The role of the $GABA_A$ receptor δ subunit domains on agonist binding kinetics

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Abstract

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The $GABA_A$ receptor is a ligand-gated ion channel that conveys inhibitory signals in neurons. It is a pentameric receptor assembled from combinations of 19 subunits, however, most receptors are composed of α-, β- and γ-subunits. The properties of the receptor depend on its composition. A typical receptor contains αxβxγ2 and is located in the synapse. The focus of this project is the α6β3δ receptor which is extrasynaptic and provides a basal inhibitory tone rather than the rapid phasic action of its γ2-containing cousin. The main property of the δ-type is a high affinity for low concentrations of GABA. The differences between receptors containing δ- and γ2-subunits were explored by creating chimeric constructs containing sequences from both subunits using polymerase chain reaction-mediated site-directed mutagenesis. The properties of the chimaera-containing receptor were compared to those of the wild type δ-containing receptor by measuring the binding kinetics of the $GABA_A$ agonist ^{3}H-muscimol. The final construct contained δ sequences with the exception of the amino acids between S238-V264 taken from the corresponding γ2 sequence. It was found that the chimaera was equivalent to the wild type in terms of both association and dissociation kinetics of ^{3}H-muscimol. This indicates that the critical site for slow binding kinetics is not in the first transmembrane domain even though this site is essential for channel function according to previous studies. Further research mutating the extracellular domains is recommended.

Key words: $GABA_A$, radioligand, GABA, muscimol

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1. Introduction

GABAergic signalling is the principal generator of fast inhibitory potentials in the central nervous system. The neurotransmitter γ-aminobutyric acid (GABA) and several endogenous neurosteroids act on $GABA_A$ and $GABA_B$ receptors to hyperpolarise cell membranes and decrease the odds of action potentials firing. The $GABA_A$ receptor is a ligand-gated chloride ion channel which acts very quickly, in the span of milliseconds. Once GABA molecules have bound to the receptor, it allows chloride ions to flow into the cell. The resting membrane potential of the cell is -70 mV which is close to the reversal potential for chloride. The opening of chloride channels will push any excited cells back to the resting potential and prevent excitation. $GABA_B$ receptors are G-protein-coupled receptors that slowly hyperpolarise the postsynaptic cell by coupling to secondary messenger G_i to open potassium channels. The potassium will leak out of the cell and the loss of positive charge hyperpolarises the membrane. These inhibitory signals are essential in the central nervous system (CNS) to prevent excitotoxicity. There used to be a receptor group called $GABA_C$ as well, but that group has since been incorporated into the $GABA_A$ family as the ρ subunits which are unique in the family. The $GABA_A$ receptor is frequently found in synapses of interneurons where it creates negative feedback loops to prevent overexcitation. It can also be found extrasynaptically where it acts as a system-wide checkpoint against overexcitation. (For a comprehensive review see Olsen and Sieghart, 2009).

1.1. Receptor structure

The $GABA_A$ receptor bears great similarity to the nicotinic receptor, the main progenitor of excitatory signals in the periphery. The receptors are both members of the Cys-loop superfamily, so-called because of their characteristic consensus sequence of a 15 amino acid loop formed by a disulphide bridge created from cysteine-cysteine interactions (Simon et al., 2004). All the family members form pentamers whose subunit composition is vital to the characteristics of the finished receptor(Nayeem et al., 1994). There are 19 different subunits of the $GABA_A$ receptor: α1-6, β1-3, γ1-3, δ, ε, θ, π and ρ1-3 (Collingridge and Olsen, 2009). The subunits have high homology across species barriers and rodent genes can be safely used to study the receptor as almost all the subunits are equivalent,

although avian species have some differences: the human ε subunit is represented by γ4 and likewise the counterpart of θ is β4 (Simon et al., 2004). The rat gene was used in this paper both in illustrations of the sequence and in the wet lab as it is functionally identical to the human gene.

The nicotinic receptor responds to acetylcholine and creates excitatory currents whereas the $GABA_A$ receptor responds to GABA and results in inhibitory currents but the structure is similar enough that the nicotinic receptor and bacterial homologues of the nicotinic receptor were used for homology modelling of the $GABA_A$ receptor since there were very few crystal structures available of $GABA_A$ receptors until recently. Homology modelling is by no means perfect and does not provide the full picture. Without high-resolution crystal structures of the numerous subtypes, it is very challenging to determine structure-activity relationships for the domains of the $GABA_A$ receptor. Currently, there is a crystal of the artificial β3 homopentamer and a few native compositions such as β3 & α5 combinations and α1β2γ2. For rarer subtypes like the δ-containing receptors, one can only perform homology modelling based on the β3 pentamer and make an educated guess regarding the conformation of the receptor. Thanks to these recent 3D structures, assumptions based on the structures of related receptors like nicotinic receptor can finally be confirmed. (Miller and Aricescu, 2014).

Despite the existence of 19 subunits, there are not 19^5 viable subunit combinations. In fact, subunits can be very picky about which other subunits they will associate with. The stoichiometry of the subunits in the complete receptor is crucial to its function. The complete receptor must contain 5 subunits in a legal combination or it will be unable to leave the endoplasmic reticulum and get recycled. The bare minimum for a receptor to be accepted is a β or ρ subunit, only those can reach the cell membrane with the help of chaperone proteins (Connolly et al., 1996). Generally, a receptor will contain 2 α and 2 β subunits and the fifth member of the pentamer is allowed more variety. It is usually a γ subunit. To respond to GABA and be functional, a receptor has to contain an α subunit next to a β subunit. Their interface creates the binding site for GABA. (Olsen and Sieghart, 2009).

Homopentamers are rare and it seems like β and ρ are the only valid ones. The β subunit is capable of forming the necessary hydrogen bonds with itself whereas the ρ subunits are genetic isolates and very distinct from the other $GABA_A$ subunits both in their genetic makeup and function. They were, understandably, regarded as a separate entity for many years (Bormann and Feigenspan, 2001). Like the other genetically distinct subunit δ, the ρ receptors have a high affinity for GABA with slow kinetics and resists desensitisation (Feigenspan and Bormann, 1994). The ρ receptors mainly appear in the retina. It can also be detected in smaller concentrations in other parts of the CNS such as the caudate nucleus and corpus callosum (López-Chávez et al., 2005). The artificial β homopentamer discussed earlier with regards to homology modelling is most likely not a naturally occurring receptor subtype but can be expressed in recombinant systems.

