Functional properties and pharmacology of extrasynaptic GABA-A receptors

by

Thomas Patrick McGee

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy of the of Imperial College London

December 2011

Biophysics Section
Division of Cell and Molecular Biology
The Blackett Laboratory
Imperial College London
South Kensington
London
SW7 2BW

Abstract

The “ambient” GABA that is present in the extracellular space surrounding all neurons of the brain is believed to be capable of persistently activating high-affinity extrasynaptic GABA-A receptors to generate a tonic membrane conductance. This generates a form of shunting inhibition that is capable of influencing cellular and network excitability. Extrasynaptic δ subunit-containing GABA-A receptors are known to generate this form of tonic inhibition in a number of defined brain regions and these are emerging as important clinical drug targets for the treatment of a number of neurological conditions. This thesis examines the functional and pharmacological properties of recombinant and native GABA-A receptors that allow them to function as ambient GABA detectors. Surprisingly, the data presented in this Thesis shows that the behaviour of these extrasynaptic GABA-A receptor populations is dramatically influenced by the steady-state GABA concentration they experience. For example, recombinant α4βδ and α6βδ receptor populations are shown to exhibit profound levels of desensitization in the presence of low ambient GABA levels that will limit their ability to respond to changes in ambient GABA. We also find that the action of certain sedative/hypnotic drugs on extrasynaptic GABA-A receptors expressed in cerebellar granule neurons is critically dependent upon the concentration of ambient GABA. For example, we show that the intravenous anaesthetic propofol will only enhance tonic inhibition when ambient GABA levels are below 100 nM. Similarly, we show that the GABA-A receptor agonist Gaboxadol is not capable of enhancing tonic inhibition when ambient GABA levels are high. In contrast to the behaviour of drugs like propofol and Gaboxadol, we find that neurosteroid enhancement of tonic inhibition will occur regardless of ambient GABA levels. This issue will be important when considering therapeutic strategies to target tonic inhibition in the treatment of neurological disorders. Furthermore, we show for the first time, that copper ions can potently block extrasynaptic GABA-A receptors, suggesting that copper may provide a means to selectively block tonic inhibition in the brain, and may even represent a novel source of extrasynaptic GABA-A receptor modulation *in vivo*.

Acknowledgements

First of all I must thank my supervisors, Alastair Hosie and Stephen Brickley, who guided me through my PhD. I began my time in Biophysics with Alastair, who sadly has since passed away. During what can be a tough transition into PhD research Alastair's patient and expert support made this a productive and rewarding time. Moreover Alastair's enthusiasm and generosity made this period a lot of fun. He is sorely missed. I would also like to thank Stephen Brickley, who very kindly took me under his wing. His guidance and encouragement have been invaluable.

I've also been very fortunate to share my time in Biophysics with many other exceptional people, without whose support this PhD would've been much harder. One such is Catriona Houston. Her insights, patch-clamp wisdom and biscuits gave me encouragement throughout. I would also like to thank Georgina MacKenzie, for my initial induction into electrophysiology and her company during tea breaks, Anna Zecharia for help with viruses and always being a friendly face, and Raquel Yustos for her tireless technical assistance and beautiful HEK cells. Thanks also to Bill Wisden for his advice on molecular biology and careers.

My gratitude also goes to many other people in the Biophysics department who provided such a friendly and fun atmosphere: Nick Franks, Rob Dickinson, Paul, Grace, Saffi, David, Amar, Eoin, Rowan, Scott, Katie, Trevor, Thom and Daniel to name a few.

Finally, I must thank my nearest and dearest for all their support through the years, and mostly for putting up with me, I really appreciate it. Vicki, I couldn't have done it without you! Mum, Dad, Mia, Clare, you've been perfect.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

GABA	γ-Aminobutyric acid
GABA-AR	γ -Aminobutyric acid type A receptor
GFP	green fluorescent protein
SEP	Super ecliptic pHlorin
δ-SEP	δ GABA-AR receptor subunit conjugated to the SEP tag
CGN	Cerebellar granule neuron
CNS	Central nervous system
RT-PCR	Reverse transcriptase polymerase chain reaction
IPSC	Inhibitory postsynaptic current
IPSP	Inhibitory post synaptic potential
ER	Endoplasmic reticulum
Gaboxadol	4,5,6,7-tetrahydroisoxazolo[5,3-c]pyridine-3-ol
DMSO	Dimethyl sulfoxide
EtOH	Ethanol
REM	Rapid eye movement
SV40	Simian virus 40
CMV	Cytomegalovirus
PBS	Phosphate buffered saline
ACSF	Artificial cerebral spinal fluid
DRN	Dorsal raphe nucleus
EEG	Electroencephalogram
SEM	Standard error of the mean
C-terminal	Carboxyl terminus

HSA	Human serum albumin
WT	Wild-type
Vm	Membrane potential
Best-1	Bestrophin-1 anion channel
$\delta^{-/-}$ mice	Mice in which the δ subunit gene is deleted
AAV	Adeno-associated virus

1. Introduction

1.1 GABA mediated neuronal inhibition

The functional precision of the adult mammalian brain relies heavily on a delicate balance maintained between excitatory and inhibitory drives at the cellular level. Neuronal transmission thus involves neurons often receiving multiple inputs from diverse sources; some excitatory, drawing the neuron closer to firing threshold, and some inhibitory which will attenuate action potential generation. There are a number of inhibitory neurotransmitters at work within this orchestrated interplay of neuronal inputs, but the most prevalent of these in the mammalian central nervous system (CNS) is GABA, which mediates the majority of inhibitory neuronal drive.

GABA (γ-aminobutyric acid) mediated inhibition, which represents the focus of this body of work, plays a major role in virtually all neurophysiological functions and represents a target for numerous classes of drugs (Hevers and Luddens 1998; Belelli and Lambert 2005). GABAergic cells communicate through diverse sets of inhibitory processes, the complexity of which is highlighted by the array of functionally distinct interneurons which have been identified in recent years. It is also becoming increasingly clear that individual GABA-AR properties and their regional and subcellular locations provide an important source of diversity in GABA-mediated signalling. GABA's actions are mediated by three classes of GABA-ARs known as GABA-A, GABA-A rho (formerly GABA-C), and GABA-B. GABA-B receptors are metabotropic and therefore stimulate ion channels via intermediary G-proteins and relatively slow response, while the ubiquitously expressed GABA-A receptors mediate fast inhibitory neurotransmission involving the activation of intrinsic ion channels.

1.1.1 GABA-A receptors

GABA-A receptors are members of the cysteine-loop ligand-gated ion channel family and share structural homology with other members of that family which includes glycine, 5-hydroxytryptamine (5-HT) type 3 and nicotinic acetylcholine receptors (Schofield, Darlison et al. 1987; Olsen and Sieghart 2009). Ligand binding to GABA-A receptors causes channel

activation and increased permeability to chloride and bicarbonate ions, with a relative permeability (P HCO_3^-/P Cl^-) of about 0.2 (Bormann, Hamill et al. 1987). This typically results in chloride ions conducting down their concentration gradient into the cell via a central pore, with an overall net effect on most mature neurons of hyperpolarising the membrane and creating the inhibitory post-synaptic potential (IPSP) (Mody and Pearce 2004; Farrant and Nusser 2005). The central ion conducting pore is surrounded by individual GABA-A subunits which combine to form a pentameric structure (Chiu, Jensen et al. 2002). Each of these subunits possesses 4 hydrophobic transmembrane domains (TM 1-4), a large N-terminus extracellular domain, and a large intracellular loop between TM3 and TM4 (figure 1). Subunits are thought to link up via sites on respective TM2 domains which line the central pore. The N-terminus region provides the site for GABA binding as well as binding sites for other psychoactive drugs such as benzodiazapines (BZs), while the cytoplasmic loop provides the site for various protein interactions as well as for various post-translational modifications that modulate receptor activity (figure 1) (Moss, Jacob et al. 2008).

Receptors assemble from a range of subunits from eight different classes: $\alpha_{(1-6)}$, $\beta_{(1-3)}$, $\gamma_{(1-3)}$, δ, π, ε, θ and $\rho_{(1-3)}$, with typically 30-40 % homology between subunit families and 70-90 % between subunit isoforms (Smith and Olsen, 1995; Korpi et al., 2002). In addition, many of these subunits have multiple splice variants (Moss and Smart, 1996). Thus this subunit diversity permits a large number of putative receptor isoforms (Olsen and Sieghart 2009). However, only a few dozen combinations have been shown to exist which may imply that there are some basic 'rules' of assembly. Critically these isoforms display unique biophysical and pharmacological properties which have been shown to vary considerably depending on subunit composition (Macdonald and Olsen 1994; Barnard, Skolnick et al. 1998; Hevers and Luddens 1998). Further, receptor isoforms differ in abundance and distribution throughout the nervous system and thus they differ in functional roles within the circuits involved (Olsen and Sieghart 2009).

Identification of GABA-A receptor subtypes and their regional and cellular distributions has involved a combination of *in situ* hybridisation studies (Persohn, Malherbe et al. 1992; Wisden, Laurie et al. 1992), immunohistochemical studies (Fritschy, Benke et al. 1992;

Sperk, Pirker et al. 2000), radioligand binding assays (Quirk, Gillard et al. 1994; Quirk, Whiting et al. 1995; Quirk, Blurton et al. 1996) and electron microscopic studies (Nusser, Roberts et al. 1995; Somogyi, Fritschy et al. 1996). From this body of work we have learned that most GABA-A receptors are composed of 2α;2;β and 1γ, δ, or ε subunit (Moss, Jacob et al. 2008), and that the most abundant isoform is composed of α1β2γ2 subunits (Sperk, Pirker et al. 2000). It is also clear that while certain subunit combinations are expressed ubiquitously throughout the CNS, such as α1, β1, β2, β3 and γ2 subunits, others such as α2-6, γ1 and δ subunits have much more restricted distributions (Laurie, Seeburg et al. 1992; Wisden, Laurie et al. 1992). Accordingly some neurons, such as those of the neocortex and hippocampus, display complex GABA-A receptor subunit expression patterns, while other brain regions such as the cerebellum express just a few different GABA-A receptors. Further, there are distinct GABA-A subunit expression patterns at different stages of development, with marked effects on form and function of GABA mediated inhibition (Laurie, Wisden et al. 1992; Persohn, Malherbe et al. 1992; Wisden, Laurie et al. 1992).

Specific preferred subunit combinations have also come to light, such as the co-distribution of α2 and β3 in the amygdala and hypothalamus and of α1, β2 and γ2 all over the brain (Wisden, Laurie et al. 1992). However, perhaps the most marked of these combinations is represented in the compartmentalisation and co-distribution of δ subunits, which are found with the α4 subunit throughout much of the forebrain and exclusively with the α6 in the cerebellum (Sperk, Pirker et al. 2000). Interestingly it appears that isoforms compartmentalisation takes place even within the same cell, as different subunits exhibit distinct subcellular distributions. For example, in cerebellar granule cells α1, α6, β2, β2 and γ2 are concentrated at GABAergic synapses, while in the same cell δ subunits were detected exclusively at extra- and perisynaptically (Nusser, Sieghart et al. 1998). Equally, α4βδ containing receptors are exclusive to extrasynaptic locations the thalamus, dentate gyrus, striatum and outer layers of the cortex (Farrant and Nusser 2005). While in hippocampal pyramidal cells the α2 subunit is localised to the soma/axon initial segment (AIS), while α1 subunits are found more distally to the cell body (Moss, Tretter et al. 2008; Triller, Bannai et al. 2009).

Thus the intrinsic functional diversity within the GABAergic system arising from a host of distinct GABAergic neurons and an array of GABA-A receptors with distinct properties as well as specific regional and subcellular distributions provide an expansive and malleable tool set for precisely regulating neuronal excitability. However there remains a further source of diversity within this system, which is the mode of receptor activation. This we will explore in the next section.

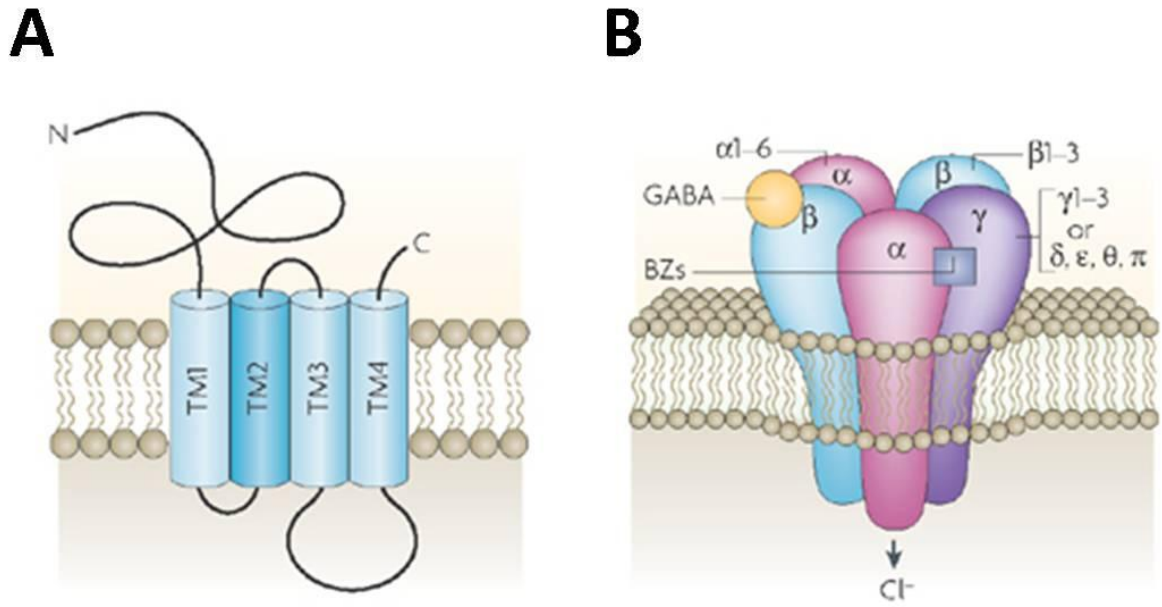

Figure 1. The GABA-A receptor. A, GABA-A receptors (GABA-ARs) are members of the ligand-gated ion-channel superfamily. GABA-AR subunits consist of four hydrophobic transmembrane domains (TM1–4), with TM2 believed to line the pore of the channel. The large extracellular amino terminus is the site of GABA binding, and also contains binding sites for psychoactive drugs, such as benzodiazepines (BZs). Each receptor subunit also contains a large intracellular domain between TM3 and TM4 that is the site for various protein interactions as well as for various post-translational modifications that modulate receptor activity. **B,** Five subunits from eight subunit subfamilies (α, β, γ, δ, π, ε, θ and ρ) assemble to form a heteropentameric Cl^--permeable channel. Despite the extensive heterogeneity of the GABA-AR subunits, most GABA-ARs expressed in the brain consist of two α subunits, two β subunits and one γ subunit; the γ subunit can be replaced by a δ, π, ε, θ. Binding of the neurotransmitter GABA occurs at the interface between the α and β subunits and triggers the opening of the channel, allowing the rapid influx of Cl^- into the cell. BZ binding occurs at the interface between the α (1, 2, 3 or 5) and γ subunits and potentiates GABA-induced Cl^- flux. Modified from Jacob et al., 2008.

1.1.2 Modes of GABA-A receptor activation

Up until relatively recently our understanding of GABA-mediated inhibition has focused on stereotypical synaptic transmission. Known as *phasic* inhibition, this type of point to point action potential driven signalling is seen throughout the CNS and is characterised as a spatially and temporally discreet transmission of a pre-synaptic signal into a post-synaptic response. However, in the last few decades we have begun to appreciate another type of GABA mediated inhibition known as *tonic*. This is spatially and temporally separated from vesicular GABA release and is characterised by a persistent and uninterrupted chloride conductance, reflecting the steady-state activation of GABA-A receptors found outside of synapses. These functionally distinct and diverse modes of neuronal transmission represent another source of heterogeneous GABAergic regulation of neuronal excitability.

1.1.2.1 Phasic inhibition

An action potential at a GABAergic nerve terminal triggers a local calcium influx, which in turn causes pre-synaptic vesicle fusion and the release of thousands of GABA molecules (>1mM) into the synaptic cleft. These diffuse across the synaptic cleft and bind post-synaptic GABA-A receptors clustered on the membrane, causing a near synchronous opening of many ion channels and thus an increase in membrane permeability to chloride and bicarbonate (Mody, De Koninck et al. 1994; Farrant and Nusser 2005). GABA subsequently rapidly diffuses away from the release site over a short time course of ~100μs (Mozrzymas, Zarmowska et al. 2003), or is taken up by GABA transporters (GAT), thus shutting off the channel activation. This describes classical *phasic* inhibition, the defining feature of which is the short duration of the GABA evoked currents and their temporal and spatial association with the vesicular GABA release. These discrete conductance changes are fundamental to information transfer in the brain have important roles in neuronal signal integration and regulation of network oscillations (Freund and Katona 2007; Klausberger and Somogyi 2008), however the acute effect of *phasic* receptor activation depends on the distribution of chloride and bicarbonate ions across the membrane relative to the membrane potential. In the vast majority mature neurons this involves the net influx of anions down their concentration gradient into the cell, and a classic inhibitory post-synaptic current (IPSC) (figure 2a). However, we should note that in some rare cases GABA-A receptor activation can prove

excitatory. For example, when the chloride extruding potassium-chloride transporter KCC2 is absent, such as in early stages of development, the chloride equilibrium potential may become more positive than the resting membrane potential (V_m). In such instances GABA-A receptor activation may depolarise the neuron (Marty and Chavas 2003; Nicoll and Stein 2003; Rivera, Voipio et al. 2005). Moreover, sometimes a hyperpolarising GABA evoked response triggers excitatory conductances leading to 'rebound' spikes (Marty, Chavas et al. 2004).

Another, albeit slower, form of phasic signalling is known as "spillover". In this instance the diffusion of vesicular GABA causes the activation of GABA-A receptors outside the immediate post-synaptic cluster. These might be either beneath the same bouton (Raman, Telgkamp et al. 2004), in pre-synaptic structures, in adjacent synapses (Wei, Zhang et al. 2003) or in the extrasynaptic membrane (Brickley, Revilla et al. 2001). This kind of response typically manifests in the current record as slower and larger averaged IPSC waveforms (figure 2b), and has been observed in many different brain regions including the cerebellum (Brickley, Cull-Candy et al. 1996) and thalamus (Brickley, Cull-Candy et al. 1996; Bright, Renzi et al. 2011).

There are many GABA-A receptor subtypes involved in synaptic/*phasic* transmission, but the most prevalent are α1 β2/3 γ2, which are highly enriched in most GABAergic synapses (Mody, De Koninck et al. 1994). While the α and β subunits can be interchangeable, it seems that γ2 subunit is a necessary for clustering of post-synaptic GABA-A receptors, in a synaptic complexing process which also involves the scaffolding protein gephyrin (Essrich, Lorez et al. 1998). This likely explains why GABA-A receptors containing subunits which substitute γ2, such as δ, are excluded from synapses.

1.1.2.2 Tonic Inhibition

Tonic inhibition, in contrast to *phasic*, is dissociated both temporally and spatially from presynaptic GABA release, and is characterised by a persistent, uninterrupted chloride conductance reflecting the activity of extrasynaptic GABA-A receptors (GABA-AR). Tonic GABA currents were first described in whole-cell voltage-clamp experiments on cerebellar granule neurons (CGNs), where application of a GABA-AR antagonist such as bicuculline or gabazine resulted in a decrease of background noise and of the 'holding current', consistent with a block of persistently open ion channels (Kaneda, Farrant et al. 1995; Brickley, Cull-Candy et al. 1996). Since then, tonic conductances have been described in a large variety of principal neurons and interneurons throughout the nervous system, including the hippocampus, cortex, thalamus and spinal cord (Semyanov, Walker et al. 2004; Farrant and Nusser 2005; Glykys and Mody 2007), and has been shown to alter neuronal excitability both *in vitro* (Brickley, Revilla et al. 2001) and *in vivo* (Chadderton, Margrie et al. 2004).

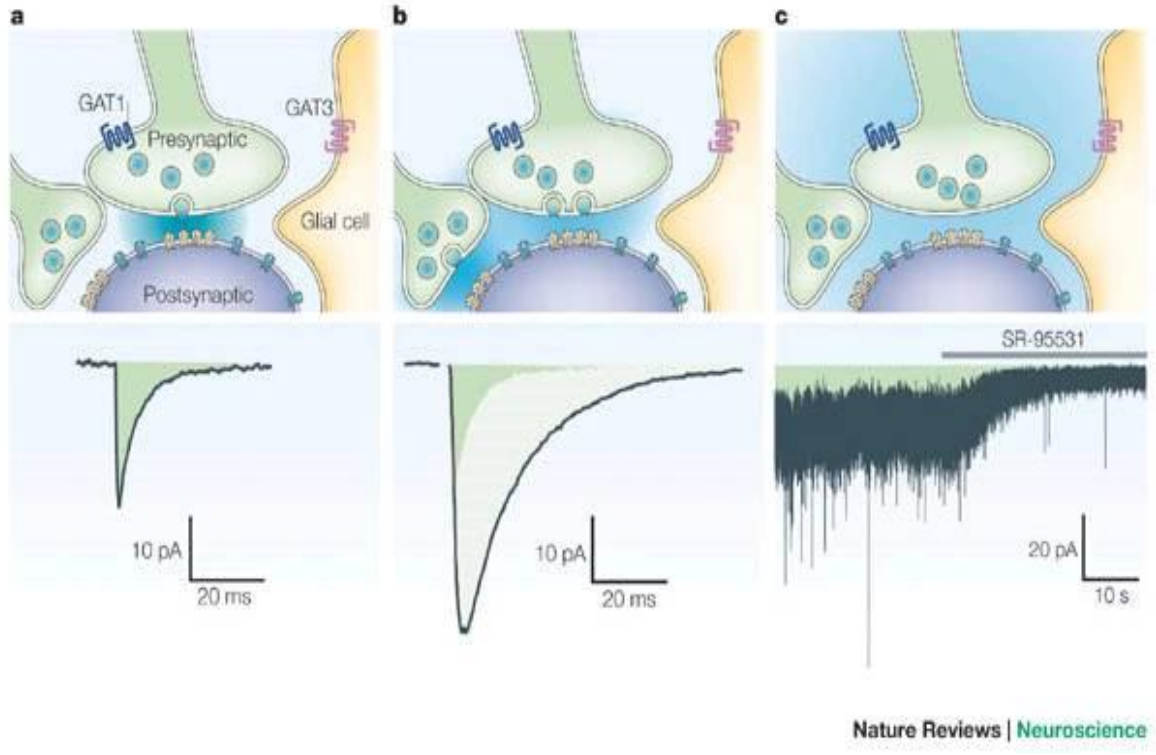

Figure 2. Modes of GABA-A receptor activation. a, Phasic: the release of a single vesicle from a presynaptic terminal activates only those postsynaptic GABA-AR (γ-aminobutyric acid type A receptors) that are clustered in the membrane immediately beneath the release site (yellow). The diffuse blue shading indicates the spread of released GABA. The current record shows an averaged waveform of miniature inhibitory postsynaptic currents (mIPSCs) recorded in the presence of the sodium channel blocker tetrodotoxin. The area beneath the record is shaded to indicate the charge transfer. GAT, GABA transporter. **b,** Action potential-dependent release of multiple vesicles or evoked release from several terminals promotes GABA 'spillover', and activates both synaptic receptors and perisynaptic or extrasynaptic receptors (blue). The current record shows the larger and much slower averaged waveform of IPSCs evoked by electrical stimulation. The area of the mIPSC is superimposed for comparison. **c**, A low concentration of ambient GABA, which persists despite the activity of the neuronal and glial GABA transporters (GAT1 and GAT3), tonically activates high-affinity extrasynaptic receptors. The trace shows the 'noisy' tonic current that results from stochastic opening of these high-affinity GABA-ARs, with superimposed phasic currents (in this case, the synaptic events would be arising at sites not depicted in the schematic diagram). A high concentration (10 μM) of the GABA-AR antagonist gabazine (SR-95531) blocks the phasic IPSCs and tonic channel activity, causing a change in the 'holding' current and a reduction in current variance. The infrequent phasic events that remain in SR-95531 are glutamatergic excitatory postsynaptic currents. The shaded area beneath the current record before SR-95531 application represents the charge carried by tonically active GABA-ARs. The current records are from whole-cell patch-clamp recordings of granule cells in acute cerebellar slices from adult mice. The recordings were made with symmetrical chloride concentrations at a holding voltage of -70mV and a temperature of 25°C. pA, pico amp. Taken form Farant & Nusser 2005.

1.2 Tonic inhibition: a molecular perspective

Tonic inhibition represents a type of paracrine interneuronal communication described as volume transmission. This type of neurotransmission forms a kind of intermediate between fast synaptic signalling and slow endocrine responses, which reflects the involvement of receptors which are found distant from neurotransmitter release sites (0.1 – 100's μm) (Mody 2001). It has become increasingly clear in recent years that the extrasynaptic GABA-A receptors which mediate this type of inhibition are distinct in form and function from their synaptic counterparts, and that they are involved in generating distinct conductances. Consistent with these different functions, synaptic and extrasynaptic receptors have distinct biophysical properties and pharmacology, the elucidation of which has started to provide novel insights into the regulation of neuronal excitability.

1.2.1 Extrasynaptic GABA-A receptors

In contrast to synaptic GABA-A receptors which see fast decaying GABA levels in the mM range, the extrasynaptic receptors which mediate tonic inhibition sense much lower levels of ambient GABA which exist in extracellular space. As such the receptors involved must fulfil a few central criteria, namely: a very high affinity for GABA and an extrasynaptic localisation. To date, two main groups of GABA-A receptors meet these criteria: αβδ- and α5βγ- containing isoforms (figure 3).

1.2.1.1 Extrasynaptic receptor isoforms which mediate tonic inhibition

EM immunogold studies have provided insight into the sub-cellular distribution of particular receptor subtypes. For example, these works have highlighted the clustering of αβγ receptors within synaptic specialisations in a host of brain regions including the cerebellum, hippocampus and neocortex (Nusser, Roberts et al. 1995; Essrich, Lorez et al. 1998; Nusser, Sieghart et al. 1998; Rudolph, Fritschy et al. 1998). This work has also demonstrated the exclusively extrasynaptic location of certain receptors, notably those containing the δ subunit. Gold labelling of cerebellar granule neurons (Nusser, Sieghart et al. 1998) and of granule cells in hippocampus (Wei, Zhang et al. 2003) demonstrate this subunit is excluded from

synapses. In the cerebellum the δ subunits were distant from synaptic junction (>100 μm), while in the hippocampus they were found somewhat closer, in perisynaptic locations around the outside edge of the synapse (~30 μm). The reason for the exclusion of δ-containing receptors from synaptic clusters is likely the lack of γ2. Studies have demonstrated that a γ2 deletion leads to a profound reduction in GABA-A receptor clustering and IPSC frequency, highlighting a critical role for the γ2 subunit in promoting synaptic clustering (Essrich, Fritschy et al. 1998; Luscher, Essrich et al. 1998).

The δ-subunit has been shown to combine preferentially with α6 and β2/3 in the cerebellum (Sieghart, Jechlinger et al. 1998; Sieghart, Poltl et al. 2003), dispersed throughout the dendritic and somatic extrasynaptic membranes (Nusser, Sieghart et al. 1998). Deletion of the α6 or δ subunit genes abrogated the tonic conductance in cerebellum (Brickley, Revilla et al. 2001; Stell, Brickley et al. 2003) and the delayed expression of α6 and δ in post-natal development parallels changes in the levels of tonic inhibition (Brickley, Cull-Candy et al. 1996; Wall and Usowicz 1997). In the forebrain the δ-subunit is thought to preferentially combine with α4 and β2/3 (Sperk, Pirker et al. 2000). Deletion of the δ gene causes a concomitant loss of the α4 subunit (Houser, Peng et al. 2002), and has also been shown to attenuate the levels of tonic inhibition in the hippocampus and thalamus (Porcello, Huntsman et al. 2003; Stell, Brickley et al. 2003). Taken together these results highlight a significant role in α4βδ- and α6βδ-containing GABA-A receptors in mediating tonic inhibition in the forebrain and cerebellum respectively (figure 4).

While δ-containing GABA-A receptors dominate the scene of tonic inhibition, there is another player: α5βγ2, which has been implicated in mediating tonic currents in CA1 and CA3 pyramidal cells (Glykys and Mody 2006; Vogt, Prenosil et al. 2006). Despite the presence of the γ2 subunit, this receptor isoform does not cluster within synapses, and shows a diffuse surface distribution more akin to δ-containing receptors (Fritschy, Brunig et al. 2002). Further, deletion of the α5 attenuates the tonic conductance in cultured hippocampal neurons (Caraiscos, Elliott et al. 2004). Although interestingly, a residual tonic conductance remains in α5 knock-out animals due to an upregulation of δ subunits in these pyramidal cells (Glykys and Mody 2006).

Thus we have our current picture of the GABA-A receptor isoforms that mediate tonic inhibition, which is to say α4/α6 βδ containing receptors and the α5βγ2 isoforms. However, very few receptors isoforms are exclusively synaptic, indeed even the α1β2/3γ2 containing isoform which is highly enriched at synaptic specialisations, are also found in abundance outside synapses (Nusser, Roberts et al. 1995), and yet they do not appear to contribute significantly to tonic conductances. As such it seems likely that distinct differences in receptor properties underlie these distinct functional roles, and therefore in the next sections I will discuss the biophysical properties and pharmacology of GABA-A receptors, with a focus on contrasting γ and the δ-containing subtypes.

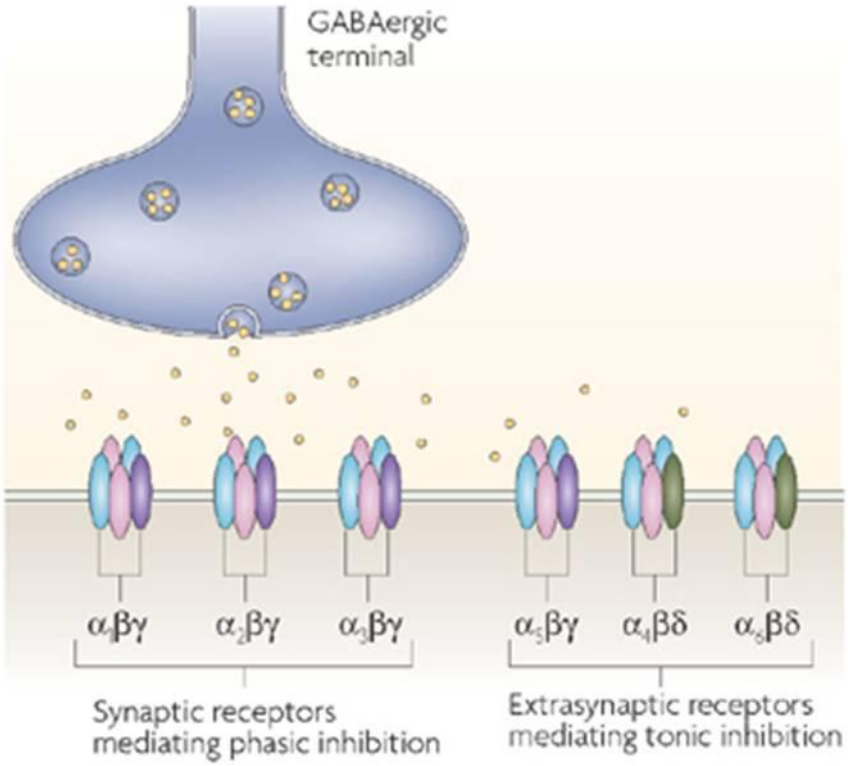

Figure 3. GABA-A receptor neuronal localisation. GABA-ARs composed of α (1–3) subunits together with β and γ subunits are thought to be primarily synaptically localized, whereas α5βγ receptors are located largely at extrasynaptic sites. Both these types of GABA-AR are BZ sensitive. By contrast, receptors composed of α (4 or 6) δ are BZ insensitive and localized at extrasynaptic sites. Taken from Jacob *et al.*, 2008.

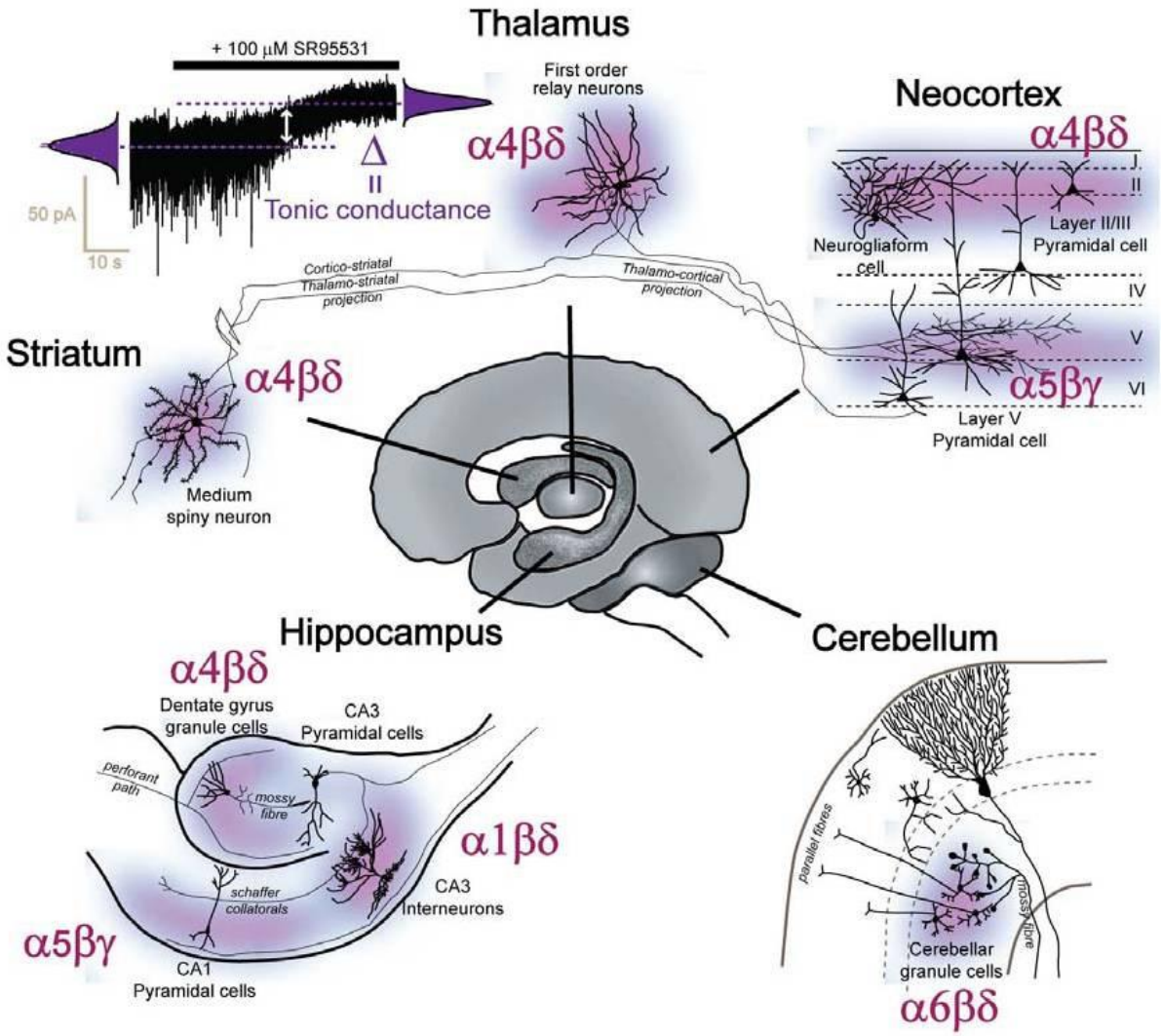

Figure 4. A tonic GABA-A receptor mediated conductance is found in key regions of the adult brain. The trace at the top left shows the recording of the transmembrane ion current in a voltage-clamped adult thalamic relay neuron illustrating how application of the GABA-AR antagonist SR95531 blocks a large fraction of the current. The magnitude of the tonic GABA-AR-mediated conductance is quantified as the difference current (Δ) from Gaussian fits to all-point histograms constructed from the holding current before and after GABA-AR blockade. The other diagrams illustrate key brain regions found to contain a tonic GABA-AR-mediated conductance of the type shown in the top trace. For each brain region, some of the specific neuronal types are indicated that are thought to express the tonic conductance. We have also highlighted the subunit composition of the extrasynaptic GABA-AR populations that are believed to generate the conductance in each neuronal type.

1.2.1.2 Properties of synaptic and extrasynaptic GABA-A receptors

The selective participation of specific GABA-A receptor subtypes in phasic and tonic inhibition undoubtedly relies in part on distinct isoform properties. Consistent with this; compared with synaptic γ-containing, extrasynaptic δ-containing receptors display a higher affinity for GABA, smaller macroscopic current amplitudes, slower desensitization and increased outward rectification (Fisher and MacDonald 1997; Fisher and Macdonald 1997; Belelli, Harrison et al. 2009). These observed macroscopic phenomena reflect unique microscopic properties such as agonist binding and unbinding rates, rate constants of transitions between open and closed states (Farrant and Nusser 2005).

As stated earlier, a key pre-requisite for tonically active extrasynaptic receptors is a high sensitivity for GABA, which enables these receptors to sample the low levels of GABA present in the extracellular space. Accordingly, δ-containing receptors display significantly lower GABA EC_{50}'s than their γ-containing counterparts (Brown, Kerby et al. 2002). Indeed of all the recombinant isoforms examined α6β3δ and α4β3δ had the lowest GABA EC_{50}'s averaging ~300-700nM, while the synaptic α1β3γ had a 10-fold greater EC_{50} at ~3-7μM (Feng and Macdonald 2004; Luddens, Bohme et al. 2004). What these EC_{50} values reflect are both GABA affinity (binding) and the efficacy of the ligand (gating). Interestingly, studies which examined the GABA efficacy of gating found that the inclusion of the δ subunit actually decreases mean open times and the duration of bursts of channel openings some 5-fold (Macdonald and Fisher 1997). Similarly, examination of α4β3δ single channel properties suggest it to displays relatively low efficacy GABA evoked gating (Akk, Bracamontes et al. 2004). Thus this suggests that GABA acts with comparatively very high affinity yet low efficacy at δ-containing receptors.

To determine the contributions of particular receptor subunits to the rapid kinetic properties of GABA-A receptor currents a lot of work has been carried out in recombinant systems. Results from these suggest that activation, deactivation, and desensitization are profoundly affected by the subunits which make up the functional receptor. Activation rates were 2-fold faster in α1β3γ compared with δ-containing receptors, and deactivation rates were slower in γ versus δ (Macdonald and Haas 1999). These rates were also highly dependent on the α

subunit involved, with α1β3δ displaying significantly slower deactivation rates than α6β3δ (Bianchi, Haas et al. 2002).

The desensitization of GABA-A receptors, i.e. entry into long periods of closed non-conducting states in the presence of GABA, is also highly isoform dependent. Much of the dogma surrounding extrasynaptic GABA-A receptors suggests that these receptors are non- or at least minimally desensitizing compared with their synaptic counterparts (Macdonald and Haas 1999; Bianchi, Haas et al. 2002; Wohlfarth, Bianchi et al. 2002), a suggestion that intuitively fits well with their proposed role as an 'always on' receptor capable or responding to GABA 'spillover'. However, this has proved an over-simplification, as in recent years groups studying relevant α4- and α6βδ in recombinant systems have suggested that these receptor subtypes are in fact largely desensitized in the presence of EC_{50} GABA concentrations (Macdonald, Feng et al. 2009; Smart, Mortensen et al. 2010; Bright, Renzi et al. 2011). This implies, and indeed groups have demonstrated, that extrasynaptic δ-containing receptors are not suited to responding to GABA spillover (Bright, Renzi et al. 2011). However, the synchronised stochastic opening of a pool of desensitized extrasynaptic receptors still account for the tonic currents.

Thus synaptic and extrasynaptic receptors have distinct microscopic properties, consistent with their contrasting conditions of activation. Somewhat counter-intuitively, GABA acts with very high affinity but with low efficacy at δ-containing receptors compared with γ-containing, while the single channel conductances are similar (~25-28pS) (Macdonald and Fisher 1997; Farrant, Brickley et al. 1999). The usefulness of such a property is unclear, although we might surmise that the modulation of extrasynaptic receptor gating properties may represent a critical level of regulation of tonic inhibition, which could dramatically increase the range of activity and introduce extra levels of heterogeneity of function. Consistent with this there are a number of processes, such as phosphorylation which can modulate function (Moss and Kittler 2003). And in addition to this there are a number of endogenous and exogenous agents which increase the efficacy of GABA gating.

1.2.1.3 Synaptic and extrasynaptic GABA-A receptor pharmacology

As with biophysical properties, the pharmacology of extrasynaptic δ-containing and synaptic γ-containing GABA-A receptors are distinct. GABA-A receptors are the site of action of a number of clinically relevant drugs, and while we are still trying to tease apart the key players which mediate specific drug actions, it is clear that certain drugs elicit heterogeneous responses from different receptor isoforms. For example, δ-containing receptors display an increased sensitivity to a number of modulators including barbiturates (Feng, Bianchi et al. 2004), zinc (Saxena and Macdonald 1994), certain anaesthetics (Orser, Bai et al. 2001; Jia, Yue et al. 2008), neurosteroids (Belelli and Lambert 2005), ethanol (Biggio, Concas et al. 2007) and the GABA analogue Gaboxadol (4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridine-3-ol). A few of which we will consider in more detail below.

Differential antagonist activity is sometimes observed at synaptic and extrasynaptic GABA-A receptors, partly due to isoform subunit combination, but also due to the differing modes of activation and in the case of competitive antagonists due to the relative affinities for GABA. Experimentally the most widely used GABA-A receptor antagonists are SR-95531(Gabazine), picrotoxin and bicuculline, all of which will block all GABA-A type receptors, although at lower concentrations gabazine (<1μM) proves selective for synaptic inhibition. (Kullmann, Semyanov et al. 2003). Furosemide also shows clear subunit selectivity, as an antagonist which targets α6 containing GABA-ARs specifically it has been used experimentally to selectively block tonic GABA in cerebellar granule neurons (Korpi, Kuner et al. 1995). Finally the divalent cation Zn^{2+}, is a well established negative allosteric inhibitor of binary GABA-A receptor complexes. While 1μM zinc has been reported to effectively curtail GABA responses in αβ receptors, this dose has little effect in ternary conformations (Draguhn, Verdorn et al. 1990; Borghese, Storustovu et al. 2006), thus zinc is often used as a diagnostic in recombinant expression systems to discriminate between binary and ternary isoforms.

One of the clearest examples of GABA-A agonist heterogeneity is the classical benzodiazepine response, which sees a clear potentiation of γ-containing GABA-A receptors containing α1, α2, α3 or α5 while exhibiting no activity at extrasynaptic δ-containing

isoforms (Farrant and Nusser 2005). The structural basis for this selectivity lies in the fact that the benzodiazepine binding site lies in the α/γ interface, thus replacing the γ subunit with a δ eliminates this activity, as does replacing the α with a α4 or α6 subunit (Barnard, Skolnick et al. 1998). Accordingly, benzodiazepines selectively enhance synaptic transmission within brain regions where tonic is mediated by δ-containing receptors (Hamann, Rossi et al. 2002; Nusser and Mody 2002). Consistent with this, where γ-containing receptors are responsible for tonic inhibition, i.e. in CA1 pyramidal neurons, currents are sensitive to benzodiazepine site manipulation (Caraiscos, Elliott et al. 2004).

As with virtually all general anaesthetics, barring xenon and cyclopropane, the intravenous anaesthetic propofol (2,6-di-isopropylphenol) positively modulates GABA-A receptors at clinical concentrations. This mechanism involves a leftward shift in the GABA dose response curve i.e. they increase the apparent affinity of the GABA-A receptor for its endogenous ligand. However, at higher concentrations (~30μM) propofol can also directly activate GABA-A receptors (Franks 2008). These effects have been seen in recombinant γ-containing receptors, where propofol enhanced the GABA evoked Cl^- currents, purportedly by increasing the frequency of channel openings (Belelli, Callachan et al. 1996; Krasowski, OShea et al. 1997), and at native synaptic receptors, in which propofols effects manifest as a prolongation the decay phase of IPSCs (Orser, Wang et al. 1994). Additionally, propofol has been implicated in the modulation of extrasynaptic GABA-A isoforms, with low concentrations shown to enhance α6βδ and α4βδ mediated currents in recombinant expression systems (Orser, Wang et al. 1994; Brown, Kerby et al. 2002; Feng and Macdonald 2004; Wallner, Meera et al. 2009). While in neocortical pyramidal cells and in cultured hippocampal neurons low dose propofol also enhanced the tonic conductances (Orser, Bai et al. 2001; Drasbek, Hoestgaard-Jensen et al. 2007). While propofol likely has numerous effects, and the relative contributions of phasic and tonic inhibition to the *in vivo* actions of propofol remain to be established, these results have led many to speculate that many of the behavioural effects of this drug are mediated by δ-containing receptors.

The GABA mimetic, Gaboxadol (4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridine-3-ol) also known as Gaboxadol, was initially investigated as an analgesic, and anti-anxiety drug, as well as for the treatment of diverse conditions such as Huntington's, epilepsy and schizophrenia.

However studies showed that Gaboxadol acts as a sleep promoter, enhancing slow wave and non-REM sleep and causing sedation (Faulhaber, Steiger et al. 1997; Lancel 1997; Lancel 1999) and therefore it has subsequently been developed as a powerful hypnotic (Wafford and Ebert 2006). At low doses (<1μM) proposed to represent the therapeutic range, Gaboxadol acts preferentially at δ-containing GABA-A receptors, with little no activation of γ-containing receptors (Storustovu and Ebert 2006). This is proposed to be due in part because while Gaboxadol behaves as a partial agonist at αβγ containing receptors it is thought to act as a full-, often termed 'super-agonist' at αβδ receptors (Brown, Kerby et al. 2002). Thus representing one of the few agonists with a preference of extrasynaptic versus synaptic GABA-A subtypes, Gaboxadol has historically proved useful as a diagnostic tool for discriminating between these two receptor subsets.

Another clinically important drug long suspected to act via the GABAergic system is ethanol. While there are a number of proposed molecular targets of alcohol, however at physiological concentration the inhibition of NMDA receptors (Otton, Janssen et al., 2009; Woodward 1999) and the activation of GABA-AR are widely considered to be the major sites. Behavioural evidence shows that alcohol shares many pharmacological effects with prototypical GABA-A receptor agonists such as benzodiazepines (Olsen, Hanchar et al. 2007). Many of the behavioural effects of acute alcohol intoxication have been linked to the enhancement of GABA-A receptor activity, including the rewarding effects that can lead to addiction (Chester and Cunningham 2002), moreover single ethanol exposures can cause long-lasting potentiation of GABAergic synapses *in vivo* (Melis *et al*., 2002), while chronic drinking has been shown to cause GABA-A receptor plasticity that very likely underlies the increases in anxiety and seizure susceptibility that characterise withdrawal (Santhakumar, Hanchar et al. 2006; Biggio, Concas et al. 2007). Further, genetic linkage studies suggest a connection between a number of polymorphisms in different GABA-A receptor genes and alcoholism (Covault, Gelernter et al. 2004; Edenberg, Dick et al. 2004; Pierucci-Lagha, Covault et al. 2005). However, evidence of direct actions of alcohol in the GABAergic system has proved harder to come by, and has often proved controversial. Studies examining native synaptic receptors using ethanol at physiologically relevant doses (<30mM) have yielded contradictory results (Weiner and Valenzuela 2006). While analysis on CA1 pyramidal neurons suggest EtOH increases the frequency of spontaneous IPSCs (Carta,

Ariwodola et al. 2003; Ariwodola and Weiner 2004), as well as enhancing post-synaptic GABA-A receptor mediated IPSCs (Sanna, Talani et al. 2004; Proctor, Diao et al. 2006; Wu, Coultrap et al. 2006), investigations in heterologous expression systems suggest ethanol does not directly activate GABA-A receptor subtypes found at synapses (Aguayo, Peoples et al. 2002). Work carried out on extrasynaptic isoforms has proved even more contentious. Some groups have suggested that extrasynaptic α4βδ and α6βδ receptors expressed in a recombinant system are directly activated by ethanol (3-30mM) (Sundstrom-Poromaa, Smith et al. 2002; Wallner, Hanchar et al. 2003), and studies in native tissue have shown that tonic conductances mediated by δ-containing receptors, e.g. in the hippocampal and cerebellar neurons, are also enhanced by physiologically relevant concentrations of ethanol (Carta, Mameli et al. 2004; Mody, Wei et al. 2004). However, some of these findings proved hard to replicate, and some groups failed to see direct ethanol induced enhancement of these isoforms (Borghese, Storustovu et al. 2006; Borghese and Harris 2007; Sigel, Baur et al. 2009). Thus ethanol's action on extrasynaptic receptors remains contentious, and it remains unclear precisely how alcohol acts within the GABAergic system.

