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REVIEW

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The importance of cache domains in $\alpha_2\delta$ proteins and the basis for their gabapentinoid selectivity

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ABSTRACT

In this hybrid review, we have first collected and reviewed available information on the structure and function of the enigmatic cache domains in $\alpha_2\delta$ proteins. These are organized into two double cache (dCache_1) domains, and they are present in all $\alpha_2\delta$ proteins. We have also included new data on the key function of these domains with respect to amino acid and gabapentinoid binding to the universal amino acid–binding pocket, which is present in $\alpha_2\delta$-1 and $\alpha_2\delta$-2. We have now identified the reason why $\alpha_2\delta$-3 and $\alpha_2\delta$-4 do not bind gabapentinoid drugs or amino acids with bulky side chains. In relation to this, we have determined that the bulky amino acids Tryptophan and Phenylalanine prevent gabapentin from inhibiting cell surface trafficking of $\alpha_2\delta$-1. Together, these novel data shed further light on the importance of the cache domains in $\alpha_2\delta$ proteins.

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Voltage-gated calcium channel; gabapentinoid; cache domain; amino acid; $\alpha_2\delta$ protein

Introduction

The identification of a human variant in a Cache domain within $\alpha_2\delta$-1 that contributes to a phenotype severely affecting neural development and function [1] has prompted this review of $\alpha_2\delta$ structure and function, in order to further understand the function of the cache domains in these multi-domain proteins. In addition, we present further experimental data related to the specificity and importance of amino acid and gabapentinoid binding to the amino acid–binding site in the first double cache domain.

Classical role of $\alpha_2\delta$ in a complex within calcium channels

Voltage-gated calcium channels were first purified and the genes cloned from skeletal muscle in the 1980s [2,3]. The $\alpha_2\delta$ subunit was identified as one of the subunits, which was associated with the dihydropyridine receptor (α1 subunit) that was identified as a pore-forming subunit of the skeletal muscle calcium channel. Once the $\alpha_2\delta$ subunits were purified and cloned [4,5], they were also found to associate with N-type and P/Q-type channels, as well as other L-type channels [6,7]. The $\alpha_2\delta$ subunits are now known to associate with and affect the function of all Ca_V1 and Ca_V2 channels [8–11].

$\alpha_2\delta$ subtypes

The skeletal muscle $\alpha_2\delta$ protein, termed $\alpha_2\delta$-1, is encoded by *CACNA2D1*, which was the first $\alpha_2\delta$ gene to be cloned [12,13]. Four $\alpha_2\delta$ subunit genes were eventually cloned: *CACNA2D2*, encoding $\alpha_2\delta$-2, was identified as a result of finding spontaneous mouse mutations leading to cerebellar ataxia and absence epilepsy [14,15]. *CACNA2D3* and *CACNA2D4* encoding $\alpha_2\delta$-3 [11] and $\alpha_2\delta$-4 [16], respectively, were then identified by homology to the $\alpha_2\delta$-1 sequence.

$\alpha_2\delta$ distribution and functions

The skeletal muscle $\alpha_2\delta$ protein, $\alpha_2\delta$-1, was found also to be present extensively in other mainly excitable cell types, including those in the heart, smooth muscle, and brain [17]. In neurons, it is particularly concentrated presynaptically, and it is involved in presynaptic functions including transmitter release, homeostatic plasticity, and synaptic organization [18–21]. In contrast, the tissue distribution of $\alpha_2\delta$-2 was found to be

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mainly in the brain, particularly the cerebellum, but also in other tissues [11,14], and $\alpha_2\delta$-3 was expressed widely in the brain, particularly in the caudate-putamen [11]. The selective distribution and importance of $\alpha_2\delta$-4 in retinal function was elucidated by virtue of its mutation in hereditary retinal dysfunction [22,23].