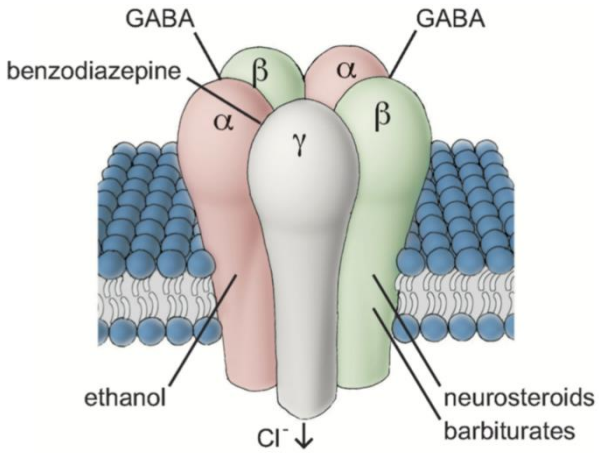

Figure 1 The most common $GABA_A$ receptor subtype. The 5 subunits form a pore through the plasma membrane and the interfaces between subunits create binding pockets for ligands. Art by Petri Vainio-Ketola, commissioned for Uusi-Oukari & Korpi (2010), permission granted by Mikko Uusi-Oukari.

The common subtype comprises α1β2γ2 and follows this order α1-β2-α1-β2-γ2 when looked at clockwise. The γ2 subunit is not very picky about its partners and will assemble with any of the α and β subunits (Sieghart and Sperk, 2002). This receptor subtype creates a phasic response when bound to GABA where it very quickly produces strong inhibition but swiftly desensitises and stops sending the signal. With its three types of subunits, it creates binding sites for the endogenous ligand GABA in the α- and β-interfaces. With the inclusion of the γ2 subunit, it also creates binding pockets for endogenous neurosteroids and the benzodiazepine drug group between the α and γ subunits (Sigel and Buhr, 1997). This subtype and

its binding sites are shown in more detail in fig. 1. This receptor subtype is absolutely essential for normal brain function and knock-out mice will die a few days after birth as the CNS fails to develop normally (Günther et al., 1995).

Receptors containing only α and β subunits do also exist. Around half of the α4 subunits in the brain are found in α4βxδ pentamers, part of them assemble with γ subunits but the rest form complete receptors without any δ or γ subunits. These receptors are either α4βx or α4βx with one of the rarer subunits discussed below (Bencsits et al., 1999). All of them cannot be rare subunit combinations though since approximately 10 % of the extrasynaptic receptors in the hippocampus reportedly contain just α and β subunits (Mortensen and Smart, 2006).

The rarer subunits are ε, θ, π. The ε and π subunits behave in the same way as δ and simply replace γ2 in the typical composition. The ε subunit is mainly found in the hypothalamus and telencephalon (Moragues et al., 2002). The π subunit breaks the mould and is very rare in the CNS, it is mainly found in the uterus, prostate, lungs and thymus (Hedblom and Kirkness, 1997). The θ subunit replaces a β subunit. As mentioned earlier it is analogous to the β4 subunit in avian brains. It is mainly located in the striatum as α2β1γ1θ (Bonnert et al., 1999). Using transcriptome analysis it was also found in the dentate gyrus where it was co-localised with α2, β1, γ1, and ε subunits. It most likely forms the aforementioned α2β1γ1θ pentamer here as well (Sequeira et al., 2019).

The δ subunit is quite picky about its subtype partners and the only functional δ-receptors contain α4 and α6 and the β subunits. (Laurie et al., 1992). The α6 receptor mainly appears in extrasynaptic receptors and differs from the more common α1 in its lack of benzodiazepine sensitivity and increased affinity for GABA (Korpi and Lüddens, 1993). This cements its connection to the δ subunit. δ can be found in the thalamus as α4β2δ, in the cerebral cortex as α4β2/3δ, hippocampal dentate gyrus granule cells as α4β2/3δ, caudate putamen and in the nucleus accumbens as α4β3δ and the cerebellar granule cells as α6βxδ (Jechlinger et al., 1998; Pirker et al., 2000; Sur et al., 1999; Pöltl et al., 2003). The stoichiometry of the δ-containing receptor is uncertain but there is some evidence that it simply replaces γ2 in the aforementioned αxβxγ2 pentamer based on studies

on binding pockets (Feng and Forman, 2018). Even if the subunit composition has been determined, the exact stoichiometry of the assembled unit can be difficult to confirm. The order in which the subunits assemble in the pentamer will determine which subunit interfaces are created and which binding sites are formed. Recombinant concatamer receptors provide a vital tool in this task. By joining the subunits with linkers they are forced to assemble in the assigned order. By comparing the assigned orders with native receptors one can determine the true native conformation once a match in properties is found.

The δ subunit is found extrasynaptically and it confers unique properties. As mentioned with regards to the special ρ subunits, it does not desensitise easily and is responsible for tonic inhibition, in other words, sustained inhibition (Nusser et al., 1998). The receptors have a particularly high affinity for GABA (Brown et al., 2002). This high affinity for ligands allows them to respond to excess GABA overflowing from nearby synapses and decrease the excitation in the system. This inhibition is key in epilepsy treatment where the basal state is too excited (Mody and Pearce, 2004). This subtype poses an interesting target as it could provide central inhibition without losing its effect over time as it resists desensitisation. This subtype is also very responsive to ethanol and general anaesthetics (Wallner et al., 2003). Knock-out mice were found to be less responsive to the effects of ethanol and less likely to seek out ethanol. The mice were also less responsive to endogenous neurosteroids (Mihalek et al., 2001).

1.2. Subunit structure

The $GABA_A$ receptor can be divided into 5 subunits and each subunit can be divided up into smaller structures. The subunit's sequence starts in the N-terminus of the amino acid chain with an extracellular structure, it will then loop around the plasma membrane with 4 transmembrane domains (TM) before exiting into the extracellular domain once more and ends in the C-terminus, as shown in the illustration fig. 2. Each subunit's extracellular domain consists of 10 beta-strands and 2 alpha-helices. The extracellular domain also contains 6 loops, loops A, B and C comprise the + interface whereas loops D, E and F make up the - interface (Ernst et al., 2005). The binding sites are often located in the interfaces between subunits but may also be located in the pore, see fig. 1. Anaesthetics like propofol

bind to the transmembrane domains rather than the extracellular sites (Sigel and Ernst, 2018). The 4 transmembrane domains are alpha-helices that create the ion channel. TM2 lines the internal side of the pore. The tilting of TM2 determines if the channel is open, closed or desensitised. There is an intracellular loop between TM3 and TM4 that allows the receptor to be modulated from the inside, the cell can phosphorylate this domain to affect the receptor. This loop determines receptor trafficking and will decide how long the receptor is retained in the synapse before it moves on to the extrasynaptic spaces (Hannan et al., 2019). Synaptic subtypes are trapped in the synapse using anchoring proteins such as radixin and gephyrin that interact with the intracellular loop (Kowalczyk et al., 2013; Tretter et al., 2012; Hausrat et al., 2015). The extrasynaptic receptors are not bound in the same way and can freely diffuse out of the synapse. The connecting loop between TM1 and TM2 can also form an intracellular domain, the γ2 and β2 subunits come in long and short splice variants with differing lengths of the intracellular loop but there seems to be little difference between the variants (Miller and Aricescu, 2014; Simon et al., 2004). The Cys-loop in the extracellular domain is essential for transmitting the signal. Once the agonist has bound to the receptor the Cys-loops have to interact with each other to tilt the second transmembrane domains to widen the channel (Kash et al., 2003).

