# Structural requirement of the calcium-channel subunit $\alpha_2\delta$ for gabapentin binding

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Gabapentin [Neurontin, 1-(aminomethyl)cyclohexaneacetic acid] is a novel anticonvulsant drug with a high binding affinity for the $Ca^{2+}$-channel subunit $\alpha_2\delta$. In this study, the gabapentin-binding properties of wild-type and mutated porcine brain $\alpha_2\delta$ proteins were investigated. Removal of the disulphide bonds between the $\alpha_2$ and the $\delta$ subunits did not result in a significant loss of gabapentin binding, suggesting that the disulphide linkage between the two subunits is not required for binding. Singly expressed $\alpha_2$ protein remained membrane associated. However, $\alpha_2$ alone was unable to bind gabapentin, unless the cells were concurrently transfected with the expression vector for $\delta$, suggesting that both $\alpha_2$ and $\delta$ are required for gabapentin binding. Using internal deletion mutagenesis, we mapped two regions [amino acid residues 339–365 ($\Delta$F) and 875–905 ($\Delta$J)] within the $\alpha_2$ subunit that are not required for gabapentin binding. Further, deletion of three other individual regions [amino acid residues 206–222 ($\Delta$D), 516–537 ($\Delta$H) and 583–603 ($\Delta$I)] within the $\alpha_2$ subunit disrupted gabapentin binding, suggesting the structural importance of these regions. Using alanine to replace four to six amino acid residues in each of these regions abolished gabapentin binding. These results demonstrate that region D, between the N-terminal end and the first putative transmembrane domain of $\alpha_2$, and regions H and I, between the putative splicing acceptor sites ($Gln^{511}$ and $Ser^{601}$), may play important roles in maintaining the structural integrity for gabapentin binding. Further single amino acid replacement mutagenesis within these regions identified $Arg^{217}$ as critical for gabapentin binding.

Key words: anticonvulsant, $Ca^{2+}$ channel, co-transfection, disulphide bond, GABA.

## INTRODUCTION

Voltage-dependent $Ca^{2+}$ channels are essential for control of $Ca^{2+}$-linked cellular processes, such as muscle excitation–contraction coupling, hormone secretion, neurotransmitter release, and plasticity [1,2]. Several classes of $Ca^{2+}$ channels have been characterized based on their electrophysiological and pharmacological properties [3,4]. Voltage-dependent $Ca^{2+}$ channels consist of $\alpha_1$ (170 kDa), $\alpha_2\delta$ (175 kDa), $\beta$ (52 kDa), and sometimes a transmembrane $\gamma$ (95 kDa) subunit [5]. The $\alpha_1$ subunit forms the ion-pore structure, binds $Ca^{2+}$-channel blockers, and functions as both a channel and a voltage sensor [6]. The $\beta$ subunit appears to be important for expression of the kinetic characteristics of the channel [7]. The precise role of the $\alpha_2\delta$ protein for $Ca^{2+}$-channel function is only partially understood. Several studies suggest that $\alpha_2\delta$ may be involved in the insertion of the $\alpha_1$ subunit into the membrane [8] and, conversely, expression of $\alpha_1$ appears to be important for proper targeting and distribution of $\alpha_2$ [9]. Co-expression of $\alpha_2\delta$ with $\alpha_1$ subunit stimulates the inward current amplitude of the $Ca^{2+}$ channel and modulates $\omega$-conotoxin GVIA binding to $\alpha_1$ [8,10]. The interaction between $\alpha_1$ and $\alpha_2\delta$ is thought to occur through their extracellular loops [11,12]. The two heavily glycosylated polypeptides $\alpha_2$ and $\delta$ are proteolytic products of a common precursor encoded by a single gene [13]. The $\alpha_2$ and the $\delta$ subunits are linked by disulphide bonds [14]. An isoform of $\alpha_2\delta$ cDNA has recently been cloned from a porcine brain cDNA library. This $\alpha_2\delta$ protein shows 96% amino acid sequence identity to those cloned from rat, human and mouse brains [10,15,16], and 95% to that from rabbit skeletal muscle [13]. Although hydropathy plots predict three putative transmembrane domains (TMs) [13],

two in $\alpha_2$ (TMI and TMII) and one in $\delta$ (TMIII), biochemical evidence supports the model that there is only a single TM in the $\alpha_2\delta$ complex [17,18]. Isoforms of $\alpha_2\delta$ have been cloned from different species or tissues and all are believed to be splice variants resulting from combinations of three alternatively spliced regions [8,10,13,15,16,19]. The two upstream alternatively spliced regions are consecutive stretches of 19 and 5 amino acids, respectively, and the third region consists of 8 amino acids [16]. Porcine and rat brain $\alpha_2\delta$ have identical splicing patterns, in which the 19 amino acids region is missing but the 5 ($Asn^{506}$–$Gln^{510}$) and the 8 ($Ser^{601}$–$Asp^{608}$) amino acids insertions are conserved [15].

Gabapentin is a novel anticonvulsant drug that was synthesized as a mimetic of the inhibitory neurotransmitter $\gamma$-aminobutyric acid (GABA) [20]. In contrast with GABA, gabapentin is well absorbed by the gut and crosses the blood–brain barrier through system L-amino acid transporters [21,22]. Gabapentin does not interact with $GABA_A$ or $GABA_B$ receptors or any other known neurotransmitter receptors [23]. It does not have any effect on the metabolism, turnover or uptake of GABA in brain [24,25]. These previous studies indicate that gabapentin exerts its anticonvulsant action by a mechanism, which is distinct from most known GABA-derived anti-epileptic drugs. In addition, recent studies have shown that gabapentin also exhibits effects that are antihyperalgesic [26], anxiolytic-like [27], and neuroprotective [28]. These newly discovered gabapentin actions might be mechanistically distinct from its anticonvulsant efficacy. The mechanisms underlying all such diverse actions of gabapentin remain unclear. The complexity of the gabapentin pharmacology implies that there might be multiple biochemical events triggered by this drug [23].

