Structural determinants and regulation of spontaneous activity in $GABA_A$ receptors

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Declaration

I, Craig Sexton, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Rapid inhibition in the central nervous system predominantly occurs through the activation of $GABA_A$ receptors. These are anion-selective ligand-gated ion channels permeable to chloride ions, and activation typically results in the inhibition of neuronal activity through phasic and tonic mechanisms. Some $GABA_A$ receptor subtypes are capable of opening in the absence of GABA to contribute towards tonic inhibition, but the mechanism by which this occurs and the effect on neuronal activity is not well understood.

A variety of recombinant $GABA_A$ receptor isoforms were examined for spontaneous activity by expression in HEK cells. Only those receptors containing the β3 subunit showed demonstrable levels of spontaneous activity, and key residues underpinning this were identified within the β3 extracellular domain. To investigate how spontaneous activity may be regulated *in vivo*, we examined phosphorylation of the β3 subunit using mutagenesis and by manipulating kinase activity, demonstrating a role for phosphorylation in regulating spontaneous current. Furthermore, the effect of various allosteric modulators, including neurosteroids, general anaesthetics and benzodiazepines, may exert a substantive part of their modulatory action through potentiation of spontaneous $GABA_A$ receptor activity.

We demonstrate that the β3 subunit is also important for spontaneous activity of receptors expressed in hippocampal neurons in culture by both expressing subunits *via* transfection and by knock-down of native subunits using β3-selective shRNAs. Examination of hippocampal and thalamic areas in adolescent rat brain slices identified varying levels of spontaneous current contributing to tonic inhibition, consistent with variable expression of the β3 subunit. Finally, mutations of the β3 subunit linked with epilepsy were studied and found to impact on spontaneous currents.

Overall, these data demonstrate the profound importance of the β3 subunit in controlling neuronal excitability, including the mechanisms by which neurons may alter levels of tonic inhibition through regulation of spontaneous $GABA_A$ receptor currents.

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Impact statement

$GABA_A$ receptors are expressed ubiquitously across the central nervous system and are vital for regulating neuronal activity and information processing by a variety of cellular networks. In some brain regions, tonic inhibition, *via* the activation of diffuse extrasynaptic $GABA_A$ receptors, may mediate greater charge transfer than the transient activation of clusters of receptors at inhibitory synapses. Although the activation of these receptors is linked to synaptic activity through transmitter spillover, a proportion of $GABA_A$ receptors activate in the absence of GABA.

A significant number of studies have focused on the effects that tonic inhibition has on neuronal computation and its role in pathological states, but the current knowledge of the impact of spontaneous activation on these processes is largely unknown. By studying spontaneous activity in greater detail using recombinant receptors, transfected neurons in culture and native culture/slice receptors, we have expanded our knowledge of this largely unexplored facet of tonic inhibition.

Manipulation of $GABA_A$ receptor currents is used in therapeutic strategies for a variety of pathologies, and $GABA_A$ receptors themselves have been implicated in many more. The high number of receptor isoforms permits the possibility of selectively targeting $GABA_A$ receptor subtypes, allowing a subpopulation of receptors, either in a particular brain region or even subcellularly, to be targeted and thus reduce the severity of off-target effects. By showing a dependence of spontaneous receptor gating on receptor subtype, we demonstrate how these spontaneous receptors may be selectively targeted in order to have region-selective effects on tonic inhibition. In addition, the identification of key molecular determinants of spontaneous gating within receptor subunits allows us to propose certain subunit interfaces which may represent future targets for therapeutic treatments.

Contents

Tables and figures

List of Abbreviations

4-PIOL	5-(4-piperidyl)-3-isoxazolol
BIS-I	Bisindolylmaleimide I
CaMKII	Ca2+/calmodulin-dependent protein kinase II
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
cryo-EM	Cryogenic electron microscopy
DAG	Diacylglycerol
DGGC	Dentate gyrus granule cell
dLGN	Dorsal lateral geniculate nucleus
DMEM	Dulbecco's modified Eagle's medium
DNTK	β2 subunit residues Asp170, Asn172, Thr178 and Lys179
DS2	Delta selective compound 2
EC50	Half maximal effective concentration
ECD	Extracellular domain
EPSP	Excitatory postsynaptic potential
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
GABA	γ-aminobutyric acid
$GABA_A$, $GABA_B$	Type A/B GABA receptor
GABARAP	$GABA_A$ receptor-associated protein
GARLH	$GABA_A$ receptor regulatory LHPFL protein
GAT	GABA transporter
GKER	β3 subunit residues Gly171, Lys173, Glu179, and Arg180
GPCR	G protein-coupled receptor
HBS	HEPES-buffered saline
HBSS	Hank's balanced salt solution
HEK	Human embryonic kidney cells
IPSC	Inhibitory postsynaptic current
M1-4	Transmembrane domains 1-4
MAPK	Mitogen-activated protein kinase

mGluRs	Metabotropic glutamate receptor
mIPSC	Miniature inhibitory postsynaptic current
mPR	Membrane progesterone receptor
NMDA	*N*-Methyl-*D*-aspartate
PCR	Polymerase chain reaction
PI3-K	Phosphoinositide 3-kinase
PKA, PKC, PKG	cAMP-dependent protein kinase, Protein kinase A
PKC	Protein kinase C
PKG	cGMP-dependent protein kinase, Protein kinase G
PKI	PKA inhibitor
PNK	Polynucleotide kinase
PRIP-1/2	Phospholipase-C-related catalytically inactive proteins 1/2
PS	Pregnenolone sulphate
ROI	Region of interest
shRNA	Short hairpin ribonucleic acid
sIPSC	spontaneous inhibitory postsynaptic current
THDOC	Tetrahydro-deoxycorticosterone , 5α-pregnan-3α,21-diol-20-one
THIP	Tetrahydroisothiazolo-[5,4-c]pyridin-3-ol
TLE	Temporal lobe epilepsy
γ2S/L	Short/Long isoforms of the $GABA_A$ receptor γ subunit

Chapter 1: Introduction

1.1. The $GABA_A$ Receptor

γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the central nervous system (CNS) and binds to two classes of receptor - the ionotropic Type A ($GABA_A$) receptors and the metabotropic Type B ($GABA_B$) receptors (Bowery & Smart, 2006). Activation of the $GABA_B$ receptor negatively regulates adenylate cyclase to reduce cAMP levels, inhibits voltage-gated Ca^{2+} channels, directly interferes with presynaptic vesicle release and, at postsynaptic sites, activates G protein-coupled inwardly rectifying potassium channels (GIRKs), inhibiting neuronal excitability (Lüscher et al., 1997; Sakaba & Neher, 2003; Xie et al., 2010; Yoon et al., 2007). The receptor is an obligate heterodimer, composed of $GABA_{B1}$ and $GABA_{B2}$ subunits (Gassmann & Bettler, 2012). $GABA_A$ receptors are pentameric ligand-gated ion channels (Corringer et al., 2012), and upon binding of GABA they undergo a conformational change which allows the flow of negatively-charged chloride (Cl^-) and bicarbonate (HCO_3^-) ions (Thompson et al., 2010). In most neurons in the adult brain, this results in both membrane hyperpolarisation and an increase in the membrane conductance (shunting inhibition) which dampens excitability (Mitchell & Silver, 2003; Farrant & Nusser, 2005). The receptor is composed from a large variety of subunits, giving rise to receptors with diverse kinetics and physiological roles, and this diversity is further enhanced by post-translational modifications, allowing the receptor to fulfil several roles in the CNS (Kittler & Moss, 2003; Olsen & Sieghart, 2009; Sigel & Steinmann, 2012). Inhibitory currents take the form of either phasic or tonic inhibition, both of which are mediated by distinct receptor subtypes. Phasic inhibition originates from receptors localised to synapses, and results in brief, large amplitude postsynaptic events caused by high concentrations of presynaptically-released GABA. Tonic inhibition, mediated by receptors outside synaptic areas, is caused by spill-over of GABA from synaptic clefts and represents a continuous inhibitory current controlled by low concentrations of ambient GABA (Mody, 2001; Luscher & Keller, 2004; Farrant

& Nusser, 2005; Brickley & Mody, 2012; Cherubini, 2012). These two distinct forms of inhibition are generally mediated by different receptor isoforms, different protein interactions, and can both be modulated by post-translational modifications to control excitability in the brain.

1.1.1. Subunit and receptor structure

$GABA_A$ receptors are ligand-gated ion channels composed of five subunits. Each subunit has a large hydrophilic amino (N)-terminal domain, four membrane spanning α-helices (M1-M4) and a large intracellular domain between M3-M4 (Sigel & Steinmann, 2012). The M2 helix of each of the five subunits forms the transmembrane region of the channel pore (Miller & Aricescu, 2014). Various protein interactions occur at the M3-M4 intracellular loop, including the binding of scaffold proteins at synapses, and proteins mediating post-translational modifications, such as protein kinases and phosphatases (Moss & Smart, 2001; Tretter et al., 2012; Nakamura et al., 2015). The structure of an individual $GABA_A$ receptor subunit, derived from the cryogenic electron microscopy (cryo-EM) structure (Laverty et al., 2019) is shown in Fig. 1.1.

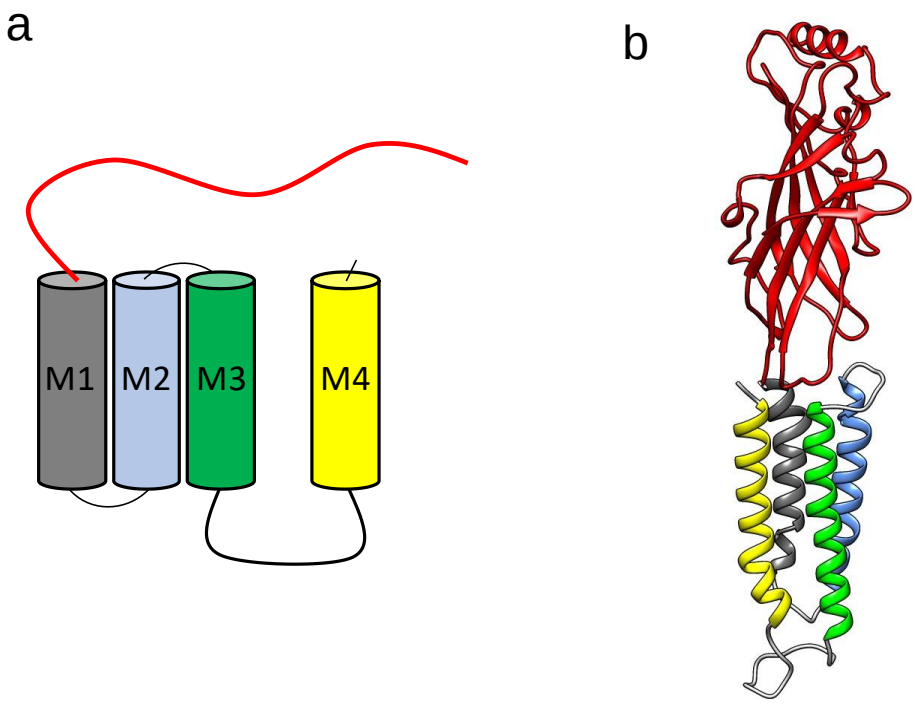

Figure 1.1: The structure of $GABA_A$ receptor subunits. A schematic diagram of a subunit (a), displaying the large hydrophilic extracellular domain (red), four transmembrane domains (M1-M4), and a large intracellular loop between M3-M4. The structure of the α1 subunit as it appears in the pentameric receptor is shown (b), utilising the same colour scheme. The M2 domain lines the transmembrane pore region. The model is derived from the cryo-EM structure produced by Laverty et al. (2019).

Currently, 19 subunits have been identified in mammals (α1-6, β1-3, γ1-3, δ, θ, ε, π and ρ1-3) (Sieghart, 1995; Olsen & Sieghart, 2009; Sigel & Steinmann, 2012). As the receptor is pentameric there is the potential for thousands of receptor subtypes, however only a relative few occur physiologically and receptors follow certain rules of assembly (Olsen & Sieghart, 2009). Most receptors have a stoichiometry of 2α, 2β and a single γ or δ subunit, formed into the clockwise sequence α-β-α-β-γ/δ surrounding a central pore (Baumann et al., 2002). Assembly of receptors occurs in the endoplasmic reticulum (ER) and requires chaperone proteins, including calnexin and immunoglobulin heavy-chain-binding protein (BiP) (Connolly et al., 1996). Numerous amino acid residues in the extracellular domain of subunits have been implicated in promoting the correct assembly of receptors (Sarto-Jackson & Sieghart, 2008). For example, residues 58-67 in the α1 subunit, when replaced with those from ρ1, prevent oligomerisation with β3

subunits (Taylor et al., 2000), whilst residues 76-89 of the β3 subunit appear to be highly important for association with α1 subunits (Ehya et al., 2003). Certain amino acid residues are required for selective assembly, for example R66 on α1 is essential for the assembly of α1β2 receptors, but not α1β1 or α1β3 receptors (Bollan et al., 2003), whilst four amino acids in β3: G171, K173, E179 and R180, are not required for assembly and surface expression of β3γ2 receptors, but are essential for the expression of β3 homomeric receptors (Taylor et al., 1999). All residues are numbered according to the mature murine protein.

The many assembly motifs in individual subunits give rise to a surprisingly small number of viable receptors found physiologically (Olsen & Sieghart, 2009). Preferential assembly of subunits results in two broad populations of receptors: those found at synapses and those found extrasynaptically. The former generally consists of α1-3, β and γ subunits, whilst the latter consists of α4/6, β and δ subunits (Stell et al., 2003; Farrant & Nusser, 2005). Receptors known to cluster at synapses are also found extrasynaptically, where they represent a pool of receptors that diffuse in and out of synaptic areas (Thomas et al., 2005; Bogdanov et al., 2006). There is also evidence for diheteromeric αβ receptors occurring physiologically, with estimates that these account for up to 10% of extrasynaptic receptors in hippocampal pyramidal neurons (Brickley et al., 1999; Sieghart & Sperk, 2002; Mortensen & Smart, 2006). Receptors consisting of α5, β and γ subunits can be found located both at synapses and in the extrasynaptic domain (Caraiscos et al., 2004; Serwanski et al., 2006; Ali & Thomson, 2008). Homomeric receptors also exist *in vivo*. The presence of homomeric ρ receptors in the retina is well established (Cutting et al., 1991; Naffaa et al., 2017), although there is also evidence that the ρ subunit is able to form heteromeric receptors with other subunits in different brain areas (Milligan et al., 2004), and possibly with structurally-related glycine receptor subunits (Pan et al., 2000). The murine β3 subunit, and to a lesser extent the β1 subunit, is also capable of forming homomeric receptors (Krishek et al., 1996b; Wooltorton et al., 1997). Such homomers are not gated by GABA but can be activated by the barbiturate pentobarbital and blocked by the non-competitive antagonist

picrotoxin (PTX). The abundance of these homomeric receptors in the brain is unknown, but as receptors have a high tendency to form heteromers, they are unlikely to account for many receptors occurring physiologically.

The crystal structures of, initially, the β3 homomer and later more physiological αβγ heteromers have been solved in various states and with various ligands bound (Miller & Aricescu, 2014; Laverty et al., 2017; Miller et al., 2017; Laverty et al., 2019; Masiulis et al., 2019). A model of a $GABA_A$ receptor derived from the recent $(α1)_2(β3)_2γ2$ receptor cryo-EM structure (defined here-on as α1β3γ2) is shown in Fig. 1.2 (Laverty et al., 2019). The receptor is approximately 110 Å in height, with over half (65 Å) protruding into the extracellular space (the extracellular domain, ECD) and has a diameter ranging from 60 to 80 Å. The large ECD of the receptor is formed of an α-helix (α1) and 10 β-strands, with a second α-helix (α2) between β-strands 3-4 under α1. Four α-helices from each subunit form the transmembrane domain (M1-M4), with M2 from each subunit lining the transmembrane region of the channel pore. A large vestibule is formed by the ECDs of each subunit, which narrows further down towards the ion channel. A ring of positively-charged residues at the cytosolic end of the ion channel contributes to a selectivity filter to ensure anions are able to pass (Keramidas et al., 2004). The pore narrows to its smallest diameter (3.15 Å when closed) at the -2' position, too narrow for Cl^- to pass unless the channel is opened (Carland et al., 2004).

Extracellular interfaces are formed between subunits, with the opposing subunit faces labelled as principal (+) and complementary (-), particularly in regards to the orthosteric binding site (Fig. 1.2c). The GABA-binding site is located at the $β^+$-$α^-$ interface, formed between adjacent β and α subunits (Miller & Smart, 2010). This results in the presence of two binding sites in the more common triheteromeric receptor, and both these sites must be occupied for full receptor activation (Macdonald et al., 1989; Mozrzymas et al., 2003; Petrini et al., 2011). The ρ homomer has five identical agonist binding sites, but not all of these need to be occupied for the receptor to adopt an open conformation (Amin & Weiss, 1996). Despite the apparent equivalency of the two binding sites in triheteromeric receptors, they do have measurably distinct differences in their affinity for GABA due to the presence of different subunits flanking the

binding site. One of the β^+-α^- interfaces is flanked by an α and a γ/δ subunit, whilst the other is flanked by a γ/δ and a β subunit (Baumann et al., 2003). The GABA binding sites are composed of six domains, 'loops' A-F (Fig. 1.2c). Loops A-C are provided by the β(+) subunit, and D-F by the α(-) (Miller & Smart, 2010; Miller & Aricescu, 2014). GABA docks into the binding site initially through a cation-π interaction with a tyrosine residue in loop A (Padgett et al., 2007). A conformational change then occurs, originating in the ECD but transmitting down the receptor to the channel pore, which subsequently opens. This step originates with a 'closing' of loop C, trapping the agonist within the binding site (Miller & Smart, 2010; Masiulis et al., 2019). This closing conformation is not observed with receptor antagonists, indicating its importance in the gating process (Miller & Smart, 2010). Loop C closure upon GABA binding causes rotation of the subunit ECDs, resulting in a stronger alignment between the β^+ and α^- interfaces. Additionally, conformational changes of the ECD β-sheets affect the transmembrane domains, which rotate and widen the channel pore, allowing ion translocation (Miller & Smart, 2010; Masiulis et al., 2019).

The intracellular loop is the site of binding for a wide variety of proteins involved in trafficking, localisation and modulation of activity (Luscher & Keller, 2004; Luscher et al., 2011). Proteins such as $GABA_A$ receptor-associated protein (GABARAP) (Wang et al., 1999) and Phospholipase-C-related catalytically inactive proteins (PRIP-1/2) (Terunuma et al., 2004) are involved with trafficking the receptor to the membrane, whilst proteins such as gephyrin localise these receptors to synapses (Tretter et al., 2008; Mukherjee et al., 2011; Tretter et al., 2011; Kowalczyk et al., 2013). The loop is also the site of phosphorylation by serine/threonine and tyrosine kinases, which affect trafficking and receptor activity (Luscher et al., 2011; Nakamura et al., 2015).

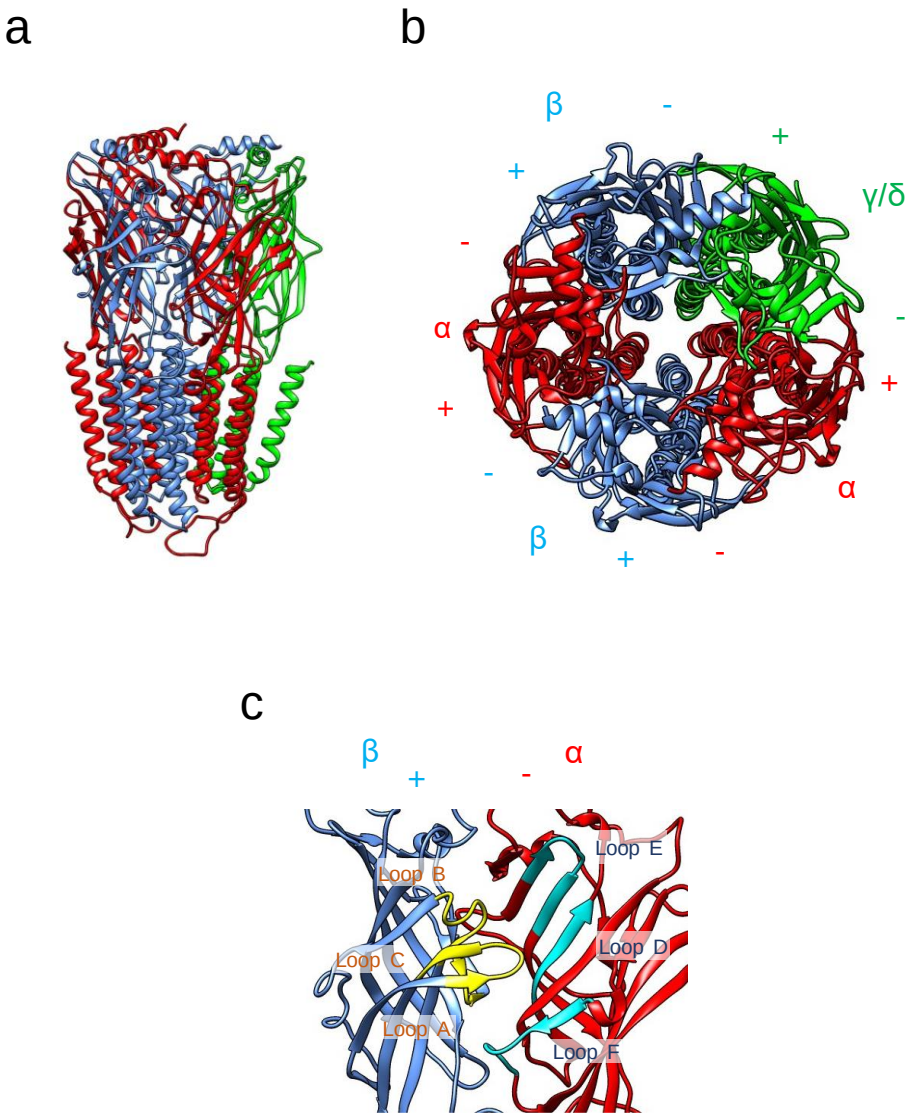

Figure 1.2: The structure of the heteropentameric $GABA_A$ receptor. a) Horizontal (membrane) view of a homology model of the α1β3γ2 receptor, derived from the cryo-EM structure (Laverty et al., 2019). The large ECDs are evident, as well as the transmembrane domains forming the channel. The α, β and γ subunits are shown in red, blue and green, respectively. b) Vertical (synaptic) view of the homology model, clearly showing the pore and M2 domains which form it, one from each subunit. The principal (+) and complementary (-) interfaces are indicated, which represent binding sites for multiple compounds. c) The orthosteric GABA-binding β^+-α^- interface with principal and complementary binding 'loops' highlighted in yellow (+) and cyan (-).

1.1.2. Expression and trafficking of $GABA_A$ receptors

Each subunit is expressed differentially throughout the brain. Immunohistochemical studies have shown variable distributions of individual subunits, indicating that receptors in distinct brain regions will vary in their subunit composition and properties (Pirker et al., 2000; Sieghart & Sperk, 2002). Pirker et al. (2000) performed immunohistochemical staining to determine the localisation of key $GABA_A$ receptor subunits in the adult rat brain (Fig. 1.3). Of the α subunits, α1 was expressed throughout the brain, whilst α2 was also widely-expressed but slightly more localised to certain areas, including the olfactory bulb, dentate gyrus, hippocampal area CA3 and amygdala. The α3 subunit was sparsely expressed, with higher expression levels in the amygdala. Immunoreactivity for the α4 subunit was most evident in the thalamus and dentate gyrus. α5 was most highly expressed in the hippocampus, whilst α6 was almost exclusively expressed in the cerebellum. Of the β subunits, all three were widely expressed. Of note, differential distribution in the hippocampus indicated that in dentate gyrus granule cells, β2 expression was relatively low whilst β3 was highly expressed. There were also other nuclei where different ratios of the β subunits occurred, including notably higher β3 in the striatum and higher β2 in the thalamus. Of the γ subunits, only γ2 was heavily expressed throughout the brain. The γ1 subunit was largely concentrated to sub-cortical areas, whilst γ3 was expressed at very low levels throughout. The δ subunit was more highly expressed in the thalamus and dentate gyrus, as well as the cerebellum. Similar patterns of subunit mRNA expression were obtained from *in situ* hybridisation studies (Laurie et al., 1992; Wisden et al., 1992).

Immunohistochemistry has also been performed to map expression of less common subunits. The θ subunit was localised to certain cortical areas, hypothalamus, amygdala, hippocampus and substantia nigra (Bonnert et al., 1999). The mRNA of the ε subunit was detected in the amygdala and thalamus (Davies et al., 1997a). The π subunit has not been observed in the brain, but has been identified in peripheral tissues, most abundantly in the uterus (Hedblom & Kirkness, 1997). Finally, ρ subunits are found in the retina, as well

as other areas involved in the processing of visual information, including the superior colliculus and thalamus (Boue-Grabot et al., 1998; Martinez-Delgado et al., 2010).