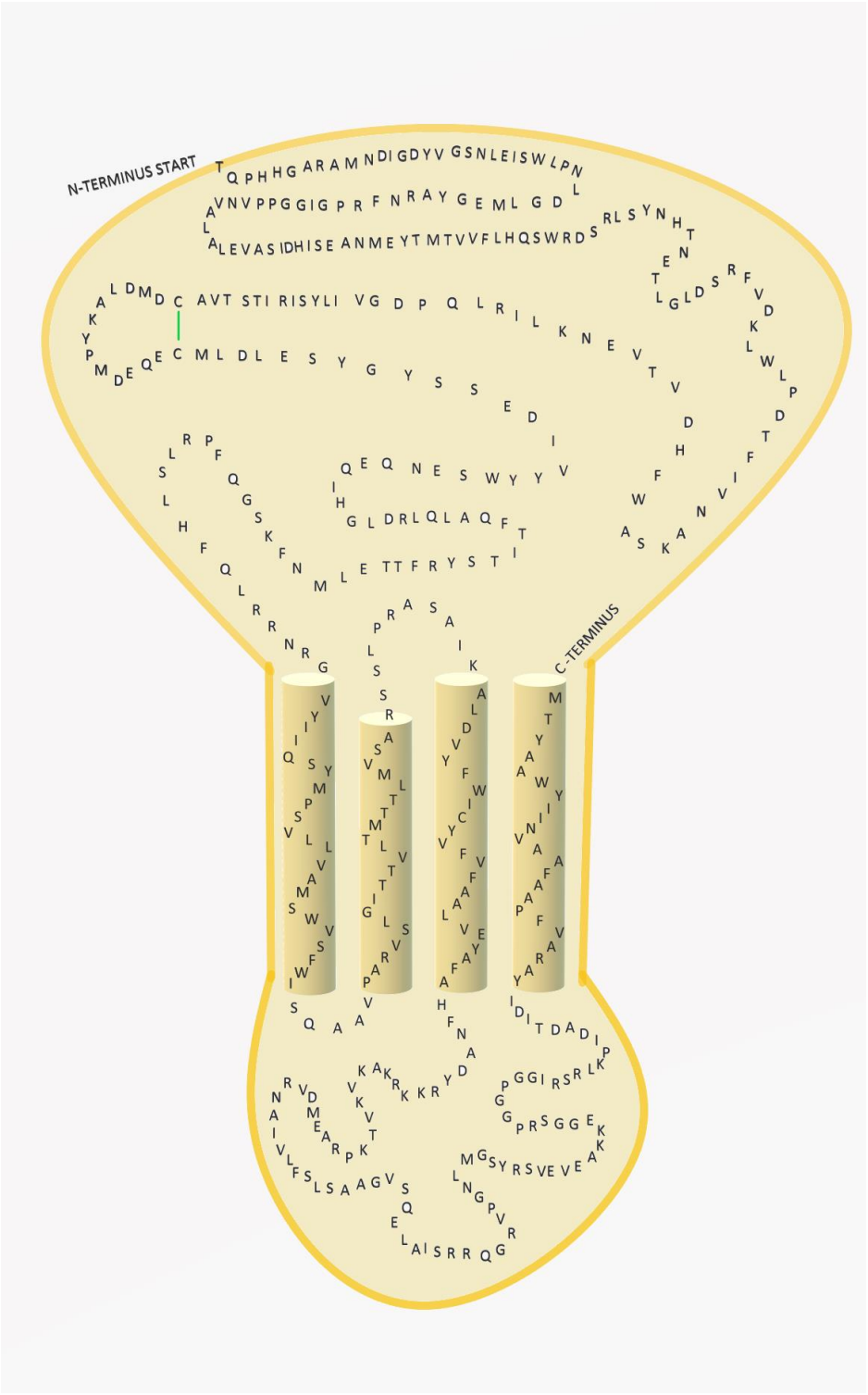

Figure 2 Internal structures of the δ subunit. For illustrative purposes, the amino acid sequence of the rat δ gene is drawn as a chain throughout the protein. The sequence begins with the N-terminus forming the extracellular face; the green line denotes the cysteine-loop. The following part of the sequence is the first transmembrane domain. The transmembrane domains (yellow cylinders) are connected with linkers; between TM3 and TM4 one can see the intracellular loop, an extended linker that regulates the subunit through phosphorylation sites. The sequence ends in the C-terminus on the extracellular side, but the δ subunit illustrated here has a very short C-terminus. N.B. this drawing is an approximation and does not show the exact location of individual amino acids.

1.3. Clinical implications

The differential binding sites created by the receptor subtypes provide fertile ground for pharmaceutical development. The subtypes can be targeted with specific drugs and produce a plethora of responses. For example, benzodiazepines can only act on a receptor containing γ2 joined with an α subunit that is not α4 or α6 (Korpi and Lüddens, 1993). Even though a myriad of drugs target the GABA system they can produce wildly different effects: targeting α1-containing receptors leads to sedation, α5 is related to memory and α2/3 affects anxiety. Generally, drugs act as allosteric modulators that bind to one or a few receptor subtypes. This is why benzodiazepines, Z-drugs like zopiclone and β-carbolines have such vastly different effects (Rudolph et al., 1999). Apart from the clinical use, this also allows researchers to detect subunits by using drugs with known affinities for the subtypes. The δ subunit can be detected by using agonists with a particularly high affinity for it, such as gaboxadol (also known as THIP) or muscimol. Muscimol is a natural product from the mushroom *Amanita muscaria* which historically has been used to induce hallucinations. It is structurally similar to GABA but shows a higher preference for the extrasynaptic receptors (Krogsgaard-Larsen et al., 1981). Gaboxadol is a synthetic derivative of muscimol with partial agonistic properties at $GABA_A$ but has been found to be a superagonist specifically at δ-containing receptors (Chandra et al., 2006).

Apart from the established pharmacopoeia discussed above the clinical interest in the $GABA_A$ receptor is also extensive. There are also over 60 point mutations among the subunits that cause epilepsy (Baulac et al., 2001; Hernandez and Macdonald, 2019). The δ subunit that this paper focuses on has also been found to be crucial in Angelman's syndrome (AS). AS, named after the physician that described it, is a developmental disorder where the children are slow to learn speech and motor skills and eventually develop ataxia and seizures (Clayton-Smith and Pembrey, 1992). It is caused by a mutation on chromosome 15q11-13 that encodes the UBE3A gene which is expressed in the hippocampus and cerebellum, hence the ataxia (Albrecht et al., 1997). Using knock-out mice it was found that the lack of normal UBE3A expression led to a decrease in tonic inhibition suggesting that the gene is implicated in δ-type $GABA_A$ receptor activity. The postulated mode

of action is through overexpression of GAT1, the transporter that removes GABA from the synapse and decreases the availability of extrasynaptic GABA (Egawa et al., 2012). Another connection to GABAergic signalling is the GABRB3 gene which encodes the β3 subunit. It has been found that if the maternal copy of the gene fails to pass on to the child then that child is much more likely to develop AS (Mercer et al., 2016). As the pathology of AS is caused by the loss of tonic inhibition due to GAT1 overexpression or β3 malfunction, the positive modulation of extrasynaptic $GABA_A$ receptors has been investigated as a solution. Currently, gaboxadol, an experimental tool that acts as a specific agonist for δ, is undergoing phase 2 trials for AS (Hoffman, 2019). Another drug, ganaxolone, showed promise in UBE3A knockout mice. Ganaxolone is a positive allosteric modulator of both synaptic and extrasynaptic $GABA_A$ receptors. The mice treated with the drug experienced less ataxia and anxiety and showed no loss of effect after 4 weeks of treatment (Ciarlone et al., 2017).