Interestingly, a high-affinity binding site for gabapentin was found in brain tissues and the corresponding binding protein was later identified as the $\alpha_2\delta$ subunit of the $Ca^{2+}$ channel [29]. The pathophysiological relevance of gabapentin binding by the $\alpha_2\delta$ protein to the pharmacological functions of gabapentin needs to be explored. To better understand how the $\alpha_2\delta$ subunit may be involved in some of these gabapentin actions, it is important to establish the structural requirements for gabapentin binding. In the present study we investigated the correlation between the structural integrity of $\alpha_2\delta$ protein and its ability to bind gabapentin.

## MATERIALS AND METHODS

### Materials

[³H]Gabapentin (63 Ci/mmol) was synthesized by Amersham. Tissue culture media and transfection reagents were purchased from Gibco BRL. Restriction enzymes were purchased from Gibco BRL or New England Biolabs (Beverly, MA, U.S.A.). The Vent DNA polymerase and other reagents for PCR were purchased from New England Biolabs. DNA purification reagents were obtained from Qiagen. The oligonucleotides were synthesized by Genosys (The Woodlands, TX, U.S.A.). The ECL® kit for developing Western blots was from Amersham. Horseradish-peroxidase-linked anti-mouse IgG was purchased from Transduction Laboratories (San Diego, CA, U.S.A.). The $Ca^{2+}$-channel $\alpha_2$ monoclonal antibody mAb 20A was provided by Dr. Mary Morton (Holy Cross College, Worcester, MA, U.S.A.) or purchased from Affinity Bioreagents, Inc. (Golden, CO, U.S.A.). Immobilon-P transfer membranes for Western blotting were purchased from Millipore (Bedford, MA, U.S.A.). Protease inhibitors were purchased from Boehringer Mannheim. GF/B filters for binding assays were purchased from Whatman (Clifton, NJ, U.S.A.). All other chemicals were purchased from Sigma.

### Mutagenesis

All constructs were made from the full-length porcine brain $\alpha_2\delta$ cDNA by PCR and subcloned into plasmid pcDNA3 (Invitrogen) for expression. To construct a vector expressing mature porcine $\alpha_2$ subunit (pcDNA3P$\alpha_2$), a PCR product was made with a T7 primer corresponding to the vector sequence adjacent to the $\alpha_2\delta$ cDNA, and a downstream primer corresponding to the sequence (5′-TGCTTCAAGAAGTCGTGG-3′) at the 3′-terminus of $\alpha_2$. The downstream primer contains an in-frame stop codon. To facilitate manipulation, a *Xho*I site was introduced between the primer sequence and the stop codon and an *Apa*I site was introduced after the stop codon. The PCR product was cloned into pcDNA3 vector by *Hin*dIII and *Apa*I ligation. To make a $\delta$-expressing vector (pcDNA3P$\delta$), a PCR product containing the $\delta$ cDNA with an *Eag*I site at the 5′ end was made. The upstream primer contained an *Eag*I site and the downstream primer contained a *Xho*I site. The PCR product was ligated in-frame to the *Eag*I site immediately downstream of the signal sequence of the $\alpha_2\delta$ cDNA. The DNA fragment containing the signal sequence followed by the $\delta$ cDNA was then cloned into pcDNA3. Deletion mutants were constructed by making two PCR products corresponding to the sequences upstream and downstream of the deleted regions. A unique *Age*I restriction site was introduced at the 3′ end of the upstream product and the 5′ end of the downstream product. The two PCR products were ligated at the *Age*I sites, resulting in an in-frame fusion. The double mutants were made by ligating the two cDNA fragments containing both deletions into the expression vector. All PCR reactions were

carried out with Vent DNA polymerase with 20 cycles as follows: 94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min.

Single amino acid mutagenesis was carried out using an Exsite site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions.

### Cell culture and transfection

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal bovine serum, penicillin (50 units/ml) and streptomycin (50 μg/ml) at 37 °C in a humidified air/$CO_2$ (19:1) atmosphere. The media were changed every 2–3 days and cells were split in a ratio of 1 to 10 upon reaching confluence. Before transfection, cells were seeded at approx. $4.5 \times 10^6$ per Petri dish (150 mm diameter). The cells were incubated at 37 °C in a $CO_2$ incubator for 16–18 h. The cells were washed with DMEM and transfected with 20 μg of plasmid DNA per Petri dish by the Lipofectamine-mediated transfection method. The transfection was carried out for 6–12 h and then the cells were shifted to DMEM containing 10% foetal bovine serum. After incubation for 48 h, the cells were harvested for membrane preparation.

### Gel electrophoresis and Western blotting

The 4−20% (w/v) gradient gels were used and run at a constant voltage (120 V). After SDS/PAGE, proteins were electrophoretically transferred to Immobilon-P membranes at 55 V for several hours in the cold room. Blots were washed with TBST [20 mM Tris/HCl, pH 7.6, containing 137 mM NaCl and 0.5% (v/v) Tween-20], blocked in blocking buffer [TBST containing 5% (w/v) dry milk] for 1 h at room temperature, and incubated with primary antibody for 1 h at room temperature. Blots were washed extensively with TBST before incubation with horseradish-peroxidase-conjugated secondary antibody. After 1 h, blots were washed as above and developed with an ECL® kit according to the manufacturer's instructions. All incubations and washes of blots were performed on an orbital shaker.

### Membrane preparation and radioligand binding assays

Cells, on 150 mm plates, were washed with 10 ml of cold PBS, pH 7.4 and harvested in 6 ml of Tris/EDTA (5 mM Tris/HCl, 5 mM EDTA, pH 7.4, containing 100 μM PMSF, 20 μM leupeptin and 20 μM pepstatin A) using a cell scraper. After 15 min incubation on ice, the cells were homogenized for 30–60 s using a Polytron homogenizer and then sonicated for 30–60 s. The homogenate was centrifuged for 10 min at 750–1000 **g** and the supernatant was transferred to fresh centrifuge tubes and centrifuged at 50000 **g** for 30 min. The pellet was resuspended in 50 mM Mops, pH 7.4, containing 100 μM PMSF, 20 μM leupeptin and 20 μM pepstatin A. All procedures were performed at 4 °C.