Biochemistry and domains within $\alpha_2\delta$

All $\alpha_2\delta$ proteins have similar topology, biochemical properties, and domain architecture (Figure 1). Both α_2 and δ are highly N-glycosylated with up to 16 glycosylation sites [13,24,25], in agreement with their extracellular topology. All are proteolytically cleaved into two polypeptides, the larger α_2, and the smaller δ [26]. These remain disulfide-bonded together [13]. The C-terminal hydrophobic domain is present in all $\alpha_2\delta$ pre-protein sequences [26]. Although this hydrophobic domain was originally predicted to be transmembrane [25], it was found to contain key glycosylphosphatidylinositol (GPI)-anchor signal motifs for all the $\alpha_2\delta$ sequences [27], which was confirmed in biochemical, functional, and structural studies [27,28]. Thus, the C-terminal hydrophobic domain and short putative intracellular sequence, translated in the $\alpha_2\delta$ pre-protein are removed by processing in the endoplasmic reticulum, being replaced by a lipid anchor, and are therefore absent from the mature $\alpha_2\delta$ protein present in the calcium channel complex [28,29].

There is also a von Willebrand factor-A (VWA) domain in α_2, [30] which is a well-recognized protein–protein interaction domain, that is also present in many other extracellular proteins, including integrins [31]. The VWA domain in $\alpha_2\delta$ proteins is required for enhancement of calcium current function [9]. In $\alpha_2\delta$-1 and $\alpha_2\delta$-2 subunits, the VWA domains have a characteristic completely intact metal ion-dependent adhesion site (MIDAS) motif [30,32]. In other VWA domains, such as those in integrins, this MIDAS motif co-ordinates binding to another protein ligand, which occurs in the presence of a divalent cation, and which results in a conformational change [31]. In $\alpha_2\delta$-1 and $\alpha_2\delta$-2, disruption of the MIDAS motif prevents the ability of these $\alpha_2\delta$ subunits to enhance calcium channel currents [9,18]. The main corresponding interaction of the $\alpha_2\delta$ MIDAS motif with the Ca_V channels involves an aspartate in extracellular loop I of domain I of the α1 subunit, which coordinates with the $\alpha_2\delta$ MIDAS motif [28,33]. However, the structure also shows an additional interaction between a loop of the first Cache domain of $\alpha_2\delta$-1 with the top of pore loop 5 in domain III, which forms part of the extracellular entrance to the channel pore [28].

The importance of cache domains in $\alpha_2\delta$ proteins

The $\alpha_2\delta$ proteins were found to contain domains related to those in bacterial chemoreceptors that were termed Cache domains [34], and it was identified structurally that four Cache domains were present in $\alpha_2\delta$-1 [28]. In $\alpha_2\delta$ proteins, as in some prokaryotic proteins, these were found to be organized into double Cache domains (dCache_1), and in bacteria, they are involved in amino acid nutrient binding in chemoreceptors and other signal transduction proteins, leading to intracellular signaling [35,36]. Although these domains are widely found in bacteria and archaea, where they have well-studied roles in nutrient sensing, the only animal proteins in which these dCache domains have been identified are $\alpha_2\delta$ proteins (Figure 1), and the novel $\alpha_2\delta$-like protein Cachd1 [35], which is a transmembrane protein with some $\alpha_2\delta$-like properties [33,37,38]

A conserved structural motif including several key residues was found to be essential for amino acid binding in all these dCache_1 domains, including in the first dCache_1 domain in $\alpha_2\delta$-1 [35]. This

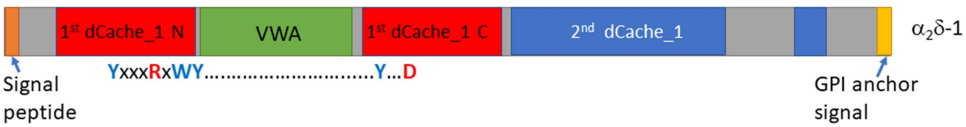

Figure 1. Domain structure of $\alpha_2\delta$-1 The amino acid–binding site motif in the first dCache_1 domain is shown beneath the linear domain representation .[35]. The R and D drawn in red in the motif have been mutated in binding studies described here. Modified from Figure 3c in [35].

dCache_1 domain is split in $\alpha_2\delta$-1, with the VWA domain inserted into it. The presence of the VWA domain also splits the amino acid–binding motif. The motif (using the single letter amino acid code) consists of YxxxRxWY in the first cache domain and Y … D in the second cache domain (Figure 1). The Arg (R) in this motif (in red in Figure 1) was previously identified as being the third Arginine in the triple-Arg sequence that was found to be essential for gabapentin binding and for the function of gabapentinoids in alleviating neuropathic pain [39,40].

