Neuronal nicotinic acetylcholine receptors of *Drosophila melanogaster*: the α-subunit Dα3 and the β-type subunit ARD co-assemble within the same receptor complex

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Abstract **Dα3 is a functional α-subunit of *Drosophila melanogaster* nicotinic acetylcholine receptors (nAChRs). Here, we produced Dα3-specific antibodies to study which other nAChR subunits can co-assemble with Dα3 in receptor complexes of the *Drosophila* nervous system. Immunohistochemical studies revealed that Dα3 is co-distributed with the β-subunit ARD in synaptic neuropil regions of the optic lobe. Both subunits can be co-purified by α-bungarotoxin affinity chromatography. Dα3 antibodies co-immunoprecipitate Dα3 and ARD proteins and, vice versa, anti-ARD antibodies co-precipitate ARD and Dα3. These data demonstrate that one type of fly nAChRs includes these two subunits as integral components.**

Key words: Insect; Nervous system; Neurotransmitter receptor; Synapse; Quaternary protein structure; Immunoprecipitation

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are an important class of excitatory neurotransmitter receptors in the central nervous system (CNS) of insects [1]. Multiple subunits of insect nAChRs have been identified by cloning their genes or cDNAs [2–7]. However, despite their significance, the quaternary structure of these receptors is still unknown.

From the fruit fly *Drosophila melanogaster*, five neuronal nAChR subunits have been characterized in some detail (for review see [2,7,8]). These are the α-type subunits ALS, Dα2/SAD, Dα3 and the β-type subunits ARD and SBD. ALS and ARD are part of high-affinity binding sites for the snake venom component α-bungarotoxin (αBgt) and therefore were thought to be components of the same receptor complex [9,10]. Both subunits show a similar distribution in the *Drosophila* CNS [11], however, a direct physical interaction has not been shown. More recently it was shown that Dα2 has a similar distribution to ALS and ARD [12] and co-assembly of ALS and Dα2 could be confirmed by co-immunoprecipitation and co-expression studies in frog oocytes [13]. Whereas the α-subunits can form functional receptors in heterologous expression systems when combined with β-subunits of vertebrate neuronal nAChRs, all attempts to functionally express *Drosophila* β-subunits have failed so far [5,14,15]. Therefore our current information about the subunit composition of fly nAChRs is circumstantial and incomplete.

Dα3 is an αBgt binding subunit predicted to consist of 773 amino acid residues, thus being the largest of the *Drosophila* nAChR proteins identified to date [5]. Here we have generated a Dα3-specific antiserum to further characterize the protein and to identify subunits co-assembling with Dα3. Co-precipitation and co-localization studies demonstrate that this subunit can be found in receptor complexes together with ARD.

2. Materials and methods

2.1. D. melanogaster strains

Wild-type flies (Canton S) were used for immunohistochemistry and the mutant strain w^{1118} for biochemical studies.

2.2. Generation of antibodies

The mouse monoclonal antibody (mAb) 3d2 was produced against the recombinant protein LAC305-444 comprising the large cytoplasmic loop of ARD fused to β-galactosidase [10] applying previously described procedures [16]. Rat polyclonal anti-ARD antibodies (pAb) were described previously [11]. The rabbit antiserum Dα3-85 was generated against the His-tagged fusion protein Dα3/HIS391–562, which includes part of the highly variable cytoplasmic region of the Dα3 protein. The recombinant protein was produced from an expression DNA construct that was generated by introducing an *Eco*RI and *Bam*HI restriction fragment (nucleotides 1528–2039) of the Dα3 cDNA [5] into the pTrcHisB1 expression vector (Invitrogen). The fusion protein was affinity-purified on Ni-NTA agarose (Qiagen) and 100 μg was used to immunize rabbits followed by two booster injections. Antibody production was performed by Eurogentec.

ARD and Dα3 antibodies were purified on protein G-Sepharose (Pharmacia) following the manufacturer's instructions.