Cell plasma membranes (0.1–0.2 mg of protein) were incubated with 20 nM or 100 nM [³H]gabapentin in 10 mM Hepes, pH 7.4, at room temperature. After 30 min, membranes in the reaction mixture were filtered on to GF/B filters under vacuum. Filters containing the plasma membranes were washed 5 times with 3 ml of ice-cold 10 mM Hepes and used for liquid-scintillation counting. For non-specific binding, the binding assays were performed in the presence of 1000-fold excess of non-labelled gabapentin. The specific binding was obtained by subtracting the radioactivity due to non-specific binding from the total radioactivity. The same procedures were employed for the $K_d$ determination except that the binding was carried out at various

[³H]gabapentin concentrations. Protein concentration was determined using serum albumin as a standard.

### Reduction of disulphide bonds in membrane proteins

Purified cell membranes were diluted to a protein concentration of 1 mg/ml in Mops containing protease inhibitors. Dithiothreitol (DTT) was added to a final concentration of 100 mM and the membranes were incubated for 15 min at room temperature. The membrane suspension was centrifuged at 20000 **$g$** and the supernatant was discarded. The membrane pellet was resuspended in 100 volumes of Mops and subjected to a similar centrifugation. After several rounds of resuspension–centrifugation–washing steps, the membranes were suspended in Mops for binding assays and Western analysis.

## RESULTS

### The disulphide linkage between the $\alpha_2$ and the $\delta$ subunits is not required for gabapentin binding

Previous studies have shown that the $\alpha_2\delta$ subunit is located in the membrane fraction of transfected cells [29]. To study gabapentin
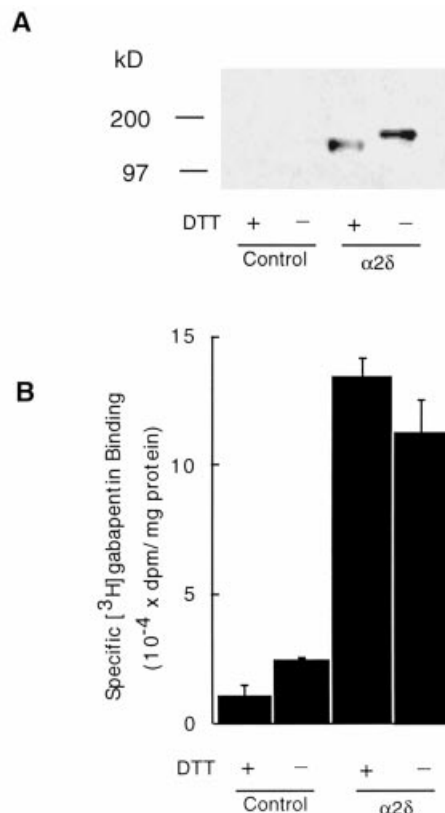


**Figure 1    Effects of disulphide-linkage disruption on gabapentin binding**

COS-7 cells were transfected with 20 $\mu$g of pcDNA3P$\alpha_2\delta$ or pcDNA3 (control) and the membranes were prepared (see the Materials and methods section). The membranes were incubated with DTT as described in the Materials and methods section and subjected to Western blot analysis or gabapentin-binding assays. (**A**) An equal amount of membrane protein (10 $\mu$g) from each sample was resolved on a non-reducing SDS gel and transferred to an Immobilon membrane. The blot was probed with an anti-$\alpha_2$ monoclonal antibody. The positions of marker proteins are indicated to the left. (**B**) After DTT treatment, the membranes were assayed for [³H]gabapentin-binding activity. The assay was performed in a final volume of 0.5 ml with 100 $\mu$g of membrane protein as described in the Materials and methods section. The final [³H]gabapentin concentration was 100 nM. Values are the means $\pm$ S.D. ($n = 3$).
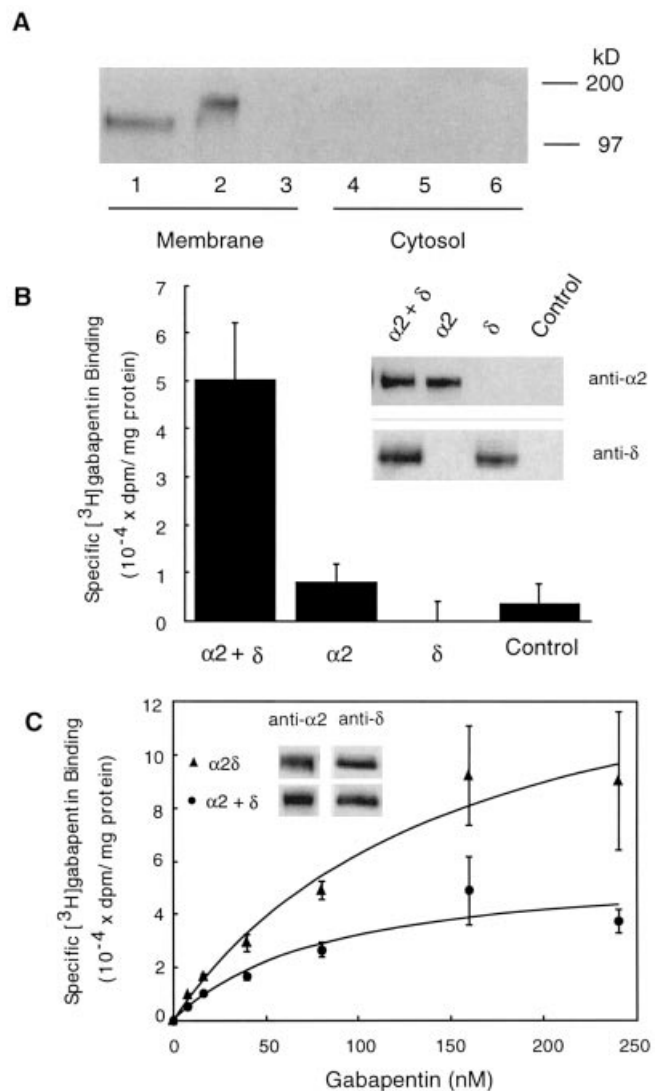