Another interaction which has received much interest, and is better understood, is between GABA-A receptors and neurosteroids. Endogenous neurosteroids such as tetetrahydrodeoxycorticosterone (THDOC) and Allopregnanolone (ALLO) are synthesized in the brain in times of stress and act directly and potently at GABA-ARs to enhance GABA mediated inhibition. This results in sedation, anxiolysis, analgesia and anaesthetic effects and is thought represent an important homeostatic mechanism in the regulation of anxiety and responses to stress (Belelli and Lambert 2005; Biggio, Concas et al. 2007; Belelli, Harrison et al. 2009). Consequently this has been exploited to develop a number of clinically relevant drugs which target the GABAergic system. The effects at GABA-A receptors reflects an allosteric interaction which involves two discrete highly conserved binding sites, one of which is found on the α subunit and mediates the potentiation of GABA currents, and one on the α/β interface which initiates direct activation (Hosie, Wilkins et al. 2006). Neurosteroids have been shown to act directly at synaptic GABA-A receptors where they enhance the charge transfer by prolonging the decay phase of IPSCs (Lambert, Belelli et al. 2003). This effect is seen throughout the brain, although there is a heterogeneity of response seen between regions and neurons, for example, while nanomolar concentrations affect IPSCs in

the hippocampus and cerebellum (Vicini, Losi et al. 2002; Lambert, Harney et al. 2003), the same effects requires micromolar levels in the hypothalamus (Brussaard, Koksma et al. 2003). As with other drugs, this heterogeneity of action likely reflects different GABA-A isoforms involvement, and this is certainly the case when considering extrasynaptic subtypes. Low, physiologically relevant concentrations of THDOC have been shown to robustly enhance δ-mediated tonic inhibition. In fact in mouse cerebellar granule neurons and hippocampal dentate granule cells THDOC potentiated the tonic current without influencing IPSCs (Stell, Brickley et al. 2003; Farrant and Nusser 2005), leading many to suggest that tonic represents their preferred site of action. Consistent with this studies in $\delta^{-/-}$ mice showed an attenuation of behavioural responses to THDOC (Mihalek, Banerjee et al. 1999), and tonic currents were insensitive to THDOC in $\delta^{-/-}$ slice experiments (Stell, Brickley et al. 2003).

1.2.2 Ambient GABA levels

Critical to the levels of tonic inhibition, and as we will go on to propose to the potency of certain clinically relevant drugs, is the concentration of GABA in the extracellular space. This 'ambient' GABA activates extrasynaptic receptors giving rise to the tonic conductance, and the level of this conductance is enhanced when GABA is raised by exogenous GABA application, GABA transporter block, modulation of GABA synthesis or block of GABA catabolism (Nusser and Mody 2002; Glykys and Mody 2006; Klaassen, Glykys et al. 2006). The proposed concentration of ambient GABA comes down to best estimates, but theoretical studies based on the stochiometry of the GABA transporter (Wu, Wang et al. 2007) and microdialysis experiments (Bianchi, Ballini et al. 2003; Nyitrai, Kekesi et al. 2006; Wu, Wang et al. 2007) agree that the range at rest is from 10-400nM, although one study did suggest that this could rise to as high as 800nM in the hippocampus when animals where exploring (Bianchi, Ballini et al. 2003).

The source of this GABA and how it might be regulated has come under much scrutiny, and still remains up for debate. Various proposals have been put forward including action

potential mediated vesicular release (Brickley, Cull-Candy et al. 1996; Bright, Aller et al. 2007), non-vesicular release (Attwell, Barbour et al. 1993), GABA transporter reversal (Richerson, Gaspary et al. 1998; Richerson and Wu 2003) and astroctytic release (Kozlov, Angulo et al. 2006; Lee, Lee et al. 2010). A number of groups have used TTX to block action potential driven GABA release and have shown that the magnitude of the tonic conductance is lowered, although other investigations carried out under similar conditions have suggested that there isn't a relationship between synaptic release and tonic levels, the reason for this discrepancy remains unclear (Attwell, Barbour et al. 1993; Brickley, Revilla et al. 2001; Glykys and Mody 2007). There is also strong evidence that GABA transporters (GAT) can reverse under certain conditions, and provide a source rather than a sink for GABA. Moreover, that this doesn't just happen in pathological conditions but has a dynamic role under normal conditions in modulating tonic inhibition in response to neuronal activity (Richerson and Wu 2003; Richerson, Wu et al. 2003; Wu, Wang et al. 2007). There is also evidence for a dynamically regulated astrocytic source of GABA. A recent report from Lee et al (2010) suggests that GABA permeates through the bestrotrophin-1 (Best-1) anion channel in astrocytes in the cerebellum and enhances tonic inhibition in adjacent granule cells. Any and all of these mechanisms may represent source of ambient GABA regulation in the brain, understanding this further will not only provide insights into how neuronal excitability is regulated but will also highlight targets for therapeutic drugs modulation of tonic inhibition.

1.3 Tonic inhibition: roles and modulation

The contrasting modes of phasic and tonic inhibition imply they are involved in profoundly different levels of control over neuronal networks. Whereas phasic inhibition reflects the activation of a relatively small number of GABA-A receptors giving rise to spatially and temporally discrete changes in conductance, tonic inhibition reflect s a temporally dispersed activation of receptors distributed across the neuronal surface. The effects of these different types of inputs on neuronal excitability and the adaptive significance of modulating tonic inhibition are only now being explored, and clues are emerging as to the significance of this phenomenon.

1.3.1 The role of tonic conductance in regulating neuronal excitability

The functional consequences of phasic inhibition at individual neurons have been explored extensively, and we have developed an understanding of how IPSCs affect neuronal excitability and contribute to information processing. Namely, IPSCs transiently inhibit neurons providing 10-100ms unitary conductance changes. This provides an inhibitory shunt to incoming excitatory inputs and can thus set precise temporal windows for synaptic integration (Scanziani and Pouille 2001; Semyanov, Walker et al. 2004). The persistent chloride conductance which characterises tonic inhibition has perhaps been explored less extensively, but recent studies have provided information on the effect this phenomenon has on neuronal excitability and computations. Simply put a chloride leak represents a steady-state increase in the membrane input conductance. This provides a shunt, decreasing the magnitude and duration of the voltage response (EPSP) to a given current (EPSC), and increasing the reduction of this voltage with distance. Thus increased tonic inhibition narrows the spatial and temporal window over which synaptic integration can occur (Kullmann, Semyanov et al. 2003; Silver and Mitchell 2003), decreasing the likelihood that an action potential will occur. Consistent with this, in *in vitro* experiments the block extrasynaptic receptors and removal of tonic inhibition in cerebellar granule neurons increased the size and duration of measured EPSPs in response to a given current injection (Brickley, Revilla et al. 2001). Subsequently Chadderton *et al*., (2004) carried out similar experiments *in vivo.* This group exploited sensory evoked excitatory mossy fibre inputs into

cerebellar neurons and explored the impact of altering tonic inhibition on firing rates. Consistent with the findings *in vitro* they discovered that the tonic conductance attenuated excitatory voltage responses and sensory evoked spike bursts increased in frequency when extrasynaptic tonically active GABA-A receptors were blocked, highlighting the dampening effect of tonic inhibition *in vivo*.

This work also hinted at the potential for tonic inhibition to influence neuronal computations, as its modulation profoundly altered firing patterns (Chadderton, Margrie et al. 2004). The processing of rate coded information in neurons relies on the relationship between the excitation input and the output firing rate (I-O relationship), which in turn relies heavily on the electrophysiological properties of the membranes. The I-O relationship represents neuronal computation at its most basic level, and can be modified by a change in offset (threshold) or a change in gain (slope), with distinct effects on signal integration and processing. A change in offset will affect the input detection threshold, while an increase in gain makes a neuron more sensitive to changes in excitatory inputs (Silver and Mitchell 2003; Pavlov, Savtchenko et al. 2009). Whether tonic inhibition has the power to affect these kinds of computations has primarily been investigated in CGNs, in part because they display a powerful tonic conductance, but also because as a small cell with minimal processes this neuron is considered as a single electrical compartment with uniform properties.

A number of studies investigated the neuronal output in relation to step injections of constant conductances, and showed that blocking tonic inhibition in CGNs reduced the current injection needed to generate an action potential, thus demonstrating that tonic inhibition can offset the firing threshold of neurons in the cerebellum (Brickley, Cull-Candy et al. 1996; Attwell, Hamann et al. 2002). This is equivalent to a subtractive operation, where the same level of inhibition is evident at all levels of excitation, reflecting a rightward shift in the input-output relationship along the x-axis (see figure 5). Further, and interestingly, when the input excitation was mediated not by step injections but by trains of synaptic conductances, tonic inhibition served to change the overall gain of the input-output relationship (Silver and Mitchell 2003). By using dynamic current clamp to input random synaptic waveforms Mitchell et al illustrated that tonic inhibition can not only elicit a rightward shift in the input-output relationship but can also modulate the slope, what is equivalent to a divisive

mathematical operation (figure 5). This powerful scaling operation means tonic inhibition can attenuate the level of threshold excitation as well as the neuronal sensitivity to increases in input frequency, and suggests that any modulation of tonic levels will have profound effects on baseline excitability and input processing. It should however be noted that this tonic inhibition-mediated gain and offset modulation observed in CGNs doesn't necessarily represent a general role for this form of inhibition, as a recent study in hippocampal pyramidal cells has shown that tonic mediated by α5βγ receptors affect offset independently of gain (Pavlov, Savtchenko et al. 2009). Thus the precise effect of tonic on neuronal excitability may have to be considered on a cell by cell basis.

As well as impacting neuronal excitability at the level of individual cells, tonic inhibition should be considered in the context of neuronal networks. For example, within the cerebellar granule cell layer where excitatory mossy fibre inputs synapse at both granule cells and Golgi interneurons, a shift in the level of tonic inhibition can have complex consequences. An increase in Golgi firing secondary to mossy fibre drive, will increase the level of tonic inhibition at granule cells secondary to increases in ambient GABA. This will in turn scale down the granule cell response to the excitatory mossy fibre inputs, and maintain sparse granule cell activation even when levels of excitatory drive are high. Such a mechanism allows granule cells to maintain firing within the operational range without saturating, and maintain sparse coding onto Purkinje cells, a critical feature of the classical theory cerebellar function (Semyanov, Walker et al. 2004). There are further insights to be gleaned from tonics impact on networks within the hippocampus, where tonic inhibition within interneurons contributes to modulating network dynamics (Freund 2003). Recently it has been demonstrated that increased tonic inhibition in hippocampal CA3 interneurons attenuated gamma oscillation frequencies in that region, highlighting a broader role of tonic inhibition in regulating rhythm generation (Mann and Mody 2010). Thus we are beginning to understand the power and complexity of the effect of tonic inhibition on neuronal excitability. Amongst our next challenges will be to try to understand the roles of these mechanisms within health and disease.

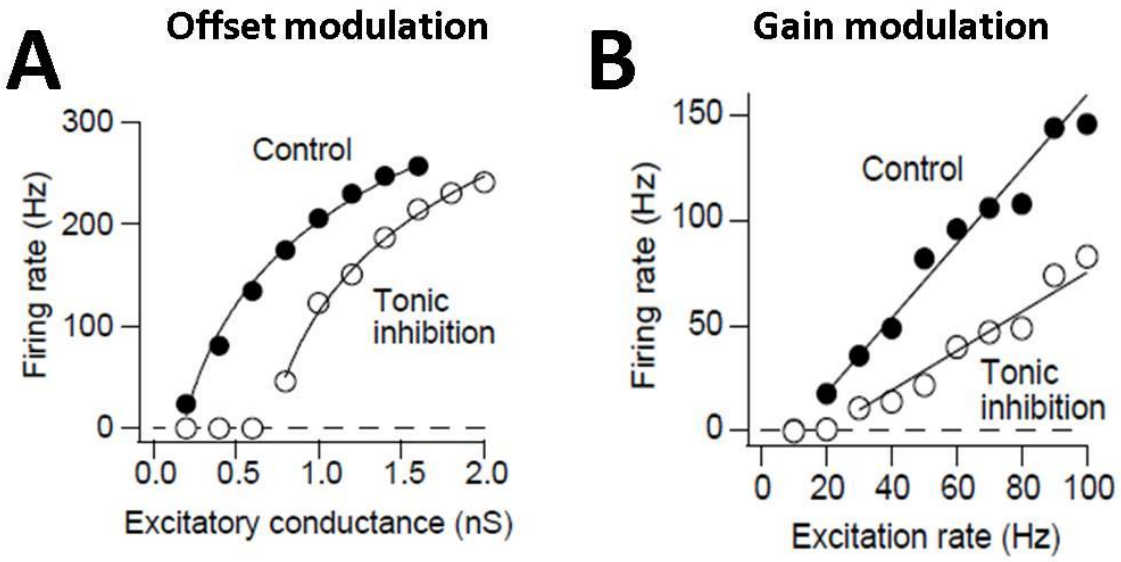

Figure 5. Mathematical operations performed by tonic inhibition. **A,** Relationship between output firing frequency and excitatory conductance amplitude under control conditions and in the presence of tonic inhibition. Tonic inhibition performed a subtractive operation on the input–output relationship under these conditions. The shift to left representing a decrease in firing threshold. **B,** Relationship between output frequency and excitation frequency under control conditions and in the presence of tonic inhibition. In contrast to (A), the gain of the relationship at each output frequency was reduced by tonic inhibition. Tonic inhibition therefore performed a multiplicative scaling operation on the input–output relationship under conditions that mimic rate-coded inputs in vivo. Taken from Semyanov et al., 2004.

1.3.2 Tonic inhibition in health and disease

Given that extrasynaptic δ-containing GABA-A receptor mediated tonic currents make a major contribution to the total inhibitory charge in many cell types (Brickley, Cull-Candy et al. 1996; Nusser and Mody 2002), and that levels of tonic conductance are dynamic, it's perhaps unsurprising that this mechanism has been implicated in a number of fundamental neurological processes. Among these include transitions between sleep states (Wafford and Ebert 2006) synaptic plasticity (Caraiscos, Newell et al. 2004) and memory formation (Dawson, Maubach et al. 2006). The enhancement of tonic conductances mediated by α4βδs in thalamic relay neurons have been shown to promote burst firing (Cope, Hughes et al. 2005), a pattern which has been implicated in instigating sleep relevant corticothalamic oscillations and transitions between sleep states (Wafford and Ebert 2006). Consistent with this it is thought that the actions of the powerful hypnotic Gaboxadol, which works to prolong sleep by enhancing periods of slow-wave and non-REM sleep (Faulhaber, Steiger et al. 1997), are mediated specifically via extrasynaptic δ-containing receptors (Brown, Kerby et al. 2002). Indeed, when δ are absent the anaesthetic potency of this drug is abrogated (Boehm, Ponomarev et al. 2006). Further, anaesthesia, which is often thought of as a sleep like state and shares a lot of molecular targets with sleep (Franks 2008), can be induced through tonic receptor activation via modified ambient GABA levels (Katayama, Irifune et al. 2007).

Extrasynaptic GABA-A receptor activation has also been implicated in learning and memory. It has long been appreciated that GABAergic inhibition acts to retard long-term potentiation (LTP), a mode of neuronal synaptic plasticity which underlies the processes of learning and memory (Wigstrom and Gustafsson 1983; Lu, Mansuy et al., 2000). Studies which block tonic receptors specifically have provided insight into their specific role. For example, the attenuation of tonic currents mediated by α5βγ receptors in hippocampal regions led to enhanced cognitive performance in rats. Researchers used an inverse agonist selective for α5 containing receptors known as L-655,708, and the animals demonstrated enhanced learning in the Morris water maze test, a hippocampal dependent memory task (Chambers, Atack et al. 2002; Chambers, Atack et al. 2003). Moreover, α5-null mutant mice also exhibited improved learning in studies using similar paradigms (Collinson, Kuenzi et al. 2002).

Further insights into the importance and roles of tonic inhibition in normal physiology come from observing the sequelae when levels are dysregulated, and altered levels of tonic inhibition have been observed in a number of different pathologies. Perhaps the most intuitively appropriate of these is epilepsy. Given the importance of maintaining appropriate levels of tonic inhibition in the control of network behaviour (Vida, Bartos et al. 2006; Mann and Mody 2010) we might expect that a break down in this would give rise to the kind of network hyper-excitability that characterises epileptic seizures. And this appears to be the case. Altered levels of tonic inhibition in the thalamus has been reported in a number of rodent models of genetic absence epilepsy (Cope, Di Giovanni et al. 2009), and several other mouse models of epilepsy have been linked to changes in δ receptor expression or function in the hippocampus (Spigelman, Li et al. 2002; Peng, Huang et al. 2004; Zhang, Wei et al. 2007). While in human epilepsies too, genetic linkage studies have implicated mutations in the δ subunit gene (Dibbens, Feng et al. 2004; Mulley, Scheffer et al. 2005).

Tonic inhibition may also have a role in the progression of certain stress and psychiatric disorders. Particularly strongly supported is the suggestion that a change in tonic levels underlie the anxiety which characterises premenstrual dysphoric disorder (PMDD). This is thought to involve the dysregulation of neurosteroid modulation of extrasynaptic GABA-A receptors, reflecting a discrepancy between δ-containing receptor number and circulating neurosteroids observed post partum (Smith, Gong et al. 1998; Maguire, Stell et al. 2005; Maguire and Mody 2008). Studies in mice showed that this dysregulation manifests as decrease in tonic levels, and that if the recovery from this is delayed the animals showed severe depressive like symptoms, while conversely symptoms could be ameliorated by administering a drug which targets δ-containing receptor specifically (i.e., Gaboxadol) (Maguire and Mody 2008). Stress, anxiety and mood disorders have also been more circumstantially linked to tonic inhibition; forced social isolation in rats led to enhanced tonic inhibition in the hippocampus reflecting an upregulation of the δ-subunit gene (Serra, Mostallino et al. 2006), and there is a link between the δ gene (GABRD) and childhood onset mood disorders (Feng, Kapornai et al. 2010). Further, alleles of the α5 gene have been associated with both major depression and schizophrenia (Kato 2007; Maldonado-Aviles, Curley et al. 2009).

1.3.3 The modulation of tonic inhibition by clinically relevant drugs

The role of tonic inhibition in the processes and pathologies described above make it a very attractive drug target. Indeed, already there are a number of clinically important drugs which elicit their actions in part or solely by altering the levels of tonic inhibition, and a number more in the developmental pipeline. When trying to alter the levels of tonic there are two strategies we can employ. Firstly, we can alter the level of ambient GABA which pervades the extracellular space, either by blocking GABA uptake, blocking catabolism of GABA or by increasing GABA synthesis or release. In this way more extrasynaptic receptors will be GABA bound and activated, giving rise to a greater chloride flux. Secondly, we can target the extrasynaptic receptors more directly with compounds which can bind and directly enhance receptor activity, either allosterically or by direct gating.

In terms of the former strategy there are already a number of prescribed drugs available which increase the concentration of extracellular GABA. Abrogated GABA uptake at the by GABA transporters (GAT1-3) has been shown to increase ambient GABA and enhance tonic inhibition (Chiu, Brickley et al. 2005). Drugs such as tiagabine can exploit this by selectively blocking GAT-1 (Borden, Dhar et al. 1994; Stahl 2004), and this is widely used as an anti-convulsant, anxiolytic and neuropathic analgesic (Pollack, Van Ameringen et al. 2005; Pollack, Roy-Byrne et al. 2005). Gabapentin on the other hand is thought to raise ambient GABA by modulating glutamic acid decarboxylase and thus increasing GABA synthesis (Taylor 1997), and this drug is used in the treatment of a range of disorders including depression, epilepsy and neuropathic pain. While another important anti-epileptic: vigabatrin, blocks GABA breakdown through the inhibition of GABA transaminase (Gidal, Privitera et al. 1999), and therefore similarly increases the available pool of GABA.

There are also a number of available drugs which elicit a change in tonic inhibition by directly modulating extrasynaptic receptor activity. Among these include a number of neurosteroid like drugs. Neurosteroids are key endogenous regulators of extrasynaptic GABA-A receptor function, acting as a powerful allosteric modulator and causing the selective enhancement of the tonic conductance. At low concentrations neurosteroids bind

GABA-A receptors and modulate enhance GABA evoked conductances, while at higher levels they can directly activate the channels (Lambert, Belelli et al. 2003) (for further detail on neurosteroid pharmacology at δ-containing GABA-A receptors see section 1.2.1.3). This gives rise to behavioural effects including anxiolysis, analgesia, anaesthesia and anti-convulsion, and has thus been exploited to develop drugs to target a number of disorders including epilepsy and anxiety as well as induce general anaesthesia (Belelli and Lambert 2005). One such is Alfaxalone, originally used as an anaesthetic and sedative in human patients (Stewart, Dobb et al. 1983; Winter, Nadeson et al. 2003) it has since been withdrawn, but is still widely used as a veterinary anaesthetic. Another is Ganaxolone. Acting similarly via extrasynaptic receptors ganaxalone has shown beneficial effects in pharmacoresistant epileptic patients, and being well tolerated in clinical trials is expected to enter the market soon for the treatment of catamenial epilepsy (Biagini, Panuccio et al. 2010). Propofol is a widely used intravenous anaesthetic for the induction and maintenance of general anaesthesia, and while it has a complex pharmacological profile it too is known to allosterically modulate extrasynaptic GABA-A receptors and tonic inhibition (see section 1.2.1.3). Another drug, Gaboxadol, is a GABA analogue and orthosteric agonist which acts specifically at δ-containing GABA-A receptors. Originally trialled as a powerful hypnotic, Gaboxadol was since removed from Phase III due to a poor risk-to-benefit ratio (Saul S, 2007, in press). There are also drugs which can block tonic inhibition, although typically selectivity has been a problem. However, a range benzodiazepine inverse agonists have shown selectivity for α5 containing receptors, and therefore for tonic inhibition in CA1 hippocampal and layer 5 neocortical regions. These include L-655, 708, α5IA and RO-493851, all of which have been shown to enhance learning, memory or cognition (Chambers, Atack et al. 2004; Dawson, Maubach et al. 2006; Ballard, Knoflach et al. 2009). However, due to anxiogenic and likely proconvulsant effects these drugs are currently contraindicated in human patients (Navarro, Buron et al. 2002). These drugs represent the vanguard in what will likely be a long campaign to find means of modulating tonic inhibition in clinical settings. A number conditions could very likely benefit from tonic enhancement or attenuation including stress and psychiatric disorders, sleep disorders, age related memory and learning deficits a number of epilepsies. As such it seems inevitable that researchers and drugs companies will continue to exploit these therapeutic strategies.

1.4 Rationale for current study

The low levels of ambient GABA which persists in the extracellular space surrounding all neurons can activate high affinity extrasynaptic GABA-A receptors expressed in a number of brain regions. This gives rise to an uninterrupted 'tonic' conductance which has profound effects on neuronal and network excitability, and has been implicated in a number of fundamental neuronal processes and pathologies. It is therefore unsurprising that the properties and pharmacology of the receptors involved have borne some scrutiny in the two decades since their discovery.

For the most part a specific subset of GABA-A receptors containing the δ-subunit (αβδ) are responsible for mediating tonic inhibition. These receptors are distinctly suited to this role due to their exclusively extrasynaptic location and some unique biophysical properties, paramount amongst which is a very high affinity for GABA. The functional properties of these receptors have been investigated in some detail over the years; however for the most part studies in recombinant systems have looked at receptor responses to rapidly applied high concentrations of GABA, a condition which may mimic synaptic receptor activation but bears little resemblance to the slow diffusion of GABA through the extrasynaptic space. With this research project we sought to examine δ-containing GABA-A receptors under physiologically relevant steady-state GABA conditions, both in a recombinant system and in native tissue. Another issue with past research in this area in that the δ-subunit has proved difficult to express, and as αβ will form functional binary receptors these could easily have been mistaken for αβδ's in past literature. Indeed this may represent the reason for some contradictory and often confounding results. To ensure that the δ-subunit was incorporated into functional GABA-A receptors, we have used a pH-sensitive GFP tag for visualization of δ surface expression (see section 3.1), and zinc application as a functional assay exploiting the differential sensitivity of αβ and αβδ containing GABA-ARs.

The concentration of GABA in the extracellular space determines the level of extrasynaptic receptor occupancy, and therefore contributes to the amplitude of tonic conductance. Crucially, this level fluctuates, with dramatic effects on neuronal excitability. The other main

variable in determining the amplitude of tonic inhibition is the extent of receptor modulation by compounds such as neurosteroids which can enhance receptor activity. While the effects of these variables have been examined independently in past research, they have as yet not been examined in concert. Thus as part of this set of experiments we have examined the relationship between the level of GABA occupancy and the potency of both endogenous modulators and clinically relevant drugs which target δ-containing receptors.

We also wished to find ways to study some of these interactions within a broader cellular context as well as in *in vivo* settings. One such interaction we wished to examine is the neurosteroid effects at GABA-ARs. Thus we have worked to develop viruses which can deliver neurosteroid insensitive GABA-A receptor mutants into neurons *in viv*o, so that we might examine its pharmacology in an intact system and try to tease apart the involvement of specific GABA-A receptor sub-populations in mediating neurosteroid effects.

In summary we have sought to investigate the properties and pharmacology of extrasynaptic αβδ-containing properties under physiologically relevant conditions, by combining studies on recombinant receptors as well as native receptor subsets. We also sought to test a specific thesis: that fluctuating levels of ambient GABA may affect the ability of certain drugs to modulate tonic conductances. And finally we have developed viral techniques with a view to investigating the effects of these interactions *in vivo*. In this way we hope to gain fundamental insights into how these receptors function and how they are modulated, and in turn gain a greater understanding of the actions of certain clinically relevant drugs.

2 Materials and Methods

2.1 Human embryonic kidney (HEK) cell culture

Recombinant GABA-AR expression and subsequent electrophysiology experiments were carried out using a modified Human Embryonic Kidney Cells (HEK293) cell line designated tSA-201 (DuBridge, Tang et al. 1987) (European Collection of Cell Cultures (ECACC) catalogue no. 96121229). This cell line has been transformed with the T antigen from the simian virus 40 (SV40) which acts as a transcription factor on plasmid DNA containing an SV40 promoter to facilitate high output episomal DNA replication. Cells were maintained at 37°C and 5% CO_2 in humidified incubators (Hereus, Germany). Sterility was maintained with standard aseptic techniques used throughout. Lab coats and latex gloves were worn at all times, and hands were sprayed with 70% ethanol when using incubators. Further, incubators where cleaned regularly with disinfectant (Virkon, VWR). All procedures were carried inside a laminar flow hood and when using the hood: hands, surfaces and any other items introduced were sprayed with 70% ethanol to maintain sterility.

HEK cells were grown as a monolayer in 25 or 75cm^2 sterile tissue culture flasks in a supplemented DMEM culture medium (see 2.1.3 for recipe). These were routinely passaged when their density reached approximately 90% confluency, which meant cells were typically passaged twice per week. Briefly the procedure is as follows: culture media was poured off and cells were dissociated from the bottom of the flask by adding 3ml of trypsin-EDTA (Sigma) and incubating for 10 mins at 37°C and 5% CO_2 in humidified incubators. Cells were fully dislodged mechanically, and 3ml of culture media added to neutralise the trypsin. The cell suspension was then transferred to a 15ml falcon tube and spun at 100g (Thermo Electron IEC CL31R multispeed) for 3mins. Supernatant was discarded and the cell pellet resuspended in 5ml of media. This suspension was then further diluted 1:9 to 1:15 in a series of three 25 cm^2 flasks, and incubated under standard conditions. Remaining cells were plated for use in electrophysiology experiments or cryopreservation.

2.1.1 Cryopreservation and reanimation of cells

After around 30-40 passages the HEK cell viability for electrophysiology experiments deteriorated, with "older" cells exhibiting decreased transfection efficiencies and fragility. Thus frozen stocks of a low passage were maintained and reanimated intermittently. To freeze stocks, cells were pelleted as described above and resuspended in cryopreservation media at a density equal to or greater than 10^6 cell/ml. The cells were then aliquoted into 1ml cryovials (Nunc, Denmark) and stored in liquid nitrogen. For reanimation cells were rapidly thawed in a 37°C water-bath before being transferred to a 15ml falcon containing 9ml culture medium. The suspension was then spun at a 100g for 3mins (Thermo Electron IEC CL31R multispeed) and the supernatant was poured off. Finally the pellet resuspended in 5ml of culture medium and this was then added to a $25cm^2$ sterile tissue culture flasks and maintained under standard conditions.

2.1.2 Cell plating for experimentation

For electrophysiology, HEK cells were placed on glass coverslips within 4-well culture plates. Prior to use cover slips were sonicated for 30 mins and stored in ethanol to decontaminate. Subsequently these were flame sterilised and coated with poly-D-lysine (PDL,Sigma) to allow for cell adhesion. PDL was applied as follows: 1mg/ml solution was added to each well using a Pasteur pipette and incubated for 30mins. Excess PDL was then removed and the plates were sterilized by exposure to ultra-violet light for 1-2 hours. The HEK cell suspension from continuous culture (described above) was diluted 1:20 to 1:30 depending on required density. Of this suspension 0.5ml/well was added to the 4 well plates (Nunc) containing 13mm round glass coverslips coated with poly-D-lysine (PDL). Cells were then incubated overnight under standard conditions before being transfected.

2.1.3 Solutions for cell culture

All solutions were maintained under strictly sterile conditions and stored at 4°C. Ingredients were obtained from Sigma-Aldrich.

HEK Cell growth medium (tSA-201)

- 425 ml Dulbecco's modified Eagle's medium (DMEM)

- 50 ml heat-inactivated foetal bovine serum (HIFBS)
- 5 ml penicillin/streptomycin (10000 U/ml penicillin, 10 mg/ml streptomycin)
- 10 ml non-essential amino acids (NEAA)
- 10 ml 200mM L-Glutamine

Cryopreservation media

- 90% HIFBS
- 10% DMSO

Phosphate buffered saline (PBS)

- 137 mM NaCl
- 2.7 mM KCl
- 4.3 mM Na_2HPO_4
- 1.47 mM KH_2PO_4

2.2 Calcium phosphate transient transfection

HEK293 cells were transiently transfected with cDNA encoding GABA-AR subunits (and GFP where appropriate) to facilitate the recombinant cell surface expression of various GABA-AR subtypes (for list of plasmids used see table 1). Briefly: 100ng/well of each appropriate plasmid (e.g. α6, β2 and δ_{SEP}) and 5.6 µl/well of $CaCl_2$ (2.5M stock) (280mM final $conc^n$) was added to an eppenndorf (A) and made up to 25 µl/well with MilliQ water. Into a separate eppendorf (B): 0.45 µl/well phosphate buffer (100mM Na_2HPO_4 stock)(900µM final $conc^n$) and 25 µl/well phosphate free HEPES buffered saline was added. Subsequently the contents of eppendorf B were added to the contents of eppendorf A, mixed gently and incubated for 10mins at room temperature (allowing $CaPO_4$/DNA precipitate to

form). 50 µl of the transfection reagent was added to each well, drop wise, and plates were incubated for 8 hrs in a humidified incubator at 3% CO_2 and 37 °C to allow for optimal transfection efficiency (Chen & Okayama 1987). Post incubation the media was decanted off and the cells were washed twice with phosphate buffered saline (PBS). Fresh culture medium was then added and the cells incubated (5% CO_2 and 37°C) overnight prior to use in electrophysiology experiments.

2.2.1 Transfection solutions

Transfection solutions were filter sterilized using a 0.22 µm syringe filter, and subsequently maintained under sterile conditions at 4°C. Ingredients were obtained from Sigma-Aldrich. Solutions were made in bulk and aliquoted into 50 ml falcons and stored long-term at -20°C.

HEPES buffered saline

- 280mM NaCl
- 50mM HEPES
- pH to 6.95 with 1M NaOH

Phosphate buffer

- 100mM Na_2HPO_4

$CaCl_2$ 2.5M

2.3 Routine molecular biology

Plasmid DNA encoding GABA-A receptor subunits were kindly provided by Dr. Alastair Hosie (Imperial College London) and included: $\alpha_{1\text{-}6}$, β_2, β_3 (mouse), δ_{SEP} (rat) and γ_{2S} (human) (see table 1). These genes were subcloned into either pRK5 or pcDNA 3.1 vectors (see figures 6 & 7). These plasmid backbones confer ampicillin resistance for selection of bacterial transformants, and contain a cytomegalovirus (CMV) promoter which facilitates high gene expression in mammalian systems.

Further the GABA-AR subunit δ_{SEP} was modified to include GFP tag known as a super-ecliptic pHlourin (SEP). This tag was produced by the semi-rational mutagenesis of a number of GFP residues leading to a pH sensitive mutant in which fluorescence is quenched below physiological pH (Ashby, Ibaraki et al. 2004). Thus it exploits the difference in pH at the plasma membrane, endoplasmic reticulum and Golgi apparatus (pH 7) from the transport vesicles (pH5.5), and allows us to visualise and distinguish receptor subunits on the surface of a cell from those within. As such SEP fluorescence showed we had δ expressed on the extracellular surface, presumably forming part of a functional GABA-AR complex.

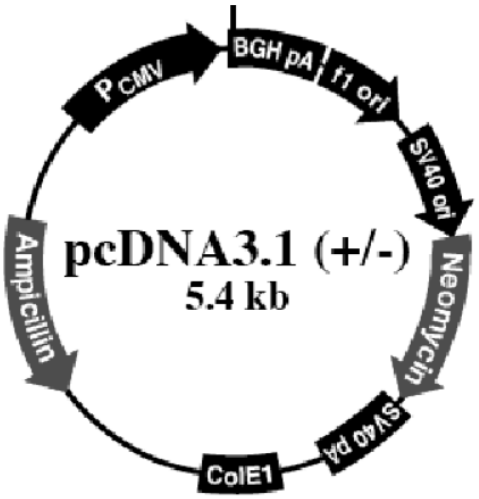

Figure 6. pcDNA 3.1 vector. The pcDNA 3.1 vector is designed for high-level, constitutive expression in a variety of mammalian cell lines. The vector offers the following features: Cytomegalovirus (CMV) enhancer-promoter for high-level expression, large multiple cloning site, bovine Growth Hormone (BGH) polyadenylation signal and transcription termination sequence for enhanced mRNA stability, SV40 origin for episomal replication and simple vector rescue in cell lines expressing the large T antigen (i.e., COS-1 and COS-7), ampicillin resistance gene and pUC origin for selection and maintenance in E. Coli, geneticin resistance marker for stable selection in mammalian cells.

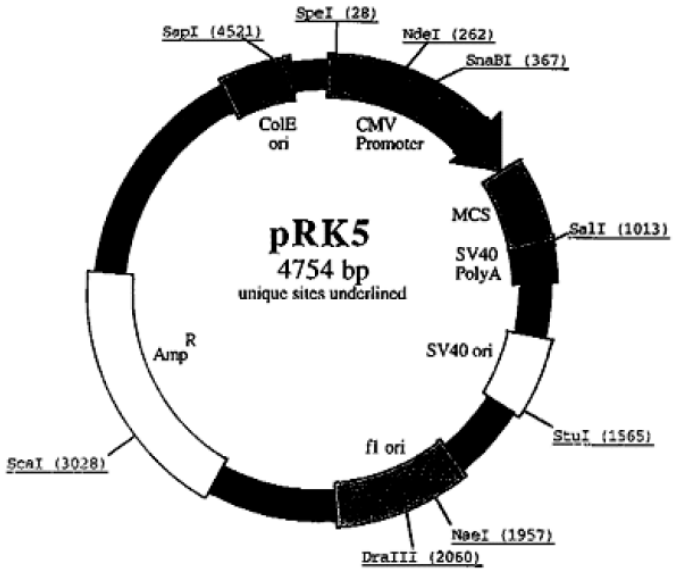

Figure 7. pRK5 vector. The pRK-5 vector contains a powerful cytomegalovirus promoter and is designed for high level expression of cloned genes in cultured mammalian cells. The pRK-5 vector offers the following features: powerful promoter/enhancer domain from the major immediate-early region of the human cytomegalovirus, a multiple cloning region (MCS), SV40 polyadenylation signals for RNA processing in mammalian cells, SV40 origin for episomal plasmid amplification in COS cells, bacteriophage f1 origin of replication for production of single-stranded plasmid DNA, ampicillin-resistant (AmpR) gene for amplification in *E. coli* bacterial strains.

2.3.1 DNA plasmid preparation

To replenish cDNA stocks chemically competent *Escherichia coli* (Stratagene) were used to amplify the plasmids. Bacterial cells were first transformed by heat shock as follows: 2µl of plasmid DNA was added to 50µl of competent cells and incubated on ice for 30mins. Next the sample was placed in a 42°C water bath for 30s before being placed directly on ice for a further 2mins (*heat-shock*). 200µl of super optimal broth was then added into the mix and the cells incubated at 37°C for 1hr. Subsequently cells were streaked onto agar plates containing ampicillin (50µg/ml) to select for transformants, and incubated at 37°C for 12-16 hours. Following incubation a few bacterial colonies were picked with a flame sterilized tungsten wire loop and used to inoculate 5-100ml of Luri-Bertani broth (LB, Sigma) also containing

50μg/ml ampicillin. Inoculated cultures were incubated overnight at 37°C on a shaker (225rpm). The following day cultures were spun down and pellets treated for DNA extraction and purification using Qiagen mini, midi or maxi prep kits according to the respective manuals. DNA was eluted with standard elution buffer (Qiagen) and aliquots stored at -20°C, while working stocks were maintained at 4°C.

GABA-AR Subunit	Gene name	Species	Genebank accession number	Vector Backbone
α1	GABRA1	*Homo Sapiens*	NM_000806.5	pcDNA 3.1
α2	GABRA2	*Mus Musculus*	NM_008066.3	pRK5
α4	GABRA4	*Mus Musculus*	NM_010251.2	pRK5
α6	GABRA6	*Mus Musculus*	NM_001099641.1	pRK5
β2	GABRB2	*Mus Musculus*	NM_008070.3	pRK5
β3	GABRB3	*Mus Musculus*	NM_008071	pRK5
γ2s	GABRG2	*Homo Sapiens*	NM_000816.3	pcDNA 3.1
δSEP	GABRD	*Rattus norvegicus*	NM_017289.1	pRK5

Table 1. GABA-A receptor subunits clones utilised in these experiments. Combinations were transiently transfected into HEK293 to examine the receptor properties in electrophysiology experiments. All were provided courtesy of Dr. Alastair Hosie (Imperial College London).

2.3.2 Analysis of DNA constructs

Plasmid preparations were routinely analyzed by digestion with specific restriction enzymes, using protocols as per the respective manuals (New England Biolabs). The resulting DNA fragment patterns were assessed using agarose gel electrophoresis. Agarose gels were

prepared using 1% agarose (Sigma-Aldritch, UK) dissolved in TAE running buffer (tris-acetate-EDTA: 40mM Tris-acetate, 1mM EDTA) and were cast in a container with multiple wells for samples. Once cooled and set, the gels were fully immersed in running buffer, and the wells were loaded with DNA samples mixed with loading buffer (30% glycerol, 0.25% Bromophenol Blue, 0.25% xylene cyanol FF). Further, a 'DNA ladder' of appropriate size, was loaded in parallel to allow comparative assessment of fragment sizes. Samples were then electrophoresed for 40-60 minutes at 80V, before the gel was removed from the chamber and stained using ethidium bromide (1µg/l, Promega). Following a rinse in tap water, the gel could be placed in a UV illuminator (BDH, UK) to allow for visualisation of the DNA bands, images of which were captured using a Kodak EDAS 290 camera (Kodak, UK).

DNA concentrations were assessed by ultraviolet spectroscopy using the NanoDrop1000 (Thermo Scientific, US). This measured the absorption (in OD units) of wavelengths of 260nm, which nucleic acids absorb, and 280nm which proteins, in particular aromatic amino acids absorb. Thus the degree of absorption at 260nm could be used to calculate DNA concentrations in aqueous solution, while the amount of 280nm light gave a measure of protein contamination.

2.3.3 Solutions for routine molecular biology

Agarose gel (1%)

1. 1 g of agarose was added per 100 ml of TAE running buffer
2. Mixture was heated until the agarose dissolved
3. The container was stoppered with a paper towel during heating to prevent evaporation

50x TAE running buffer

- 121g Tris base
- 28.55 ml glacial acetic acid
- 50 ml 0.5 M EDTA (pH 8.0)

Make up to a volume of 500 ml in Milli-Q water

2.4 Preparation of acute cerebellar slices

All experiments were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986 and have been approved by the Ethical Review Committee of Imperial College London. Male (4-5 weeks old) C57Bl/6J mice were sacrificed by cervical dislocation followed by decapitation. The skin was peeled back to expose the skull and surgical scissors were used to cut around the skull on each side. Subsequently, surgical forceps were used to carefully separate the skull from the brain tissue and a small spatula used to lift the brain from the skull cavity. The tissue was immediately placed in ice-cold, carbogen (95% O_2, 5% CO_2)-bubbled slicing solution (see table 2. for recipe) with the dissection plate resting on ice. To maintain healthy slices it is imperative that these initial steps are carried out as quickly as possible.

The cerebellum was separated from the forebrain using a blade (Wilkinson) to cut coronally in front of the cerebellum and subsequently remove the brain stem. Then, the left and right cerebellar hemispheres were carefully cut away sagitally to isolate the vermis. The vermis was then flipped on its side and lifted out of the ice-cold solution on a large spatula and any excess liquid was removed with a paper tissue. The tissue block was then carefully placed onto the centre of the metal slicing tray, upon which a thin layer of superglue (Loctite) had been applied just previously. The vermis was always placed with the cerebellar side down. Note, prior to the dissection, the vibratome tissue-slicer (Campden instruments) had been prepared by packing the outside of the main chamber with ice in order to allow it to cool down and a clean slicing blade was fitted. The chamber was then fixed to the slicer and ice-cold slicing solution was carefully poured to fill the metal slicing tray. Previously prepared ice cubes of slicing solution were added and the solution was bubbled with carbogen throughout slicing.

Sagittal slices of 250 μm thickness were cut (0.05mm/sec) and then transferred to a carbogen-bubbled slice chamber containing the slicing solution at room temperature (figure 8). The slices were then warmed to 34˚C by placing the entire chamber into a heat-block for 1 hour. Subsequently the slices were placed at room temperature and the slicing solution was slowly

replaced with external recording solution using a peristaltic pump (Masterflex C/L). The 50ml of slicing solution was replaced by a 150ml of perfusion solution over approximately 30 minutes. Cerebellar slices were then ready for use in electrophysiology experiments.

	Slicing (mM)	Perfusion (mM)
NaCl	0	125
KCl	2.5	2.5
$NaH_2PO_4.H_2O$	1.25	1.25
$NaHCO_3$	26	26
$CaCl_2$	1	2
$MgCl_2$	5	2
Glucose	0.5	11
Glycerol	125	0
Kynurenic acid	1	1

Table 2. Composition of solutions used in slice electrophysiology experiments. Ice cold slicing solution was used to maintain the tissue during the slicing procedure. This was subsequently slowly exchanged for room temperature perfusion solution for patch-clamp recordings. All solutions were continuously bubbled with carbogen throughout, or sealed in airtight containers to maintain oxygen saturation.

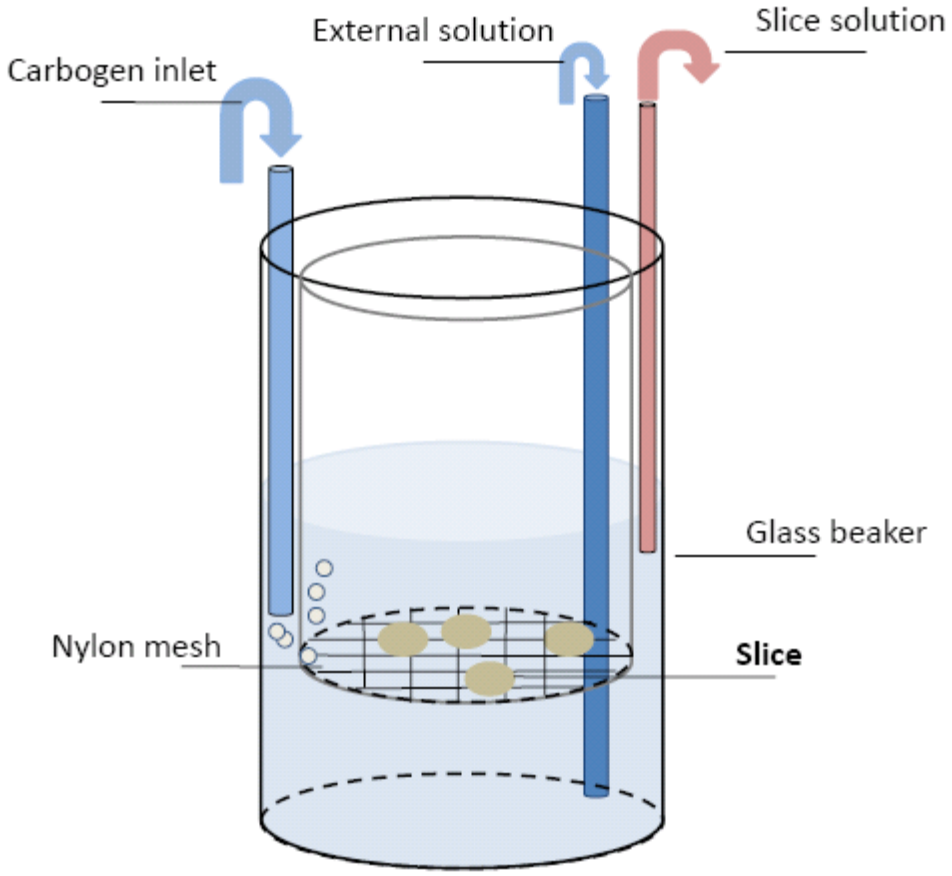

Figure 8. Slice chamber. The slice chamber was custom built by fixing a piece of fine nylon mesh to the base of a hollow plastic tube. The tube was placed within a glass beaker, suspended and fixed. Prior to slicing, the chamber was half-filled with slicing solution and bubbled continuously with carbogen. As cut, each slice in turn was placed into the solution and onto the mesh base. After slicing, the chamber was placed into a heat block and warmed to 34°C. A peristaltic pump was then used to slowly remove the slicing solution and exchange it with external recording solution.

2.5 Patch-clamp electrophysiology

The properties of GABA-AR receptors were investigated using patch-clamp electrophysiology in both a mammalian cell line and cerebellar slices. For the most part this involved patch-clamping in whole-cell configuration (Hamill et al., 1981), but also included use of excised outside-out patches. All experiments were carried out at room temperature (21-22 °C).