Splicing creates variation in cache domains of $\alpha_2\delta$ proteins

Several different splice variants of the $\alpha_2\delta$ proteins have been identified [12,41,42]; these have been investigated most extensively in $\alpha_2\delta$-1 and involve the cache domains. There are three regions of splicing in $\alpha_2\delta$-1, termed A, B, and C; A and C are cassette exons, and B is introduced via an alternative splice acceptor site [43]. A and B are situated in the distal half of the first dCache_1 domain in a loop between β-sheet 6 and α-helix 7, whereas the third splice insertion, region C, is at the start of the second dCache_1 domain (see Figure 2 and Fig. S11, in [35]).

The three splice insertions in $\alpha_2\delta$-1 are differentially expressed in different tissues [43,44]. These studies showed region A to be expressed exclusively in skeletal muscle from all the tissues examined. The rat skeletal muscle variant is +A + B ΔC, whereas in the rat brain the main splice variant is ΔA + B + C. A minor splice variant of $\alpha_2\delta$-1 lacking region C (ΔA + B ΔC) is differentially up-regulated in rat dorsal root ganglion neurons following neuropathic injury, and it shows lower affinity for gabapentin [43]. The importance of the different splice insertions is unknown; it remains to be determined whether they are important for $\alpha_2\delta$-1 structure and interaction with specific calcium channels such as, in the case of region A, the skeletal muscle channel α1S, or for interaction with other potential binding partners of $\alpha_2\delta$-1 [45]. In this regard, it is of great interest that exogenous expression in hippocampal neurons of an $\alpha_2\delta$-2 splice variant lacking exon 23, which is in an equivalent position to splice site C in $\alpha_2\delta$-1 (see alignment in Fig. S11 in [35]), triggers aberrant synapse formation in tissue culture [46].

Importance of $\alpha_2\delta$ proteins in disease in mouse and other animal models: Relevance to cache domains

Knockout mice have been generated for the different $\alpha_2\delta$ isoforms. From these studies, it is clear that the observed phenotype of particular $\alpha_2\delta$ knockout mice depends on the cell types and developmental stages associated with selective expression of the particular isoform, which may then become indispensable. The $\alpha_2\delta$-1 knockout mice have a mild phenotype of reduced cardiac function, as $\alpha_2\delta$-1 is strongly expressed in ventricular myocytes [47]. They also have a reduced sensation of mechanical pain [48], associated with the finding that $\alpha_2\delta$-1 is strongly expressed in sensory neurons and is upregulated following neuropathic injury [49–51]. Furthermore, upregulated $\alpha_2\delta$-1 mediates an increase in the trafficking of $Ca_V2.2$ particularly in low threshold mechanoreceptors involved in hyperalgesia and allodynia [52]. Related to this, $\alpha_2\delta$-1 knockout mice also exhibit delayed development of neuropathic pain-related responses [48]. Furthermore, transgenic mice that constitutively over-express $\alpha_2\delta$-1 by random insertion [53] show spontaneous epileptiform behavior observed on EEG [54], and constitutive pain-like behavior [53]. In addition, autoantibodies recognizing $\alpha_2\delta$-1 are present in cases of autoimmune encephalitis [55] and amyotrophic lateral sclerosis with type 2 diabetes [56].

In contrast, $\alpha_2\delta$-2 knockout mice [57] have a similar severe phenotype to the spontaneously arising *Ducky* and *entla* mutants, including cerebellar ataxia and epilepsy [14,15]. This phenotype relates to the fact that $\alpha_2\delta$-2 is very strongly expressed in cerebellar Purkinje cells [14,58]. The phenotype of $\alpha_2\delta$-3 knockout mice was more subtle, and included impaired acoustic startle response and hearing disruption [59].

Drosophila melanogaster has two $\alpha_2\delta$ orthologs, the skeletal muscle ortholog, *Ca-Ma2d*, and the $\alpha_2\delta$-3 ortholog, *straightjacket* (*stg*) or $d\alpha_2\delta$-3, which is important in neurotransmission [60]. Knockdown of *stg* gene expression results in impaired heat sensitivity [61]. Furthermore, single nucleotide polymorphisms (SNPs) in the human gene *CACNA2D3* have been associated with reduced behavioral noxious thermal sensitivity, likely via a central impairment [61].

Mutations in *Cacna2d4* result in disruption of retinal ribbon synapses in mice, as a result of both rod and cone dysfunction [23].