2.3. Immunoprecipitation experiments

Detergent extracts of *Drosophila* head membranes were prepared as previously described [9]. GammaBind plus Sepharose was equilibrated in buffer A (10 mM Tris–HCl, pH 8.0; 10 mM NaCl; 1% (v/v) Triton X-100). 50 μl of 1:1 GammaBind plus Sepharose/buffer A suspension was incubated for 2 h at 4°C with 10 μg of antibody. Detergent extract (2.5 mg protein) was incubated overnight with antibody-coupled GammaBind plus Sepharose in a final volume of 1 ml buffer A. In competition experiments, a 1000-fold molar excess (as compared to antibody molecules) of Dα3 or ARD fusion proteins, which served as antigen for the production of Dα3- or ARD-specific antibodies, was included in the respective incubation mixture. Immunoprecipi-

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tates were collected by centrifugation and pellets were washed twice with buffer A, twice with buffer B (buffer A containing 1 M NaCl) and once with buffer C (50 mM Tris–HCl, pH 6.8). Proteins were eluted from the GammaBind plus Sepharose with gel loading buffer, subjected to SDS–PAGE and electro-blotted as described [17]. Immunoblots were developed using the ECL detection system (Amersham).

2.4. Affinity chromatography with αBgt

αBgt from *Bungarus multicinctus* (10 mg; Sigma) were bound to tresyl-activated agarose (Schleicher and Schuell) according to the manufacturer's instructions except that coupling buffer of pH 9.0 was used. Aliquots of αBgt-agarose (500 μl) were equilibrated with buffer 1 (50 mM Tris–HCl, pH 8.5; 220 mM NaCl; 1% (v/v) Triton X-100; 0.6% deoxycholate; 10% (v/v) glycerin). Detergent extracts of *Drosophila* head membranes (250 mg) were incubated overnight at 4°C with equilibrated αBgt-agarose. After binding the gel matrix was washed with 20 ml buffer 2 (137 mM NaCl, 2.7 mM KCl, 7.4 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.1% (v/v) Triton X-100), 20 ml buffer 3 (buffer 2 with 0.5 M NaCl) and 10 ml with buffer 1. αBgt binding proteins were eluted with 100 μl 1% SDS, lyophilized and subjected to Western blot analysis.

2.5. Immunohistochemistry

Immunohistochemical studies were performed essentially as described previously [11], except that fluorescent secondary antibodies were used for immunodetection. Cy5-coupled anti-rabbit (Dianova) and anti-mouse (Dianova) antibodies were used in a 1:100 dilution.

3. Results

3.1. Identification of the Dα3 protein

Rabbit antibodies (pAb Dα3-85) were generated against a part of the large cytoplasmic domain of Dα3, which displays basically no sequence similarity with other *Drosophila* nAChR subunits [5,8]. Probably due to the low abundance of Dα3 protein present in detergent extracts of fly head membranes, the antibodies do not recognize any specific protein on immunoblots of these extracts (Fig. 1A, lane 1). Therefore we tested whether the antibodies can immunoprecipitate their antigen from solubilized membranes. A major protein band migrating at ~100 kDa and a second band at >160 kDa are detected on immunoblots of these precipitates (Fig. 1A, lane 4). The precipitation of both proteins is specifically blocked if an excess of the recombinant protein, which served as antigen to generate the Dα3 antiserum, is present during the antibody–antigen binding reaction (Fig. 1A, lane 5). The protein core of Dα3 has a calculated M_r of 85 421 [5]. As the protein is expected to be glycosylated and as membrane proteins do not always migrate exactly according to their molecular mass in SDS–PAGE, we assume that the 100 kDa protein band represents Dα3.