Another condition of interest is fragile X syndrome. It is also a developmental disorder that causes intellectual disability and is associated with autism and social anxiety. It is characterised by a brittle region at Xq27.3 (Yu et al., 1991). This fragile namesake of the syndrome is caused by expanded repeats that disrupt gene transcription. The loss of the gene product, Fragile X Mental Retardation Protein (FMRP), causes disinhibition of mRNA translation in the dendrites (Aschrafi et al., 2005). The altered protein synthesis in the dendrites affects neuron survival and signalling. The GABAergic signalling is affected and decreased even though the amount of GABA released in the synapse remains the same. The FMRP knock-out mice have less tonic inhibition, therefore, the syndrome might be alleviated using drugs that target extrasynaptic GABA receptors (Martin et al., 2014). In the FMRP knock-out mice, there have been experiments with neuroactive steroids and it was found that the drug treatment restored tonic inhibition (Modgil et al., 2019). So far only allopregnanolone, a neurosteroid and positive allosteric modulator of $GABA_A$ receptors, has been trialled for fragile X syndrome but it has shown some potential (Napoli et al., 2019; Wang et al., 2017).

2. Aims and hypothesis

This project focused on the α6β3δ receptor due to its unique properties and clinical significance outlined above. The effect of a domain in the first transmembrane region on ^{3}H-muscimol kinetics was investigated using site-directed mutagenesis. This domain of the δ subunit was previously investigated and found significant with regards to channel opening by You and Dunn (2007). By creating chimeric receptors of the δ and γ2 subunit they managed to narrow down the essential property conferring domain to the amino acids S238-V264 of the δ subunit. This domain was chosen for further studies and radioligand binding tests were performed on δ/γ2 chimaeras with γ2 amino acids in the positions 233-234 or 238-264.

3. Results

3.1. Mutant construct preparation

The identity of the constructs was confirmed using digestion reactions and electrophoresis. As the desired length of the PCR product was known it could be cut and measured on a gel with a DNA ladder as reference. The product was also checked by running a PCR cycle with the product and a primer complementary to the mutated sequence. A visible product in the agarose gel confirmed that the mutation was present. The PCR products were also sequenced by the Institute for Molecular Medicine Finland and their identity was ascertained.

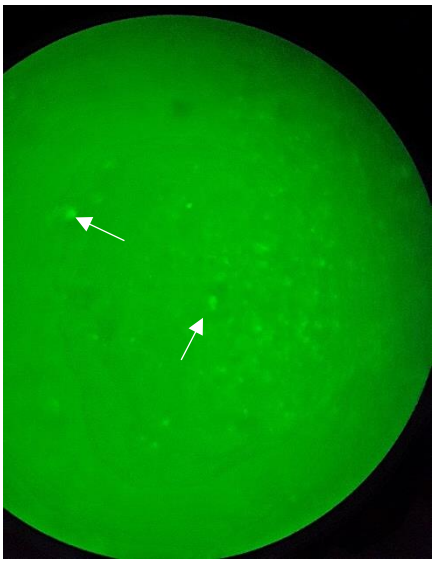

Figure 3 Image of GFP-transfected cells taken using a fluorescent microscope. The white arrows indicate strong fluorescent signals from transfected cells.

3.2. HEK 293 culture

Several batches of human embryonic kidney (HEK 293) cells were used. Initially, cells were obtained from Orion, Turku, Finland. Their identity was confirmed through sequencing at the Institute for Molecular Medicine Finland on Nov 14th 2018. Five groups of different passage numbers were used but all of them were found to be fragile and did not express plasmids efficiently. The GFP expression test was performed and some fluorescence was observed but not enough for our purposes. Three of the batches were also mycoplasma positive hence the cell line was abandoned.

New cells were obtained from Sigma-Aldrich, Espoo, Finland. The identity was assured by the supplier. These cells had a lower passage number but were very slow to grow initially and the cells rarely survived the thawing process. Once a culture had been established with the freshly prepared medium the cells thrived and performed well in the GFP transfection test, the results can be seen in fig. 3 where the strongest signals are marked with arrows. The cells scored positive for mycoplasma which may affect cell culture viability. As mentioned earlier, the cells were initially growing poorly and some of the mutated cells died after transfection. The survivability issue was overcome after a few passage numbers and the transfected cells thrived and grew enough to complete binding studies.

The incubators were cleaned regularly and the cells were handled in the same laminar hood each time. No other cell lines were cultured in the same laminar hood.

3.3. Association binding studies

The association experiment was performed three times with quadruplicate data points. The concentration of the radioligand was 10 nM in each well. The EC50, the concentration required to reach half of the maximum effect, for the δ-containing receptor is expected to be around 2 nM, therefore, maximum occupancy should be achieved at the selected dose (Benkherouf et al., 2019).

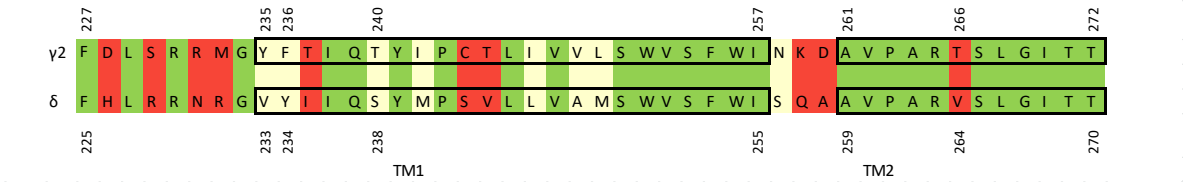

Figure 4 Alignment of the amino acid sequence of the δ and γ2 subunits. Matched amino acids are coloured green, amino acids that do not match but have similar properties are marked in pale yellow and mismatched pairs are coloured red. There is high homology between the two subunits in the transmembrane domains, whose amino acids are encompassed by the black borders, and more divergence in the linkers and extracellular domains. The larger mutated construct incorporated amino acids from γ2 at the locations marked 238(240) to 264(266). The γ2 sequence is off by 2 amino acids from the δ one. The smaller construct only swaps 233-234(235-236).

The first mutant construct, which had two mutated amino acids at 233-234, was used in 2 binding experiments but the initial results, not shown here, suggested that there was no difference between the mutant and the wild type. The first construct was abandoned and the chimaera with the larger mutated segment was used in all further tests.