2.5.1 Experimental set-up

Electrophysiological recordings are highly sensitive to electrical and mechanical disturbances. As such the patch-clamp rig had to be carefully isolated. Electrical isolation was accomplished by shielding the recording equipment within a Faraday cage, and by grounding all components to a single point which was then connected to the amplifier earth point (Axopatch 200B, Molecular Devices, CA). Mechanical isolation was achieved by mounting the set-up on an anti-vibration air table (63-530 series, TMC, MA) (refer to figure 9).

For patch-clamp experiments HEK cells and slices alike were placed within a clear plastic bottomed recording chamber/bath with an approximate volume of 3 ml and maintained at room temperature (19-23°C). This chamber was continuously perfused with oxygenated external solution in a gravity-feed flow system and solutions were removed from the bath using peristaltic pump action (Watson Marlow SciQ400). Flow rates were thus maintained at 2-3 ml/min. To prevent movement of samples due to bath flow glass coverslips were immobilised on the bottom of the bath by applying a small amount of laboratory grease to the edge of the coverslip, while cerebellar slices were held in place using a ‘harp’ made from a flattened platinum disc lined with individual strands of nylon attached by glue.

Some drugs were bath applied, but for fast local drug applications a small diameter 8 unit manifold (QMM-8 Quartz Micromanifold) was positioned close to target cells using a set of manual micromanipulators (LBM-7, Scientifica, Uckfield, UK) (figure 9D). PTFE tubing carried drug solutions from sealed glass syringes via a custom built 8 unit solenoid driver

switch box to the micromanifold. The solenoid switches were driven by TTL pulses via WinEDR freeware (John Dempster, Strathclyde University). Refer to figure 9.

Samples were visualised using a fixed stage upright microscope (Olympus: PatchPro system, Scientifica) fitted with an immersion objective (Olympus 60x) and a non-immersion objective (10x), a fluorescent light source and infrared differential interference contrast (IR-DIC). Also attached was a digital camera (Olympus) (fig. 9G) linked up to a viewing screen (Samsung) (fig.9 H).

Patch pipettes were fabricated from thin-walled filamented borosilicate glass (1.5mm outer diameter (o.d.), 0.86 mm inner diameter, Harvard Apparatus, GC150F-10) using a two-stage vertical puller (Narishige PP-830). Pipette open tip resistance when filled with intracellular solution was typically between 4-6 MΩ for HEK cells and 8-10MΩ when patching cerebellar granule cells. During experiments the patch pipette was positioned using a Patchstar micromanipulator (Scientifica, Uckfield, UK)(fig 9C) which performed fine and coarse movements as required.

The patch-clamp recording set-up was in a standard configuration. Briefly: a silver chloride coated wire within an internal ionic solution electrically connected the bath with the amplifier headstage (figure 9F). The bath solution (fig 9E) was earthed by an immersed silver chloride pellet (Harvard Apparatus, UK) which was connected back to the headstage earth point to complete the circuit. The headstage in turn was connected to the Axopatch 200B amplifier (Molecular Devices, Foster City, CA). The analogue amplifier output was filtered at 10 kHz (–3 dB, 8-pole low-pass Bessel) and digitized via a National Instruments digitization board (NI-DAQmx, BNC-2110 National Instruments, Austin, Texas) itself linked to a PC (figure 9.J). Data recording and analysis were performed using WinEDR software (Version 3.0.9), courtesy of John Dempster (© John Dempster, University of Strathclyde).

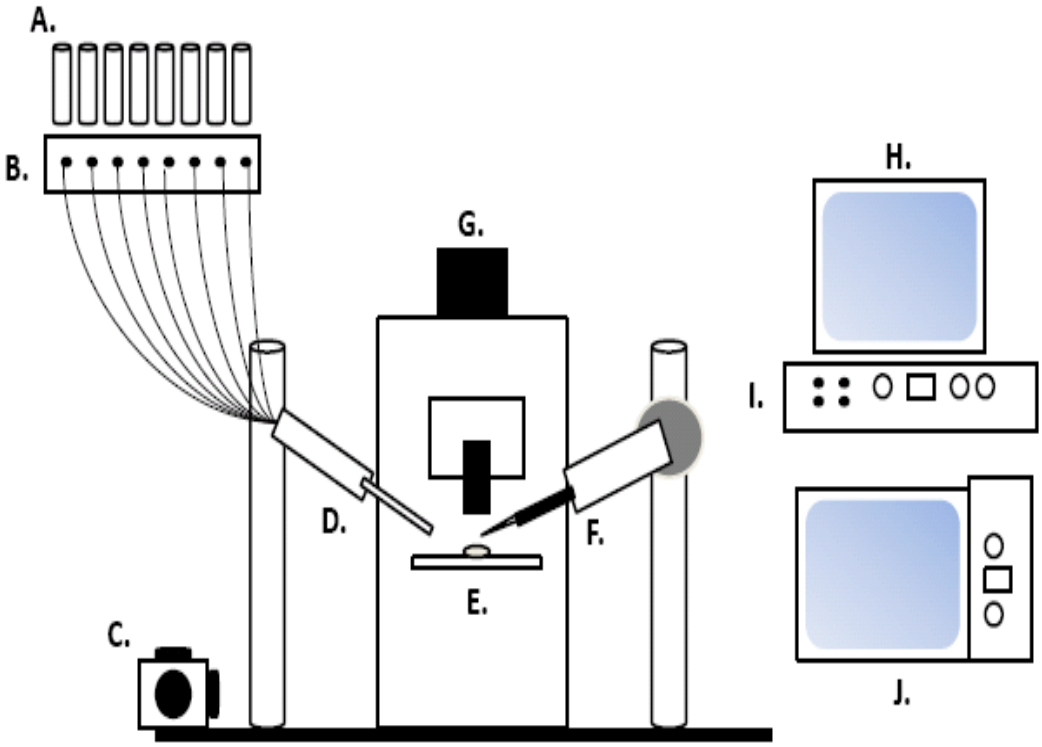

Figure 9. Experimental set-up electrophysiological recordings. **A**. Glass syringes containing sample solutions **B.** Solenoid driver triggered with TTL pulses via PC **C**. Micromainpulator to control headstage/electrode positioning **D**. Micromanifold used for local drug applications **E**. Bath containing brain slices **F**. Headstage and electrode for making electrophysiological measurements **G**. Digital camera for capturing samples and targeting electrodes **H.** Screen for viewing samples **I**. Axopatch 200B amplifier connected to headstage and PC **J**. PC for recording data.

2.5.2 Experimental procedures

2.5.2.1 Whole-cell patch-clamp

The recording pipette was filled with a caesium chloride based internal solution (140mM CsCl, 4mM NaCl , 0.5mM CaCl2, 10mM HEPES, 5mM EGTA, 2mM Mg-ATP; pH adjusted to 7.3 with CsOH) and attached securely to the electrode holder so that the tip of the silver wire was contacting the solution. Positive pressure was applied within the patch pipette to maintain a clear open tip, and the electrode was lowered into the bath. The liquid junction potential between pipette and external solutions was neutralised using amplifier offset controls. The pipette resistance (i.e. tip size) was also determined by utilising a 'seal-test' function in WinEDR, which simply applied 5 mV square wave in voltage-clamp and determines the resistance through the use of Ohm's law (V=IR).

Using the coarse controls on the micromanipulator the pipette was moved into position over the target cells. The manipulator was then switched to fine function and the pipette tip moved slowly, diagonally onto the cell surface. When the pipette was in position close to the cell the cell surface could be seen to dimple due to the positive pressure within the pipette. When the cell surface wasn't visible an increase in the open tip resistance was used to indicate proximity to target. When the pipette was thus positioned the positive pressure was released and a gentle negative pressure applied by suction through a 1 ml syringe attached with tubing to the pipette holder side-port. This gave rise to a tight seal between pipette tip and cell membrane. At this point the holding voltage was set to -60 mV and the resistance was monitored using the 'seal test' function until a high resistance seal (>3 GΩ: gigaseal) was formed, and any capacitive transients associated with the pipette were neutralized on the amplifier.

Next, in order to 'breakthrough' and achieve 'whole-cell' configuration, a short sharp suction was manually applied. This ruptured the cell membrane so that the internal pipette solution was now contiguous with the cell interior, thus allowing access to and measurement of cellular membrane properties. Capacitive transients were again evident when the 5 mV step pulse was applied and now reflected the cells membrane capacitance. This was recorded for each cell, as was a measurement of series resistance in 'whole-cell'.

During experiments some drugs were bath applied and allowed to perfuse the whole tissue. However, for local drug applications, a micromanifold was positioned over the target cells opposite the pipette position. This allowed fast switches between various drugs and wash during whole-cell recording. During whole-cell recording, wash solutions were bath applied at a flow rate of 6 ml/min, resulting in an exchange time of 1 min. To measure whole-cell peak and steady-state responses GABA was applied locally through the manifold device (placed ~150 μm from the recorded cell. Solutions could be gravity fed via the solenoid valve system controlled using WinEDR software. Liquid junction currents, recorded in response to application of a 5% diluted solution across the open tip of a pipette, were used to estimate the solution exchange times (10–90% rise-time 308 ± 39 ms, n= 22).

2.5.2.2 Outside-out patch-clamp

To achieve 'outside-out patch' configuration, whole-cell access was first achieved precisely as described above. The pipette was then slowly pulled away from the cell diagonally upward using the fine motion on the micromanipulator. This eventually caused a small 'patch' of membrane to tear off the cell and reseal over the pipette, forming a bilayer across the pipette tip in which what was the outside of the cell membrane faces outward from the pipette. The success of this patch formation was evident from the 5 mV step pulse, as the large capacitive transients which reflected the whole-cell membrane disappear while a seal is maintained. For drug applications onto the patch, the pipette was moved up and away from the tissue, to stand in front of the micromanifold outlet. Drug and wash steps could thus be variously applied.

2.6 Data analysis

Online data acquisition and offline analysis were performed using the Strathclyde Electrophysiology software WinEDR/WinWCP (Version 3.3.3, © John Dempster, University of Strathclyde, UK). Graphs were plotted and statistics performed using OriginPro 8.5 (Microcal,Century City, CA), at a 95% confidence level. All other analyses were carried out using Microsoft Excel (Microsoft Corp. US).

Peak and steady-state drug responses were measured in voltage-clamp by constructing all-points histograms from 500ms segments of current trace, and fitting to a Gaussian function of the form $y(x) = (a/\sqrt{(2\pi\sigma 2)})*exp(-(x-\mu)2/(2*\sigma 2))$ where a is the percentage of the total area under the curve, μ is the mean current and ς is the standard deviation about the mean.

2.6.1 Resistance and capacitance measurements

For each cell the seal resistance, R_{seal}, was calculated using the steady state response to hyperpolarising steps (-5mV) applied in the cell attached configuration, utilizing Ohm's law (V=IR). Recordings were only subject to additional analysis if R_{seal} was >2 GΩ. The series resistance, R_S, was similarly taken from the amplifier or calculated offline using membrane current recordings and the relationship of V (mV)/current (pA). This was calculated at breakthrough and monitored throughout the experiment. Further, the voltage error (V_{err}) was calculated for each cell as the sum of series resistance and the initial holding current ($V_{err} = I \times R_S$). To control for experimental variation in series resistance, conductance estimates were normalised to V_{err}.

The membrane capacitance (c_m) was either taken from the amplifier or estimated offline from voltage-clamp data according to the relationship $c_m = \frac{Q}{\Delta V}$, where Q is the charge transfer measured from the current record during a hyperpolarising 5 mV step in the amplifiers command voltage (ΔV). This was calculated from an average of 10 consecutive current transients recorded immediately following breakthrough into whole-cell configuration at a command voltage of -60mV. Specifically the current responses to a 5 mV hyperpolarising voltage step were used in the following relationship: C_m = area under curve (pA.ms).peak

current (pA))/series resistance. The resulting estimate of c_m directly correlates with membrane surface area when ΔV influences all regions of the cell uniformly and assuming that the lipid bilayer has a similar thickness in all membrane compartments. Considering this is suggested to be true for both HEK cells and cerebellar granule cells, all conductance estimates were normalised to c_m in order to control for differences in cell size.

2.6.2 Tonic conductance measurements

Membrane currents were periodically sampled before and after cell breakthrough in order to estimate membrane capacitance, series resistance and tonic GABA-AR-mediated conductance throughout the experiment. Following break through into whole-cell configuration and subsequent exchange of the cell interior with the pipette solution a number of things happen. Most importantly for this study, a standing inward current is generated as the cell interior shifts from a low to a high chloride concentration: 'chloride load'. Chloride loading of the cell immediately following breakthrough was used to estimate the tonic conductance at the beginning of the recording session and this estimate was compared to the tonic GABA conductance measured from the blocking action of 100 μM SR95531 (Sigma, UK) at the end of the recording. In order to calculate the tonic conductance, all-point histograms of the current record were constructed at a command voltage of -60 mV and differences in the amplitude measured from a single Gaussian fit were used to calculate the amplitude of the standing inward current before and after breakthrough and also in the presence of 100 μM SR95531.

2.6.3 Drug concentration-response relationships

Drug responses were normalised to a maximum concentration response, and to account for the cell-cell variability, to membrane capacitance and V_{err} also. Values were then used to construct concentration-response curves which were fitted with a the Hill equation according to the following formula: $y = min + (max\text{-}min)/1 + 10(logEC50 – x)n$. Where n = hill slope and EC50 = the concentration of agonist which induces 50% of the maximal agonist evoked response.

3. Results

3.1 Basic properties of αβδ containing GABA-ARs

As the critical role of extrasynaptic GABA-A receptor mediated tonic inhibition in controlling neuronal excitability becomes increasingly apparent, so has the requisite for reliable data on the functional properties of the receptor subtypes responsible. However, this has often proved elusive, and much of the dogma surrounding these receptors remains controversial. With this set of experiments we sought to examine the functional properties of α4βδ and α6βδ GABA-A receptors, the subtypes responsible for mediating *tonic* inhibition in the thalamus/hippocampus and in the cerebellum respectively (Brickley, Revilla et al. 2001; Porcello, Huntsman et al. 2003; Stell, Brickley et al. 2003; Farrant and Nusser 2005). Using patch-clamp analysis on recombinant receptors expressed in a mammalian expression system, as well as on native α6βδ receptors in cerebellar granule neurons (CGNs), we examined GABA potency, receptor desensitisation and constitutive activity.

3.1.1 Recombinant δ subunit GABA-AR expression

Some of the controversy and contradictions regarding the properties of recombinant αβδ GABA-ARs is thought to reflect the difficulty in incorporating the δ subunit, and the concern that binary αβ receptors may (Meera, Olsen et al. 2011 ; Wagoner and Czajkowski 2010) predominate in expression systems. To avoid this, many laboratories transfect the δ in excess of other subunits, often up to ratios of 1:1:10 (α:β:δ) (Meera, Olsen et al. 2011 ; Wallner, Hanchar et al. 2003; Borghese, Storustovu et al. 2006). However it has been suggested that this can force the generation of abnormal subunit assemblies with differing functional and pharmacological properties (Wagoner and Czajkowski 2010). Thus, while we wished to avoid arbitrarily increasing the ratio of δ expression, we also wanted to ensure and monitor its incorporation into functional receptors. To this end the δ subunit was conjugated to a pH sensitive fluorescent tag known as a super ecliptic pHlourin (SEP). This tag was produced by the semi-rational mutagenesis of a number of GFP residues leading to a pH sensitive mutant in which fluorescence is quenched below physiological pH (Ashby et al., 2004). Thus it exploits the difference in pH at the plasma membrane, endoplasmic reticulum and Golgi

apparatus (pH 7) from the transport vesicles (pH5.5), and allows us to visualise and distinguish receptor subunits on the surface of a cell from those within. In this way we were able to choose experimental target cells which had the δ_{SEP} unit inserted in the membrane, and forming part of a functional GABA-A receptor complex.

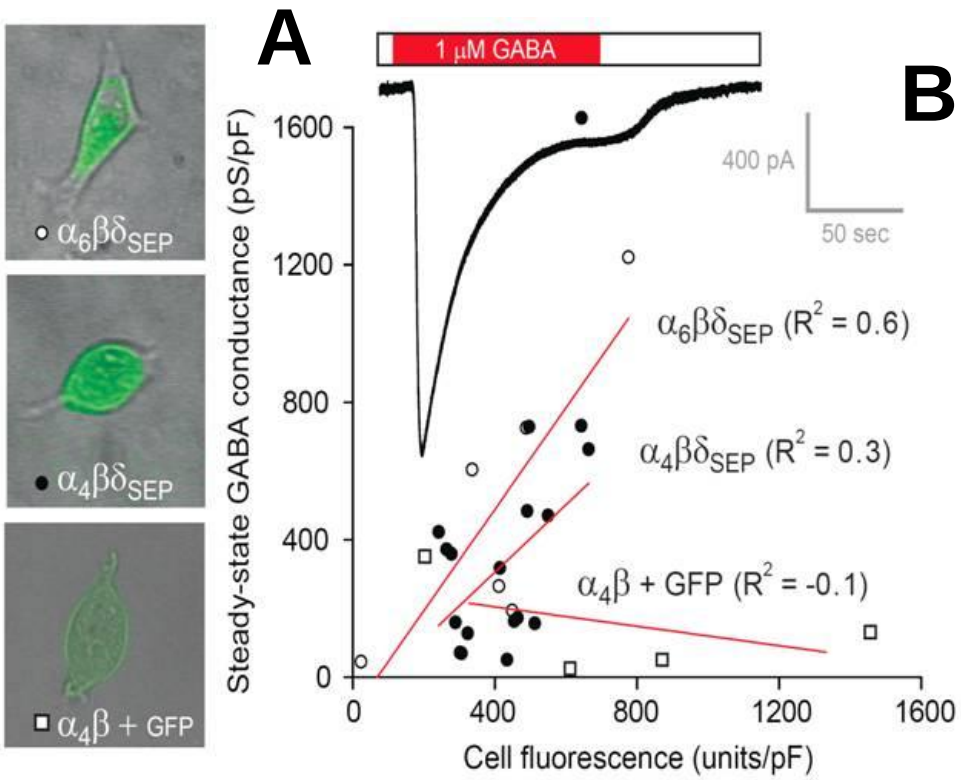

Figure 10. δSEP fluorescence correlates with peak conductances induced by 1µM GABA applications. **A**, representative images of HEK 293 cells transfected with $\alpha_4\beta_2\delta$ (closed circles), $\alpha_6\beta_2\delta$ (open circles) and $\alpha_4\beta_2$ (+GFP) (open squares) respectively. Green fluorescence in the case of the δ containing receptors is associated with the modified GFP tag known as SEP **B**, Comparison of relationship between steady-state conductances following 1µM GABA applications, and cell fluorescence, both normalised to capacitance. R^2 values (measure of correlation) relating to each data set suggest that the degree of SEP fluorescence correlates with the magnitude of GABA responses, while the degree of GFP fluorescence does not. *Inset* is a representative continuous whole-cell recording of a 1µM GABA response in a HEK cell voltage-clamped at -60mV.

To verify that δ_{SEP} represents a good measure of functional αβδ expression we examined the relationship between the cell/SEP fluorescence (units of green signal normalised to capacitance) and the steady-state receptor activation induced by 1μM GABA (figure 10). This relationship was evaluated for correlate and an R^2 value (square of correlation coefficient) generated for each data set. The $\alpha_6\beta\delta_{SEP}$ and $\alpha_4\beta\delta_{SEP}$ receptor populations exhibited R^2 values of 0.6 (n=6) and 0.3 (n=14) respectively, suggesting a correlation between GABA evoked conductances and SEP fluorescence in these cells. Analysis of binary $\alpha_4\beta$ receptors co-transfected with GFP, showed no correlation between fluorescence and GABA currents (R^2 value of -0.1) (n=4) (see figure 10). Thus this data suggests that the degree of δ_{SEP} fluorescence is related to the magnitude of GABA responses while GFP fluorescence is not. And by extension, that the level of δ_{SEP} fluorescence is indicative of functional αβδ GABA-A receptor expression.

To further verify δ_{SEP} surface expression and optimise δ_{SEP} incorporation, we evaluated the level of SEP fluorescence in relation to external pH shifts. As fig 11A demonstrates for four individual cells, the shift in extracellular pH elicited by bath applied external solutions, resulted in a concomitant shift in SEP fluorescence. From a high at pH 8, the level of fluorescence was quenched in acidifying solutions, before recovering again as the pH was raised towards neutral. Internal acidification was minimal reflected by the fact that some cell fluorescence always remains. Thus this demonstrates the extracellular localization of the pH sensitive δ_{SEP}.

We exploited this phenomenon to compare the effect of transfecting different ratios of cDNAs on δ_{SEP} surface expression. In separate experiments we transfected cells with $\alpha\beta\delta_{SEP}$ cDNA in ratios of 1:1:0.1, 1:1:1 or 1:1:10, and examined the extracellular pH induced shifts in SEP fluorescence (see fig 11B). Figure 11B shows the average pH shifts for cell populations the respective conditions, normalised to the fluorescence at pH7.3. The mean fluorescence shifts when the extracellular solution was altered from pH8 to pH5 was -25.4 ± 4.9% (n = 10), -25.6 ± 7.9% (n = 7) and -36.6 ± 7.3% (n = 8) for 1:1:0.1, 1:1:1 and 1:1:10 cDNA ratios respectively. There was no significant difference in these levels between the three groups ($P > 0.05$ Kruskal-Wallis test), suggesting that the ratio of cDNA starting

material does not affect the level of surface expression of δ_{SEP}. On the the basis of these observations the equimolar 1:1:1 cDNA ratio was used in all future HEK cell experiments.

Finally, we confirmed δ incorporation and contribution to functional GABA-A receptors pharmacologically, by using zinc chloride. Relative resistance to zinc block of αβδ subunit containing receptors compared with binary αβ is well established (Borghese, Storustovu et al. 2006; Storustovu and Ebert 2006), providing a selective pharmacological δ diagnostic in electrophysiological experiments. As figure 12 illustrates cells transfected with the αβδ subunits displayed only a modest 1μM zinc block of 1μM steady-state GABA conductances averaging -33 ± 24 pS/pF (n=9) -42 ± 23 pS/pF (n=3) for $\alpha_4\beta\delta$ and $\alpha_6\beta\delta$ receptors respectively. In contrast, receptors made up of binary $\alpha_4\beta$ receptors averaged a more marked -245 ± 56 pS/pF (n=3) block of steady-state conductances.

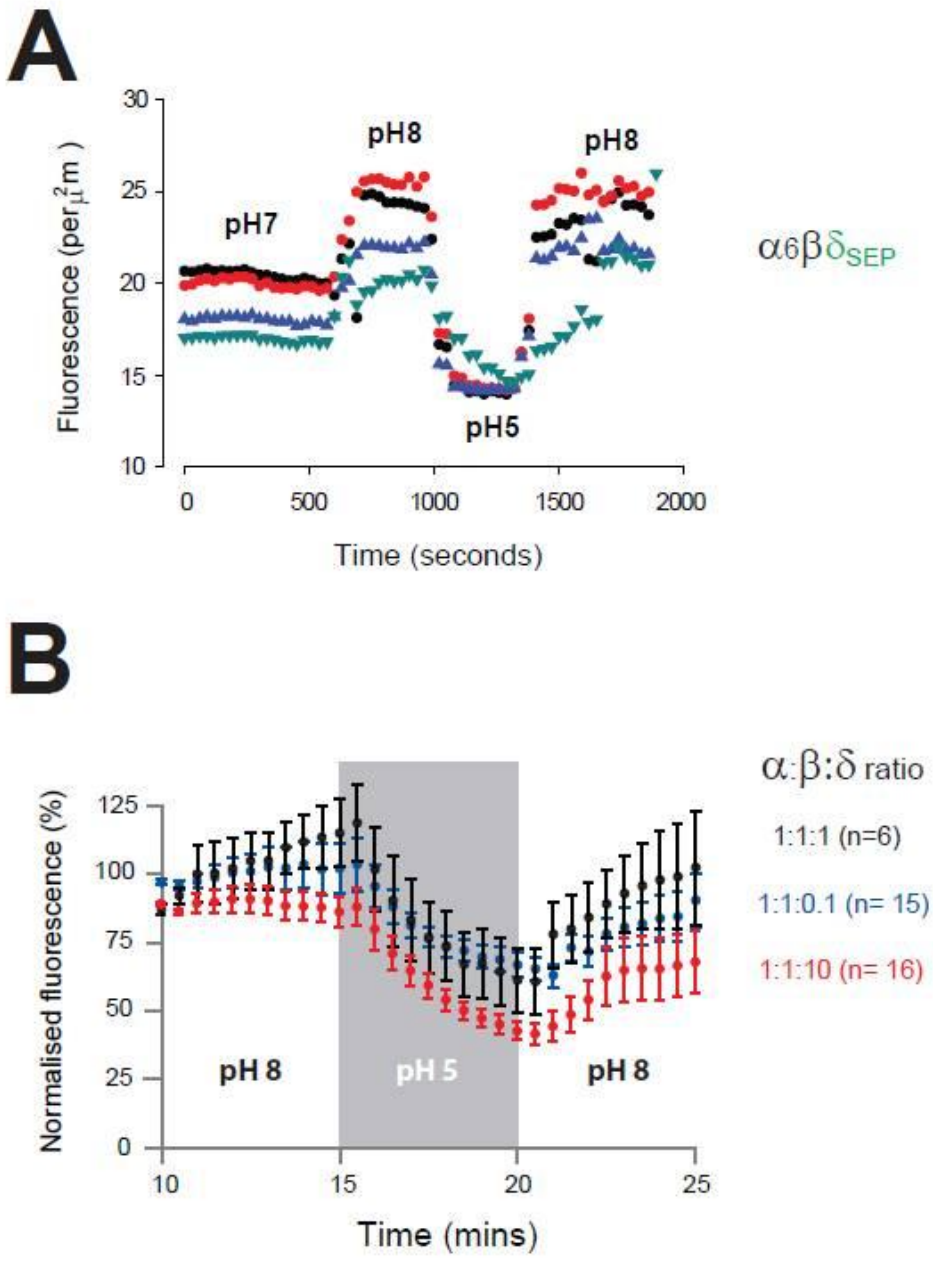

Figure 11. There is no difference in cell surface expression levels between the α6β2δ cDNA transfection ratios of 1:1:0.1, 1:1:1 and 1:1:10. Alterations in extracellular pH and subsequent changes in fluorescence intensity can be used as an assay for cell surface expression. ***A***, The pH dependent shift in fluorescence for each transfection ratio is shown. Each time course represents a single cell. The different pH levels are highlighted in by the black line above. To avoid experimenter bias the fluorescence was measured for an entire field of view with the background subtracted. ***B,*** The percentage pH shift with time for all cells in all conditions normalised to pH7. Although there was some quenching of the fluorescent signal with time there was still a clear difference in intensity between pH 5 and pH 8 and no difference between transfection ratios. All plots are an average of 7 to 10 experiments. Error bars reflect the standard error of the mean.

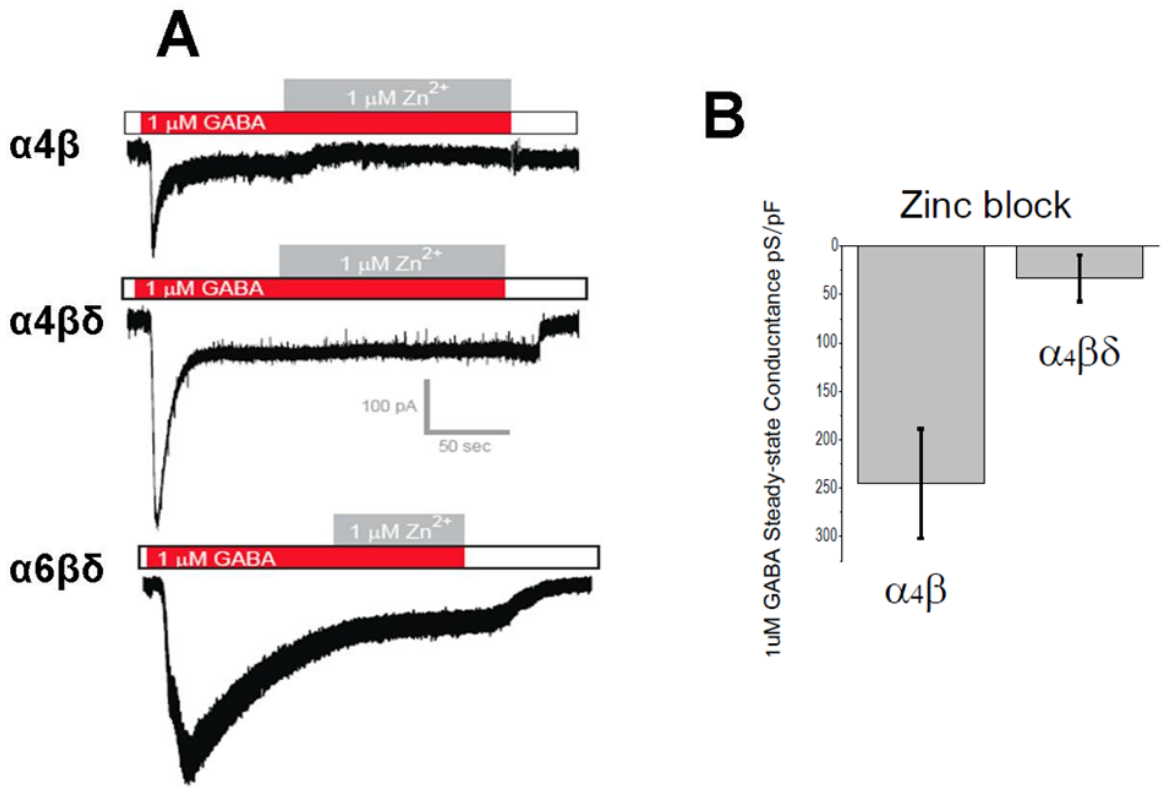

Figure 12. αβδ and αβ exhibit distinct zinc sensitivities. **A,** Representative continuous whole-cell recordings on $\alpha_4\beta_2$, $\alpha_4\beta_2\delta$ and $\alpha_6\beta_2\delta$ transfected HEK293 cells respectively, clamped at -60mV. Duration of 1μM GABA application indicated by red bars. 1μM GABA + 1μM zinc chloride application indicated by thick grey bars. Block of GABA induced currents by zinc is evident in receptors composed of αβ, but not in those containing δ subunit, highlighting the contribution of the δ containing receptors in those cells. **B,** Average zinc block of 1μM GABA steady-state conductances. Error bars represent SEMs. α6β are not shown as they did not form functional receptors.

3.1.2 Constitutive activity of α4βδ and α6βδ GABA-ARs

It has been suggested that certain GABA-A receptors will open spontaneously in the absence of agonist binding (Sigel, Baur et al. 1989; Lindquist, Dalziel et al. 2004; Hadley and Amin 2007), and that this property in extrasynaptic GABA-A receptor subtypes may underlie the generation of tonic inhibition (Birnir, Everitt et al. 2000; Hadley and Amin 2007). This has however proved controversial, and thus we wished to address this question by examining recombinant α4βδ and α6βδ GABA-A receptors for constitutive activity using a mammalian expression system (HEK293 cells). HEK293 cells were transfected with $\alpha_4\beta\delta$ or $\alpha_6\beta\delta$

GABA-A subunits and analysed in whole-cell patch-clamp mode. As a diagnostic for δ subunit expression cells were first exposed to Gaboxadol (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol), a potent GABA-A receptor agonist with a preference for δ containing subtypes (Adkins, Pillai et al. 2001; Brown, Kerby et al. 2002; Meera, Wallner et al. 2011). Subsequently receptors were examined for constitutive activity using picrotoxin, a non-competitive antagonist at GABA-A receptors (Krishek, Moss & Smart, 1996), at saturating concentrations (50μM).

As illustrated by figure 13 both $\alpha_4\beta\delta$ and $\alpha_6\beta\delta$ receptors were robustly activated by 1μM Gaboxadol, giving rise to characteristic desensitising current transients (red bars indicate Gaboxadol application). On average, 1μM Gaboxadol evoked larger conductances from $\alpha_6\beta\delta$ than $\alpha_4\beta\delta$ GABA-ARs, 711 ± 297 pS/pF and 123.5 ± 54 pS/pF (see fig B), and had no effect on binary $\alpha_4\beta$ (10 ± 6 pS/pF) or untransfected HEK cells (-1 ± 2 pS/pF). Thus Gaboxadol application illustrated the expression of functional δ containing GABA-A receptor expression. Cells were then washed and currents allowed to return to baseline before picrotoxin was applied. If these receptors were indeed constitutively active we might expect picrotoxin to elicit a decrease in holding current, reflecting a block of active receptors. However, picrotoxin application had little or no effect on holding current levels (figure 13A blue bars). Note inset on figure 13A: representative currents under each condition described with a single Gaussian fit to the corresponding all points amplitude histogram: control (black), Gaboxadol application (red) and after picrotoxin block (blue), which illustrate the lack of picrotoxin sensitivity of either receptor subtypes. Persistent conductances, defined as the picrotoxin block of holding current (pA) normalised to cell capacitance and voltage error, averaged 2 ± 4 pS/pF (n=7) and 8 ± 7 pS/pF (n=10) for $\alpha_4\beta\delta$ and $\alpha_6\beta\delta$ respectively, and 18 ± 24 pS/pF (n=6) for binary $\alpha_4\beta$ receptors (figure 13C). Thus these results suggest recombinant δ-containing GABA-A receptors expressed in HEK 293 cells show no constitutive activity in the absence of agonist.

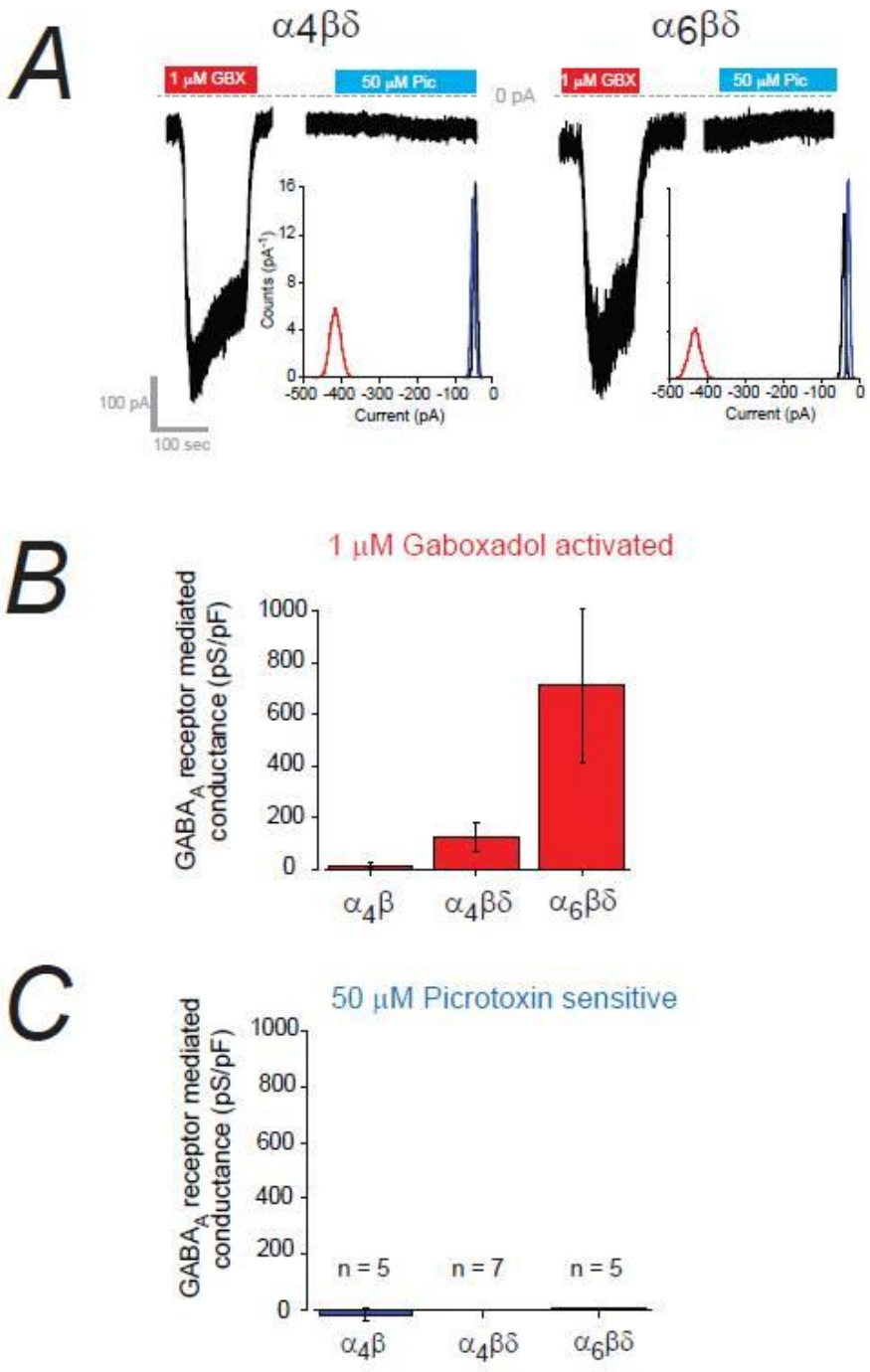

Figure 13. αβδ GABA-A receptors are not constitutively active. **A**, Representative continuous whole-cell recordings on $\alpha_4\beta_2\delta$ and $\alpha_6\beta_2\delta$ transfected HEK293 cells respectively, clamped at -60mV. The δ agonist Gaboxadol was used as a diagnostic of δ expression and the GABA-AR blocker picrotoxin used as a measure of constitutive receptor activity. *inset* average currents at control (black), after Gaboxadol application (red) and after picrotoxin block (blue), described with a single Gaussian fit to the corresponding all points amplitude histogram. **B,** Comparison of Gaboxadol (1μM) evoked peak currents (normalised to capacitance and voltage error). Both populations transfected with δ exhibited Gaboxadol evoked currents. **C,** Comparison of picrotoxin (50μM) sensitivity. Neither of the populations examined exhibited significant picrotoxin sensitivity, illustrating that these receptor subtypes are not constitutively active.

3.1.3 GABA potency at α4βδ and α6βδ GABA-ARs

GABA potency at δ containing GABA-A receptors was evaluated using whole-cell patch-clamp electrophysiology on HEK293 and measuring responses to locally applied GABA. cDNA of mouse wild-type α_4, α_6, β_2 and rat wild-type δ subunits were transfected into HEK 293 cells in either $\alpha_4\beta\delta$ or $\alpha_6\beta\delta$ combinations. The following day GABA activated currents were recorded using a GABA concentration range from 10nM to 10μM. Figure 14A shows representative GABA evoked currents in HEK 293 cells transfected with αβδ at various GABA concentrations. As we can see, GABA activated GABA-A receptors in a dose dependent manner. Responses were comparable for $\alpha_4\beta\delta$ and $\alpha_6\beta\delta$ receptors, with GABA evoked currents observable across a similar concentration range of around 50nM to a maximal at around 1mM. The size of response was also similar for both subtypes: 50nM GABA eliciting peaks measuring 337 ± 11 pS/pF (n = 6) and 180 ± 139 pS/pF (n = 4) for $\alpha_4\beta\delta$ and $\alpha_6\beta\delta$ respectively, while 1mM evoked 3124 ± 1830 pS/pF (n = 7) and 2652 ± 673 pS/pF (n = 12). Peak responses to GABA applications were normalised to the maximal peak conductance, and used to construct concentration response curves for each receptor subset (figures 14B & C). EC_{50} values (a GABA concentration eliciting half-maximal current) were subsequently calculated from the dose response data which was fitted to the Hill equation according to the following formula: $G = [GABA]^n / EC_{50}^n + [GABA]^n$. $\alpha_6\beta\delta$ yielded an EC_{50} of 430nM (n=9) while $\alpha_4\beta\delta$ containing receptors exhibited an EC_{50} of 300nM (n = 7).

While examining these whole-cell responses it became apparent that, contrary to expectations, GABA evoked macro-desensitizing currents in cells expressing αβδ receptors (figure 14A). This previously unappreciated property could have important implications in how we understand and examine these receptors, and thus warranted a more comprehensive examination.

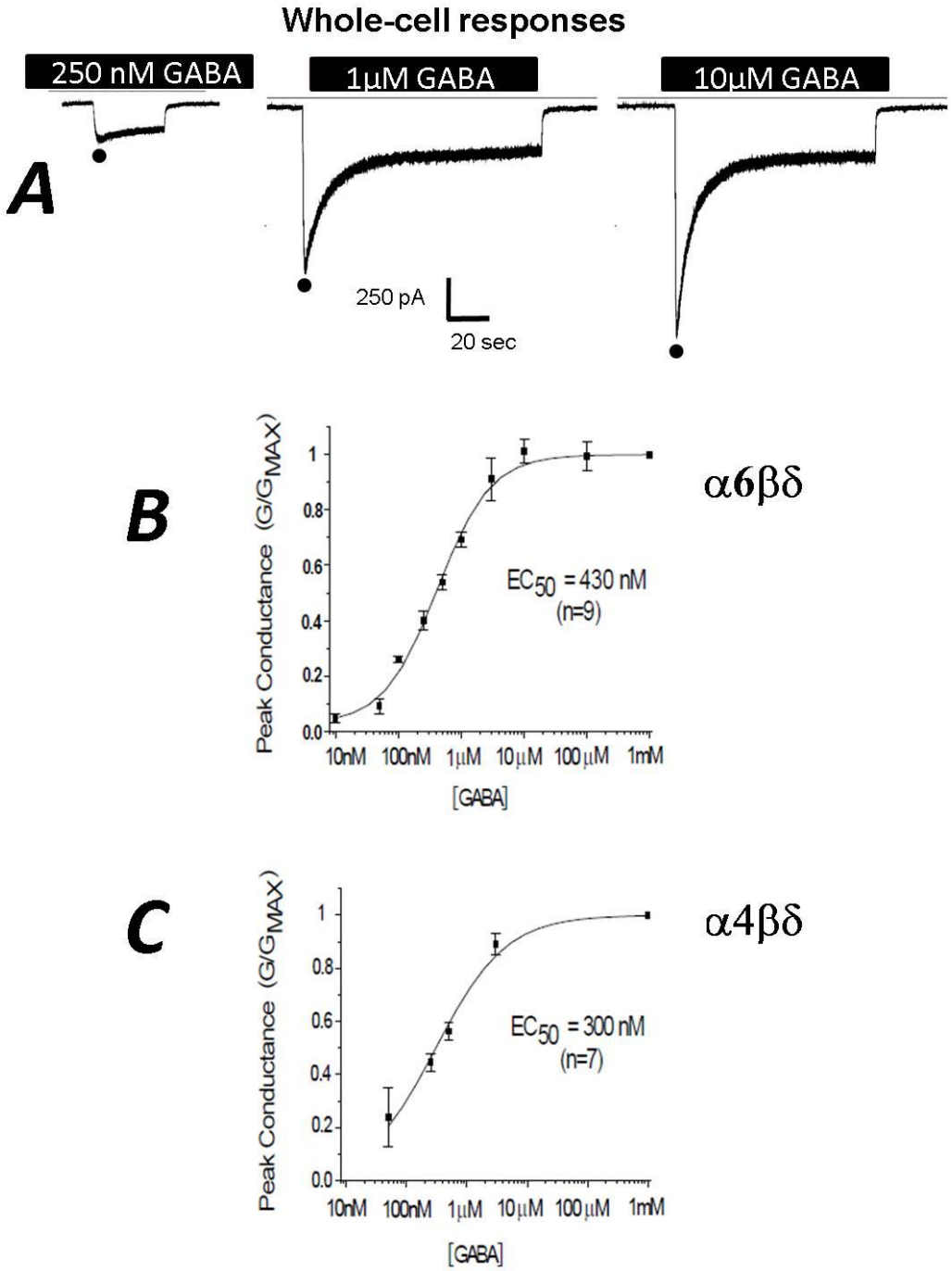

Figure 14. GABA concentration-response relationships for α4β2δ and α6βδ GABA-A receptors. **A,** continuous whole-cell recordings on $\alpha_6\beta_2\delta$ transfected HEK293 cell clamped at -60mV. GABA applications indicated by black bars respectively. **B,** Peak concentration-response relationship of normalised GABA conductance at $\alpha_6\beta_2\delta$ receptors. Error bars represent SEM. *Inset:* peak EC_{50} value for $\alpha_6\beta_2\delta$ of 430nM. ***C***, Peak concentration-response relationship of normalised GABA conductance at α4βδ receptors. Error bars represent SEM. *Inset:* peak EC_{50} value for $\alpha_4\beta_2\delta$ of 300nM.

3.1.4 α4βδ and α6βδ GABA-ARs are profoundly desensitizing

For ligand-gated ion channels, such as glutamate or synaptic GABA-A receptors, it is well established that steady-state exposure to low concentration of agonist can promote receptor desensitization and thus remove a proportion of receptors from the conducting pool (Overstreet, Jones et al. 2000; Lagrange, Botzolakis et al. 2007). However, this property has largely been ignored in δ containing GABA-A in spite of the fact that these receptors are persistently activated by GABA, because it was believed that they exhibit only very modest desensitization (Farrant and Nusser 2005). Here we present data clearly illustrating (Fig 15A) that αβδ receptors are in fact profoundly desensitizing at GABA concentrations that are well within the range of expected *in vivo* ambient GABA levels (Farrant and Nusser 2005; Santhakumar, Hanchar et al. 2006; Wu, Wang et al. 2007). Fig 15A shows a typical 250nM and 1μM GABA response at a α6βδ transfected HEK cell. Note the peak and steady-state equilibrated response (marked on trace) reflecting the removal of a large percentage of receptors from the chloride conducting pool, and the current decay which was well described by a single exponential (averaged τ equalled 54.4 ± 7 seconds (n = 8)) (figure 18B). The level of desensitization was concentration dependent, with 50nM GABA exposures eliciting 32 ± 3 % (n=14) desensitization, peak to steady-state, while 250nM and 1μM displayed 66 ± 4 % (n=14) 1μM 73 ± 4 % (n=20) respectively. However, macroscopic desensitization peaked with 10μM GABA at 78 ± 3 %, suggesting that even under saturating GABA conditions steady-state conductance remains. Similar analyses were carried out on recombinant α4βδ (figure 17) and we found that these too exhibited rapid desensitization with 1μM GABA responses reduced by 87 ± 6% (*n* = 8) at steady state (Figure 17), and a time course described by a single exponential averaging (τ) 20.9 ± 3 s (n=17) (Figure 18).