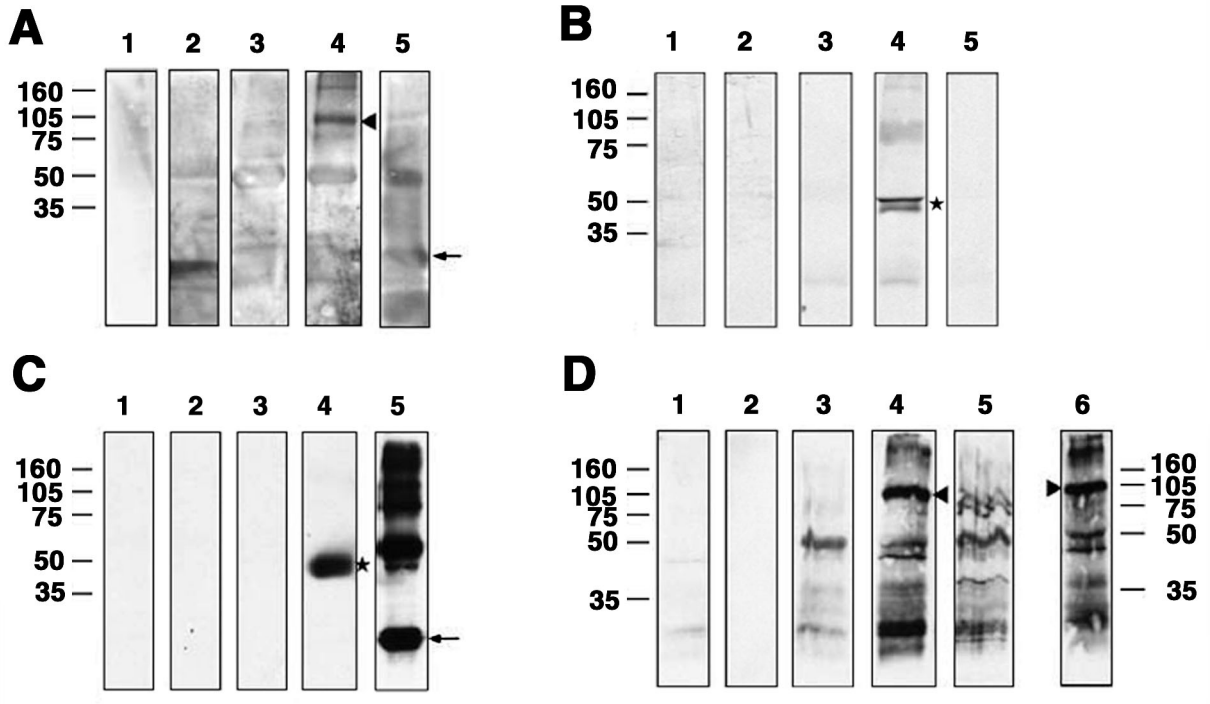

Fig. 1. Identification and co-immunoprecipitation of Dα3 and ARD *Drosophila* nAChR subunits. Immunoprecipitations were performed with pAb anti-Dα3 antibody Dα3-85 (A,B) and anti-ARD antibodies pAb RII (C,D) or mAb 3d2 (D, lane 6). Immunodetections on Western blots were performed with anti-Dα3 Dα3-85 (A,D) and mAb 3d2 (B,C). A: Dα3 antibody immunoprecipitates Dα3 (triangle). Lane 1: detergent extracts of head membranes; lane 2: material absorbed by GammaBind plus Sepharose in the absence of pAb Dα3-85; lane 3: eluate of Dα3-85 coupled to GammaBind plus Sepharose without detergent extract. Immunoprecipitated Dα3 (lane 4) is competed by an excess of recombinant cytoplasmic Dα3 fragment (arrow) (lane 5). Note that, as the same rabbit antiserum was used for precipitation and immunodetection, immunoglobulin heavy chains are also detected. B: Dα3 antibody co-immunoprecipitates ARD protein (asterisk). Lanes 1–3 as in A. Immunoprecipitated ARD (lane 4) is competed by an excess of recombinant cytoplasmic Dα3 fragment (lane 5). C: ARD antibody RII immunoprecipitates ARD protein (asterisk). Lane 1: detergent extract of head membranes; lane 2: material absorbed by GammaBind plus Sepharose in the absence of pAb RII; lane 3: RII bound to GammaBind plus Sepharose without detergent extract; immunoprecipitated ARD (lane 4) is competed by an excess of recombinant cytoplasmic ARD fragment (arrow) (lane 5). D: ARD antisera co-immunoprecipitate Dα3 subunit (triangle). Lanes 1–3 as in C. Immunoprecipitated Dα3 (lane 4) is competed by an excess of recombinant cytoplasmic ARD fragment (lane 5); anti-ARD mAb 3d2 co-immunoprecipitates Dα3 subunit (triangle) (lane 6).

3.2. Dα3 and ARD are co-localized in the fly CNS

The Dα3-85 antiserum was used to study the distribution of Dα3 in the adult fly brain by immunohistochemistry. As exemplified for the optic lobe (Fig. 2A) the distribution of this subunit is similar to that of ARD, ALS and Dα2 (Fig. 2B [11,12]). Interestingly, Dα3 antibodies recognize distinct structures in the distal lamina (Fig. 2A,C), which are also stained by anti-ARD antibodies (Fig. 2B,D) but not by antibodies against ALS and Dα2 [11,12]. This Dα3/ARD-immunoreactive structure is also stained with antibodies against choline acetyltransferase confirming that it includes cholinergic synapses [18].