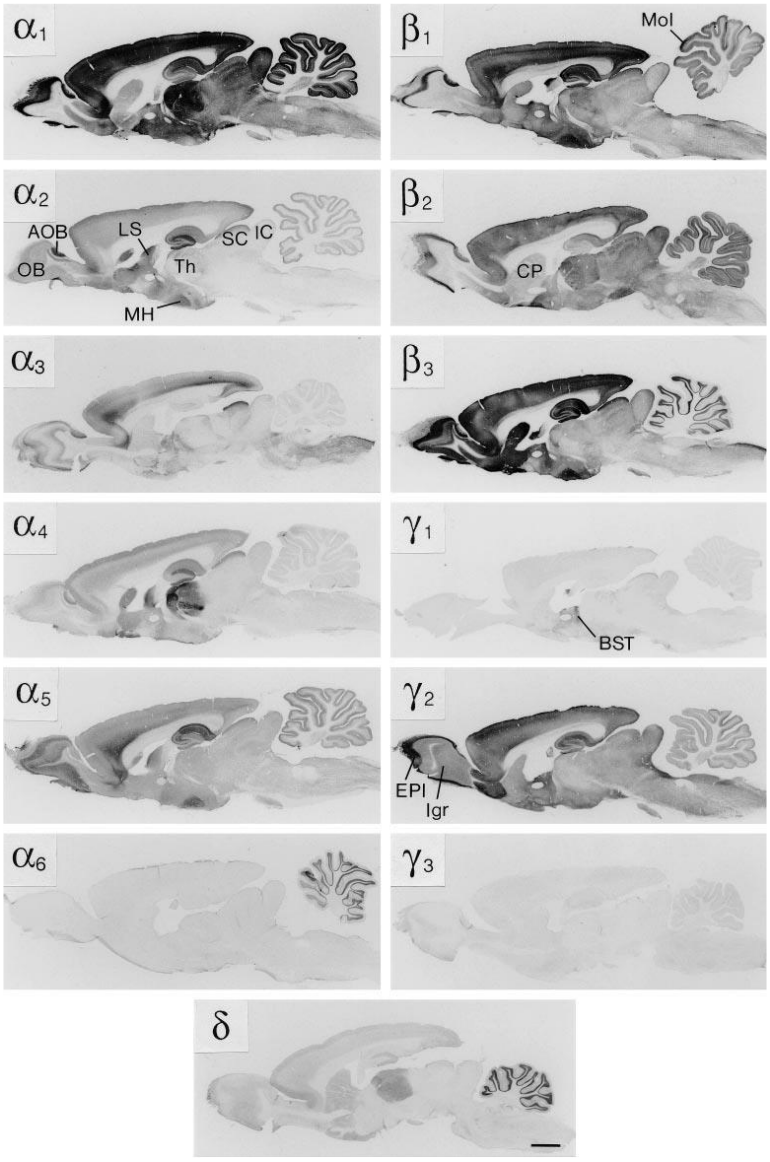

Figure 1.3: Regional diversity in the expression of $GABA_A$ receptor subunits in the adult rat brain, determined by immunohistochemistry. Reproduced from Pirker et al. (2000). AOB: Accessory olfactory bulb, OB: Olfactory bulb, LS: lateral septal nucleus, MH: medial hypothalamic nuclei, Th: thalamus, SC: superior colliculus, IC: inferior colliculus, Mol: molecular layer of the cerebellum, CP: caudate putamen (striatum), BST: bed nucleus of the stria terminalis, EPI: external plexiform layer of the olfactory bulb, Igr: internal granular layer of the olfactory bulb. Scale bar: 2mm.

$GABA_A$ receptors display diverse expression patterns in different neurons and different brain areas. They also display significant diversity within single neurons. This is most evident when comparing receptors clustered at synapses with those found extrasynaptically. Receptors are initially inserted into the membrane at extrasynaptic locations and diffuse laterally along the membrane, before then being held at the synapse through interaction with synaptic proteins (Thomas et al., 2005; Bogdanov et al., 2006). Trafficking of the receptor is controlled *via* interactions of the large intracellular loop of $GABA_A$ receptor subunits with a wide variety of proteins, for example GABARAP and PRIP-1/2 (Chen & Olsen, 2007). GABARAP interacts with the intracellular loop of the γ2 subunit (Wang et al., 1999), but also with elements of the cytoskeleton, including microtubules and microfilaments (Wang & Olsen, 2000), and with N-ethylmaleimide-sensitive factor (NSF) (Kittler et al., 2001) indicating a role in the transport of $GABA_A$ receptors. Interestingly, GABARAP was not localised to synapses, meaning other proteins must be responsible for organisation of the synaptic architecture (Kittler et al., 2001). PRIP-1/2 both interact with GABARAP but also directly with all β subunits and, weakly, with γ2 (Kanematsu et al., 2002; Terunuma et al., 2004). PRIP-1 and PRIP-2 double knock-out studies revealed impaired expression of γ-containing receptors, as determined by reduced benzodiazepine binding, and also reduced interaction between the receptor and GABARAP, suggesting that these proteins likely act as a 'bridging molecule' between GABARAP and $GABA_A$ receptors (Mizokami et al., 2007).

Once receptors have been trafficked through the cell and inserted into the membrane, they can be clustered at synapses or remain in extrasynaptic locations depending on their subunit composition. There is constant diffusion of receptors in and out of synapses, with reduced movement and temporary clustering caused by transient binding to synaptic proteins (Thomas et al., 2005; Bogdanov et al., 2006). An important clustering protein at inhibitory synapses is gephyrin (Groeneweg et al., 2018). Hippocampal neurons in culture derived from gephyrin knock-out mice displayed no synaptic clustering of $GABA_A$ receptors, and reduced GABAergic currents were observed with higher levels of internalised receptor (Kneussel et al., 1999). A direct

interaction of gephyrin with the intracellular loops of α1-3 (Tretter et al., 2008; Mukherjee et al., 2011; Tretter et al., 2011) and β2/3 (Kowalczyk et al., 2013) has been identified as important for synaptic clustering. Gephyrin not only directly binds to and localises $GABA_A$ receptor subunits, but is also an integral part of a network of proteins that maintain inhibitory synapses, including essential interactions with collybistin and neuroligins (Groeneweg et al., 2018).

Despite the importance of gephyrin in certain inhibitory synapses for receptor clustering, there is also evidence for gephyrin-independent clustering. Staining for α1 and α5 showed unaltered puncta formations in gephyrin knock-out mice in the spinal cord (Kneussel et al., 2001) and, in contrast to earlier studies, clustering has been observed in hippocampal neurons of these mice (Kneussel et al., 1999; Levi et al., 2004). Radixin has been identified as binding and clustering α5-containing $GABA_A$ receptors, as injection of antisense oligonucleotides targeted to knock-down radixin dramatically reduced the number of α5 clusters, as was also evident from hippocampal slices of radixin knock-out mice (Loebrich et al., 2006). Interestingly, these clusters predominantly occur outside of synapses, which may represent pools of receptors available to be recruited to synapses during changes associated with synaptic plasticity (Hausrat et al., 2015). A more recent discovery has identified the tetraspanin protein LHFPL4 (Lipoma HMGIC Fusion Partner-Like 4) as an important auxiliary protein required for synaptic clustering of $GABA_A$ receptors in hippocampal excitatory neurons, but not interneurons (Davenport et al., 2017; Yamasaki et al., 2017). These proteins, termed GARLH ($GABA_A$ receptor regulatory LHPFL) proteins, are not involved in surface trafficking of receptors, but instead aid the synaptic clustering in conjunction with other synaptic proteins, including gephyrin. In GARLH knock-out mice, the formation of both receptor and gephyrin clusters was significantly impaired, with subsequent effects on inhibitory postsynaptic currents (IPSCs).

1.1.3. Subunit composition and biophysical properties of $GABA_A$ receptors

The location of receptors within a neuron is largely defined by their subunit composition, as these determine interactions with intracellular proteins. Synaptic receptors typically exist as clustered α1-3βγ receptors, whilst extrasynaptic receptors exist as diffuse receptor populations and are typically composed of α4/6βδ, along with a population of αβγ receptors in transit to and from synapses (Thomas et al., 2005; Bogdanov et al., 2006). Despite the presence of the γ subunit, α5βγ receptors are found at extrasynaptic locations but also exhibit synaptic clustering (Caraiscos et al., 2004; Serwanski et al., 2006; Ali & Thomson, 2008). The γ subunit is a strong determinant of receptor localisation and clustering, as knock-out of this subunit almost entirely removed synaptic clustering of $GABA_A$ receptors (Essrich et al., 1998). Interestingly, the area required for clustering has been mapped to M4 of the γ2 subunit, although the intracellular loop facilitates the recruitment of gephyrin to the receptor and is required for inhibitory synaptic transmission (Alldred et al., 2005), explaining the importance of both gephyrin and γ2 in mediating synaptic accumulation of receptors. A schematic representation of the distribution of $GABA_A$ receptors at synaptic and extrasynaptic zones is shown in Fig. 1.4.

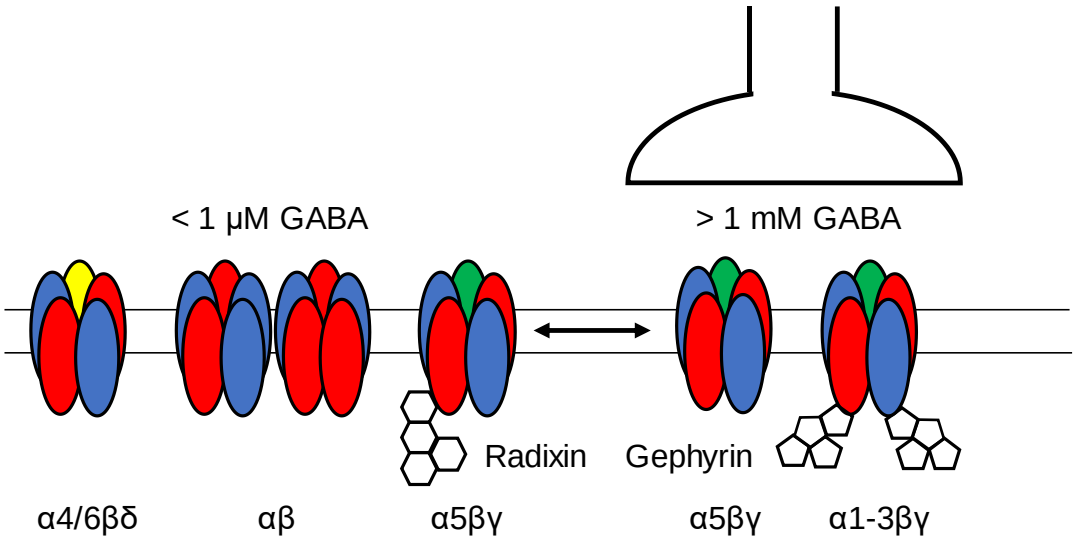

Figure 1.4: Schematic representation of the subcellular distribution of various $GABA_A$ receptor isoforms. α1-3βγ receptors are predominantly localised to synapses and anchored by the synaptic scaffold protein gephyrin binding to α and β subunits, aided by the γ subunit. Synaptic α5βγ receptors also exist, clustered by gephyrin. These synaptic receptors mediate phasic inhibition by responding to high concentrations of GABA released into the synaptic cleft from the presynaptic terminal. The α5βγ receptors are also found clustered in extrasynaptic sites by the protein radixin, binding to the α5 subunit. Other known extrasynaptic receptors include α4/6βδ and αβ receptors, which respond to low ambient concentrations of GABA.

The subunit composition of receptors determines not only their subcellular localisation, but biophysical properties and the role of those receptors. Synaptic receptors, those containing α1-3, β and γ, typically have a lower affinity for GABA than the extrasynaptic receptors consisting of α4/6, β and δ (Farrant & Nusser, 2005). This is consistent with the role of synaptic receptors, which are exposed to high concentrations of GABA (> 1 mM) released presynaptically, whilst extrasynaptic receptors are exposed to low levels of ambient GABA, and so must be more sensitive to respond. The identity of the α subunit is a key determinant of GABA potency, with EC_{50} values ranging from 1 μM (α6β3γ2) to 48 μM (α3β3γ2) (Bohme et al., 2004; Picton & Fisher, 2007; Mortensen et al., 2011). The α3 subunit confers a much lower potency of GABA compared to the other subunits, with the second-lowest, α5β3γ2, having an EC_{50} of 11.6 μM. The order of GABA potency at

these receptors is therefore α6 > α1 > α2 > α4 > α5 >> α3. Exact EC_{50} values vary between experimental conditions, in particular this has been noted when comparing expression in *Xenopus* oocytes and mammalian cell lines (Zeng et al., 2020), and other potency ranks have been suggested, such as α6 > α5 > α4 > α1 > α3 > α2 (Mortensen et al., 2011). Differential expression of α subunits will therefore impact on the sensitivity of receptor isoforms to GABA and affect neuronal excitability. This same study by Mortensen et al. (2011) identified effects on GABA potency by the identity of the β subunit, with a rank order of potency being β3 > β2 > β1 in α1βγ2 receptors.

The presence and identity of the third subunit, typically either γ or δ, also has significant impacts on GABA potency. The α4β3δ receptor displays a five-fold lower EC_{50} than α4β3γ2 (0.49 μM against 2.57 μM, respectively) (Brown et al., 2002). This is consistent with the extrasynaptic localisation of δ-containing receptors, which are only exposed to low concentrations of ambient GABA. The presence of the δ subunit also affects the efficacy of GABA at the receptor. GABA acts only as a partial agonist at α4β3δ receptors but a full agonist at α4β3γ2 receptors, determined using the full GABA agonist 4,5,6,7-tetrahydroisothiazolo-[5,4-c]pyridin-3-ol (THIP/gaboxadol) (Wafford et al., 1996; Brown et al., 2002; Storustovu & Ebert, 2006; Mortensen et al., 2010). GABA is therefore a high affinity, low efficacy agonist at extrasynaptic δ-containing receptors. The low efficacy permits a dynamic range in the effect of ambient GABA, allowing the receptor to be more efficiently modulated by compounds which increase the effective GABA efficacy, such as neurosteroids (Stell et al., 2003; Houston et al., 2012).

The rates of activation, deactivation and desensitisation are also affected by subunit composition. Using fast application of saturating GABA concentrations, α1β3γ2L receptors were shown to be activated faster than α1β3δ receptors (0.46 ms compared to 2.4 ms) (Haas & Macdonald, 1999). These γ-containing receptors were slower to deactivate than their δ-containing counterparts (76.1 ms compared to 42.8 ms), but showed significantly higher levels of desensitisation (92.4% compared to 55.6%) at a faster rate (462 ms compared to 1260 ms). The reduced desensitisation of δ-containing receptors makes them ideal for persistent GABAergic currents, whereas the presence of

the γ subunit allows these receptors to provide much shorter and more controlled currents, consistent with their roles as tonic and phasic receptors, respectively. Despite slow and incomplete desensitisation of extrasynaptic δ-containing receptors, there is evidence that, at physiological temperatures, a majority of these receptors exist in a desensitised state due to their high sensitivity to GABA (Bright et al., 2011). This then makes these receptors less reactive when there is increased synaptic activity resulting in enhanced GABA spillover, almost disconnecting the tonic inhibition from synaptic activity (Mortensen et al., 2010; Bright et al., 2011; Lagrange et al., 2018). Changes to the concentration of ambient GABA, for example through high-frequency synaptic activity, are still likely to influence tonic inhibition, however, as prevention of GABA uptake through inhibition of GABA transporters (GATs) increases recorded tonic current (Jin et al., 2011).

Even within subunit families there can be significant differences between activation, deactivation and desensitisation properties (Picton & Fisher, 2007). The deactivation rate of α1β2γ2L is faster than that of α2β2γ2L (Dixon et al., 2014) and α3β2γ2 (Barberis et al., 2007). The α3β2γ2 receptor also displayed lower levels of desensitisation, which resulted in greater recovery in paired-pulse tests (Barberis et al., 2007), and also has a very slow activation rate which likely reduces temporal precision of phasic inhibition (Lagrange et al., 2018). The subunit composition of receptors will therefore impact on IPSC waveform and fluctuations in tonic currents, with subsequent effects on neuronal activity (Lagrange et al., 2018).

1.1.4. Post-translational modifications: Phosphorylation

Further diversity in receptor function is provided by post-translational modifications. Both tyrosine kinases and serine/threonine kinases phosphorylate $GABA_A$ receptor subunits, attaching a phosphoryl group (PO_3^-) to selected amino acid residues (Ardito et al., 2017). This can alter the interaction of residues within the receptor, as well as interactions with other proteins, affecting channel function, trafficking, surface expression, clustering

and pharmacology (Nakamura et al., 2015). Known phosphorylation sites are described in Table 1.1.

Subunit	Residue	Physiological effect	Kinase	Ref.
α1	T375	Reduced expression	ERK/MAPK	(Bell-Horner et al., 2006; Mukherjee et al., 2011)
α4	S443	Increased expression	PKC	(Abramian et al., 2010)
β1	T227, Y230	-	-	(Kang et al., 2011)
	S384	-	CaMKII	(McDonald & Moss, 1994)
	S409	Reduced expression, slowed desensitisation	PKA, PKC, PKG, CaMKII	(Moss et al., 1992a; Moss et al., 1992b; Krishek et al., 1994; McDonald & Moss, 1994)
β2	Y215, T439	-	-	(Kang et al., 2011)
	S410	Reduced expression	PKA, PKC, PKG, CaMKII	(Kellenberger et al., 1992; McDonald & Moss, 1997)
	Y372, Y379	Increased expression	PI3-K	(Vetiska et al., 2007)
β3	T282, S406	-	-	(Kang et al., 2011)
	S408, S409	Single phosphorylation reduced expression, phosphorylation of both sites increased expression. Additional evidence for a direct effect on channel gating/conductance	PKA, PKC, PKG, CaMKII	(McDonald et al., 1998; Brandon et al., 2000; Kittler et al., 2005; Adams et al., 2015)
	S383	Enhanced receptor expression	CaMKII	(Houston et al., 2007; Saliba et al., 2012)
γ2S	Y365, Y367	Enhanced receptor activity	Src	(Moss et al., 1995)
	S327	Reduced receptor activity	PKC	(Kellenberger et al., 1992; Krishek et al., 1994)
	S348, T350	-	CaMKII	(McDonald & Moss, 1994)
γ2L	S343	Reduced currents	PKC, CaMKII	(Moss et al., 1992a; Krishek et al., 1994)

Table 1.1: Sites of phosphorylation within $GABA_A$ receptor subunits. Ser/Thr and Tyr residues previously identified as being phosphorylated are listed. Where known, the physiological impact is detailed, with the phosphorylating kinases. Residue numbering is as provided by the original authors.

The role of gephyrin in clustering receptors at synapses and facilitating inhibitory synaptic transmission has been previously discussed, but the binding efficiency of gephyrin can be modulated by the phosphorylation state of the receptor. Mutating a potential phosphorylation site, T375, of the α1

subunit affected synaptic clustering through alterations in gephyrin binding (Mukherjee et al., 2011). Phosphorylation of this residue reduces association of the subunit with gephyrin, and indeed mutating this residue to the phosphomimetic aspartate (D) significantly reduced synaptic clustering and miniature IPSC (mIPSC) amplitude (Mukherjee et al., 2011). The T375 residue is thought to be targeted by proline-directed kinases, including mitogen-activated protein kinase (MAPK) and cyclin-dependent protein kinase (CDK) due to analysis of the phosphorylation consensus sequences (Mukherjee et al., 2011). Indeed, extracellular-signal regulated kinase (ERK), part of the MAPK pathway, has been previously shown to reduce currents from α1β2γ2 receptors (Bell-Horner et al., 2006). Unsurprisingly, the T375 residue and other phosphorylated residues are localised to the large intracellular loop, which is the predominant location of protein-protein interactions. Alteration of the phosphorylation status of residues in this area will therefore have multiple effects on protein binding.

Other phosphorylation sites of α subunits have been associated with altering receptor stability on the cell membrane. The α4 subunit is phosphorylated at S443 within the large intracellular loop by protein kinase C (PKC). Phosphorylation of this site increases the stability of α4 within the ER and hence drives receptor assembly and membrane insertion (Abramian et al., 2010). Phosphorylation of this residue has an intriguing interplay with neurosteroid potentiation of $GABA_A$ receptor activity, as 10 min application of the neurosteroid tetrahydro-deoxycorticosterone (THDOC) potentiates PKC phosphorylation of this site, driving receptor insertion into the membrane (Abramian et al., 2014).

The β subunits are major targets for kinases and phosphatases, with multiple phosphorylation sites that vary between the subunits. Mass spectrometry has revealed multiple phosphorylation sites across this subunit family, although most have yet to be investigated for functional effects (Kang et al., 2011). These include T227 and Y230 (β1), Y215 and T439 (β2) and T282 and S406 (β3). Other sites within the β subunits have been investigated for functional effects and the kinases that phosphorylate those sites have been identified. The β1 subunit is phosphorylated at S409 by cAMP-dependent

protein kinase A (PKA) (Moss et al., 1992b) and PKC (Krishek et al., 1994), which has the effect of reducing α1β1γ2 receptor currents and reducing the fast component of desensitisation of α1β1 receptors expressed in HEK cells (Moss et al., 1992a; Moss et al., 1992b). S409 was also shown to be phosphorylated by protein kinase G (PKG) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (McDonald & Moss, 1994). This same study identified a second phosphorylation site within the intracellular loop of β1, S384, which was phosphorylated by CaMKII (McDonald & Moss, 1994).

The β2 subunit is phosphorylated at S410, a site homologous to S409 in β1. This site is phosphorylated by a range of kinases, with the first identified being PKC, which downregulates $GABA_A$ receptor currents (Kellenberger et al., 1992). Further investigations revealed phosphorylating effects of PKA, PKG and CaMKII (McDonald & Moss, 1997). In addition, the β2 subunit has tyrosine residues within the loop which can be phosphorylated by the non-receptor tyrosine kinase c-Src at Y372 and Y379 (Vetiska et al., 2007). Insulin can enhance mIPSCs through increasing $GABA_A$ receptor surface expression, mediated by binding of phosphoinositide 3-kinase (PI3-K) to these phosphorylated residues.

As with the other β subunits, β3 is phosphorylated at the homologous site within the M3-M4 loop corresponding to S409 and S410 in β1 and β2, respectively. However, uniquely among the subfamily, it has a second, adjacent phosphorylation site, and is therefore phosphorylated at residues S408 *and* S409 (McDonald et al., 1998). The actions of PKA have opposing effects on α1β3γ2 receptors and α1β1γ2 receptors; it potentiated the former current but reduced the latter. This difference was mediated by the single S408 phosphorylation site, which, when removed through serine to alanine mutations, was able to recapitulate the inhibitory effect seen with β1. This was also mirrored by an introduction of a second, adjacent, phosphorylation site into β1, which translated the effect of PKA into a potentiating effect at $\alpha1\beta1^{A408S}\gamma2$ receptors (McDonald et al., 1998). This effect on $GABA_A$ receptor currents resulted from changes to trafficking of these receptors, as phosphorylation of β3 reduces binding of the clathrin adaptor protein AP2, thereby preventing receptor internalisation and increasing surface expression

(Kittler et al., 2005). Similarly to the S443 phosphorylation of α4, there is an interplay between the S408 and S409 phosphorylation sites on β3 and neurosteroid potentiation, as the magnitude of potentiation caused by THDOC was enhanced when either of these residues was phosphorylated (Adams et al., 2015). A second phosphorylation site exists in the loop of β3, S383, which is phosphorylated by CaMKII to enhance $GABA_A$ receptor currents (Houston & Smart, 2006; Houston et al., 2007).

The γ2 subunit is also phosphorylated at numerous sites within its intracellular loop. Tyrosine kinase phosphorylation sites include Y365 and Y367 on the short isoform of the subunit (γ2S) (homologues residues Y373 and Y375 on the longer isoform, γ2L) which are phosphorylated by the tyrosine kinase Src to enhance $GABA_A$ receptor currents (Moss et al., 1995). Interestingly, CaMKII in NG108-15 cells activates this tyrosine kinase, resulting in phosphorylation of both S383 of β3 and Y365 and Y367 of γ2 in α1β3γ2S receptors to further enhance $GABA_A$ receptor currents (Houston et al., 2007). S327 in γ2S is phosphorylated by PKC to reduce $GABA_A$ receptor currents (Kellenberger et al., 1992; Krishek et al., 1994). S348 and T350 have also been identified as phosphorylation sites of CaMKII on γ2 (McDonald & Moss, 1994). The 8 amino acid insert in the loop that distinguishes γ2L from γ2S (LLRMFSFK) provides a unique phosphorylation site, S343, for the long isoform. This is phosphorylated by CaMKII and PKC to reduce $GABA_A$ receptor currents (Moss et al., 1992a; Krishek et al., 1994).

1.1.5. Post-translational modifications: N-*linked glycosylation and palmitoylation*

N-linked glycosylation of proteins is a common post-translational and co-translational event which attaches a dolichol oligosaccharide to an asparagine residue of a protein, mediated by oligosaccharyltransferase and dependent on the consensus sequence Asn-X-Ser/Thr, where X is a neutral residue position (Mohorko et al., 2011). This can facilitate the correct assembly of a protein, promote trafficking through the cell, affect protein degradation and

downstream components of a protein's signalling pathways (Helenius & Aebi, 2004).

Early experiments showed that applying tunicamycin, an inhibitor of *N*-linked glycosylation, could significantly reduce surface expression of $GABA_A$ receptors expressed in oocytes (Sumikawa et al., 1988). *N*-linked glycosylation of the α1 subunit has been observed using mass spectrometry of purified $GABA_A$ receptors from rat neocortex (Chen et al., 2012). Removing these sites using mutagenesis to produce $\alpha1^{N10Q}$ and $\alpha1^{N110Q}$ compromised surface expression of α1β1γ2 receptors in HEK 293 cells (Buller et al., 1994). Glycosylation is therefore a key modification which promotes cell surface expression of α1-containing receptors.

The β2 subunit also exhibits glycosylation sites at N32 (homologous to the previously described N10 within α1), N104 and N173. Mutagenesis studies in HEK cells suggest that all three of these residues are glycosylated, with effects on assembly and trafficking and also on channel gating properties (Lo et al., 2010). This study showed an effect of glycosylation on assembly and stability in the ER, with N173 being important for single subunit stability, whilst the N104 glycosylation site was more important for the formation of pentameric α1β2 receptors. Levels of surface expression of heteromeric receptors were not significantly altered by N104Q, but were significantly impaired by N173Q. Despite equivalent levels of surface expression of $\alpha1\beta2^{N32Q}$ and $\alpha1\beta2^{N173Q}$ compared to wild-type, these receptors, along with $\alpha1\beta2^{N104Q}$, showed impaired peak responses to GABA with reduced frequency of prolonged receptor openings at the level of the single channel (Lo et al., 2010).

The process of palmitoylation represents a further method of modulating $GABA_A$ receptor function. The reversible attachment of the long-chain fatty acid palmitate to cysteine residues by the actions of palmitoyltransferases is a common feature among receptors to regulate assembly, trafficking and localisation (el-Husseini Ael & Bredt, 2002; Naumenko & Ponimaskin, 2018).

The first palmitoyltransferase found to interact with and palmitoylate a $GABA_A$ receptor subunit was the Golgi apparatus-specific protein with a DHHC

zinc finger domain (GODZ) causing palmitoylation of the γ2 subunit (Keller et al., 2004). Multiple cysteine residues in the large intracellular loop of the γ2 subunit appear to be the target for palmitoylation, and the application of 2-BrP, an inhibitor of palmitoylation, significantly reduced the targeting of γ2-containing receptors to inhibitory synapses (Rathenberg et al., 2004). This therefore represents an important determinant of receptor localisation and surface expression. Interestingly, palmitoylation also occurs at the postsynaptic protein gephyrin, a key regulator of $GABA_A$ receptor clustering, and disruption of this also interferes with inhibitory synaptic signalling (Dejanovic et al., 2014; Groeneweg et al., 2018).