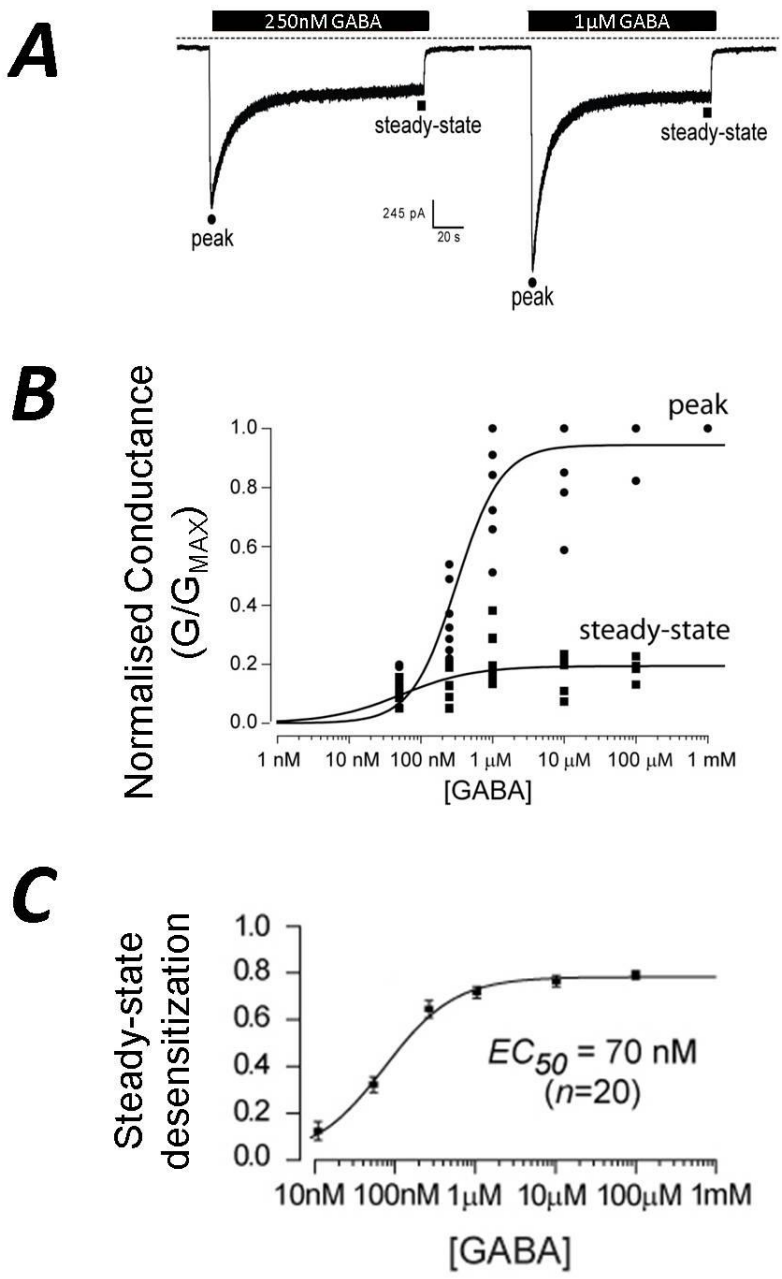

Figure 15. Steady-state desensitisation of α6βδ GABA-A receptors A, continuous whole-cell recording on α6βδ transfected HEK293 cell voltage-clamped at -60mV. GABA applications indicated by black bars. Receptors displayed marked desensitisation from peaks to a steady-state level, marked beneath respective points on trace. Dashed line represents the zero current level. **B**, the concentration–response relationship based on peak (circles) and steady state (squares) responses. Lines represent a Hill equation fit to all the data points, normalised to the 1mM peak response. **C**, plot of *α6βδ* steady-state desensitisation, reflecting the ratio of the peak to steady-state currents across a range of GABA concentrations. The solid line is a fit of the data to the Hill equation, giving rise to an EC_{50} value of 70nM GABA (*inset*). Error bars reflect ± standard error of the mean (SEM).

In a similar vein to their synaptic counterparts, extrasynaptic GABA-ARs have largely been examined during brief exposures to GABA (Brown et al, 2002; Feng & Macdonald, 2004; Mortensen et al, 2010; Saxena & Macdonald, 1994; Wallner et al, 2003). This was in spite of the fact that extrasynaptic receptors do not see fast GABA transients, but justified partly due to the erroneous belief that αβδ containing receptors are non-desensitizing and therefore that peak and steady-state responses are effectively equivalent. In light of our findings we wished to re-evaluate the relationship between GABA and αβδ receptors under steady-state conditions, and provide a more appropriate reference for future studies of this receptor population. Thus, peak and steady-state response data were used to generate and compare concentration response curves for α6βδ, which were well described by a Hill equation. Peak data gave rise to an EC_{50} of 430nM and compared with a steady-state EC_{50} 70 nM (figure 15B). EC_{10} and EC_{90} values for each condition (peak 5nM and 613nM; steady-state 65nM and 1.5μM) were also markedly different, suggesting the dynamic range over which receptors are responsive is different in each condition. The ratio of peak to steady-state conductances was used to construct a steady-state desensitization curve (figure 15C), reflecting the percentage of non-conducting desensitized receptors across a range of concentrations, and giving rise to an EC_{50} of desensitisation for α6βδ of 70nM (n=20) (*inset* figure 15C). Similar analyses were carried out on α4βδ. Again, the steady-state concentration–response relationship was well described by a Hill equation and resulted in an EC_{50} of 243 ± 5 nM (EC_{20} 92 ± 3 nM, EC_{80} 645 ± 40 nM) (Fig. 17A, *inset*), while the steady-state desensitization curve gave rise to an EC_{50} of desensitisation of just 60nM (*inset* figure 17C).

With a view to confirming that native αβδ receptors also desensitize, outside-out patches were pulled from CGNs, and currents in response to GABA or Gaboxadol analyzed. As we can see from figure 16 100μM GABA gave rise to distinctive desensitizing transients in outside-out patches, with an average of 81% of peak current decaying to steady-state levels (57 ± 11 pA peak versus 11± 1.5 pA steady-state). Similarly, Gaboxadol was applied to patches at a concentration known to be αβδ selective (500nM), and this also evoked rapidly decaying GABA currents (see fig 16B). On average 500nM Gaboxadol elicited 60% receptors desensitisation (n=31) (14 ± 3 pA peak versus 4 ± 0.5 pA steady-state). Thus this data illustrates that native δ-containing GABA-ARs are also desensitising, confirming that this property is not an artefact associated with recombinant expression.

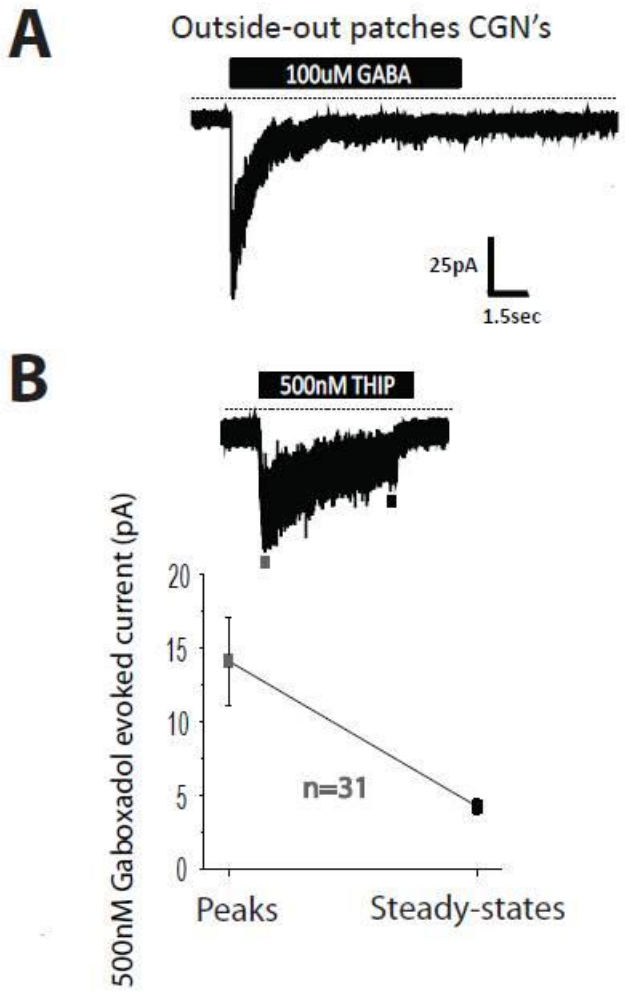

Fig 16. Desensitising α6βδ GABA-A receptors in cerebellar granule neurons (CGN)s. A, Continuous recording from a CGN derived outside-out patch voltage-clamped at -60mV. GABA application indicated by black bar. Dashed line represents zero current level. **B**, 500nM Gaboxadol, a concentration known to be δ selective, was applied to the patch. Note the current decay from a peak to a steady-state, reflecting the desensitization of native *α6βδ* GABA-A receptors. Averaged data below illustrates peak and steady state current level (n=31 patches), confirming that native *α6βδ* receptors desensitize.

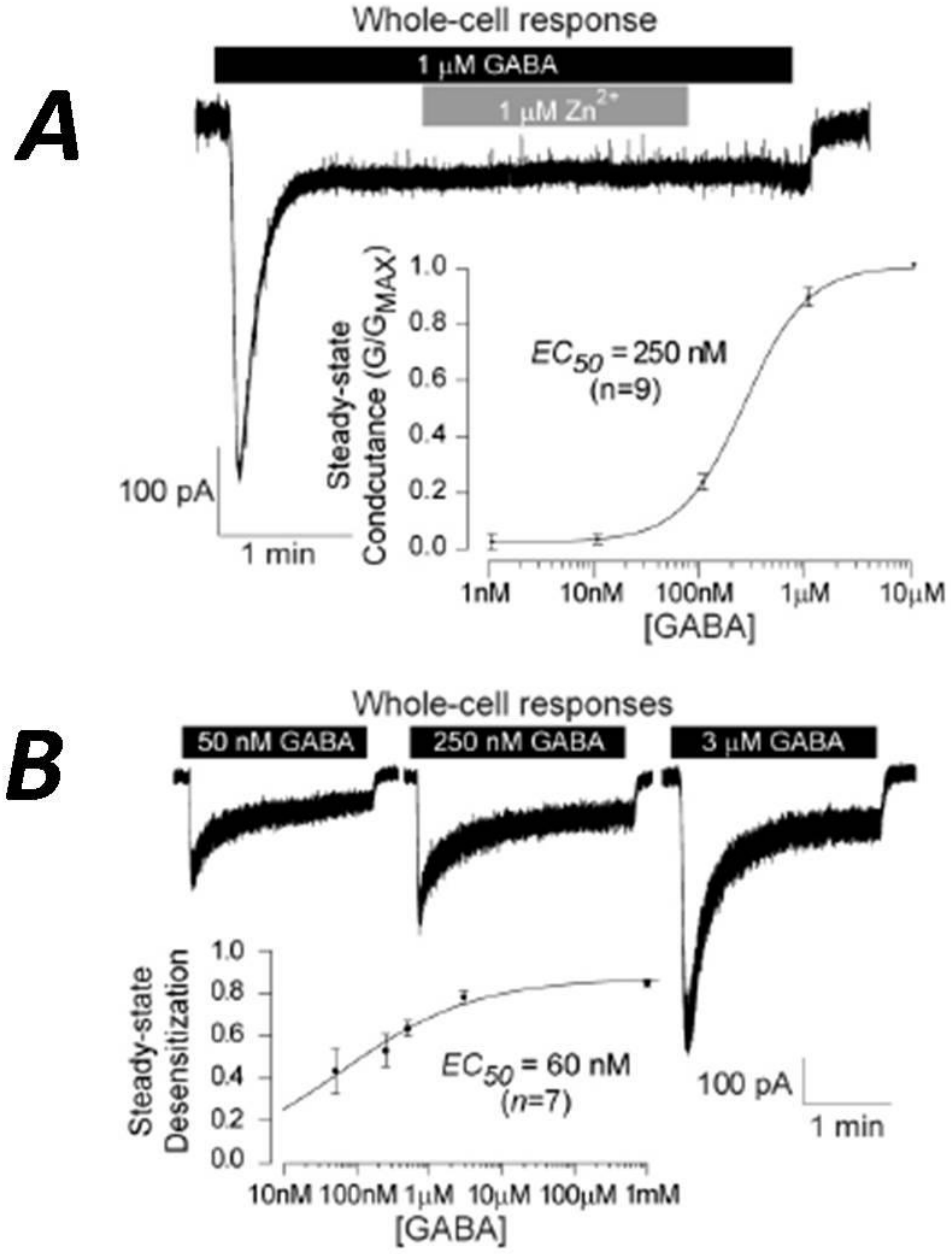

Figure 17. Steady-state desensitisation of α4βδ GABA-A receptors. A, continuous whole-cell recording on α4βδ transfected HEK293 cell voltage-clamped at -60mV. GABA and zinc applications indicated by black and grey bars respectively. Block of GABA induced currents by zinc is evident in receptors composed of binary α4β, but not in those containing δ subunit, highlighting the contribution of the δ containing receptors in those cells. *Inset*, the concentration–response relationship based on steady-state responses to a range of GABA concentrations.. Lines represent a sigmoidal best fit to the data points, normalised to the maximal response (1mM GABA). **B**, plot of *α4βδ* steady-state desensitisation, reflecting the ratio of the peak to steady-state currents across a range of GABA concentrations. The solid line is a fit of the data to the Hill equation, giving rise to an EC_{50} value of 60nM GABA (*inset*). Error bars reflect ± standard error of the mean (SEM).

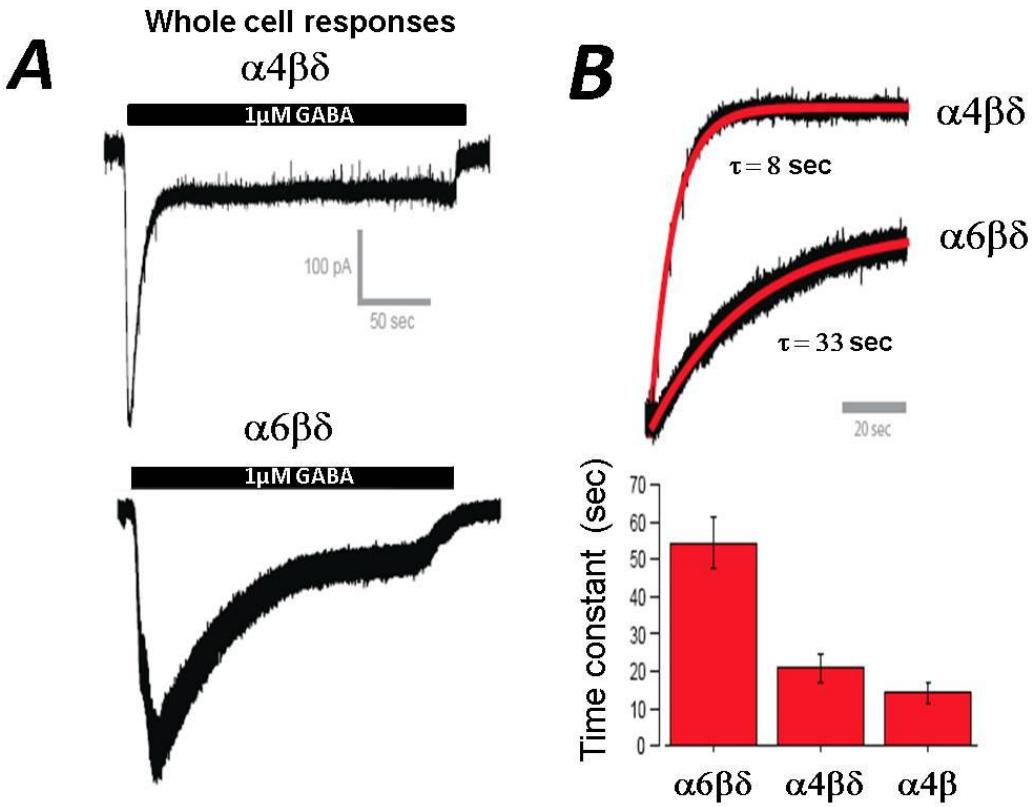

Figure 18. α4βδ and α6βδ exhibit distinctly different desensitisation kinetics. A, representative continuous whole-cell recordings *α4βδ and α6βδ* transfected HEK293 cells respectively, clamped at -60mV. Duration of 1μM GABA application indicated by black bars. **B, top,** representative trace showing the current decay following 1μM GABA applications on *α4βδ and α6βδ* cells respectively. The decay was fitted with a single exponential and the time constant (τ) calculated from the line (in s). Result illustrates the larger time constant and therefore apparently slower rate of desensitisation exhibited by *α6βδ* receptors. **B, bottom,** comparison of averaged time constants for the rate of current decay following 1μM GABA applications on *α4βδ, α6βδ* and binary *α4β*. Error bars represent SEMs. Overall, larger time constants and therefore slower receptor desensitisation rates are exhibited by the *α6βδ* subtype.

Studies in the past have suggested that α6βδ receptors display a voltage dependence to the extent of receptor desensitization, and that they desensitise more significantly at positive potentials (Bianchi, Haas et al. 2002). To test this we examined the peak and steady-state responses of recombinant α6β3δ GABAR to 1μM GABA at different holding potentials. Illustrated in figure 19A are representative whole-cell responses in HEK cells voltage-clamped at -40, +40 and -100mV respectively. All showed robust 1μM GABA responses

averaging peaks of -1654 ± 248 pA, 2023 ± 292 pA and -2915 ± 593 respectively, and showed marked macroscopic desensitization. Contrary to reports however, the extent of desensitization at each potential was not significantly different (P>0.05 Krustal-Wallis test), as illustrated in panel B of the same figure. Averaged levels of desensitization are expressed as the percentage of peak reduced to steady-state, and were comparable at around 70% (-40mV: 68 ± 2 %, +40mV: 68 ± 3% and -100mV 74 ± 2 %).

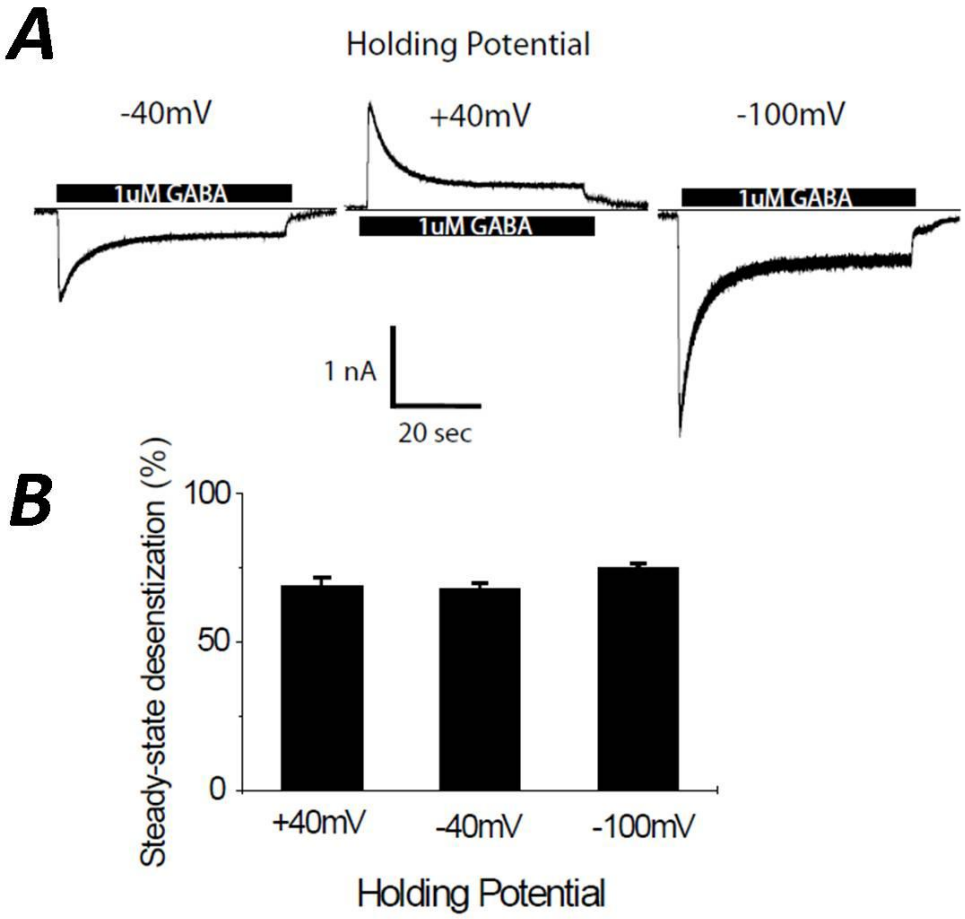

Figure 19. The extent of α6βδ GABA-AR desensitisation is not voltage dependent A, representative continuous whole-cell recordings from *α6βδ* transfected HEK293 cells, clamped at -40mV, +40mV and -100mV respectively. Duration of 1μM GABA application indicated by black bars. **B,** Comparison of averaged levels of desensitisation, calculated as the percentage of peaks reduced to steady-state, at different holding potentials. Levels are not significantly different (P>0.05 Kruskal-Wallis test), suggesting that the holding potential does not affect desensitization.

3.1 Summary

Studies on recombinant αβδ receptors have proved problematic, with many independent research groups struggling to reliably express functional δ containing GABA-A receptors in recombinant systems. As such we sought to start out on this set of experiments by confirming and monitoring δ subunit surface expression and its contribution to GABA evoked currents. To this end we demonstrated δ incorporation functionally by using zinc to selectively block binary receptor mediated conductances and confirm the presence of δ. Further, we utilised a pH sensitive fluorophore (SEP) tag to visualise surface δ expression and allow for the targeting of appropriate cells for electrophysiological analysis. Analyses confirmed a correlation between SEP fluorescence and αβδ mediated currents, suggesting SEP is a good assay of functional αβδ receptor expression. Analyses also confirmed that a 1:1:1 αβδ cDNA ratio is sufficient to ensure optimal δ expression. Thus, confident that we were seeing recombinant αβδ in our studies, we worked to more comprehensively characterise their properties and pharmacology.

With a view to investigating and comparing the potency of GABA at α4βδ and α6βδ we first carried out some basic pharmacology, locally applying GABA and recording whole-cell responses in voltage-clamp mode. Thus we could visualise the concentration-response relationships of each subtype as shown in section 3.1.3. As well as examining peak concentration response relationships as is the norm, we also applied GABA to steady-state equilibrium, conditions which more closely mimic those found *in vivo* at extrasynaptic sites. Thus we present a more relevant GABA dose response relationship, and one which provided a more appropriate reference for future experiments. While carrying out this analysis, we also noted something of potentially greater significance: that, contrary to the dogma surrounding δ containing GABA-A receptors, these receptors are profoundly desensitizing. More comprehensive analysis examined and compared the rates of macroscopic desensitization and the voltage dependence of desensitization, allowing us to better characterise this property. Critically, we have also shown that this phenomenon is evident at the low GABA concentrations that are thought to exist *in vivo*, and that native αβδ expressed in cerebellar granule neurons (CGNs) showed similar responses. Thus we provide strong evidence that steady-state desensitization of these receptor subtypes is physiologically relevant.

3.2 Pharmacological modulation of αβδ GABA-ARs

While our understanding of extrasynaptic receptors and tonic inhibition is just fifteen years in the making, certain dogma surrounding the properties and pharmacology of this receptor population is firmly entrenched, while conversely there is considerable controversy to be resolved. Our insight into how δ-containing GABA-ARs work centres around a few basic tenets. Notably, that in response to GABA, αβδ receptors are high affinity, low efficacy and minimally desensitizing receptors (Farrant and Nusser 2005). In the previous section we call into question the assertion that these receptors are minimally desensitizing and highlight the importance of using physiologically relevant steady-state GABA concentrations while examining αβδ GABA-ARs. With this set of experiments, we sought to extend our understanding of the pharmacology of extrasynaptic receptors under relevant conditions, and have examined responses to ethanol, neurosteroids, Gaboxadol and copper.

3.2.1 Ethanol does not modulate recombinant α6βδ GABA-ARs

The primary mechanisms by which alcohol enhances inhibitory transmission remain unclear, and the specific molecular targets of ethanol remain enigmatic. There is however robust genetic and pharmacological evidence suggesting a role for the GABAergic system, and more specifically that extrasynaptic receptor mediated tonic inhibition may represent the mechanism responsible for many of the behavioural effects of alcohol (Biggio, Concas et al. 2007). How this modulation is affected, however, remains poorly understood. It has been suggested that recombinantly expressed αβδ containing GABA-A receptors are directly modulated by ethanol (Sundstrom-Poromaa, Smith et al. 2002; Wallner, Hanchar et al. 2003), however this has proved controversial with several other independent research groups not observing ethanol sensitivity of these receptor subtypes (Borghese, Storustovu et al. 2006; Storustovu and Ebert 2006; Yamashita, Marszalec et al. 2006). Here we have examined the sensitivity to physiologically relevant ethanol concentrations of α6βδ containing GABA-A receptors expressed in HEK293 cells.

Ethanol, at levels thought known to elicit behavioural effects *in vivo* (Alifimoff, Firestone et al. 1989; Harris, Trudell et al. 2008), was co-applied with a 250nM GABA dose (figure 20A)

in order to test whether α6βδ mediated GABA currents were enhanced. Steady-state experiments involved the application of GABA to steady-state equilibrium, followed by the co-application of ethanol at increasing concentrations (figure 20A, *top*). As is clear from figure 20A while 250nM GABA evoked a robust steady-state conductance (401 ± 233 pS/pF steady-state) (n=9), but the subsequent co-application of ethanol up to 100mM did little to augment this (10mM EtOH: 393 ± 81 pS/pF, 30mM EtOH: 436 ± 90 pS/pF, 100mM EtOH: 429 ± 101 pS/pF) (fig 20B)(red bars). Similarly, analysis of peak responses showed that GABA evoked currents (1698 ± 233 pS/pF)(n=9) were not enhanced when GABA solutions were supplemented with ethanol (10mM EtOH: 1735 ± 336 pS/pF, 30mM EtOH: 1552 ± 282 pS/pF, 100mM EtOH:1583 ± 266 pS/pF)(fig 20B)(white bars). Statistical analysis examining variance confirmed that ethanol did not alter averaged conductances in either experimental conditions ($P>0.05$ Kruskal-Wallis test), confirming that ethanol has no direct effect on either peak or steady-state GABA evoked currents at α6βδ containing GABA-A receptors.

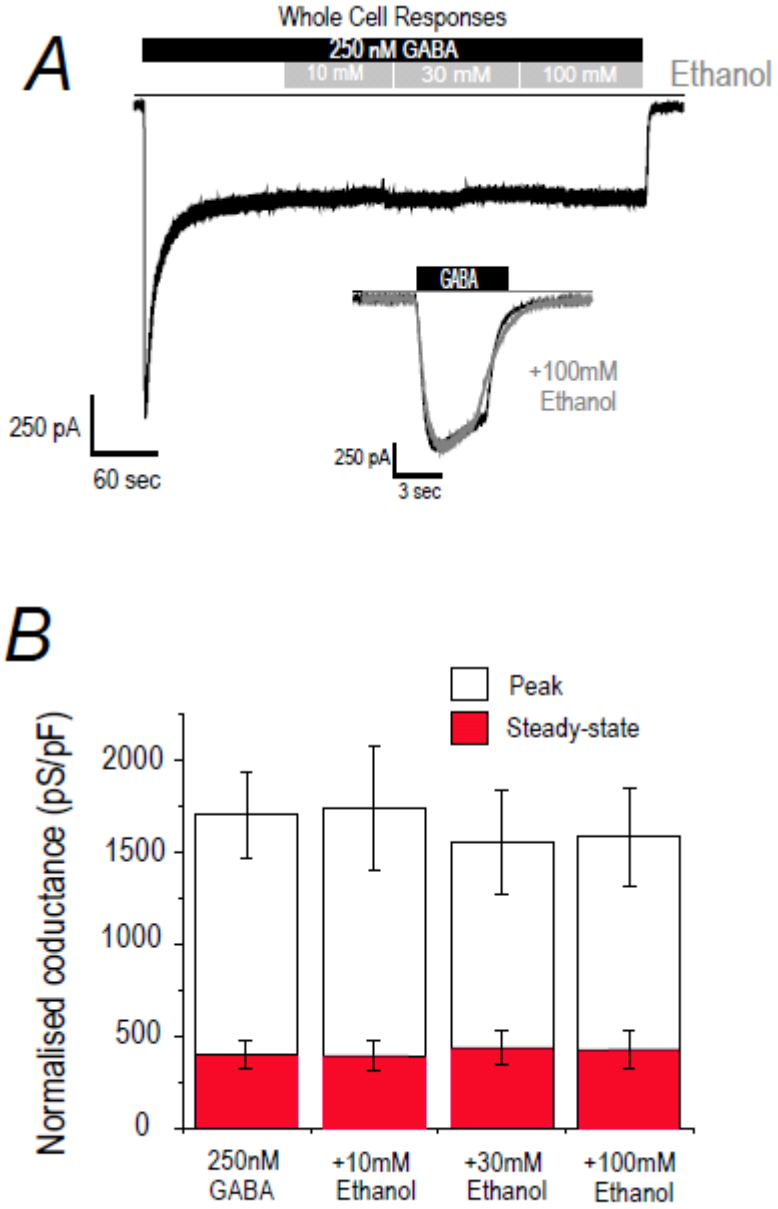

Figure 20. Ethanol does not modulate recombinant α6βδ GABA-A receptors. A, representative continuous whole-cell recording from an *α6βδ* transfected HEK293 cell, voltage-clamped at -60mV. The black bar marks a 250nM GABA application, while the grey bars beneath indicate co-application of increasing levels of ethanol (10-100mM) at steady-state GABA equilibrium. Note there is no apparent ethanol evoked potentiation of steady-state. *Inset* peak responses: 250nM GABA response (black) is overlaid 250nM GABA + 100mM EtOH. Note traces are comparable. **B,** Graph compares peaks (white bars) with steady-state (red bars) conductances evoked by 250nM GABA with 250nM GABA plus ethanol. Error bars represent SEMs. Data suggests there is no effect of ethanol of any concentration on peak or steady-state GABA responses in *α6βδ* containing GABA-ARs (P>0.05 Kruskal-Wallis test).

3.2.2 Neurosteroids potently enhance peak and steady-state α6βδ mediated GABA-A currents

Neuroactive steroids (neurosteroids) are able to rapidly alter neuronal excitability through the stereoselective binding and potentiation of GABA-A receptors (Belelli and Lambert 2005). Furthermore, studies suggest extrasynaptic GABA-ARs are the primary target of neurosteroids and the enhancement of tonic inhibition has been described for a number of different neurosteroids in a number of different brain regions (Belelli, Casula et al. 2002; Wohlfarth, Bianchi et al. 2002; Stell, Brickley et al. 2003; Cope, Halbsguth et al. 2005; Maguire and Mody 2007). Thus, in a similar manner as described previously for ethanol, we have tested the effect of neurosteroids on GABA evoked peak and steady-state currents in α6βδ expressing HEK cells.

Alfaxalone is a potent neurosteroid anaesthetic. Here we have examined its effects on GABA mediated conductances at 30nM, thus avoiding the direct receptor activation evoked by higher concentrations >100nM (Belelli and Lambert 2005). Figure 21A (left) illustrates a typical 250nM GABA response (black), and overlaid is a 250nM GABA response in the presence of 30nM Alfaxalone (grey): note a clear potentiation of both peak and steady-state currents, averaging 109 ± 33 % enhancement of steady-state currents (n=7). To further highlight this observation the average data for GABA and alfaxalone has been normalised to the 100μM GABA peak response (G_{MAX}) and plotted in Figures 21B and C. It is clear from a Hill fit to these data that the peak responses for α6βδ GABA-A receptors resulted in an enhanced G_{MAX} in the presence of Alfaxalone (grey fit): 130% (n=6) when compared to GABA alone. Interestingly however, this did not involve a shift to the left in the dose-response relationship as the GABA EC50 values for peak responses were comparable in both conditions: 430nM versus 400nM (see figure 21B). The pattern is evidently the same when examining the steady-state GABA dose-response relationship, with and without Alfaxalone (figure 21C), as the Hill fits clearly show 30nM Alfaxalone at steady-state enhances the GABA evoked currents across the range of concentrations (G_{MAX} 123% of control) without significantly influencing the EC50 (74nM verus 44nM) (see figure 21C). Thus, consistent with published findings, we have see that the neurosteroid Alfaxalone can positively modulate GABA responses at α6βδ, and critically, that this effect is seen at both peak and

steady-state. Interestingly, these data also suggest that the mechanism of action does not involve GABA binding, as the potency of GABA was not affected.

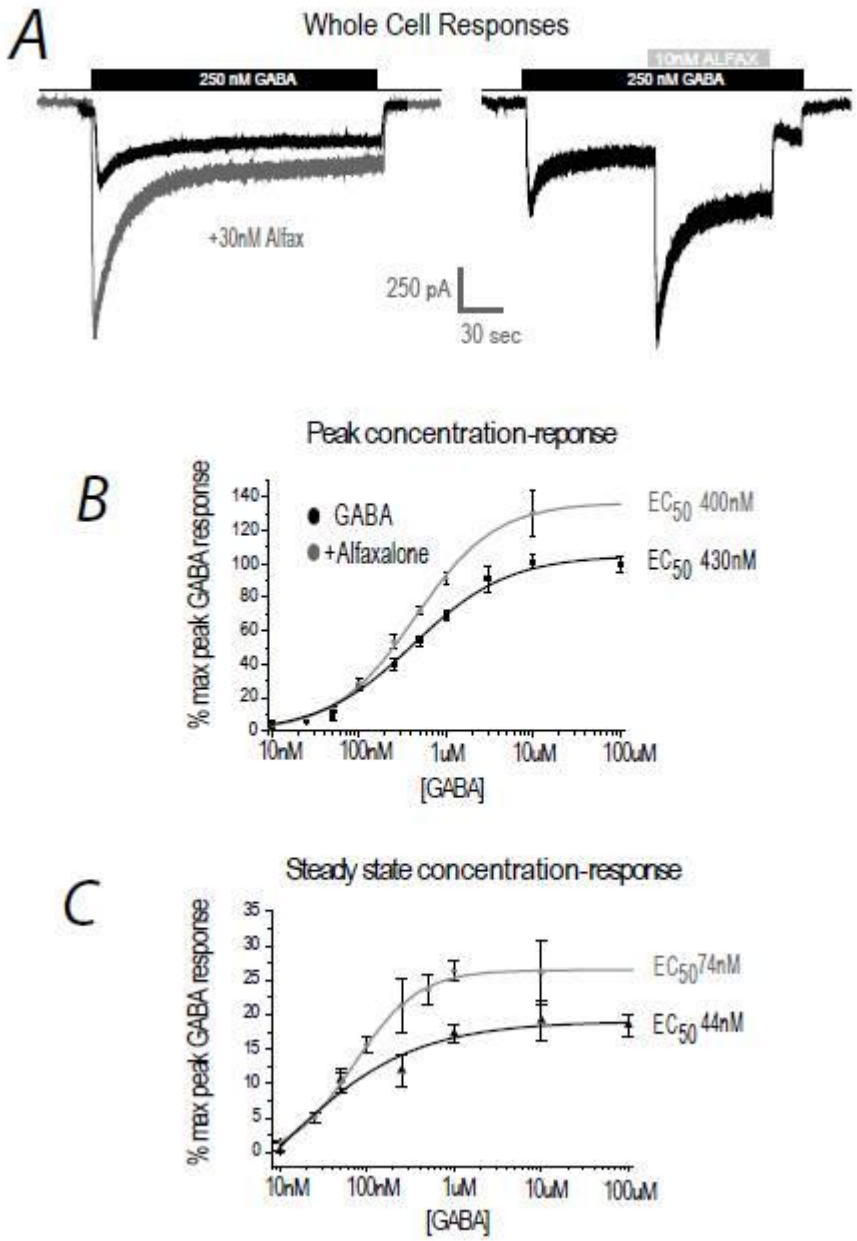

Figure 21. Alfaxalone potently enhances GABA evoked peak and steady-state currents in HEK cells expressing α6βδ GABA-ARs. A, Representative whole-cell recording voltage-clamped HEK cell. A, *left*, 250nM GABA response (black) over laid by a GABA +30nM alphaxalone response. Note alphaxalone evoked a marked increase in the peak and steady-state current levels. **B,** Peak GABA concentration-response relationships comparing responses with (grey) and without (black) 30nM alphaxalone. Note an increase in maximal responses with little effect on EC50. **C,** equivalent to B, comparing steady-state conductances. Results show alfaxalone potentiates *α6βδ* mediated GABA responses without influencing GABA affinity.

We also tested the endogenous neurosteroid Tetrahydrodeoxycorticosterone (THDOC) in a similar experimental paradigm. Here too we see that at low nM concentrations, well beneath the level needed for direct receptor activation, THDOC enhances GABA evoked currents. Figure 22A shows a typical 250nM GABA response allowed to reach steady-state equilibrium before the co-application with 10nM THDOC. The THDOC robustly enhances the α6βδ GABA-AR mediated steady-state currents, from an average of 602 ± 27 pS/pF (n=7) in 250nM GABA alone to 974 ± 184 pS/pF (n=9) in GABA plus 10nM THDOC. Similarly THDOC potentiated peak GABA currents at these receptors, enhancing average GABA conductances from 1455 ± 255 pS/pF to 2348 ± 393 pS/pF (see figure 22B). To examine the effect of THDOC on the GABA dose-response relationship, we normalised THDOC and GABA responses to the 1 mM GABA response (G_{MAX}) and generated curves using a Hill fit to the data (Figure 22C). Results show that, similar to alfaxalone, 10nM THDOC potently enhanced GABA currents across the concentration range, increasing the G_{MAX} to 152% of GABA alone, while having little effect on the GABA potency (EC50 500nM with THDOC (grey line) versus 430nM for GABA alone(black line)).

THDOC and alfaxalone data confirm that neurosteroids act as potent modulators of GABA evoked currents at α6βδ containing GABA-ARs. However, they also suggest that this mechanism does not involve GABA affinity as is the case with many other allosteric modulators of GABA-ARs. It has been suggested that neurosteroids may exert their effects by altering levels of receptor desensitization, and in light of our discovery that these extrasynaptic δ-containing receptors are profoundly desensitized in physiological conditions this may represent an optimal means of potentiating their activity. To test this prediction, we compared peak and steady-state responses of GABA and GABA + 10nM THDOC respectively (Figure 23A). Fitting this data, peaks in black and steady-state in red, supports the suggestion that THDOC may reduce the level of steady-state desensitization, i.e. the ratio of peak to steady-state across the concentration range is decreased. To better illustrate this, the ratio's of peak to steady-state (steady-state desensitization) is plotted against GABA concentrations in figure 23B. Fitting this data with a Hill equation showed that, as expected, the level of steady-state desensitization is decreased across the concentration range, resulting in a THDOC induced 10-fold shift in the EC50 of desensitization (80nM versus 1μM). This extends to high GABA concentrations, so that at full GABA-A receptor occupancy, the

steady-state receptor desensitization was reduced from 80% in control to only 60% in the presence of 10 nM THDOC.

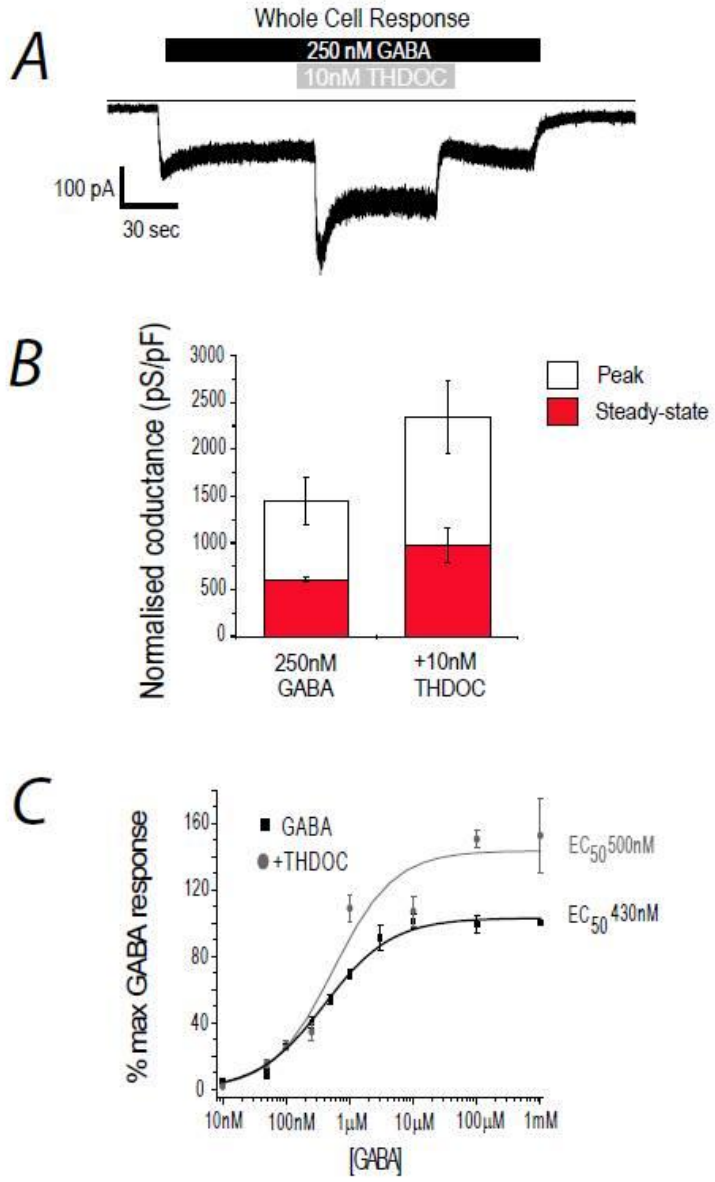

Figure 22. THDOC potently enhances GABA evoked peak and steady-state currents in HEK ells expressing α6βδ GABA-ARs. A, Representative whole-cell recording voltage-clamped HEK cell. 250nM GABA was locally applied (black bar) and allowed to reach steady-state equilibrium, before 10nM THDOC (grey bar) was then co-applied. Note THDOC evoked a marked increase in the steady-state current level. **B,** Graph shows averaged GABA and GABA +THDOC evoked conductances, comparing peak (white bars) with steady-state (red bars) levels. Error bars represent SEMs. Note co-application of THDOC enhances both the peaks and steady-states. **C,** GABA concentration-response relationships comparing responses with THDOC (grey) and without (black). Note an increase in maximal responses with little effect on EC50.

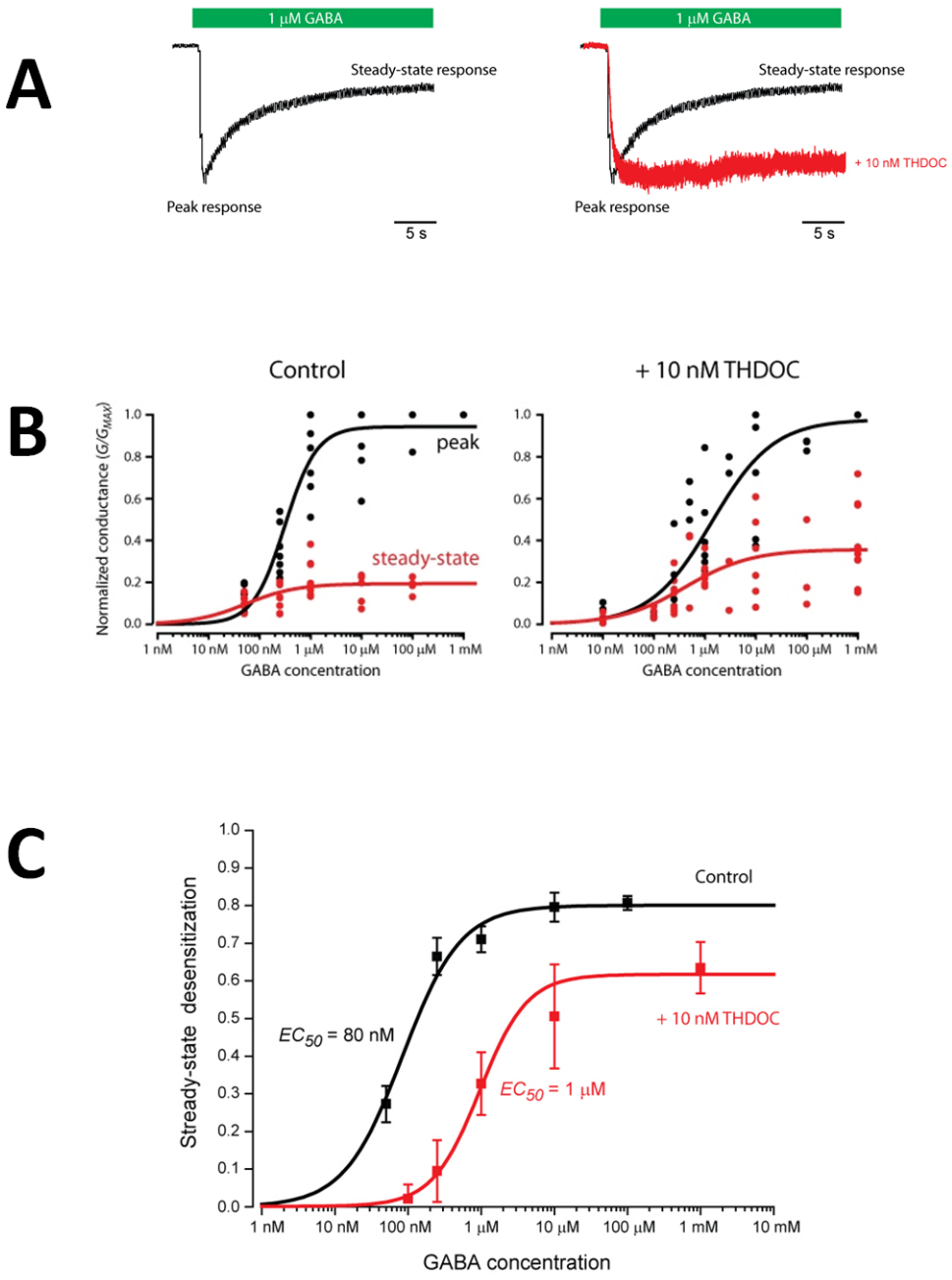

Fig 23. Steady-state desensitisation of α6βδ GABA-A, with and without 10nM THDOC. **A,** Representative whole-cell recording of a voltage-clamped HEK cell. 1μM GABA was applied with and without 10nM THDOC. Note, in the presence of THDOC responses are markedly less desensitizing. ***B***, the steady-state concentration–response curve for the peak (black) and steady state (red) response. The left graph showing GABA responses, and the right GABA responses in the presence of 10nM THDOC. The solid lines are a fit of the Hill equation to all the data points normalised to the 1mM peak response, ***C***, The ratio of the peak to steady-state current was used to construct the steady-state desensitisation plot. The solid line is a fit of the Hill equation, giving a desensitisation EC50 in control conditions of 80 nM, while in the presence of THDOC this increased to 1μM. Error bars reflect ± standard error of the mean (SEM).

3.2.3 Gaboxadol is not a super-agonist at α6βδ GABA-ARs under steady-state conditions

Gaboxadol (THIP: 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol), is a powerful hypnotic with selectivity for extrasynaptic δ-containing GABA-A receptors, where it is thought to act with high potency and supramaximal efficacy, earning it the mantle of 'super agonist' (Brown, Kerby et al. 2002; Stórustovu and Ebert 2006). However, as these past studies have relied on peak response data in evaluating this relationship, we sought re-examine Gaboxadol's effects at extrasynaptic GABA-A receptors under steady-state equilibrium conditions. Thus we have re-evaluated and compared the concentration-response relationships of GABA and Gaboxadol at recombinant α6βδ GABA-A receptors. Figure 24A illustrates typical response to 10μM GABA, 1μM Gaboxadol and 10μM Gaboxadol respectively, in which the peak and steady-state responses have been marked. The peak 10μM GABA responses averaged 4.0 ± 0.9 nS/pF (n = 22) while the peak Gaboxadol evoked conductances stood at 11.4 ± 5.2 nS/pF (n = 10). Therefore consistent with published reports, Gaboxadol has supramaximal efficacy at α6βδ receptors and can be considered a super agonist. However when we compared this with the steady-state responses as illustrated in the same figure, we saw that the desensitzation is such that the steady-state conductance elicited by 10μM Gaboxadol was no larger than that evoked by 10μM GABA: 633 ± 160 pS/pF (n=10) versus 600 ± 84 pS/pF (n=13). Concentration-response curves for peak and steady-states were constructed to investigate this phenomenon further, with average data normalised to a maximal 1mM GABA response (figure 24B). Here too it is evident that while Gaboxadol displays superagonism compared to GABA at peaks, this is not the case at steady-state. In fact, a Hill fit to these data suggest that the steady-state responses for for Gaboxadol at α6β2δ GABA-A receptors, which resulted in a G_{MAX} of 0.1, was smaller than that seen for GABA (G_{MAX} of 0.2). Therefore, contrary to the situation for peak responses, we suggest Gaboxadol should not be considered a 'super agonist' under steady-state conditions.