**Figure 2    Effects of subunit composition on gabapentin binding**

(**A**) Membrane (lanes 1–3) or cytosolic (lanes 4–6) proteins from cells transfected with pcDNA3P$\alpha_2$ (lanes 1 and 4), pcDNA3P$\alpha_2\delta$ (lanes 2 and 5) and pcDNA3 (lanes 3 and 6) were resolved on a non-reducing SDS gel, transferred to an Immobilon membrane and probed with an anti-$\alpha_2$ monoclonal antibody. (**B**) [³H]Gabapentin binding by membranes from cells transfected with $\alpha_2$ and $\delta$ jointly, $\alpha_2$ alone and $\delta$ alone. The same amount of $\alpha_2$ and $\delta$ DNA was used in each transfection. Membranes from the cells transfected with the corresponding expression vectors were subjected to binding analysis as described in the Materials and methods section. The final [³H]gabapentin concentration was 100 nM. Values are the means $\pm$ S.D. ($n = 3$). The immunoblots with the anti-$\alpha_2$ and anti-$\delta$ antibodies are shown (inset) to monitor the expression levels in each transfection. (**C**) [³H]Gabapentin binding by membranes from cells transfected with $\alpha_2\delta$ or co-transfected with $\alpha_2 + \delta$. An equal amount of $\alpha_2$ and $\delta$ DNA (10 $\mu$g each) was used in the $\alpha_2 + \delta$ co-transfection and 10 $\mu$g $\alpha_2\delta$ DNA plus 10 $\mu$g blank vector DNA (to compensate for the amount of DNA used in the co-transfection) was used in the $\alpha_2\delta$ transfection. Levels of $\alpha_2$ and $\delta$ in both transfections are shown by immunoblotting (inset).

binding by the Ca²⁺-channel subunit $\alpha_2\delta$, a transient transfection system was used to express the recombinant porcine $\alpha_2\delta$ protein. Membranes were prepared from cells transfected with pcDNA3P$\alpha_2\delta$ (the $\alpha_2\delta$ expression vector) and pcDNA3 (control) and resolved by SDS/PAGE under non-reducing conditions. Western blotting showed a protein band corresponding to 175 kDa in the membranes from pcDNA3P$\alpha_2\delta$-, but not
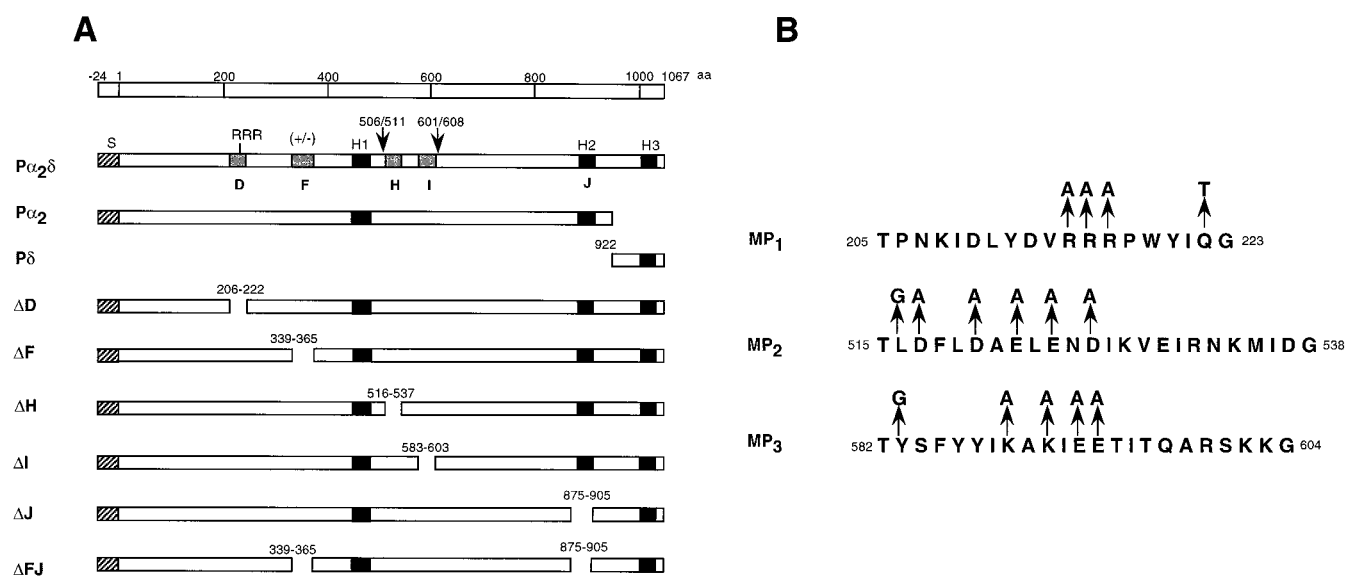
**A**



**B**



**Figure 3    Diagram of the full-length porcine $\alpha_2\delta$ subunit and the mutants used in the study**

(**A**) Deletion mutants. The amino acid numbering starts from the N-terminus of the mature $\alpha_2\delta$ protein. The arrows indicate the predicted alternative splicing sites. S denotes the signal sequence; H$_1$, H$_2$ and H$_3$ represent three predicted hydrophobic domains. The shaded areas are the deletion regions as indicated (D, F, H, I, and J). The RRR motif in region D and the heavily charged region F ($+/-$) are also indicated. Abbreviation: aa, amino acids. (**B**) Details of the multiple point mutations of $\alpha_2$ used for the binding assays and Western blotting analysis in Figure 5. Except for P$\alpha_2$ and P$\delta$, all of the above mutations were constructed in the pcDNA3$\alpha_2\delta$ vector.

pcDNA3-transfected cells, indicating the expression of the $\alpha_2\delta$ subunit (Figure 1A). Hill plot analysis revealed that there is no co-operativity or heterogeneity in gabapentin binding.

Since the disulphide bonds between the $\alpha_2$ and the $\delta$ subunits are thought to be important for maintaining the structural conformation of the $\alpha_2\delta$ protein [11,12], we determined whether this linkage is critical for gabapentin binding. The cell membranes from control and $\alpha_2\delta$-expressing cells were preincubated with high concentrations of DTT to disrupt disulphide bonds and subjected to Western blotting and binding assays. Consistent with a previous report [14], under such reducing conditions, the $\alpha_2\delta$ protein band shifted to a position predicted for $\alpha_2$, suggesting that the disulphide bonds linking the $\alpha_2$ and the $\delta$ subunits were completely disrupted by DTT (Figure 1A). Binding analysis showed that both untreated and DTT-treated membranes from the $\alpha_2\delta$-expressing cells exhibited an approx. 3-fold increase in gabapentin binding over that of the control (Figure 1B), suggesting that the disulphide bonds between the $\alpha_2$ and the $\delta$ subunits are not required for gabapentin binding.

