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Atomic Force Microscopy Reveals the Stoichiometry and Subunit Arrangement of the $\alpha_4\beta_3\delta$ GABA_A Receptor

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Running title page

Running title: Subunit Arrangement of the $\alpha_4\beta_3\delta$ GABA_A Receptor

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Abbreviations: AFM, atomic force microscopy; HA, hemagglutinin.

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ABSTRACT

The GABA_A receptor is a chloride-selective ligand-gated ion channel of the Cys-loop superfamily. The receptor consists of five subunits arranged pseudosymmetrically around a central pore. The predominant form of the receptor in the brain contains α_1 -, β_2 -, and γ_2 -subunits in the arrangement $\alpha\beta\alpha\gamma\beta$, counter-clockwise around the pore. GABA_A receptors containing δ - instead of γ -subunits, although a minor component of the total receptor population, have interesting properties, such as an extrasynaptic location, high sensitivity to GABA and potential association with conditions such as epilepsy. They are therefore attractive targets for drug development. Here we addressed the subunit arrangement within the $\alpha_4\beta_3\delta$ form of the receptor. Different epitope tags were engineered onto the three subunits, and complexes between receptors and anti-epitope antibodies were imaged by atomic force microscopy. Determination of the numbers of receptors doubly decorated by each of the three antibodies revealed a subunit stoichiometry of $2\alpha:2\beta:1\delta$. The distributions of angles between pairs of antibodies against the α - and β -subunits both had peaks at around 144° , indicating that these pairs of subunits were non-adjacent. Decoration of the receptor with ligands that bind to the extracellular domain (i.e. the lectin concanavalin A and an antibody that recognizes the β -subunit N-terminal sequence) showed that the receptor preferentially binds to the mica extracellular face down. Given this orientation, the geometry of complexes of receptors with both an antibody against the δ -subunit and Fab fragments against the α -subunits indicates a predominant subunit arrangement of $\alpha\beta\alpha\delta\beta$, counter-clockwise around the pore when viewed from the extracellular space.

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The GABA_A receptor, responsible for fast inhibitory transmission in the CNS, is a member of the Cys-loop ligand-gated ion channel superfamily, together with the nicotinic acetylcholine receptor, the 5-HT₃ receptor and the glycine receptor (Karlin, 2002; Lester et al., 2004). The receptor exists as a heteromeric complex of five subunits, arranged pseudo-symmetrically around a central Cl⁻ ion channel (Sieghart, 1995). Electron microscopy of samples of purified GABA_A receptor reveals a cylinder of external diameter 7 nm with a central pore of diameter 2-3 nm (Nayeem et al., 1994). Nineteen GABA_A receptor subunit isoforms have so far been identified (Barnard et al., 1998). The predominant form of the receptor in the brain contains α_1 -, β_2 -, and γ_2 -subunits in the stoichiometry 2 α :2 β :1 γ (Farrar et al., 1999). Previous work, in which various combinations of concatenated subunits were expressed in *Xenopus* oocytes, indicated a subunit arrangement of $\alpha\beta\alpha\gamma\beta$, reading counter-clockwise around the pore when viewed from the extracellular face of the membrane (Baumann et al., 2002; Baur et al., 2006).

It is now clear that GABA_A receptors are found not only at the synapse but also extrasynaptically, where they act as sensors for extracellular GABA, mediating tonic inhibition (Brickley et al., 2001; Nusser and Mody, 2002; Stell et al., 2003). A significant population of extrasynaptic receptors appear to contain at least one δ -subunit, in combination with α_4 - or α_6 - and β_1 - or β_3 -subunits (Nusser et al., 1998; Brickley et al., 2001; Nusser and Mody, 2002; Stell et al., 2003). The δ -containing receptors are particularly sensitive to the natural agonist GABA, the response to which shows no evidence of the positive cooperativity that is displayed by the most ubiquitous member of the family comprising α_1 -, β_2 - and γ_2 -subunits; the δ -containing receptors also desensitize significantly more slowly (Brown et al., 2002).

Pharmacologically, the δ -containing receptors lack sensitivity to the classical

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benzodiazepines, and THIP is a more efficacious agonist than GABA (Adkins et al., 2001; Brown et al., 2002; Stórustova and Ebert, 2006; Chandra et al., 2006). While the expression of the δ -subunit is restricted both in density and distribution, there is evidence that both exogenous and endogenous stimuli result in significant plastic changes in its expression. Decreases in the level of expression of δ -containing receptors have been found in various animal models of epilepsy (Schwarzer et al., 1997; Zhang et al., 2007), while increases have been observed in mice during adolescence (Shen et al., 2007) and in rats during late-diestrus (Lovick et al., 2005), suggesting potential roles for these receptors in epilepsy, emotional stress in teenagers and pre-menstrual psychological disturbances in women. Thus, although the δ -containing receptors represent only a minor component of the total GABA_A receptor population, their atypical properties render them attractive novel targets for drug development. Exploration of receptor attributes at the molecular level has been facilitated by the construction of homology models of the $\alpha_1\beta_2\gamma_2$ receptor, using the nicotinic acetylcholine receptor as the structural template (Ernst et al., 2005; Mokrab et al., 2007). However, as recognition sites for agonists, antagonists and allosteric agents are found at subunit interfaces within this family, it is important to determine not only the stoichiometry but also the architecture of the subunits within the pentamer if useful homology models are to be constructed for other receptor subtypes.

We have developed a method, based on atomic force microscopy (AFM) imaging, for directly determining the arrangement of subunits within multimeric proteins. So far, we have applied this method to the architecture of the $\alpha_1\beta_2\gamma_2$ GABA_A receptor (Neish et al., 2003), the 5-HT₃ receptor (Barrera et al, 2005a) and the P2X receptor (Barrera et al., 2005b; Barrera et al, 2007). The method involves engineering epitope tags onto the receptor subunits and expressing the receptors exogenously in a suitable

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cell line (tsA 201). Receptors isolated from the transfected cells are then incubated with antibodies to the tags, and the resulting receptor-antibody complexes are imaged by AFM. The geometry of the complexes reveals the receptor architecture. Here we have used this method to determine the subunit arrangement within the $\alpha_4\beta_3\delta$ GABA_A receptor.