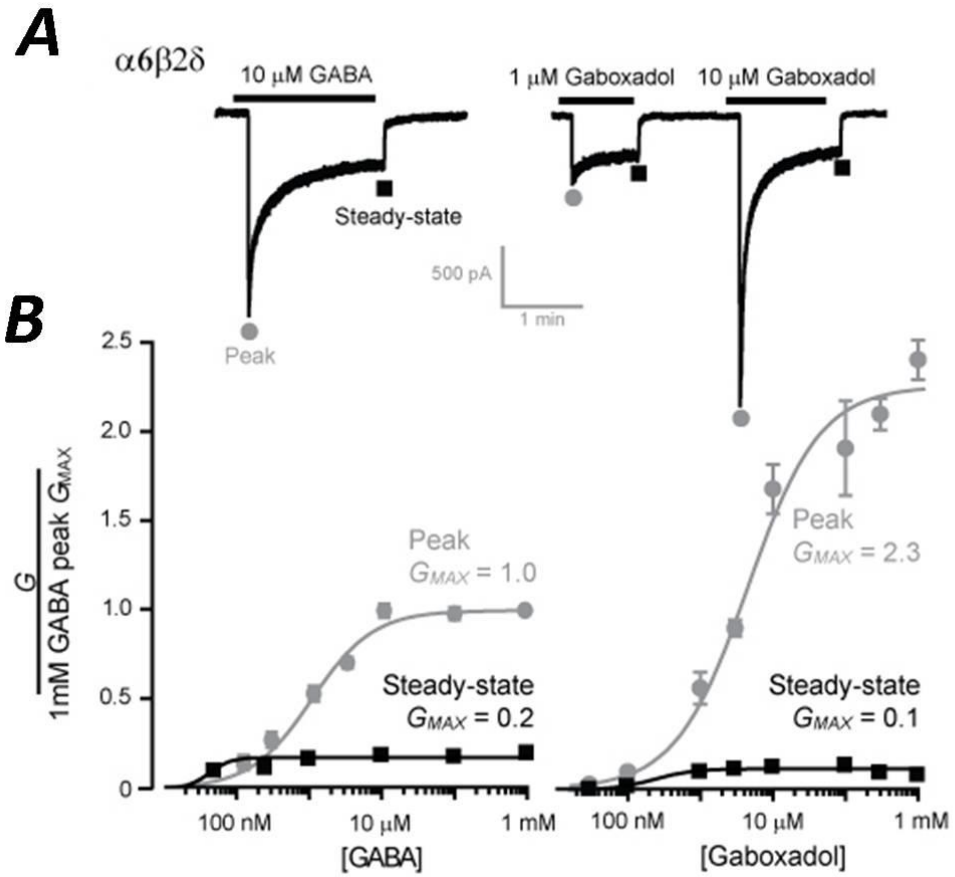

Figure 24. Gaboxadol is a super-agonist under peak but not steady-state GABA conditions. **A,** Typical responses to 10μM GABA, 1μM Gaboxadol and 10μM Gaboxadol, with peak and steady-state responses marked by grey circles and black squares respectively. **B,** Concentration-response relationships of GABA and Gaboxadol respectively, at *α6βδ* GABA-ARs. Responses are normalised to the 1mM GABA. Both peak (grey line) responses and steady-state (black) as well as peak responses have been plotted. As is clear from a Hill fit to steady-state data, the steady-state responses for α6βδ GABA-A receptors resulted in a G_{MAX} of 0.1 for Gaboxadol, compared to a G_{MAX} of 0.2 for GABA. Therefore, contrary to the situation for peak responses, Gaboxadol should not be considered a 'super agonist' under steady-state conditions.

3.2.4 High affinity Copper block of α6βδ containing GABA-ARs

For the next set of experiments we wished to investigate the modulation of α6βδ containing GABA-A receptors by Propofol. However, during the course of control experiments we observed the anomalous GABA evoked currents, with unknown cause. An example of how these currents manifested can be seen in the middle grey trace on figure 25A. These currents were smaller than the typical GABA responses (see overlaid on same figure) and gave rise to apparently non-desensitizing currents without peaks. We investigated further and discovered that the abberant GABA responses were only evident when the GABA was applied via one of the available lines, and critically this line had recently been changed to include a metal connector. Thus we concluded that metal ion contamination which was leaching from the metal connector into our drug solutions was affecting the GABA evoked responses at α6βδ receptors. Investigations of the metal composition as well as trial applications onto cells revealed this contaminant as copper (Cu^{2+}) (see figure 25A, *right*).

The inhibition of synaptic GABA-A receptors by Cu^{2+} has been appreciated for some time (Narahashi, Ma et al. 1994; Trombley and Shepherd 1996; Sharonova, Vorobjev et al. 1998), although it seems extrasynaptic δ-containing GABA-ARs have so far been over looked. Here we co-applied GABA and 1μM Cu^{2+} and observed an signifcant attenuation of peak and steady-state 10μM GABA evoked responses. Further experiments concentrated on the effect of Cu^{2+} on steady-state GABA currents (see figure 25B). Application of Cu^{2+} robustly and dose-dependently blocked steady-state GABA currents, displaying a relatively slow association, and an even slower wash off. In fact in most cases a component of the copper block remains even after minutes of wash application (see figure 25B).

The Cu^{2+} block was evident at low nanmolar concentrations, 10nM Cu^{2+} eliciting 10.5 ± 1.5 % (n=12) block of steady-state currents, and this maxed out at 10μM Cu^{2+} resulting in 65 ± 6 % (n=7). Thus even at saturating Cu^{2+} concentrations a steady-state GABA evoked current persists. To further investigate the potency of this effect we applied copper, across a concentration range, to 1μM GABA evoked steady-state responses, and calculated the percentages of inhibition. Thus, in a steady-state equilibrium paradigm, which is arguably most similar to the environ *in vivo*, we generated a dose response relationship for copper at α6βδ (see figure 25C). Fitting this curve with a Hill equation, as seen with the solid line in figure 25C, gives rise to an IC_{50} for this interaction of 900nM (n = 12).

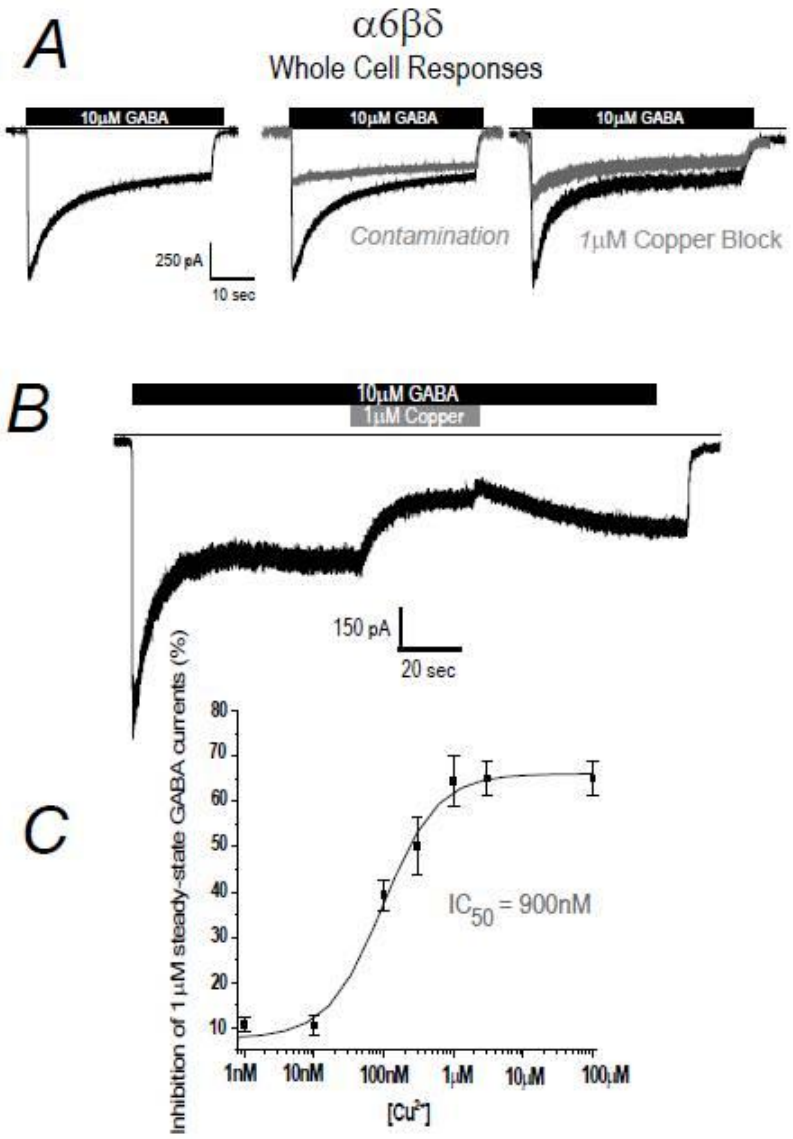

Figure 25. Contamination highlights copper block of α6βδ GABA-A receptors. A, 10μM GABA responses. Contamination significantly attenuated GABA currents (grey, middle), investigations revealed this to be copper ions, and the effect could be replicated by exogenous application 1μM copper in the extracellular solution (grey, right). **B,** 10μM GABA was applied to steady-state equilibrium (black bar) before co-application of 1μM copper (grey bar). As illustrated, copper robustly blocks steady-state GABA currents in a dose-dependent manner. ***C,*** Steady-state inhibition of GABA currents by a range of copper concentrations were used to examine the concentration-response relationship at α6βδ. The solid line is a fit of the Hill equation, giving rise to an IC_{50} of 900 nM.

To understand the nature of the copper block at α6βδ and ascertain if GABA binding is effected, we investigated the GABA dose-response relationship on the presence of an IC_{50}

dose of Cu^{2+} (i.e. 1μM). Consistent with earlier findings GABA evoked peak and steady-state conductances were markedly smaller in the presence of 1μM Cu^{2+}. For example where as peak 10μM GABA responses averaged 4.0 ± 0.9 nS/pF (n = 22) and steady-states 633 ± 160 pS/pF (n=10), the addition of 1μM Cu^{2+} reduced these to 908 ± 114 pS/pF (n=8) and 318 ± 44 pS/pF (n=8) respectively (see figure 26A). Analyses were carried out across the GABA concentration range and responses were normalised to the maximal currents, susequently a dose-response curve was generated using a Hill fit to this data (Figure 26B). From this fit peak and steady-state EC_{50} values were calculated as 350nM and 130nM respectively. This compares with GABA EC_{50} values when Cu^{2+} is not present of 430nM and 70nM for peaks and steady-states respectively, thus suggesting the effect of copper on α6βδ does not impact GABA affinity.

Past studies examining Cu^{2+} block at γ-containing GABA-A receptors in a recombinant system suggested an IC_{50} in the tens of μM range (Narahashi, Ma et al. 1994; Fisher and Macdonald 1998). Here we suggest a sub-micromolar IC_{50} association with δ-containing GABA-ARs, a full order of magnitude lower than those reported for synaptic receptors, and consequently pose the question of whether Cu^{2+} might selectively target one receptor subset over another. To that end we examined the copper block of αβγ-containing GABA-A receptors in the steady-state paradigm previously described, and compared the dose-response relationships of both receptors subtypes. At submicromolar Cu^{2+} there was no discernable block of αβγ receptor mediated steady-state currents (n = 11), compared with a 11 ± 1.5 % block of αβδ mediated currents by 100nM Cu^{2+}. In αβγ a small suppression of GABA currents was evident in 1μM Cu^{2+}: 5 ± 3 % (n = 8), while the same concentration at αβδ containing GABA-A receptors blocked a full 39 ± 3 % (n = 8) of the 1μM GABA evoked current. To further highlight this phenomenon a full copper inhibition curve was constructed, fitted a Hill equation (figure 27B). This was used to calculate the Cu^{2+} IC_{50} at αβγ receptors at 85 μM, 2 orders of magnitude higher than that seen at αβδreceptors (900nM). Thus suggesting that at low concentrations Cu^{2+} will selectively block δ-containing GABA-A receptors.

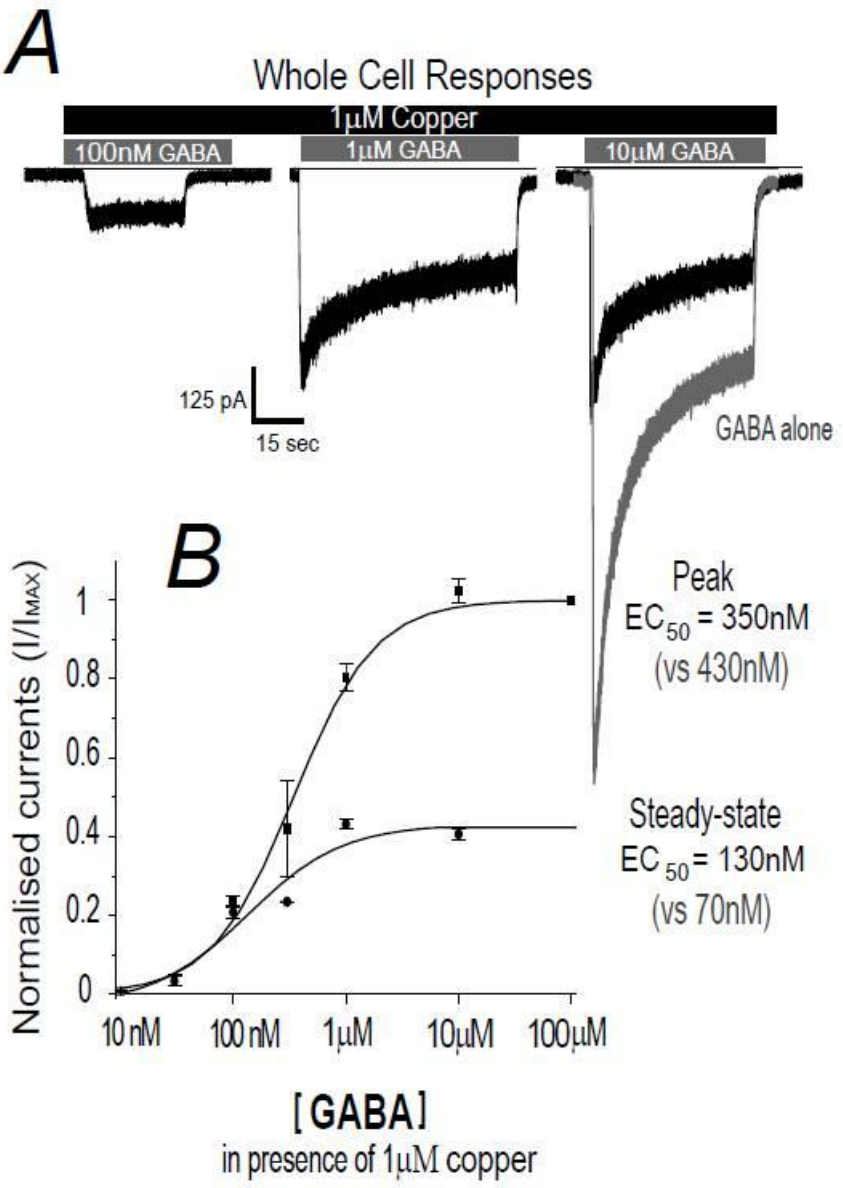

Figure 26. Peak and steady-state GABA dose response relationships at α6βδ in the presence of 1μM copper. **A**, representative continuous whole-cell recordings from *α6βδ* transfected HEK293 cells, voltage-clamped at -60mV. The black bar marks the 1μM copper application, while the grey bars beneath indicate co-application of different GABA concentrations (100nM-10μM). Overlaid the 10μM GABA response is a typical GABA response without copper, note the larger peak and steady-state conductances. **B,** GABA currents in the presence of copper were used to generate a GABA dose response curve at α6βδ. The solid line is a fit of the Hill equation, giving rise to a peak EC_{50} of 350nM and a steady-state EC_{50} of 130nM. This is comparable with GABA EC_{50}'s generated without copper present (bracketed EC_{50}s), suggesting the copper block mechanism does not involve GABA binding.

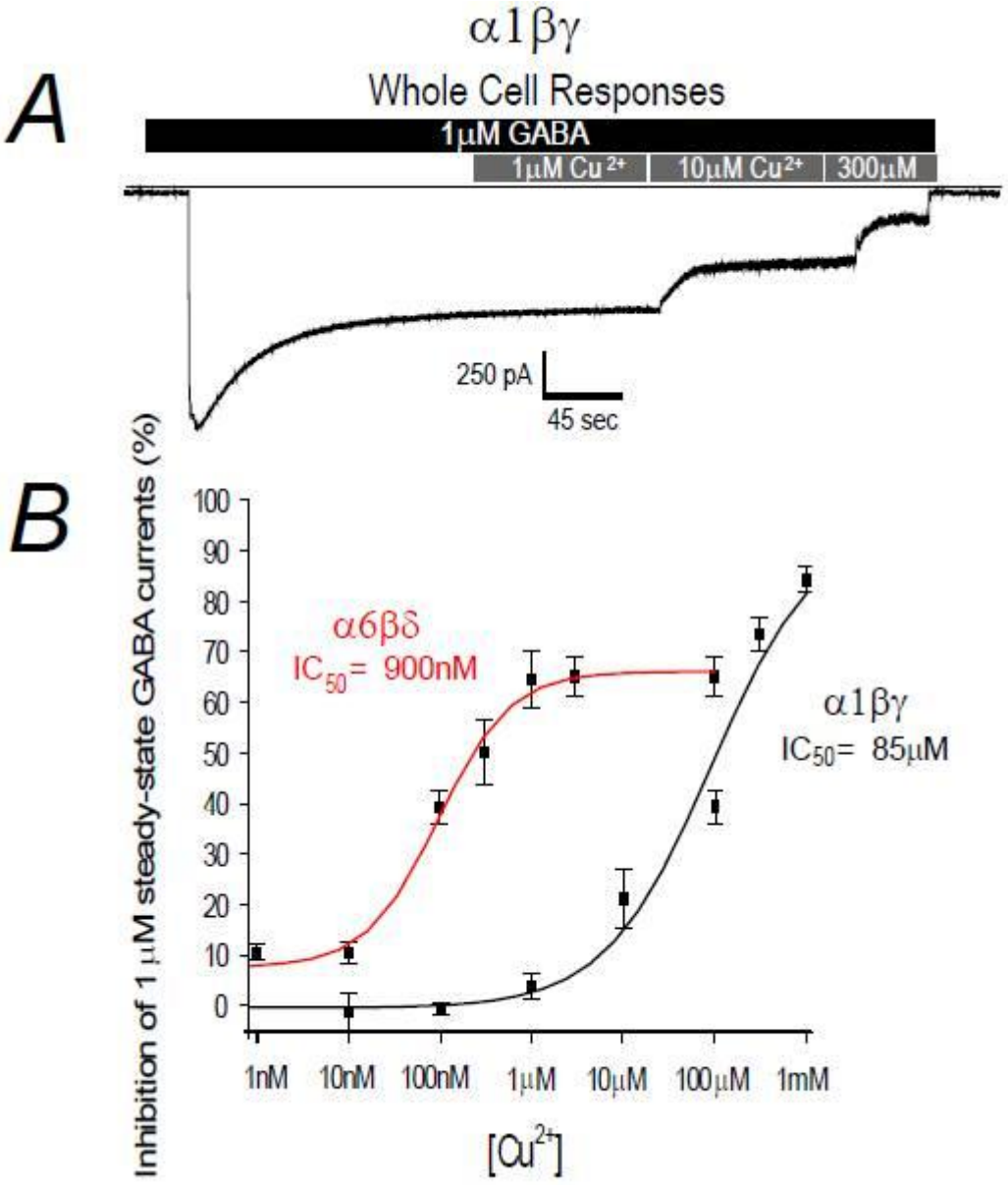

Figure 27. α1βγ are less sensitive to copper block than α6βδ. **A**, representative continuous whole-cell recordings from a *α6βδ* transfected HEK293 cell, voltage-clamped at -60mV. 1μM GABA was applied to steady-state (black bar), subsequently different copper concentrations were co-applied (1μM -300μM). Note that 1μM copper did little to suppress the GABA current, while 10μM and upwards showed significant block. . **B,** Steady-state inhibition of GABA currents by a range of copper concentrations were used to examine the concentration-response relationship at *α1βγ*. The solid black line is a fit of the Hill equation to the *α1βγ* data, giving rise to an IC_{50} of 85μM. Compare this with the red curve fitting the *α6βδ* equivalent data with an IC_{50} of 900nM, suggesting that at low to sub-micromolar concentrations copper will selectively block *δ*-containing GABA-A receptors.

3.2 Summary

With this set of experiments we sought to examine the pharmacology of recombinant α6βδ containing GABA-A receptors using local drug applications and patch-clamp electrophysiological techniques. In contrast to past studies we have focused not just on peak responses but on the effect of these drugs at steady-state equilibrated levels. And in this way we have gained insights into the effects of ethanol, Gaboxadol, neurosteroids and copper on this receptor subtype. Ethanol modulation of peak and steady-state GABA evoked responses was examined, and contrary to published findings we have shown that ethanol does not directly potentiate δ-containing GABA-A receptors. Next we examined the neuroactive steroids THDOC and alfaxalone, which are known to be potent enhancers of GABA-A receptors. Here we demonstrated that, even at low nM concentrations, these steroids potentiate both peak and steady-state GABA currents. We also suggest a mechanism of neurosteroid enhancement, which was to decrease the extent of GABA induced receptor desesenstization and thus increase the input conductance. Gaboxadol is a potent hypnotic and is believed to act as a 'superagonist' at δ-containing GABA-A receptors. Here we examined peak and steady-state Gaboxadol induced conductances, and can report that while this drug does display supramaximal efficacy at peaks, it is in fact not a 'superagonist' under physiologically relevant steady-state conditions. Finally we sought to examine propofol, but during experiments a chance contamination led to the novel disovery that Cu^{2+} ions potently block α6βδ receptors. Further examination revealed this Cu^{2+} block at α6βδ displayed nM affinity, in contrast to αβγ containing receptors which are much less sensitive to this effect.

Building on these investigations of the pharmacolgy α6βδ-containing GABA-A receptors, in the next section we investigated the influence of ambient GABA concentrations on the modulation of extrasynaptic receptors by certain drugs.

3.3 Ambient GABA concentrations can influence the potency of certain drugs at αβδ containing GABA-ARs

Tonic GABA-mediated inhibition is thought to reflect the persistent activation of extrasynaptic GABA-A receptors by the low levels of ambient GABA present within the extracellular space. This conductance, while persistent, is not static, and fluctuates secondary to variations of ambient GABA levels, both in normal physiology and in certain pathologies. Indeed in recent years disturbances in this tonic conductance have been implicated in a number of neurological disorders, and consequently is beginning to emerge as an important clinical target for a number of drugsincluding: anaesthetics, anti-epileptics and sleep promoters. With this next set of experiments we sought to investigate what effect, if any, variations in the levels of ambient GABA has on the potency of these drugs at extrasynaptic GABA-A receptors. To this end we examined α6βδ-containing GABA-A receptors in HEK 293 cells and in CGNs, and monitored the effect of varying ambient GABA levels on the modulation of these receptors by Propofol, Gaboxadol and neurosteroids.

3.3.1 Propofol modulation of α6βδ GABA-ARs is critically dependent on the level of ambient GABA

Propofol is a short-acting, intravenously administered hypnotic which is widely used for the induction and maintenance of general anaesthesia. While its molecular targets are numerous it is believed that many of its behavioural effects are mediated by synaptic and extrasynaptic GABA-A receptors where it acts potently to enhance GABA mediated neuronal inhibition (Brown, Kerby et al. 2002; Feng and Macdonald 2004; Franks 2008; Wallner, Meera et al. 2009). However, this effect has not been examined in the presence of fluctuating levels of physiologically relevant steady-state GABA, and thus with this set of experiments we set out to investigate the effect that variations in ambient GABA concentration have on the propofol induced potentiation of α6βδ receptors.

Propofol is a small, relatively apolar molecule and is thought to bind preferentially to pre-formed cavities within receptors, causing no significant structural rearrangements. At

physiologically relevant concentrations (<2μM) the effect of propofol binding is to enhance channel opening by stabilising the GABA bound state causing a prolongation of deactivation and a parallel leftward shift in the dose-response curve (Orser, Wang et al. 1994; Orser, Bai et al. 1999). Thus it has been suggested that propofol potentiates GABA-A receptor populations simply by shifting the equilibrium in favour of these anaesthetic bound states (Franks 2008). Consistent with this profile we hypothesised that propofol enhancement of GABA-A receptor mediated currents would be proportionally less pronounced as the level of GABA occupancy increase. Using a simple model generated in collaboration with Dr. Stephen Brickley we confirmed the validity of this prediction (see figure 28A). In this kinetic scheme, based on the classical Jones and Westbrook model (Jones, Sahara et al. 1998), propofol was treated as simply binding to all the open and desensitized states with equal affinity (KD = 1 μM). In this form this scheme predicts the key effects of propofol which are observed experimentally, i.e. a large prolongation of deactivation and a parallel leftwards shift in the GABA dose-response curve (figure 28B). Consistent with these effects, this model predicts that propofol enhancement of GABA evoked currents will be critically dependent on ambient GABA concentrations (see figure 28C and D).

To test this prediction experimentally we first examined recombinant α6βδ receptors in HEK293 cells. As figure 29 illustrates a range of GABA concentrations from 15nM to 1μM were applied in whole-cell patch-clamp mode, and once the GABA response reached equilibrium, 1.5μM propofol was co-applied. At low nM (sub EC_{50}) GABA concentrations, i.e. 15, 30 and 50nM, propofol robustly enhanced GABA evoked currents by 34.5 ± 11.3% (*n*=4), 30.0 ± 12.5 % (*n*=5) and 29.7 ± 11.5 % (*n*=6) respectively. However, at 250nM and 1μM, concentrations which are well within the expected levels of ambient GABA *in vivo*, the propofol induced potentiation was largely ablated: just 3.4 ± 11.1 % (*n*=6) and 1.4 ± 14.4 % (*n*=6) respectively. Thus, consistent the model driven prediction, propofol enhancement of α6βδ mediated GABA currents is attenuated as GABA levels are raised.

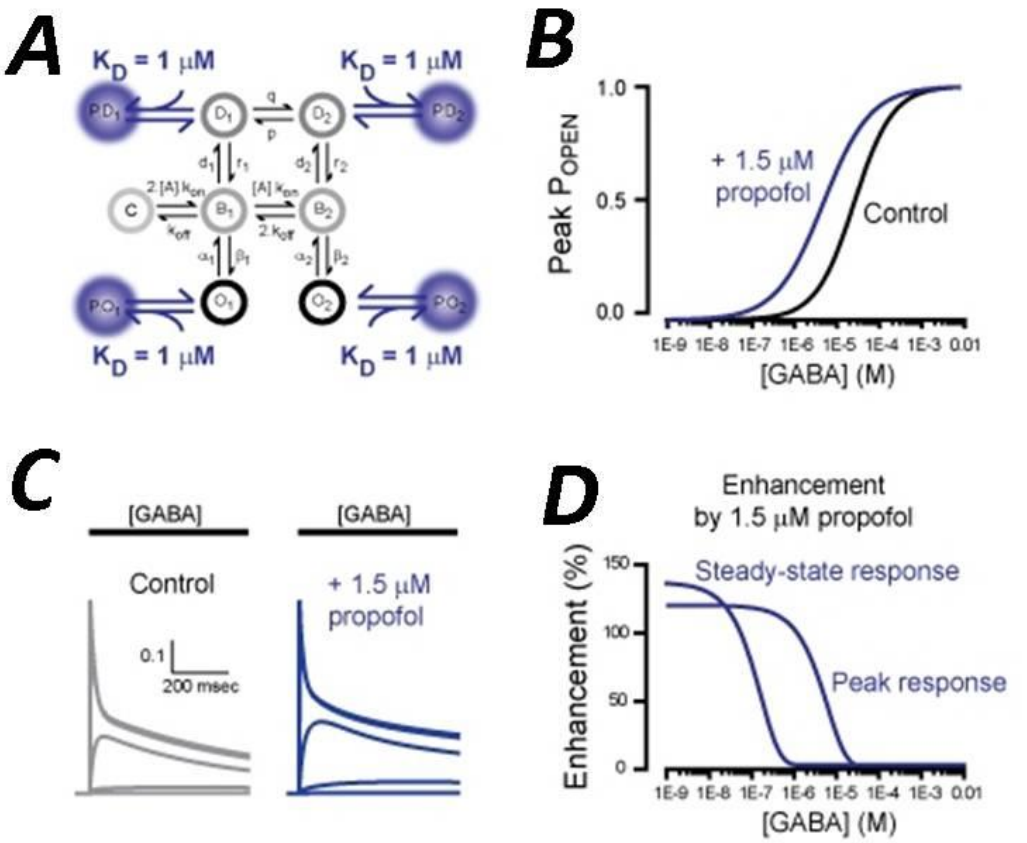

Figure 28. GABA model predicts the attenuation of the Propofol induced enhancement of GABA-A receptors by increasing concentrations of ambient GABA. **A,** Kinetic scheme based on the Jones and Westbrook (1998) quantitative model of a GABA-A receptor. The model has two GABA binding sites (B1 and B2) from which the receptor can enter either a desensitised (closed) state (D1 or D2), an open conductive state (O1 and O2) or enter the unbound closed state (C). Propofol is treated as binding with equal affinity (K_D = 1 μM) to both of the desensitised and open states (P.D1, P.D2, P.O1 and P.O2) causing an increased open probability. **B,** GABA dose response relationship in the presence and absence of 1.5 μM Propofol. Note the increase in GABA potency in the presence of Propofol. **C**, Open probabilities (P_{open}) of a model GABA-A receptor in response to incremental increases in GABA concentration. Illustrating that the P_{open} increases upon the addition of 1.5 μM Propofol. ***D,*** The model predicts that both peak propofols enhancement of peak and steady-state is dependent on the ambient GABA concentration. Simulations were performed in Channelab (version 2, Synaptosoft, Decatur, GA) and data exported and analysed in Origin 6 (Microcal, MA).

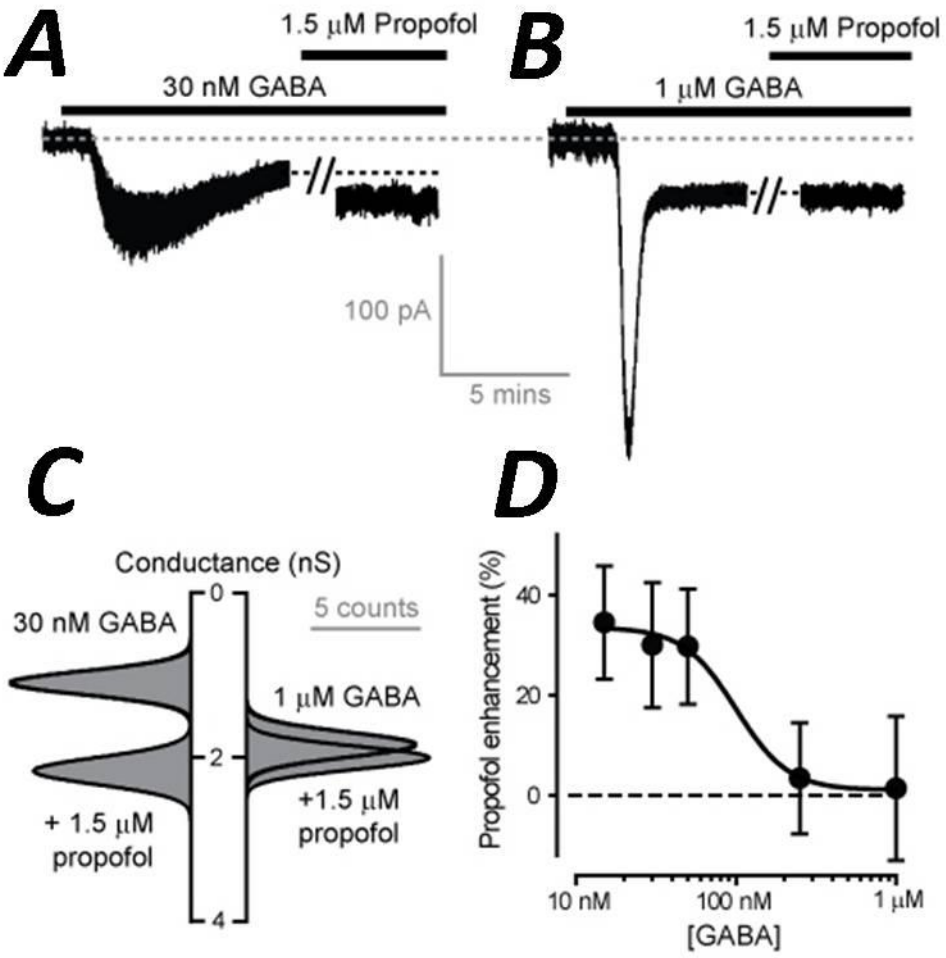

Figure 29. Increasing GABA occupancy attenuates propofol enhancement of steady-state currents at α6βδ containing GABA-A receptors. A, representative continuous whole-cell recordings from a *α6βδ* expressing HEK cell. Cells were exposed to 30nM GABA and once the response reached steady-state 1.5μM propofol was co-applied. Note at this GABA concentration propofol enhances the current. **B,** In contrast, when the same experiment was carried out in 1μM GABA, the propofol effect was abolished. **C,** All points histograms, fitted with a single Gaussian function were generated to further highlight the effect of raised GABA levels on propofol modulation of tonic conductance. ***D,*** The relationship between steady-state ambient GABA levels and propofol induced enhancement. Averaged data was fitted with a Hill equation (solid line), error bars represent SEMs. Note the reduction in response at supra-EC_{50} GABA levels.

We next needed to confirm that this phenomenon extends to native α6βδ containing GABA-A receptors. To this end we targeted CGNs, in which α6βδ-containing GABA-A receptors mediate tonic inhibition. Acute slices of the murine cerebellum were prepared and whole-cell voltage-clamp recordings were made from CGNs located deep within the granule cell layer. As we were 'blind patching', CGNs were identified by their biophysical properties and pharmacology, namely a low capacitance (<8.5pF) and SR95531 (Gabazine) (>50 μM) sensitivity. To establish a control response 1.5μM propofol was applied to CGNs, and as figure 30A illustrates elicited a robust enhancement of the tonic conductance, averaging an increase of 102 ± 26 %. We then wished to bias the ambient GABA levels, and to begin with did so simply through exogenous local application. 100μM was applied and the response allowed to reach steady-state, at which point 1.5μM propofol was co-applied. Contrary to propofol application alone, 1.5μM propofol in the presence of saturating GABA, did not enhance the steady-state current (figure 30B). In fact, averaged results suggest propofol attenuated the tonic conductance under these conditions: -25 ± 4 % (n=6)(figure 30C). However, exogenous application of a high GABA concentration could have multiple effects within the slice, and we therefore wished to alter ambient GABA by more subtle means, to within more physiologically relevant levels. To this end we used 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and tetrodotoxin (TTX). These drugs respectively increase and decrease vesicular GABA release, concomitantly increasing and decreasing ambient GABA levels. (TTX, 0.5 +/- 0.1 Hz, n = 13; ACSF 1.3 +/- 0.4 Hz, n = 14; CNQX, 1.9 +/- 0.5 Hz, n = 16: ANOVA, P=0.04). The mean tonic conductance was 73.5 +/- 7.7 pS/pF in TTX (n = 30) and this significantly increased to 109.1 +/- 13.9 (n = 23) in CNQX (ANOVA, P=0.04).

Under control conditions IPSC frequency averaged 1.3 +/- 0.4 Hz, (n = 14). 500nM TTX blocked action potential dependent GABA release resulting in a reduced mean IPSC frequency of 0.5 ± 0.1 Hz (*n*=13), while 5 μM CNQX increased it to 1.9 +/- 0.5 Hz (n = 16) (p = 0.04 ANOVA). This 4-fold increase in frequency in CNQX compared with TTX was reflected in a significantly increased tonic conductance, from 73.5 +/- 7.7 pS/pF in TTX (n = 30) to in TTX to 109.1 +/- 13.9 (n = 23) when samples were pre-incubated in CNQX (ANOVA, P=0.04) (see figure 31). Thus confident that TTX and CNQX can be used to alter ambient GABA levels, we used these drugs to test our hypothesis that propofol enhancement

of α6βδ receptors is dependent on GABA levels. In normal ACSF (control) 1.5μM propofol increased tonic GABA conductances by an average of 102 ± 26 % (*n*=6). However, in line with previous observations, lowering ambient using 500nM TTX, increased the level of enhancement to 163 ± 29 % (*n*=7), and when GABA levels were raised using 5μM CNQX the level of propofol induced enhancement was halved to 49 ± 3 % (*n*=6) (see figure 32). Thus these results, consistent with our findings in a recombinant system, show that propofols potentiation of extrasynaptic α6βδ is critically dependent on GABA-A receptor occupancy.

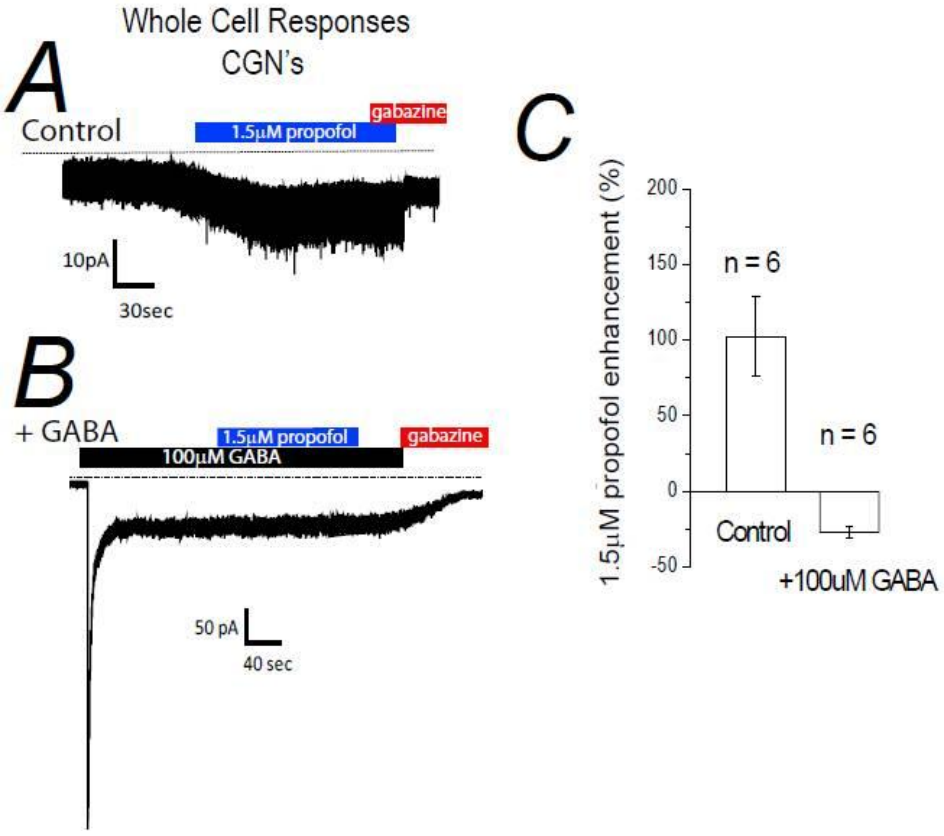

Figure 30. Exogenous GABA application attenuates propofols enhancement tonic currents in cerebellar granule neurons (CGNs). **A,** representative continuous whole-cell recordings from a *α6βδ* expressing CGNs. To obtain control propofol responses, 1.5μM propofol was locally applied, resulting in a robust enhancement of the tonic conductance (left trace). We then raised ambient GABA by applying 100μM to the medium. Once the response reached a steady-state, propofol was co-applied. Gabazine was applied at end of experiment to provide a measure of tonic GABA conductance. As is evident, the raised ambient levels abrogate the propofol response (right trace). **B,** Comparison of 1.5μM propofol induced tonic conductances in control conditions and following 100 μM GABA application. On average, propofol actually inhibited the 100μM GABA invoked steady-state current (-25%). Suggesting that an increase in ambient GABA levels at extrasynaptic receptors may influence their modulation by propofol.

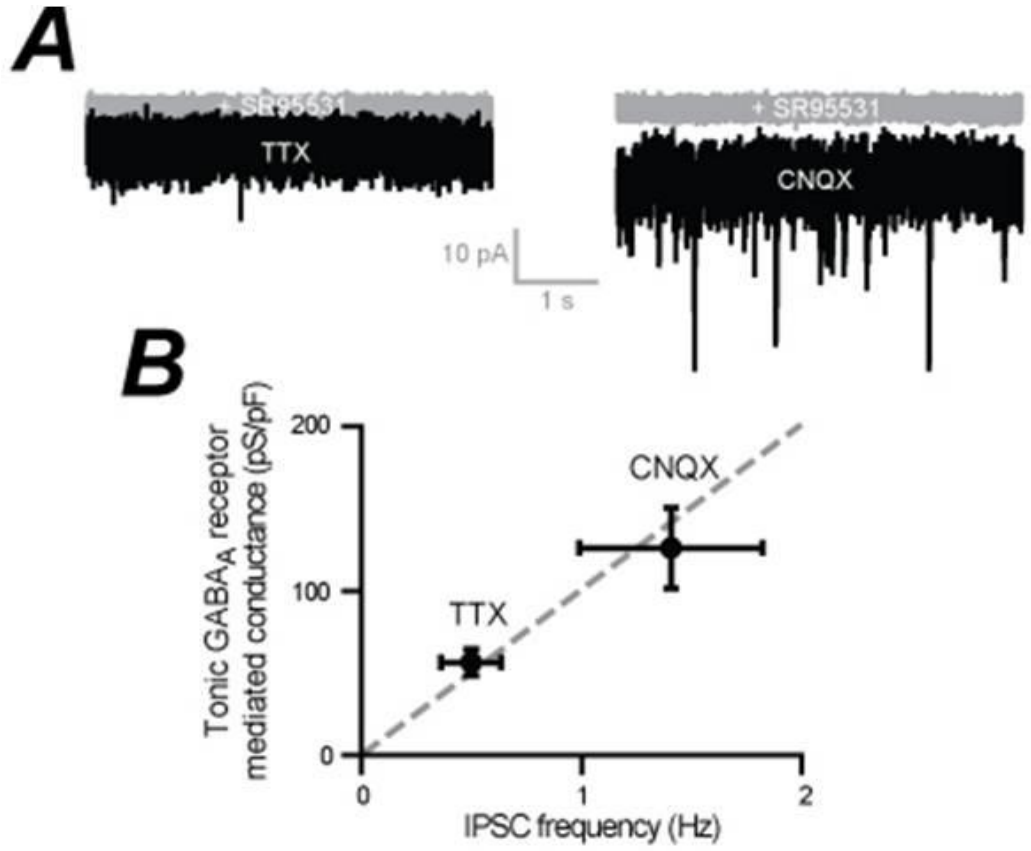

Figure 31. The Modulation of vesicular GABA release by TTX and CNQX alters tonic GABA mediated conductance. It has been reported that ambient GABA concentration and therefore tonic conductance can be influenced by levels of vesicular release. To test this we compared the magnitude of the tonic conductance after vesicular release was decreased by TTX and conversely increased by CNQX. ***A,* (left)** representative whole-cell recording in a CGN bathed in 500nM TTX, voltage-clamped at -60mV (black trace). Above this is the same recording following application of SR95531, a GABA-A receptor blocker (grey trace). The difference between the two represents the tonic current. ***A, (right)*** similarly in 5μM CNQX. Note an increase the tonic conductance in cells bathed in CNQX versus TTX. ***B,*** Relationship between IPSC frequency (Hz) and tonic conductance (pS/pF). Graph suggests TTX and CNQX induced alterations in GABA release frequency will impact ambient GABA levels and tonic inhibition.

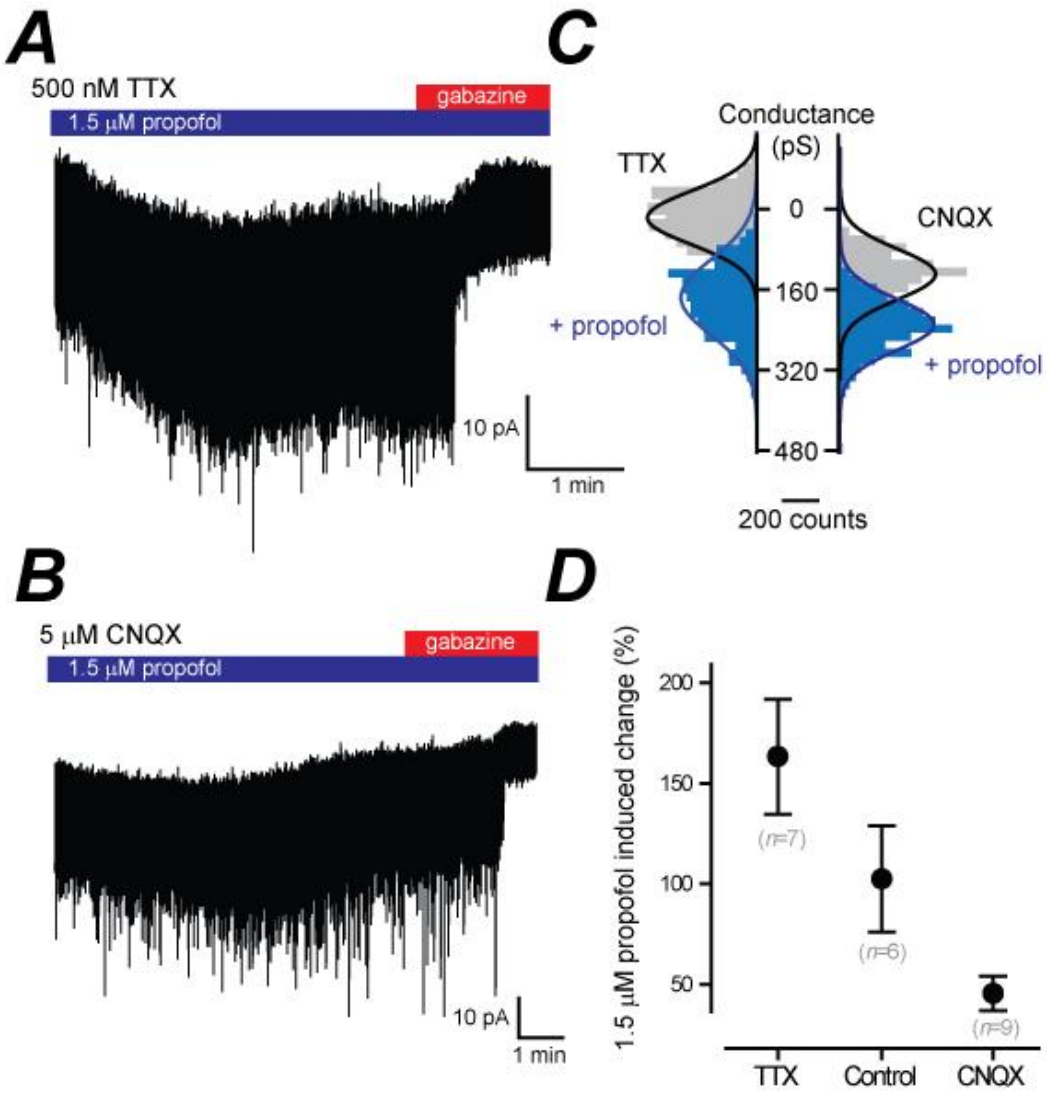

Figure 32. Propofol modulation of GABA-A receptors on CGNs is highly sensitive to fluctuations in ambient GABA. **A**, Representative whole-cell recording of a CGN voltage-clamped at -60mV. The acute cerebellar slice was incubated in 500nM TTX which blocks action potential dependent vesicular GABA release and lowers ambient GABA levels. 1.5μM propofol was locally applied resulting in a robust enhancement of the tonic conductance. Gabazine was applied at the end of the experiment to provide a measure of tonic GABA conductance. **B**, Equivalent to A described above, excepts cells were pre-incubated in CNQX which increases vesicular GABA release and raises ambient GABA levels. Again 1.5μM propofol was applied, however in the case the tonic enhancement was reduced. **C**, conductances described with a single Gaussian fit to the corresponding all points amplitude histogram. Note in grey the control holding current, and in blue the respective propofol induced conductances. **D**, Relationship between the 1.5μM propofol induced enhancement of tonic conductance with drug induced changes in ambient GABA levels. Note that lowering ambient GABA using TTX increases the propofol induced conductance while conversely raising ambient GABA using CNQX largely abrogates attenuates propofols effects.