The larger mutated construct incorporated γ2 amino acids in positions 238-264. However, it performed in much the same way as the more conservative 233-234 mutation in that it was indistinguishable from the wild type. In fig. 4 the mutation sites are tagged with their amino acid numbers and one can see that the locations incorporated unique amino acids that should have altered the structure of TM1, either the start of it with the 2 amino acid switch or the whole of TM1 and part of TM2 with the larger construct. When measuring the binding as compared to their own maximum at 15 minutes they behaved in the same way but when comparing the disintegration per minute (DPM) counts of the samples the wild type consistently reached higher values. This behaviour, as demonstrated in the graphs below, was consistent in all the experiments, both association and dissociation.

Upon regarding fig. 5 one can see that the error bars for the two groups are always touching each other so the results cannot be regarded as significantly different from each other.

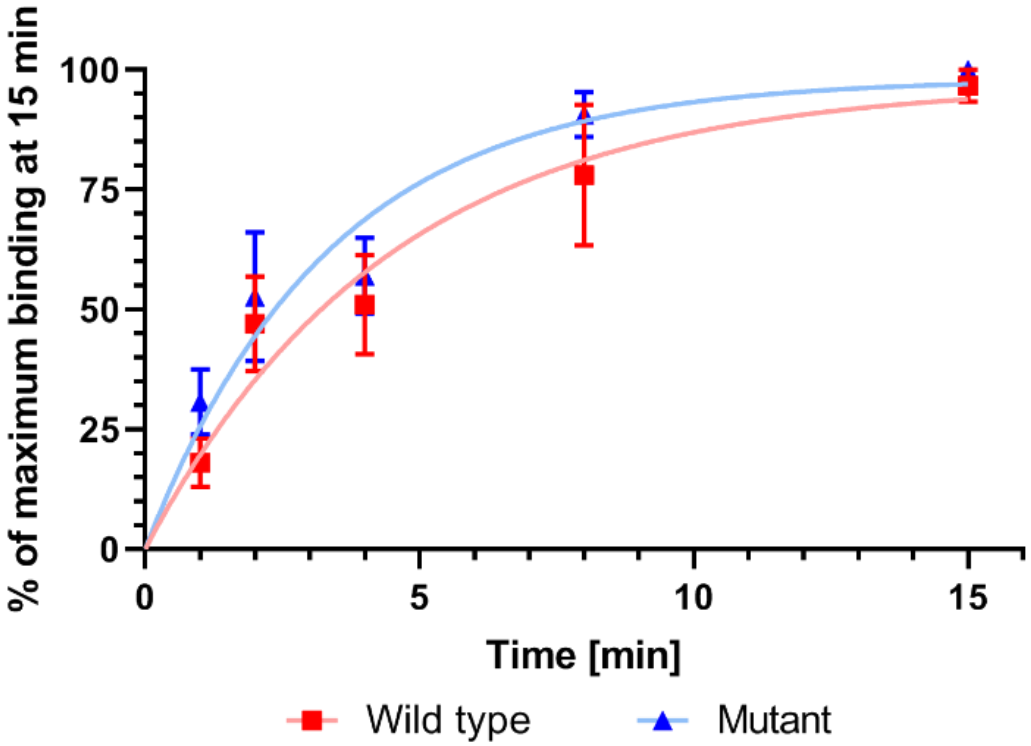

Figure 5 ^{3}H-muscimol association measured in α6β3δ and the chimaera. The data points are the means of the 3 experiments and the error bar is the standard error of mean.

3.4. Dissociation binding studies

In the dissociation experiments, there were a total of 3 successful experiments performed with quadruplicate samples. They painted a similar picture to the association studies above. As shown in fig. 6 the two groups followed the same trend. When looking at the total DPM counts the wild type started at a much higher value than the mutant.

The data points' error bars are in such proximity that one must conclude that the results are not significantly different.

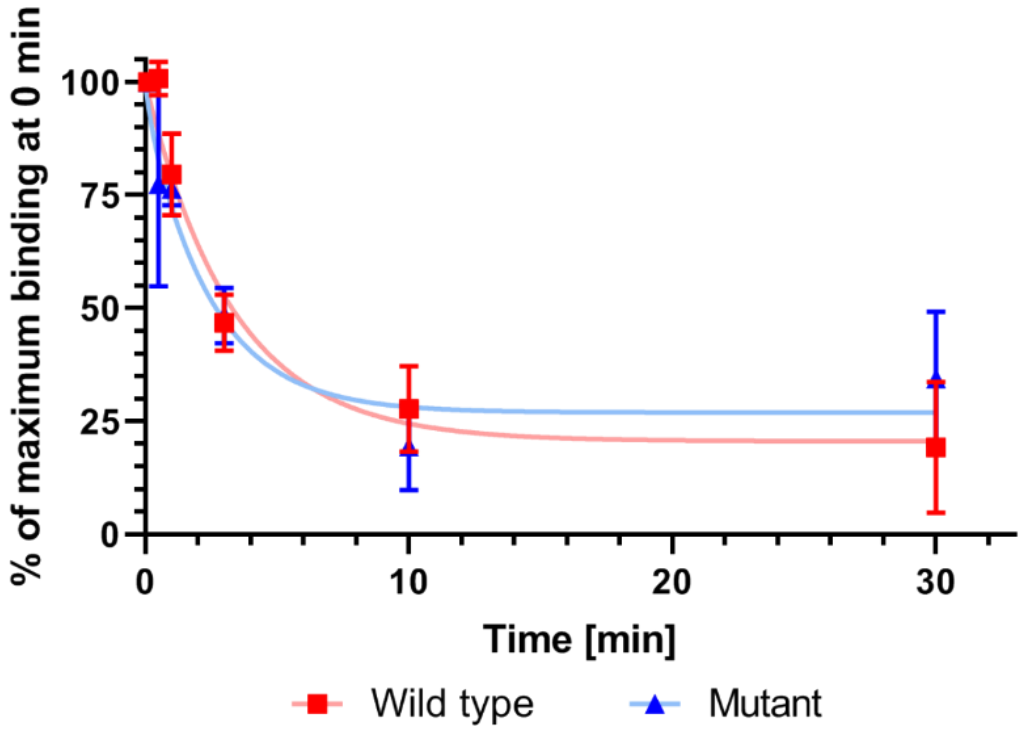

Figure 6 ^{3}H-muscimol dissociation after binding for 15 minutes. The ligand was displaced by adding GABA and washing the samples. The data points are the means of the three experiments and the error bars are the standard error of mean.

3.5. Affinity

Using GraphPad Prism 8 the association rate (Kon), dissociation rate (Koff) and equilibrium dissociation constant (Kd) were calculated. The dissociation curves were analysed using the two-phase decay formula and the fast decay constant was used in the formula to determine Kon. The chimaera's best-fit Kon was 5.3×10^{7} M^{-1} x min^{-1} and the wild-type's was 4.2×10^{7} M^{-1} x min^{-1}. The program also calculated the best-fit Koff values for the chimaera as 0.18 min^{-1} and for the wild-type as 0.18 min^{-1}.