3.3. Dα3 and ARD are co-immunoprecipitated by anti-Dα3 and anti-ARD antibodies

The co-distribution of Dα3 and ARD immunoreactivities prompted us to test whether these two subunits are components of the same receptor complex. As Dα3 is an αBgt binding subunit [5] we tested whether both subunits can be co-purified from head membrane extracts by affinity chromatography with this snake toxin. Eluates of αBgt columns contain the 100 kDa protein recognized by anti-Dα3 antibody (Fig. 3, lane 4), which is not detectable in extracts (see above), flow-through or wash fractions. A double band at 50/45 kDa is specifically detected with two different anti-ARD antibodies (Fig. 3, lanes 5 and 6). As reported previously, ARD migrates at about 50 kDa in SDS–PAGE [11]. Presently it is unclear why ARD in some instances migrates as a doublet. The co-enrichment of Dα3 and ARD in eluates of αBgt columns further supports, but does not prove, that the two subunits co-assemble into the same receptor.

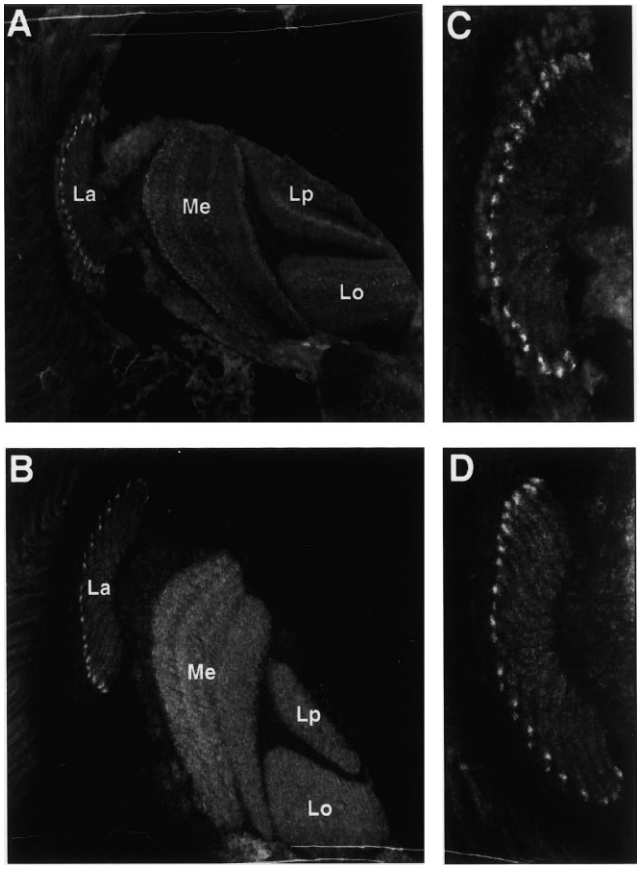

Fig. 2. Co-distribution of ARD and Dα3 subunits in the optic lobe of adult flies. Immunohistochemistry was performed with pAb anti-Dα3 antibody Dα3-85 (A,C) and mAb anti-ARD antibody 3d2 (B,D). Staining with both antibodies is co-localized in the neuropils of the lamina (La), the medulla (Me), the lobula (Lo) and the lobula plate (LP). Punctate structures in the lamina (C,D) constitute a striking example of exact co-localization.

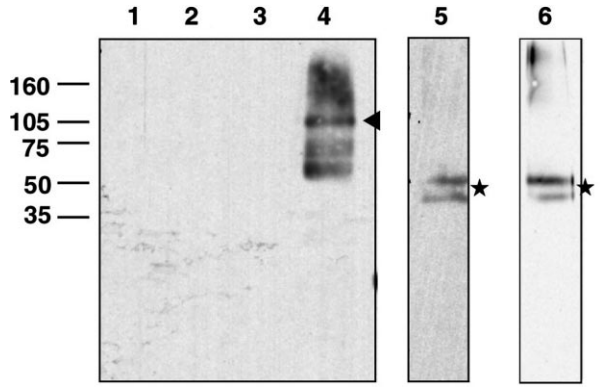

Fig. 3. Co-purification of Dα3 and ARD proteins by αBgt affinity chromatography. Immunoblots of detergent extract of head membranes (lane 1), flow-through (lane 2) and wash fraction (lane 3) and eluate (lanes 4–6) of αBgt column. Lanes 1–4 were probed with pAb Dα3-85, lane 5 with mAb 3d2 and lane 6 with antiserum RII. The triangle indicates the Dα3 protein, the asterisks the ARD double band.