Effect of human mutations in *CACNA2D* genes and relevance to cache domains

Neurological disease

Several recent reviews cover the involvement of Ca_V channels in neurological and psychiatric disorders [62,63] and only a summary of recent studies relating to *CACNA2D* genes is provided here. In *CACNA2D2*, rare biallelic loss-of-function variation has been reported in individuals with developmental epileptic encephalopathy, including cerebellar atrophy [64–67]. Rare homozygous truncating mutations of *CACNA2D4* have been reported, which result in recessive, slowly progressing cone dystrophy and hereditary night blindness [22].

In *CACNA2D1*, biallelic loss-of-function mutations have also recently been reported in two patients with developmental epileptic encephalopathy, which is associated with cerebral cortical rather than cerebellar atrophy [1]. These individuals were also reported to be insensitive to pain. In one patient, there was a homozygous frameshift mutation, resulting in a marked reduction in *CACNA2D1* mRNA measured in the patient fibroblasts. The other patient was compound heterozygous for a very early frameshift mutation on one allele, and a point mutation (Gly209-Asp) on the other allele. This Gly209 was in a highly conserved residue in the first dCache_1 domain of $\alpha_2\delta$-1 [1]. We found that this mutation rendered $\alpha_2\delta$-1 nonfunctional, in that the mutant protein did not traffic to the cell surface. Our evidence further suggested that the mutant $\alpha_2\delta$-1 was retained in the endoplasmic reticulum, since it was not proteolytically cleaved into α_2 and δ, a process that occurs mainly in the Golgi apparatus [1,68].

Genetic variation in *CACNA2D1*: Implications for cardiac disease in humans

In humans, heterozygous missense variations in *CACNA2D1* have previously been associated with cardiac dysfunction, with Brugada [69] and short QT [70] syndromes. However, these dominant associations with cardiac dysfunction have recently been called into question [1].

Mechanism of action of gabapentinoid drugs and basis for their selectivity with respect to $\alpha_2\delta$ proteins

Gabapentin and pregabalin were first developed in drug discovery programs to identify novel antiepileptic drugs mimicking or promoting the function of the inhibitory neurotransmitter GABA [71]. These drugs were then identified to bind to $\alpha_2\delta$-1 rather than their originally intended mechanism of action [72]. Mutational analysis then found the Arg mentioned above to be involved in gabapentinoid binding and function [39,40,73]. More recently, a key aspartate (Asp, D, Figure 1) was also identified as being essential to coordinate amino acid binding in this binding pocket, which is in the dCache_1 domain of $\alpha_2\delta$-1 [35].

Regarding the mechanism of action of the gabapentinoids, we identified that gabapentin reduced the trafficking of $\alpha_2\delta$-1 and $\alpha_2\delta$-2 [74,75] and also disrupted the trafficking of associated calcium channels, and their function [75–78]. Within $\alpha_2\delta$-1, both the key Arg241 [75,78] and also Asp491 [35] residues in the dCache_1 amino acid–binding site of $\alpha_2\delta$-1 are important for the ability of gabapentin to inhibit $\alpha_2\delta$-1 trafficking and function.

Of interest, $\alpha_2\delta$-3 (and also $\alpha_2\delta$-4) does not contain the triple Arg sequence that was thought to be implicated in gabapentin binding (it is Arg-Asn-Arg in $\alpha_2\delta$-3), and neither $\alpha_2\delta$-3 nor $\alpha_2\delta$-4 binds gabapentin [79]. Furthermore, $\alpha_2\delta$-3 is not recycled to the plasma membrane via a Rab11-dependent pathway [80].

Our analysis of the structures modeled by AlphaFold [81] shows that $\alpha_2\delta$-3 and $\alpha_2\delta$-4 do contain an amino acid–binding site, in an analogous position to that identified in the first dCache domain of $\alpha_2\delta$-1 [35] (Figure 2). We conducted molecular docking in AutoDock Vina [82] with AlphaFold models of $\alpha_2\delta$-2, $\alpha_2\delta$-3 and $\alpha_2\delta$-4 proteins using gabapentin, pregabalin, mirogabalin, and amino acids, and found that in case of $\alpha_2\delta$-3 and $\alpha_2\delta$-4, only small amino acids bind to the pocket, while gabapentinoids and bulky amino acids do not (structural models of $\alpha_2\delta$-2, $\alpha_2\delta$-3, and $\alpha_2\delta$-4 proteins with docked ligands and docking simulation parameters can be found at this link: https://