1.2. The roles of the $GABA_A$ receptor

GABA is the predominant inhibitory neurotransmitter within the CNS, acting through $GABA_A$ receptors to mediate both fast, transient phasic inhibition and continuous tonic inhibition (Farrant & Kaila, 2007). These two modes of inhibitory neurotransmission arise from distinct subunit configurations of the $GABA_A$ receptor separated by their subcellular localisation. The actions of $GABA_A$ receptors, both phasic and tonic, act to regulate neuronal activity. $GABA_A$ receptors are permeable to Cl^- and HCO_3^- which, in most adult neurons, results in a net influx of anions depending on the GABA reversal potential (E_{GABA}), hyperpolarising the membrane potential and so reducing the excitability of the neuron. They also reduce neuronal excitability through shunting inhibition, a local effect of channel opening which reduces the membrane resistance, thereby reducing the depolarising effect of excitatory currents (Staley & Mody, 1992; Mann & Paulsen, 2007). Shunting inhibition will also impact on synaptic waveform kinetics; a reduction in membrane resistance caused by open channels causes a simultaneous reduction in the membrane time constant and hence increases the rate of the voltage change of excitatory postsynaptic potentials (EPSPs).

1.2.1. Phasic inhibition

Phasic inhibition represents the synaptic component of $GABA_A$ receptor activity. A presynaptic depolarisation results in the influx of Ca^{2+} and subsequent vesicular-release of GABA into the synaptic cleft, which activates $GABA_A$ receptors clustered on the postsynaptic membrane. The time-course of the resulting IPSC is determined by a variety of factors; GABA concentration in the cleft, rate of removal of neurotransmitter through diffusion or uptake, and the deactivation and desensitisation properties of the $GABA_A$ receptor isoform expressed postsynaptically (Scimemi & Beato, 2009).

The synaptic cleft is approximately 20 nm wide, resulting in a saturating concentration of GABA, likely in the magnitude of > 1 mM, which binds to postsynaptic receptors, resulting in rapid activation and current flux. Estimates of the time GABA is present in the cleft predict approximately 500 µs, with an exponential decay time estimated at 0.3 – 0.6 ms (Scimemi & Beato, 2009; Barberis et al., 2011). For GABA, this is caused by diffusion out of the cleft, to be taken up by either glia or neurons *via* GATs (Borden, 1996). Rapid exposure and removal of GABA results in the activation of receptors and IPSCs with rapid rise times. The decay-phase of the IPSC is determined by the deactivation rate, which in turn is determined by the receptor isoform, as discussed earlier. The extent of receptor desensitisation also impacts on the decay of IPSCs. Outside-out patches of hippocampal neurons stimulated with brief GABA pulses mimicking synaptic transmission showed rapid entry into a desensitised state, followed by recovery into a bound and open conformation. At the level of the single channel, this was represented by initial activity, followed by a quiescent, desensitised phase, followed by re-activation (Jones & Westbrook, 1995). The interplay between activation, deactivation and desensitisation kinetics therefore plays a significant role in determining IPSC waveforms and subsequent information processing by the neuron.

Phasic inhibition results in large but brief IPSCs at the synapse, an example recording of which can be seen in Fig. 1.5. The effect of this on neuronal activity varies depending on the location of the synapse (He et al., 2015). In the hippocampus, different interneurons synapse onto distinct

membrane domains of principal cells (Klausberger & Somogyi, 2008). Basket cells typically make synaptic connections with the soma and proximal dendrites, whilst chandelier cells make contact with axons (hence their alternative name of axo-axonic cells). Various other interneurons make contact with dendrites, including bistratified cells, O-LM (Oriens Lacunosum-Moleculare) cells and neurogliaform cells (Klausberger & Somogyi, 2008; Kullmann, 2011; Pelkey et al., 2017). Action potentials result from the summation of excitatory and inhibitory potentials at the axon initial segment. If the overall voltage reaches the threshold for the activation of voltage-gated Na^+ channels then a regenerative current, the action potential, is generated which travels down the axon. Inhibitory potentials therefore significantly impact on how likely a neuron is to produce an action potential. Interneurons that make contact with the soma or perisomatic areas as part of feed-forward inhibition in the hippocampus set a very short interval (< 2 ms) over which excitatory events can be summated (coincidence detection). In contrast, feed-forward inhibition targeting the dendrites does not produce such strict temporal requirements for summation and action potential generation (Pouille & Scanziani, 2001). Phasic inhibition therefore has a range of effects on neuronal excitability depending on the location of synapses, as well as the number and type of $GABA_A$ receptors expressed postsynaptically.

Phasic inhibition has important effects on network synchronicity. The coincidence detection and spike timing conferred on neurons by somatic and perisomatic phasic inhibition operates as part of a network of interneurons to ensure principal cells all coordinate their firing to generate various types of synchronous oscillatory activity (Mann & Paulsen, 2007; Mann & Mody, 2010). This network of interneurons is connected by chemical and electrical synapses (gap junctions) which, when disrupted, alter theta oscillations and impair short-term spatial memory (Allen et al., 2011). Network oscillations are associated with certain behaviours and are also indicative of communication between brain areas (Salinas & Sejnowski, 2001).

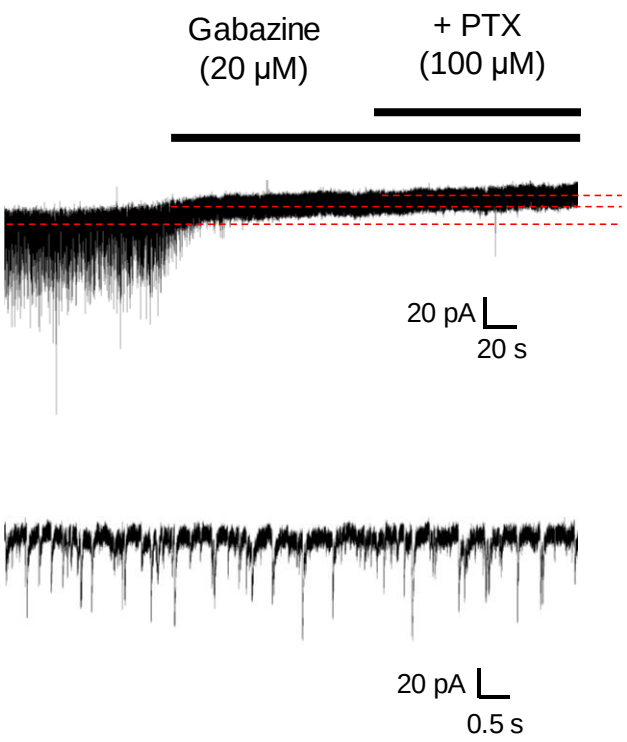

Figure 1.5: Phasic and tonic inhibition in thalamic relay neurons. Tonic inhibition (top panel) mediated by extrasynaptic $GABA_A$ receptors is revealed by the antagonists gabazine (20 μM) and picrotoxin (PTX, 100 μM), resulting in a reduction in the holding current, as indicated by the dashed markers. Phasic inhibition (lower panel) encompasses activity at synapses, resulting in discrete, brief events due to quantal release of GABA from presynaptic terminals.

1.2.2. Tonic inhibition

Tonic activity of $GABA_A$ receptors results in a continuous inhibitory current distinct from the short IPSCs which characterise phasic activity (Brickley et al., 1996; Brickley & Mody, 2012). This tonic inhibition is mediated by receptors outside of synapses which respond to ambient levels of GABA, of which predominant isoforms are α4/6βδ and α5βγ receptors (Mody, 2001; Glykys et al., 2008). The δ-containing receptors are suited to respond to low-levels of ambient GABA due to their high affinity and slow desensitisation rate. High expression levels of the δ subunit are associated with higher tonic activity, for example in dentate gyrus granule cells, thalamic areas such as the dorsal lateral geniculate nucleus and ventrobasal thalamus, and cerebellar granule cells (Brickley et al., 1996; Nusser et al., 1998; Pirker et al., 2000; Stell

et al., 2003; Cope et al., 2005). Tonic inhibition in neuronal recordings is evident during application of $GABA_A$ receptor antagonists, which reduce the holding membrane current, indicative of a population of continually active $GABA_A$ receptors, as shown in Fig. 1.5.

The source of GABA in extracellular compartments, typically 100 nM – 1 µM, originates from overspill from synapses, particularly during high-frequency activity resulting in multi-vesicular release (Bright et al., 2007; Glykys & Mody, 2007). Glia as a source of GABA mediating tonic inhibition has also been suggested (Yoon et al., 2011; Yoon & Lee, 2014). Astrocytes in culture display facilitated diffusion of GABA out of the cell through GABA transporters (GATs). This release could be partially inhibited by GAT inhibitors and Ca^{2+} chelators (Lee et al., 2011a). GABA is also released through the anion bestrophin channels (Lee et al., 2010; Diaz et al., 2011; Yoon & Lee, 2014). Both of these mechanisms occur through diffusion and therefore require a high intracellular concentration of GABA and, indeed, astrocytes express the synthesising enzyme glutamic acid decarboxylase (GAD) and intracellular GABA concentrations of approximately 2.32 mM (Lee et al., 2011b). GATs traditionally cause the uptake of GABA molecules through facilitated diffusion, driven by the Na^+ gradient generated by the Na^+/K^+-ATPase, and requires the co-transport of two Na^+ and one Cl^- ions (Scimemi, 2014). In the presence of high concentrations of intracellular GABA, these transporters instead act to expel GABA into the extrasynaptic space. The GABA released from glial sources directly impacts on tonic inhibition and affects motor coordination *in vivo* (Woo et al., 2018).

Tonic inhibition generates a persistent elevation in the conductance of the membrane, resulting in smaller voltage changes associated with excitatory currents and a greater attenuation of EPSPs as they propagate towards the soma, however they also increase the rate of voltage change in response to current. The overall effect of this reduces the temporal window over which EPSPs can summate to generate an action potential (Farrant & Nusser, 2005). Indeed, tonic inhibition plays a role in the processing of rate-coding information by modulating the gain of input-output curves (Mitchell & Silver, 2003; Silver, 2010). However, there is also evidence for tonic inhibition to modulate the

offset, independent of changes in gain. Due to outward rectification, some $GABA_A$ receptors display small tonic currents at the resting potential, with minimal impact on sub-threshold excitatory inputs. When the neuron depolarises and reaches threshold for action potential generation, there is a relatively greater activity of $GABA_A$ receptors and a greater impact of tonic inhibition. The result of this is to alter the input-output curve offset, but without changing the shape of the curve (Pavlov et al., 2009).

There is also evidence for a tonic GABAergic component controlling oscillatory activity in neuronal networks. The frequency of induced gamma oscillations in CA3 neurons *in vitro* could be potentiated in an NMDA receptor-dependent manner, but was counteracted by the actions of tonic δ-containing $GABA_A$ receptors (Mann & Mody, 2010). Tonic inhibition may also bi-directionally modulate the firing rate of interneurons which determine oscillatory activity (Pavlov et al., 2014). Tonic inhibition in cerebellar granule cells significantly shifts the input-output relationship between current injection and action potential frequency, promoting low spontaneous firing rates and enhancing the fidelity of sensory information transmission in the cerebellum (Chadderton et al., 2004; Duguid et al., 2012). Tonic currents in cerebellar granule cells develop over time in postnatal animals, indicating either a developmental role or changing requirements for tonic inhibition over time (Brickley et al., 1996).

The importance of tonic inhibition as a mechanism of neuronal plasticity and homeostatic plasticity was initially identified in animals lacking the α6 subunit and therefore showing impaired tonic inhibition. The higher membrane conductance, usually caused by extrasynaptic $GABA_A$ receptors, was compensated for by the upregulation of a two-pore-domain potassium channel, causing a persistent leak current, thereby restoring the normal membrane conductance (Brickley et al., 2001). The impact of tonic $GABA_A$ receptor activity on neuronal excitability is therefore important, but why are they present when a potassium channel can serve a similar function? By relying on a ligand-gated ion channel, it allows greater network control over neuronal excitability, in particular if the extracellular GABA derives from synaptic spillover during high frequency activity. This adaptive regulation

therefore provides tight control of network activity. Further examples of activity-dependent plasticity have been shown by CaMKII-dependent phosphorylation (strongly associated with activity-dependent plasticity) of the β3 subunit at S383, which enhanced insertion of α4β3δ receptors into the membrane, and thereby enhancing tonic inhibition (Saliba et al., 2012).

In addition to a tonic inhibitory current mediated by $GABA_A$ receptors, a component of the tonic current can be inhibited by the glycine receptor antagonist strychnine. This glycine receptor-dependent tonic current is present in several forebrain structures, including the striatum, hippocampus and prefrontal cortex, and is dependent on the glycine receptor α3 subunit (Salling & Harrison, 2014; McCracken et al., 2017). In the prefrontal cortex, a mixed population of α4βδ receptors and glycine receptors likely exist, producing two separate persistent currents, both of which contribute to tonic inhibition (Salling & Harrison, 2014).

1.3. Receptor pharmacology and disease

The wide range of $GABA_A$ receptors, with their diverse kinetics and localisation, is complemented by an equally diverse pharmacology. These include experimental drugs, used to probe the activity and properties of $GABA_A$ receptors, but also a significant number of therapeutic drugs used to treat conditions associated with an excess of excitability, most prominently in epilepsy (Korpi et al., 2002). $GABA_A$ receptors are involved in a number of disease states, and thus also represent viable therapeutic targets for treatments (Macdonald et al., 2010; Braat & Kooy, 2015). The binding sites for a variety of compounds are shown in Fig. 1.6, the majority of which are located at subunit interfaces, again promoting the concept that subunit identity heavily determines receptor properties, both kinetic and pharmacological.

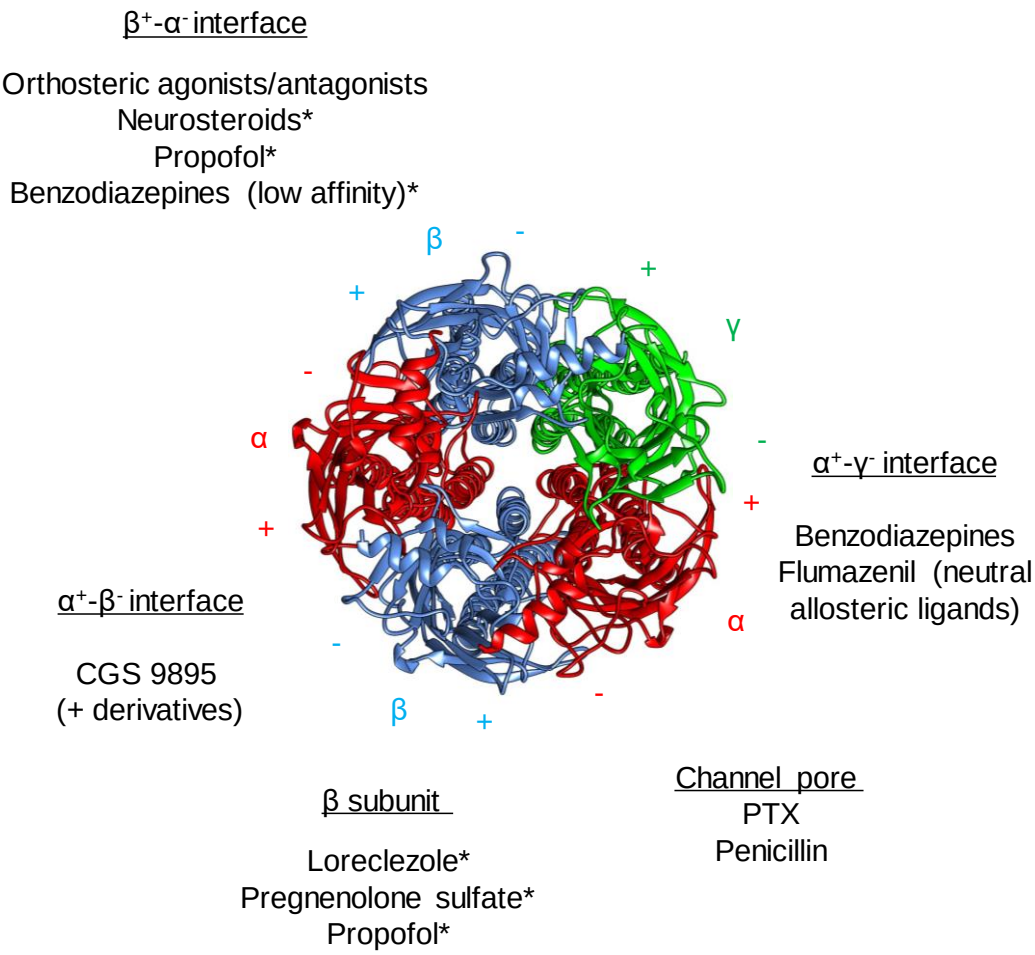

Figure 1.6: Pharmacology of the $GABA_A$ receptor. Most compounds bind at subunit interfaces, introducing the potential for subtype selectivity. Compounds denoted by * indicate binding within the transmembrane domains, others bind at the extracellular domain except PTX and penicillin, which bind within the channel pore (Wieland et al., 1992; Sanna et al., 1996; Xue et al., 1996; Hosie et al., 2009; Varagic et al., 2013b; Yip et al., 2013; Laverty et al., 2017; Olsen, 2018; Masiulis et al., 2019). The homology model was derived from the cryo-EM structure produced by Laverty et al. (2019).

1.3.1. $GABA_A$ receptor agonists and antagonists

A significant number of GABA homologues have been developed which activate the receptor with different affinities and efficacies, dependent on the subunit composition of the receptor. The orthosteric agonist muscimol acts as a full agonist at αβγ receptors and a super-agonist at αβδ receptors, showing peak currents 120-140% the size of saturating GABA responses (Mortensen et al., 2010; Johnston, 2014). Muscimol is also a more potent agonist than

GABA, with EC_{50} values approximately three-fold smaller (Ebert et al., 1994; Mortensen et al., 2010).

Another orthosteric agonist is THIP, and this too has an activity profile dependent on the presence of the γ or δ subunit. At γ-containing receptors, THIP is a partial agonist with a significantly lower affinity for the receptor than GABA. However, at δ-containing receptors, it retains its low affinity but acts as a super-agonist, displaying currents in excess of 200% those of GABA (Mortensen et al., 2010).

The action of these agonists can be examined at the level of the single channel. The single channel conductance elicited by each of GABA, muscimol and THIP is unchanged, with each agonist eliciting three conductance states, with the largest, 26-31 pS dominating (Mortensen et al., 2010). In other studies, two conductance states have been reported when applying GABA to recombinant receptors (Brickley et al., 1999; Keramidas & Harrison, 2010). GABA activation of α1β3γ2 receptors displayed higher open probabilities than α4β3δ, with longer mean open times. However, application of THIP generated a significantly higher open probability in α4β3δ receptors, with prolonged open times within bursts, explaining its super-agonist properties (Mortensen et al., 2010).

Typical synaptic (γ2-containing) and extrasynaptic (δ-containing) receptors can therefore be distinguished based upon their potency and efficacy of activation by THIP, but more selective ligands are required to examine in greater detail the roles of these different receptors. One such family of ligands are derived from 5-(4-piperidyl)-3-isoxazolol (4-PIOL), a low-affinity, weak partial agonist at the $GABA_A$ receptor (Mortensen et al., 2002). 4-PIOL itself may have use as a diagnostic tool to probe synaptic and extrasynaptic receptors. Tonic current in the cerebellum is mediated by extrasynaptic α6βδ receptors (Nusser et al., 1998; Brickley et al., 2001), but this was unaffected by the application of 4-PIOL (Patel et al., 2016). In contrast, application of 4-PIOL caused a bi-directional modulation of tonic currents in CA1 hippocampal neurons, where tonic current is mediated by α5βγ, α4βδ and αβ receptors (Caraiscos et al., 2004; Mortensen & Smart, 2006; Glykys et al., 2008), the

polarity of which is likely to be determined by ambient GABA concentration (Patel et al., 2016). The 4-PIOL derivative 5-(4-piperidyl)-3-isothiazolol (Thio-4-PIOL) acts as a partial agonist at extrasynaptic α4β3δ, α5β3γ2S and α6β3δ receptors, but displays almost no efficacy at synaptic α1-3β3γ2S receptors (rendering it a competitive antagonist at these receptors). Indeed, application to hippocampal CA1 and thalamic slices, known to express a significant tonic current mediated by α5 and δ-containing receptors, resulted in a silencing of IPSCs, but an increase in tonic current and RMS noise (Hoestgaard-Jensen et al., 2013). Another derivative, 4-(3,3-diphenylpropyl)-5-(4-piperidyl)-3-isoxazolol hydrobromide (DPP-4-PIOL), is able to selectively antagonise tonic currents in dentate gyrus granule cells with minimal effects on IPSCs (Boddum et al., 2014).

Antagonists of $GABA_A$ receptors are also used to probe functionality. Orthosteric antagonists commonly used include gabazine (SR-95531) and bicuculline, and these act by competing with GABA for the binding site but, with no efficacy of their own, do not cause channel activation to significant levels (Johnston, 2013). Neither of these antagonists shows significant selectivity between receptor subtypes, with calculated pK_i, the concentration of antagonist required for 50% receptor occupancy, not significantly different between receptors composed of varying α and β subunits (Krishek et al., 1996a; Ebert et al., 1997). This non-selective activity of gabazine has resulted in the proposal that it could be used to distinguish synaptic and extrasynaptic receptors, as the former have significantly lower affinity for GABA and will therefore be more sensitive to competition by gabazine (Stell & Mody, 2002). Intriguingly, both gabazine and bicuculine show negative allosteric properties (Ueno et al., 1997). Bicuculine is the more potent negative allosteric modulator, which has led to gabazine being proposed as a more appropriate compound to selectively abolish, at low concentrations, IPSCs without affecting spontaneous tonic currents (McCartney et al., 2007).

Picrotoxin (PTX) is a non-competitive antagonist composed of two components, the largely inactive picrotin, and the more active picrotoxinin. PTX shows use-dependency, with studies showing a likely binding site within the channel pore, accessible when the channel is open. Recent cryo-EM

studies have confirmed that PTX binds in this location and induces conformational changes associated with an allosteric modulatory effect on currents rather than just a simple pore blockade (Masiulis et al., 2019). This is in agreement with earlier studies, in which PTX has also been hypothesised to not only physically occlude the channel pore, but to have allosteric effects on the receptor to promote and stabilise an agonist-bound closed or desensitised state (Smart & Constanti, 1986; Newland & Cull-Candy, 1992; Krishek et al., 1996a; Korshoej et al., 2010). Structural modelling has identified a putative secondary binding site at the interface of the ligand binding domain and transmembrane regions, which could promote this non-conducting state (Carpenter et al., 2013).

1.3.2. Allosteric modulators

A variety of allosteric modulators exist, both endogenously and as therapeutic treatments, which target the $GABA_A$ receptor. The benzodiazepines, such as diazepam, were highly used as anti-epileptic, anxiolytic and hypnotic therapeutics but have, to an extent, fallen out of favour due to an increasing realisation of their associated tolerance and dependency (Olsen, 2018). Benzodiazepines enhance GABA-mediated currents without directly activating the receptor; at the level of the single channel they potentiate $GABA_A$ receptor currents by increasing the number of bursts, thereby increasing total open frequency, without affecting burst duration or single channel conductance (Rogers et al., 1994). The actions of benzodiazepines are highly dependent on subunit composition of receptors. The γ2 subunit is essential for activity and if replaced by δ there is no potentiating effect (Pritchett et al., 1989; Gunther et al., 1995). The identity of the α subunit is also critical, as those associated with extrasynaptic receptors, α4 and α6, show no potentiation, even if expressed as α4/6βγ receptors. This selectivity arises from the location of the benzodiazepine binding site at the α^+-γ^- interface, at a site analogous to the GABA-binding site at the β^+-α^- interface (Olsen, 2018). A histidine residue on the α subunit (H101 in α1) is critical for the potentiating effect, and indeed mutation of this to arginine, the homologous

residue in α4/6, prevents benzodiazepine potentiation (Wieland et al., 1992; Rudolph et al., 1999). In addition to this selectivity of conventional benzodiazepines, non-benzodiazepine 'Z-drugs', including zolpidem and zopiclone, bind to the benzodiazepine site but have greater affinity for α1-containing $GABA_A$ receptors. Due to this selectivity, they are widely used as effective sedatives and hypnotics (Nutt & Stahl, 2010). Alongside the conventional binding site of the 'benzodiazepine receptor', recent evidence suggests other, low affinity, benzodiazepine binding sites at the homologous site at the α^+-β^- interface (Ramerstorfer et al., 2011) and within the transmembrane domains of the orthosteric β^+-α^- interface (Walters et al., 2000; Masiulis et al., 2019).

Other positive allosteric modulators are, instead, used to selectively probe extrasynaptic δ-containing receptors. DS2 (delta selective compound 2) potentiates maximum $GABA_A$ receptor currents, with an effect partially dependent on the identity of the α subunit, with α4/6-containing $GABA_A$ receptors showing significantly higher potentiation than α1 (Wafford et al., 2009; Jensen et al., 2013; Lee et al., 2016). This compound enhances tonic currents of thalamic relay neurons and cerebellar granule cells, with no effect on synaptic currents (Wafford et al., 2009).

A major endogenous enhancer of $GABA_A$ receptors are the neurosteroids. These modulators are synthesised *de novo* in the brain from cholesterol and from precursors synthesised in the periphery that then cross the blood-brain barrier due to their lipophilic properties (Carver & Reddy, 2013). The enzymes 5α-reductase and 3α-hydroxysteroid dehydrogenase sequentially catalyse the generation of the pregnane steroids 5α-pregnan-3α-ol-20-one (allopregnanolone) and 5α-pregnan-3α,21-diol-20-one (THDOC) from progesterone and deoxycorticosterone, respectively. These are the two most commonly studied neurosteroids, but a host of others exist (Baulieu, 1998; Reddy, 2010). This production occurs in principal neurons and has autocrine and paracrine effects on $GABA_A$ receptor signalling (Agis-Balboa et al., 2006). At the level of the single channel, neurosteroids increase both the opening frequency and burst duration, resulting in a potentiation of $GABA_A$ receptor currents (Twyman & Macdonald, 1992). Neurosteroids bind to and

enhance all $GABA_A$ receptors through binding to a subunit interfacial (β^+-α^-) site within the transmembrane domains that harbours a key glutamine residue from the α subunit, conserved across all classes (Q241 and Q246 in α1 and α4, respectively) (Hosie et al., 2006; Hosie et al., 2007; Hosie et al., 2009; Laverty et al., 2017). Despite the binding site at the β^+-α^- interfaces, neurosteroids have greater potentiating effects on receptors containing the δ subunit, and so they have a proportionally larger effect on tonic inhibition (Belelli et al., 2002; Pillai et al., 2004). Indeed, in δ knock-out animals, neuronal sensitivity to neurosteroid significantly decreased (Mihalek et al., 1999). When neurosteroids are applied acutely to brain slices, effects on both phasic and tonic inhibition are observed. Holding currents are significantly increased, representing enhancement of tonic currents, whilst IPSCs recorded from CA1 neurons show prolonged decay times (Harney et al., 2003; Stell et al., 2003). Over a longer time-scale, neurosteroids also increase the expression of α4 and δ-containing receptors, possibly through a phosphorylation-dependent mechanism as noted earlier at the S443 site of the α4 subunit (Hsu et al., 2003; Shen et al., 2005; Abramian et al., 2014). This interplay with phosphorylation is also observed with the adjacent phosphorylation sites on the β3 subunit, S408 and S409, which, when phosphorylated, enhance the level of potentiation caused by THDOC at both synaptic and extrasynaptic-type receptors (Adams et al., 2015). Neurosteroids have been shown to promote the phosphorylation of these two key residues through activation of the membrane progesterone receptor (mPR) in a PKA- and PKC-dependent manner. The selective agonist of these receptors, ORG OD 02-0, which displayed no acute effects on tonic current, instead increased it after prolonged (15 min) exposure through its actions on the mPR and stimulation of kinases (Parakala et al., 2019).

