oocytes, where expression of D\(\alpha\)7 alone generates functional nAChRs but it fails to do so when co-expressed with D\(\alpha\)6. This may reflect a tendency for D\(\alpha\)6 and D\(\alpha\)7 to assemble into non-functional complexes. The one situation where this tendency is not dominant is when all three subunits (D\(\alpha\)5 + D\(\alpha\)6 + D\(\alpha\)7) are co-expressed with RIC-3 in oocytes, where they are able to form a functional ‘triplet’ nAChR with high apparent affinity for acetylcholine.
The present findings suggest that the environment provided by the host cell exerts a substantial effect on the assembly of these nAChR subtypes, a phenomenon that has been reported previously for other nAChRs [47,55,56]. Previous studies by another research group [40] support the conclusion that co-assembly of Da5+Da6 nAChRs is somewhat inefficient. Not only was functional expression of the Da5+Da6 subunit combination found to be inconsistent in the previous study, but it also appeared to be dependent on the ratio of cRNAs injected [40]. Perhaps this inconsistent functional expression reflects a tendency for some subunit combinations to assemble into non-functional complexes and that this may be more prevalent in certain subunit stoichiometries. It is possible that, in the native cellular environment, factors determining efficiency of subunit assembly and maturation may differ, perhaps as a consequence of a different array of endogenous chaperone proteins. This conclusion is supported by previous studies that have indicated that influence of RIC-3 on maturation of nAChRs is influenced by the host cell [39] and may help to explain the differences that we have observed in the ability of some subunit combinations to assemble into nAChRs in different expression systems.

The data obtained from expression studies in Drosophila and human cell lines is broadly similar. However, successful expression in human cells required incubation at a temperature lower than would normally be maintained at (25°C, rather than 37°C) [note: Drosophila S2 cells are routinely maintained at 25°C]. Previous studies have demonstrated that the folding and assembly of the nAChRs from insects [17] and from some other non-insect species, such as the cold-water ray Torpedo [57], can be influenced by temperature. This temperature dependence appears to be a consequence of inefficient protein folding and/or subunit assembly at higher temperatures. Previously, due to difficulties in expression of Da6 and Da7 nAChR subunits, we examined the ability of subunit chimeras to assemble into complexes capable of binding $^{125}$I-α-bungarotoxin [15]. From such studies, it was possible to conclude that the Da6 and Da7 subunits were capable of heteromeric co-assembly. In the present study the data from subunit chimeras is less clear cut. Although higher levels of $^{125}$I-α-bungarotoxin were seen consistently when the Da5 chimera was co-expressed with either the Da6 and Da7 chimeras, it was not clear in all cases whether this was greater than an additive effect. Nevertheless these findings are consistent with the conclusion that Da5 is able to co-assemble into heteromeric complexes. For all subunit combinations examined, responses to acetylcholine were completely blocked by α-bungarotoxin, a finding that is consistent with previous studies conducted with native nAChRs purified from Drosophila which demonstrated that Da5 is part of an α-bungarotoxin binding nAChR [46].

As mentioned above, a previous study has reported the functional expression of heteromeric Da5+Da6 nAChRs (co-expressed with RIC-3) in Xenopus oocytes and also the inability of either Da5 or Da6 to form functional homomeric nAChRs [40]. Significantly, the authors of this earlier study describe substantial difficulties in achieving reliable functional expression. In the present study, despite demonstrating the functional expression of several combinations of the Da5, Da6 and Da7 subunits, we have also encountered a much lower success rate than is typically achieved with other nAChRs. In both transfected cell lines and in Xenopus oocytes, we occasionally failed to detect evidence of radioligand binding or functional expression, despite success with other nAChRs that were expressed as positive controls (for example the mammalian a7 nAChR). The difficulties that we and others have encountered may be associated with a tendency for these subunits to co-assemble into non-functional complexes. It is possible that this may reflect a requirement for additional chaperone proteins. Indeed, a study conducted with a C. elegans nAChR has demonstrated a requirement for three different chaperone proteins for efficient functional heterologous expression [58].

**Conclusions**

In summary, whereas it has been reported previously that Da5 and Da6 can form a functional heteromeric nAChR (albeit inefficiently) when expressed in Xenopus oocytes [40], this is the first evidence that either Da5 or Da7 can form functional homomeric nAChRs. It is also the first demonstration that Da7 can form a functional heteromeric nAChR. Of particular interest is the evidence that the three subunits examined in this study can co-assemble to form a functional triplet (Da5+Da6+Da7) nAChR with a high apparent affinity for acetylcholine.