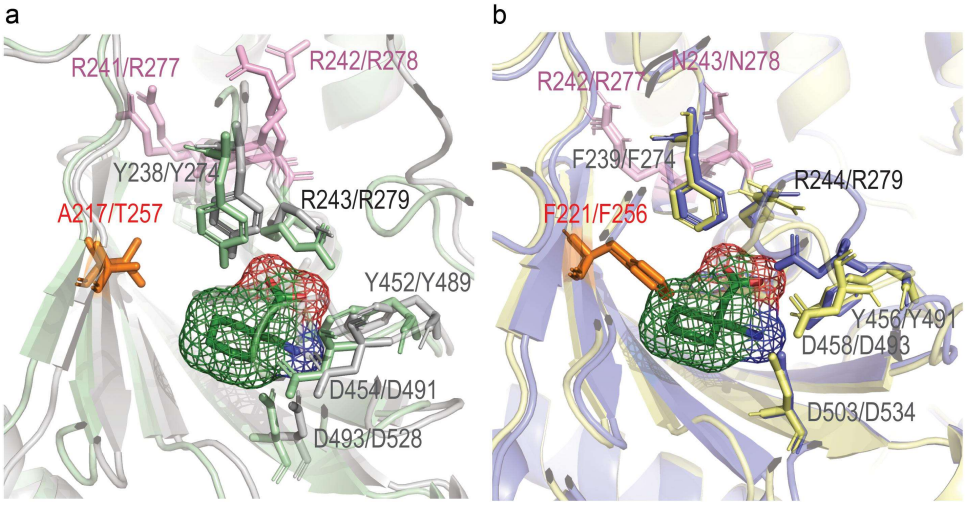

Figure 2. Gabapentinoid and amino acid–binding pockets of $\alpha_2\delta$-1 – $\alpha_2\delta$-4. Phe221/Phe256 (F221/F256) creates steric hindrance in the ligand-binding pocket of $\alpha_2\delta$-3 and $\alpha_2\delta$-4 proteins. Superimposed structures of $\alpha_2\delta$-1 and $\alpha_2\delta$-2 (a) and $\alpha_2\delta$-3 and $\alpha_2\delta$-4 (b) ligand-binding pockets. $\alpha_2\delta$-1 is the rabbit protein cryo-EM structure, $\alpha_2\delta$-2 to $\alpha_2\delta$-4 are the AlphaFold models. Gabapentin is docked to the binding pocket of $\alpha_2\delta$-1. Each of $\alpha_2\delta$-2 to $\alpha_2\delta$-4 was superimposed on $\alpha_2\delta$1; $\alpha_2\delta$-3 and $\alpha_2\delta$-4 then were extracted and placed on panel B for clarity. A figure with all the proteins simultaneously superimposed on $\alpha_2\delta$-1 can be found on GitHub at the following link https://github.com/ToshkaDev/Alpha2Delta-proteins-review. In pink font the first two residues of the RRR (in $\alpha_2\delta$-1 and $\alpha_2\delta$-2)/ RNR (in $\alpha_2\delta$-3 and $\alpha_2\delta$-4) sequence are shown – as can be seen they are not involved in ligand binding. Other residues, except for the residue corresponding to Asp454 (D454) in $\alpha_2\delta$-1, denote the amino acid–binding motif .[35]

github.com/ToshkaDev/Alpha2Delta-proteins-review). Interestingly, all proteinogenic amino acids and gabapentinoids were bound to $\alpha_2\delta$-2, but tryptophan (Trp) was found to bind only in a certain pose and with low affinity, which contrasts with its high affinity binding to $\alpha_2\delta$-1. Our structural analysis shows that the first two Arg residues of the above-mentioned triple-Arg motif are directed away from the pocket and in fact do not directly contribute to the formation of the ligand-binding interface (Figure 2a). Only the third Arg in this sequence, which is part of the universal amino acid–binding motif, is directed toward the inside of the pocket and binds ligands (Figure 2b, [35]). Thus, the two first residues of the triple-Arg sequence do not play a role in ligand binding and, therefore, replacement of the second Arg to Asn in this motif observed in $\alpha_2\delta$-3 and $\alpha_2\delta$-4 is not the reason for their inability to bind gabapentinoids. Our subsequent examination allowed us to identify the "culprit" – Phenylalanine (Phe) at a specific position within the ligand-binding pocket of $\alpha_2\delta$-3 and $\alpha_2\delta$-4 that creates a steric hindrance interfering with the binding of bulky ligands (Figure 2b). In $\alpha_2\delta$-1 and $\alpha_2\delta$-2, alanine (Ala217) and threonine (Thr257), respectively, are located at this position (see Figure 2), and they do not impede ligand binding.