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Materials and Methods

GABA_A Receptor Subunit Constructs. The rat cDNA sequences used in these studies were those reported in the NCBI database: α_4 NM-080587, β_3 NM-017065.1, and δ NM-017289. cDNA encoding the GABA_A receptor α_4 -subunit, with a C-terminal FLAG epitope tag, was subcloned into the vector pcDNA3.1/V5-His A using *HindIII* and *AgeI*, so as to delete the V5 epitope tag. cDNA encoding the β_3 -subunit, was subcloned into the same vector using *KpnI* and *XbaI*. cDNA encoding the GABA_A receptor δ -subunit, with a C-terminal HA epitope tag, was subcloned into the same vector using *KpnI* and *AgeI*, so as to delete the V5 epitope tag.

Transient Transfection of tsA 201 Cells. Transfections of tsA 201 cells (a subclone of human embryonic kidney 293 cells stably expressing the SV40 large T-antigen) were carried out using the CalPhos™ mammalian transfection kit, (Clontech). After transfection, cells were incubated for 24-48 h at 37°C, to allow expression of the receptors.

Solubilization and Purification of His₆-tagged Receptors. The solubilization/purification procedure was as described previously for P2X receptors (Barrera et al., 2005b). Briefly, a crude membrane fraction prepared from transfected tsA 201 cells was solubilized in 1% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, and the solubilized material was incubated with Ni²⁺-agarose beads (Probond, InVitrogen). The beads were washed extensively, and bound protein was eluted with increasing concentrations of imidazole. Samples were analyzed by SDS-polyacrylamide gel electrophoresis, and protein was detected by immunoblotting, using mouse monoclonal antibodies against the His₆ tag, present on all subunits (Research Diagnostics Inc.), the FLAG tag on the α_4 -subunit (Sigma), the

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V5 tag on the β_3 -subunit (InVitrogen), or the HA tag on the δ -subunit (Covance), as appropriate. An anti-Myc antibody (Boehringer) was used as a negative control.

AFM Imaging of Receptors and Receptor-Antibody Complexes. GABA_A receptors were imaged either alone or following incubation for 14 h at 4°C with a 1:2 molar ratio (approximately 0.2 nM receptor concentration) of either anti-FLAG, anti-V5, anti-HA or anti-His₆ monoclonal antibodies. An anti-Myc antibody (Boehringer) was used as a negative control. Receptors were also incubated with Fab fragments of the anti-FLAG antibody, generated by papain digestion (ImmunoPure Kit; Pierce). Proteins were diluted to a final concentration of 0.04 nM, and 45 μ l of the sample was allowed to adsorb to freshly cleaved, poly-L-lysine-coated mica coverslips (Sigma). After a 10-min incubation, the sample was washed with MilliQ-water and dried under nitrogen. Imaging was performed with a Multimode atomic force microscope (Digital Instruments). Samples were imaged in air, using tapping mode. The silicon cantilevers used had a drive frequency \sim 300 kHz and a specified spring constant of 40 N/m (Mikromasch). The applied imaging force was kept as low as possible (target amplitude \sim 1.6-1.8 V and amplitude setpoint \sim 1.3-1.5 V).

The molecular volumes of the protein particles were determined from particle dimensions based on AFM images. After adsorption of the receptors onto the mica support the particles adopt the shape of a spherical cap. The heights and half-height radii were measured, and the molecular volume was calculated using the following equation:

$$V_m = (\pi h/6)(3r^2 + h^2) \quad (1)$$

where h is the particle height and r is the radius (Schneider et al., 1998).

Molecular volume based on molecular weight was calculated using the equation

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$$V_c = (M_0/N_0)(V_1 + dV_2) \quad (2)$$

where M_0 is the molecular mass, N_0 is Avogadro's number, V_1 and V_2 are the partial specific volumes of particle and water, respectively, and d is the extent of protein hydration (Schneider et al., 1998).

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Results

Exogenous Expression of $\alpha_4\beta_3\delta$ GABA_A Receptors. Rat $\alpha_4\beta_3\delta$ GABA_A receptors were expressed in tsA 201 cells by transfection with the appropriate cDNAs. The α_4 -subunit bore a FLAG-His₆ tag; the β_3 -subunit bore a V5-His₆ tag, and the δ -subunit bore a hemagglutinin- (HA-)His₆ tag. All tags were at the C-termini of the subunits. In cells transfected with cDNAs for all three subunits (at a 1:1:1 ratio by weight), anti-His₆, anti-FLAG, anti-V5 and anti-HA antibodies all gave positive immunofluorescence signals, but an anti-Myc antibody was negative (Fig. 1A). In cells transfected with cDNA for the β_3 - and δ -subunits, the anti-FLAG antibody gave no signal, as expected. Similarly, the anti-V5 antibody gave no signal in cells transfected with the α_4 - and δ -subunits, and the anti-HA antibody gave no signal in cells transfected with the α_4 - and β_3 - subunits. These results indicate that all three subunits are expressed in the tsA 201 cells and are detected specifically by the appropriate anti-epitope antibodies. The receptors were located both at the plasma membrane and in internal membranes.

Receptor Isolation. Crude membrane fractions of the transfected cells were solubilized in the detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (1%), and the receptors were isolated through their binding to Ni²⁺-agarose columns via their His₆ tags. As shown in Fig. 1B, the isolated GABA_A receptor gave positive signals on immunoblots with anti-FLAG, anti-V5 and anti-HA antibodies. The major bands in all three lanes ran at 50-60 kDa in size order $\alpha_4 > \beta_3 > \delta$, consistent with the expected sizes of the three subunits, and their previously reported mobilities on gels (Bencsits et al., 1999). An anti-Myc antibody gave no signal with the isolated receptor; furthermore, none of the antibodies gave a signal with fractions isolated from mock-transfected cells. These results show that the

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isolation procedure used results in the production of receptors containing all three subunits. The double bands seen in the lanes showing the α_4 -, β_3 - and δ -subunits likely represent different glycosylation states. It has been shown that inhibition of N-linked glycosylation by tunicamycin does not affect receptor assembly (Connolly et al., 1996). Hence the presence of subunits at various stages of glycosylation is unlikely to influence the results of our experiments.