Most widely studied neurosteroids have potentiating effects on $GABA_A$ receptors, but there is also a class that has inhibitory effects: the sulfated neurosteroids (Shen et al., 2000; Akk et al., 2001). Pregnenolone sulfate (PS) shows inhibitory effects on GABAergic currents, with a higher efficacy of block at δ-containing receptors, suggesting that, just as with the potentiating

neurosteroids, the inhibitory neurosteroids also have a significant role in modulating tonic inhibitory currents (Seljeset et al., 2018).

1.3.3. Disease states and treatments manipulating inhibitory neurotransmission

An imbalance between excitation and inhibition is a hallmark of many disease states (Fritschy, 2008; Lopatina et al., 2019; Sohal & Rubenstein, 2019). Some have a direct link with $GABA_A$ receptor function, for example point mutations linked with epilepsy, whilst others are caused by factors such as brain injury or drug abuse (Leach et al., 2012; Møller et al., 2017; Sohal & Rubenstein, 2019). The diverse pharmacology of the $GABA_A$ receptor results in it being a target for current and future therapies aimed at altering both phasic and tonic inhibition (Sigel & Steinmann, 2012).

Epilepsy is characterised by uncontrolled excitability in the brain leading to seizures. Many therapies for epilepsy target $GABA_A$ receptor signalling. For status epilepticus, benzodiazepines are given to suppress seizures; however this increasingly becomes less effective over time (Niquet et al., 2016; Burman et al., 2019). Changes in subunit composition of $GABA_A$ receptors have been extensively identified in epilepsy, for example there is upregulation of α4βγ receptors at synapses during epileptogenesis, the generation of an epileptic basal state. This is accompanied by a downregulation of total δ subunit expression in dentate gyrus granule cells, but interestingly no loss of tonic inhibition, likely through compensatory mechanisms, but this does lead to lower neurosteroid potentiation which may contribute to the epileptogenic process (Zhang et al., 2007). Indeed, a role for neurosteroid synthesis and activity in epileptogenesis has been identified by an upregulation of the P450scc enzyme, a rate-limiting step in synthesis, after induction of status epilepticus and proportional to the duration of the initial seizure. This upregulation was then associated with prolonging the latency before chronic seizures started. Finasteride, a 5α-reductase inhibitor, blocks neurosteroid synthesis and subsequently reduced the latency time (Biagini et al., 2006).

Neurosteroids are anti-epileptic, and ganaxolone, a 3β-methyl allopregnanolone derivative, is currently in clinical trials for treatment of epilepsy (Reddy & Estes, 2016). The methylation of the 3β position prevents metabolism, rendering ganaxolone a more stable compound with greater bioavailability (Yawno et al., 2017). Supporting the idea that disruptions in tonic inhibition can be integral to epileptogenesis (Qi et al., 2006), δ knock-out animals show lower threshold for seizure generation, indicating tonic and possibly neurosteroid potentiation is important (Spigelman et al., 2002). Many epilepsy treatments involve enhancing levels of tonic current, for example tiagabine prevents GABA uptake through GAT-1, thereby increasing ambient GABA. Other therapies increase the production of GABA, such as vigabatrin, which inhibits the catabolic GABA-transaminase (Treiman, 2001). Other treatments have been hypothesised to selectively enhance tonic inhibition to treat epilepsy, for example THIP and DS2, which have greater efficacy at δ-containing receptors (Storustovu & Ebert, 2006; Wafford et al., 2009; Brickley & Mody, 2012).

Psychiatric disorders have also implicated tonic inhibition in their pathology. Reduced levels of mRNA of the δ subunit have been reported in post-mortem studies of schizophrenic patients (Maldonado-Aviles et al., 2009) and abnormal gamma oscillations in the hippocampus of schizophrenic patients, which are regulated by tonic inhibition, suggest a role for δ-containing receptors in schizophrenia (Uhlhaas & Singer, 2010; Brickley & Mody, 2012). However, the exact role of GABA, and specifically tonic inhibition, in schizophrenia is unclear, as no reduction in α4 mRNA was observed, and there are deficiencies in both GABA uptake and synthesis in schizophrenic brains (Maldonado-Aviles et al., 2009; de Jonge et al., 2017). Postpartum depression is another psychiatric illness associated with alterations in $GABA_A$ receptor signalling. After birth, levels of neuroactive steroid decline rapidly, potentially leading to pathological changes in inhibitory signalling (Schüle et al., 2014). To counter this, allopregnanolone, marketed as brexanolone, has recently been approved for treatment of postpartum depression (Scarff, 2019).

Tonic inhibition plays a significant role in neuroprotection during and after brain injury. In the striatum, muscimol-induced tonic currents reduced the

hallmarks of neuronal damage after insult with NMDA in wild-type, but not δ knock-out, animals (Santhakumar et al., 2010). In some cases, tonic inhibition may actually impede recovery after injury, as artery occlusion in the motor cortex of rats resulted in reduced levels of α4β3δ receptors, improving recovery of motor performance, but also resulting in post-stroke epileptic seizures (Jaenisch et al., 2016). In other studies, tonic inhibition in the surrounding area of photothrombotic stroke in the motor cortex was actually increased due to impairment of GAT function (Clarkson et al., 2010). The effects of stroke on tonic inhibition therefore likely vary depending on the type of lesion (Jaenisch et al., 2016).

1.4. Spontaneous gating of $GABA_A$ receptors

Spontaneous activity of Cys-loop receptors has long established the presence of certain receptors which can open in the absence of agonist. This varies between subunit composition, expression system and species (Jackson, 1984; Jackson et al., 1990). Continuous low-level spontaneous activity represents a form of tonic inhibition. Tonic inhibition plays an essential role in neuronal physiology at all levels of brain function, from regulating oscillatory activity to controlling the excitability and output of individual neurons. Discussion of tonic inhibition generally assumes GABA-mediated activity, but there is considerable evidence for a component mediated by the spontaneous agonist-independent opening of receptors. Indeed, $GABA_A$ receptors have been noted for their high constitutive activity compared to other ligand-gated ion channels (Bera & Akabas, 2005; Nayak et al., 2019).

1.4.1. Receptor subtypes that activate spontaneously

Investigations using heterologous expression systems first identified a constitutive component to $GABA_A$ receptor activity. Expression of β homomers alone in HEK 293 cells or oocytes showed the formation of homomeric receptors for β1, β2 and β3 subunits (Sigel et al., 1989; Cestari et al., 1996;

Krishek et al., 1996b; Wooltorton et al., 1997). Expression of rat β1 homomers was evident in oocytes which showed significant outward currents when the antagonist PTX was applied, indicating the expression of spontaneously active β1 homomeric receptors (Sigel et al., 1989), and this was later observed for the β3 subunit (Cestari et al., 1996; Wooltorton et al., 1997). Formation and expression of homomeric β subunits is far from consistent, as other groups have shown homomeric β3 expression but with no spontaneous activity (Davies et al., 1997b; Miko et al., 2004), and is likely affected by the expression system, cDNA species and experimental protocol.

The expression of β homomers is unlikely to occur physiologically in the presence of other subunits and, to date, no evidence of β homomeric receptor expression has been observed in neurons. However, $GABA_A$ heteromers have also been identified which show spontaneous activity. Diheteromeric receptors composed of α4 and β1 subunits were expressed in oocytes and found to be highly spontaneously active, more so than α1β1 receptors (Khrestchatisky et al., 1989). These diheteromeric receptors are more likely to exist physiologically, and indeed they have been estimated to contribute up to 10% of the extrasynaptic receptors in hippocampal pyramidal neurons (Mortensen & Smart, 2006). Another less common subtype of $GABA_A$ receptor that nevertheless is likely to be expressed in neurons are the αβε receptors. These receptors show significant levels of spontaneous activity when expressed as α1β3ε and α3β1ε, as determined by blockade by PTX and/or Zn^{2+} (Neelands et al., 1999; Ranna et al., 2006). In-situ hybridisation experiments indicate that the mRNA of the ε subunit is found in the amygdala, thalamus and subthalamic nuclei (Davies et al., 1997a; Whiting et al., 1997), although immunohistochemistry shows that the protein was only significantly detectable in the hypothalamus and areas of the hippocampus (Whiting et al., 1997). The overall levels of ε-containing receptors relative to other, more common, $GABA_A$ receptor classes is unknown, but this receptor could contribute to tonic inhibition through its spontaneous activity if expressed at appreciable levels.

More common $GABA_A$ receptors have also been shown to display significant levels of spontaneous activity. The α1β1γ2S receptor was examined for spontaneous activity using the use-dependent channel blocker

penicillin. In the absence of agonist, penicillin appeared to cause the gradual accumulation of spontaneously active receptors in an open conformation but with the ion pore blocked, with spontaneity being revealed by a large inward current upon removal of penicillin (Lindquist et al., 2004). The α4β3δ and α4β3γ2L receptors have also displayed spontaneous currents revealed by PTX (Tang et al., 2010). The former is the most common type of extrasynaptic receptor in the dentate gyrus, whilst the latter may contribute to perisynaptic receptors during epileptogenesis after down-regulation of δ subunits (Peng et al., 2004; Pavlov & Walker, 2013).

1.4.2. Physiological role of spontaneous activity and its regulation

The role of spontaneous currents in tonic inhibition has been probed using competitive antagonists which also display negative allosteric properties. Both bicuculline and gabazine competitively antagonise GABA but also antagonise allosteric agonists such as pentobarbital, and inhibit spontaneous activity. In receptors expressing mutant β subunits that show spontaneous activity, both bicuculine and gabazine inhibited spontaneous currents (Ueno et al., 1997). However, gabazine applied to HEK 293 cells expressing α1β3γ2 receptors could not block propofol-induced currents, in contrast to bicuculline (McCartney et al., 2007). In outside-out patches pulled from hippocampal neurons, gabazine weakly blocked propofol-induced currents (McCartney et al., 2007). The effects of gabazine and bicuculline on spontaneous activity are likely dependent on receptor isoform and post-translational modifications such as phosphorylation (Ueno et al., 1997; Tang et al., 2010), as well as other factors such as interacting proteins which may be present in neurons but not in systems expressing recombinant receptors (Moss & Smart, 2001; Tretter et al., 2012).

These differential effects of gabazine and bicuculline have been used to probe spontaneous activity of receptors in neurons. Gabazine at low concentrations was unable to antagonise tonic currents, but bicuculline revealed a significant tonic component in cultured hippocampal neurons

(McCartney et al., 2007). This could potentially be explained by the higher potency of GABA at extrasynaptic receptors (Stell & Mody, 2002), although the authors used concentrations of gabazine that should antagonise these receptors if they were GABA-activated. Their conclusion was that hippocampal neurons express spontaneously-gating receptors that cause tonic currents. This is in agreement with an earlier study that performed cell-attached recordings of CA1 hippocampal neurons, which identified spontaneous openings of $GABA_A$ receptors which could be potentiated by benzodiazepines (Birnir et al., 2000). These receptors contain the γ2 subunit and are clustered at synapses, but are also expressed extrasynaptically, and so both populations of these receptor types may contribute to the spontaneous tonic current (Alldred et al., 2005; Thomas et al., 2005).

This was further investigated by looking at hippocampal brain slices. Dentate gyrus granule cells express significant levels of tonic inhibition and this was investigated for spontaneous activity, with gabazine having minimal effect on tonic currents, in contrast to PTX which caused significant reductions (Wlodarczyk et al., 2013). Intriguingly, very low concentrations (100 nM) of extracellular GABA were estimated for the dentate gyrus *in vivo,* determined using zero-net-flux microdialysis (Wlodarczyk et al., 2013). The authors concluded that the majority of tonic inhibition in dentate gyrus granule cells is therefore mediated by spontaneous activity rather than GABA-mediated. Spontaneous activity therefore likely plays a large role in regulating activity in the dentate gyrus and, subsequently, CA3 and CA1 areas.

The presence of a spontaneous component to tonic currents is likely to occur *in vivo*, but would require regulation in order to effectively control levels of tonic inhibition. A mechanism through which this can be achieved is through the activity of protein kinases. Activation of PKA increased the level of spontaneous current recorded from HEK 293 cells expressing both α4β3δ and α4β3γ2L receptors, but did not affect currents recorded in the presence of 1 μM GABA (Tang et al., 2010). This could represent a phosphorylation-dependent mechanism through which tonic inhibition can be regulated during periods of low ambient GABA, but when GABA is present the extracellular concentration is the predominant determinant of tonic current amplitude.

Kinases would therefore allow modulation of tonic currents even in the absence of GABA.

1.5. Aims

1.5.1. Structural determinants of spontaneous receptor activity

No previous study has performed a screen of the spontaneous activity of different $GABA_A$ receptors, or shown a dependence on subunit composition for agonist-independent gating. Our first aim, therefore, was to quantify spontaneous activity through electrophysiological techniques, recording from a variety of recombinant receptors expressed in HEK 293 cells. With this information, and using mutagenesis to create chimeric subunits, we then aimed to show domains within individual subunits that could be responsible for the spontaneous receptor activity, and to suggest a mechanism by which these contribute to, or cause, the spontaneous phenotype.

1.5.2. Regulation of spontaneous activity

$GABA_A$ receptor activity is modified by phosphorylation and modulatory compounds. There is evidence for a phosphorylation-dependence to the spontaneous activity, but the modulatory kinases and their targets have not been fully examined. We therefore aimed, through mutagenesis and pharmacological manipulation of kinases, to determine which residues within the receptor pentamer are targets for phosphorylation and to suggest how this could impact on the spontaneous activity. We also used heterologous expression of spontaneous receptors to determine the effects of modulatory compounds on the spontaneous current through acute application of neuroactive steroids, general anaesthetics and benzodiazepines.

1.5.3. The physiological impact of spontaneous receptor activity

Our previous aims were focused on investigating the properties of spontaneously-active $GABA_A$ receptors in isolation by using heterologous expression. We therefore needed to validate our findings in a neuronal environment. This was achieved by using transfection of subunit cDNA or knockdown of native receptor subunits to modify expression in hippocampal neurons. We also investigated how manipulation of subunit expression and spontaneous activity could impact on neuronal excitability, with the aim of providing evidence for a role of spontaneous tonic activity in network activity and information processing.

From recombinant receptor expression and neuronal studies we hypothesised that subunit expression was a key determinant of spontaneous activity. To this end, we selected two brain areas which displayed different subunit expression patterns and recorded the GABA-evoked and spontaneous components to the tonic current from *ex vivo* slices.

Finally, we aimed to examine the potential role of spontaneous activity in pathological states through recombinant expression of mutant $GABA_A$ receptors implicated in epilepsy. Our previous data suggested that spontaneous receptor activity could contribute significantly to tonic inhibition, and so we proposed that subunit mutations that had pathological phenotypes likely impacted on spontaneous activity.

1.5.4. Summary of aims

- To determine which $GABA_A$ receptor isoforms display spontaneous currents and to define structural domains within subunits which contribute to or cause spontaneous activity. This will allow us to suggest a kinetic model of action to explain spontaneous receptor gating.

- To show how spontaneous currents *in vivo* may be modulated by investigating the impact of phosphorylation and kinase activity on spontaneous receptor activity.

- To demonstrate the effects of allosteric modulators on the spontaneous current, showing how regulation can occur through both endogenous means, and to suggest novel mechanisms of actions of current and future therapeutics.

- To investigate how manipulation of spontaneous currents can impact on neuronal excitability and network information processing.

- To extrapolate our findings to predict the reliance of tonic inhibition on spontaneous receptor activity across different brain areas.

Chapter 2: Materials and methods

2.1. Mutagenesis

2.1.1. Site-directed mutagenesis

Site-directed mutagenesis was used to generate point mutations in $GABA_A$ receptor subunits using the PCR protocol described in Table 2.1. Phusion polymerase (ThermoFisher) or Q5 polymerase (New England Biolabs, NEB) were used as described by the manufacturers. Briefly, a PCR mix was made consisting of 0.5 µM forward and reverse primer, 10 ng of template cDNA, 1 µl dNTP, 10 µl polymerase buffer and 0.5 µl polymerase, made up to 50 µl in water. The PCR protocol consisted of an initial denaturing step at 98˚C for 30 s, followed by 25-35 cycles of sequential denaturing, annealing and extension phases, as described in Table 2.1. Forward and reverse primers (Sigma) for all mutant subunits created are displayed in Table 2.2. Mutant subunits that are discussed in the Results sections but not included in the table were already available from other projects in the lab.

The PCR product was run on 0.8% agarose gel for 45 mins at 100 V to examine the effectiveness of the PCR protocol. DNA was labelled using either ethidium bromide or GelRed (Biotium). A 1 kb ladder was run alongside for molecular weight sizing and bands were picked from the gel product corresponding to the correct size for the plasmid. DNA was extracted from the gel as described by the kit manufacturers (Qiagen and NEB): bands of the appropriate size were cut out of the gel, and were weighed and dissolved in an appropriate volume of dissolving buffer at 50˚C. The solution was inserted into a filter column and centrifuged to collect the plasmid, which was washed and dissolved again in 35 µl of water. DNA was then ligated, using T4 polynucleotide kinase (PNK) and DNA ligase, as described by the manufacturers (NEB): 16 µl of the gel extract was kept at 72˚C for 5 mins, before cooling on ice for 1 min and the addition of 2 µl of T4 ligase buffer (NEB) and 1 µl T4 PNK. This was incubated at 37˚C for 40 mins before the addition

of 1 µl of T4 ligase. The solution was kept at 4˚C overnight, before transformation the following day.

The ligation product was used to transform competent *E. coli* cells. A small volume (1-3 µl) of the ligation product was gently added to 25 µl *E. coli* and left on ice for 30 mins. *E. coli* then underwent heat-shock at 42˚C for 30 s and returned to ice for 1 min, before the addition of 100 µl SOC medium. Cells were left gently shaking at 37˚C for 30 mins. Bacteria were plated for growth on LB agar dishes containing ampicillin (100 µg/ml) for selection and left at 37˚C overnight. Individual colonies were picked the next day and grew overnight. DNA was extracted using miniprep kits (Qiagen and NEB). These DNAs were sequenced (Source Bioscience) and if the mutation was evident, bacterial colonies were further grown and midiprep and maxiprep kits (Qiagen) were used to extract the DNA. The whole subunit cDNA was then sequenced using SP6 forward primer and P5 reverse primer.

Step	Temperature (˚C)	Time
Initial denaturation	98	30 s
(25 – 35 cycles)		
Denaturation	98	30 s
Annealing	50-72	30 s
Extension	72	5 mins
Final extension	72	5 mins

Table 2.1: Polymerase chain reaction protocol. An initial denaturation step was performed, followed by 25-35 cycles of denaturation, annealing and extension steps, before a final extension step. Samples were maintained at 4˚C after the PCR protocol was completed.

2.1.2. Restriction-free cloning

For generating chimeric subunits, such as the $\beta3^{\beta2(ECD)}$ and $\beta2^{\beta3(ECD)}$ subunits (Ch. 3), restriction-free cloning was used to exchange cDNA sequences from different $GABA_A$ receptor subunit plasmids. Primers were generated and PCR conditions determined using online resources (https://www.rf-cloning.org). This tool designed large primers complementary to both the DNA fragment to be inserted and the DNA sequence surrounding the insertion area of the template DNA. For example, the sequence of the forward primer for the production of the $\beta3^{\beta2(ECD)}$ subunit:

CCGGGGCGCGGCGAAGGGATGTGGAGAGTCCGGAAAAG

This contains the sequence for amplification of the β2 ECD (in blue) but also the complementary sequence for the surrounding residues in the β3 plasmid (in yellow).

PCR was performed to amplify short DNA sequences of the fragment to be inserted, which was analysed by running the PCR product on a 1.5% agarose gel. This ‘mega primer’ was then used in a second PCR protocol to insert the foreign subunit cDNA into the target. The DpnI restriction enzyme (NEB) was then added to the PCR product (2 µl at 37˚C for 2 hrs) to degrade methylated plasmids, and the product was used to transform competent *E. coli* cells. Colonies were harvested and the DNA extracted as described above.

Subunit	Forward primer	Reverse primer
$\alpha4^{\alpha1(\text{loop C})}$	CATTGGGCAGACTGTATCAAGCGAGATTGTTCAGTCCAGTACTGGAGAA	TCTGAATCATAAAGTAGCCCATCTTCCGTCTCAAGTGGAAGTGAGTCGTCATA
$\alpha1^{\alpha4(\text{loop C})}$	CTTCTTGGACAAACAGTTGACTCTGGAACTATCAAATCTATTACAGGTGAATACA	TATGTTTGAATAACAAAGTAGCCAATTTTTCTCTTGAGGTGGAAGTAAACCGTCAT
$\beta2^{\beta3(\text{loop})}$	TGGCCCTTCTGGAATACGCTTTGGTCAACTACATTTTCTTTGGAAGAGGTCCC	CACAGGGAAGAAAATGCGGGACCATCTGTCTATGGCATTCACATCGGT
$\beta3^{\beta2(\text{loop})}$	CTGGCACTTCTGGAGTATGCCTTTGTCAACTACATCTTCTTTGGGAGAGGAC	GAATGGAAACACGATGCGGGACCACCGATCAATGGCATTCACATCAG
$\beta2^{\beta3(\text{ECD})}$	TCCTCAATCCCACCAGAACCTAAAGGGATGTGGGGCTTTGCGGGAG	CAGAATTGATGGCATGTATGTCTGCAGGATGAAGTACCCGATATTTCTCTTCAACCGAA
$\beta3^{\beta2(\text{ECD})}$	CCGGGGCGCGGCGAAGGGATGTGGAGAGTCCGGAAAAG	ATTGAGGGCATATACGTCTGCAGAATGAAGTAGCCAATGTTCCTTTTCAGC
$\alpha4^{\alpha1(\text{ECD})}$	CTCGGTTCTATCGATTGAATTCGCCACCATGAAGAAAAGTCGGGGTCTCTC	CTGTCATGATGCATGGGATATACGTCTGAATCATAAAGCCAATTTTTCTCTTCAAGTGGAAG
$\alpha1^{\alpha4(\text{ECD})}$	CCGAGCTGTGCAAGCCCGTGATGGTTTCTGTCCAGAAGGTACCC	CATTATGCACGGCAGATATGTTTGAATAACAAAGTAGTAGCCCATCTTCCGTCTGAGG
$\alpha4^{\text{R100H}}$	TACTTTCTTCCATAATGGAAAGAAATCTGTCTC	TCAGGGGTCCAAACTTTG
$\beta3^{\text{A201S,A204S}}$	CACAGGTTCCTATCCTCGAC	GAGAAGACAACATTCCTGGA
$\beta3^{\text{TK}}$	AAAGATCGAGCTCCCACAGTTCTC	GTCACGCCAGTGACAGCCTT
$\beta3^{\text{DN}}$	GACAATGCTGTCACTGGCGT	ATCGCCACGCCAGTAAAATT
$\beta3^{\text{DNTK}}$	ACATTGAATTTTACTGGCGTGGCGATGACAATGCAG	GAGAACTGTGGGAGCTCGATCTTTGTCACTCCTGTGA
$\beta2^{\text{GKER}}$	ACATTGAGTTTTACTGGCGCGGCGGGGACAAGGCTG	GAGAACTGAGGAAGCTCAATCCTTTCCACGCCAGTGA
$\delta^{\text{Y2L(loop C)}}$	CCAGTTCACTATCACCAGTTACCGCTTCACAACTGAAGTAGTGAAGACAACTTCTGGTGACTAT	CGGTTCCTCCGAAGCTGGAAGTGTAAAGACATCACCACATAGTCACCAGAAGTTGTCTTCACTA
$\delta^{\text{Y2L(ECD)}}$	GCACCTCGGTTCTATCGATTGAATTCGCCATGAGTTCGCCAAATACATGGAGC	AGAGGGCATGTAAGACTGGATGATGTAGACGCCCATTCTTCTGCTCAGATCG
$\beta3^{\text{L170R}}$	AGATACCCACGGGATGAGCAAAA	TCTGAGGTCCATCATGCAC
$\beta3^{\text{T185I}}$	TATGGCTACATTACGGATGAC	GCTTTCAATTTCCAAAGTG
$\beta3^{\text{R429Q}}$	CCCACCTACAGAGGAGGTCT	TCTTCTTGTGCGGGATGC
$\beta3^{\text{K279T}}$	AAATTCCCTATGTCAcAGCCATCGACATGTACCTG	TGGGTAGAGTCTCCCGAAGGTGAGTGTTGATGGTT
$\beta2^{\text{A409S}}$	CAACTGAAAATCACCATCCCC	AGAGGAACGTCTCCTCAGGC
$\beta3^{\text{S408D,S409D}}$	CAGCTCAAAATCAAAATCCC	GTCATCCCTCCTCCGTAGGT

Table 2.2: Primer sequences used for mutagenesis of $GABA_A$ receptor subunits. Nucleotide sequences of the primers used in PCR for the generation of mutant receptor subunits, denoted 5' to 3'. Mutant subunits are either point mutants or, if denoted by a subunit followed by a subunit area in brackets, chimeric subunits. Residue numbering is based upon the mature murine protein, or as previously defined in the literature.

2.2. HEK cell culture and electrophysiology

2.2.1. Cell culture and transfection

HEK 293 cells were used for heterologous expression studies of recombinant $GABA_A$ receptors (Thomas & Smart, 2005). HEK cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v fetal calf serum, 100 units/ml penicillin-G and 100 µg/ml streptomycin and incubated at 37˚C in humidified 95% air/5% CO_2. All components were obtained from Life Technologies. At 80% confluency, HEK cells were washed with Hank's Balanced Salt Solution (HBSS) and dissociated using 0.05% (w/v) trypsin-EDTA for 1-2 mins, before the addition of DMEM and centrifugation (1000 x g, 2 mins) followed by resuspension in fresh DMEM.