3.3.2 Neurosteroid modulation of α6βδ GABA-ARs is not influenced by ambient GABA levels

While it is clear that propofol will not modulate tonic inhibition when extrasynaptic receptors are fully occupied, we surmised it was possible that other allosteric modulators were not similarly sensitive. One such candidate is neurosteroids. As suggested in section 3.2.2, neurosteroids enhance tonic conductances by decreasing the extent of steady-state receptor desensitization at extrasynaptic GABA-A receptors, thus we hypothesized that these drugs might prove effective irrespective of ambient GABA levels and receptor occupancy. To test this prediction we first examined the effects of the endogenous neurosteroid THDOC on recombinant α6βδ containing GABA-A receptors. As figure 33A illustrates, 10nM THDOC robustly enhanced α6βδ mediated steady-state currents in the presence of 50nM GABA (~EC_{10}), averaging 67 ± 5 % (n = 7). Interestingly, and in contrast to the previously outlined propofol results, when GABA was increased to saturating levels (i.e. 10μM) (fig 33B), THDOC was still able to augment the steady-state current: 37 ± 4 %. Indeed, our results suggest that that across the entire GABA concentration range tested (10nM - 10μM) THDOC was able to enhance the tonic currents to a similar degree (10nM: 57 ± 8 %, 250nM: 55 ± 15 %, 1μM: 39 ± 7%) (P>0.05 ANOVA) (fig 33C).

To determine if this was also true for clinically relevant neuroactive steroids, we examined the anaesthetic alfaxalone and the anti-epileptic drug Ganaxalone. Alfaxalone was tested first on recombinant receptors, where the level of 30nM Alfaxalone induced enhancement was compared in the presence of an EC_{10} (50nM) and an EC_{50} (250nM) GABA dose (figure 34). As figure 34 shows, the GABA response was allowed to reach steady-state before Alfaxalone was then co-applied, and the increased conductance assayed. Consistent with the THDOC data and in sharp contrast with the propofol findings, the level of alfaxalone induced enhancement of tonic was the same irrespective of the ambient GABA concentration: 114 ± 18 % (n = 7) vs 109 ± 33 % (n = 4) (P>0.05, unpaired *t*-test).

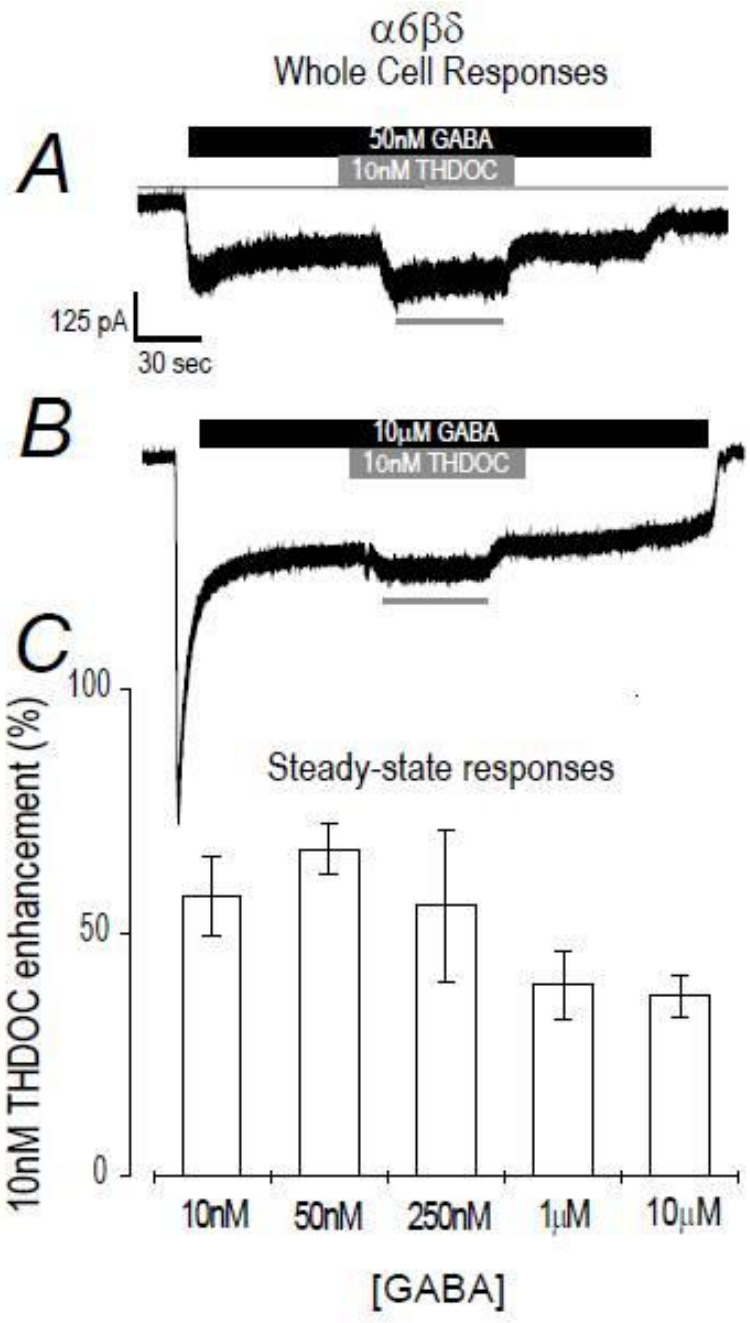

Figure 33. THDOC enhances steady-state GABA currents irrespective of GABA concentration. **A**, Representative whole-cell recording of α6βδ expressing HEK293 cell voltage-clamped at -60mV. 50nM GABA was applied, at steady-state 10nM THDOC was co-applied causing an increase in steady-state conductance. **B**, 10μM GABA was applied to steady-state, at which point 10nM THDOC was co-applied. Note even at this saturating GABA concentration THDOC elicits an enhancement of the steady-state current. **C**, Relationship between ambient the GABA concentration and THDOC modulation of steady-state currents. Averaged results suggest that the endogenous neurosteroid THDOC will enhance GABA evoked currents regardless of ambient GABA level. Populations are not significantly different, P>0.05 ANOVA.

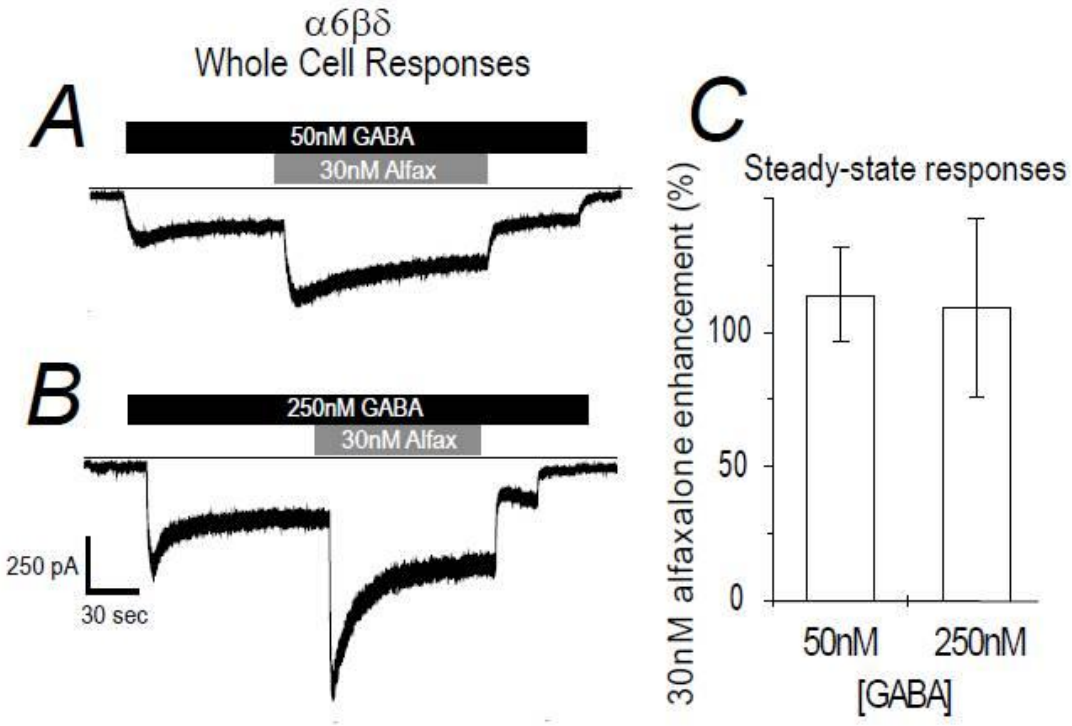

Figure 34. Alfaxalone enhances steady-state GABA currents irrespective of GABA concentration. **A**, Representative whole-cell recording of α6βδ expressing HEK293 cell voltage-clamped at -60mV. 50nM GABA was applied, at steady-state 30nM Alfaxalone was co-applied causing an increase in steady-state conductance. **B**, 250nM GABA was applied to steady-state, at which point 30nM Alfaxalone was co-applied. In contrast to propofol modulation, this EC_{50} GABA dose does not affect neurosteroid enhancement of the steady-state current. **C**, Relationship between ambient GABA concentration and Alfaxalone modulation of steady-state currents. Averaged results suggest that the raising ambient GABA will not influence the extent to which Alfaxalone enhances GABA evoked currents regardless of level. Populations are not significantly different, P>0.05 unpaired *t* test).

Next we needed to confirm these neurosteroid observations extended to native receptors. Therefore we compared the effect of altering vesicular GABA release on alfaxalone modulation of tonic inhibition on cerebellar granule neurons (CGN) (figure 35). Consistent with recombinant findings, the level of tonic enhancement by 60nM alfaxalone was the same under control conditions (64.6 ± 10.5 pS/pF, n=6)(figure 35A) and when GABA was either raised using CNQX evoked vesicular GABA release (72.2 ± 29.1 pS/pF, n=6) or conversely lowered using TTX to block action potential dependent release (36.8 ± 10.1 pS/pF, n=5) (fig 35B). There was no significant difference in the magnitude of alfaxalone induced conductance under these different conditions. ($P>0.05$ ANOVA).

With the final set of experiments in this range we sought to confirm that the neurosteroid Ganaxalone is equally unaffected by ambient GABA levels. To get a control response 100nM Ganaxalone was locally applied onto CGNs and the tonic conductance monitored (figure 36A). We then raised the ambient GABA levels that surround cerebellar granule cells by supplementing the recording ACSF with 10μM GABA, which significantly enhanced the tonic conductance by 125 ± 33 pS/pF (n=12). 100nM Ganaxalone was then applied and tonic conductance monitored and compared with control. Consistent with THDOC and alfaxalone data, results suggest that the level of conductance elicited by Ganaxalone was not affected by raised GABA levels. Control responses were as follows: 116 ± 37 pS/pF (n=9) compared with 122 ± 29 pS/pF (n=10) when GABA levels were augmented ($P>0.05$ unpaired *t*-test) (figure 36C).

Thus, in contrast to the actions of the anaesthetic propofol, we suggest that neurosteroid modulation of tonic inhibition is possible even when extrasynaptic GABA-A receptors are fully occupied by GABA. And further, that this observation extends to endogenous neurosteroids and clinically relevant analogues such as alfaxalone and Ganaxalone. In the next section we examined the actions of Gaboxadol, an orthosteric agonist of GABA-A receptors, and again tested whether raising the level of receptor occupancy by GABA would influence this type of drug.

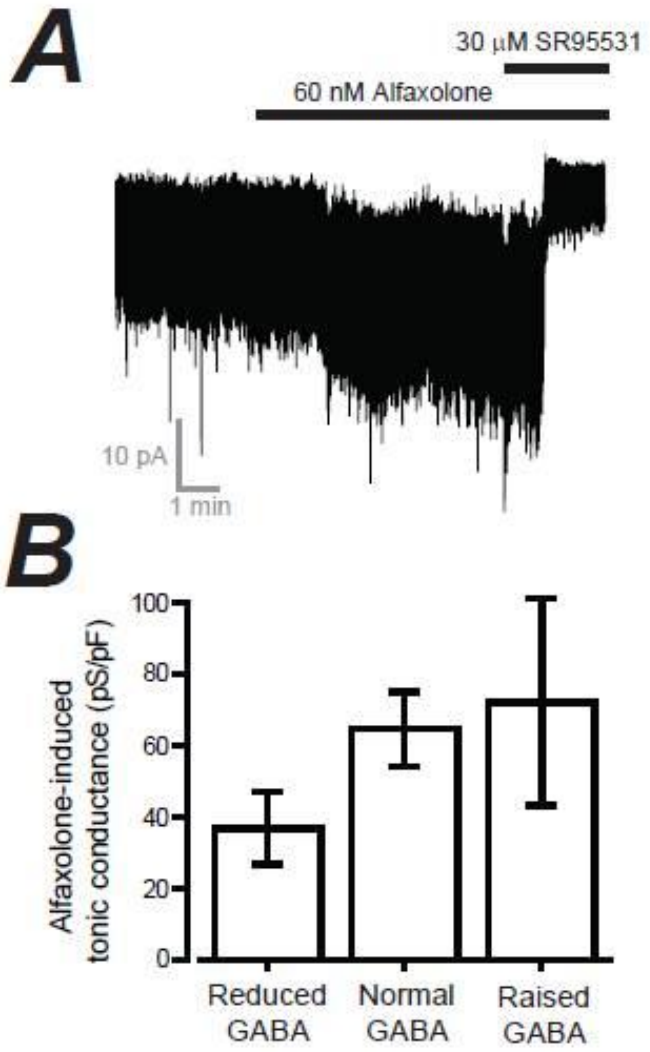

Figure 35. The modulation of tonic inhibition by the neurosteroid Alfaxalone is not sensitive to fluctuations in ambient GABA. **A**, Representative whole-cell recording of a CGN voltage-clamped at -60mV. 60nM Alfaxalone was applied causing an enhancement of the tonic conductance. Similar protocols were carried out in the presence of 500nM TTX which served to lower ambient GABA levels, and 5μM CNQX which raised it. **B**, Comparison of alfaxalone evoked tonic conductances under conditions of raised (CNQX) and reduced (TTX) ambient GABA levels. Averaged results suggest that Alfaxalones effects on tonic inhibition are not sensitive to fluctuating GABA levels. Populations are not significantly different ($p > 0.05$, ANOVA). Error bars represent SEMs. Carried out in collaboration with Dr. Catriona Houston.

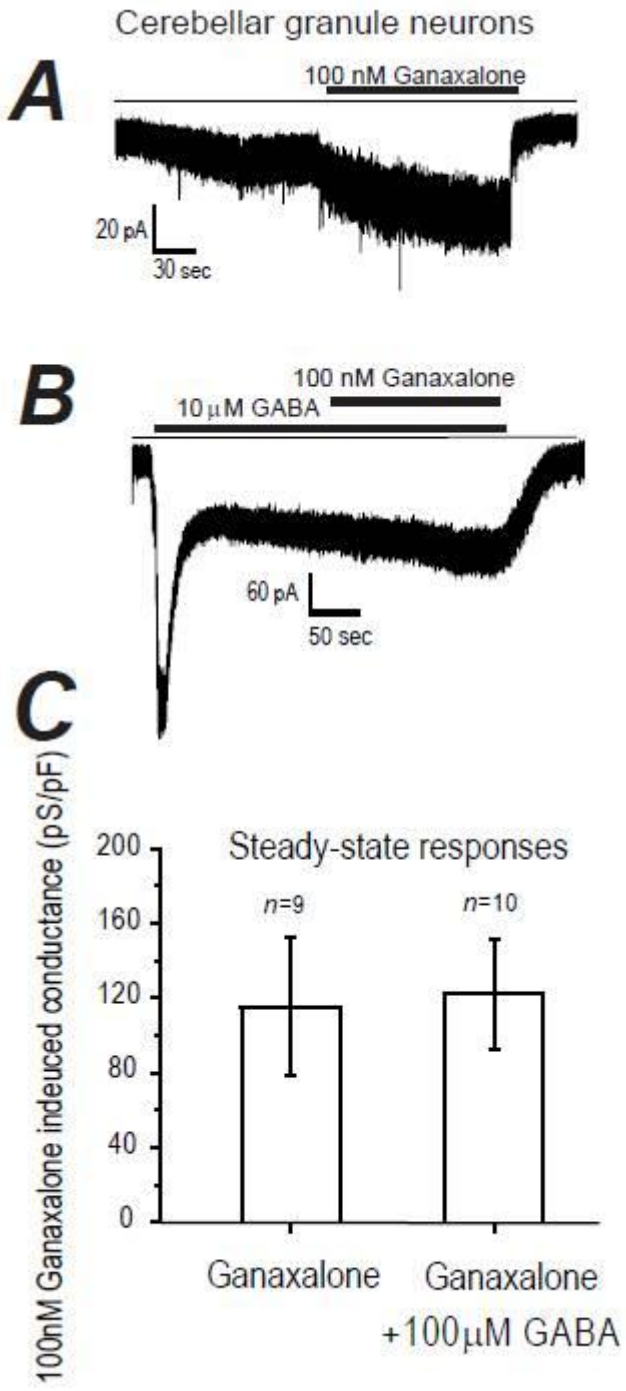

Figure 36. The neurosteroid Ganaxalone enhances tonic inhibition irrespective of ambient GABA concentration. **A,** Representative whole-cell recording of a CGN voltage-clamped at -60mV. 30nM and 100nM Ganaxalone (GNX) was applied, resulting in a dose-dependent enhancement of tonic inhibition. **B**, 10μM GABA, a saturating dose, was applied locally to the slice. Once the GABA response reached steay-state 100nM Ganaxalone was co-applied causing an increase in the steady-state conductance. **C**, Comparison of GNX induced tonic under control conditions and when GABA is raised through exogenous application. Averaged results suggest that the raising ambient GABA will not influence the extent to which GNX enhances tonic inhibtion. Populations are not significantly different; P>0.05 unpaired t-test.

3.3.3 Gaboxadol modulation of tonic inhibition is sensitive to fluctuations in ambient GABA

Gaboxadol is a sleep promoting drug which has been shown to act preferentially at extrasynaptic δ-containing GABA-A receptors and enhance tonic inhibition (Wafford and Ebert 2006). Its potent effects at extrasynaptic receptors are thought to reflect a highly efficacious interaction, so much so that Gaboxadol is considered a super-agonist with respect to GABA (Smart, Mortensen et al. 2010). Under such conditions we might've expected Gaboxadol to enhance tonic conductances under all conditions but complete GABA occupancy. However, as we have shown in section 3.2.3, while Gaboxadol is a superagonist at peak responses, it should not be considered such under more physiologically relevant steady-state conditions. Thus under these revised conditions we predicted that this drug may indeed be sensitive to fluctuating GABA levels as competition for the GABA binding site becomes the defining interaction.

To test this prediction we again examined recombinant α6βδ-containing GABA-A receptors expressed in HEK293 cells. In whole-cell patch-clamp configuration and using local drug applications, we measured the Gaboxadol activated conductance under varying ambient GABA conditions ranging from 10nM to 1μM. 500nM Gaboxadol, a concentration which has been shown to be within the therapeutic range (Storustovu and Ebert 2006) and to selectively target δ-containing receptors (Smart, Mortensen et al. 2010), caused a robust direct activation of α6βδ receptors with a mean steady-state conductance of 200 ± 36 pS/pF (n = 24)(fig A&B). However, in the presence of just 50nM GABA, this effect is attenuated by a full 70%, giving rise to a steady-state conductance of just 60 ± 30 pS/pF (n = 9) (figA), while at 1μM GABA Gaboxadol's effects are completely ablated with a mean steady-state conductance of just -9 ± 7 pS/pF (n = 7). The relationship of Gaboxadol induced conductance across a GABA concentration range is illustrated in part B of figure 36, note an almost linear relationship suggesting that Gaboxadol's action at α6βδ receptors is exquisitely sensitive to fluctuations in GABA occupancy.

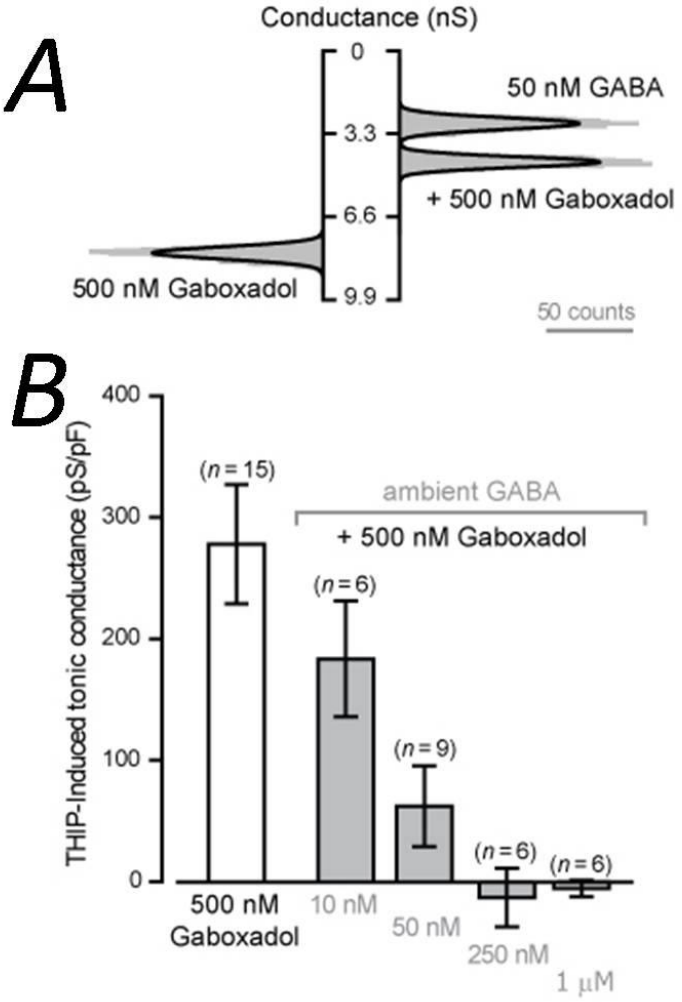

Figure 37. Gaboxadol activation of α6βδ containing GABA-A receptors is attenuated by raised ambient GABA levels. A, Comparison of 500nM Gaboxadol induced conductance with and without 50nM GABA. HEK cells expressing *α6βδ* were voltage-clamped at -60mV and whole currents measured. Note pre-incubation in 50nM GABA significantly reduces Gaboxadols effect on the *α6βδ* mediated conductance. Histograms were fitted with a single Gaussian function using OriginPro software (Microcal, MA) **B**, Averaged results comparing Gaboxadol induced conductance in the presence of different levels of ambient GABA. Note an almost linear relationship between raised ambient GABA levels and a reduced Gaboxadol response, suggesting that Gaboxadol modulation of *α6βδ* GABA-A receptors is highly sensitive to fluctuations on ambient GABA.

The δ subunit selectivity of 500 nM gaboxadol was illustrated in a recombinant system as it gave no significant response at α1βγ2s-containing GABA-AR receptors (32 ± 96 pS/pF; n = 7; data not shown). But before testing our hypothesis on native GABA-A receptors we wanted to confirm that the same selectivity was evident within native neurons. To this end

Gaboxadol was applied to α6βδ expressing CGNs, and as a control to cells known to express γ but not δ subunits, namely Purkinje neurons of the cerebellum. Consistent with previous findings as well as our own recombinant data, 500nM Gaboxadol locally applied to CGNs elicited a robust enhancement of the tonic conductance averaging 208 ± 20 pS/pF (n = 42) (figure 37A), while the same dose applied to αβγ containing Purkinje cells did not significantly alter the holding current (mean Gaboxadol-activated conductance: 2.8 ± 2 (n=6)) (see figure 37B).

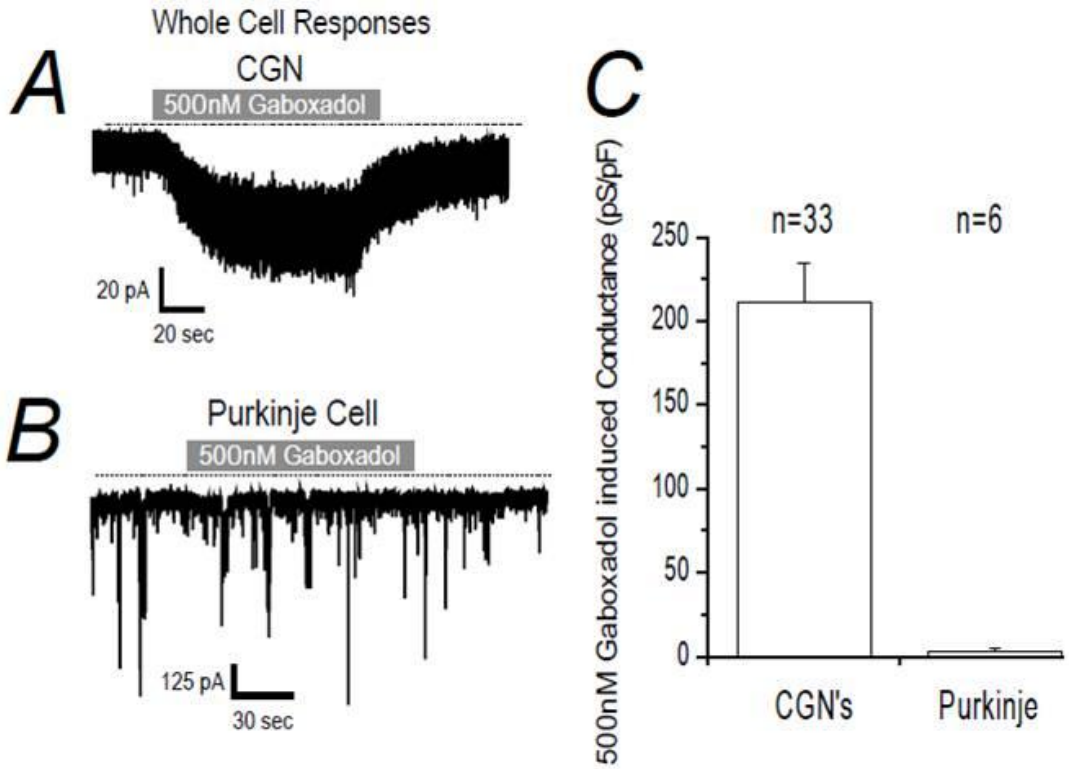

Figure 38. 500nM Gaboxadol selectively activates α6βδ containing GABA-A receptors to enhance tonic conductance in cerebellar granule neurons (CGN). A, Whole cell recording of CGN voltage-clamped at -60mV. 500nM Gaboxadol activates *α6βδ* and robustly enhances the tonic conductance in CGNs. Grey bar marks the duration of application. **B**, Purkinje cells which lack *α6βδ* receptors and a tonic conductance were used to examine the ability of gaboxadol to selectively activate these receptors. Note Gaboxadol has no effect on the whole-cell current. **C**, Comparison of Gaboxadol induced conductances in CGNs and Purkinje cells. Averaged results suggest 500nM Gaboxadol is selective for native *δ*-containing GABA-A receptors. Error bars represent SEMs.

Thus confident we were seeing α6βδ mediated responses in CGNs we next raised the ambient levels by exogenously adding GABA as previously described. As figure 38 illustrates, 500nM Gaboxadol applications significantly enhanced the tonic current (208 ± 20 pS/pF; n = 42 (figure 38A)). However, as in a recombinant system, this effect was dramatically attenuated by GABA in a dose dependent manner; 1μM GABA supplementation giving a mean gaboxadol activated steady-state conductance of 159 ± 18 pS/pF (n = 13), and 10μM GABA 134 ± 34 pS/pF (n = 12)(figure B). In fact, in 100μM GABA, Gaboxadol actually seemed to block the GABA activated current, eliciting a mean steady-state conductance of -50 ± 17 pS/pF(n = 8) (fig 38C). This is consistent with data shown in section 3.2.3 which suggests a larger steady-state G_{MAX} for a GABA-activated conductance compared to a gaboxadol-activated steady-state conductance.

Thus, by adding GABA we can attenuate the Gaboxadol induced conductance in recombinant receptors and in native tissue. However, as with previous experiments, we wished to explore whether this effect was replicated when we alter vesicular GABA release pharmacologically. Again TTX and CNQX were used and Gaboxadol-activated conductances measured under each condition. As figure 39 shows the mean 500nM Gaboxadol induced conductance (123.6 ± 21.5 pS/pF; *n*=9) was increased when vesicular GABA release was attenuated using TTX (154.3 ± 28.6 pS/pF; *n*=8) and conversely decreased when release was raised using CNQX (61.8 ± 20.5 pS/pF; *n*=9). The differences under these conditions was significant (P=0.02 ANOVA). Thus, in line with recombinant data and in a similar manner to propofols interactions, the level of Gaboxadol activation of α6βδ receptors and its modulation of tonic inhibition will critically depend on the level of ambient GABA in the extracellular space.

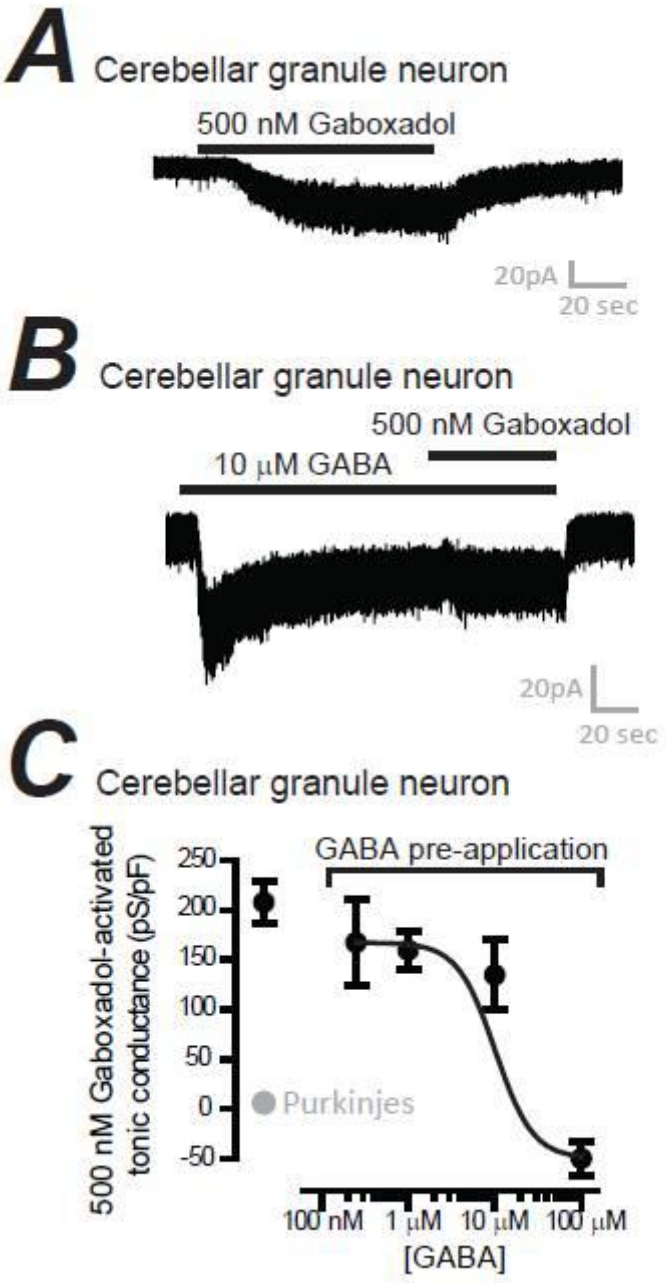

Figure 39. Gaboxadol modulation of tonic conductance in cerebellar granule neurons (CGNs) is attenuated by raised ambient GABA levels. **A**, Representative whole-cell recording of a CGN voltage-clamped at -60mV. 500nM Gaboxadol activates *α6βδ* and robustly enhances the tonic conductance in CGNs. **B**, Ambient GABA was raised through exogenous application. In this representative trace 10μM GABA was applied and once the response reached steady-state 500nM Gaboxadol was co-applied. Note, under these high GABA steady-state conditions Gaboxadol does not enhance the tonic conductance. **C**, Relationship between Gaboxadol evoked conductances in CGNs and the level of ambient GABA. Solid line represents a fit of the data to the Hill equation. Error bars represent SEMs. Averaged results suggest that raising ambient GABA levels attenuates Gaboxadol activated conductances.

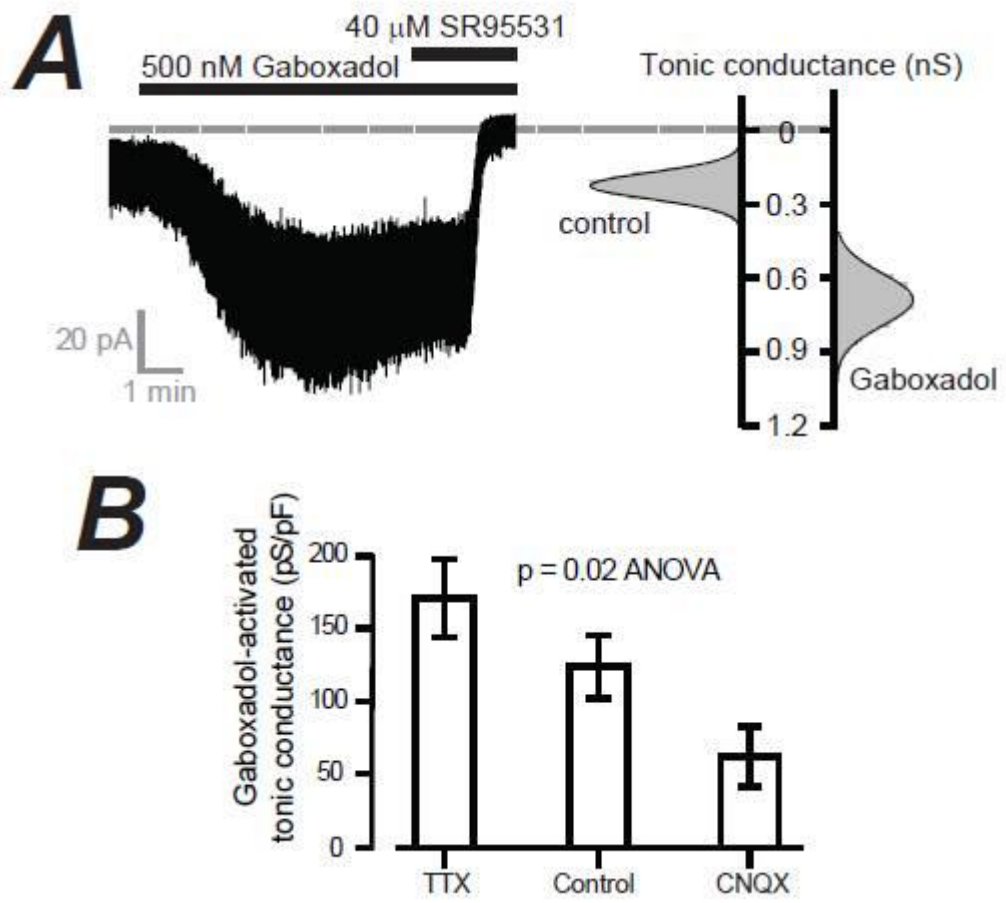

Figure 40. Gaboxadol activated tonic currents are sensitive to fluctuations in ambient GABA. A, representative whole-cell recording of a cerebellar granule neuron voltage-clamped at -60mV. 500nM Gaboxadol activates $\alpha 6\beta\delta$ and robustly enhances the tonic conductance in CGNs. Corresponding all points histograms were fitted with single Gaussians (left) to highlight Gaboxadol induced increase in conductance. **B**, Gaboxadol activated conductances in CGNs were compared when ambient GABA was lowered using TTX and conversely raised using CNQX. Results illustrate that when we raise ambient GABA Gaboxadols enhancement of tonic conductance is attenuated. Statistical analyses confirm these populations are significantly different (p=0.02 ANOVA).

3.3 Summary

A number of clinically relevant drugs act at extrasynaptic αβδ-containing GABA-A receptors to modulate tonic inhibition. In this section we have demonstrated that the potencies of two of these are profoundly affected by fluctuations in the ambient GABA concentration. Using a model of GABA-A receptor allosteric modulation, we predicted that the intravenous anaesthetic Propofol will only enhance tonic inhibition when GABA occupancy is low. We then went on to prove this experimentally in both a recombinant system and in native tissue. Using identical techniques we also showed that the effects of the sleep promoting drug Gaboxadol at αβδ-containing receptors is equally dependent on ambient GABA levels. Finally we examined a number of different neurosteroids, and in contrast, report that these can enhance tonic inhibition irrespective of ambient GABA concentration. These results highlight the usefulness of neurosteroid-like drugs, and more broadly may shape the strategies of drug development to target extrasynaptic GABA-A receptors.

In the following section I will describe the development of viruses which will be used to manipulate the neurosteroid sensitivity of GABA-A receptors *in vivo*. I will also outline the development optogenetic tools and techniques which we will combine with viral delivery methods to investigate the source of ambient GABA, and attempt to exert optogenetic control over its release.

4 Discussion

This study aimed to build on our understanding of the functional properties and pharmacology of α6β2δ-containing GABA-A receptors, and thus gain insights into how tonic inhibition functions and is modulated *in vivo*. To this end whole-cell patch-clamp was employed to examine recombinant and native αβδ-containing GABA-A receptors. An important premise of these experiments was that steady-state drug responses should be examined as opposed to peaks, as we suggest this may represent conditions more akin to those *in vivo*. Using this paradigm we present novel data showing that contrary to previous reports α6βδ are not constitutively active and contrary to dogma are profoundly desensitizing. Also contrary to recent reports we show that recombinant α6βδ receptors are not sensitive to ethanol at physiologically relevant doses and that while the hypnotic Gaboxadol is a 'super-agonist' in terms of peak responses it is not such under steady-state conditions. We also present the novel discovery that copper ions will reversibly block α6βδ receptors. These findings will be discussed in relation to the mechanistic underpinnings of these interactions and in the broader context of possible physiological implications. We then went on to test a more specific thesis: that ambient GABA levels influence the potency of certain clinically relevant drugs. This paradigm has never been investigated, and considering ambient GABA levels are known to fluctuate, represents a significant gap in our knowledge. The actions of the anaesthetic propofol and the sleep promoter Gaboxadol, both of which are known to modulate tonic inhibition in the adult brain, were significantly reduced when ambient GABA levels were raised. In contrast however, the anti-epileptic Ganaxalone and the anaesthetic Alfaxalone enhanced tonic inhibition irrespective of ambient GABA concentrations. These findings will be discussed in relation to known changes of ambient GABA.

4.1 Insights gained into the properties of αβδ GABA-ARs

To investigate the properties of extrasynaptic δ-containing GABA-A receptors we first looked to a heterologous expression system, so that we might examine these receptor properties in a controlled and isolated environment. HEK293 cells are a mammalian cell line derived of embryonic kidney cells which are particularly useful for electrophysiology experiments due to their amenability to transfection and efficiency of expression, as well as their small size which makes them appropriate for voltage-clamp experiments (Thomas and Smart 2005).

4.1.1 Expression of the recombinant αβδ receptors

The full incorporation of the δ-subunit into recombinant receptors has been a matter of some concern, particularly because it is reported that α and β subunits will readily form functional binary GABA-A receptors (Smart, Moss et al. 1991; Thompson, Bonnert et al. 2002). Indeed it has been suggested that heterogeneous binary and ternary receptor populations are the reason for certain inconsistencies in past recombinant data (Meera, Olsen et al. 2010). To mitigate this and attempt to obtain a homogenous ternary receptor population experimenters have typically transfected the δ-subunit cDNA in excess of α and β, often at a 1:1:10 ratio (Sundstrom-Poromaa, Smith et al. 2002; Wallner, Hanchar et al. 2003; Borghese and Harris 2007; Smart, Mortensen et al. 2010). However, it is by no means conclusive that more cDNA starting material promotes increased δ incorporation, indeed some groups suggest that maximal αβδ expression is obtained with 1:1:0.1 ratio (You and Dunn 2007; Barrera, Betts et al. 2008).

In our hands, and consistent with past findings, the δ-deficient α6β receptor combination did not give rise to functional receptor complexes which were sensitive to GABA (Storustovu and Ebert 2006). Moreover, examination of HEK cells expressing the δ-subunit alone or αδ, βδ combinations did not give rise to functional GABA-A receptors (data not shown). As such, the formation of binary by-products when working with α6βδ's is not a crucial problem. α4β combinations provide more of an issue as these do form functional GABA-A receptors

(Meera, Olsen et al. 2010). To confirm δ-subunit surface expression and incorporation when working with both combinations, we used a modified pH sensitive tag (SEP) conjugated to the δ-subunit, which allowed visualisation of δ-subunit surface expression (Sankaranarayanan, De Angelis et al. 2000; Ashby, Ibaraki et al. 2004). We also pharmacologically assayed δ expression by exploiting zinc's differential sensitivity for αβ and αβδ. Consistent with past reports the inclusion of the δ-subunit conferred a very low sensitivity to zinc block (<20% block of 1μM GABA currents) (Borghese, Storustovu et al. 2006; Storustovu and Ebert 2006), while a correlation between GABA evoked current amplitude and δ-SEP fluorescence provided functional confirmation of δ's incorporation in recombinant receptors.

We have shown that altering the cDNA ratio in our system had no effect on δ surface expression. This runs contrary to some reports in *Xenopus* oocytes (You and Dunn 2007) and likely reflects differences within these expression systems. For example, the high efficiency of protein expression and trafficking in HEK293 cells combined with the small surface area, means that even small amounts of cDNA can be transcribed, translated and trafficked to quickly occupy much of the cell surface, and thus may negate the effects of different levels of starting material. The differences in transfection procedures may also be significant. The calcium phosphate method displays an inherent inconsistency, in that not all of the starting material will necessarily be taken up by the cells and transcribed, and therefore the starting ratios by no means represent precisely the levels of protein produced. Direct injection into oocytes on the other hand means the cellular machinery sees all the genetic material, and therefore the imposed relative ratios may be more directly represented at the protein level.

Thus we feel confident that δSEP was expressed, trafficked to the cell surface and forming part of functional GABA-A receptors in this system, and that the cDNA ratios used were appropriate. However, as with any recombinant system, we can't rule out the formation of receptors with abnormal/unnatural receptor stoichiometry, although intuitively we would suggest that an equimolar DNA start point helps negate this risk. We also can't be sure, at least when dealing with α4βδ, that we have a truly homogeneous receptor population,

although these results do suggest that ternary δ-containing receptors are favoured and ultimately predominate in this expression system.

4.1.2 δ-containing GABA-A receptors are not constitutively active

It has been shown, both in recombinant systems (Sigel, Baur et al. 1989; Lindquist, Dalziel et al. 2004; Hadley and Amin 2007) and in native tissue (Birnir, Everitt et al. 2000; McCartney, Deeb et al. 2007), that certain GABA-A receptor subtypes will open spontaneously in the absence of agonist. Hadley *et al*., examined α6β2δ containing GABA-A receptors expressed in *Xenopus* oocytes and reported persistent activity, which corresponded with tonic inhibition (Hadley and Amin 2007). The results presented in this thesis challenge that finding. Applying picrotoxin to HEK cells expressing α6β2δ, in concentrations in excess of those used by Hadley *et al*. (50μM versus 20μM), we found negligible levels of persistent activity. While Hadley & Amin reported spontaneous currents in $\alpha_6\beta\delta$ representing approximately 23% of total attainable current (GABA-evoked I_{max}), we observed a persistent conductance for $\alpha_6\beta\delta$ averaging 8 ± 7 pS/pF with maximal conductances exceeding 1 nS/pF. We further demonstrated that the holding current in cells clamped at -60mV never exceeded -50pA, suggesting very little current background leak at rest, the opposite of what one might expect with constitutively open channels. This inconsistency likely reflects the different experimental expression systems. In the report by Hadley and Amin the authors suggest that $\alpha_6\beta\delta$ have two separate agonist affinity states, one of which displays spontaneous activity. And that the receptor population can be biased in favour of the persistently active state by increasing the amount of DNA injected. In light of our contradictory finding we suggest that the spontaneous activity may be artefact of receptor over-expression, evidenced by the fact that it is not observed when lower levels of DNA were used. The mechanism of such an activity is unclear but we might surmise that excess protein may force unnatural receptor subunit conformations/stoichiometry, or that interaction of properly assembled receptors tightly packed into the cell membrane may alter their properties. Indeed clustering of extrasynaptic receptors cultured hippocampal neurons has been shown to modify receptor gating properties (Cherubini, Petrini et al. 2003). These opposing results may also reflect differences in the way proteins are processed in these systems. The suggestion that the oocyte and HEK293 expression systems may not produce functionally identical products is

not without precedent (Egebjerg and Heinemann 1993). Indeed, it is by no means certain that these two expression systems do not differ in, for example: the efficiency of cDNA translation pathways, in post-translational modifications such glycosylation or phosphorylation, in the manner in which receptor complexes are inserted into the membrane, or in the assembly of receptor complexes. Furthermore some reports have suggested that endogenous oocyte gene expression can give rise to unwanted receptor subsets (Buller & White, 1990; Hedin et al., 1996).

These results demonstrate that the findings of this earlier study carried out in *Xenopus* oocytes (Hadley and Amin 2007) are not necessarily relevant to a mammalian system, and may well represent an artefact of *Xenopus* expression. As well as examining α6β2δ, we also confirmed that α4β2δ are similarly not spontaneously active. Thus, consistent with our current understanding of extrasynaptic δ-containing receptor activation, we suggest low levels of ambient GABA are necessary to elicit receptor activation and to mediate tonic inhibition.

4.1.3 Steady-state desensitization of δ-containing GABA-A receptors

The dogma surrounding extrasynaptic GABA-A receptors suggests they have a very high affinity for GABA and are minimally desensitizing, consistent with their role in persistently responding to low levels of ambient GABA (Glykys and Mody 2007). Our results show that while these receptors have indeed a high GABA affinity, they are profoundly desensitizing. Examination of GABA potencies at α4βδ and α6βδ GABA-A receptors suggest comparatively high affinity GABA interactions, with half-maximal GABA responses in the low 100's of nM range, broadly in line with past reports in which recorded GABA peak responses in recombinant expression systems (Saxena and Macdonald 1996; Smith, Shen et al. 2007). Unexpectedly, whole-cell patch-clamp examination also revealed a significant degree of macroscopic receptor desensitization. As illustrated in section 3.1 both α4βδ and α6βδ containing GABA-A receptors gave rise to desensitizing GABA transients, even when exposed to low nM GABA concentrations. Indeed results suggest half-maximal desensitizing responses at 60 and 70nM respectively, levels which are at the lower end of plausible

estimates for ambient GABA levels *in vivo* (Rossi and Hamann 1998; Bianchi, Ballini et al. 2003; Wu, Wang et al. 2007). Furthermore, examination of native α6βδ receptors in outside-out patches also showed a significant degree of desensitization. There are a few possible reasons for the apparent inconsistency between these findings and the previous understanding of δ receptor desensitization; firstly much of the original recombinant experiments examined α1βδ rather than α6 or α4 containing receptors, indeed when early experiments compared α1βδ and α6βδ, data clearly illustrates that while α1 may be minimally desensitizing, α6 containing δ receptors showed a maximal 75% macroscopic desensitization (Bianchi, Haas et al. 2002; Feng and Macdonald 2004), consistent with our own data. Secondly, as is typical, past analyses have focused on GABA evoked peak responses, and as such have not prolonged the GABA applications long enough to make an appropriate evaluation of steady-state desensitization. This is fundamental when dealing with extrasynaptic GABA-A receptor subtypes, as slow equilibrium to ambient GABA steady-states is the nature of their environment, as opposed to fast GABA transients which reflect synaptic environs. Even from superficial analysis of some of the past literature examining recombinant α4βδ and α6βδ desensitization we can see that responses are far from steady-state, and while the speed of desensitization may be comparatively slower than synaptic counterparts it is by no means convincing that the extent of desensitization is different (Brown, Kerby et al. 2002; Wallner, Hanchar et al. 2003). Thirdly, some differences may stem from the expression systems used, differing post-translational modifications and regulatory pathways could lead to altered receptor properties. Phosphorylation mechanisms which may be different across expression systems, has been shown to alter α4β3δ receptor desensitization (Borghese and Harris 2007; Tang, Hernandez et al. 2010), furthermore receptor clustering which may also be expression system dependent has been shown to affect desensitization kinetics (Cherubini, Petrini et al. 2003). Finally, we would suggest that this dogma likely survived partly due to its tidy fit into the proposed role of δ-containing GABA-A receptors in tonic inhibition, namely that if they are minimally desensitizing they would be uniquely disposed to mediate both tonic conductance and phasic spillover currents (Attwell, Hamann et al. 2002).