### Co-existence of the $\alpha_2$ and the $\delta$ subunits is necessary for gabapentin binding

Previous studies suggest that the $\delta$ subunit is a transmembrane protein, and the $\alpha_2$ subunit is entirely extracellular but linked to the $\delta$ subunit via disulphide bonds [17,18,30]. To test if singly expressed $\alpha_2$ subunit is still in the membrane fraction, we expressed a mutant form lacking the $\delta$ subunit. When membranes from cells transfected with the $\alpha_2$ expression vector were probed with the anti-$\alpha_2$ monoclonal antibody, the Western blot clearly revealed a single immunoreactive protein band (Figure 2A) with the size (approx. 143 kDa) predicted for $\alpha_2$. In addition, Western blotting did not show any detectable $\alpha_2$ protein in the cytosolic fraction (Figure 2A). Thus the $\alpha_2$ subunit is in the membrane

fraction in the absence of $\delta$. Identification of the $\alpha_2$ subunit in the membrane fraction allowed us to evaluate gabapentin binding by the $\alpha_2$ subunit using the membrane fraction from cells transfected with $\alpha_2$ cDNA.

To examine whether co-existence of the $\alpha_2$ and the $\delta$ subunits is required for gabapentin binding, we further evaluated the binding activity in cells transfected with cDNAs for $\alpha_2$ and $\delta$ alone or together. As shown in Figure 2B the transfectants expressing either $\alpha_2$ or $\delta$ alone did not exhibit a significant increase in gabapentin binding over the pcDNA3-transfected control cells. In contrast, when the cells were co-transfected with both $\alpha_2$ and $\delta$ expression plasmids, a marked increase in binding activity was observed. The binding activity for the transfectants, expressing $\alpha_2$ plus $\delta$, was further evaluated at increasing concentrations of [$^3$H]gabapentin (Figure 2C). At similar expression levels the ($\alpha_2+\delta$) transfectants exhibited lower gabapentin binding than the transfectants with intact $\alpha_2\delta$ (Figure 2C). It should be noted that the expression level of $\alpha_2\delta$ in Figure 2C was lower than that in Figure 1B due to the difference in the amount of $\alpha_2\delta$ DNA used (see the legends to Figures 1 and 2). As a result, the specific binding by $\alpha_2\delta$ here is lower than that in Figure 1B.

### Regions Pro[206]–Gln[222], Leu[516]–Asp[537] and Tyr[583]–Lys[603] are important for gabapentin binding

To map the structural requirements for gabapentin binding, a series of internal deletion mutants were constructed within the $\alpha_2$ protein (Figure 3A). It has been proposed from the $\alpha_2\delta$ cDNA sequence that there are three putative hydrophobic domains: H$_1$ (TMI, residues 422–444), H$_2$ (TMII, residues 882–906), and H$_3$ (TMIII, residues 1043–1062) [13]. Later work showed that only H$_3$ is a TM and H$_1$ and H$_2$ are entirely extracellular [17]. However, it is interesting to investigate the roles of the hydrophobic regions and the regions nearby in gabapentin binding. The second hydrophobic domain H$_2$ is deleted in the $\Delta$J mutant.
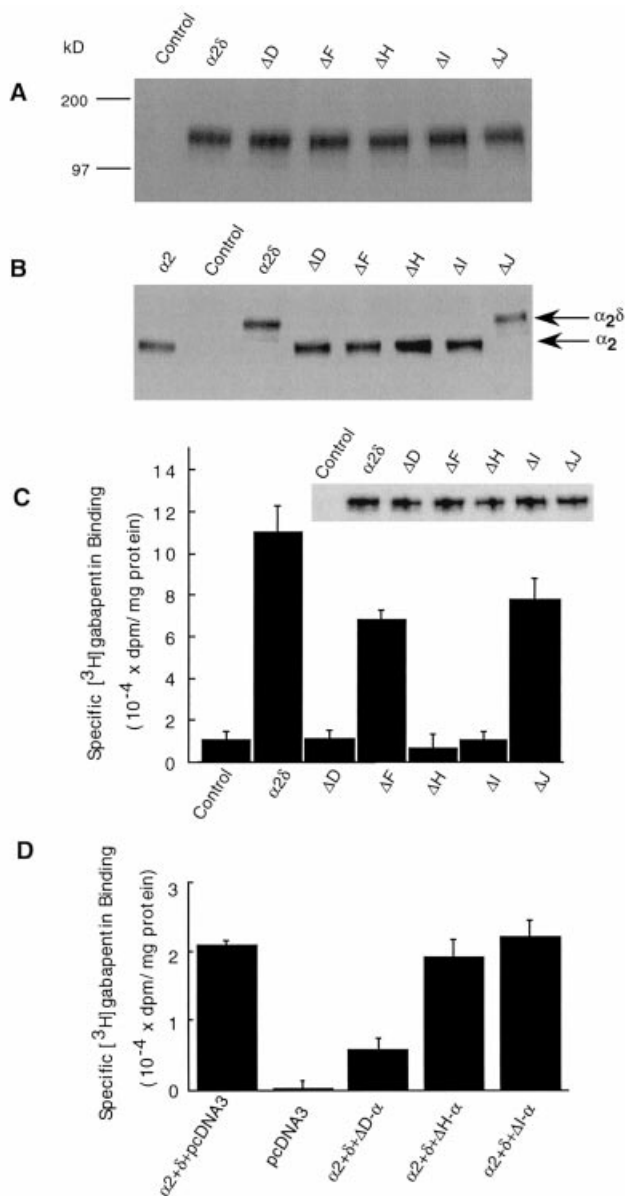
**Figure 4    Characterization of the internal deletion mutants of $\alpha_2\delta$ and $\alpha_2$**

(**A**) Western blot of the deletion mutants on a reducing gel. Membrane proteins from cells transfected with pcDNA3 (lane 1), pcDNA3P$\alpha_2\delta$ (lane 2), $\Delta$D (lane 3), $\Delta$F (lane 4), $\Delta$H (lane 5), $\Delta$I (lane 6) and $\Delta$J (lane 7) were resolved by reducing SDS/PAGE, transferred to an Immobilon membrane and probed with an anti-$\alpha_2$ monoclonal antibody. The positions of marker proteins are indicated to the left. (**B**) Western blot of the deletion mutants resolved on a non-reducing gel. A membrane preparation from the cells expressing $\alpha_2$ alone was also included. (**C**) [³H]Gabapentin binding. The binding analysis was carried out as described in the Materials and methods section. The final [³H]gabapentin concentration was 100 nM. Values are the means $\pm$ S.D. (n = 3). The expression levels were monitored by immunoblotting. (**D**) [³H]Gabapentin binding by membranes from the indicated co-transfections. Totally, 24 $\mu$g of DNA was used in each co-transfection; 8 $\mu$g of each DNA was used in the triple combinations.