Dissociated HEK cells were plated onto 22 mm glass coverslips (VWR) coated in poly-L-lysine (Sigma). Cells were transfected > 1 hr later using a calcium phosphate protocol whereby a transfection solution was prepared consisting of 20 µl of 340 mM $CaCl_2$, 24 µl of 2x HBS (50 mM HEPES, 280 mM NaCl, 2.8 mM Na_2HPO_4) and 4 µg cDNA per coverslip. All cells were transfected with equal amounts of $GABA_A$ receptor subunit cDNA, and with eGFP cDNA for transfections other than those using δ cDNA, which included a super-ecliptic pHluorin at its N-terminus (Patel et al., 2014). Mouse α, β and γ subunit cDNA, and rat δ subunit cDNA were used. Cells were recorded from 24-48 hrs later.

2.2.2. Voltage-clamp protocols

For all electrophysiological experiments, cells were visualised using a Nikon Eclipse E600FN microscope. Whole cell currents were recorded using an Axopatch 200B amplifier and digitised using a Digidata 1322A (Axon Instruments). Currents were acquired with Clampex ver. 10.2 and analysed with Clampfit ver. 10.7 (Axon Instruments) on a Dell Optiplex 960M computer. Recordings were sampled at 20 kHz and Bessel filtered at 2 kHz (80

dB/Decade). All recordings were performed at room temperature (20˚C). Input resistance, series resistance and membrane capacitance were calculated from the current response to a 10 mV hyperpolarising voltage step. Series resistance compensation was ~70%, and cells were rejected if series resistance was too high (> 20 MΩ) and if the leak current was greater than 250 pA. For prolonged recordings, cells were rejected if the series resistance changed > 20% over the recording time.

HEK cells were recorded from using thin-walled borosilicate glass electrodes (World Precision Instruments, TW150-4) with resistances ~3-5 MΩ when filled with an internal solution containing, in mM: 1 $MgCl_2$, 140 KCl, 11 EGTA, 10 HEPES, 1 $CaCl_2$, 2 K_2ATP, pH 7.2. Osmolarity was 300 ± 20 mOsm/L, measured using a vapour pressure osmometer (Model 5520, Wescor Inc). Cells were superfused with, and drugs suspended in, a modified Kreb's solution containing, in mM: 140 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 11 glucose, 5 HEPES, pH 7.4. Cells were voltage-clamped at -40 mV, unless stated otherwise.

Drugs were applied using a fast-application Y-tube (Mortensen & Smart, 2007). Kreb's solution was constantly superfused at a slow rate onto the cells through the Y-tube, with drug solution flowing into waste under vacuum pressure. During drug application, the solenoid controlling the drug flow closed, removing the vacuum pressure and allowing the solution to rapidly exit into the bath (10-90% current rise time of GABA acting upon a variety of $GABA_A$ receptors was consistently 30-70 ms).

2.2.3. Quantification of spontaneous activity

Spontaneous activity in transfected HEK cells was quantified by applying a brief saturating concentration of GABA (500 µM) to elicit a maximal whole cell current ($I_{GABA\text{-}MAX}$). Once the current had returned to baseline, a separate saturating concentration of PTX (100 µM) was then applied and the current recorded (I_{PTX}). Spontaneous currents were quantified as the basal

spontaneous current as a percentage of total current in response to a saturating concentration of agonist (I_{spont}, %), as described by equation 1:

Equation 1:

$$I_{spont}\ (\%) = \left[\frac{I_{PTX}}{I_{PTX} + I_{GABA\text{-}MAX}}\right] \times 100$$

Example recordings are shown in Fig. 3.1.

Due to the inability of homomeric $GABA_A$ receptors to bind GABA, experiments involving homomeric receptors were not normalised to the peak current in response to saturating concentrations of agonist, and instead the I_{PTX} was normalised to cell capacitance.

2.2.4. Concentration-response curves and fitting

Increasing concentrations of GABA were applied to HEK cells transiently expressing $GABA_A$ receptors to generate GABAergic currents. These data were plotted and fitted using a non-linear least-squares method with the Hill equation, expressed in equation 2:

Equation 2:

$$y = y_{max}\left[\frac{A^n}{EC_{50}{}^n + A^n}\right]$$

Where A, y_{max}, EC_{50} and n represent the GABA concentration, the maximum GABA current, the GABA EC_{50}, and the Hill coefficient, respectively. The data

was then normalised to the fitted y_{max}. Curves were of agonist-activated currents only and did not account for any holding spontaneous current.

2.2.5. Calculation of desensitisation kinetics

A saturating concentration of GABA (1 mM) was applied to HEK cells expressing receptor variants for 30 s. A two-term exponential curve was fitted to the current decay using the Levenberg–Marquardt algorithm (increasing the number of terms did not significantly decrease the sum of squared residuals). From this fit, the fast and slow phases of desensitisation were estimated. These were then used to calculate a weighted tau (τ_w) using equation 3:

Equation 3:

$$\tau_w = \frac{(A1 \times \tau1) + (A2 \times \tau2)}{A1 + A2}$$

Where A1 and A2 represent the amplitude of the fast and slow components, and $\tau1$ and $\tau2$ represent the time constants of the fast and slow components.

The extent of desensitisation was calculated as the steady-state current expressed as a percentage of maximum GABA-activated current using equation 4:

Equation 4:

$$\text{Desensitisation (\%)} = \left[1 - \frac{I_{SS}}{I_{GABA\text{-}MAX}}\right] \times 100$$

Where the I_{SS} is the amplitude of the steady-state current occurring at maximum desensitisation, and $I_{GABA\text{-}MAX}$ is the peak response to saturating 1 mM GABA.

2.3. Primary hippocampal culture and electrophysiology

2.3.1. Cell culture and transfection

Rat hippocampal cultures were prepared from E18 Sprague-Dawley embryos as previously described (Hannan et al., 2011). Dissociated cells were plated onto 18 mm glass coverslips coated in poly-L-ornithine (Sigma) in a plating medium consisting of minimum essential media supplemented with 5% v/v heat-inactivated fetal calf serum, 5% v/v heat-inactivated horse serum, 100 units/ml penicillin-G, 100 µg/ml streptomycin, 2 mM L-glutamine and 20 mM glucose; and incubated at 37˚C in humidified 95% air/5% CO_2. This media was replaced 2 hours later with a maintenance media consisting of neurobasal-A media supplemented with 1% v/v B-27, 100 units/ml penicillin-G, 100 µg/ml streptomycin, 0.5% v/v glutamax and 35 mM glucose.

These cells were transfected 6-8 days later using calcium phosphate or effectene protocols. For calcium phosphate transfection, a transfection solution consisting of 60 µl 2x HEPES-buffered saline (HBS; 274 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 15 mM D-glucose, 42 mM HEPES), 2.5 µl of 2.5 M $CaCl_2$, 4 µg cDNA and made up to 120 µl with TE buffer was incubated at room temperature for 30 mins, being vortexed every 5 mins. Neurons were washed with HBSS and the media replaced with neurobasal-A supplemented with 2 mM kynurenic acid. 60 µl of the transfection solution was added dropwise to each coverslip, and the neurons were left for 30 mins before cells were washed and the media replaced with fresh maintenance media. For effectene transfection, per coverslip, a transfection solution was made using a total of 0.8 µg of cDNA, 6.4 µl enhancer and 100 µl buffer and left for 5 mins. 10 µl of effectene was then added and the solution left for a further 10 mins, before mixing the solution in 1 ml of maintenance media. The neurons were washed with HBSS and the media replaced with the transfection solution. 2 hrs later the neurons were washed with HBSS and the media replaced with maintenance media. Neurons were transfected with equal amounts of subunit cDNA and eGFP. Neurons were used for electrophysiological recording or confocal imaging 5-9 days later.

2.3.2. Voltage-clamp protocols

Neurons were recorded from using thin-walled borosilicate glass electrodes (World Precision Instruments, TW150-4) with resistances ~3-5 MΩ when filled with an internal solution containing, in mM: 140 CsCl, 2 NaCl, 10 HEPES, 5 EGTA, 2 $MgCl_2$, 0.5 $CaCl_2$, 2 Na_2ATP, 0.5 Na_2GTP, 2 QX-314, pH 7.3. Osmolarity was 300 ± 20 mOsm/L.

Cells were superfused with a modified Kreb's solution containing, in mM: 140 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 11 glucose, 5 HEPES, 2 kynurenic acid, pH 7.4. Neurons were voltage-clamped at -60 mV. Drugs were applied through a Y-tube as described in Section 2.2.2.

2.3.3. Calculating sIPSC frequency and amplitude

Neurons were held at -60 mV for 5-10 mins and spontaneous IPSCs (sIPSCs) were recorded. Data were imported into WinEDR (John Dempster, University of Strathclyde) for sIPSC analysis. A current deviation of -8 pA that was maintained for at least 1 ms was set as the detection threshold for events. The average rate and amplitude of the detected events was then calculated.

2.3.4. Current-clamp protocols

Neurons were recorded from using thin-walled borosilicate glass electrodes (World Precision Instruments, TW150-4) with resistances ~3-5 MΩ when filled with an internal solution consisting of, in mM: 137 potassium gluconate, 3 KCl, 10 HEPES, 5 EGTA, 0.5 $CaCl_2$, 2 $MgCl_2$, 2 Na_2ATP, and 0.5 Na_2GTP, pH 7.3. Osmolarity was 300 ± 20 mOsm/L. Cells were superfused with a modified Kreb's solution containing, in mM: 140 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 11 glucose, 5 HEPES, 2 kynurenic acid, pH 7.4. The estimated junction potential was 15 mV. Constant current was manually injected to maintain cells at -70 mV, if required, for appropriate comparison

between neurons. Current steps were injected in 20 pA increments for 300 ms, increasing from -100 to 500 pA, with a 700 ms recovery time between steps. Action potentials were manually counted for each current step until events became unresolvable.

2.4. Brain slice electrophysiology

2.4.1. Slice preparation

Adolescent male rats (P21 to P28) were anaesthetised by inhalation with 5% isoflurane before decapitation in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986. The brain was rapidly isolated and immersed in an ice-cold slicing solution containing, in mM: 85 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 75 sucrose, 1 $CaCl_2$, 4 $MgCl_2$, 25 glucose and 2 kynurenic acid, (pH 7.4 when bubbled with 95% O_2/5% CO_2). Horizontal (hippocampal) and coronal (thalamic) sections (all 350 µm thick) were cut using a Leica VT1200S vibratome before transfer to a holding chamber at 37˚C. The solution was slowly exchanged over 1 hr with aCSF containing, in mM: 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$, 25 glucose and 2 kynurenic acid (pH 7.4 when bubbled with 95% O_2/5% CO_2) before maintaining the slices at room temperature prior to recording.

2.4.2. Voltage-clamp protocols

Neurons were recorded from using thin-walled borosilicate glass electrodes (World Precision Instruments, TW150-4) with resistances ~3-5 MΩ when filled with an internal solution consisting of, in mM: 140 CsCl, 2 NaCl, 10 HEPES, 5 EGTA, 2 $MgCl_2$, 0.5 $CaCl_2$, 2 Na_2ATP, 0.5 Na_2GTP, 2 QX-314, pH 7.3. Osmolarity was 300 ± 20 mOsm/L. Slices were superfused with the aCSF described above, and drugs were bath-applied. Neurons were voltage-clamped at -60 mV.

2.5. Pharmacological tools

A list of pharmacological compounds used is shown in Table 2.3.

Compound	Source	ID
GABA	Sigma	A5835
PTX*	Sigma	P1675
THIP	Sigma	T101
Gabazine	Tocris	1262
Staurosporine*	Cell guidance system	SM97-1
PKI*	Calbiochem	476485
8-Br cAMP	Cayman	14431
Bisindolylmaleimide I*	Cayman	21180
KT 5823*	Cambridge Bioscience	CAY10010965
KN-62*	Generon	BS0104
THDOC*	Sigma	P2016
Allopregnanolone*	Sigma	P8887
Pregnenolone sulfate*	Sigma	P162
Propofol*	Sigma	W505102
Flurazepam	Cerilliant	F-003
Concanamycin A*	Insight Biotechnology	C685080
Kynurenic acid	Sigma	K3375
QX-314	Tocris	1014

Table 2.3: Pharmacological tools and their origin. Drugs used during experimentation are shown, with origin and identifier. Compounds dissolved and stored in DMSO vehicle are denoted by *. For all drugs, DMSO volume in final solution did not exceed 0.2% v/v, and solvent controls had no effect on results.

2.6. shRNA and immunocytochemistry

For short hairpin RNA (shRNA)-mediated knockdown (silencing) of native β3 subunits in hippocampal neuronal cultures, we used cotransfection of two pGIPZ-based constructs (Open Biosystems V2LMM_62992: TTTAAGAAATATGTGTCGG and V2LMM_65176: TTCATTGTGAACATCCATC) that selectively target rat β3. Control neurons were transfected with a pGIPZ construct containing a scrambled shRNA sequence (TCTCGCTTGGGCGAGAGTAAG). pGIPZ constructs also encoded for GFP to allow transfected cell identification. A basic schematic is shown in Fig. 2.1.

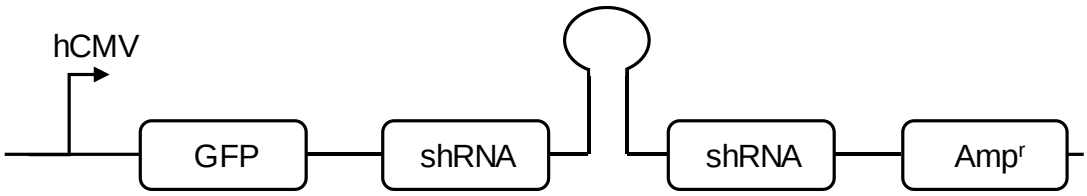

Figure 2.1: Schematic representation of the shRNA plasmid. The shRNA sequence is depicted with the 'hairpin' of the transcription product. Both the GFP and shRNA sequences are under the control of the human cytomegalovirus (hCMV) promoter. Amp^r represents the ampicillin-resistance gene.

Seven days after transfection with shRNA constructs, neurons were washed with ice-cold PBS and fixed with paraformaldehyde (4% PFA/ 4% sucrose/ PBS pH 7) for 5 mins before extensive washing in PBS. Cells were then permeabilised using Triton X-100 (0.1% in PBS) for 5 mins and then washed in PBS. Cells were incubated in a blocking solution (10% goat serum in PBS) for 30 mins before brief washing and subsequent incubation for 1 hr in 3% goat serum with a mouse anti-β3 primary antibody (NeuroMab, 1:500). Cells were then washed and incubated with a goat anti-mouse secondary

antibody conjugated to Alexa Fluor 555 (1:500) for 1 hr. After washing, coverslips were mounted on slides with Prolong Glass mounting medium (ThermoFisher Scientific) for confocal imaging.

2.7. Generation of myc-tagged β3 subunits and immunocytochemistry

The mutant $\beta3^{L170R}$ and $\beta3^{T185I}$ subunits were generated through site-directed mutagenesis, as described in Section 2.1.1. For analysis of their surface expression levels in transfected HEK cells, a codon sequence encoding a myc-tag (EQKLISEEDL) was inserted into the mutant plasmids at the 5' end of the subunit cDNA, corresponding to the amino-terminal domain of the translated subunit. The $\beta3^{myc}$ subunit was available in the lab, and restriction enzyme digest was used to insert the tag into the mutant subunit cDNAs. The restriction enzymes AccI and BstBI (NEB) were used to generate two fragments of each of $\beta3^{myc}$, $\beta3^{L170R}$ and $\beta3^{T185I}$ cDNAs: a small fragment containing the mutant site and a larger backbone fragment containing the tag site (depicted in Fig. 2.2). Solution for the digestion of plasmids: cDNA (2 µg), restriction buffer (2 µl), 10% BSA (2 µl), AccI (1 µl), BstBI (1 µl), made up to 20 µl with water. The solution was incubated at 37˚C for 2 hrs. The generation of fragments was confirmed by gel electrophoresis (Section 2.1.1). The band corresponding to the large myc-containing backbone and the smaller bands corresponding to the $\beta3^{L170R}$ and $\beta3^{T185I}$ fragments were extracted. The large backbone was mixed with the smaller mutant-containing fragments in a roughly 1:3 ratio (4 and 13 µl, respectively), and ligated using T4 ligase buffer (2 µl) and T4 ligase (1 µl). This solution was kept at 4˚C overnight, before transformation of *E. coli* cells the following day and subsequent DNA extraction and sequencing (Section 2.1.1.).

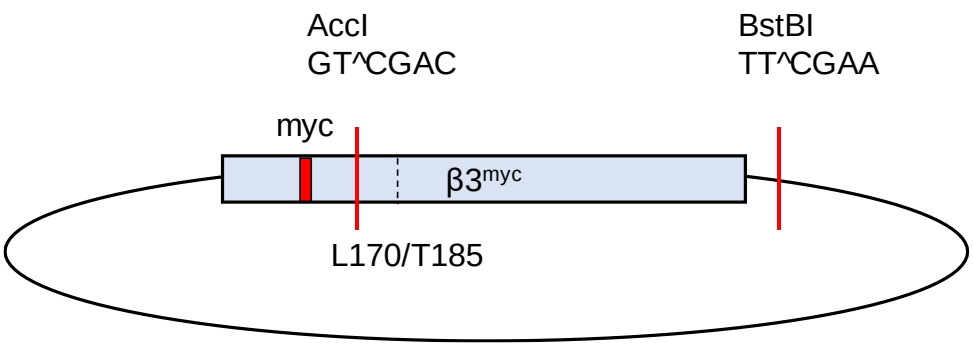

Figure 2.2: Schematic representation of the β3^{myc} plasmid and the location of cleavage by restriction enzymes. The β3^{myc} cDNA is shown in light blue as part of the plasmid. The myc tag is depicted in solid red, and the site of the L170/T185 residue is shown as a dashed line. The cleavage sites of AccI and BstBI are represented by the solid red lines and their nucleotide sequence is also depicted. The actions of these two restriction enzymes generated two fragments: a smaller fragment containing the L170/T185 site, and a larger fragment containing the myc nucleotide sequence with the rest of the plasmid.

Once tagged mutant subunit cDNAs had been generated, these were transfected into HEK cells, along with α4 and δ subunit cDNAs, in order to examine the surface expression of these receptors. eGFP cDNA was also transfected. After 24-48 hours, the HEK cells were washed with ice-cold PBS and fixed with paraformaldehyde (4% PFA/ 4% sucrose/ PBS pH 7) for 5 mins before extensive washing in PBS. Cells were incubated in a blocking solution (10% Goat serum in PBS) for 30 mins before brief washing and subsequent incubation for 1 hour in 3% goat serum with a mouse anti-myc primary antibody (Abcam, 1:500). Cells were then washed and incubated with a goat anti-mouse secondary antibody conjugated to Alexa Fluor 555 (1:500) for 1 hr. After washing, coverslips were mounted on slides with Prolong Glass mounting medium (ThermoFisher Scientific) for confocal imaging.

2.8. Confocal microscopy

Cells from at least three different preparations were identified through expression of GFP and were imaged using a Zeiss 510 confocal microscope with a 40x oil objective (1.4 NA). Images were digitally captured using Zeiss LSM software with excitation at 488 nm for GFP and 555 nm for Alexa Fluor 555. 8-bit images were generated with 1024x1024 pixels and analysed using ImageJ (ver. 1.52p).

Neurons were imaged for Alexa Fluor 555-conjugated antibodies labelling native β3 subunits (Section 2.6). For each neuron, regions of interest (ROI) were manually defined around 2-4 proximal dendrites (< 50 μm from the soma). For each dendritic ROI, the mean background-subtracted fluorescence intensity was determined. The fluorescence intensity of all ROIs for a single neuron were then averaged, which was then normalised to the average fluorescence intensity of all control neurons for that preparation.

HEK cells were imaged for surface expression of myc-tagged β3 subunits (Section 2.7). The ROI was manually defined around the membrane of transfected cells, demarcated by eGFP fluorescence. The mean background-subtracted fluorescence intensity of each ROI was determined and normalised to the average fluorescence intensity of all control cells for that preparation.

2.9. Generation of homology models

This was performed by Dr M. Mortensen in the lab. The homology model of the murine α4β3δ $GABA_A$ receptor was based on the cryo-EM structure of human α1β3γ2L receptors (Laverty et al., 2019) (pdb: 6I53). Complementary mature amino acid sequences were first aligned using ClustalW. Modeller (ver. 9.19) was then used to generate 50 different models of murine α4β3δ, using human α1β3γ2L (6I53) as the structural template. The model quality estimation webtool for membrane proteins, QMEANBrane (http://swissmodel.expasy.org/qmean/), was used to rank all 50 models to

obtain the most appropriate structures, which were subsequently processed in SCRWL4 (Krivov et al., 2009) to optimise side-chain configurations. Further model optimization was performed using MOLProbity (http://molprobity.biochem.duke.edu/) where Asn/Gln/His residues were flipped if clear evidence was provided for protonation. The α4β3δ $GABA_A$ receptor homology model was then structure minimized in Chimera, before final refinement using MOLProbity where protein geometry scores showed successful continued model optimization leading to the final homology model. All structural images were visualized and rendered using the molecular graphics systems PyMOL (Schrodinger) and UCSF Chimera.

2.10. Statistical analysis

Data are represented as the mean ± standard error of the mean (SEM). Individual data points are included within figures where appropriate. Data were analysed and statistical tests performed using Origin 2019 (OriginLab). All datasets were tested for normality using the Kolmogorov-Smirnov test with Lilliefors correction. If data were normally distributed, unpaired t-tests, paired t-tests and ANOVAs with Tukey's post hoc tests were used as indicated. For non-Gaussian distributed data, Mann-Whitney, paired samples Wilcoxon and Kruskal-Wallis with Dunn's post hoc tests were used. A p-value of < 0.05 was set as the level of significance and denoted by *. When $p < 0.01$ or $p < 0.001$, this is represented by ** and *** respectively.

Chapter 3: Structural determinants of spontaneous activity

3.1. Introduction

A significant number of recombinant $GABA_A$ receptors of diverse subunit compositions have been expressed and their spontaneous activity described (Khrestchatisky et al., 1989; Wooltorton et al., 1997; Neelands et al., 1999; Tang et al., 2010). From these studies, there appears to be very little pattern to the subunit identity of those receptors displaying spontaneous currents. Homomeric receptors formed by β subunits show spontaneous activity, although this was inconsistent between studies, likely due to difficulties in expressing functional homomeric receptors, as well as differences in the expression system used (Sigel et al., 1989; Cestari et al., 1996; Krishek et al., 1996b; Wooltorton et al., 1997). The β1, but not β3, homomeric receptor has been described as spontaneous (Sigel et al., 1989; Miko et al., 2004), although in different studies the β3 homomer *has* been shown to be spontaneously active (Cestari et al., 1996; Wooltorton et al., 1997). The β2 subunit has also been proposed to form homomeric receptors with some spontaneous activity (Boileau et al., 2002), but studies of the assembly of homomeric and heteromeric receptors showed retention in the ER of the β2 subunit when expressed alone (Taylor et al., 1999). The key determinant permitting ER exit and homomer formation was identified as a four amino acid motif (GKER) in the extracellular domain (ECD) present on the β3 subunit, but not β2 (Taylor et al., 1999). Diheteromeric receptors, present in extrasynaptic areas (Mortensen & Smart, 2006), have also been identified as spontaneously active, with a dependence on the identity of the α subunit. The α4β1 receptor has shown spontaneous activity, but not the α1β1 receptor (Khrestchatisky et al., 1989). Finally, triheteromeric receptors displaying spontaneous activity have also been identified, with α4β3δ and α4β3γ2L both showing significant levels of spontaneous activity (Tang et al., 2010).

The generation of mutant $GABA_A$ receptor subunits has been used to investigate binding and gating properties of the receptor, with many of these subunit changes resulting in spontaneous activity (Boileau et al., 2002; Scheller & Forman, 2002; Patel et al., 2014). The $\beta2^{L99C}$ mutant has been reported to form spontaneously gating homomeric receptors (Boileau et al., 2002). This mutation lies in the GABA-binding site (loop A, Fig. 1.2c), and so residues directly at, or surrounding, ligand-binding interfaces may impact on spontaneously gating properties in wild-type receptors. Mutations in the pore-lining M2 transmembrane region also affect spontaneous activity (Miko et al., 2004). The mutation of the 9' leucine residue in the second transmembrane domain of α4, β3, γ2L and δ subunits, present at the narrowest area of the closed pore and constituting the activation gate, changes the kinetics of receptor desensitisation and gating, but also introduces significant levels of spontaneous activity (Bianchi & Macdonald, 2001; Miller & Aricescu, 2014; Patel et al., 2014; Masiulis et al., 2019). Mutations in M1 and M3 of the β1 subunit (P228H and L285R respectively) have also been shown to increase spontaneous activity, and to drive alcohol-seeking behaviour in rodents through influences on tonic inhibition in the nucleus accumbens (Anstee et al., 2013). Spontaneous activity in wild-type receptors could therefore depend on residues at ligand-binding interfaces, areas involved in the transduction of ligand binding to channel opening, or other areas which influence gating (Boileau et al., 2002; Scheller & Forman, 2002). Diversity in residues at these possibly critical sites between subunits could result in the identification of wild-type receptor compositions which display significantly more or less spontaneous activity, important to consider when examining receptor distribution in the brain.

To investigate the role that subunit composition plays in determining the spontaneous activity of $GABA_A$ receptors, a variety of recombinant receptors were expressed in HEK 293 cells and their spontaneous activity measured. This was calculated by the fast application of a saturating concentration of GABA to elicit a maximal $GABA_A$ receptor response, and subsequent application of the non-competitive antagonist PTX to block spontaneously open receptors (Patel et al., 2014). Once we had assayed the spontaneous

activity of the most common $GABA_A$ receptors, more in-depth analysis was used to identify key areas on individual subunits which underlie the observed spontaneous activity. We also examined the link between spontaneous activity and the apparent affinity of the receptor for GABA, and propose a mechanism through which spontaneous activity occurs in a subset of $GABA_A$ receptors.

3.2. Results

3.2.1. The subunit composition of receptors is a key determinant of spontaneous gating

We first screened a variety of the most common isoforms of the $GABA_A$ receptor for spontaneous activity by recording from HEK cells transiently expressing synaptic-type receptors (α1β2/3γ2L), extrasynaptic-type receptors (α4/6β2/3δ), and those localised both synaptically and extrasynaptically (α5β2/3γ2L) (Mody, 2001; Glykys et al., 2008; Olsen & Sieghart, 2009).