AFM Imaging of Receptors. The GABA_A receptor preparation was adsorbed to a mica support, dried and subjected to AFM imaging in air. In a control experiment, a sample from mock-transfected cells was imaged. As shown in Fig. 2A, this sample was almost featureless. In contrast, the GABA_A receptor population appeared as a spread of particles (Fig. 2B). The difference in the appearances of the samples from mock-transfected and transfected cells indicates that the vast majority of the imaged particles represent isolated receptors or receptor subunits. The heights and radii of a large number of receptor particles from each sample were determined, as described (Neish et al., 2003; Barrera et al., 2005a,b, 2007). Particle radii were measured at half the maximal height to compensate for the tendency of AFM to overestimate this parameter when the radii of both particle and scanning tip are similar (i.e. in the nanometer range; Schneider et al., 1998). The dimensions were used to calculate molecular volumes using equation 1. The frequency distribution of the calculated molecular volumes (Fig 2C) had two clear peaks, at 120 ± 4 (S.E.M.) nm^3 , (n=431) and 593 ± 31 nm^3 (n=110). Each particle in Fig. 2B can be assigned on the basis of its size to one of the two peaks, with an arbitrary division being made at 420 nm^3 . Arrowheads indicate particles with a molecular volume $>420 \text{ nm}^3$; the remaining particles (unmarked) are $<420 \text{ nm}^3$. Clearly, the larger molecular volume (593 nm^3) is approximately five times the smaller molecular volume (120 nm^3), suggesting that the

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two peaks correspond to monomers and pentamers. The predicted molecular volume of a GABA_A receptor subunit, based on a molecular mass of 55 kDa, is 105 nm³ (Equation 2), close to the measured volume of 120 nm³. This close correspondence suggests that the particles imaged are indeed GABA_A receptor subunits and pentameric receptors. The small discrepancy between the predicted and the measured volumes is likely accounted for by the presence of some detergent bound to the isolated proteins, as has been observed for other ionotropic receptors (Neish et al., 2003; Barrera et al., 2005a,b, 2007).

Determination of the Subunit Stoichiometry by AFM Imaging of Receptor-Antibody Complexes. The GABA_A receptor was next imaged following incubation with mouse monoclonal antibodies against its epitope tags - FLAG on the α_4 -subunit, V5 on the β_3 -subunit, HA on the δ -subunit and His₆ on all three subunits. As shown in Fig. 3A, the receptor alone appeared as a heterogeneous spread of particles. Anti-FLAG antibody samples showed a more homogenous population of particles, as expected. Samples resulting from co-incubations of receptors and anti-FLAG antibodies appeared very heterogeneous. Nevertheless, there were examples of large particles (molecular volume >420 nm³) that were decorated by one (arrowhead) or two (arrows) smaller particles (presumably antibodies). Fig. 3B-D show galleries of images of individual receptors either undecorated, or decorated with one or two anti-FLAG (Fig. 3B), anti-V5 (Fig. 3C) and anti-His₆ (Fig. 3D) antibodies. For each anti-epitope antibody, many datasets were analyzed and the status (i.e. undecorated, or singly or double decorated) of the larger (>420 nm³) particles was assessed (Table 1). To ensure that the apparent receptor-antibody complexes were genuine and not simply a consequence of large and small particles settling close together on the mica surface, a control experiment was carried out, in which the receptor was incubated with an

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anti-Myc antibody. In this case, 2.0% of a total of 248 large particles appeared to have one small particle attached, and only one large particle appeared to be doubly decorated by small particles. In contrast, when the receptors were incubated with anti-FLAG, anti-V5, anti-HA or anti-His₆ antibodies, a substantial proportion (25.7-32.8%) of the large particles were decorated by single antibodies. Significantly, the proportion of the large particles that were doubly decorated by antibodies was in the range 7.1-7.8% for the anti-FLAG, anti-V5 and anti-His₆ antibodies, but was much smaller (0.7%) for the anti-HA antibody. These results indicate that the subunit stoichiometry of the receptor is 2 α :2 β :1 δ .

Given a 2 α :2 β :1 δ stoichiometry, there are six possible arrangements of subunits, reading counter-clockwise around the receptor rosette: $\alpha\beta\alpha\delta\beta$, $\alpha\beta\alpha\beta\delta$, $\alpha\alpha\beta\beta\delta$, $\alpha\alpha\delta\beta\beta$, $\alpha\delta\alpha\beta\beta$ and $\alpha\alpha\beta\delta\beta$. Note that the first two of these arrangements are mirror images of each other, and that the last four have either the two α -subunits or the two β -subunits (or both) adjacent. To narrow down the possible arrangements, receptors decorated with two anti-FLAG, anti-V5 or anti-His₆ antibodies were identified, and the angles between the pairs of bound antibodies were calculated by joining the height peaks of the particles. The angles were used to construct the frequency distributions shown in Fig. 3E-G. For both anti-FLAG and anti-V5 antibodies, the distributions had single peaks, at 137 ± 4 (S.E.M.) $^\circ$ (n=54) and 142 ± 4 (n=54), respectively, indicating that both the α - and the β -subunits were separated by another subunit (expected angle 144°). In contrast, the angle distribution for the anti-His₆ antibody, which should bind to all three types of subunit, had two peaks, at 78 ± 4 (n=23) and 139 ± 3 (n=31), indicating that it was binding to either adjacent or non-adjacent subunits. Based on these results, the last four subunit arrangements (above) can be ruled out, leaving two remaining possibilities: $\alpha\beta\alpha\delta\beta$ and $\alpha\beta\alpha\beta\delta$.