The region immediately upstream of the hydrophobic domain $H_1$ is deleted in the $\Delta$F mutant. Region F contains a high number of charged amino acids (5 positive and 6 negative out of a total of 27 amino acids). Deletion mutant $\Delta$D was selected as a region that is further away from $H_1$ and contains an Arg-Arg-Arg motif (residues 215–217). Deletion of region H was made because this region is immediately downstream of the second alternatively

spliced site (between residues 506 and 507) [16,19]. In addition, a previous study with antibodies against a 19-amino-acid peptide (residues 509–527), which overlaps with region H (by 13 residues), suggested the importance of this region in Ca²⁺-channel-mediated dopamine release [18,19]. Region I (residues 583–603) is immediately upstream of the third alternatively spliced region (residues 601–608) with a three-residue overlap [16,19]. As shown in Figure 4, all the mutants co-migrated with the $\alpha_2\delta$ protein on a reducing gel (Figure 4A). The sizes of $\Delta$D, $\Delta$F, $\Delta$H, and $\Delta$I remained approx. the same as that of $\alpha_2$ (Figure 4B) on a non-reducing gel. These data suggest that the disulphide linkages between $\alpha_2$ and $\delta$ in $\Delta$D, $\Delta$F, $\Delta$H, or $\Delta$I were disrupted. However, the disulphide linkage in $\Delta$J remained intact (Figure 4B). Binding assays showed that deletion of $\Delta$F or $\Delta$J resulted in only a slight decrease in gabapentin binding. Deletion of F and J simultaneously ($\Delta$FJ) had no effect on gabapentin binding (results not shown). Deletion of regions D, H and I disrupted gabapentin binding (Figure 4C). To verify if the inability of $\Delta$D, $\Delta$H, and $\Delta$I to bind gabapentin was due to the lack of the $\delta$ subunit in the membrane, the mutant expression vectors were co-transfected with the $\delta$ expression vector. In contrast with co-transfection with cDNA for wild-type $\alpha_2$, which facilitates gabapentin binding (Figure 2), co-expression of the $\delta$ subunit with $\Delta$D, $\Delta$H and $\Delta$I did not restore gabapentin-binding activity (results not shown). This data suggests that the inability of these mutants to bind gabapentin was due to the deletions in the $\alpha_2$ subunit rather than the lack of the $\delta$ subunit in the membrane. However, the decreased gabapentin binding in these mutants ($\Delta$D, $\Delta$H and $\Delta$I) could be due to the disruption of the association between the $\alpha_2$ and the $\delta$ subunits. Alternatively, these mutations could disrupt the gabapentin-binding site(s) either directly or indirectly by altering the conformation.

As shown in Figure 2B only membranes from cells transfected with the ($\alpha_2 + \delta$) pair bind gabapentin as opposed to those with $\alpha_2$ or $\delta$ alone, suggesting that there is physical interaction between $\alpha_2$ and $\delta$ in the ($\alpha_2 + \delta$) pair. This interaction appears to be critical for gabapentin binding. It is not clear whether loss of gabapentin binding in $\Delta$D, $\Delta$H or $\Delta$I was due to the disruption of the subunit interaction. To explore the roles of regions D, H and I in subunit interaction, deletion of these regions within the separately expressed $\alpha_2$ subunit was carried out to generate $\Delta$D-$\alpha$, $\Delta$H-$\alpha$ and $\Delta$I-$\alpha$, respectively. To examine whether these mutants still interact with $\delta$, the dominant negative effects of these mutants on gabapentin binding by the ($\alpha_2 + \delta$) pair were tested. Mutants $\Delta$D-$\alpha$, $\Delta$H-$\alpha$ and $\Delta$I-$\alpha$ were co-transfected with the ($\alpha_2 + \delta$) pair (triple co-transfection with $\Delta$D-$\alpha + \alpha_2 + \delta$) and gabapentin binding was examined. If $\Delta$D-$\alpha$ (or $\Delta$H-$\alpha$ or $\Delta$I-$\alpha$) interacts with the $\delta$ subunit in the pair, it should compete with $\alpha_2$ for $\delta$ and decrease the level of physically associated $\alpha_2$ and $\delta$. Based on the data for $\Delta$D (Figure 4C), the physically associated $\Delta$D-$\alpha$ plus $\delta$ do not bind gabapentin. As a result, the gabapentin binding by the ($\alpha_2 + \delta$) pair is inhibited. Thus inhibition of gabapentin binding by $\Delta$D-$\alpha$ in the triple co-transfection ($\alpha_2 + \delta + \Delta$D-$\alpha$) indirectly suggests physical interaction of $\Delta$D-$\alpha$ with $\delta$. On the other hand, if $\Delta$D-$\alpha$ (or $\Delta$H-$\alpha$ or $\Delta$I-$\alpha$) does not interact with the $\delta$ subunit, there should not be any dominant negative effect on gabapentin binding by the ($\alpha_2 + \delta$) pair. In Figure 4D, only $\Delta$D-$\alpha$ inhibits the interaction between $\alpha_2$ and $\delta$ in the ($\alpha_2 + \delta$) pair and affects gabapentin binding, suggesting that $\Delta$D-$\alpha$ still interacts with $\delta$ whereas $\Delta$H-$\alpha$ and $\Delta$I-$\alpha$ do not. These data further suggest that there is subunit interaction in $\Delta$D, whereas there is not subunit interaction in $\Delta$H and $\Delta$I. Thus loss of gabapentin binding in $\Delta$D is likely due to the disruption of the binding site. However, loss of gabapentin binding in $\Delta$H and $\Delta$I may have resulted from the disruption of subunit interaction. It should be noted that regions
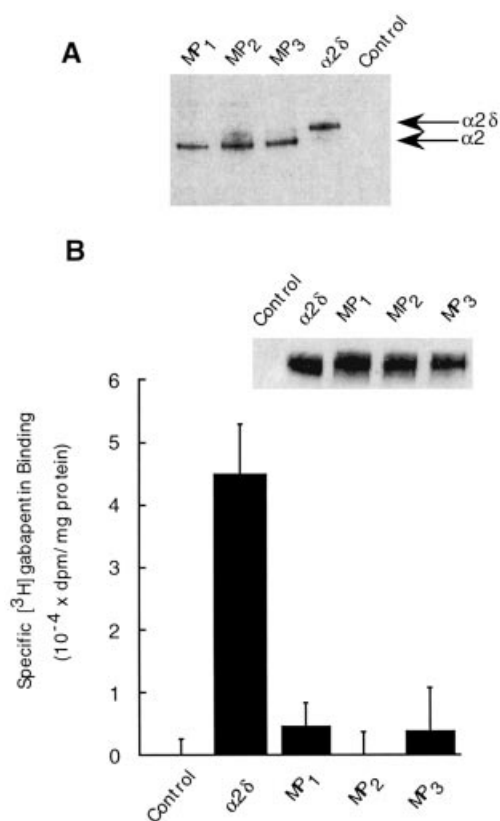
**Figure 5     Effects of multiple point mutations in regions D, H and I on gabapentin binding**