Does this new fact that δ-containing receptors are highly desensitizing draw into question the role of extrasynaptic receptors in mediating tonic conductances? No, because although a

smaller population of non-desensitizing GABA-A receptors would be needed to mediate a tonic conductance of a given magnitude, a larger population of stochastically opening desensitized receptors can sum to have the same effect. And, as our results illustrate even in saturating GABA concentrations 20% of receptors were still conducting. These results do however challenge the involvement of δ-containing GABA-A receptors in any phasic response, and indeed we have already gone on to demonstrate that δ-containing receptors do not have a role in mediating spillover currents (Bright, Renzi et al. 2011). These desensitizing transients highlighted a further implication of this finding. Namely, that if peak and steady-state levels are so profoundly different then perhaps the GABA concentration-response relationships based on these values are equally different. In fact this did turn out to be the case, charting peak versus steady-state dose response relationships of δ-containing GABA-A receptors revealed that half-maximal (EC50) responses were significantly lower, and the effective concentration range significantly narrower, when evaluated using steady-state responses. As steady-state conditions may be more physiologically relevant, this suggests that past experiments which analyzed peak responses likely underestimated the potency of these receptors while over-estimating their effective concentration range. A high and varying degree of GABA evoked desensitization may also have implications on how these receptors are modulated. Under some conditions this may limit their potential for modulatory effects. Conversely however, this may in fact offer more scope for modulation of tonic inhibition. It may not be immediately intuitive why this should be so, but consider that as these receptors have a very high affinity for GABA, thus drugs which further increase affinity have a very limited range in which to operate. In this case modulation of receptor desensitization may offer a more effective mechanism to enhance tonic inhibition (this will be explored in more detail in section 4.3.). In a similar vein, the fact that GABA is a low efficacy agonist at extrasynaptic GABA-A receptors seems biologically inefficient. However, as we are beginning to appreciate, this provides a mechanism for receptor potentiation, thus the drugs which increase the efficiency of gating elicit enhancement of tonic inhibition (Glykys and Mody 2007). In gaining insights into these fundamental molecular mechanisms we can better understand the activity and contributions of these receptors in whole systems. These insights also provide clues as to how these receptors are modulated.

4.2 Insights gained into α6βδ receptor pharmacology

4.2.1 Ethanol: δ-containing receptors are not the answer

Our results demonstrate convincingly that ethanol will not modulate recombinant α6β3δ containing GABA-A receptors. Co-applying ethanol concentrations from 10-100mM with a steady-state EC_{50} GABA dose, we observed no significant ethanol induced enhancement of peak or steady-state GABA currents. In a field in which contradiction and controversy are the norms this finding is no different, while this result finds much corroboration within the alcohol literature, it simultaneously challenges a number of reports. The reasons for such discrepancies however, are not immediately clear.

Elucidating the mechanisms underlying ethanol's intoxicating effects has proved incredibly difficult. Original theories suggested ethanol acted non-specifically by disordering lipid membranes, however most now implicate specific protein interactions. Of the many proteins implicated including a number of enzymes and ion channels, it is often even unclear whether these targets are directly modulated by ethanol or by intermediaries. The reason for difficulties in this area is due to the likely very low affinity interaction involved. This is reflected in the very high blood alcohol concentrations needed to elicit physiological effects. For example the blood concentration considered legally impairing is 17 mM while anaesthetic doses are around 190 mM (Alifimoff, Firestone et al. 1989; Harris, Trudell et al. 2008). Of these prospective ethanol targets, GABA-A receptors are particularly strongly supported. Many of the behavioural effects of alcohol are thought to result from the enhancement of inhibitory neurotransmission mediated by GABA-A receptors (Boehm, Ponomarev et al. 2004; Criswell and Breese 2005; Krystal, Staley et al. 2006), and there is considerable evidence that this plays a critical role in alcohol intoxication via reward pathways reinforce consumption and can lead to addiction (Chester and Cunningham 2002; Crabbe, Phillips et al. 2006; Melis, Camarini et al. 2002). Of the many GABA-A receptors which could have a role in mediating these effects the α4βδ and α6βδ have attracted recent attention. Behavioural experiments in δ-knockout mice lend some credence to this hypothesis as compared with wild-type δ-knockouts presented with altered responses in a number of tests including: reduced ethanol consumption and attenuated withdrawal (Mihalek, Bowers et al. 2001). The involvement of extrasynaptic receptors has been further

corroborated by recordings from δ subunit-containing native neurons, in which ethanol enhanced tonic conductances (Mody, Wei et al. 2004; Hanchar, Wallner et al. 2005; Santhakumar, Hanchar et al. 2006). Crucially and almost uniquely, a number of groups even report that these subtypes are sensitive to direct ethanol modulation in recombinant systems (Olsen, Hanchar et al. 2007). However this finding has come under considerable scrutiny, with many groups, including ourselves, presenting results which challenge this finding.

Two groups have reported that recombinant δ-containing receptors are sensitive physiologically relevant concentrations of ethanol (1-30mM) (Sundstrom-Poromaa, Smith et al. 2002; Wallner, Hanchar et al. 2003). These groups showed ethanol from as low as 1mM potentiated EC_{20} GABA responses by some 50-75%. Furthermore, the more recent of these suggested that the β subunit was a major determinant of ethanol sensitivity, and that substitution of the β2 for β3 in α4/6βδ containing receptors increased the amplitude of ethanol modulation. Our results directly contradict this finding. We also tested recombinant α6β3δ receptors across a similar GABA concentration range. Moreover, a colleague tested and compared α6β2δ and α6β3δ, and equally witnessed no effect (unpublished data). And we are not alone in this. A number of groups have also reported a lack of sensitivity to ethanol by recombinant δ-containing GABA-A receptors. One group in particular mimicked the conditions of these previous reports, namely the same cDNA's, expression system (oocyte) and ethanol dose, and yet presented contrary results (Borghese, Storustovu et al. 2006). Another recent publication even went so far as to suggest ethanol inhibits recombinant α4βδ and α6βδ receptor mediated currents (Yamashita, Marszalec et al. 2006).

These apparently contradictory findings remain a matter of discussion. The expression systems used represent a possible reason for variability. As I've described previously the host cell may influence the receptor properties, and this may be most marked when considering mammalian versus non-mammalian cells. Indeed, reports of ethanol sensitivity have often involved the *Xenopus* oocyte expression system (Sundstrom-Poromaa, Smith et al. 2002; Wallner, Hanchar et al. 2003), while our work and that of others who have observed a lack of ethanol effects have used mammalian expression systems (Storustovu and Ebert 2006; Yamashita, Marszalec et al. 2006). However, having said that, studies in oocytes have

yielded results which also challenge ethanol's direct action at δ-containing receptors (Borghese, Storustovu et al. 2006). The level of GABA used in experiments may also be a factor, although the 250nM we have used here is broadly in line with the 300nM used by Smith *et al* (2002), and Wallner *et al* (2003). Other variables may also be involved, including differences in the cDNA clones used, the level of protein expression, the levels of receptor phosphorylation and other post-translational modifications. Whatever the reasons however, it seems clear that there are crucial pieces of the puzzle missing in our understanding of ethanol's effects at extrasynaptic receptors.

4.2.2 Neuroactive steroids: potent modulators of δ-containing receptors

Neuroactive steroids (neurosteroids) are potent modulators of GABA-A receptors (Belelli and Lambert 2005). Here we have examined the effect of the endogenous neurosteroid Tetrahydrodeoxycorticosterone (3α,21-dihydroxy-5α-pregnan-20-one: THDOC) and the anaesthetic analogue Alfaxalone on recombinant α6βδ-containing GABA-A receptors, and report that both will robustly enhance GABA evoked peak and steady-state responses. Furthermore we have shown that this modulation of GABA currents does not involve a shift in GABA potency as defined by half-maximal responses (EC_{50}), but suggest that this potentiation in fact reflects a decrease in the level of GABA-stimulated macroscopic desensitization in the presence of neurosteroid binding.

It has been appreciated for some time that certain pregnane steroids potentiate synaptic GABA-A receptors (Harrison 1987). Moreover it was shown that while they will enhance GABA responses at low nM concentrations (GABA modulatory), they can also directly activate at higher concentrations: >100nM (GABA mimetic) (Callachan, Cottrell et al. 1987; Shu, Eisenman et al. 2004). An initial report suggested that extrasynaptic αβδ-containing GABA-A receptors were not sensitive to this modulatory effect (Zhu, Wang et al. 1996), however numerous groups have since observed that replacing the γ-subunit with a δ- in recombinant systems actually enhances the steroid sensitivity (Adkins, Pillai et al. 2001; Brown, Kerby et al. 2002; Wohlfarth, Bianchi et al. 2002). Furthermore, studies have gone on to show that at physiologically relevant concentrations THDOC will preferentially

enhance tonic without impacting phasic responses (Stell, Brickley et al. 2003), a modulatory effect which is impaired in mice lacking the δ-subunit (Mihalek, Banerjee et al. 1999; Spigelman, Li et al. 2003).

Thus it seems clear that αβδ-containing GABA-A receptors represent a crucial target for neurosteroids. However, the effect on steady-state GABA currents in recombinant receptors has not been investigated, and the mechanism of action remains poorly understood. Our results show THDOC and Alfaxalone will robustly enhance GABA conductances (G_{MAX}) without affecting EC_{50} values. This finding supports earlier work carried out by Wohlfarth *et al*., (2002). In line with this previous work we used the same expression system and applied comparable levels of THDOC (10nM versus 30nM), and as such saw a similar level of enhancement: GABA G_{MAX} enhanced by ~30%. Crucially, Wohlfarth et al. (2002) also saw no shift in GABA half-maximal values associated with neurosteroid effects.

In light of this we would surmise that the allosteric mechanism underlying neurosteroid GABA-modulatory effects impact intrinsic gating properties. Consistent with this hypothesis our data shows that THDOC served to robustly decrease the level of steady-state receptor desensitization. Based on our earlier findings that α6βδ are profoundly desensitized at physiologically relevant GABA concentrations, this represents an efficient mechanism for enhancing tonic conductances. In support of this, a previous publication reports that prolonged THDOC administration *in vivo* decreased the extent of GABA evoked desensitization in patches pulled from CA1 pyramidal cells (Smith and Gong 2005). However, one group has reported that THDOC actually increased the extent of receptor desensitization (Bianchi, Haas et al. 2002; Wohlfarth, Bianchi et al. 2002; Bianchi and Macdonald 2003). In these three manuscripts αβδ receptors were examined in HEK293 cells, and experimenters showed that pre-incubation with THDOC increased the GABA-evoked current amplitude, but also dramatically increased the level of macroscopic desensitization. This finding runs counter-intuitive to the role of neurosteroids in enhancing tonic inhibition, which we have shown reflects a population of already profoundly desensitized GABA-A receptors. There are a few likely reasons for this apparent discrepancy. Foremost, Bianchi *et al* used a THDOC concentration two orders of magnitude greater than ourselves: 1μM versus

10nM. This concentration far exceeds any seen physiologically, where resting levels are proposed to be between 3 and 10nM and only reach a maximum of ~100nM during parturition (Belelli and Lambert 2005), moreover 1μM THDOC will directly activate the receptor, while 10nM used in our experiment will only modulate the GABA responses. This is significant because these two effects are mediated by distinct binding sites (Hosie, Wilkins et al. 2006), and therefore it is conceivable that there are distinct underlying mechanisms. Indeed in one such report where 30nM THDOC was applied, there is no increased desensitization, however the authors do not comment on this (Wohlfarth, Bianchi et al. 2002). In addition these reports more often examined α1βδ rather than α6βδ, and used higher GABA concentrations which could account for the altered levels of receptor desensitization.

Bianchi *et al* go on to argue that the mechanism of neurosteroids action is to shift the partial agonist activation of GABA-A receptors from low- to high-efficacy gating patterns (Bianchi and Macdonald 2003). While this is generally an attractive suggestion for δ-containing receptors, it fails to fully explain the enhancement of γ-containing receptors, at which GABA already acts with high efficacy. Our results support a role for δ-containing GABA-A receptors in mediating the effects of neurosteroids. Moreover we present a novel mechanism which sees neurosteroids pull extrasynaptic GABA-A receptors out of desensitized states and thus efficiently recruit these otherwise quiescent channels to enhance tonic inhibition.

4.2.3 Gaboxadol

We provide evidence that the GABA mimetic gaboxadol is not a 'super-agonist' at α6βδ GABA-A receptors under physiologically relevant conditions. Previous studies with gaboxadol demonstrated that it interacts with all GABA-A receptors, consistent with it being a GABA analogue (Brehm, Ebert et al. 1997; Ebert, Thompson et al. 1997), but the subsequent efficacy of response was shown to be highly subunit dependent (Wafford and Ebert 2006). Specifically it was shown that gaboxadol acted as a partial agonist at α1 or α3β2γ2, a full agonist at α5β2γ2 (Ebert, Wafford et al. 1994) and a 'super-agonist' at α4βδ (Adkins, Pillai et al. 2001; Brown, Kerby et al. 2002) and α6βδ (Storustovu and Ebert

2006). However, crucially, these previous investigations in recombinant systems have involved examination of peak responses, a condition which as we've argued is not physiologically relevant when examining extrasynaptic GABA-A receptors.

We also examined peak responses to gaboxadol at α6βδ, and report that here gaboxadol acts as a super-agonist compared with GABA responses. Consistent with past findings we observed gaboxadol acted with high efficacy, eliciting maximal responses (G_{MAX}) some 200-300% of that evoked by GABA, while the peak half-maximal response values (EC_{50}) were also in line with those previously reported for this subtype: 6μM versus 8.2μM (Stórustovu and Ebert 2006). However examination under steady-state conditions revealed that gaboxadol evoked desensitization was so much greater than GABA's that steady-state G_{MAX} were actually diminished with gaboxadol. While in the proposed therapeutic range, 500nM - 2μM (Madsen, Lindeburg et al. 1983), gaboxadol's steady-state response is merely equivalent to GABA.

Combined, these results illustrate that the enhancement of tonic inhibition by gaboxadol is not due to 'super-agonism', as when steady-state conditions are considered we see no evidence that gaboxadol exhibits greater efficacy compared to GABA. Thus gaboxadol application may be analogous simply to supplementing extracellular GABA. It has been shown that gaboxadol activates tonic currents mediated by extrasynaptic receptors in the hippocampus and thalamus (Liang, Cagetti et al. 2004; Belelli, Peden et al. 2005), moreover it has been suggested that at therapeutic concentrations δ-containing GABA-A receptors were the primary target for gaboxadols hypnotic effects (Storustovu and Ebert 2006). This new understanding of gaboxadols actions at steady-state does not necessarily challenge these findings. However as GABA acts with considerably higher affinity than gaboxadol and approximately equivalent efficacy, the ability of gaboxadol to enhance tonic will likely be dependent on having low GABA concentration or an otherwise large surplus of extrasynaptic receptors (this will be explored further in section 4.3).

4.2.4 Copper – a novel extrasynaptic GABA-A receptor blocker

For the first time, we have shown that copper (Cu^{2+}) ions can potently and reversibly block α6βδ containing GABA-A receptors. Moreover we've demonstrated that this high-affinity block is more potent at δ-containing extrasynaptic receptors than at γ-containing synaptic counterparts. As such we postulate that copper represents a pharmacological tool for discriminating between phasic and tonic inhibition and consider the possible functional significance of this *in vivo*.

It has been appreciated for some time that certain cations can modulate GABA-A receptor currents. While Zinc (Zn^{2+}) in particular has received much scrutiny, it is also clear that Cu^{2+} has a significant role in regulating GABAergic inhibition. Similar to zinc, copper is ubiquitous but levels are higher in the brain than in any other organs (Hui, Davis et al. 1977). Furthermore it appears Cu^{2+} is released during synaptic activity and has been therefore proposed to have a role in synaptic transmission (Hartter and Barnea 1988; Kardos, Kovacs et al. 1989). Work in both transfected cells and native neurons from different brain regions have examined γ-containing subsets and reported a Cu^{2+} induced block of GABA evoked currents by (Narahashi, Ma et al. 1994; Fisher and Macdonald 1998; Sharonova, Vorobjev et al. 1998; Kim and Macdonald 2003), however the sensitivity to coppers action have shown some regional and developmental variability (Narahashi, Ma et al. 1994; Trombley and Shepherd 1996; Sharonova, Vorobjev et al. 2000), highlighting a subtype and indeed subunit dependence of effect (Kim and Macdonald 2003).

Here we show for the first time that extrasynaptic δ-containing receptors can be similarly modulated. Our results demonstrate that Cu^{2+} will block both peaks and crucially, steady-state δ mediated currents, suggesting extracellular Cu^{2+} will modulate tonic currents. Significantly a steady-state block was seen at low nM Cu^{2+} concentrations, indeed this acute sensitivity was evident when we observed a channel block due to low level Cu^{2+} contamination. While *in vivo* extracellular Cu^{2+} concentrations are unclear, Kardos *et al* (1989) predicted the concentration in the synaptic cleft to be 100-250 μM. As such, analogous to GABA spillover we would surmise the Cu^{2+} concentration at extrasynaptic

receptors would be much lower. Consistent with this we observed a median inhibiting Cu^{2+} concentration (IC_{50}) at α6βδ extrasynaptic receptors in the nM range, while at synaptic receptors this was much higher. Similarly, other groups have suggested Cu^{2+} half maximal values (IC_{50}) at γ-containing receptors are in the μM range. Originally reported in dissociated dorsal root ganglion neurons as 19 μM (Narahashi, Ma et al. 1994), this figure which was soon after corroborated in olfactory bulb neurons (Trombley and Shepherd 1996).

Cu^{2+} is an ubiquitous and highly reactive cation, and as such a host of mechanisms could account for its actions at GABA-A receptors. These include an allosteric modulation if channel gating, a competitive interaction at the GABA site, a channel block and that copper complexes GABA in solution. First off, our data argues against a simple competitive interaction. GABA concentration-response curves were not shifted in the presence of Cu^{2+} suggesting GABA binding was not affected, also consistent with this there was a lack of a full block even at saturating Cu^{2+} concentrations. The likelihood that coppers effects on GABA currents reflects the complexing of GABA is also low, evidenced by a clearly non-linear concentration response and again by the lack of complete block in very high Cu^{2+}. Moreover the fact that we see a block when Cu^{2+} was not in the GABA solutions, i.e. when just the wash line was contaminated or copper pre-applied, supports this conclusion. Cu^{2+} may elicit an open channel block, although again the fact that pre-application seems to elicit a block before channel activation makes this unlikely. Moreover work from other labs showed that chelators such as histidine were able to quickly and robustly reverse the Cu^{2+} block of GABA-ARs suggesting Cu^{2+} was bound on the surface of the cell (Sharonova, Vorobjev et al. 2000). Thus the properties of Cu^{2+} block are most consistent with an allosteric mode of action, where Cu^{2+} binds to a distinct site on the GABA-A receptor complex and modulates gating properties. This model of action has been previously suggested for cation modulation of GABA-A receptors, indeed Hosie *et al* (2003) defined distinct zinc binding sites on the N-terminus of the GABA-A receptor and it has been suggested that Zn^{2+} and Cu^{2+} may this (Ma and Narahashi 1993; Kim and Macdonald 2003). Moreover Kim *et al* (2003) implicated specific residues within the αβ interface in Cu^{2+} actions, however they also showed that inclusion of the γ–subunit altered this effect and therefore it is conceivable that the mechanisms of Cu^{2+} block are not the same for δ-containing GABA-A receptors.

Thus our observations highlight Cu^{2+} as a potent modulator of α6βδ containing GABA-A receptors, and that consistent with past results GABA-A receptors display differential sensitivity to this effect. As a consequence, Cu^{2+} may represent a useful diagnostic tool to identify receptors underlying specific currents or effects, as indeed Zn^{2+} is used to identify αβ containing receptor subtypes (Mathie, Sutton et al. 2006). Further, from a physiological standpoint this variability may also be useful in allowing neurons to respond differently depending on receptor expression levels. The high sensitivity of extrasynaptic receptors in particular may serve as a critical mechanism in regulating neuronal excitability, if, as proposed, copper is released during depolarization, this will likely spillover into the extracellular space, and as we have demonstrated only trace amounts of Cu^{2+} will be necessary to elicit a profound effect on neuronal excitability. Moreover, glia have been shown to be copper rich, and thus may represent a source of Cu^{2+} released into the extracellular space, perhaps in a separate excitability independent mechanism (Barnes, Tsivkovskii et al. 2005).

Copper's actions on the GABAergic system, may also have significance to the progression of certain pathologies. Wilson's disease for example is an inherited disorder of copper transport which results in a build up of copper in tissues. Associated neurological abnormalities are focused in the striatum and cerebellum (Brewer and Yuzbasiyangurkan 1992; Yarze, Munoz et al. 1992), both of which express high levels of δ-containing receptors. Further, these abnormalities often manifest as EEG epileptiform activity which could well reflect impaired GABAergic activity (Chu, Chu et al. 1991). While behavioural symptoms including uncontrolled movement and tremor, analogous to Parkinsonian manifestations, may well also have its roots in the GABergic system (Vlastelica 2011). While copper's physiological roles are numerous and therefore the effects of its actions and dysregulation will be equally so, we suggest the very high sensitivity of extrasynaptic GABA-A receptors to Cu^{2+} block provides compelling circumstantial evidence that this interaction is physiologically significant and likely plays an important role in the pathogenesis of certain diseases.

4.3 Drugs that modulate tonic inhibition may be dramatically influenced by ambient GABA levels

In section 3.3 we explored the effects of ambient GABA concentrations on the actions of three classes of clinically relevant drugs that are believed to enhance tonic inhibition in the brain. Results suggested that the action of the intravenous anaesthetic propofol and the hypnotic GABA-mimetic gaboxadol were attenuated when ambient GABA levels were raised. In contrast, neuroactive steroids could enhance tonic inhibition even at saturating GABA concentrations. These findings will be discussed in terms of the mechanisms that underpin these contrasting effects, and in relation to the known changes of ambient GABA concentrations.

The physiological significance of these observations depends on the level of ambient GABA *in vivo*. Theoretical studies based on the stoichiometry of the GABA transporter suggest extracellular GABA levels are between 10 and 400 nM range (Attwell, Barbour et al. 1993;Richerson, Wu et al. 2003; Wu, Wang et al. 2007). Microdialysis studies seem to corroborate this and place resting ambient GABA concentrations between 30nM (de Groote and Linthorst 2007) up to 300 nM (Richter, Schmidt-Garcon et al. 1999; Bianchi, Ballini et al. 2003; Xi, Ramamoorthy et al. 2003). This wide range could reflect inherent inconsistencies between microdialysis experiments stemming from the invasive and potentially damaging nature of this technique, however it also reflects real cell type and regional concentration differences (Mody, Glykys et al. 2007). Moreover it probably reflects differences in neuronal excitability. Activity dependent vesicular release and the diffusion of GABA away from the synaptic cleft is widely believed to influence ambient GABA concentrations (Mitchell and Silver 2000; Bright, Renzi et al. 2011). A number of groups have shown that blocking action potential dependent GABA release with the voltage-gated sodium channel blocker TTX reduced the tonic conductance recorded from adult cerebellar granule neurons (Carta, Mameli et al. 2004; Bright, Renzi et al. 2011). Conversely, increased synaptic release from golgi cell terminals enhances the tonic conductance in these neurons (Brickley, Cull-Candy et al. 1996; Carta, Mameli et al. 2004). Increasing GABAergic interneuron activity in the hippocampus has been shown to increase the tonic conductance levels in both the interneurons and neighbouring pyramidal cells (Frerking, Petersen et al.

1999; Kullmann, Semyanov et al. 2003). This dependence of tonic GABA levels on neuronal activity is also reflected in shifts of tonic associated with certain behaviours. Ambient GABA levels can rise to ~800nM in the ventral hippocampus during exploration and in response to stress (Bianchi, Ballini et al. 2003; de Groote and Linthorst 2007). While in the thalamus extracellular GABA concentrations are almost double during non-REM sleep than during REM or waking states (Kekesi, Dobolyi et al. 1997). Thus we have a picture of physiological GABA levels ranging from 10nM up to 800nM, however it is also possible that this range will extend even further under certain pathological conditions. For example an increase in ambient GABA levels has been reported in the peri-infarct zone following stroke in mice (Clarkson, Huang et al. 2010), while in Parkinson's patients significantly enhanced GABA levels in the striatum were observed (Kish, Rajput et al. 1986).

Propofol induced enhancement of recombinant α6βδ receptors was completely abrogated at GABA concentrations above 100nM. This was consistent with our model driven prediction and further corroborated by the finding that propofols ability to enhance native α6βδ in cerebellar granule cells could be reduced by increasing the rate of vesicular GABA release. This phenomenon can be explained in terms of propofols action on GABA responses. Observations based on peak responses suggest that propofol modulates synaptic GABA-A receptors by increasing channel opening and prolonging macroscopic deactivation while causing little change in desensitisation, manifesting as a leftward shift in the GABA concentration-response curve (Orser, Bai et al. 1999; Franks 2008). As illustrated in section 3, steady-state conditions produce a narrower and leftward shifted GABA dose-response as compared with peaks, as such even modest increases in GABA levels will push responses close to maximal conductances. Under these conditions, where a response 'ceiling' has been reached the ability of propofol to further enhance current levels is attenuated. As outlined above, there are many physiological and pathological conditions which could see extracellular GABA levels rise beyond 100nM, and therefore place clear limits on conditions in which propofol may enhance tonic inhibition. The main implication of this finding is one of two things: either, propofol is only an effective anaesthetic when ambient GABA is very low or, δ-containing receptors are not the primary target for propofols anaesthetic effects.

While propofols molecular targets are numerous, there is strong evidence that the GABAergic system represents a primary target (Franks 2008). Sanna et al (1995) expressed an array of many different GABA-A subunit constructs in *Xenopus laevis* oocytes and reported that propofol modulated GABA-evoked chloride currents in all combinations (Sanna, Mascia et al. 1995). Work in native tissue also bore this out, showing that propofol could enhance the synaptic responses in a number of brain regions (Taylor and Collins 1988; Hales and Lambert 1991; Peduto, Concas et al. 1991). More telling still comes from work carried out by Jurd *et al* (2003), which showed that point mutated β3 N265M knock-in mice exhibited a reduction in the duration of propofol induced loss of righting reflex and a complete lack of immobilisation upon administration of noxious stimuli. Extrasynaptic receptors too have been implicated in a number of studies (Franks 2008), and as we've demonstrated propofol at physiological levels is a potent modulator of α6βδ containing GABA-A receptors. Combined, this wealth of data has led many to suggest that GABA-A receptors are the primary target for propofols effects *in vivo*, however, the relative contributions of synaptic and extrasynaptic receptors remains to be established.

We would suggest that the fact that propofol modulation of extrasynaptic receptors is so vulnerable to modest increases in ambient GABA makes it less likely that that tonic inhibition is responsible for propofols anaesthetic action. Anecdotal evidence would seem to support this, i.e. the fact that propofol is one of the world's most widely used IV anaesthetics and has reported anaesthetic induction success rates of up to 95.5% (Brimacombe 1996; Joo and Perks 2000) would argue for a very robust and reliable mechanism of action. Also consistent with this, the examination of δ knock-out mice showed that the ability of propofol and etomidate to increase sleep time were not altered (Mihalek, Banerjee et al. 1999). While it is possible that extrasynaptic tonically active γ-containing receptors could mediate some of these affects, our current understanding of where anaesthetic action is initiated in the brain does not implicate these receptors. Taken together, our findings suggest that propofols effects on the GABAergic system are mediated primarily through phasically active synaptic GABA-A receptors.

Gaboxadol's effects on α6βδ containing receptors were similarly affected. At just 50nM GABA, a concentration at the low end of plausible estimates of GABA levels *in vivo*, the

ability of gaboxadol to enhance recombinant receptors was reduced by around 70%, while in native neurons an increase in synaptic GABA release was also able to attenuate gaboxadols enhancement of tonic. Predictions regarding this effect where mixed. On the one hand the robust so-called "super-agonism" attributed to gaboxadol may be expected to elicit an increase in tonic under any conditions. On the other the competition for the same binding site may prove critical. However, as discussed, our previous results highlight that gaboxadol is in fact no more efficacious than GABA at δ-containing receptors when steady-state conditions are considered, and as such under physiological conditions should not be considered a super-agonist. As such it is unsurprising that gaboxadol's effects are attenuated in high GABA, explained by gaboxadols inability to compete with GABA higher affinity binding.

Unlike propofol, the evidence that gaboxadol exerts its clinical effect through extrasynaptic receptors is significantly more clear cut (Wafford and Ebert 2006), borne out by the fact the ability of gaboxadol to induced slow EEG oscillations and enhance non-REM sleep was reduced δ-knock out animals (Tobler, Winsky-Sommerer et al. 2007). Taken together, our findings would seem to place clear constraints on the conditions under which gaboxadol will be clinically effective. One caveat to consider here however is that in order to fully block gaboxadols effects the receptor population must be fully occupied, as we were able to demonstrate in HEK cells and using exogenous application of high dose GABA in the slice. However, during pharmacological manipulations of ambient GABA gaboxadol always elicited an increase in conductance, all be it reduced in CNQX-evoked high GABA conditions. Thus this may suggest that *in vivo* the number of extrasynaptic GABA-A receptors far exceeds the number of GABA molecules. In which case if GABA concentrations increased only modestly, there would still be sufficient sites for gaboxadol to bind and enhance the tonic levels. In support of this, observations in our lab showed no correlation between cerebellar granule neuron surface area and the magnitude of tonic conductance (unpublished), consistent with a high receptor to ligand ratio. Of course we can't know to what level we were raising GABA in the slice, and it may still be very low.

Our results predict that the physiological hypnotic effect of gaboxadol could be highly variable. Enhanced tonic GABA conductance mediated by α4βδ receptors promotes burst firing of thalamic relay neurons (Cope, Hughes et al. 2005; Bright, Aller et al. 2007), a key

requirement in the generation of slow 1-4 Hz EEG rhythms during non-REM sleep. It is via this mechanism that gaboxadol likely exerts its hypnotic effects. In light of our findings it is possible that increases in thalamic ambient GABA, such as those seen during non-REM sleep may well serve to abrogate gaboxadol's effects at α4βδ GABA-A receptors (Tobler, Winsky-Sommerer et al. 2007). Indeed we might speculate that this phenomenon underlies the lack of efficacy cited by Merck and Lundbeck as one of the reasons this drug was discontinued in development as an insomnia treatment. Although Gaboxadol was discontinued as a therapeutic drug during Phase III clinical trial it remains a powerful tool for investigating the roles of δ-containing GABA-A receptors. As such these new findings should be taken into account in many experimental paradigms, especially where GABA is supplemented.

In contrast to Propofol and Gaboxadol, the neuroactive steroids THDOC, Alfaxalone and Ganaxalone enhanced tonic receptors to similar extent in all ambient GABA conditions. This naturally reflects distinct mechanisms of activation, however more than that it may also highlight an ideal mechanism for tonic modulation. Propofol's allosteric modulation and gaboxadol's orthosteric activation of δ-containing receptors are both sensitive to shifts in GABA, but the mechanisms that underpin these phenomena are distinct. Equally, we have shown that neurosteroid potentiation of steady-state α6βδ currents is via a distinct mechanism which may involve decreasing the extent of steady-state desensitization. We would propose that this represents an ideal strategy with which to enhance the profoundly desensitized pool of GABA-A receptors found outside of synapses. Indeed important drugs may already exploit this mechanism. The neurosteroid analogue ganaxolone is in Phase III clinical trials for the treatment of catamenial epilepsy, a highly prevalent type of epilepsy in women that shows cyclic variations in the frequency and intensity of seizures depending on the phase of the menstrual cycle. Consistent with reported changes in δ-GABA-A receptor mediated tonic inhibition during the ovarian cycle (Maguire, Stell et al. 2005), it is believed that ganaxolone's efficacy involves tonic enhancement (Belelli and Herd 2003). Extrasynaptic δ-containing GABA-A receptors are highly sensitive to neurosteroid enhancement (Stell, Brickley et al. 2003) and the ability of ganaxolone to enhance the conductance of these likely explains why this drug protects against seizure during sensitive periods of the ovarian cycle when other drug treatments may be ineffective. Furthermore the hypnotic effects of Alfaxalone are reduced in animals where the δ-receptors are genetically removed in rodents

(Mihalek, Banerjee et al. 1999) suggesting this widely used veterinary anaesthetic has a largely extrasynaptic site of action.

Given that fluctuations in extracellular GABA concentrations are associated with many physiological and pathological conditions, the clinical significance of these results should be considered. Many clinically prescribed drugs are known to alter ambient GABA levels. The GABA uptake blocker tiagabine is approved for the treatment of partial seizures as well as anxiety disorders and neuropathic pain. Gabapentin is an anti-epileptic that is also prescribed for the treatment of neuropathic pain and major depressive disorders, and acts partly by increasing GABA synthesis by the enzyme glutamic acid decarboxylase (GAD) and thus raising ambient GABA levels (Taylor 1997). Another antiepileptic drug, vigabatrin, also raises ambient GABA levels by blocking GABA catabolism by the GABA transaminase enzyme. In light of our findings we might speculate that the use of these drugs should be contraindicated with use of GABA mimetics such as gaboxadol or allosteric modulators such as propofol. As discussed ambient GABA can also be altered secondary to pathologies. This may include stress disorders as during periods of enforced stress increased ambient GABA was raised by upwards of 250% in the hippocampus of rodents (de Groote and Linthorst 2007). Thus under such conditions the efficacy of some drugs may be drastically reduced. The fact that GABAergic agonists are often employed in treating stress disorders lends this further significance. Taken together our results highlight the efficacy and usefulness of neurosteroid drugs already available such as ganaxolone and alfaxalone. However they may also have a broader significance in drug design strategy, put simply, if you wish to design a drug to enhance tonic inhibition, make it more like neurosteroids and less like gaboxadol or propofol.

References

Adkins, C. E., G. V. Pillai, et al. (2001). "α4β3δ GABAA Receptors Characterized by Fluorescence Resonance Energy Transfer-derived Measurements of Membrane Potential." Journal of Biological Chemistry **276**(42): 38934-38939.

Agis-Balboa, R. C., G. Pinna, et al. (2006). "Characterization of brain neurons that express enzymes mediating neurosteroid biosynthesis." Proc Natl Acad Sci U S A **103**(39): 14602-14607.

Aguayo, L. G., R. W. Peoples, et al. (2002). "GABA(A) receptors as molecular sites of ethanol action. Direct or indirect actions?" Curr Top Med Chem **2**(8): 869-885.

Akk, G., J. Bracamontes, et al. (2004). "Activation of GABA(A) receptors containing the alpha 4 subunit by GABA and pentobarbital." Journal of Physiology **556**(2): 387-399.

Alifimoff, J. K., L. L. Firestone, et al. (1989). "Anesthetic Potencies of Primary Alkanols - Implications for the Molecular Dimensions of the Anesthetic Site." British Journal of Pharmacology **96**(1): 9-16.

Ariwodola, O. J. and J. L. Weiner (2004). "Ethanol potentiation of GABAergic synaptic transmission may be self-limiting: role of presynaptic GABA(B) receptors." Journal of Neuroscience **24**(47): 10679-10686.

Ashby, M. C., K. Ibaraki, et al. (2004). "It's green outside: tracking cell surface proteins with pH-sensitive GFP." Trends in Neurosciences **27**(5): 257-261.

Attwell, D., B. Barbour, et al. (1993). "Nonvesicular Release of Neurotransmitter." Neuron **11**(3): 401-407.

Attwell, D., M. Hamann, et al. (2002). "Tonic and spillover inhibition of granule cells control information flow through cerebellar cortex." Neuron **33**(4): 625-633.

Ballard, T. M., F. Knoflach, et al. (2009). "RO4938581, a novel cognitive enhancer acting at GABA(A) alpha 5 subunit-containing receptors." Psychopharmacology **202**(1-3): 207-223.

Barrera, N. P., J. Betts, et al. (2008). "Atomic force microscopy reveals the stoichiometry and subunit arrangement of the alpha(4)beta(3)delta GABA(A) receptor." Molecular Pharmacology **73**(3): 960-967.

Barnes, N., R. Tsivkovskii, et al. (2005). "The copper-transporting ATPases, Menkes and Wilson disease proteins, have distinct roles in adult and developing cerebellum." Journal of Biological Chemistry **280**(10): 9640-9645.

Barnard, E. A., P. Skolnick, et al. (1998). "International Union of Pharmacology. XV. Subtypes of gamma-aminobutyric acid(A) receptors: Classification on the basis of subunit structure and receptor function." Pharmacological Reviews **50**(2): 291-313.

Belelli, D., H. Callachan, et al. (1996). "Interaction of positive allosteric modulators with human and Drosophila recombinant GABA receptors expressed in Xenopus laevis oocytes." Br J Pharmacol **118**(3): 563-576.

Belelli, D., A. Casula, et al. (2002). "The influence of subunit composition on the interaction of neurosteroids with GABA(A) receptors." Neuropharmacology **43**(4): 651-661.

Belelli, D., N. L. Harrison, et al. (2009). "Extrasynaptic GABAA receptors: form, pharmacology, and function." Journal of Neuroscience **29**(41): 12757-12763.

Belelli, D. and M. B. Herd (2003). "The contraceptive agent Provera enhances GABA(A) receptor-mediated inhibitory neurotransmission in the rat hippocampus: evidence for endogenous neurosteroids?" Journal of Neuroscience **23**(31): 10013-10020.

Belelli, D. and J. J. Lambert (2005). "Neurosteroids: endogenous regulators of the GABA(A) receptor." Nat Rev Neurosci **6**(7): 565-575.

Belelli, D., D. R. Peden, et al. (2005). "Extrasynaptic GABAA receptors of thalamocortical neurons: a molecular target for hypnotics." Journal of Neuroscience **25**(50): 11513-11520.

Biagini, G., G. Panuccio, et al. (2010). "Neurosteroids and epilepsy." Current Opinion in Neurology **23**(2): 170-176.

Bianchi, L., C. Ballini, et al. (2003). "Investigation on acetylcholine, aspartate, glutamate and GABA extracellular levels from ventral hippocampus during repeated exploratory activity in the rat." Neurochemical Research **28**(3-4): 565-573.

Bianchi, M. T., K. F. Haas, et al. (2002). "Alpha1 and alpha6 subunits specify distinct desensitization, deactivation and neurosteroid modulation of GABA(A) receptors containing the delta subunit." Neuropharmacology **43**(4): 492-502.

Bianchi, M. T. and R. L. Macdonald (2003). "Neurosteroids shift partial agonist activation of GABA(A) receptor channels from low- to high-efficacy Gating patterns." Journal of Neuroscience **23**(34): 10934-10943.

Biggio, G., A. Concas, et al. (2007). "Stress, ethanol, and neuroactive steroids." Pharmacology & Therapeutics **116**(1): 140-171.

Birnir, B., A. B. Everitt, et al. (2000). "Spontaneously OpeningGABA(A) Channels in CA1 Pyramidal Neurones of Rat Hippocampus." Journal of Membrane Biology **174**(1): 21-29.

Boehm, S. L., 2nd, I. Ponomarev, et al. (2004). "gamma-Aminobutyric acid A receptor subunit mutant mice: new perspectives on alcohol actions." Biochemical Pharmacology **68**(8): 1581-1602.

Boehm, S. L., 2nd, I. Ponomarev, et al. (2006). "From gene to behavior and back again: new perspectives on GABAA receptor subunit selectivity of alcohol actions." Advances in Pharmacology **54**: 171-203.

Borden, L. A., T. G. M. Dhar, et al. (1994). "Tiagabine, Sk-and-F 89976-a, Ci-966, and Nnc-711 Are Selective for the Cloned Gaba Transporter Gat-1." European Journal of Pharmacology **269**(2): 219-224.

Borghese, C. M. and R. A. Harris (2007). "Studies of ethanol actions on recombinant delta-containing gamma-aminobutyric acid type A receptors yield contradictory results." Alcohol **41**(3): 155-162.

Borghese, C. M., S. I. Storustovu, et al. (2006). "The delta subunit of gamma-aminobutyric acid type A receptors does not confer sensitivity to low concentrations of ethanol." Journal of Pharmacology and Experimental Therapeutics **316**(3): 1360-1368.

Bormann, J., O. P. Hamill, et al. (1987). "Mechanism of Anion Permeation through Channels Gated by Glycine and Gamma-Aminobutyric-Acid in Mouse Cultured Spinal Neurons." Journal of Physiology **385**: 243-286.

Brehm, L., B. Ebert, et al. (1997). "Structure and pharmacology of 4,5,6,7-tetrahydroisothiazolo[5,4-c]pyridin-3-ol (Thio-THIP), an agonist/antagonist at GABA(A) receptors." European Journal of Medicinal Chemistry **32**(4): 357-363.

Brewer, G. J. and V. Yuzbasiyangurkan (1992). "Wilson Disease." Medicine **71**(3): 139-164.

Brickley, S. G., S. G. Cull-Candy, et al. (1996). "Development of a tonic form of synaptic inhibition in rat cerebellar granule cells resulting from persistent activation of GABAA receptors." The Journal of Physiology **497**(Pt 3): 753-759.

Brickley, S. G., V. Revilla, et al. (2001). "Adaptive regulation of neuronal excitability by a voltage-independent potassium conductance." Nature **409**(6816): 88-92.

Bright, D. P., M. I. Aller, et al. (2007). "Synaptic release generates a tonic GABA(A) receptor-mediated conductance that modulates burst precision in thalamic relay neurons." J Neurosci **27**(10): 2560-2569.

Bright, D. P., M. Renzi, et al. (2011). "Profound desensitization by ambient GABA limits activation of delta-containing GABAA receptors during spillover." Journal of Neuroscience :**31**(2): 753-763.

Brimacombe, J. (1996). "Analysis of 1500 laryngeal mask uses by one anaesthetist in adults undergoing routine anaesthesia." Anaesthesia **51**(1): 76-80.

Brown, N., J. Kerby, et al. (2002). "Pharmacological characterization of a novel cell line expressing human α4β3δ GABAA receptors." British Journal of Pharmacology **136**(7): 965-974.

Brussaard, A. B., J. J. Koksma, et al. (2003). "Oxytocin regulates neurosteroid modulation of GABA(A) receptors in supraoptic nucleus around parturition." Journal of Neuroscience **23**(3): 788-797.

Buller AL, White MM (1990) Functional acetylcholine receptors expressed in *Xenopus* oocytes after inhection of *Torpedo* β, γ and δ RNAs are a consequnce of endogneous oocyte gene expression. Molecluar Pharmacology **37**:423-428.

Callachan, H., G. A. Cottrell, et al. (1987). "Modulation of the GABA-A Receptor by Progesterone Metabolites." Proceedings of the Royal Society of London Series B-Biological Sciences **231**(1264): 359-369.

Caraiscos, V. B., E. M. Elliott, et al. (2004). "Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by alpha5 subunit-containing gamma-aminobutyric acid type A receptors." Proc Natl Acad Sci U S A **101**(10): 3662-3667.

Caraiscos, V. B., J. G. Newell, et al. (2004). "Selective enhancement of tonic GABAergic inhibition in murine hippocampal neurons by low concentrations of the volatile anesthetic isoflurane." Journal of Neuroscience **24**(39): 8454-8458.

Carta, M., O. J. Ariwodola, et al. (2003). "Alcohol potently inhibits the kainate receptor-dependent excitatory drive of hippocampal interneurons." Proc Natl Acad Sci U S A **100**(11): 6813-6818.

Carta, M., M. Mameli, et al. (2004). "Alcohol enhances GABAergic transmission to cerebellar granule cells via an increase in Golgi cell excitability." Journal of Neuroscience **24**(15): 3746-3751.

Chadderton, P., T. W. Margrie, et al. (2004). "Integration of quanta in cerebellar granule cells during sensory processing." Nature **428**(6985): 856-860.

Chambers, M. S., J. R. Atack, et al. (2002). "6,7-Dihydro-2-benzothiophen-4(5H)-ones: a novel class of GABA-A alpha5 receptor inverse agonists." Journal of Medicinal Chemistry **45**(6): 1176-1179.

Chambers, M. S., J. R. Atack, et al. (2003). "Identification of a novel, selective GABAA alpha 5 receptor inverse agonist which enhances cognition." Journal of Medicinal Chemistry **46**(11): 2227-2240.

Chambers, M. S., J. R. Atack, et al. (2004). "An orally bioavailable, functionally selective inverse agonist at the benzodiazepine site of GABAA alpha5 receptors with cognition enhancing properties." Journal of Medicinal Chemistry **47**(24): 5829-5832.

Cherubini, E., E. M. Petrini, et al. (2003). "Declusterization of GABA(A) receptors affects the kinetic properties of GABAergic currents in cultured hippocampal neurons." Journal of Biological Chemistry **278**(18): 16271-16279.

Chester, J. A. and C. L. Cunningham (2002). "GABA(A) receptor modulation of the rewarding and aversive effects of ethanol." Alcohol **26**(3): 131-143.

Chiu, C. S., S. Brickley, et al. (2005). "GABA transporter deficiency causes tremor, ataxia, nervousness, and increased GABA-induced tonic conductance in cerebellum." Journal of Neuroscience **25**(12): 3234-3245.

Chiu, C. S., K. Jensen, et al. (2002). "Number, density, and surface/cytoplasmic distribution of GABA transporters at presynaptic structures of knock-in mice carrying GABA transporter subtype 1-green fluorescent protein fusions." Journal of Neuroscience **22**(23): 10251-10266.

Chu, N. S., C. C. Chu, et al. (1991). "Eeg Spectral-Analysis and Topographic Mapping in Wilsons-Disease." Journal of the Neurological Sciences **106**(1): 1-9.

Clarkson, A. N., B. S. Huang, et al. (2010). "Reducing excessive GABA-mediated tonic inhibition promotes functional recovery after stroke." Nature **468**(7321): 305-U193.

Collinson, N., F. M. Kuenzi, et al. (2002). "Enhanced learning and memory and altered GABAergic synaptic transmission in mice lacking the alpha 5 subunit of the GABA(A) receptor." Journal of Neuroscience **22**(13): 5572-5580.

Cope, D. W., G. Di Giovanni, et al. (2009). "Enhanced tonic GABA(A) inhibition in typical absence epilepsy." Nature Medicine **15**(12): 1392-U1396.

Cope, D. W., S. W. Hughes, et al. (2005). "GABAA receptor-mediated tonic inhibition in thalamic neurons." Journal of Neuroscience **25**(50): 11553-11563.