Spontaneous activity was quantified by calculating the amplitude of the basal holding current as a percentage of the maximal response to a saturating concentration of agonist, as described in the Methods (Section 2.2.3). Briefly, separate applications of saturating concentrations of GABA (500 µM) and PTX (100 µM) provided GABA-activated ($I_{GABA\text{-}MAX}$) and picrotoxin-sensitive (I_{PTX}) currents, respectively (Fig. 3.1). PTX is a channel blocker and allosteric antagonist of $GABA_A$ receptors, and so is able to effectively antagonise constitutively active receptors and block spontaneous currents (Smart & Constanti, 1986; Newland & Cull-Candy, 1992; Tang et al., 2010; Masiulis et al., 2019). Both $I_{GABA\text{-}MAX}$ and I_{PTX} were then used to calculate the spontaneous current (I_{spont}) expressed as the percentage of total (GABA-activated + spontaneous) $GABA_A$ receptor current. An example recording is provided in Fig. 3.1.

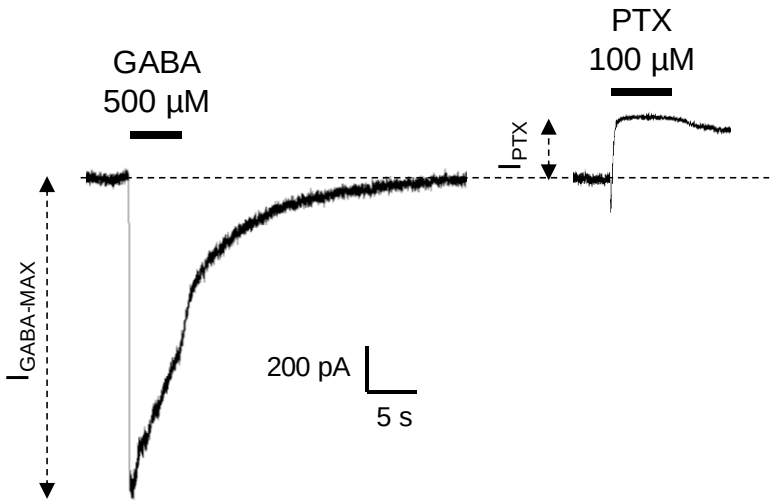

Figure 3.1: Calculating the percentage of receptors spontaneously active. A saturating concentration of GABA (500 µM) was applied to HEK cells expressing α4β3δ $GABA_A$ receptors to generate a maximal current ($I_{GABA-MAX}$). When the current had returned to a basal level following washout of GABA, a saturating concentration of the use-dependent antagonist PTX (100 µM) was applied, revealing the amplitude of spontaneous activity (I_{PTX}). These values were used to calculate I_{spont}, the spontaneous current expressed as a percentage of maximum agonist-activated current, as described in the Methods.

The results of these experiments are shown in Fig. 3.2a. From the eight different $GABA_A$ receptor constructs analysed, only those receptors containing the β3 subunit displayed a significant spontaneous current (α1β3γ2L: 1.5 ± 0.33%, n = 6; α5β3γ2L: 4.0 ± 1.1%, n = 9; α4β3δ: 19.1 ± 2.1%, n = 27; α6β3δ: 15.4 ± 3.6%, n = 21). Switching β3 for β2 subunits was sufficient to eliminate all spontaneous gating, independent of the identities of co-assembled subunits (α1β2γ2L: 0.13 ± 0.04%, n = 7; α5β2γ2L: 0.27 ± 0.15%, n = 7; α4β2δ: 0.02 ± 0.02%, n = 6; α6β2δ: 0.17 ± 0.13%, n = 7). This loss of spontaneous activity was also observed when expressing α4β1δ receptors (0.16 ± 0.06%, n = 8; Fig. 3.2b,c).

In addition to this differentiation between β1/2- and β3-containing receptors, δ-containing receptors (with co-assembled β3) showed significantly higher levels of spontaneous current than their γ-containing counterparts (Fig.

3.2a). These extrasynaptic δ-$GABA_A$ receptors (α4β3δ and α6β3δ) are considered critical for the generation of tonic currents in various brain areas (Brickley et al., 2001; Glykys et al., 2008; Lee & Maguire, 2014) and might be expected to show more constitutive activity than synaptic receptors. GABA acts as a partial agonist at δ-containing receptors, but a full agonist at γ-containing receptors (Brown et al., 2002; Storustovu & Ebert, 2006; Mortensen et al., 2010); possibly resulting in an over-estimate of the level of spontaneous activity. To investigate this, and to allow a more appropriate comparison between γ- and δ-containing receptors, the experiments were repeated using saturating concentrations of THIP (3 mM, Fig. 3.2d); classed as a super-agonist at δ-containing receptors (Brown et al., 2002; Storustovu & Ebert, 2006; Wafford et al., 2009; Mortensen et al., 2010). The estimate of I_{spont} of α4β3δ receptors using THIP was slightly, but significantly, smaller than when GABA was used (12.2 ± 2.9% and 16.6 ± 3.1%, respectively; n = 8). There was no difference between the calculated I_{spont} for the α1β3γ2L receptor using either THIP or GABA (2.7 ± 1.3% and 2.0 ± 0.8%, respectively; n = 6). Comparing the calculated I_{spont} using THIP for α4β3δ and GABA for α1β3γ2L receptors showed the former still maintained a significantly larger spontaneous profile, therefore not altering the conclusion that the extrasynaptic α4β3δ receptor is significantly more spontaneous than the synaptic α1β3γ2L receptor. Tonic inhibition in the brain, mediated by these extrasynaptic receptors, may therefore be provided through a combination of GABA-mediated and GABA-independent activity (Bright et al., 2007; Brickley & Mody, 2012; Bright & Smart, 2013a; Wlodarczyk et al., 2013).

Having established the importance of β3 subunits for spontaneous activity of αβγ and αβδ receptors, it was also necessary to ascertain whether other subunits in the pentameric complex performed similar roles. Our initial screen of recombinant receptors suggested that incorporation of the δ subunit increased the level of spontaneous activity, and that the identity of the α subunit in the receptor may also affect agonist-independent gating (Fig. 3.2a). To investigate, we quantified spontaneous currents from cells expressing α1β3δ and α4β3γ2L receptors (Fig. 3.2e). In comparison to α4β3δ, these two receptors exhibited negligible spontaneous activity (α4β3δ: 19.1 ± 2.1%, n =

27; α1β3δ: 0.49 ± 0.08%, n = 7; α4β3γ2L: 0.67 ± 0.11%, n = 10); indicating that incorporation of both α4 (or α6) and δ in conjunction with β3 is necessary to facilitate agonist-independent receptor gating. Expression of recombinant α4β3γ2L has been previously demonstrated to show high levels of spontaneous activity (Tang et al., 2010). To confirm the results that α4β3γ2 receptors do not show significant levels of spontaneous activity under our experimental conditions, the short isoform of the γ2 subunit (γ2S) was expressed as α4β3γ2S receptors; but again these showed very little spontaneous activity (0.35 ± 0.06%, n = 9; Fig. 3.2e).

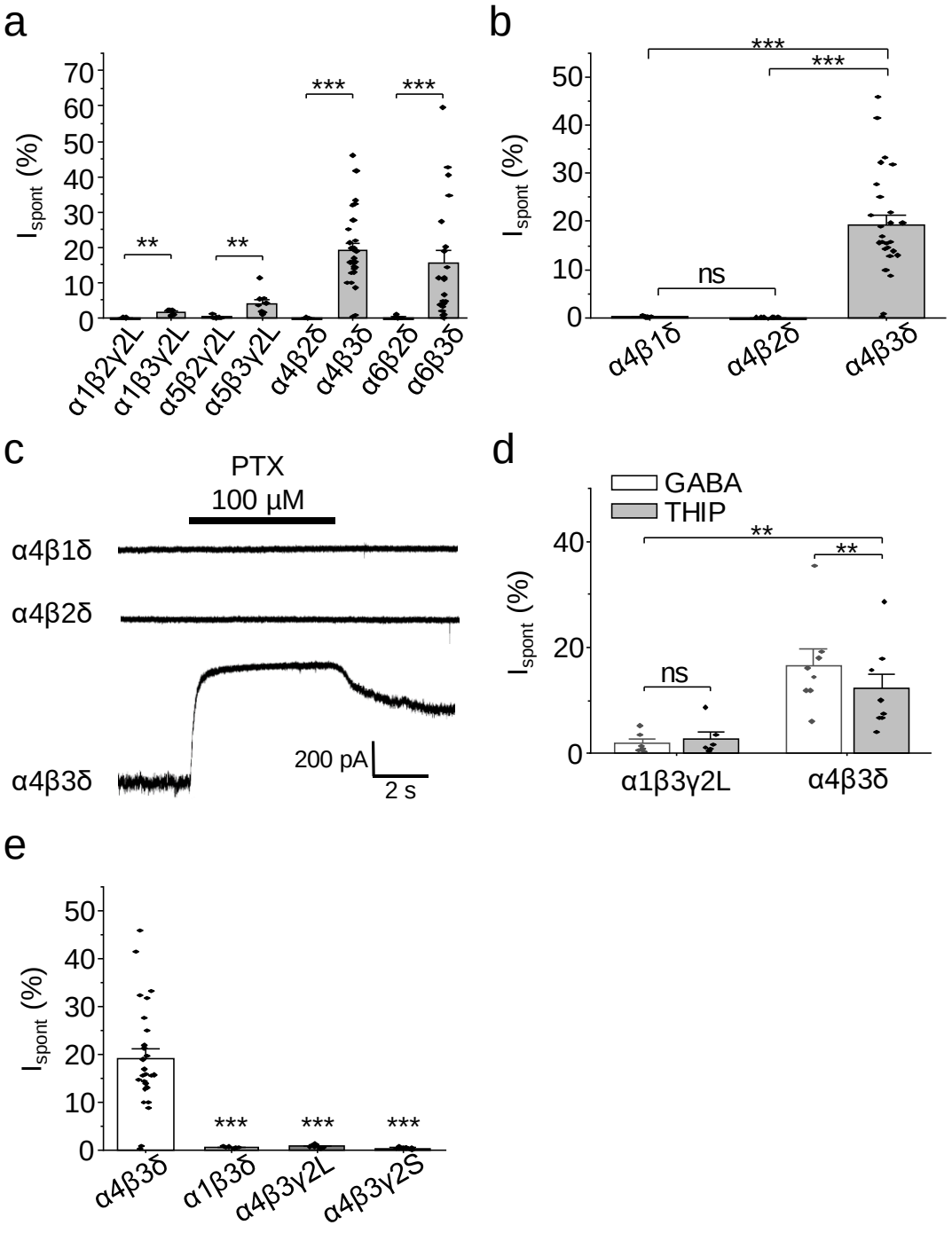

Figure 3.2: The spontaneous activity of wild-type $GABA_A$ receptors is dictated by subunit composition. a) Spontaneous activity of a variety of wild-type synaptic- and extrasynaptic-type $GABA_A$ receptors expressed in HEK cells (n = 6-27; unpaired t-test and Mann-Whitney test between groups). b) Calculation of the I_{spont} of α4βδ receptors composed of different β subunits (n = 6-27; Kruskal-Wallis with Dunn's post hoc test). c) Representative recordings of α4βδ receptors during PTX application. d) Comparison of estimated I_{spont} values of α1β3γ2L and α4β3δ receptors using the agonists GABA and THIP (n = 6; paired t-test within groups, unpaired t-test between groups). e) Comparison of I_{spont} values for the extrasynaptic-type α4β3δ receptor and receptors where a single subunit was exchanged for a synaptic-type subunit (n = 7-27; ANOVA with Tukey's post hoc test). For all graphs, columns and error bars represent the mean and SEM, respectively (*p<0.05; **p<0.01; ***p<0.001; ns: no significance).

These data provide evidence of the importance of β3 subunits in allowing $GABA_A$ receptors to constitutively gate. However, unlike most other subunits, β3 can form homomeric receptors and these too are spontaneously active (Cestari et al., 1996; Wooltorton et al., 1997). To investigate whether β3 homomers were contributing to the spontaneous current, we performed several control experiments. I_{spont} was not calculated during experiments involving homomeric receptors as GABA is unable to activate them. Instead, I_{PTX} was normalised to cell capacitance. When expressing β3 subunits alone or as α4β3 diheteromers, the spontaneous current density was significantly lower compared to those observed with α4β3δ triheteromers (α4β3δ: 7.1 ± 0.6 pA/pF, n = 25; α4β3: 0.58 ± 0.14 pA/pF, n = 7; β3: 0.96 ± 0.33 pA/pF, n = 11; Fig. 3.3a). Furthermore, we also noted that the competitive $GABA_A$ receptor antagonist gabazine (1 μM) behaved as a negative allosteric modulator at triheteromeric receptors, albeit less effective than PTX ($I_{gabazine}$ was 33% the size of I_{PTX}), but was ineffective at blocking spontaneous currents of β3 homomers due to the lack of an orthosteric GABA binding site (Fig. 3.3b) (Ueno et al., 1997; Wooltorton et al., 1997). Together, these results suggest that the spontaneous currents observed following transient transfection with α4, β3 and δ cDNAs predominantly represent constitutively active αβδ triheteromeric receptors.

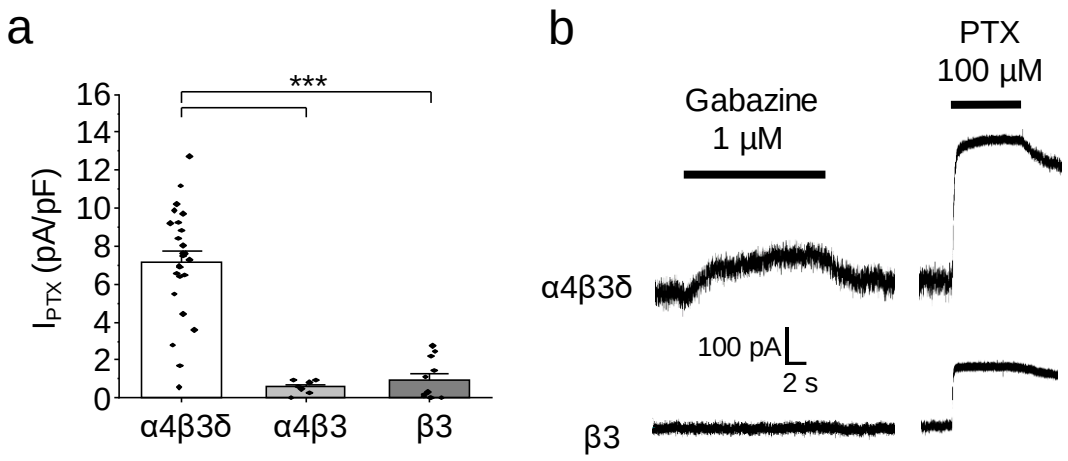

Figure 3.3: Spontaneous currents recorded from HEK cells expressing three $GABA_A$ receptor subunits represent spontaneous activity of triheteromeric receptors. a) The spontaneous current density, as determined from PTX (100 μM) application, of HEK cells expressing triheteromeric (n = 25), diheteromeric (n = 7), or homomeric (n = 11) receptors. Columns and error bars represent the mean and SEM, respectively (Kruskal-Wallis with Dunn's post hoc test; ***p<0.001). b) The orthosteric antagonist gabazine (1 μM) had negative allosteric effects at triheteromeric, but not homomeric, receptors; providing further evidence that spontaneous activity is predominantly mediated by triheteromeric receptors in these cells.

3.2.2. Structural motifs within the β3 subunit are essential for spontaneous activity

Key differences in structure must exist between β2 and β3 subunits to explain the clear requirement for β3, but not β2, to produce spontaneously active $GABA_A$ receptors. To identify the structural domains involved, we created $GABA_A$ receptor domain-chimeras; initially exchanging the entire ECD up to the first transmembrane (M1) domain between β2 and β3 subunits (Fig. 3.4a). This ECD exchange completely switched the spontaneously active properties of these receptors (Fig. 3.4a). Replacement of the β2 ECD with that of β3 in the $α4β2^{β3(ECD)}δ$ receptor gave rise to similar levels of spontaneous activity as wild-type α4β3δ receptors (22.7 ± 4.6%, n = 9; and 27.5 ± 2.8%, n = 14, respectively) whilst the converse exchange resulted in $α4β3^{β2(ECD)}δ$ receptors that showed negligible spontaneous currents, in accordance with the behaviour of wild-type α4β2δ receptors (0.41 ± 0.18%, n = 8; and 0.36 ± 0.17%, n = 7, respectively).

The next aim was to identify sub-areas of the ECD which differ between β2 and β3 subunits that could cause such a discrepancy in their ability to initiate receptor spontaneity. One clear difference in subunit properties is the ability of β3, but not β2, to form functional homomers at the cell surface (Taylor et al., 1999). This homomeric assembly has previously been attributed to four residues in the ECD of β3 subunits: Gly171, Lys173, Glu179, and Arg180 – the GKER motif (Taylor et al., 1999). Exchanging this GKER motif with the equivalent residues in β2: Asp170, Asn172, Thr178 and Lys179 – the DNTK motif, permitted homomeric β2 expression ($\beta2^{GKER}$), whilst the converse exchange ablated homo-oligomerisation of β3 subunits ($\beta3^{DNTK}$) (Taylor et al., 1999). In addition to this assembly role, we postulated that the GKER motif might also contribute to the spontaneous activity of α4β3δ receptors, as β3 homomeric receptors are spontaneously active (Wooltorton et al., 1997; Cestari et al., 2000). We therefore expressed the exchanged motif receptor isoforms $\alpha4\beta3^{DNTK}\delta$ and $\alpha4\beta2^{GKER}\delta$ (Fig. 3.4b). For $\alpha4\beta2^{GKER}\delta$ receptors, similar levels of spontaneous activity were measured compared to wild-type α4β3δ (19.3 ± 4.4%, n = 8; and 15.9 ± 3.1%, n = 8, respectively). By contrast, $\alpha4\beta3^{DNTK}\delta$ receptors exhibited negligible spontaneous activity and not significantly different from wild-type α4β2δ receptors (0.92 ± 0.63%, n = 7; and 0%, n = 7, respectively). Thus, this ECD assembly motif plays a critical role in permitting spontaneous activity in $GABA_A$ receptors.

To attempt to distinguish between the roles of this motif in mediating homo-oligomerisation and spontaneous gating in heteromeric receptors, we created β3 subunits where each half of the GKER motif was separately mutated to its β2 equivalent (GK to DN and ER to TK). Intriguingly, we found that splitting the DNTK motif in heteromeric $\alpha4\beta3^{DN}\delta$ and $\alpha4\beta3^{TK}\delta$ receptors substantially reduced, but did not abolish, the level of spontaneous activity compared to α4β3δ receptors (α4β3δ: 20.7 ± 3.1%, n = 19; $\alpha4\beta3^{DN}\delta$: 4.9 ± 1.2%, n = 8; $\alpha4\beta3^{TK}\delta$: 8.1 ± 3.3%, n = 10; Fig. 3.4c). However, there was no apparent disruption to homo-oligomerisation when these mutant subunits were expressed as β3 homomers ($\beta3^{DN}$ or $\beta3^{TK}$), as indicated by the unaltered spontaneous current density determined by PTX (100 μM) application compared to wild-type β3 assemblies (β3: 3.2 ± 0.4 pA/pF, n = 19; $\beta3^{DN}$: 2.2

± 0.7 pA/pF, n = 8; $\beta3^{TK}$: 3.0 ± 0.6 pA/pF, n = 8; $\beta3^{DNTK}$: 0.06 ± 0.06 pA/pF, n = 5; Fig. 3.4d). Therefore, at least three GKER residues are required for full spontaneous activity in α4β3δ heteromers, whilst either pair of these residues is sufficient for β3 homomeric assembly and spontaneous gating. From this it can be concluded that homo-oligomerisation of β3 subunits and spontaneous gating of heteromeric α4β3δ receptors depend differentially on the GKER motif.

Our homology model of the α4β3δ receptor, derived from the cryo-EM structure of the α1β3γ2 receptor (Laverty et al., 2019), is shown with the GKER motif highlighted (Fig. 3.5). These residues are located at the non-GABA-binding α^{+}-β^{-} and δ^{+}-β^{-} interfaces, connecting ECD β sheets 8 and 9 (Thompson et al., 2010; Laverty et al., 2019).

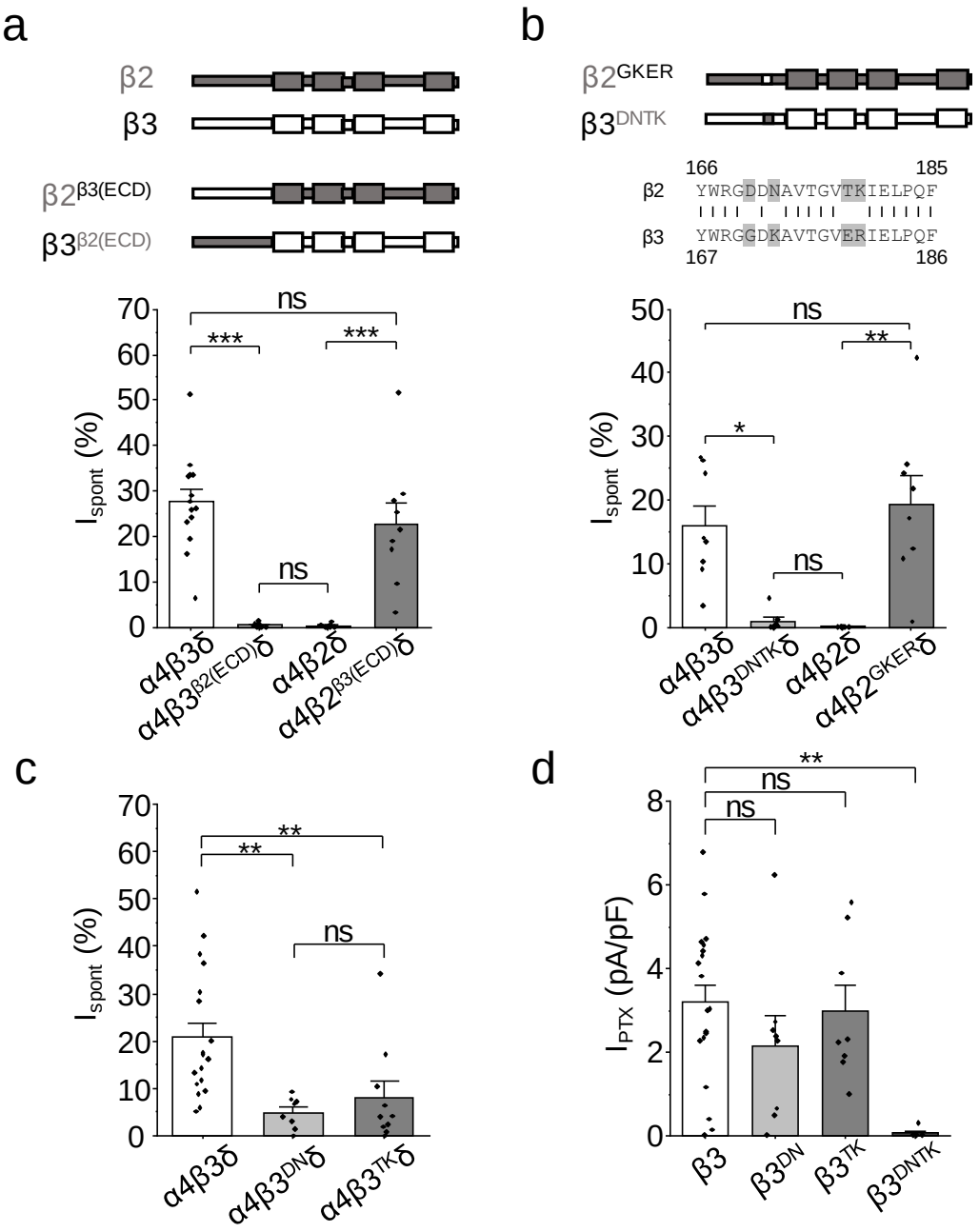

Figure 3.4: Residues in the extracellular domain of the β3 subunit are responsible for spontaneous activity of heteromeric GABA$_A$ receptors. a) Upper panel: schematic representation of the generated β3 chimeras with the exchanged ECDs. Lower panel: calculation of I_{spont} of wild-type and chimeric α4βδ receptors expressed in HEK cells (n = 7-14). b) Upper panel: schematic representation of GKER/DNTK motif-exchange between the two subunits, with the amino acid sequence of β2 and β3 below. The GKER and DNTK residues are highlighted. Lower panel: calculation of I_{spont} of wild-type and motif-exchanged α4βδ receptors expressed in HEK cells (n = 7-8). c) Calculation of I_{spont} of receptors expressing partial exchange of the GKER/DNTK motif (n = 8-19). d) Measurement of spontaneous current density of wild-type β3 homomeric receptors and those containing a partially or fully exchanged GKER/DNTK motif (n = 5-27). For all graphs, columns and error bars represent the mean and SEM, respectively (Kruskal-Wallis with Dunn's post hoc test for all comparisons; *<0.05; **p<0.01; ***p<0.001; ns: no significance).

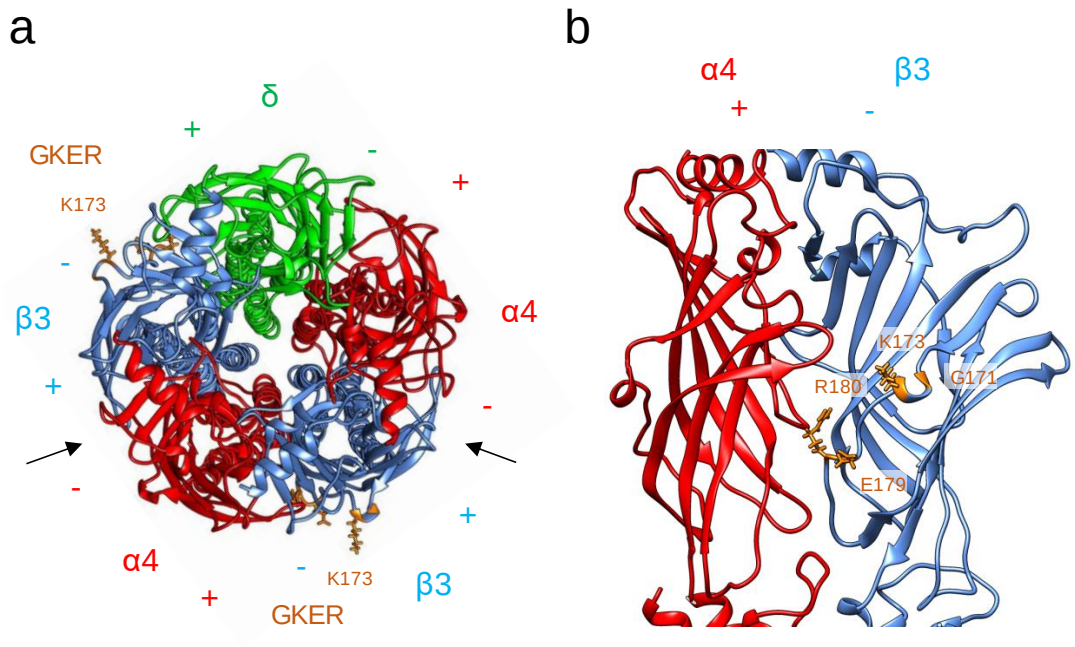

Figure 3.5: The location of the GKER motif on the α4β3δ receptor. The GKER residues are located at the $\alpha4^{+}$-$\beta3^{-}$ and δ^{+}-$\beta3^{-}$ interfaces, separate from the GABA-binding sites at the two $\beta3^{+}$-$\alpha4^{-}$ interfaces (indicated by the arrows). Plan (a) and side (b) views of the receptor ECD are shown. The α4, β3 and δ subunits are displayed in red, blue and green, respectively. The GKER residues are displayed in orange.