From the Kon and Koff values, the Kd could be calculated. The chimaera had a Kd best-fit value of 3.49 nM and the wild-type was 4.31 nM. They were within one power of 10 from each other and can be regarded as very similar. A Kd value of this magnitude agrees well with previous data on the characteristics of the δ containing receptor (Benkherouf et al., 2019).

4. Discussion

As seen in fig. 5 and 6 of the results section the slopes of the mutant and wild type are incredibly similar. This is peculiar as the mutated block was one of the areas examined by You and Dunn, 2007. In their electrophysiology studies, the channel function was markedly altered by exchanging the amino acids in the first transmembrane domain with its γ2 counterpart. Here it was found that the association and dissociation constants, and therefore the affinity, of the mutant receptor, remained unchanged with regards to muscimol.

From this one could conclude that the changes in channel activity observed in previous studies were independent of receptor affinity. The drug can bind to the receptor just as easily but may not activate it in the same way. As there was no electrophysiology aspect of this project any functional changes remain unconfirmed.

It was found during the experiments that the total DPM recorded was higher among the wild type samples than the mutants. This may be an assembly issue. It was observed among the early cell batches that the mutant cells were less viable than the wild type ones and did not grow as vigorously. In the data presented cell colonies with similar density were used but the increased mortality suggests that the mutant construct is toxic or difficult for the cell to express. If there is a lower expression of receptors in the mutant group that could account for the consistently lower radioactivity signal.

In a few instances, the total radioactivity was lower in the 15 min association than the 8 min one. Since there should be no discernible desensitisation with δ containing receptors this peculiarity could be due to protein degradation as these samples were exposed to room temperatures longer than the others.

Despite the negative results, could this site still be the determinant for muscimol affinity? If the answer is yes, why was it not detected in this assay? One possibility is the state of the cells. They were infected with mycoplasma which affects cell growth and plasmid expression. However, after the first few batches, there was no issue with cell proliferation and the mutant and wild type transfections survived equally well. It could be an issue with the assay. Alterations and optimisations were

made during the course of the project and the results from earlier experiments may not be comparable with the later ones. Samples that required longer incubation with ligands were kept refrigerated unlike their peers with shorter timeframes. The filters were not given enough time to dissolve in the scintillation liquid which may affect readings. There was a software problem that caused the Hidex scintillation reader to skip vials which meant that the racks had to be read multiple times, some tubes had their radioactivity measured within the first 24h of the experiment whereas others may not be measured until several days later.

An issue with studying channel properties is time. There are two main tools for elucidating channel properties: radioligand binding studies and patch-clamp studies. The problem is that they operate on different time frames. Patch-clamp recordings deal with milliseconds whereas binding studies are measured in seconds and minutes. In the timespan of milliseconds, during a patch-clamp experiment, a receptor with only slow desensitisation like δ will not immediately trap the agonist in a prolonged deactivation process like the γ2 receptors that immediately desensitise and hold on to the agonist. The δ receptor, as a property of its desensitisation, will have fast dissociation of the agonist during a patch-clamp experiment, therefore the desensitisation properties are directly related to kinetics. However, during radioligand binding experiments the time frame is much larger and the steady-state association and dissociation are measured and here it is found that δ containing receptors have slow dissociation kinetics. The connection is nonetheless present and any domains involved in desensitisation should be examined in terms of their effect on binding kinetics as well. (Bianchi and Macdonald, 2001).

Due to all these sources of errors, one cannot rule out that the selected site could affect binding kinetics but if these results are to be taken at face value it begs the question of where the putative site could be. One could postulate that the sites more likely to affect the receptor affinity may be the extracellular domains rather than TM1.

The transmembrane domains of different subunits have high homology with each other. Even when the amino acids of δ and γ2 were different they often shared the

same electrical properties as seen in fig. 4. There are very few red amino acids marking complete mismatches. This makes the far more unique N-terminus the more likely suspect The N-terminus can be split up into 2 alpha helices, 10 beta-strands and connecting loops. The characteristic Cys-loop transmits the binding signal to the pore-lining which makes it responsible for efficacy and unlikely to be involved with affinity. Other loops form interfaces with the neighbouring subunits. Loop A, B and C form the + side interface and loop D, E and F form the - interface (Miller and Aricescu, 2014; Ernst et al., 2005). Among the α subunits, there is variation in terms of GABA affinity with α6, α1 and α2 having a particularly high affinity for GABA in the nanomolar range with α4 and α5 trailing closely behind and α3 with a significantly lower affinity. Among this group, it was found that a 4 amino acid motif in loop E was responsible for the differing affinities. This site has proximity to the GABA binding site but affects the affinity indirectly through allosteric means. However this was only regarding GABA, the site did not affect muscimol affinity (Böhme et al., 2004). The δ subunit is not directly involved in the muscimol binding site but one could still make a case for its E-loop having an allosteric effect on the complete receptor's configuration and affinity. Theoretically, the δ subunit could also be directly involved in the binding. Putative binding sites for GABA have been found in the β+/δ- interface (Lee et al., 2016) and potential sites for hops compounds are in the β-/δ+ interface (Somborac, 2019).

There are papers that support the theory that the unique properties of the δ subunit are conferred by TM1 as assumed in this paper. Bianchi *et al.* (2001) found that a γ2 subunit with transplanted δ sequence in TM1 and TM2 behaved like δ in terms of its desensitisation. Normally a γ2-containing receptor has 4 phases of desensitisation: a fast phase that lasts 10 ms, an intermediate phase of 150 ms, a slow phase up to 1500 ms and a very slow phase beyond that. In contrast, a δ containing receptor has 2 phases, namely the slow and very slow phases. They could not find any single motif or amino acid responsible for this behaviour but found that the N-terminus in concert with TM1 and TM2 were responsible. With this in mind, it is perfectly possible that the section examined in this project is partially responsible for unique δ properties such as desensitisation and slow binding kinetics but that the γ-like behaviour will not be detected unless cooperating sites in

the N-terminus are co-mutated (Bianchi and Macdonald, 2002). This may at first seem like an insurmountable challenge as there are many non-homologous regions in the vast N-terminus but there are already implicated amino acids. When swapping regions, the same group found that swapping out V233-Y234 in TM1 together with the entire N-terminus could alter the δ containing receptor (Bianchi et al., 2001). This site, 233-234, is conserved in most other subunits as YF but the desensitisation resistant subunits ρ and δ have different amino acids. The mutation used here was 238-264 so if one assumes that both of the unique properties of δ, i.e. slow desensitisation and high muscimol affinity, are conferred by the same motif then these amino acids near the start of TM1 could be responsible. It is worth considering the similarities between δ and the rather unique ρ subunits. They share the resistance to desensitisation that δ has. If one equates desensitisation resistance to the characteristic slow binding kinetics then the domains that affect the ρ subunits desensitisation may have equivalent effects on the δ subunit. It has been found that ρ1 will lose its resistance if a point mutation is introduced in the intracellular loop at amino acid 349. If the threonine is replaced by a lysine from the α1 sequence then the ρ1 subunit will desensitise like α1 (Gielen et al., 2015). The intracellular loop has mainly been implicated in receptor trafficking and internalisation (Hannan et al., 2019). It has phosphorylation sites that are involved in receptor modulation (Uusi-Oukari and Korpi, 2010) and it is possible that receptor desensitisation is partially controlled at this site. All of this is assuming that the desensitisation property is related to the slow kinetics, but there is a rationale behind connecting the two.