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Determination of the Subunit Arrangement. To determine the absolute subunit arrangement, we used two different approaches in tandem: (1) decoration of the receptor simultaneously with recognizably different ligands for either α - and δ - or β - and δ -subunits, and (2) determination of the orientation of the receptor on the mica substrate. As a first step, we produced Fab fragments of the anti-FLAG antibody, using papain digestion. When receptors were incubated with these Fab fragments, receptor-Fab complexes were produced. A frequency distribution for angles between pairs of bound Fabs is shown in Fig. 4A. The distribution has a single peak, at $135 \pm 5^\circ$ ($n=41$), again indicating that the α -subunits are non-adjacent. We then incubated the receptors with both anti-FLAG Fabs and anti-HA antibodies, to decorate both α - and δ - subunits. We identified 14 receptors that had clearly been decorated with two Fabs and one antibody. Representative images are shown in Fig. 4B, along with the subunit arrangements indicated by the patterns of decoration. Note that the Fabs are smaller than the antibodies, as expected. For each of the 14 complexes we determined the progression of angles between bound ligands, beginning with the whole antibody (bound to the δ -subunit) and reading counter-clockwise around the receptor. The angles from whole antibody to Fab, and from Fab to Fab, for individual decorated receptors are shown in Fig. 4C. The mean angles are 83° for antibody-Fab and 122° for Fab-Fab; furthermore, the Fab-Fab angles are larger than the antibody-Fab angles for 11 of the 14 pairs. A non-parametric Mann-Whitney test revealed that the Fab-Fab angle is significantly larger than the antibody-Fab angle ($P < 0.05$). These results indicate that the receptors had a preferred orientation on the mica, and that the predominant subunit arrangement was $\alpha\beta\alpha\beta\delta$, counter-clockwise.

To determine the orientation of the receptors, we coated the mica with either poly-L-lysine (as usual) or poly-L-glutamate, to give either a positively- or a negatively-

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charged surface. We then bound receptors to the two surfaces and incubated them with either concanavalin A, which should bind to the oligosaccharides on the extracellular face of the receptor (Im et al., 1989), or monoclonal antibody bd17, which recognizes an epitope at the N-terminus of the β -subunit (Richards et al., 1987). We reasoned that changing the charge of the mica surface would change the predominant orientation of the receptors, depending on the charge distribution over the receptor surface. According to the sequence database, the α_4 -subunit has three consensus glycosylation sites. Mapping the sequence of the α_4 -subunit onto a model of the structure of the $\alpha_1\beta_2\gamma_2$ form of receptor (Mokrab et al., 2007) indicates that these sites are 0.7 nm, 2.6 nm and 4.7 nm from the most extracellular part of the protein. The β_3 -subunit also has three consensus glycosylation sites, which are 1.4 nm, 1.6 nm and 7.0 nm from the most extracellular part of the receptor. Finally, the δ -subunit has two consensus glycosylation sites, which are 1.2 nm and 1.5 nm from the most extracellular part of the receptor. Assuming a subunit stoichiometry of $2\alpha:2\beta:1\delta$, therefore, there are 14 potential glycosylation sites, of which 10 are within 2.6 nm of the most extracellular part of the receptor. Concanavalin A, a tetramer of molecular mass 102 kDa, has a molecular diameter of about 8 nm ($2 \times 0.483 \times (\text{molecular mass})^{0.386}$; Venturoli and Rippe, 2005). Hence, we would assume that only the 4 sites that are either 4.7 nm or 7.0 nm from the most extracellular part of the receptor could possibly become tagged if the receptor were bound extracellular face down. Hence, receptors bound in this orientation would have the majority of their binding sites for concanavalin A occluded, reducing the probability of receptor decoration by the lectin. Their binding sites for antibody bd17 would also be occluded. Receptors decorated with smaller particles were observed following incubation with either concanavalin A or antibody bd17, irrespective of the mica coating. The decorated

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receptors shown in Fig. 4D are presumably resting on their sides, allowing the ligand to attach to the extracellular face. As shown in Table 2, for concanavalin A, 18/154 receptors were tagged at least once when bound to poly-L-lysine, compared with 37/180 when bound to poly-L-glutamate. According to the Chi-Square test, the proportion of receptors tagged by concanavalin A was significantly larger for poly-L-glutamate coated mica than for poly-L-lysine coated mica ($P < 0.05$). Similarly for antibody bd17, 14/161 receptors were tagged when bound to poly-L-lysine, compared with 37/217 when bound to poly-L-glutamate. Once again, the Chi-Square test indicates that the proportion of receptors tagged by antibody bd17 was significantly larger for poly-L-glutamate coated mica than for poly-L-lysine coated mica. Hence, more extracellular faces were occluded when the mica was coated with poly-L-lysine. We conclude that the receptor normally binds predominantly extracellular face down to poly-L-lysine coated mica, probably because the negatively charged oligosaccharides bind to the positively charged surface. In contrast, when the surface is coated with poly-L-glutamate, the receptor binds preferentially intracellular face-down, likely because the intracellular domain of this receptor carries a net positive charge (+54 for the stoichiometry described above; the charge totals are $\alpha 4$: 2D, 8E, 10R, 13K; $\beta 3$: 10D, 5E, 12R, 12K; δ : 5D, 5E, 11R, 9K, using single-letter amino acid code). With a receptor bound mainly extracellular face-down, the tagging pattern seen with the anti- α -subunit Fabs and the anti- δ -subunit antibody (above) indicates a predominant subunit arrangement of $\alpha\beta\alpha\delta\beta$, counter-clockwise, when viewed from the extracellular space, with some 21% (i.e. 3/14) of the population exhibiting a distinct subunit arrangement.

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Discussion

The $\alpha_4\beta_3\delta$ GABA_A receptor appears in our samples as a mixed population of assembled receptors and single subunits. The distribution of imaged particles between the two molecular volume peaks indicates that 20% of the particles are assembled receptors, or, put another way, 56% of the total subunits are present in assembled receptors. Whether the unassembled subunits are isolated in this state from the transfected cells, or whether receptors undergo partial disassembly during isolation, is unclear. It has been shown previously that exogenous expression of α_1 -subunits results in the production of monomers that remain in the ER and are quickly degraded (Connolly et al., 1996). In contrast, co-expression of both α_1 - and β_2 -subunits allowed the production of assembled receptors that accessed the cell surface. Assembly of other Cys-loop receptors, such as the nicotinic receptor (Green and Claudio, 1993) also occurs in the endoplasmic reticulum soon after polypeptide synthesis. If the $\alpha_4\beta_3\delta$ receptor behaves similarly, it is likely that, even though we are using a crude membrane fraction as our starting material, and are therefore isolating receptors from intracellular membranes as well as the plasma membrane, we will be isolating a mixture of correctly assembled receptors and unassembled subunits; however, we have no information about the relative extents of these two populations. It should be noted that the unassembled subunits seen here were not seen in our previous AFM imaging studies on the $\alpha_1\beta_2\gamma_2$ GABA_A receptor (Neish et al., 2003) and the 5-HT₃ receptor (Barrera et al., 2005a). This might suggest that the $\alpha_4\beta_3\delta$ receptor is particularly unstable, and partially disassembles during isolation. Alternatively, one of the three subunits might be produced in excess, despite the use of equal amount of the three cDNAs for the transfections, leading to the generation of a pool of subunits that cannot be incorporated into assembled receptors. Recent studies have suggested that