Pentameric ligand-gated ion channels bind their orthosteric ligands through interaction with six binding 'loops' (A-F; Fig. 3.6a), three of which (A-C) are provided by, in the $GABA_A$ receptor, the β subunit principal (+) face, and three (D-F) by the α subunit complementary (-) face (Miller & Smart, 2010; Thompson et al., 2010). The GKER motif of β3 is located within loop F, which is not involved in the direct binding of GABA since it locates to the non-binding β complementary interface. However, structural changes here may conceivably propagate to the GABA binding site or the ion channel activation gate, affecting the activation state of the receptor (Williams et al., 2010). At the orthosteric binding site, of the loops A-C, only loop C differs between β2 and β3, with two alanine residues in β3 (A201 and A204) replaced by two serines in β2 (S200 and S203; Fig 3.6b). To determine whether differences within the GABA-binding site could affect spontaneous currents, we generated a mutant subunit, $\beta3^{A201S,A204S}$, to mimic the β2 subunit GABA-binding loop C. However,

when expressed in heteromeric α4β3^{A201S,A204S}δ receptors, there was no effect on the level of spontaneous activity compared with wild-type α4β3δ receptors (24.1 ± 3.3%, n = 8; and 18.3 ± 3.6%, n = 8, respectively, Fig. 3.6b). Therefore, whilst the GKER motif within loop F at the non-binding interface of β3 subunits is a key determinant of spontaneous receptor gating, the GABA-binding principal interface of β3 (loops A-C) does not underlie the difference between β2 and β3-containing receptors. However, this does not preclude this area of the ECD from affecting spontaneous activity, as demonstrated from previous mutagenesis studies (Boileau et al., 2002).

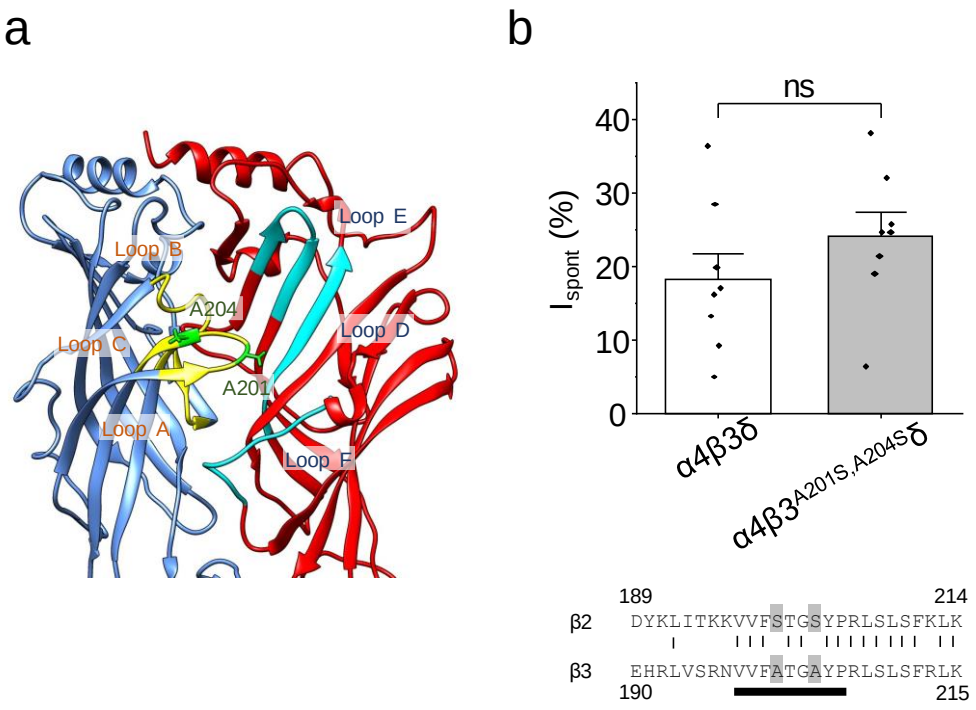

Figure 3.6: Residues in the GABA-binding loops do not underlie differences in spontaneous activity between β2 and β3 subunits. a) The binding 'loops' forming the orthosteric GABA-binding domain. Loops A-C on the β3 subunit are highlighted in yellow, whilst loops D-F on the α subunit are shown in cyan. The A201 and A204 residues of β3, which differ with S200 and S203 on β2, are shown with their side chains in green. b) Top panel: calculation of I_{spont} of α4β3δ receptors (n = 8) and those expressing the mutant α4β3^{A201S,A204S}δ receptor (n = 8). Lower panel: sequence alignment of residues surrounding loop C. The mutated residues are highlighted. The residues comprising the approximate area of loop C are shown by the black bar. Columns and error bars represent the mean and SEM, respectively (unpaired t-test; ns: no significance).

3.2.3. Investigating molecular determinants of spontaneous activity in non-β subunits

The previous results show that there is a high dependency on the subunit configuration to allow receptors to spontaneously gate, with only extrasynaptic-type α4/6β3δ receptors showing significant levels of spontaneous activity. The replacement of an 'extrasynaptic' α4/δ subunit with that of a 'synaptic' α1/γ2 subunit resulted in complete removal of spontaneous activity (Fig. 3.2e). To determine if the ECDs of these non-β subunits were important for spontaneity, we generated new chimeras which exchanged the ECDs in α4 and δ subunits with those from α1 ($\alpha4^{\alpha1(ECD)}$) and γ2L ($\delta^{\gamma2L(ECD)}$) subunits, respectively. Expressing these chimeric subunits in $\alpha4^{\alpha1(ECD)}\beta3\delta$ and $\alpha4\beta3\delta^{\gamma2L(ECD)}$ receptors revealed reduced, but not abolished, levels of spontaneous activity (α4β3δ: 17.3 ± 1.5%, n = 29; $\alpha4^{\alpha1(ECD)}\beta3\delta$: 10.6 ± 1.2%, n = 12; $\alpha4\beta3\delta^{\gamma2L(ECD)}$: 6.3 ± 1.3%, n = 8; Fig. 3.7a). These mutant receptors did not show the same reductions in spontaneous current as wild-type α1β3δ and α4β3γ2L receptors (Fig. 3.2e), suggesting that the ECDs in α4 and δ are unlikely to be solely responsible for affecting spontaneity.

However, given the key role of the β3 GKER motif, we further interrogated residues in the ECD of the α4 and δ subunits that might interact with the GKER amino acids across the α^+-β^- and δ^+-β^- interfaces. We focused on loop C and surrounding residues of the α, δ and γ2L subunits, as this structure projects across the subunit-subunit interface and could interact with juxtaposed β subunits (Laverty et al., 2019). Accordingly, we exchanged all of loop C and some of the surrounding residues of the α4 subunit (amino acids T200 to L217) for the equivalent domain residues in α1 subunits (I201 to L218), and performed similar mutagenesis on the δ subunit loop C and surrounding residues (T206 to S222) to their equivalents in γ2L (T209 to S224). The differing residues are highlighted in Fig. 3.7b,c.

Differing effects were observed on spontaneous activity depending on the expressed mutant subunit (Fig. 3.7d). Compared with α4β3δ receptors, no effect on spontaneity was observed by switching the α4 loop C and surrounding residues with that from α1 ($\alpha4^{\alpha1(loop\ C)}\beta3\delta$ receptors). However, a

significant reduction was evident in spontaneous currents when the δ loop C and surrounding residues were replaced with the γ2L equivalent in $\alpha4\beta3\delta^{\gamma2L(\text{loop C})}$ receptors (α4β3δ: 22.0 ± 2.5%, n = 14; $\alpha4^{\alpha1(\text{loop C})}\beta3\delta$: 28.2 ± 2.6%, n = 8; $\alpha4\beta3\delta^{\gamma2L(\text{loop C})}$: 10.6 ± 2.1%, n = 8). This is consistent with an analysis of the amino acid homology of this domain between α1 and α4 (61%), and γ2L and δ (29%) subunits, where the latter comparison shows more non-conserved residues (Fig. 3.7b,c).

To examine other structural determinants within the ECD that could account for why α4 subunits are more effective at inducing spontaneous activity than α1 subunits, we referred to the α4β3δ homology model to explore residue differences between the α subunits that could potentially alter their interactions with the GKER residues on β3 subunits. One notable area of potential difference was an arginine residue (R100) in loop A of α4 which is replaced by a histidine (H101) in α1 (Fig. 3.7e). Expressing $\alpha4^{R100H}\beta3\delta$ receptors showed that indeed this mutant had significantly lower levels of spontaneous activity than the wild-type α4β3δ control (8.2 ± 2.5%, n = 8; and 22.3 ± 3.0%, n = 8, respectively, Fig. 3.7f), indicating that the interaction between α4 R100 and the β3 GKER motif (possibly through an arg-arg interaction) may be involved in regulating spontaneous receptor activity. Intriguingly, the H101 residue of α1 is critical for the binding of benzodiazepines at the α^{+}-γ^{-} interface (Wieland et al., 1992; Rudolph et al., 1999), providing further evidence of this residue's importance in modulating receptor activity.

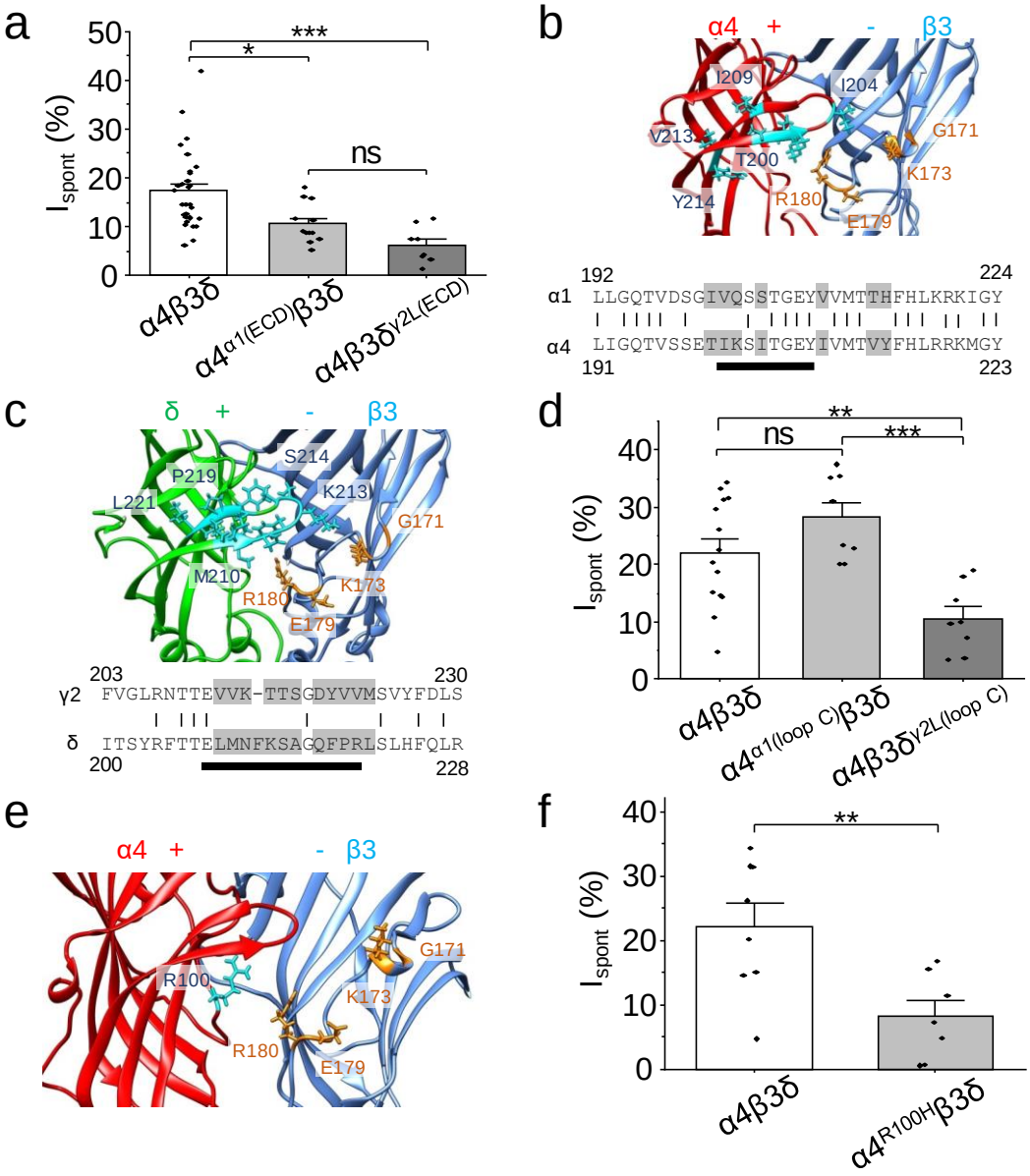

Figure 3.7: The α4 and δ extracellular domains are partially involved in permitting spontaneous activity, possibly by interacting with the GKER motif of the β3 subunit. a) Calculation of I_{spont} values of α4β3δ receptors (n = 29) and those where the ECD of the α4 (n = 12) or δ (n = 8) subunits were replaced by the equivalent residues from α1 and γ2L, respectively (ANOVA with Tukey's post hoc test). Residues within and surrounding loop C which were different between α4 and α1, and between δ and γ2L, were then exchanged. The residues which were mutated are shown in the homology models of the α4 (b) and δ (c) subunit interfacial site opposing the β3 subunit. Residues highlighted in the sequence alignment below represent the mutated residues and are shown in cyan with their side chains. Black bars represent approximate constituent residues of loop C. The GKER residues of β3 are shown in orange. d) The calculation of I_{spont} of α4β3δ receptors (n = 14) and those receptors containing chimeric α4 (n = 8) and δ (n = 8) subunits described in (b) and (c; ANOVA with Tukey's post hoc test). e) The $\alpha4^{+}$-$\beta3^{-}$ subunit interface showing the α4 R100 residue in cyan. f) I_{spont} calculation for wild-type α4β3δ receptors (n = 8) and receptors composed of mutant α4 subunits expressing the R100H mutation, homologous to α1 subunits (n = 8; unpaired t-test). For all graphs, columns and error bars represent the mean and SEM, respectively (*p<0.05; **p<0.01; ***p<0.001; ns: no significance).

3.2.4. GABA is a more potent agonist at spontaneously active receptors

To explore the possible mechanisms underlying spontaneity, the relationship between ligand-gating and spontaneous activity was next investigated. If receptors are more able to open in the absence of agonist, then it is plausible that they may also be more responsive to agonist with a lower threshold for activation (Campo-Soria et al., 2006; Akk et al., 2018). To investigate, GABA concentration-response curves were constructed for wild-type α4β3δ receptors, and for corresponding mutant receptors that affected the level of spontaneous activity. The first receptor variant studied was $\alpha4\beta3^{DNTK}\delta$, which we have shown to ablate spontaneous activity (Fig. 3.4b). The GABA concentration-response curve for these non-spontaneous $\alpha4\beta3^{DNTK}\delta$ receptors displayed a rightward-shift compared with wild-type α4β3δ receptors, as indicated by the near 5-fold increase in GABA EC_{50} (α4β3δ: 0.35 ± 0.07 μM, n = 9; $\alpha4\beta3^{DNTK}\delta$: 1.49 ± 0.14 μM, n = 10; $p < 0.001$; Fig. 3.8a). Thus, GABA is a more potent agonist at the spontaneous variant of this extrasynaptic-type $GABA_A$ receptor. We also compared the concentration-response curve of the $\alpha4\beta3^{DNTK}\delta$ receptor with that of the α4β2δ receptor. Interestingly, the EC_{50} of the α4β2δ receptor was significantly lower than that of the $\alpha4\beta3^{DNTK}\delta$ receptor (0.57 ± 0.04 μM, n = 6; $p < 0.001$), however the slopes of the two curves, as defined by the Hill coefficient, were similar and quite different to the slope for the spontaneous α4β3δ receptor (α4β3δ: 0.52 ± 0.02, n = 9; $\alpha4\beta3^{DNTK}\delta$: 0.99 ± 0.04, n = 10; α4β2δ: 1.14 ± 0.02, n = 6) suggesting possible reduced cooperativity for receptor activation for α4β3δ, though it is important to note that the Hill equation is empirical and not based on a reaction mechanism. The α4β3δ receptor showed similar responses to higher concentrations of GABA compared with the α4β2δ receptor, but at concentrations relevant to tonic inhibition (< 1 μM), the α4β3δ receptor showed significantly larger responses, possibly related to a lower threshold energy requirement for gating conferred on the receptor by the β3 subunit.

To probe the link between receptor spontaneity and GABA potency further, the mutation K279T was created in the β3 M2-M3 linker, since this mutant has previously been shown to markedly increase the potency of GABA

in α1β3γ2L receptors (Fisher, 2002). When expressed as α4β3^{K279T}γ2L receptors, very high levels of spontaneity were observed compared to α4β3γ2L which, as noted earlier (Fig. 3.2e), displays no spontaneous activity (α4β3γ2L: 1.4 ± 0.4%, n = 6; α4β3^{K279T}γ2L: 74 ± 5.0%, n = 6; Fig. 3.8b,c). The mutant α4β3^{K279T}γ2L receptor was assessed for GABA potency and, as for the α1β3^{K279T}γ2L construct (Fisher, 2002), the GABA EC_{50} was markedly lower by ~40-fold (0.37 ± 0.16 µM, n = 6) compared to wild-type (14.2 ± 3.71 µM, n = 5; $p < 0.05$), exhibiting a threshold GABA concentration requirement of just 1 nM instead of 1 µM for the wild-type receptor (Fig. 3.8d).

These data suggest that receptors which are spontaneously active are also more sensitive to activation by GABA and therefore likely to show lower activation energy for channel opening, either in the presence or absence of agonist. This is also consistent with the observation that extrasynaptic δ-containing receptors are both more sensitive to GABA and exhibit higher levels of spontaneous activity compared to their synaptic γ-containing counterparts (Fig. 3.2a,d).

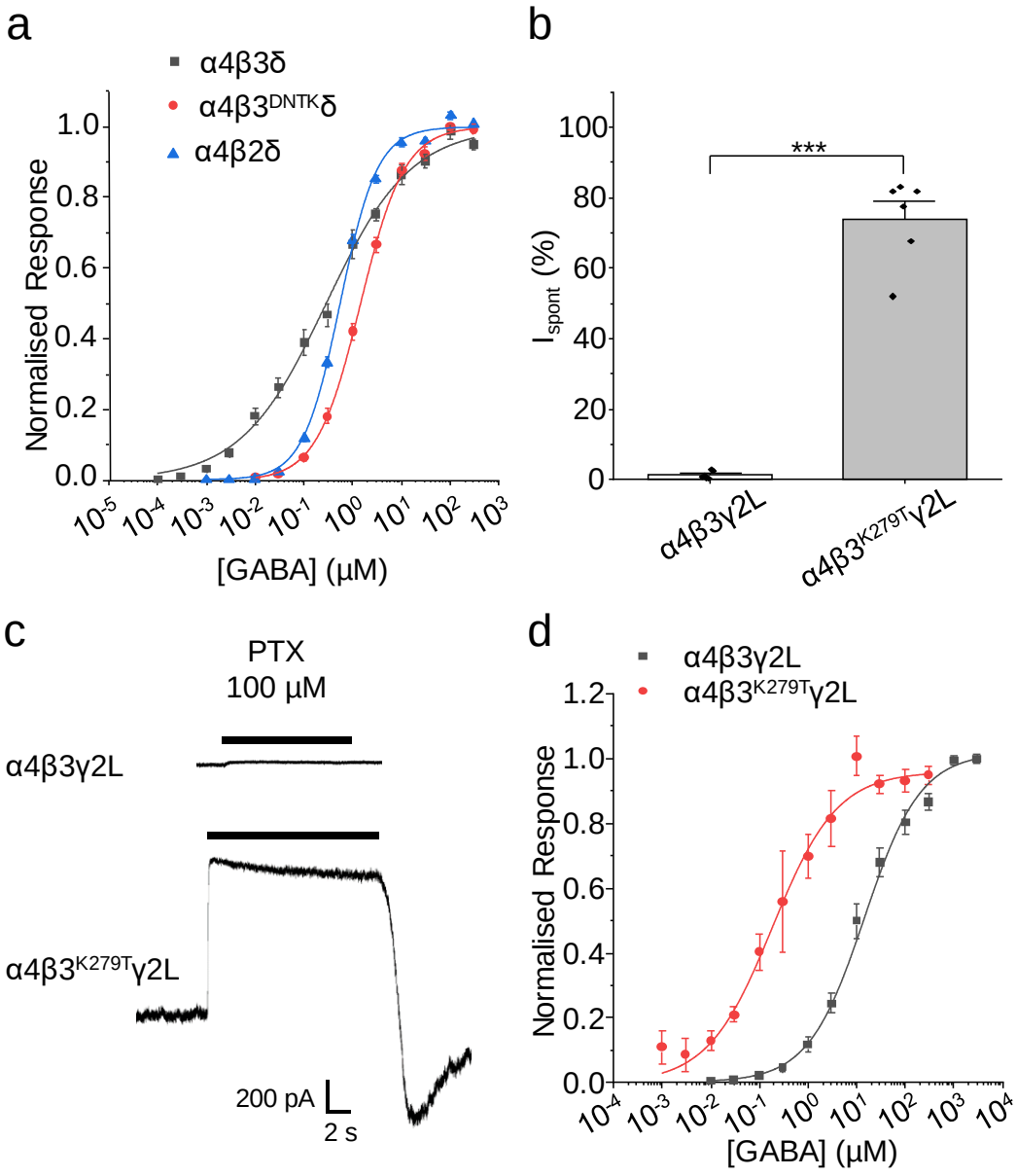

Figure 3.8: The potency of GABA is linked to the capacity of $GABA_A$ receptors to gate spontaneously. a) Concentration-response curves for the α4β3δ receptor, and the non-spontaneous α4β3^{DNTK}δ and α4β2δ receptors. The EC_{50} of the α4β3^{DNTK}δ receptor (1.49 ± 0.14 μM, n = 10) was significantly smaller than that of the α4β3δ (0.35 ± 0.07 μM, n = 9; one-way ANOVA, $p < 0.001$) and α4β2δ (0.57 ± 0.04 μM, n = 6; $p < 0.001$) receptors. The Hill coefficient of the α4β3^{DNTK}δ receptor (0.99 ± 0.04, n = 10) was significantly different than for the α4β3δ (0.52 ± 0.02, n = 9; one-way ANOVA, $p < 0.001$) and α4β2δ (1.14 ± 0.02, n = 6; $p < 0.001$) receptors, but more closely resembled the latter. b) Measurement of I_{spont} for wild-type α4β3γ2L receptors (n = 6) and those expressing the β3 K279T mutation (n = 6). Columns and error bars represent the mean and SEM, respectively (unpaired t-test, ***$p<0.001$). c) Representative recordings of wild-type and α4β3^{K279T}γ2L receptors during PTX application, revealing spontaneous current. d) Concentration-response curves of wild-type α4β3γ2L and mutant α4β3^{K279T}γ2L receptors. EC_{50} values for mutant receptors (0.37 ± 0.16 μM, n = 6) were significantly smaller than for wild-type receptors (14.2 ± 3.71 μM, n = 5; unpaired t-test, $p<0.05$).

3.3. Discussion

3.3.1. Extrasynaptic-type $GABA_A$ receptors display the highest level of spontaneous activity

In this study, we first quantified the level of spontaneous activity for a variety of $GABA_A$ receptors. To do this, we examined synaptic receptors responsible for phasic inhibition (α1β2/3γ2L), extrasynaptic receptors responsible for tonic currents (α4/6β2/3δ) and receptors found at both synaptic and extrasynaptic locations, contributing to both phasic and tonic inhibition (α5β2/3γ2L) (Stell et al., 2003; Alldred et al., 2005; Serwanski et al., 2006; Ali & Thomson, 2008). We identified two clear requirements for maximum spontaneous activity: the incorporation of the β3 subunit, over β1 and β2, and the presence of α4/6 and δ subunits, instead of α1/5 and γ2L.

The two distinct roles of phasic and tonic inhibition lend credence to the much higher levels of spontaneous current of α4β3δ over α1β3γ2L receptors. Phasic inhibition relies on rapid hyperpolarising and shunting currents which promote coincidence-detection and oscillatory activity (Pouille & Scanziani, 2001; Cardin et al., 2009), whilst tonic currents represent a continual hyperpolarisation which has wide-scale effects on neuronal excitability (Brickley et al., 2001; Mitchell & Silver, 2003). The extrasynaptic δ-containing $GABA_A$ receptors (α4β3δ and α6β3δ) are considered critical for the generation of tonic currents in various brain areas (Brickley et al., 2001; Mody, 2001; Glykys et al., 2008; Lee & Maguire, 2014). Spontaneous activity results in consistent activation of receptors in a stochastic manner and so represents a form of tonic inhibition, consistent with the proposed role of extrasynaptic α4β3δ receptors. Tonic inhibition in the brain may therefore be provided through a combination of GABA-mediated and GABA-independent activity.

The observation that α5β3γ2L receptors did not show as much spontaneous activity as α4/6β3δ receptors is intriguing. The α5βγ2 receptor is responsible for most tonic inhibition in CA1 neurons and might have been

expected to show significantly more spontaneous activity (Caraiscos et al., 2004; Bonin et al., 2007). Instead, it displayed similar levels of spontaneous activity to the largely synaptic α1β3γ2L receptor. This could indicate differences between tonic currents in CA1 regions and the dentate gyrus, which displays tonic currents originating from α4βδ receptors (Stell et al., 2003; Glykys et al., 2008). Tonic inhibition in the CA1 region may be more sensitive to fluctuations in ambient GABA, whilst tonic inhibition in the dentate may be more consistent due to a lower dependence on ambient GABA resulting from spontaneous receptor activity.