The scheme for each mutation is shown in Figure 3. (**A**) Western blot of mutants MP$_1$, MP$_2$ and MP$_3$. The cells were transfected with vectors for MP$_1$ (lane 1), MP$_2$ (lane 2), MP$_3$ (lane 3), pcDNA3P$\alpha_2\delta$ (lane 4) and pcDNA3 (lane 5), and cell membranes were prepared. The membranes were resolved on a non-reducing SDS gel, transferred to an Immobilon membrane and probed with an anti-$\alpha_2$ monoclonal antibody. (**B**) [$^3$H]Gabapentin binding. Membranes from the cells transfected with the corresponding expression vectors were used for binding assays as described in the Materials and methods section. The final [$^3$H]gabapentin concentration was 100 nM. Values are the means $\pm$ S.D. (n = 3). The expression levels were monitored by immunoblotting (inset).

H and I might be involved in both subunit interaction and maintaining binding pocket structure. Whereas region D is not involved in subunit interaction, it may be involved in maintaining gabapentin-binding-pocket structure.

To further explore the importance of regions D, H and I, multiple point mutations were introduced in these regions to form constructs MP1, MP2 and MP3, respectively (Figure 3B). In each construct four to six residues were replaced, and in each case all but one replacement involved charged amino acids. Expression of the mutant $\alpha_2$ proteins was confirmed by Western blotting (Figure 5). Similar to the result for the deletion mutants, gabapentin-binding ability was substantially lost, suggesting that these three regions might be important for binding. To identify the roles of the individual charged amino acids in gabapentin binding and subunit interaction, mutants with single amino acid substitutions were generated (Figure 6A). Conversion of the charged residues in regions D (Pro$^{206}$–Gln$^{222}$), H (Leu$^{516}$–Asp$^{537}$) and I (Tyr$^{583}$–Lys$^{603}$) did not disrupt the disulphide linkage between the $\alpha_2$ and the $\delta$ subunits (Figure 6B). Alanine substitutions at residues Asp$^{520}$, Lys$^{589}$, Lys$^{591}$, Glu$^{593}$ and Glu$^{594}$ did not affect gabapentin binding (Figure 6C). However, the
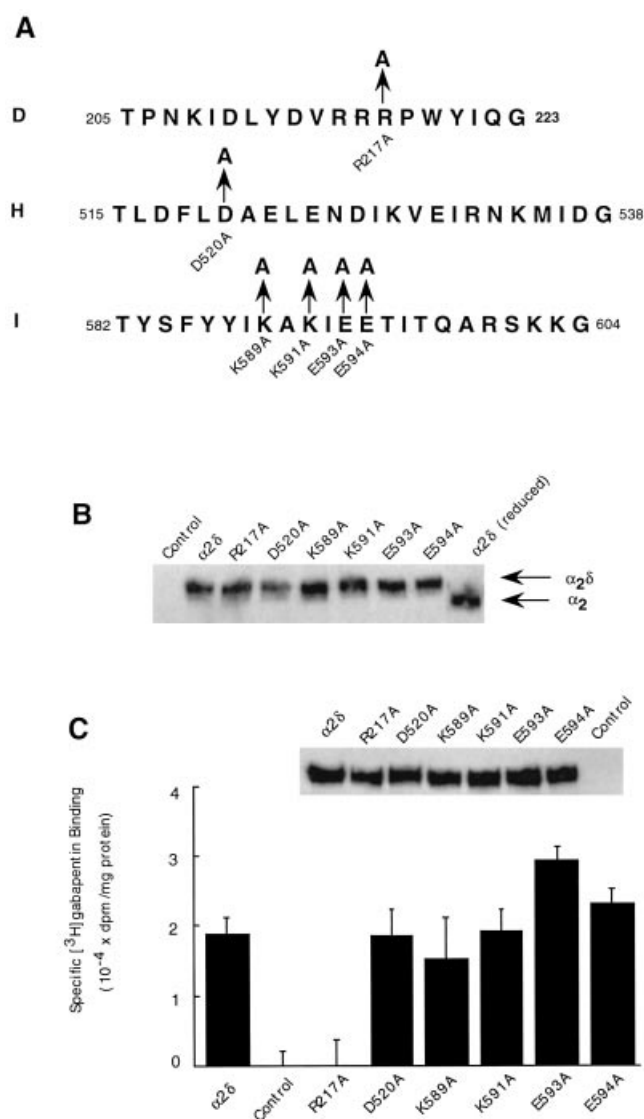


**Figure 6     Effects of single point mutations on gabapentin binding**

(**A**) Amino acid sequences in regions D, H and I with the indicated point mutations. (**B**) Western blot of the indicated single point mutants. Membranes from cells transfected with the indicated mutant DNAs were resolved on a non-reducing SDS gel, transferred to an Immobilon membrane and probed with an anti-$\alpha_2$ monoclonal antibody. (**C**) [$^3$H]Gabapentin binding. Membranes from the cells with the indicated transfection were used for binding assays as described in the Materials and methods section. The final [$^3$H]gabapentin concentration was 100 nM. Values are the means $\pm$ S.D. (n = 3). The expression levels were monitored by immunoblotting (inset).

mutant containing the Ala substitution at residue Arg$^{217}$ completely lost gabapentin-binding ability, suggesting that residue Arg$^{217}$ is critical for gabapentin binding by $\alpha_2\delta$ (Figure 6C).