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the expression of α_1 - and β_2 -subunit oligomers in HEK393T cells is reduced nearly ten-fold by coexpression of the δ -subunit; maximal surface expression of all three subunits occurred at $\alpha_1:\beta_2:\delta$ cDNA ratios of 1:1:0.1 (Botzolakis et al., 2007), suggesting that the appearance of single subunits in our study may indeed be associated with inefficient receptor assembly caused by the presence of the δ -subunit.

The stoichiometry of the $\alpha_4\beta_3\delta$ receptor subtype is 2:2:1 and the α - and β -subunits are non-adjacent. Simultaneous decoration of receptors with anti- α -subunit Fab fragments and an anti- δ -subunit whole antibody revealed that the receptor has a predominant subunit arrangement of $\alpha\beta\alpha\delta\beta$, in a counter-clockwise direction when viewed from the extracellular space. This arrangement is the same as that of the abundant $\alpha_1\beta_2\gamma_2$ receptor, except for the γ - δ subunit substitution. Since agonist activation sites are predicted to lie at the β - α interfaces, both receptors would have two such sites. It should be noted, however, that of the 14 receptor-antibody complexes analyzed here (Figure 4C), three (21%) have a Fab-Fab angle smaller than the antibody-Fab angle, suggesting that a minority of the receptors might have the alternative counter-clockwise arrangement $\alpha\beta\alpha\beta\delta$, when viewed from the extracellular space, which would yield only a single agonist recognition site.

Hill slopes for GABA activation of the $\alpha_4\beta_3\delta$ receptor show no evidence of positive cooperativity, in contrast to those for the most ubiquitous $\alpha_1\beta_2\gamma_2$ receptor (Stórustova and Ebert, 2006; Derry et al., 2007; You and Dunn, 2007). It was, therefore, an attractive possibility that differences in Hill slopes might be explained by alternative subunit arrangements of the $\alpha_4\beta_3\delta$ and $\alpha_1\beta_2\gamma_2$ receptors and hence differences in the number of agonist binding sites. However, since the predominant forms of the two receptor subtypes have the same subunit stoichiometry and

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arrangement, it must be their unique subunit compositions that underlie their distinct GABA activation properties.

Understanding of the molecular determinants of the recognition and functional characteristics of the 'classical' GABA_A receptor, $\alpha_1\beta_2\gamma_2$, has benefited significantly from the development of comparative homology models (Ernst et al., 2005; Mokrab et al., 2007). The elucidation of the subunit arrangement in the $\alpha_4\beta_3\delta$ subtype provides an opportunity to develop similar models for this receptor subtype, the importance of which in the control of neuronal excitability is becoming increasingly recognized (Farrant and Nusser, 2005).

As for the $\alpha_1\beta_2\gamma_2$ GABA_A receptor (Baumann et al., 2002; Baur et al., 2006), the *Torpedo* nicotinic receptor (Karlin et al., 1983) and the 5-HT₃ receptor (Barrera et al., 2005a), the subunits of the $\alpha_4\beta_3\delta$ receptor are apparently arranged in a preferred order to produce an assembled receptor. In contrast, it is possible to change the subunit stoichiometry of the $\alpha_4\beta_2$ nicotinic receptor (Zwart and Vijverberg, 1998; Nelson et al., 2003) and the trimeric P2X_{2/6} receptor heteromer (Barrera et al., 2007) by manipulating subunit expression levels. No attempt has yet been made to vary the expression levels of, say, the A- and B-subunits of the 5-HT₃ receptor, leaving open the possibility that its stoichiometry might also be plastic. In fact, it has been reported recently that increasing the relative amounts of cRNA for the δ -subunit in *Xenopus* oocytes expressing the $\alpha_4\beta_3\delta$ receptor caused a progressive decrease in the sensitivity of the receptor to GABA and a corresponding decrease in the Hill slope (You and Dunn, 2007). To account for this observation, it was suggested that the subunit stoichiometry of the $\alpha_4\beta_3\delta$ pentamer might depend on relative subunit expression levels. According to this scenario, one would expect more than one δ -subunit per receptor at high levels of δ -subunit expression.

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Our results for the $\alpha_4\beta_3\delta$ GABA_A receptor extend our previous studies on the architecture of ionotropic receptors. The significant technical advance reported here is the method for determining the orientation of the receptor on the mica support, which allows us to solve the structure of receptors containing three different subunits. This methodology is applicable to other ionotropic receptors and also more widely to other types of multi-subunit protein.

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Footnotes

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Figure legends

Fig. 1. Immunofluorescence and immunoblot analysis of GABA_A receptors. A, immunofluorescence detection of GABA_A receptors in transfected tsA 201 cells. Cells were transfected with the cDNA for various combinations of subunits. They were then fixed, permeabilized and incubated with monoclonal primary antibodies, followed by a Cy3-conjugated goat anti-mouse secondary antibody. Cells were imaged by confocal laser scanning microscopy. B, detection of GABA_A receptors in eluates from Ni²⁺-agarose columns. Samples, from either transfected or mock-transfected cells, were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting, using monoclonal anti-FLAG, anti-V5, anti-HA and anti-Myc primary antibodies followed by a horseradish peroxidase-conjugated goat anti-mouse secondary antibody. Immunoreactive bands were visualized using enhanced chemiluminescence. Arrows indicate receptor subunits, and arrowheads indicate molecular mass markers (kDa).

Fig. 2. AFM imaging of GABA_A receptors. A, image of a sample prepared from mock-transfected cells. B, image of a sample from cells transfected with the cDNA for all three GABA_A receptor subunits. Arrowheads indicate particles with a molecular volume >420 nm³; the remaining particles (unmarked) are <420 nm³. A shade-height scale is shown at the right. C, frequency distribution of molecular volumes of GABA_A receptors. The curve indicates the fitted double-Gaussian function. The distribution was arbitrarily divided at a molecular volume of 420 nm³, in order to calculate the errors on the means.