To confirm co-assembly of Dα3 and ARD, immunoprecipitation studies were performed with antibodies against both subunits. The antiserum pAb Dα3-85 precipitates in addition to Dα3 (Fig. 1A, lane 4) a ~50 kDa protein that is detected by the mAb 3d2 anti-ARD antibody (Fig. 1B, lane 4). The intensity of staining is significantly reduced, if recombinant Dα3 fusion protein (Dα3/HIS391–562) is added to the binding reaction (Fig. 1B, lane 5). Unfortunately, the ARD protein exactly co-migrates with the immunoglobulin heavy chain of the immunoprecipitating antibody (Fig. 1B, lane 4). Therefore the reciprocal immunoprecipitation experiment was performed with anti-ARD antibodies. Polyclonal rat anti-ARD antiserum RII specifically immunoprecipitates the ARD protein as detected with mAb 3d2 (Fig. 1C, lane 4). The 50 kDa band is not detected in solubilized membranes nor in control experiments (Fig. 1C, lanes 1–3). Addition of an excess of recombinant ARD fusion protein (ARD/MS313–439 [9]) to the incubation mixture significantly reduced the amount of ARD protein precipitated (Fig. 1C, lane 5). Both anti-ARD antibodies, pAb RII and mAb 3d2, co-immunoprecipitate Dα3 protein (Fig. 1D, lanes 4 and 6). Again precipitation is competed by the addition of bacterially expressed ARD fusion protein (ARD/MS313–439) that was used to generate the anti-ARD antibodies (Fig. 1D, lane 5).

4. Discussion

Molecular, physiological and pharmacological data have indicated that different types of nAChRs exist in the *Drosophila* CNS [2,7,19]. The data reported here show that the two nAChR subunits ARD and Dα3 are co-assembled within one type of these receptor complexes. First, immunohistochemical data indicated the presence of ARD and Dα3 in the same

receptor complex. To date the distribution of ALS, ARD, Dα2/SAD and Dα3 has been studied in the adult brain ([11,12] and this study). While all four subunits are essentially co-distributed in most synaptic neuropil regions, only ARD and Dα3, but not ALS and Dα2, immunoreactivity is detected in globular cholinergic structures in the distal lamina (Fig. 2). The identity of these structures is presently unclear [11]. Second, immunoprecipitation experiments with anti-ARD and anti-Dα3 antibodies confirmed the hypothesis that these two subunits are integral components of the same receptor complex. No ALS and Dα2 immunoreactivity was found in immunoprecipitates of anti-Dα3 antibody. Vice versa, neither anti-ALS nor anti-Dα2 antibodies precipitate Dα3 protein (not shown), suggesting that the former two α-subunits may belong to different types of receptors. Co-expression of Dα3 with other *Drosophila* nAChR subunits including ARD in *Xenopus* oocytes failed to synthesize functional receptors [5]. This suggests that other, yet unknown subunits may be required to form functional receptors together with Dα3 and ARD. Alternatively proper expression and assembly of insect receptors may be impaired in vertebrate expression systems [15].

From immunoprecipitation studies of αBgt binding sites we have previously concluded that ARD may assemble with the toxin-sensitive ALS into the same receptor complex [10]. More recently, we have shown that the two ligand binding subunits ALS and Dα2 are immunoprecipitated together from head membrane extracts and form functional trimeric receptors when co-expressed with vertebrate β2-subunit [13]. Both ALS and Dα2 are also present in eluates of αBgt columns (data not shown). However, immunoprecipitation provided no further evidence for the presence of ARD in ALS-containing receptors. Though the present data do not allow us to conclude that ARD is present exclusively in Dα3-containing receptors, we now consider it likely that Dα3, rather than ALS, is the toxin binding subunit that assembles with ARD.

Despite the fact that the complete quaternary structure of insect nicotinic receptors still remains to be elucidated, a view emerges of two receptor complexes with a similar, though not completely identical distribution in the adult synaptic neuropil regions. It will, however, require localization studies at the ultrastructural level to decide whether the two types of nAChRs are present at the same synapse (this would be reminiscent of glutamate receptor subtypes in the vertebrate CNS) or at neighboring synapses [20,21].

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