Bacterial chemoreceptors bind both agonists and antagonists at this universal amino acid–binding site within the dCache_1 domain [83,84]. For $\alpha_2\delta$-1, the amino acid leucine was found previously to bind to the same binding site and compete with gabapentin, although the function of this binding was not known [85]. In our recent study, the binding affinity of various amino acids including Trp and Phe to $\alpha_2\delta$-1 was calculated from docking analysis to be higher than that of leucine, and as high as that of the gabapentinoids [35].

We therefore examined here, using techniques already described [35], whether Trp or Phe would either inhibit $\alpha_2\delta$-1 trafficking in the same way as gabapentin or, alternatively, act as agonists and enhance its trafficking. We found that although an elevated concentration (1 mM) of either Trp or Phe alone did not affect $\alpha_2\delta$-1 cell surface expression in cultured cells, both these amino acids did inhibit the ability of gabapentin in this regard (Figure 3a-b). The cell surface expression of $\alpha_2\delta$-1 was reduced by 56% by 1 mM gabapentin, as we have described previously [78,80], whereas this reduction was prevented by the additional presence of 1 mM Trp

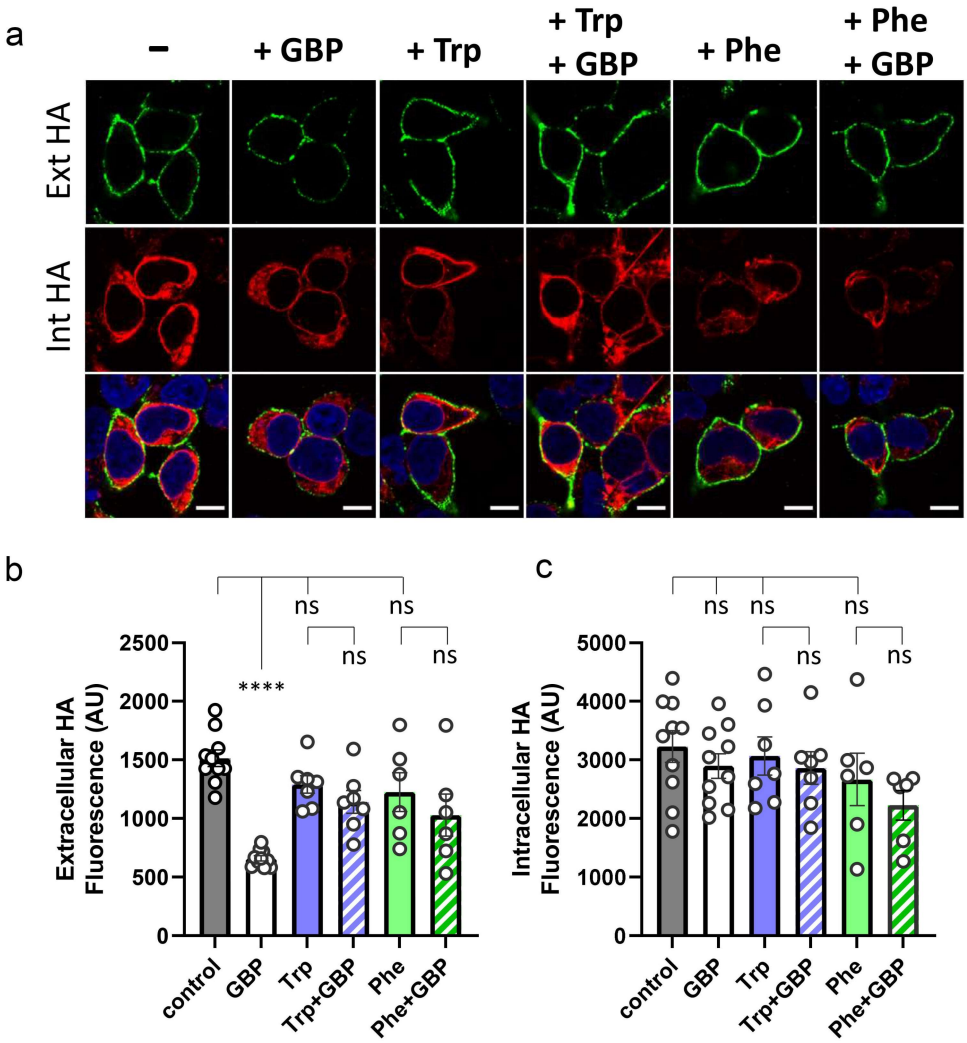

Figure 3. Tryptophan and Phenylalanine prevent the inhibition of cell surface expression of HA-tagged $\alpha_2\delta$-1 by gabapentin. Experiments were performed as described previously described [35]. (a) Representative images of tsA-201 cells expressing hemagglutinin (HA)-tagged $\alpha_2\delta$-1 subunit in the absence of gabapentin or additional amino acids (control, -) or the presence of 1 mM gabapentin (+ GBP) alone, 1 mM L-Tryptophan (+ Trp) alone, 1 mM L-Tryptophan + 1 mM gabapentin (+ Trp + GBP), 1 mM L-Phenylalanine (+ Phe) alone or 1 mM L-Phenylalanine + 1 mM gabapentin (+ Phe + GBP), incubated in serum-free media for 24 h. Top row (green, Ext HA) shows cell surface $\alpha_2\delta$-1-HA staining in the nonpermeabilized condition; middle row (red, Int HA) shows intracellular $\alpha_2\delta$-1-HA staining after permeabilization with 0.1% Triton X-100; bottom row shows merged images with the nuclei stained with DAPI (blue). Scale bars: 10 µm. (b) Bar chart (mean ± SEM, with individual data-points each showing the mean of more than 35 cells from 6–10 different transfections in three independent experiments), showing cell surface expression of $\alpha_2\delta$-1-HA