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Fig. 3. AFM imaging of complexes between GABA_A receptors and anti-subunit antibodies. A, images of receptors (*left*), anti-FLAG antibodies (*middle*), and receptor-antibody complexes (*right*). B-D, zoomed images of receptors that are uncomplexed (*top*), or bound by one (*middle*) or two (*bottom*) anti-FLAG (B), anti-V5 (C) or anti-His₆ (D) antibodies. A shade-height scale is shown at the right. E-G, frequency distributions of angles between anti-FLAG (E), anti-V5 (F) or anti-His₆ (G) antibodies. The curve indicates the fitted single (E, F) or double (G) Gaussian functions. In (G), the distribution was arbitrarily divided at an angle of 105°, in order to calculate the errors on the means.

Fig. 4. Determination of the absolute subunit arrangement. A, distribution of angles between pairs of anti-FLAG Fab fragments, bound to the α -subunits. B, representative images of receptors decorated with two anti-FLAG Fab fragments and one anti-HA antibody. The *left-hand* image indicates a subunit arrangement of $\alpha\beta\alpha\delta\beta$, counter-clockwise, and the *right-hand* image indicates an arrangement of $\alpha\beta\alpha\beta\delta$, counter-clockwise. The two subunit arrangements are illustrated above the corresponding AFM images. C, progression of angles around the receptor for 14 receptors decorated with two Fabs and one antibody. The angles are read counter-clockwise around the receptor, beginning at the δ -subunit, which is decorated by the anti-HA antibody. Lines connect pairs of angles for individual decorated receptors. The mean angles, 83° from antibody-Fab and 122° from Fab-Fab, indicate a predominant subunit arrangement of $\alpha\beta\alpha\beta\delta$, counter-clockwise. D, representative images of receptors that had been bound to mica coated with either poly-L-lysine (*top*) or poly-L-glutamate (*bottom*) and then decorated with either concanavalin A (*left*) or antibody bd17 (*right*).

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Table 1. Antibody Tagging Profile of the GABA_A Receptor

Number of particles bound to receptor	Anti-FLAG	%	Anti-V5	%	Anti-HA	%	Anti-His ₆	%	Anti-myc	%
0	511	67.1	461	64.9	488	69.2	394	56.9	242	97.6
1	196	25.7	194	27.3	211	29.9	227	32.8	5	2.0
2	54	7.1	54	7.6	5	0.7	54	7.8	1	0.4
3	1	0.1	1	0.1	1	0.1	12	1.7	0	0.0
4	0	0.0	0	0.0	0	0.0	5	0.7	0	0.0
5	0	0.0	0	0.0	0	0.0	1	0.1	0	0.0

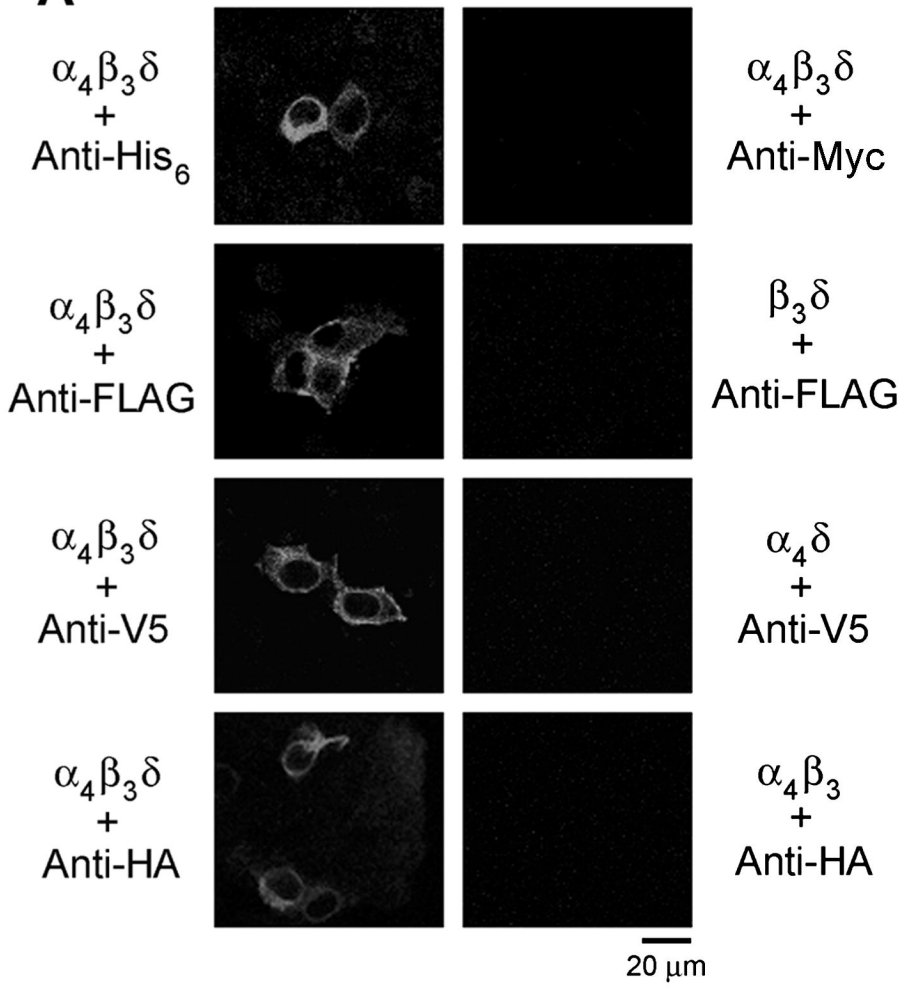
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Table 2. Profile of Decoration of GABA_A Receptor with Con A and Antibody bd17

Number of particles bound to receptor	Con A				Antibody bd17			
	Poly-lysine	%	Poly-glutamate	%	Poly-lysine	%	Poly-glutamate	%
0	136	88.3	143	79.4	147	91.3	180	82.9
1	12	7.8	27	15.0	14	8.7	32	14.8
2	6	3.9	9	5.0	0	0.0	5	2.3
>2	0	0.0	1	0.6	0	0.0	0	0.0