In conclusion, the selected site proposed by previous literature could not be confirmed as the site responsible for the high-affinity binding unique to the δ subunit-containing receptor and further research into the cooperation between the N-terminus and TM1 is recommended.

5. Methods

5.1. Mutant construct

The mutations were introduced to the δ template using custom-ordered primers. The primers were ordered from Metabion International AG, Germany. Three DNA pieces were produced using PCR amplification: the extracellular N-terminus of the δ subunit with an overhang that matches the γ2 sequence, the first transmembrane domain of the γ2 unit and the last three transmembrane domains and C-terminus of the δ subunit with a γ2 overhang. The overhangs allowed the pieces to be connected and elongated to full length by PCR and a construct consisting of the three pieces was created. This piece-by-piece method is summarised in fig. 7.

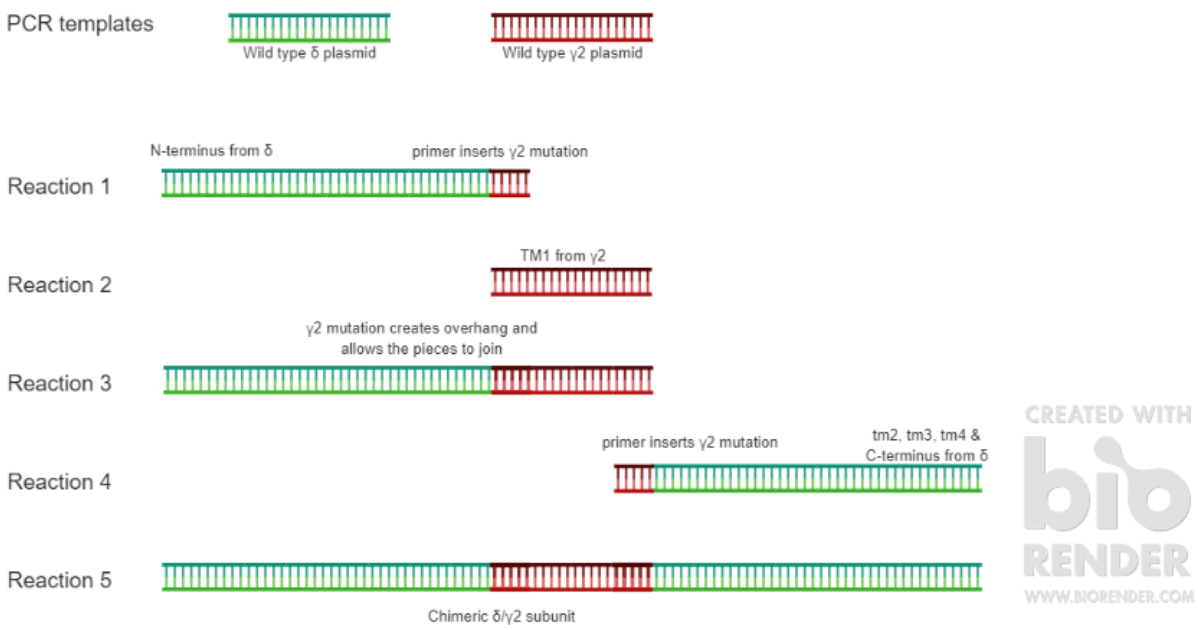

Figure 7 Summary of PCR reactions for the second chimeric construct. The template DNA was the wild type δ or wild type γ2S in circular pRK5 plasmids. The γ2 amino acids used are equivalent to 238-264 in the δ sequence. This picture was created using the drawing tool BioRender.

A smaller construct was also created using only 2 pieces. Custom primers introduced a 2 amino acid mutation into the δ sequence and the two ends were connected thanks to the overhang. The alteration was in TM1: V233Y and Y234F. The primers and free nucleotides were diluted in water and Q5 polymerase buffer.

The template DNA was added after the solution had been vortexed. The Q5 DNA polymerase enzyme was added once the mixture had been heated to 94°C and the DNA strands had separated. The hot start decreases the risk of mispriming. The PCR machine cycled between 94°C, 57°C and 72°C which allowed DNA strands to separate, anneal with primers and be elongated respectively. 30 cycles were performed.

5.2. DNA purification

The constructs were loaded onto 1 % agarose gels to confirm their appropriate size and remove impurities. The agarose was dissolved in Tris-Boric acid-EDTA buffer by heating and shaking the flask. Once the solution had cooled down ethidium bromide was added in order to stain DNA bands. For 250 ml gel 5 µl DNA stainer was added. All samples were dyed with Purple loading dye (New England Biolabs, Ipswich, United Kingdom) to track their progress. The electrophoresis proceeded for 1h at 180 V. The bands were visualised under UV light and cut out and cleaned. The gels were purified with Wizard SV gel and PCR cleanup system (Promega, Nacka, Sweden). The gel was dissolved in 1 µl binding solution per mg gel and heated to 60°C. Impurities were removed using filtered tubes, ethanol-containing washing solutions and a tabletop centrifuge as per the manufacturer's instructions. The pure DNA was eluted into nuclease-free water.

5.3. Plasmid preparation

The constructs were cut with restriction enzymes to create sticky ends. They were ligated to a pRK5 plasmid containing the wild type δ subunit whence the critical section had been cut out using the same enzymes. The enzymes were obtained from New England Biolabs, Ipswich, United Kingdom. Ligation was performed using a Quick ligation kit from the same supplier. The digested insert and backbone were diluted in ligase buffer and joined with T4 DNA ligase. The now circular plasmid was introduced to *E.coli* bacteria by transformation.

First, the bacteria had to be made transformation competent. DH5α cells were thawed out in body-temperature Luria-Bertani (LB) broth and grown on a stirring table in an incubator for 3h using a sterile container. The LB broth was made up with 10 g tryptone, 5 g yeast and 10 g NaCl dissolved in 1 L pure water. The growing colony was then transferred to a larger autoclaved flask with more LB

broth and grown overnight. The cells were spun down into pellets at 6000 g and resuspended in cold 0.1 M $CaCl_2$. The cells were once again centrifuged and the resuspension was repeated. After incubating on ice for 30 minutes the solution was centrifuged once more and the pellets were suspended in 0.1 M $CaCl_2$/20 % glycerol.