in the absence (control, gray) or presence of 1 mM GBP (white), 1 mM Trp (blue), 1 mM Trp + 1 mM GBP (blue and white stripes), 1 mM Phe (green), 1 mM Phe + 1 mM GBP (green and white stripes). Statistical significance was determined using one-way ANOVA and Šídák's multiple comparison post-hoc test; **** $P < 0.0001$, ns: no statistical significance ($P > 0.2$). (c) As for (B) but showing intracellular HA staining after permeabilization of the cells. Cell surface expression of $\alpha_2\delta$-1-HA is reduced by GBP to 44% of control levels but this reduction is not seen in the presence of additional L-Trp or L-Phe.

or Phe (Figure 3a, b). There were no effects of any of the manipulations on intracellular $\alpha_2\delta$-1 expression (Figure 3a, c).

These results indicate that, although endogenous amino acids are likely to occupy the universal amino acid–binding site in $\alpha_2\delta$-1, we were unable to detect any effect of the binding of high concentrations of Trp or Phe on cell surface expression of $\alpha_2\delta$-1, indicating that under the conditions used here they did not act alone as either agonists or antagonists, although they are able to prevent the effect of gabapentin, presumably by occupying the binding site. This may represent one mechanism that contributes to the variable efficacy of gabapentinoid drugs.

Conclusion

Within Metazoa, cache domains are only found in $\alpha_2\delta$ proteins and in Cachd1. In these proteins, the four cache domains are organized into two double Cache (dCache_1) domains, and contain a universal amino acid–binding pocket, which in $\alpha_2\delta$-1 and $\alpha_2\delta$-2 also accommodates gabapentinoid drugs. Here we have examined, from a structural point of view, why $\alpha_2\delta$-3 and $\alpha_2\delta$-4 do not bind gabapentinoids or amino acids with bulky side chains. Furthermore, we have determined that the bulky amino acids Trp and Phe prevent gabapentin from inhibiting cell surface expression of $\alpha_2\delta$-1. Altogether, this illustrates the importance of the cache domains in $\alpha_2\delta$ proteins. It also highlights that novel interactions of these cache domains are likely to be found in the future.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability

Structural data are available at https://github.com/ToshkaDev/Alpha2Delta-proteins-review. Other data will be made available upon reasonable request.

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