Covault, J., J. Gelernter, et al. (2004). "Allelic and haplotypic association of GABRA2 with alcohol dependence." Am J Med Genet B Neuropsychiatr Genet **129**(1): 104-109.

Crabbe, J. C., T. J. Phillips, et al. (2006). "Alcohol-related genes: contributions from studies with genetically engineered mice." Addict Biol **11**(3-4): 195-269.

Criswell, H. E. and G. R. Breese (2005). "A conceptualization of integrated actions of ethanol contributing to its GABAmimetic profile: a commentary." Neuropsychopharmacology **30**(8): 1407-1425.

Dawson, G. R., K. A. Maubach, et al. (2006). "An inverse agonist selective for alpha5 subunit-containing GABAA receptors enhances cognition." J Pharmacol Exp Ther **316**(3): 1335-1345.

de Groote, L. and A. C. E. Linthorst (2007). "Exposure to novelty and forced swimming evoke stressor-dependent changes in extracellular GABA in the rat hippocampus." Neuroscience **148**(3): 794-805.

Dibbens, L. M., H. J. Feng, et al. (2004). "GABRD encoding a protein for extra- or peri-synaptic GABA(A) receptors is a susceptibility locus for generalized epilepsies." Human Molecular Genetics **13**(13): 1315-1319.

Dittgen, T., A. Nimmerjahn, et al. (2004). "Lentivirus-based genetic manipulations of cortical neurons and their optical and electrophysiological monitoring *in vivo*." Proc Natl Acad Sci U S A **101**(52): 18206-18211.

Draguhn, A., T. A. Verdorn, et al. (1990). "Functional and Molecular Distinction between Recombinant Rat Gaba-a Receptor Subtypes by Zn-2+." Neuron **5**(6): 781-788.

Drasbek, K. R., K. Hoestgaard-Jensen, et al. (2007). "Modulation of extrasynaptic THIP conductances by GABAA-receptor modulators in mouse neocortex." Journal of Neurophysiology **97**(3): 2293-2300.

DuBridge, R. B., P. Tang, et al. (1987). "Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system." Molecular and Cellular Biology **7**(1): 379-387.

Dumoulin, A., P. Rostaing, et al. (1999). "Presence of the vesicular inhibitory amino acid transporter in gabaergic and glycinergic synaptic terminal boutons." Journal of Neurochemistry **73**: S6-S6.

Ebert, B., S. A. Thompson, et al. (1997). "Differences in agonist/antagonist binding affinity and receptor transduction using recombinant human gamma-aminobutyric acid type a receptors." Molecular Pharmacology **52**(6): 1150-1156.

Ebert, B., K. A. Wafford, et al. (1994). "Molecular Pharmacology of Gamma-Aminobutyric-Acid Type-a Receptor Agonists and Partial Agonists in Oocytes Injected with Different Alpha-Receptor, Beta-Receptor, and Gamma-Receptor Subunit Combinations." Molecular Pharmacology **46**(5): 957-963.

Edenberg, H. J., D. M. Dick, et al. (2004). "Variations in GABRA2, encoding the alpha 2 subunit of the GABA(A) receptor, are associated with alcohol dependence and with brain oscillations." American Journal of Human Genetics **74**(4): 705-714.

Egebjerg, J. and S. F. Heinemann (1993). "Ca2+ Permeability of Unedited and Edited Versions of the Kainate Selective Glutamate Receptor Glur6." Proc Natl Acad Sci U S A **90**(2): 755-759.

Essrich, C., J. M. Fritschy, et al. (1998). "Synaptic targeting of GABA(A) receptors: Essential roles for the gamma 2 subunit and gephyrin." European Journal of Neuroscience **10**: 185-185.

Essrich, C., M. Lorez, et al. (1998). "Postsynaptic clustering of major GABAA receptor subtypes requires the gamma 2 subunit and gephyrin." Nature Neuroscience **1**(7): 563-571.

Farrant, M., S. G. Brickley, et al. (1999). "Single-channel properties of synaptic and extrasynaptic GABA(A) receptors suggest differential targeting of receptor subtypes." Journal of Neuroscience **19**(8): 2960-2973.

Farrant, M. and Z. Nusser (2005). "Variations on an inhibitory theme: phasic and tonic activation of GABAA receptors." Nature Neuroscience Reviews **6**(3): 215-229.

Faulhaber, J., A. Steiger, et al. (1997). "The GABA(A) agonist THIP produces slow wave sleep and reduces spindling activity in NREM sleep in humans." Psychopharmacology **130**(3): 285-291.

Feng, H. J., M. T. Bianchi, et al. (2004). "Pentobarbital differentially modulates alpha1beta3delta and alpha1beta3gamma2L GABAA receptor currents." Molecular Pharmacology **66**(4): 988-1003.

Feng, H. J. and R. L. Macdonald (2004). "Multiple actions of propofol on alpha beta gamma and alpha beta delta GABA(A) receptors." Molecular Pharmacology **66**(6): 1517-1524.

Feng, Y., K. Kapornai, et al. (2010). "Association of the GABRD gene and childhood-onset mood disorders." Genes Brain and Behavior **9**(6): 668-672.

Fisher, J. L. and R. L. MacDonald (1997). "Functional properties of recombinant GABA(A) receptors composed of single or multiple beta subunit subtypes." Neuropharmacology **36**(11-12): 1601-1610.

Fisher, J. L. and R. L. Macdonald (1997). "Single channel properties of recombinant GABAA receptors containing gamma 2 or delta subtypes expressed with alpha 1 and beta 3 subtypes in mouse L929 cells." The Journal of Physiology **505 (Pt 2)**: 283-297.

Fisher, J. L. and R. L. Macdonald (1998). "The role of an alpha subtype M-2-M-3 his in regulating inhibition of GABA(A) receptor current by zinc and other divalent cations." J Neurosci **18**(8): 2944-2953.

Foust, K. D., E. Nurre, et al. (2009). "Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes." Nature Biotechnology **27**(1): 59-65.

Franks, N. P. (2008). "General anaesthesia: From molecular targets to neuronal pathways of sleep and arousal." Nature Reviews Neuroscience **9**(5): 370-386.

Freund, T. F. (2003). "Interneuron diversity series: Rhythm and mood in perisomatic inhibition." Trends in Neurosciences **26**(9): 489-495.

Freund, T. F. and I. Katona (2007). "Perisomatic inhibition." Neuron **56**(1): 33-42.

Frerking, M., C. C. H. Petersen, et al. (1999). "Mechanisms underlying kainate receptor-mediated disinhibition in the hippocampus." Proc Natl Acad Sci U S A **96**(22): 12917-12922.

Fritschy, J. M., D. Benke, et al. (1992). "5 Subtypes of Type-a Gamma-Aminobutyric-Acid Receptors Identified in Neurons by Double and Triple Immunofluorescence Staining with Subunit-Specific Antibodies." Proc Natl Acad Sci U S A **89**(15): 6726-6730.

Fritschy, J. M., I. Brunig, et al. (2002). "Intact sorting, targeting, and clustering of gamma-aminobutyric acid A receptor subtypes in hippocampal neurons in vitro." The Journal of Comparative Neurology **443**(1): 43-55.

Gadea, A. and A. M. Lopez-Colome (2001). "Glial transporters for glutamate, glycine, and GABA: II. GABA transporters." Journal of Neuroscience Research **63**(6): 461-468.

Gidal, B. E., M. D. Privitera, et al. (1999). "Vigabatrin: A novel therapy for seizure disorders." Annals of Pharmacotherapy **33**(12): 1277-1286.

Glykys, J. and I. Mody (2006). "Hippocampal network hyperactivity after selective reduction of tonic inhibition in GABA A receptor alpha5 subunit-deficient mice." Journal of Neurophysiology **95**(5): 2796-2807.

Glykys, J. and I. Mody (2007). "Activation of GABAA Receptors: Views from Outside the Synaptic Cleft." Neuron **56**(5): 763-770.

Glykys, J. and I. Mody (2007). "The main source of ambient GABA responsible for tonic inhibition in the mouse hippocampus." The Journal of Physiology **582**(Pt 3): 1163-1178.

Hadley, S. H. and J. Amin (2007). "Rat α6βδGABAA receptors exhibit two distinct and separable agonist affinities." The Journal of Physiology **581**(3): 1001-1018.

Hales, T. G. and J. J. Lambert (1991). "The Actions of Propofol on Inhibitory Amino-Acid Receptors of Bovine Adrenomedullary Chromaffin Cells and Rodent Central Neurons." British Journal of Pharmacology **104**(3): 619-628.

Hamann, M., D. J. Rossi, et al. (2002). "Tonic and spillover inhibition of granule cells control information flow through cerebellar cortex." Neuron **33**(4): 625-633.

Hanchar, H. J., M. Wallner, et al. (2005). "The potential alcohol binding site of extrasynaptic GABA(A) receptors." Alcoholism-Clinical and Experimental Research **29**(5): 93a-93a.

Harris, R. A., J. R. Trudell, et al. (2008). "Ethanol's Molecular Targets." Science Signaling **1**(28).

Harrison, N. L. (1987). "Progesterone Metabolites Enhance Gaba Activities." Trends in Pharmacological Sciences **8**(9): 334-334.

Hartter, D. E. and A. Barnea (1988). "Brain-Tissue Accumulates Copper-67 by 2 Ligand-Dependent Saturable Processes - a High-Affinity, Low Capacity and a Low Affinity, High-Capacity Process." Journal of Biological Chemistry **263**(2): 799-805.

Hedin KE, Lim NF, Clapham DE (1996) Cloning of a *Xenopus laevis* inwardly rectifying K^+ channel subunit that permits GIRK1 expression of I_{KACh} currents in oocytes. Neuron 16:423–429.

Hevers, W. and H. Luddens (1998). "The diversity of GABA(A) receptors - Pharmacological and electrophysiological properties of GABA(A) channel subtypes." Molecular Neurobiology **18**(1): 35-86.

Hosie, A. M., M. E. Wilkins, et al. (2006). "Endogenous neurosteroids regulate GABAA receptors through two discrete transmembrane sites." Nature **444**(7118): 486-489.

Hosie, A. M., M. E. Wilkins, et al. (2007). "Neurosteroid binding sites on GABA(A) receptors." Pharmacol Ther **116**(1): 7-19.

Houser, C. R., Z. Peng, et al. (2002). "GABA(A) receptor changes in delta subunit-deficient mice: Altered expression of alpha 4 and gamma 2 subunits in the forebrain." Journal of Comparative Neurology **446**(2): 179-197.

Hui, K. S., B. A. Davis, et al. (1977). "Analysis of Copper in Brain by Mass-Spectrometric Integrated-Ion-Current Procedure." Neurochemical Research **2**(5): 495-506.

Jia, F., M. Yue, et al. (2008). "Isoflurane is a potent modulator of extrasynaptic GABA(A) receptors in the thalamus." The Journal of pharmacology and experimental therapeutics **324**(3): 1127-1135.

Jones, M. V., Y. Sahara, et al. (1998). "Defining affinity with the GABA(A) receptor." Journal of Neuroscience **18**(21): 8590-8604.

Joo, H. S. and W. J. Perks (2000). "Sevoflurane versus propofol for anesthetic induction: A meta-analysis." Anesthesia and Analgesia **91**(1): 213-219.

Kaneda, M., M. Farrant, et al. (1995). "Whole-Cell and Single-Channel Currents Activated by Gaba and Glycine in Granule Cells of the Rat Cerebellum." Journal of Physiology **485**(2): 419-435.

Kardos, J., I. Kovacs, et al. (1989). "Nerve-Endings from Rat-Brain Tissue Release Copper Upon Depolarization - a Possible Role in Regulating Neuronal Excitability." Neuroscience Letters **103**(2): 139-144.

Katayama, S., M. Irifune, et al. (2007). "Increased gamma-aminobutyric acid levels in mouse brain induce loss of righting reflex, but not immobility, in response to noxious stimulation." Anesthesia and Analgesia **104**(6): 1422-1429.

Kato, T. (2007). "Molecular genetics of bipolar disorder and depression." Psychiatry and Clinical Neurosciences **61**(1): 3-19.

Kekesi, K. A., A. Dobolyi, et al. (1997). "Slow wave sleep is accompanied by release of certain amino acids in the thalamus of cats." Neuroreport **8**(5): 1183-1186.

Kim, H. J. and R. L. Macdonald (2003). "An N-terminal histidine is the primary determinant of alpha subunit-dependent Cu2+ sensitivity of alpha beta 3 gamma 2L GABA(A) receptors." Molecular Pharmacology **64**(5): 1145-1152.

Kish, S. J., A. Rajput, et al. (1986). "Elevated Gamma-Aminobutyric-Acid Level in Striatal but Not Extrastriatal Brain-Regions in Parkinsons-Disease - Correlation with Striatal Dopamine Loss." Annals of Neurology **20**(1): 26-31.

Klaassen, A., J. Glykys, et al. (2006). "Seizures and enhanced cortical GABAergic inhibition in two mouse models of human autosomal dominant nocturnal frontal lobe epilepsy." Proc Natl Acad Sci U S A **103**(50): 19152-19157.

Klausberger, T. and P. Somogyi (2008). "Neuronal diversity and temporal dynamics: The unity of hippocampal circuit operations." Science **321**(5885): 53-57.

Kneussel, M. and S. Loebrich (2007). "Trafficking and synaptic anchoring of ionotropic inhibitory neurotransmitter receptors." Biol Cell **99**(6): 297-309.

Korpi, E. R., T. Kuner, et al. (1995). "Selective Antagonist for the Cerebellar Granule Cell-Specific Gamma-Aminobutyric-Acid Type-a Receptor." Molecular Pharmacology **47**(2): 283-289.

Kozlov, A. S., M. C. Angulo, et al. (2006). "Target cell-specific modulation of neuronal activity by astrocytes." Proc Natl Acad Sci U S A **103**(26): 10058-10063.

Krasowski, M. D., S. M. OShea, et al. (1997). "alpha subunit isoform influences GABA(A) receptor modulation by propofol." Neuropharmacology **36**(7): 941-949.

Krystal, J. H., J. Staley, et al. (2006). "Gamma-aminobutyric acid type A receptors and alcoholism: intoxication, dependence, vulnerability, and treatment." Arch Gen Psychiatry **63**(9): 957-968.

Kullmann, D. M., A. Semyanov, et al. (2003). "GABA uptake regulates cortical excitability via cell type-specific tonic inhibition." Nature Neuroscience **6**(5): 484-490.

Kullmann, D. M., A. Semyanov, et al. (2003). "Tonic GABA(A) receptor mediated currents modulate cortical excitability." Journal of Neurology Neurosurgery and Psychiatry **74**(8): 1182-1183.

Lagrange, A. H., E. J. Botzolakis, et al. (2007). "Enhanced macroscopic desensitization shapes the response of α4 subtype-containing GABAA receptors to synaptic and extrasynaptic GABA." The Journal of Physiology **578**(3): 655-676.

Lambert, J. J., D. Belelli, et al. (2003). "Neurosteroid modulation of GABA(A) receptors." Progress in Neurobiology **71**(1): 67-80.

Lambert, J. J., S. C. Harney, et al. (2003). "Phosphorylation influences neurosteroid modulation of synaptic GABA(A) receptors in rat CA1 and dentate gyrus neurones." Neuropharmacology **45**(6): 873-883.

Lancel, M. (1997). "The GABA(A) agonist THIP increases non-REM sleep and enhances non-REM sleep-specific delta activity in the rat during the dark period." Sleep **20**(12): 1099-1104.

Lancel, M. (1999). "Role of GABA(A) receptors in the regulation of sleep: Initial sleep responses to peripherally administered modulators and agonists." Sleep **22**(1): 33-42.

Laurie, D. J., P. H. Seeburg, et al. (1992). "The Distribution of 13-Gaba-a Receptor Subunit Messenger-Rnas in the Rat-Brain .2. Olfactory-Bulb and Cerebellum." Journal of Neuroscience **12**(3): 1063-1076.

Laurie, D. J., W. Wisden, et al. (1992). "The Distribution of 13 Gaba(a) Receptor Subunit Messenger-Rnas in the Developing Rat-Brain." European Journal of Neuroscience: 6-6.

Lee, C. J., S. Lee, et al. (2010). "Channel-Mediated Tonic GABA Release from Glia." Science **330**(6005): 790-796.

Liang, J., E. Cagetti, et al. (2004). "Altered pharmacology of synaptic and extrasynaptic GABA(A) receptors on CA1 hippocampal neurons is consistent with subunit changes in a model of alcohol withdrawal and dependence." Journal of Pharmacology and Experimental Therapeutics **310**(3): 1234-1245.

Lindquist, C. E. L., J. E. Dalziel, et al. (2004). "Penicillin blocks human [alpha]1[beta]1 and [alpha]1[beta]1[gamma]2S GABAA channels that open spontaneously." European Journal of Pharmacology **496**(1-3): 23-32.

Lindquist, C. E. L., J. E. Dalziel, et al. (2004). "Penicillin blocks human [alpha]1[beta]1 and [alpha]1[beta]1[gamma]2S GABAA channels that open spontaneously." European Journal of Pharmacology **496**(1-3): 23-32.

Lu, YM., Mansuy, IM., et al (2000). "Calcineurin-mediated LTD of GABAergic inhibtion underlies the increased excitability of CA1 neurons associated with LTP." Neuron **26**(1):197-205.

Luddens, H., I. Bohme, et al. (2004). "Four amino acids in the alpha subunits determine the gamma-aminobutyric acid sensitivities of GABA(A) receptor subtypes." Journal of Biological Chemistry **279**(34): 35193-35200.

Luscher, B., C. Essrich, et al. (1998). "Postsynaptic clustering of major GABA(A) receptor subtypes requires the gamma 2 subunit and gephyrin." Nature Neuroscience **1**(7): 563-571.

Ma, J. Y. and T. Narahashi (1993). "Differential Modulation of Gaba-a Receptor-Channel Complex by Polyvalent Cations in Rat Dorsal-Root Ganglion Neurons." Brain Research **607**(1-2): 222-232.

Macdonald, R. L., H. J. Feng, et al. (2009). "Context-dependent modulation of alpha beta gamma and alpha beta delta GABA(A) receptors by penicillin: Implications for phasic and tonic inhibition." Neuropharmacology **56**(1): 161-173.

Macdonald, R. L. and J. L. Fisher (1997). "Single channel properties of recombinant GABA(A) receptors containing gamma 2 or delta subtypes expressed with alpha 1 and beta 3 subtypes in mouse L929 cells." Journal of Physiology **505**(2): 283-297.

Macdonald, R. L. and K. F. Haas (1999). "GABA(A) receptor subunit gamma 2 and delta subtypes confer unique kinetic properties on recombinant GABA(A) receptor currents in mouse fibroblasts." Journal of Physiology **514**(1): 27-45.

Macdonald, R. L. and R. W. Olsen (1994). "Gaba(a) Receptor Channels." Annual Review of Neuroscience **17**: 569-602.

Madsen, S. M., T. Lindeburg, et al. (1983). "Pharmacokinetics of the Gamma-Aminobutyric Acid Agonist Thip (Gaboxadol) Following Intramuscular Administration to Man, with Observations in Dog." Acta Pharmacologica Et Toxicologica **53**(5): 353-357.

Maguire, J. and I. Mody (2008). "GABA(A)R plasticity during pregnancy: Relevance to postpartum depression." Neuron **59**(2): 207-213.

Maguire, J. L., B. M. Stell, et al. (2005). "Ovarian cycle-linked changes in GABA(A) receptors mediating tonic inhibition alter seizure susceptibility and anxiety." Nature Neuroscience **8**(6): 797-804.

Maguire, J. and I. Mody (2007). "Neurosteroid synthesis-mediated regulation of GABA(A) receptors: relevance to the ovarian cycle and stress." Journal of Neuroscience **27**(9): 2155-2162.

Maguire, J. L., B. M. Stell, et al. (2005). "Experimental alterations in the function of delta subunit-containing GABA(A)R alter seizure susceptibility during the ovarian cycle in mice." Epilepsia **46**: 12-12.

Mathie, A., G. L. Sutton, et al. (2006). "Zinc and copper: Pharmacological probes and endogenous modulators of neuronal excitability." Pharmacology & Therapeutics **111**(3): 567-583.

McCartney, M. R., T. Z. Deeb, et al. (2007). "Tonically active GABA(A) receptors in hippocampal pyramidal neurons exhibit constitutive GABA-independent gating." Molecular Pharmacology **71**(2): 539-548.

Meera, P., R. W. Olsen, et al (2010). "Alcohol- and Alcohol Antagonist-Sensitive Human GABAA Receptors: Tracking δ Subunit Incorporation into Functional Receptors." Molecular Pharmacology **78**(5): 918-924.

Meera, P., M. Wallner, et al. (2011). "Molecular basis for the high THIP/gaboxadol sensitivity of extrasynaptic GABAA receptors." Journal of Neurophysiology.

Melis, M., Camarini, R., Ungless, MA, Bonci A. (2002). "Long-lasting potentiation of GABAergic synapses in dopamine neurons after a single *in vivo* ethanol exposure." Journal of Neuroscience **22**(6): 2074-84.

Mortensen, M., U. Kristiansen, et al. (2004). "Activation of single heteromeric GABA(A) receptor ion channels by full and partial agonists." Journal of Physiology **557**(Pt 2): 389-413.

Moss, S. J., V. Tretter, et al. (2008). "The clustering of GABA(A) receptor subtypes at inhibitory synapses is facilitated via the direct binding of receptor alpha 2 Subunits to gephyrin." Journal of Neuroscience **28**(6): 1356-1365.

Maldonado-Aviles, J. G., A. A. Curley, et al. (2009). "Altered Markers of Tonic Inhibition in the Dorsolateral Prefrontal Cortex of Subjects With Schizophrenia." American Journal of Psychiatry **166**(4): 450-459.

Mann, E. O. and I. Mody (2010). "Control of hippocampal gamma oscillation frequency by tonic inhibition and excitation of interneurons." Nature Neuroscience **13**(2): 205-U290.

Marty, A. and J. Chavas (2003). "Coexistence of excitatory and inhibitory GABA synapses in the cerebellar interneuron network." Journal of Neuroscience **23**(6): 2019-2031.

Marty, A., J. Chavas, et al. (2004). "Osmotic tension as a possible link between GABA(A) receptor activation and intracellular calcium elevation." Neuron **44**(4): 701-713.

Mihalek, R. M., P. K. Banerjee, et al. (1999). "Attenuated sensitivity to neuroactive steroids in gamma-aminobutyrate type A receptor delta subunit knockout mice." Proc Natl Acad Sci U S A **96**(22): 12905-12910.

Mihalek, R. M., B. J. Bowers, et al. (2001). "GABA(A)-receptor delta subunit knockout mice have multiple defects in behavioral responses to ethanol." Alcoholism-Clinical and Experimental Research **25**(12): 1708-1718.

Mitchell, S. J. and R. A. Silver (2000). "GABA spillover from single inhibitory axons suppresses low-frequency excitatory transmission at the cerebellar glomerulus." Journal of Neuroscience **20**(23): 8651-8658.

Mitchell, S. J. and R. A. Silver (2000). "Glutamate spillover suppresses inhibition by activating presynaptic mGluRs." Nature **404**(6777): 498-502.

Mody, I. (2001). "Distinguishing between GABA(A) receptors responsible for tonic and phasic conductances." Neurochemical Research **26**(8-9): 907-913.

Mody, I., J. Glykys, et al. (2007). "A new meaning for "Gin & Tonic": tonic inhibition as the target for ethanol action in the brain." Alcohol **41**(3): 145-153.

Mody, I., Y. De Koninck, et al. (1994). "Bridging the Cleft at Gaba Synapses in the Brain." Trends in Neurosciences **17**(12): 517-525.

Mody, I. and R. A. Pearce (2004). "Diversity of inhibitory neurotransmission through GABAA receptors." Trends in Neurosciences **27**(9): 569-575.

Mody, I., W. Z. Wei, et al. (2004). "Low ethanol concentrations selectively augment the tonic inhibition mediated by delta subunit-containing GABA(A) receptors in hippocampal neurons." Journal of Neuroscience**24**(38): 8379-8382.

Moss, S. J., T. C. Jacob, et al. (2008). "GABA(A) receptor trafficking and its role in the dynamic modulation of neuronal inhibition." Nature Reviews Neuroscience **9**(5): 331-343.

Moss, S. J. and J. T. Kittler (2003). "Modulation of GABA(A) receptor activity by phosphorylation and receptor trafficking: implications for the efficacy of synaptic inhibition." Current opinion in neurobiology **13**(3): 341-347.

Moss, S. J., V. Tretter, et al. (2008). "The clustering of GABA(A) receptor subtypes at inhibitory synapses is facilitated via the direct binding of receptor alpha 2 Subunits to gephyrin." Journal of Neuroscience **28**(6): 1356-1365.

Mozrzymas, J. W., E. D. Zarmowska, et al. (2003). "Modulation of GABA(A) receptors by hydrogen ions reveals synaptic GABA transient and a crucial role of the desensitization process." Journal of Neuroscience **23**(22): 7981-7992.

Mulley, J. C., I. E. Scheffer, et al. (2005). "Susceptibility genes for complex epilepsy." Human Molecular Genetics **14**: R243-R249.

Narahashi, T., J. Y. Ma, et al. (1994). "Gaba Receptor-Channel Complex as a Target Site of Mercury, Copper, Zinc, and Lanthanides." Cellular and Molecular Neurobiology **14**(6): 599-621.

Navarro, J. F., E. Buron, et al. (2002). "Anxiogenic-like activity of L-655,708, a selective ligand for the benzodiazepine site of GABA(A) receptors which contain the alpha-5 subunit, in the elevated plus-maze test." Progress in Neuro-Psychopharmacology & Biological Psychiatry **26**(7-8): 1389-1392.

Nicoll, R. A. and V. Stein (2003). "GABA generates excitement." Neuron **37**(3): 375-378.

Nusser, Z. and I. Mody (2002). "Selective modulation of tonic and phasic inhibitions in dentate gyrus granule cells." Journal of Neurophysiology **87**(5): 2624-2628.

Nusser, Z., J. D. B. Roberts, et al. (1995). "Immunocytochemical Localization of the Alpha-1 and Beta-2/3 Subunits of the Gaba(a) Receptor in Relation to Specific Gabaergic Synapses in the Dentate Gyrus." European Journal of Neuroscience **7**(4): 630-646.

Nusser, Z., J. D. B. Roberts, et al. (1995). "Relative Densities of Synaptic and Extrasynaptic Gaba(a) Receptors on Cerebellar Granule Cells as Determined by a Quantitative Immunogold Method." Journal of Neuroscience **15**(4): 2948-2960.

Nusser, Z., W. Sieghart, et al. (1998). "Segregation of Different GABAA Receptors to Synaptic and Extrasynaptic Membranes of Cerebellar Granule Cells." Journal of Neuroscience **18**(5): 1693-1703.

Nyitrai, G., K. A. Kekesi, et al. (2006). "Extracellular level of GABA and Glu: *In vivo* microdialysis-HPLC measurements." Current Topics in Medicinal Chemistry **6**(10): 935-940.

Olsen, R. W., H. J. Hanchar, et al. (2007). "GABA(A) receptor subtypes: the "one glass of wine" receptors." Alcohol **41**(3): 201-209.

Olsen, R. W. and W. Sieghart (2009). "GABA(A) receptors: Subtypes provide diversity of function and pharmacology." Neuropharmacology **56**(1): 141-148.

Orser, B. A., D. L. Bai, et al. (1999). "The general anesthetic propofol slows deactivation and desensitization of GABA(A) receptors." Journal of Neuroscience **19**(24): 10635-10646.

Orser, B. A., D. L. Bai, et al. (2001). "Distinct functional and pharmacological properties of tonic and quantal inhibitory postsynaptic currents mediated by gamma-aminobutyric acid(A) receptors in hippocampal neurons." Molecular Pharmacology **59**(4): 814-824.

Orser, B. A., L. Y. Wang, et al. (1994). "Propofol Modulates Activation and Desensitization of Gaba(a) Receptors in Cultured Murine Hippocampal-Neurons." Journal of Neuroscience **14**(12): 7747-7760.

Otton, H.J., Janssen et al. (2009) "Inhibition of rat recombinant GluN1/GluN2A and GluN1/ GluN2B NMDA receptors by ethanol at concentrations based on the US/UK drink-drive limit". European Journal of Pharmacology 6**14**(1-3): 14-21.

Overstreet, L. S., M. V. Jones, et al. (2000). "Slow Desensitization Regulates the Availability of Synaptic GABAA Receptors." Journal of Neuroscience **20**(21): 7914-7921.

Pavlov, I., L. P. Savtchenko, et al. (2009). "Outwardly Rectifying Tonically Active GABA(A) Receptors in Pyramidal Cells Modulate Neuronal Offset, Not Gain." Journal of Neuroscience **29**(48): 15341-15350.

Peduto, V. A., A. Concas, et al. (1991). "Biochemical and Electrophysiologic Evidence That Propofol Enhances Gabaergic Transmission in the Rat-Brain." Anesthesiology **75**(6): 1000-1009.

Peng, Z. C., C. S. Huang, et al. (2004). "Altered expression of the delta subunit of the GABA(A) receptor in a mouse model of temporal lobe epilepsy." Journal of Neuroscience **24**(39): 8629-8639.

Persohn, E., P. Malherbe, et al. (1992). "Comparative Molecular Neuroanatomy of Cloned Gaba-a Receptor Subunits in the Rat Cns." Journal of Comparative Neurology **326**(2): 193-216.

Pierucci-Lagha, A., J. Covault, et al. (2005). "GABRA2 alleles moderate the subjective effects of alcohol, which are attenuated by finasteride." Neuropsychopharmacology **30**(6): 1193-1203.

Pollack, M., M. Van Ameringen, et al. (2005). "Tiagabine in generalized anxiety disorder: a randomized, double-blind, placebo-controlled study." European Neuropsychopharmacology **15**: S538-S538.

Pollack, M. H., P. P. Roy-Byrne, et al. (2005). "The selective GABA Reuptake inhibitor tiagabine for the treatment of generalized anxiety disorder: Results of a placebo-controlled study." Journal of Clinical Psychiatry **66**(11): 1401-1408.

Porcello, D. M., M. M. Huntsman, et al. (2003). "Intact Synaptic GABAergic Inhibition and Altered Neurosteroid Modulation of Thalamic Relay Neurons in Mice Lacking Î´ Subunit." Journal of Neurophysiology **89**(3): 1378-1386.

Proctor, W. R., L. Diao, et al. (2006). "Differential alcohol modulation of synaptic GABAA and NMDA receptor activity in hippocampal neurons from ISS and ILS mouse brain slices." Alcoholism-Clinical and Experimental Research **30**(6): 182a-182a.

Proctor, W. R., L. Diao, et al. (2006). "Synaptic GABAergic and glutamatergic mechanisms underlying alcohol sensitivity in mouse hippocampal neurons." Journal of Physiology **575**(Pt 1): 145-159.

Quirk, K., P. Blurton, et al. (1996). "[H-3]L-655,708, a novel ligand selective for the benzodiazepine site of GABA(A) receptors which contain the alpha 5 subunit." Neuropharmacology **35**(9-10): 1331-1335.

Quirk, K., N. Gillard, et al. (1994). "Structure and Pharmacological Characterization of Delta-Containing Gaba(a) Receptors in Rat-Brain." British Journal of Pharmacology **112**: U131-U131.

Quirk, K., P. J. Whiting, et al. (1995). "Characterization of Delta-Subunit Containing Gaba(a) Receptors from Rat-Brain." European Journal of Pharmacology-Molecular Pharmacology Section **290**(3): 175-181.

Raman, I. M., P. Telgkamp, et al. (2004). "Maintenance of high-frequency transmission at Purkinje to cerebellar nuclear synapses by spillover from boutons with multiple release sites." Neuron **41**(1): 113-126.

Richerson, G. B., H. L. Gaspary, et al. (1998). "Carrier-mediated GABA release activates GABA receptors on hippocampal neurons." Journal of Neurophysiology **80**(1): 270-281.

Richerson, G. B. and Y. M. Wu (2003). "Dynamic equilibrium of neurotransmitter transporters: Not just for reuptake anymore." Journal of Neurophysiology **90**(3): 1363-1374.

Richerson, G. B., Y. M. Wu, et al. (2003). "Vigabatrin induces tonic inhibition via GABA transporter reversal without increasing vesicular GABA release." Journal of Neurophysiology **89**(4): 2021-2034.

Richter, D. W., P. Schmidt-Garcon, et al. (1999). "Neurotransmitters and neuromodulators controlling the hypoxic respiratory response in anaesthetized eats." Journal of Physiology **514**(2): 567-578.

Rivera, C., J. Voipio, et al. (2005). "Two developmental switches in GABAergic signalling: the K+-Cl- cotransporter KCC2 and carbonic anhydrase CAVII (vol 562, pg 29, 2004)." Journal of Physiology-London **564**(3): 953-953.

Rossi, D. J., M. Hamann, et al. (2003). "Multiple modes of GABAergic inhibition of rat cerebellar granule cells." The Journal of Physiology **548**(Pt 1): 97-110.

Rudolph, U., F. Crestani, et al. (1999). "Benzodiazepine actions mediated by specific gamma-aminobutyric acid(A) receptor subtypes." Nature **401**(6755): 796-800.

Rossi, D. J. and M. Hamann (1998). "Spillover-mediated transmission at inhibitory synapses promoted by high affinity alpha6 subunit GABA(A) receptors and glomerular geometry." Neuron **20**(4): 783-795.

Rudolph, U., J. M. Fritschy, et al. (1998). "Independent assembly and subcellular targeting of GABA(A)-receptor subtypes demonstrated in mouse hippocampal and olfactory neurons *in vivo*." Neuroscience Letters **249**(2-3): 99-102.

Rudolph, U., J. A. Benson, et al. (1998). "Pharmacology of recombinant gamma-aminobutyric acida receptors rendered diazepam-insensitive by point-mutated alpha-subunits." FEBS Letters **431**(3): 400-404.

Sankaranarayanan, S., D. De Angelis, et al. (2000). "The use of pHluorins for optical measurements of presynaptic activity." Biophysical Journal **79**(4): 2199-2208.

Sanna, E., M. P. Mascia, et al. (1995). "Actions of the General Anesthetic Propofol on Recombinant Human Gaba(a) Receptors - Influence of Receptor Subunits." Journal of Pharmacology and Experimental Therapeutics **274**(1): 353-360.

Sanna, E., G. Talani, et al. (2004). "Brain steroidogenesis mediates ethanol modulation of GABAA receptor activity in rat hippocampus." Journal of Neuroscience **24**(29): 6521-6530.

Santhakumar, V., H. J. Hanchar, et al. (2006). "Contributions of the GABAA Receptor Î±6 Subunit to Phasic and Tonic Inhibition Revealed by a Naturally Occurring Polymorphism in the α6 Gene." Journal of Neuroscience **26**(12): 3357-3364.

Saxena, N. C. and R. L. Macdonald (1994). "Assembly of Gaba(a) Receptor Subunits - Role of the Delta-Subunit." Journal of Neuroscience **14**(11): 7077-7086.

Saxena, N. C. and R. L. Macdonald (1996). "Properties of putative cerebellar gamma-aminobutyric acid(A) receptor isoforms." Molecular Pharmacology **49**(3): 567-579.

Scanziani, M. and F. Pouille (2001). "Enforcement of temporal fidelity in pyramidal cells by somatic feed-forward inhibition." Science **293**(5532): 1159-1163.

Schofield, P. R., M. G. Darlison, et al. (1987). "Sequence and Functional Expression of the Gaba-a Receptor Shows a Ligand-Gated Receptor Super-Family." Nature **328**(6127): 221-227.

Semyanov, A., M. C. Walker, et al. (2004). "Tonically active GABAA receptors: modulating gain and maintaining the tone." Trends in Neurosciences **27**(5): 262-269.

Serra, M., M. C. Mostallino, et al. (2006). "Social isolation-induced increase in alpha(4) and delta subunit gene expression is associated with a greater efficacy of ethanol on steroidogenesis and GABA(A) receptor function." Journal of Neurochemistry **98**(1): 122-133.

Sharonova, I. N., V. S. Vorobjev, et al. (1998). "High-affinity copper block of GABA(A) receptor-mediated currents in acutely isolated cerebellar Purkinje cells of the rat." The European journal of neuroscience **10**(2): 522-528.

Sharonova, I. N., V. S. Vorobjev, et al. (2000). "Interaction between copper and zinc at GABA(A) receptors in acutely isolated cerebellar Purkinje cells of the rat." British Journal of Pharmacology **130**(4): 851-856.

Shu, H. J., L. N. Eisenman, et al. (2004). "Slow actions of neuroactive steroids at GABA(A) receptors." Journal of Neuroscience **24**(30): 6667-6675.

Sieghart, W., M. Jechlinger, et al. (1998). "Subunit composition and quantitative importance of heterooligomeric receptors: GABA(A) receptors containing alpha(6) subunits." Journal of Neuroscience **18**(7): 2449-2457.

Sieghart, W., A. Poltl, et al. (2003). "Subunit composition and quantitative importance of GABA(A) receptor subtypes in the cerebellum of mouse and rat." Journal of Neurochemistry **87**(6): 1444-1455.

Sigel, E., R. Baur, et al. (1989). "The rat [beta]1 -subunit of the GABAA receptor forms a picrotoxin-sensitive anion channel open in the absence of GABA." FEBS Letters **257**(2): 377-379.

Sigel, E., R. Baur, et al. (2009). "Structure of alpha(6)beta(3)delta GABA(A) receptors and their lack of ethanol sensitivity." Journal of Neurochemistry **111**(5): 1172-1181.

Silver, R. A. and S. J. Mitchell (2003). "Shunting inhibition modulates neuronal gain during synaptic excitation." Neuron **38**(3): 433-445.

Smart, T. G., M. Mortensen, et al. (2010). "Distinct activities of GABA agonists at synaptic- and extrasynaptic-type GABA(A) receptors." Journal of Physiology **588**(8): 1251-1268.

Smart, T. G., S. J. Moss, et al. (1991). "Gaba-a Receptors Are Differentially Sensitive to Zinc - Dependence on Subunit Composition." British Journal of Pharmacology **103**(4): 1837-1839.

Smith, S. S., Q. H. Gong, et al. (1998). "GABA(A) receptor alpha 4 subunit suppression prevents withdrawal properties of an endogenous steroid." Nature **392**(6679): 926-930.

Smith, S. S. and Q. H. Gong (2005). "Neurosteroid administration and withdrawal alter GABA(A) receptor kinetics in CA1 hippocampus of female rats." Journal of Physiology **564**(2): 421-436.

Smith, S. S., H. Shen, et al. (2007). "Neurosteroid regulation of GABA(A) receptors: Focus on the alpha4 and delta subunits." Pharmacol Ther **116**(1): 58-76.

Somogyi, P., J. M. Fritschy, et al. (1996). "The gamma 2 subunit of the GABA(A) receptor is concentrated in synaptic junctions containing the alpha 1 and beta 2/3 subunits in hippocampus, cerebellum and globus pallidus." Neuropharmacology **35**(9-10): 1425-1444.

Sperk, G., S. Pirker, et al. (2000). "GABA(A) receptors: Immunocytochemical distribution of 13 subunits in the adult rat brain." Neuroscience **101**(4): 815-850.

Spigelman, I., Z. Li, et al. (2002). "Behavior and physiology of mice lacking the GABAA-receptor delta subunit." Epilepsia **43 Suppl 5**: 3-8.

Spigelman, I., Z. Li, et al. (2003). "Reduced inhibition and sensitivity to neurosteroids in hippocampus of mice lacking the GABA(A) receptor delta subunit." J Neurophysiol **90**(2): 903-910.

Stahl, S. M. (2004). "Anticonvulsants as anxiolytics, part 1 - Tiagabine and other anticonvulsants with actions on GABA." Journal of Clinical Psychiatry **65**(3): 291-292.

Stell, B. M., S. G. Brickley, et al. (2003). "Neuroactive steroids reduce neuronal excitability by selectively enhancing tonic inhibition mediated by δ subunit-containing GABAA receptors." Proceedings of the National Academy of Sciences **100**(24): 14439-14444.

Stewart, G. O., G. J. Dobb, et al. (1983). "Clinical-Trial of Continuous Infusion of Alphaxalone Alphadolone in Intensive-Care Patients." Anaesthesia and Intensive Care **11**(2): 107-112.

Stórustovu, S. and B. Ebert (2006). "Pharmacological characterization of agonists at delta-containing GABAA receptors: Functional selectivity for extrasynaptic receptors is dependent on the absence of gamma2." J Pharmacol Exp Ther **316**(3): 1351-1359.

Sundstrom-Poromaa, I., D. H. Smith, et al. (2002). "Hormonally regulated alpha(4)beta(2)delta GABA(A) receptors are a target for alcohol." Nature Neuroscience **5**(8): 721-722.

Tang, X., C. C. Hernandez, et al. (2010). "Modulation of Spontaneous and GABA-Evoked Tonic alpha 4 beta 3 delta and alpha 4 beta 3 gamma 2L GABA(A) Receptor Currents by Protein Kinase A." Journal of Neurophysiology **103**(2): 1007-1019.

Taylor, C. P. (1997). "Mechanisms of action of gabapentin." Revue Neurologique **153**: 39-45.

Taylor, B. L. and C. Collins (1988). "Aminophylline and Propofol - Apparent Antagonism." Anaesthesia **43**(6): 508-508.

Thomas, P. and T. G. Smart "HEK293 cell line: A vehicle for the expression of recombinant proteins." Journal of Pharmacological and Toxicological Methods **51**(3): 187-200.

Thompson, S. A., T. P. Bonnert, et al. (2002). "Overexpression of the GABA(A) receptor epsilon subunit results in insensitivity to anaesthetics." Neuropharmacology **43**(4): 662-668.

Tobler, I., R. Winsky-Sommerer, et al. (2007). "The EEG effects of THIP (Gaboxadol) on sleep and waking are mediated by the GABA(A) delta-subunit-containing receptors." European Journal of Neuroscience **25**(6): 1893-1899.

Triller, A., H. Bannai, et al. (2009). "Activity-Dependent Tuning of Inhibitory Neurotransmission Based on GABA(A)R Diffusion Dynamics." Neuron **62**(5): 670-682.

Trombley, P. Q. and G. M. Shepherd (1996). "Differential modulation by zinc and copper of amino acid receptors from rat olfactory bulb neurons." Journal of Neurophysiology **76**(4): 2536-2546.

Vicini, S., G. Losi, et al. (2002). "GABA(A) receptor delta subunit deletion prevents neurosteroid modulation of inhibitory synaptic currents in cerebellar neurons." Neuropharmacology **43**(4): 646-650.

Vida, I., M. Bartos, et al. (2006). "Shunting inhibition improves robustness of gamma oscillations in hippocampal interneuron networks by homogenizing firing rates." Neuron **49**(1): 107-117.

Vogt, K. E., G. A. Prenosil, et al. (2006). "Specific subtypes of GABAA receptors mediate phasic and tonic forms of inhibition in hippocampal pyramidal neurons." Journal of Neurophysiology **96**(2): 846-857.

Vlastelica, M. (2011). "Psychiatric Aspects of Basal Ganglia Diseases." Psychiatria Danubina **23**(2): 152-156.

Wafford, K. A. and B. Ebert (2006). "Gaboxadol - a new awakening in sleep." Current Opinion in Pharmacology **6**(1): 30-36.

Wagoner, K. R. and C. Czajkowski (2011) "Stoichiometry of Expressed α4β2δAminobutyric Acid Type A Receptors Depends on the Ratio of Subunit cDNA Transfected." Journal of Biological Chemistry **285**(19): 14187-14194.

Wall, M. J. and M. M. Usowicz (1997). "Development of action potential-dependent and independent spontaneous GABA(A) receptor-mediated currents in granule cells of postnatal rat cerebellum." European Journal of Neuroscience **9**(3): 533-548.

Wallner, M., H. J. Hanchar, et al. (2003). "Ethanol enhances Î±4Î²3Î´ and Î±6Î²3Î´ Î³-aminobutyric acid type A receptors at low concentrations known to affect humans." Proceedings of the National Academy of Sciences **100**(25): 15218-15223.

Wallner, M., P. Meera, et al. (2009). "Etomidate, propofol and the neurosteroid THDOC increase the GABA efficacy of recombinant alpha 4 beta 3 delta and alpha 4 beta 3 GABA(A) receptors expressed in HEK cells." Neuropharmacology **56**(1): 155-160.

Wei, W., N. Zhang, et al. (2003). "Perisynaptic Localization of Î" Subunit-Containing GABAA Receptors and Their Activation by GABA Spillover in the Mouse Dentate Gyrus." Journal of Neurosciencee **23**(33): 10650-10661.

Weiner, J. L. and C. F. Valenzuela (2006). "Ethanol modulation of GABAergic transmission: The view from the slice." Pharmacology & Therapeutics **111**(3): 533-554.

Wieland, H. A., H. Luddens, et al. (1992). "A Single Histidine in Gaba-a Receptors Is Essential for Benzodiazepine Agonist Binding." Journal of Biological Chemistry **267**(3): 1426-1429.

Wigstrom, H. and B. Gustafsson (1983). "Facilitated Induction of Hippocampal Long-Lasting Potentiation during Blockade of Inhibition." Nature **301**(5901): 603-604.

Winter, L., R. Nadeson, et al. (2003). "Antinociceptive properties of neurosteroids: A comparison of alphadolone and alphaxalone in potentiation of opioid antinociception." Anesthesia and Analgesia **97**(3): 798-805.

Wisden, W., D. J. Laurie, et al. (1992). "The Distribution of 13-Gaba-a Receptor Subunit Messenger-Rnas in the Rat-Brain .1. Telencephalon, Diencephalon, Mesencephalon." Journal of Neuroscience **12**(3): 1040-1062.

Wohlfarth, K. M., M. T. Bianchi, et al. (2002). "Enhanced Neurosteroid Potentiation of Ternary GABAAReceptors Containing the δ Subunit." Journal of Neuroscience **22**(5): 1541-1549.

Wu, P. H., S. J. Coultrap, et al. (2006). "Acute functional tolerance to alcohol in rat hippocampal pyramidal neurons." Alcoholism-Clinical and Experimental Research **30**(6): 183a-183a.

Wu, Y., W. Wang, et al. (2007). "Nonvesicular Inhibitory Neurotransmission via Reversal of the GABA Transporter GAT-1." Neuron **56**(5): 851-865.

Xi, Z. X., S. Ramamoorthy, et al. (2003). "GABA transmission in the nucleus accumbens is altered after withdrawal from repeated cocaine." Journal of Neuroscience **23**(8): 3498-3505.

Yamashita, M., W. Marsz3alec, et al. (2006). "Effects of ethanol on tonic GABA currents in cerebellar granule cells and mammalian cells recombinantly expressing GABA(A) receptors." J Pharmacol Exp Ther **319**(1): 431-438.

Yarze, J. C., S. J. Munoz, et al. (1992). "Diagnosing Wilson Disease." Annals of Internal Medicine **117**(1): 91-91.

You, H. and S. M. J. Dunn (2007). "Identification of a domain in the δ subunit (S238-V264) of the α4β3δ GABAA receptor that confers high agonist sensitivity." Journal of Neurochemistry **103**(3): 1092-1101.

Zhang, N. H., W. Z. Wei, et al. (2007). "Altered localization of GABAA receptor subunits on dentate granule cell dendrites influences tonic and phasic inhibition in a mouse model of epilepsy." Journal of Neuroscience **27**(28): 7520-7531.

Zhu, W. J., J. F. Wang, et al. (1996). "delta subunit inhibits neurosteroid modulation of GABA(A) receptors." Journal of Neuroscience **16**(21): 6648-6656.

Zyka, M. J., L. Campagnola, et al. (2008). "Fiber-coupled light-emitting diode for localized photo stimulation of neurons expressing channelrhodopsin-2." Journal of neuroscience methods **169**(1): 27-33.