Figure 1

A



B

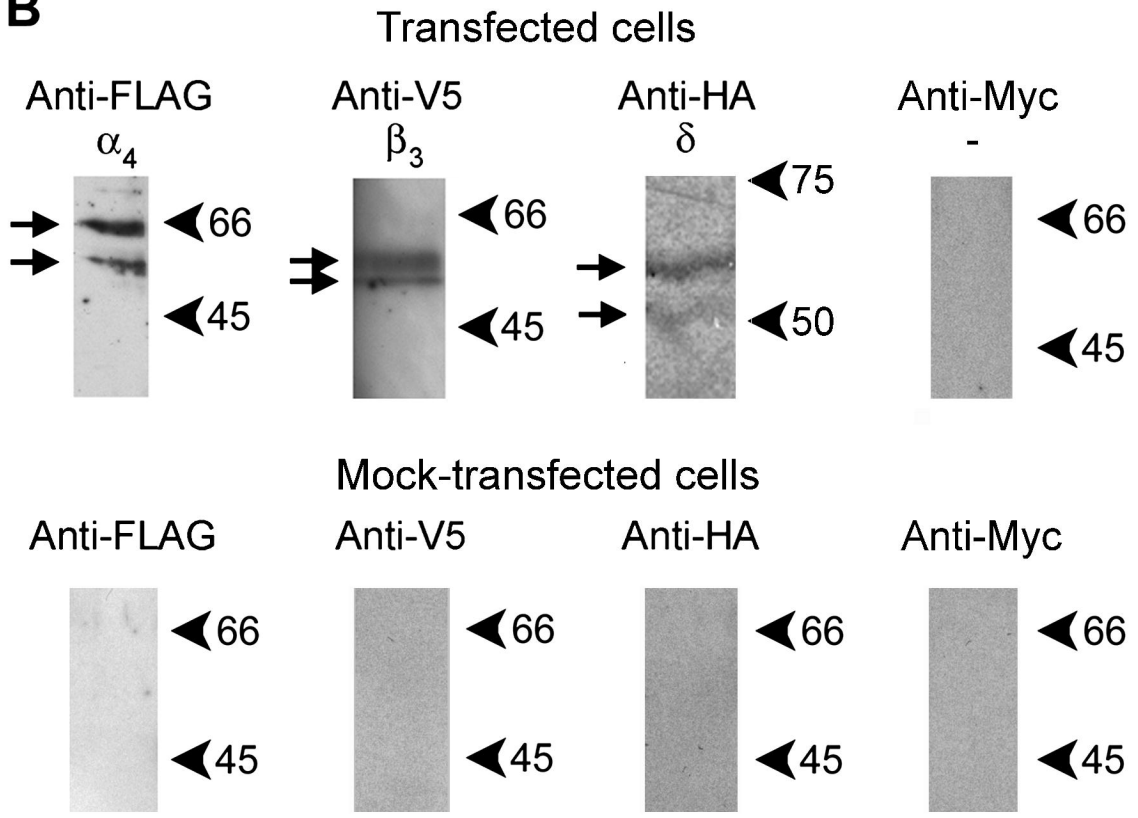


Figure 2

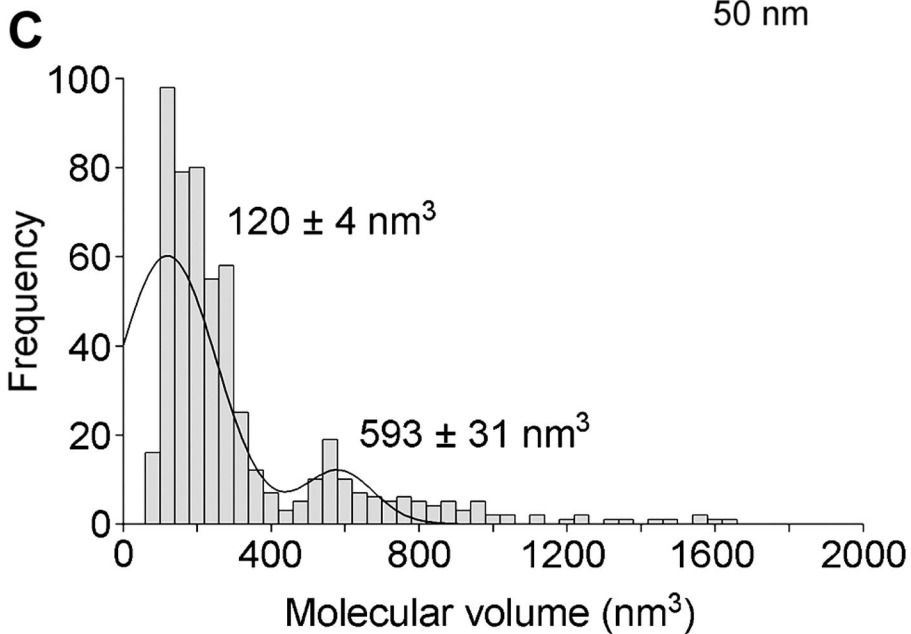
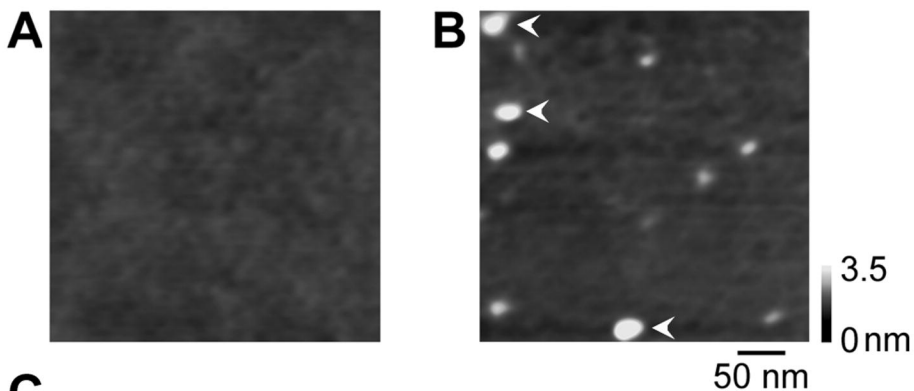