**Methods**

**Plasmids and cRNA synthesis**

Subcloning of Drosophila nAChR subunit cDNAs Da1 (ALS), Da2(SAD), Da3, Da4, Da6, Da7, DJ1(ARD), DJ2(SBD) and DJ3 [alternative subunit nomenclature in parenthesis] into expression vectors pRMHa3 and pRK5 has been described previously [15,17,59,60]. Construction and subcloning of Da6/5HT3A and Da7/5HT3A subunit chimeras has also been described previously [15]. For expression studies in Xenopus oocytes, subunit cDNAs were subcloned into pGEMHE [61] downstream of the SP6 promoter. Plasmid constructs (pGEMHE) containing nAChR subunit cDNAs were linearized with Nhel and purified with QIAQuik PCR purification kit (Qiagen). In vitro synthesis of cRNA was performed
using mMessage mMachine SP6 transcription kit (Ambion). *C. elegans* RIC-3 (CeRIC-3) cDNA [35] was provided by Millet Treinin (Hebrew University, Israel). The *D. melanogaster* RIC-3 (DmRIC-3) cDNA used in this study corresponds to the previously described splice variant DmRIC-3\(^2\text{N}9\) [39].

**Molecular cloning of Da5**

Oligonucleotide primers were synthesized which correspond to the predicted 5’ and 3’ untranslated regions of transcript CG32975 identified by the GadFly *Drosophila* genome annotation project (flybase.org). A first-strand cDNA synthesis kit (G.E. Healthcare) was used to isolate cDNA from *Drosophila melanogaster* embryo Poly A + RNA (Clontech). A 2425 bp fragment was amplified using KOD hot start polymerase (Novagen) and was subcloned into plasmid pCRII (Invitrogen). The cDNA construct was sequenced and found to correspond to the previously identified isoform B [41] (note: isoforms A and C show alternative splicing and have fewer exons than isoform B). The cDNA fragment was subcloned into the EcoRI site of pRmHa3 and pGEMHE to create pRmHa3-Da5 and pGEMHE-Da5.

**Construction of Da5/5HT3A chimera**

To construct a Da5/5HT3A chimera, a similar approach was used to that described previously for the *Drosophila* Da6 and Da7 subunits [15] and for mammalian nAChR subunits [47,62]. A BclI site was introduced into pRmHa3-Da5 by means of the QuickChange site-directed mutagenesis method (Stratagene) at a position equivalent to V201 in the previously described mammalian a7/5HT3A chimera [62]. The C-terminal region of Da5 was removed by digestion with BclI and Smal and the corresponding region of the mouse 5HT3A subunit [63] ligated to create the construct pRmHa3-Da5/5HT3A. The chimeric cDNA was subcloned into plasmid pRK5 by excising the construct from pRmHa3 with restriction enzymes EcoRI and XbaI to create pRK5-Da5/5HT3A.

**Heterologous expression in cultured cell lines**

Schneider’s *Drosophila* S2 cells [64] were obtained from Dr Thomas Bunch, University of Arizona, and grown in Shields and Sang M3 medium (Sigma) containing 12.5% heat inactivated foetal calf serum (First Link), 100U/ml penicillin and 100 μg/ml streptomycin (Invitrogen) at 25°C. Exponentially growing S2 cells were transfected by a modified calcium phosphate method as described previously [15]. Preliminary experiments were carried out to ensure that incubation times were long enough to enable radioligand binding to reach equilibrium. Amounts of total cellular protein were determined by a Bio-Rad DC protein assay using bovine serum albumin standards.

**Oocyte electrophysiology**

Adult female *Xenopus laevis* frogs were obtained from the European *Xenopus* Resource Centre (University of Portsmouth). Oocytes were isolated and defolliculated as described previously [67] following procedures that have been approved by both UCL’s Biological Services Management Group and the UK Home Office (under licences PIL70/23585 and PPL70/06819). For heterologous expression, cRNA (6–12 ng) was injected into the oocyte cytoplasm in a volume of 32.2 nl, using a Nanoject II microinjector (Drummond Scientific). Experiments were performed, typically, 2–4 days after injection of oocytes. Two electrode voltage-clamp recordings (with the oocyte membrane potential held at -60 mV) were performed essentially as described previously [67], using a Warner Instruments OC-725 C amplifier (Harvard Apparatus), PowerLab 8SP and Chart 5 software (AD Instruments). Agonists were applied to oocytes using a BPS-8 solenoid valve solution exchange system (ALA Scientific), controlled by Chart software. Data were analyzed using GraphPad Prism software. For multiple comparisons, statistical significance was determined by ANOVA with Tukey’s post-hoc test. Additional pairwise comparisons were performed by Student’s t-test.
Abbreviations
nAChR: nicotinic acetylcholine receptor.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
SJL performed the experimental work (molecular biological, pharmacological and electrophysiological), interpreted the data and helped to write the manuscript. TC performed some of the molecular biological work. JG was involved in planning experiments and assisted in interpretation of the data. NSM designed the study, assisted in interpreting the data and helped to write the manuscript. All authors read and approved the final manuscript.

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