The now plasmid-receptive cells were mutated by adding plasmids to the ice-cold cells and gently mixing them with a pipette tip. The reaction proceeded for 30 min on ice after which the tube was placed in a 42°C water bath to induce heat shock for 90 s. The cells were then returned to the ice for 5 min and the DNA entered the cells. The cells were plated onto agar plates. The agar plates were made up of LB broth and agar. The mixture had been heated to solubilise the agar powder and ampicillin was added, 100 mg per litre gel mixture. The gel had been cast into plates and allowed to cool. The cells that had successfully adopted the plasmid gained ampicillin resistance and formed colonies on the agar plates while the untransformed perished. The growing colonies were kept on a stirring table at 250 min^{-1} and 37°C overnight. The transformed colonies were selected the following day and expanded in LB broth containing ampicillin.

The plasmids were then harvested using Nucleobond Xtra Maxi Plus EF by Macherey-Nagel, Düren, Germany. The cells were spun down into pellets and resuspended with an RNase A containing solution and lysed with a NaOH and SDS solution according to the supplier's protocol. The lysis was terminated with a potassium acetate solution and the cellular waste was precipitated. The DNA containing solution was filtered and bound to anion exchange resin. The resin was washed and the DNA released with an alkaline buffer. The DNA was precipitated with isopropanol and collected in a filter. The DNA was washed with ethanol and eluted with a Tris-based buffer. To confirm the presence of DNA the eluate was precipitated once more with 2 volume units of ethanol and sodium acetate, 0.3 M in the aqueous phase. The DNA precipitated into white coils and was centrifuged. The ethanol supernatant was removed and its remnants allowed to evaporate. The DNA was resuspended in pure water. The plasmid concentration was determined spectrophotometrically using Nanodrop by Thermo-Fisher Scientific, Espoo,

Finland. The spectrophotometer measured the absorbance at 260 nm and 320 nm to detect double-stranded DNA.

5.4. Transfection

HEK 293 cells were ordered from Sigma-Aldrich, Espoo, Finland. Stock cells were quickly thawed in a 37°C water bath and diluted in cold DMEM 10 % FBS. The cells were centrifuged for 2 min at 2000 rpm. The supernatant was removed and the cells were resuspended in warm complete medium and plated. Once the cell culture had grown adequately it was split 1:3 using a hypertonic solution and gentle shaking. The newly split cells were allowed to adhere to their new plate and grow overnight before being transfected. The cells were kept in an incubator at 37°C and 5 % CO_2. The medium was prepared from Dulbecco's Modified Eagle Medium powder purchased from Gibco, Life Technologies, Espoo, Finland. The powder was dissolved in sterile water, 13.7 g/L, with sodium bicarbonate 3.7 g/L. The pH was adjusted to 7.0 with HCl and the medium was run through a sterile filter. 10 % inactivated foetal bovine serum and penicillin-streptomycin was added to each bottle.

For transfection, the calcium phosphate precipitation technique was employed. The cells were transfected with rat α6, β3 and δ subunit cDNAs each in a pRK5 plasmid or α6, β3 and the mutant construct in a 1:1:1 ratio. The cells were grown in 15 cm diameter plates and received 15 µg of each plasmid. Calcium chloride was added dropwise and the tube was shaken until white DNA complexes were visible. HEPES buffered saline was prepared using sodium chloride, Na_2HPO_2 dihydrate and HEPES. It was adjusted with HCl or NaOH to pH 7.0. The HEPES buffered saline was added dropwise and the liquid was inverted to mix the reagents. After incubating at room temperature for 1 min it was pipetted evenly onto the plate. The plates were kept in the incubator overnight to absorb the plasmids.

To confirm that the HEK cells were transfection competent GFP was used as a test plasmid. 48h after the transfection the test plate was examined with a fluorescent microscope. Only cell lines with visible GFP expression were cultured further and used for transfection.

The medium was changed 24h after the transfection and the cells were harvested after growing for an additional day. The medium was discarded and the cells were dislodged with an ice-cold buffer containing 10 mM Tris, pH 7.4, 0.15 M NaCl and 2 mM EDTA. The cells were centrifuged and resuspended in a Tris-HCl buffer with 43% glycerol and stored at -20°C. Overnight storage in glycerol was found to increase the binding according to unpublished data from Mikko Uusi-Oukari.

5.5. Binding studies

The binding of radiolabelled muscimol was measured. Unlabelled cold GABA was used to displace the radioligand and thereby determine non-specific binding. The harvested cells were thawed and the glycerol was diluted with NaCl and Tris-HCl pH 7.4 and centrifuged. The glycerol-free cells were resuspended in 10 mM Tris-HCl, pH 7.4, from here on referred to as the assay buffer.

For the association experiment, there were 4 groups: total binding in the mutant cells, non-specific binding in mutants, total binding in the wild type cells and non-specific binding in the wild types. There were 5 time points: 1 min, 2 min, 4 min, 8 min, 15 min. It was known from earlier studies that maximal binding would be achieved after 15 minutes and there would be no need to have longer time points. Shorter time points were not employed as the initial experiments showed no specific binding under 1 min. The non-specific binding was measured by exposing the cells to 100 µM cold GABA for more than 15 min before the tagged muscimol was added. The total binding was measured on cells without the presence of GABA. After the cells had received 10 nM ^{3}H-muscimol the vials were shaken to mix the drug and cells and incubated for a set amount of time. The cells and any ^{3}H-muscimol bound to them were then washed with assay buffer and absorbed into a Whatman GF/B filter (Whatman International Ltd., Maidstone, UK) using Brandel Cell Harvester model M-24, Gaithersburg, MD, USA. The filters were dissolved in 3 ml scintillation liquid (Optiphase HiSafe 3 scintillation fluid, Wallac, Turku, Finland) for 24h and the radioactivity of the samples was then measured using a Hidex 600 SL liquid scintillation counter, Turku, Finland. The disintegration per minute measured after a 5 min reading was the endpoint used in all calculations.

The dissociation studies had the same 4 groups as described above. In the non-specific binding tubes, the cells were first treated with 100 µM cold GABA whereas the total binding group was not. The labelled muscimol was pipetted into each vial and allowed to equilibrate for 15 min to reach maximum binding. Cold GABA was added to the non-specific tubes again and they were shaken, allowing the ^{3}H-muscimol and GABA to freely reach their maximal association. After 15 min the total binding tubes received their first dose of cold GABA and were washed as described earlier at the set time points: 0 min, 0.5 min, 1 min, 3 min, 10 min and 30 min. GABA should have displaced more than half of the bound ^{3}H-muscimol after 30 min and according to Dr Uusi-Oukaris experience, it is impossible to displace all of the ^{3}H-muscimol even if the incubation time is extended so 30 min was chosen as the last time point.

The results from the binding studies were analysed and presented using GraphPad Prism 8.

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7. List of Abbreviations

AS	Angelman's syndrome
CNS	Central nervous system
FMRP	Fragile X Mental Retardation Protein
GABA	γ-aminobutyric acid
HEK 293	Human embryonic kidney cells 293
Kd	Dissociation constant at equilibrium
Kon	Association rate
Koff	Dissociation rate
LB Broth	Luria-Bertani medium (tryptone, NaCl, yeast)
pRK5	Plasmid vector for mammalian cells that confers ampicillin resistance
TM	Transmembrane domain

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