Figure 3

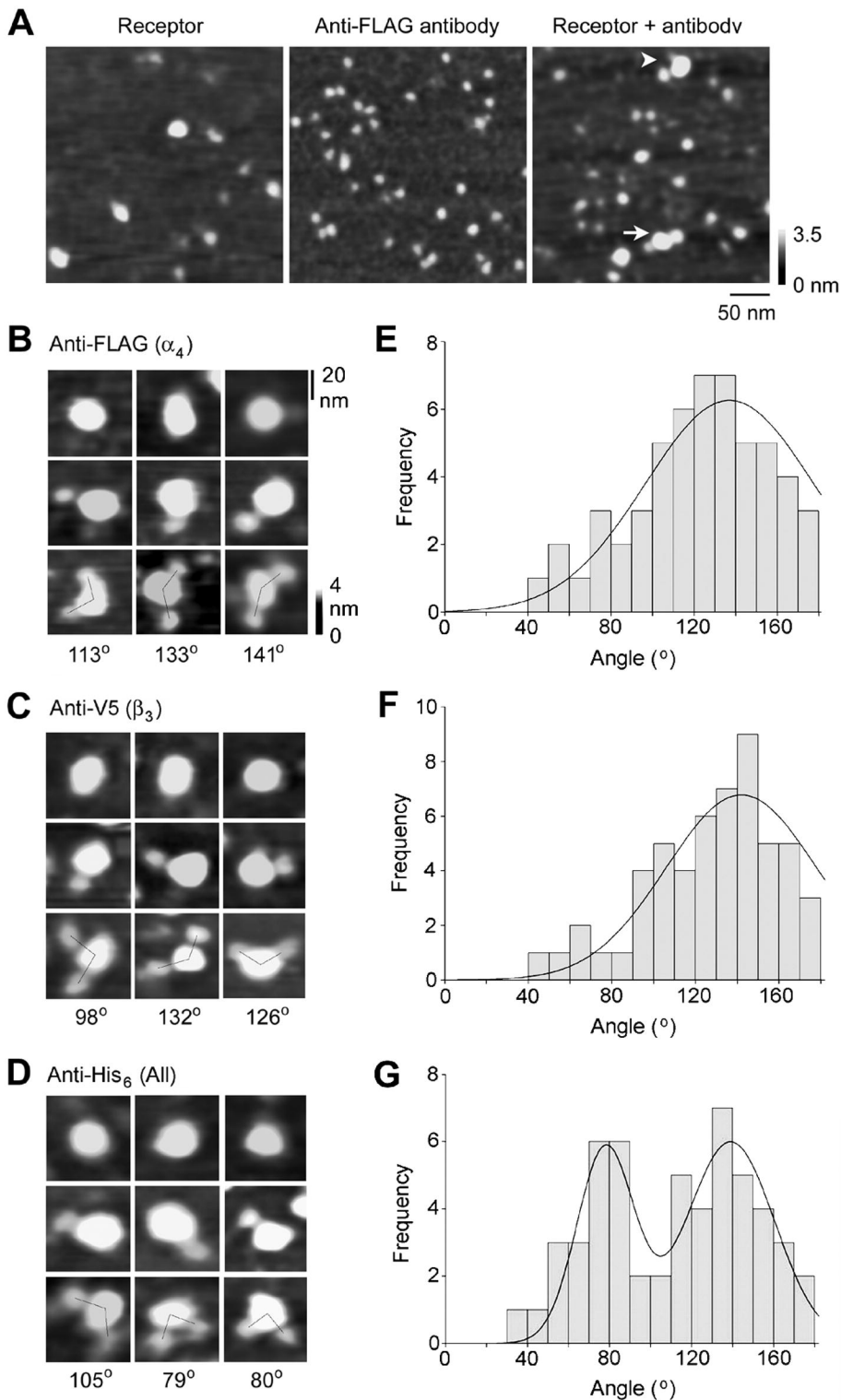
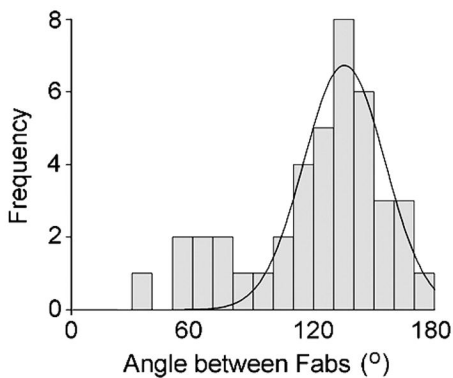
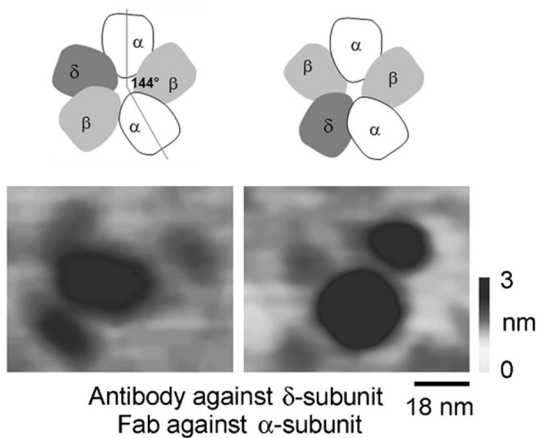


Figure 4

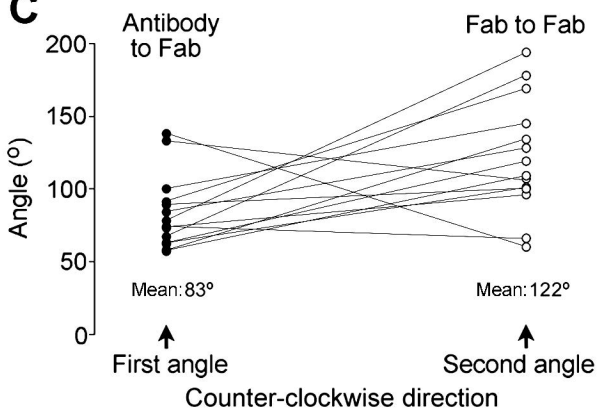
A



B



C



D