## DISCUSSION

In the present study we demonstrated that both the $\alpha_2$ and the $\delta$ subunits of Ca$^{2+}$ channel are important for gabapentin binding, though the disulphide linkage between these two subunits does not appear to be required. According to a previous report, removal of the disulphide linkage in the $\alpha_2\delta$ protein causes a conformational change [12]. It is evident that this change does not affect the binding (Figure 1). We also show that the binding

activity remains when both subunits were expressed from separate expression vectors (Figure 2). However, the $(\alpha_2 + \delta)$ pair exhibited lower gabapentin binding than that by the wild-type $\alpha_2\delta$ molecule (Figure 2C). This indicates that the $(\alpha_2 + \delta)$ pair may not fold in the native form (as does the wild-type $\alpha_2\delta$ molecule) even though the expressed pair still binds gabapentin. This could be due to the lack of disulphide linkage in the $(\alpha_2 + \delta)$ pair as we observed. Although removal of the linkage from the $\alpha_2\delta$ molecule does not appear to affect gabapentin binding (Figure 1), lack of the disulphide linkage during protein folding could affect the native structures of $\alpha_2$ and/or $\delta$. As a result, the $(\alpha_2 + \delta)$ pair exhibited less gabapentin binding.

Our result about the role of the disulphide linkage in gabapentin binding in Figure 1 differs from the previous study in which the $\alpha_2\delta$-enhanced current amplitude was abolished when the disulphide linkage was absent in *Xenopus* oocytes injected with $\alpha_2$ plus $\delta$ cRNAs [12]. The discrepancy suggests that the structural requirements of the $\alpha_2\delta$ subunit for gabapentin binding and the stimulatory function by $\alpha_2\delta$ on the $Ca^{2+}$ channel are not the same. In fact, $\delta$ alone can modulate the voltage-dependent behaviour of the L-type $Ca^{2+}$ channel [31] but cannot bind gabapentin (Figure 2B). Thus a mutant $\alpha_2\delta$ subunit that loses its stimulatory function on the $Ca^{2+}$ channel may still bind gabapentin. The present study has identified two regions in the $\alpha_2\delta$ subunit that are not required for gabapentin binding. Although about 40% of amino acids in region F ($Asp^{339}$–$Val^{365}$) are charged amino acids, deletion of this region does not dramatically affect the binding affinity. Similarly, region J ($Lys^{875}$–$Ile^{905}$) which overlaps with the predicted second TM is not critical for binding. This conclusion is further supported by our finding that the double mutant $\Delta$FJ has a nearly normal binding activity, suggesting that the structural changes introduced by deletions of these two regions are either distant from or less important to the binding pocket. On the other hand, both deletion and multiple point mutation experiments suggest that regions D ($Pro^{206}$–$Gln^{222}$), H ($Leu^{516}$–$Asp^{537}$) and I ($Tyr^{583}$–$Lys^{603}$), especially the charged amino acid residues in these regions, may be directly or indirectly involved in the binding.

The importance of the sequences adjacent to the alternatively spliced regions for $\alpha_2$ function is highlighted by two recent studies [18,19]. These authors demonstrate that the L-type $Ca^{2+}$-channel-mediated dopamine release is blocked by antibodies raised against a region corresponding to residues $Lys^{508}$–$Ile^{526}$ of porcine $\alpha_2$, which overlaps with the residues $Phe^{518}$–$Ile^{527}$ of region H in the present study. Although both sets of data are consistent in pointing to the importance of these flanking regions, it remains to be determined which amino acids in region H may play key roles for gabapentin binding as well as for $Ca^{2+}$-channel-mediated dopamine release. The overlapping structural requirements for $\alpha_2\delta$-involved dopamine release and gabapentin binding may provide a link between gabapentin-attenuated monoamine release [32,33] and $Ca^{2+}$-channel function. Although one previous study failed to show any significant effect of gabapentin on the L-, N- or T-type voltage-dependent $Ca^{2+}$ channels [34], a recent study showed that gabapentin inhibits $Ca^{2+}$ currents mediated by the L-type $Ca^{2+}$ channel in isolated rat-brain neurons [35].

The correlation between abnormal $Ca^{2+}$-channel function and the pathogenesis of seizures has been shown in cases of genetic defects in the $\alpha_1$ and $\beta$ genes [36,37]. These genetic defects are closely linked to one type of mouse seizure that is similar to human absence epilepsy. On the other hand, since $Ca^{2+}$-channel functions are directly related to neuronal excitability, gabapentin may modulate their activities by binding to $\alpha_2\delta$. In fact, the recent study by Stefani et al. showed that gabapentin inhibits $Ca^{2+}$ currents mediated by L-type $Ca^{2+}$ channels [35]. Thus it is reasonable to believe that gabapentin may exert its pharmacological actions through this pathway. It should be noted that further study is needed to clarify whether $\alpha_2\delta$ binding and the consequent effect on $Ca^{2+}$ channels mediate the physiological actions of gabapentin. The structural factors identified in this study that are either dispensable or critical for gabapentin binding could be used as a tool to examine the link between $Ca^{2+}$ channels and the pharmacological actions of gabapentin, including anti-epileptic activity, anti-hyperalgesic activity and neuroprotective effects. For example, structural changes that could disrupt gabapentin binding can help one to understand if all these effects of gabapentin are mediated by the $\alpha_2\delta$ subunit. This will in turn identify the roles of $Ca^{2+}$ channels in the physiological and pharmacological actions of gabapentin. In addition, availability of the structural features at the binding pocket in the $\alpha_2\delta$ subunit will facilitate better chemical design for more efficient and potent drugs in the future.

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