Another surprising observation was that both α4 and δ subunits must be present to form spontaneously active receptors. High spontaneous activity in α4β3γ2L has been reported previously (Tang et al., 2010), but significant levels of spontaneous activity were not observed in this study, even when incorporating the short isoform of the γ2 subunit. This discrepancy could be caused by species differences in the subunits, or experimental conditions such as age and origin of the HEK cells (Lin et al., 2014; Ponce et al., 2018). The δ subunit has significant effects on receptor properties, increasing the potency of GABA and reducing the rate and extent of desensitisation (Haas & Macdonald, 1999; Brown et al., 2002). It is therefore unsurprising that this subunit facilitates higher levels of spontaneous activity, but it is surprising that its effects are so dependent on the presence of the α4/6 subunit. This may indicate that subunit interfaces and interactions are essential for spontaneous activity.

Alongside the presence of the α4/6 and δ subunits, there was a clear requirement for the β3 subunit in order for receptors to gate spontaneously. The subcellular localisation of the β3 subunit would have a large impact on the spontaneous currents *in vivo*, as this usually correlates with the receptor isoform (Mody, 2001; Sigel & Steinmann, 2012). If predominantly found at synapses, β3 subunits preferentially form synaptic α1β3γ2 receptors, which show some but not significant levels of spontaneous activity. If, however, predominantly found at extrasynaptic locations, it may be forming α4β3δ receptors, giving rise to a significant spontaneous current. Experimental evidence on the subcellular localisation of β subunits is unclear and results

vary between brain regions, with confounding evidence provided by variable location of 'synaptic' αβγ receptors (Thomas et al., 2005; Herd et al., 2008; Kasugai et al., 2010; Nguyen & Nicoll, 2018). The three β subunit isoforms are broadly distributed across the brain but show significant differences in their expression within specific nuclei, for example β2 is less evident in the dentate gyrus granule cell layer (Pirker et al., 2000; Korpi et al., 2002). Further work should aim to delineate expression patterns of the β3 subunit to synaptic and extrasynaptic receptor isoforms, as this would provide an indication as to the extent of spontaneous $GABA_A$ receptor activity in varying brain regions.

3.3.2. The GKER motif of the β3 subunit is a key molecular determinant of spontaneous activity

The β subunits are essential for correct $GABA_A$ receptor assembly and are widely distributed throughout the brain (Connolly et al., 1996; Taylor et al., 1999; Pirker et al., 2000; Bollan et al., 2003; Sarto-Jackson & Sieghart, 2008). The principal structural determinant required for spontaneous activity in $GABA_A$ receptors was identified as a small motif within the β3 subunit ECD consisting of four residues: the GKER motif. This has previously been shown to be an assembly signal which allows the formation of β3 homomers (Taylor et al., 1999). The β2 subunit, which expresses DNTK residues instead of the GKER motif, does not show homomeric formation (Connolly et al., 1996), but incorporation of the GKER motif allows formation of functional homomeric $\beta2^{GKER}$ receptors (Taylor et al., 1999). We identified this motif as key for spontaneous activity, as exchange of GKER and DNTK residues from β3 and β2 subunits, respectively, also exchanged spontaneous properties. The $\alpha4\beta3^{DNTK}\delta$ receptor expressed very little spontaneous activity, whilst exchanging the ECD motif introduced large spontaneous currents in $\alpha4\beta2^{GKER}\delta$ receptors. A variety of control experiments, in particular the sensitivity to gabazine, ensured spontaneous currents recorded from HEK cells transfected with three subunit cDNAs derived from triheteromeric receptors and not a subpopulation of β3 homomers.

The GKER motif has a clear effect on spontaneity, but the underlying mechanism is unclear. The motif may produce an intrinsically less stable receptor with a lower energy requirement to change to an active conformation (Auerbach, 2013; Nayak et al., 2019). The GKER motif as an assembly signal strongly implies it interacts with, either directly or indirectly, residues in adjacent subunits, both α and γ/δ (Taylor et al., 1999). This interaction likely explains the requirement for α4 and δ, rather than α1 and γ2, to produce spontaneously active receptors, as differing residues on these subunits may interact differently with the GKER motif on the β subunit to affect spontaneous properties. This agrees with the finding of select residues at the interface in both α4 and δ which promote spontaneous activity. These interactions are distinct, with the loop C of δ and R100 in α4 promoting spontaneity.

The absolute requirement for both α4 and δ subunits for the generation of spontaneous currents is clear, as replacement of just one subunit was sufficient to completely remove spontaneous activity. This could be due to the location of the GKER motif at the α^+-β^- and δ^+-β^- interfaces, whereby interactions at a single interface are sufficiently important for spontaneous activity that their removal is able to prevent all spontaneity of the receptor. This gains credence when examining the $\beta3^{DN}$ and $\beta3^{TK}$ homomeric receptors. A half mutated GKER motif was sufficient to significantly reduce spontaneous currents in heteromeric receptors, but had no effect on the spontaneous currents of homomeric receptors. An impaired ability of the motif to promote spontaneity may be overcome at five β^+-β^- interfaces, but not at only two (α^+-β^- and δ^+-β^-) in heteromeric receptors.

However, this does not rule out subunit interactions elsewhere in the receptor regulating spontaneous gating. We discounted β3-dependent interactions at the GABA-binding β^+-α^- interface, with the exchange of loop C of β3 with that of β2 not impacting upon spontaneous activity. Mutations at the binding site, such as $\beta2^{L99C}$ and ρ^{Y102C}, can give rise to spontaneously active receptors (Boileau et al., 2002; Torres & Weiss, 2002), but, at least for wild-type β2 and β3-containing receptors, the modest differences at the GABA-binding interface do not underlie the clear disparity in spontaneity. Further evidence for alternative areas of subunit interactions come from the

observations surrounding the exchange of the ECD of α4 with α1 and of δ with γ2L, which did not entirely abolish spontaneous activity, as it would have if the ECD was the sole determinant, as is the case with the GKER motif of β3. Therefore, areas outside of α4 and δ ECDs also facilitate spontaneous activity, likely through interactions with adjacent subunits.

3.3.3. A possible mechanism for spontaneous activity

The GKER motif lies within loop F of the β subunit (Thompson et al., 2010; Miller & Aricescu, 2014), situated at the non-GABA-binding α^{+}-β^{-} and δ^{+}-β^{-} interfaces. The role of loop F in Cys-loop receptors is still not fully defined (Khatri & Weiss, 2010). It is involved in determining GABA potency in α subunits (Palvolgyi et al., 2018) and has been proposed to be involved in ligand binding, but not the gating process, in ρ homomers (Khatri et al., 2009). However, other evidence suggests its movement is coupled with channel activation (Zhang et al., 2009) and certain residues within loop F can influence the conformation of other nearby residues, especially in 5-HT_3 receptors, implying involvement in the gating mechanism (Thompson et al., 2006). Our data revealing the requirement of the β3 GKER motif for spontaneous receptor activity implicitly indicates a role for loop F in the spontaneous gating process of the receptor. The impact of this motif and loop F on receptor agonist sensitivity, combined with its distance from the GABA binding interface, further suggests a role in signal transduction (Hanson & Czajkowski, 2008). This is particularly evident when examining concentration-response curves; $\beta3^{DNTK}$-containing receptors displayed Hill coefficients, the slope of the curve and the sensitivity of the receptor to increasing concentrations of agonist, more similar to β2-containing (expressing the DNTK motif) than to β3-containing (GKER) receptors.

The GKER residues, compared to DNTK in β2, likely promote receptor gating by lowering the activation energy required for a conformational change associated with opening the channel pore. The binding of the agonist molecule at the orthosteric site is usually necessary to lower this energy barrier in non-

β3-containing receptors (Mozrzymas et al., 2003; Auerbach, 2013; Nayak et al., 2019). Reducing the activation barrier may explain the greater tendency of β3-containing receptors to open in the absence of agonist, and also explain the increased potency of GABA at receptors showing greater spontaneous activity (Campo-Soria et al., 2006; Akk et al., 2018). Models of receptor gating predict that GABA binding 'locks' the β^{+}-α^{-} interface into a more compact conformation, pulling the subunits closer together which results in a widening of the channel pore (Masiulis et al., 2019). This proposed gating mechanism involves movement across the length of the ECD; indeed this has already been described for an unstructured linker between loop E at the GABA-binding interface and loop A at the non-binding interface of the α subunit (Hanek et al., 2010). Given that the GKER motif is likely to make contact with residues on adjacent subunits (Taylor et al., 1999), its presence may also pull the subunits together, facilitating channel opening in the absence of agonist, explaining its importance for spontaneity. This may also explain the requirement for specific subunit composition (α4/6 and δ), as interaction with the GKER motif at two, rather than at a single, interface is necessary to lower the activation energy sufficiently to promote spontaneous opening. Additionally, loop F of the β2 subunit may actively reduce spontaneity (Williams et al., 2010). Residues within this area of the subunit stabilise the closed-state of α1β2γ2 receptors, thereby reducing the ability of the receptor to gate spontaneously (Williams et al., 2010). This study by Williams et al. (2010) showed that residues in this area of the β2 subunit could be mutated to either enhance or reduce GABA potency, and could introduce spontaneous gating. The individual mutations G170A, V175A and G177A within β2, all residues directly adjacent to the GKER/DNTK motif, increased the GABA EC_{50}, thereby indicating reduced gating efficiency of GABA. The Q185A mutation, however, both decreased the EC_{50} and introduced spontaneous gating of α1β2γ2S receptors in HEK cells.

The link between spontaneity and agonist potency has also been demonstrated in other mutagenesis studies (Akk et al., 2018). Mutation of the 9' leucine in the pore-lining M2 region of α4, β3 or δ subunits resulted in receptors with significant spontaneous activity and more potent activity of GABA (Patel et al., 2014). Notably, introduction of the mutation in the β3

subunit produced the largest increases in spontaneous activity. Additionally, $\beta1^{P228H}$ and $\beta1^{L285R}$ produces receptors which are simultaneously spontaneously active and more sensitive to GABA (Anstee et al., 2013). Even in wild-type receptors, incorporation of the β3 subunit into α1βγ2 receptors resulted in GABA acting as a more efficacious agonist than when either β1 or β2 were expressed (Mortensen et al., 2011), although GABA has been shown to have a higher potency when the β2 subunit was part of the α4βδ receptor (Lagrange et al., 2018).

The α^+-β^- and δ^+-β^- interfaces have been described as modified GABA-binding sites; they contain loops A-F but lack key residues within these structures which allows GABA binding and transduction (Ramerstorfer et al., 2011; Olsen, 2018). This is indeed the mechanism of various pharmacological compounds including benzodiazepines, which bind at the α^+-γ^- interface to potentiate $GABA_A$ receptor currents (Rudolph et al., 1999), and neurosteroids, which bind at the β^+-α^- interfaces (although within the transmembrane domains) (Hosie et al., 2006; Hosie et al., 2007; Laverty et al., 2017; Miller et al., 2017). Importantly, benzodiazepine derivatives have also been shown to bind and potentiate at the α^+-β^- interface (Baur et al., 2008; Ramerstorfer et al., 2011; Sieghart et al., 2012; Varagic et al., 2013a; Varagic et al., 2013b). Screens of compounds which bind to this interface and potentiate GABA-mediated currents were more potent at β3-containing receptors, rather than β1 or β2 (Varagic et al., 2013a), possibly linked to higher levels of intrinsic spontaneous activity. Even in the absence of pharmacological compounds, these modified binding sites may have an important role in facilitating channel opening, as indicated by the importance of the GKER motif of the β3 subunit for both spontaneous and GABA-mediated receptor activation.

3.4. Conclusion

We can conclude that the subunit identity in the pentamer is critical for the ability of the receptor to gate spontaneously. The GKER motif in the β3 subunit is a key determinant of spontaneous activity, most likely through

interactions with adjacent α4 and δ subunits at the α^{+}-β^{-} and δ^{+}-β^{-} interfaces, although other residues deeper in the α4 and δ subunits likely also facilitate spontaneous openings. Spontaneous receptors are more sensitive to GABA, possibly because they have a lower activation energy due to interactions of the GKER motif with residues on the adjacent subunit. To investigate this further, future studies should examine the single channel properties of the spontaneous openings to determine gating kinetics and rate constants, in particular at physiological temperatures.

Chapter 4: Modulation of spontaneous activity

4.1. Introduction

$GABA_A$ receptor activity is highly regulated by the presence of various modulators found endogenously. Subunit phosphorylation, a post-translational modification, is mediated by kinases and phosphatases to influence receptor expression through changes in trafficking and localisation (Terunuma et al., 2004; Luscher et al., 2011; Nakamura et al., 2015). Other modulators have a more direct impact on receptor activity and function, such as neurosteroids and endozepines, which enhance or reduce receptor currents (Belelli & Lambert, 2005; Carver & Reddy, 2013; Christian et al., 2013; Farzampour et al., 2015). These modulators have diverse effects which are strongly influenced by the subunit composition of the receptor, ranging from altering the trafficking of receptors and surface expression; to modifying current amplitude and kinetics of IPSCs (Reddy, 2010; Sigel & Steinmann, 2012; Nakamura et al., 2015).

Phosphorylation of $GABA_A$ receptors occurs at specific serine/threonine and tyrosine residues on a subset of subunits, dictated by surrounding residues (consensus sequences) (Nakamura et al., 2015). Phosphorylation represents the process by which a phosphoryl group (PO_3^-) is added to an amino acid residue (Ardito et al., 2017). This negatively charged addition alters the interaction of the amino acid with surrounding residues; potentially interfering or enhancing association with other proteins, or altering protein activity profiles (Comenencia-Ortiz et al., 2014). Phosphorylation is commonly studied by the removal or addition of phosphorylation sites through mutagenesis (Dissmeyer & Schnittger, 2011). Serine/threonine residues can be mutated to the structurally-related alanine, resulting in minimal steric effects but unable to be phosphorylated, thereby generating a phospho-null mutant (McDonald et al., 1998). Conversely, mutagenesis to a negatively charged residue, such as aspartic acid, is commonly used to mimic the effects of phosphorylation (Saliba et al., 2012). The effects of phosphorylation are

highlighted by examining protein-protein interactions which, for example, may affect surface expression of receptors, such as phosphorylation of α1 at T375. This reduces the affinity of the subunit for the synaptic clustering protein gephyrin, thereby reducing its presence at inhibitory synapses (Mukherjee et al., 2011). Phosphorylation of the β subunits has been extensively studied (McDonald et al., 1998; Kittler & Moss, 2003; Kang et al., 2011; Nakamura et al., 2015). Both β1 and β2 are phosphorylated at a single serine residue within the intracellular loop between M3 and M4: S409 and S410, respectively (Kellenberger et al., 1992; Moss et al., 1992a; Krishek et al., 1994; McDonald & Moss, 1997). The β3 subunit is phosphorylated at two adjacent serine residues: S408 and S409 (McDonald & Moss, 1997; McDonald et al., 1998). These sites are phosphorylated by a variety of kinases, including cAMP-dependent protein kinase (PKA), protein kinase C (PKC), cGMP-dependent protein kinase (PKG) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (McDonald & Moss, 1997; Brandon et al., 2002b; Houston et al., 2007). The β3 subunit, along with β1, also has a second CaMKII phosphorylation site at S383 (McDonald & Moss, 1994; McDonald & Moss, 1997; Houston et al., 2007; Saliba et al., 2012). The predominant effects of phosphorylation within the intracellular loop of receptor subunits is to control trafficking and surface expression; as demonstrated by the S408 and S409 residues of β3, at which phosphorylation reduces the binding of the trafficking protein AP2 and therefore promoting surface expression (McDonald et al., 1998; Kittler & Moss, 2003; Kittler et al., 2005). In this way, network signals which promote the activation of PKA and PKC through G protein-coupled receptors (McDonald et al., 1998; Brandon et al., 2002b; Connelly et al., 2013a), or CaMKII through activity-dependent mechanisms (Saliba et al., 2012; Ghosh et al., 2015), can alter the level of inhibition as required by the network.

Neurosteroids have a more direct impact on $GABA_A$ receptor currents, potentiating or inhibiting GABA-mediated activity. Synthesised in the brain from cholesterol precursors, neurosteroids such as THDOC and allopregnanolone enhance GABA-mediated currents at all subtypes of receptor, but have a larger effect at δ-containing receptors (Brown et al., 2002;

Stell et al., 2003; Wang, 2011; Carver & Reddy, 2013). Neurosteroids, therefore, have a large effect on tonic currents, although their effect on synaptic currents can also be significant (Twyman & Macdonald, 1992; Belelli et al., 2002; Hsu et al., 2003; Carver & Reddy, 2013). At the level of the single channel, neurosteroids both increase the number of bursts and prolong burst duration (Twyman & Macdonald, 1992). For macroscopic currents, this is usually manifested as an increase in tonic current and a prolongation of the IPSC decay (Lambert et al., 2009; Belelli et al., 2018). At higher concentrations they can directly activate the receptor, but this is unlikely at normal physiological levels (Wang, 2011). Key residues involved in the binding of the potentiating neurosteroids are present on the α subunit (Hosie et al., 2009). In particular, a conserved glutamine residue present within the M1 transmembrane domain of all α subunits (Q241 in α1 and Q246 in α4) was identified as a critical determinant of neurosteroid binding (Hosie et al., 2006; Hosie et al., 2007; Laverty et al., 2017). More recent structural studies have shown neurosteroids bound at the β^{+}-α^{-} interface within the transmembrane region, coordinated by residues contributed from M1 and M3 of the α and β subunits, respectively (Laverty et al., 2017; Miller et al., 2017). Interestingly, there is a clear interplay between neurosteroid potentiation and phosphorylation (Comenencia-Ortiz et al., 2014). Neurosteroids potentiate the PKC-dependent phosphorylation of S443 on the α4 subunit, resulting in enhanced insertion into the membrane and hence increased tonic currents (Abramian et al., 2010; Abramian et al., 2014). Phosphorylation of the adjacent S408 and S409 residues on the β3 subunit promotes the potentiation of GABAergic currents by neurosteroids (Adams et al., 2015). The interplay of these neuromodulatory mechanisms therefore sets inhibitory tone and affects neuronal excitability.

Manipulation of GABAergic transmission is also the mechanism through which various pharmacological agents operate (Sigel & Steinmann, 2012; Olsen, 2018; Sigel & Ernst, 2018). Benzodiazepines are selective potentiators of receptors containing $\alpha_x\beta\gamma2$ (x= 1, 2, 3 or 5) (Pritchett et al., 1989; Rudolph et al., 1999; Olsen, 2018). They therefore preferentially potentiate synaptic receptors, and hence IPSCs, by prolonging bursts of

activity at the level of the single channel (Rogers et al., 1994). They bind to the α^{+}-γ^{-} interface in the ECD of the receptor, dependent on the presence of a histidine residue found in all α subunits (H101 in α1) except α4 and α6 (Wieland et al., 1992; Rudolph et al., 1999). These subunits have, instead, an arginine residue, rendering these receptors insensitive to benzodiazepines (Rudolph et al., 1999). Benzodiazepines, such as diazepam, are used as a treatment for status epilepticus, a prolonged state of epileptic seizure (Niquet et al., 2016). The benzodiazepine binding site is also the site of action of the 'Z-drugs', a group of compounds that show greater selectivity for $GABA_A$ receptor α1 subunits and are widely used as hypnotics and sedatives (Nutt & Stahl, 2010). In addition to therapeutic agents acting at this site, the endogenously expressed endozepines also potentiate $GABA_A$ receptor currents (Christian et al., 2013; Farzampour et al., 2015). Barbiturates act in a similar manner to benzodiazepines by potentiating GABA-activated activity (Olsen, 2018). Pentobarbital was originally prescribed for insomnia, but due to significant risks has largely been replaced by the safer benzodiazepines, which, unlike barbiturates, do not directly activate the receptor. This difference has been explained by the different compounds having distinct allosteric effects on receptor conformation (Sancar & Czajkowski, 2011). At the level of the single channel, benzodiazepines increase the open frequency without changing open duration. Barbiturates, conversely, increase the channel open duration without affecting opening frequency. Barbiturates therefore appear to increase the rate of receptor opening once GABA is bound, whilst it has been suggested that benzodiazepines alter the association rate of the agonist for the receptor (Twyman et al., 1989).

Compounds which modulate $GABA_A$ receptor activity have been used as sedatives and general anaesthetics. The barbiturate thiopentone was used as a general anaesthetic, although this has generally been replaced by propofol (Garcia et al., 2010). Propofol binds to the transmembrane domains M1 and M2 of the β subunit and potentiates $GABA_A$ receptor currents, with a direct gating effect at higher concentrations (Yip et al., 2013). Propofol is largely non-selective in its targeting, whereas etomidate, another general anaesthetic, is selective for β2/3-containing receptors (Belelli et al., 1997).

Various modulators of $GABA_A$ receptor activity are well characterised as potentiators and, in some cases, inhibitors of GABA-mediated currents. However, relatively few studies have examined their impact on spontaneous currents (Birnir et al., 2000; Maksay et al., 2003; Jatczak-Śliwa et al., 2018; Pierce et al., 2019). Our aim here was to systematically investigate the effects of phosphorylation, neurosteroids and other modulatory agents on the spontaneous activity of $GABA_A$ receptors using mutagenic and pharmacological approaches. We identify key targets for protein kinases to phosphorylate and modulate spontaneous currents, as well as show clear modulating effects of potentiating and inhibitory neurosteroids, general anaesthetics and benzodiazepines.

4.2. Results

4.2.1. Phosphorylation of the β3 subunit regulates spontaneous activity

As part of our initial investigations into the structural determinants underlying the ability of the β3 subunit to promote the spontaneous opening of receptors, we generated subunit chimeras with exchanged M3-M4 intracellular loops between β3 and β2. The introduction of the β3 loop into the β2 subunit, when expressed as $\alpha4\beta2^{\beta3(loop)}\delta$, did not affect spontaneous activity and this receptor displayed almost no spontaneous currents (0.05 ± 0.03%, n = 6), similar to wild-type α4β2δ receptors (0.36 ± 0.17%, n = 7; Fig. 4.1a). However, the introduction of the β2 intracellular loop into the β3 subunit and the expression of $\alpha4\beta3^{\beta2(loop)}\delta$ receptors resulted in a significant reduction in the level of spontaneous activity compared to wild-type α4β3δ receptors (15.0 ± 3.5%, n = 13; and 27.9 ± 3.2%, n = 6, respectively; Fig. 4.1a). The β subunits all have several phosphorylation sites within their intracellular loops (Moss et al., 1992a; McDonald et al., 1998), and we investigated whether manipulation of these would have an effect on the level of spontaneous activity, thereby providing further evidence for the importance of the loop in modulating spontaneous currents. We first removed phosphorylation sites from the β3

subunit by generating and expressing S408A and S409A mutants, both individually and together. We observed significant reductions in the level of spontaneous activity in each of α4β3^{S408A}δ, α4β3^{S409A}δ and α4β3^{S408A,S409A}δ receptors compared to wild-type (α4β3δ: 24.2 ± 2.9%, n = 19; α4β3^{S408A}δ: 8.0 ± 1.7%, n = 16; α4β3^{S409A}δ: 4.4 ± 1.0%, n = 12; α4β3^{S408A,S409A}δ: 14.6 ± 1.6%, n = 17; Fig. 4.1b). Intriguingly, we observed significantly less spontaneous activity when only a single phosphorylation site was ablated than when both sites were. This lack of a cumulative effect was surprising, and serves to highlight the complex actions of phosphorylation at this site.

Phosphorylation of these residues is known to alter trafficking and membrane expression of these receptors (McDonald et al., 1998; Kittler et al., 2005; Comenencia-Ortiz et al., 2014; Nakamura et al., 2015). Measurement of total receptor current (I_{PTX} + $I_{GABA\text{-}MAX}$) showed slight increases for the single-site mutant α4β3^{S409A}δ and a slight but non-significant increase of α4β3^{S408A}δ receptors (α4β3δ: 2.4 ± 0.3 nA, n = 19; α4β3^{S408A}δ: 3.6 ± 0.5 nA, n = 16; α4β3^{S409A}δ: 5.1 ± 0.8 nA, n = 15; α4β3^{S408A,S409A}δ: 2.9 ± 0.5 nA, n = 18; Fig. 4.1c). This could indicate increased expression of the single phosphorylation mutant receptors. We adequately control for changes in expression by normalising the spontaneous current to the total GABA-activated current. However, the increase to total receptor current could also be explained by changes to gating kinetics introduced by the phosphorylation mutants, which could impact on our calculation of I_{spont} (Brandon et al., 2000). However, absolute spontaneous currents of these single-site mutants, determined by PTX (100 μM) application, were lower than for wild-type, although interestingly not for the double mutant (α4β3δ: 483.4 ± 54.3 pA, n = 19; α4β3^{S408A}δ: 230.6 ± 47.1 pA, n = 16, $p < 0.05$; α4β3^{S409A}δ: 278.3 ± 59.8 pA, n = 15, $p = 0.09$; α4β3^{S408A,S409A}δ: 432.8 ± 73.5, n = 18, $p = 0.93$). Therefore, despite possible changes in surface expression or gating, there is still a substantial reduction in spontaneous activity when altering the phosphorylation state of the β3 subunit.

We next investigated whether manipulating the phosphorylation of the homologous sites in β2 (A409 and S410) could introduce any spontaneous activity in β2-containing receptors. We both introduced a second serine site

(A409S), thereby creating two phosphorylation sites homologous to the site in β3, and also removed the single phosphorylation site (S410A) (McDonald et al., 1998). However, neither $\alpha4\beta2^{S410A}\delta$ (n = 6) or $\alpha4\beta2^{A409S}\delta$ (n = 8) receptors showed any differences in their spontaneous profiles from wild-type α4β2δ receptors (n = 5), remaining quiescent under basal conditions with spontaneous current < 0.5% (Fig. 4.1d).

We can conclude from these mutagenesis studies that the phosphorylation state of the β3 subunit impacts on the receptor's ability to gate spontaneously. Only a fully phosphorylated β3 subunit at both S408 and S409 shows maximal spontaneous activity. Introduction of the β3 intracellular loop into the β2 subunit and manipulation of the β2 phosphorylation sites had no impact on the receptor's ability to open spontaneously, indicating that, whilst the loop is important for modulating the extent of spontaneous activity, it does not have a direct effect on the ability of the receptor to gate spontaneously. This is consistent with the previous findings implicating the ECD of the β3 subunit and the GKER motif as key in permitting high levels of spontaneous activity (Fig. 3.4a,b).

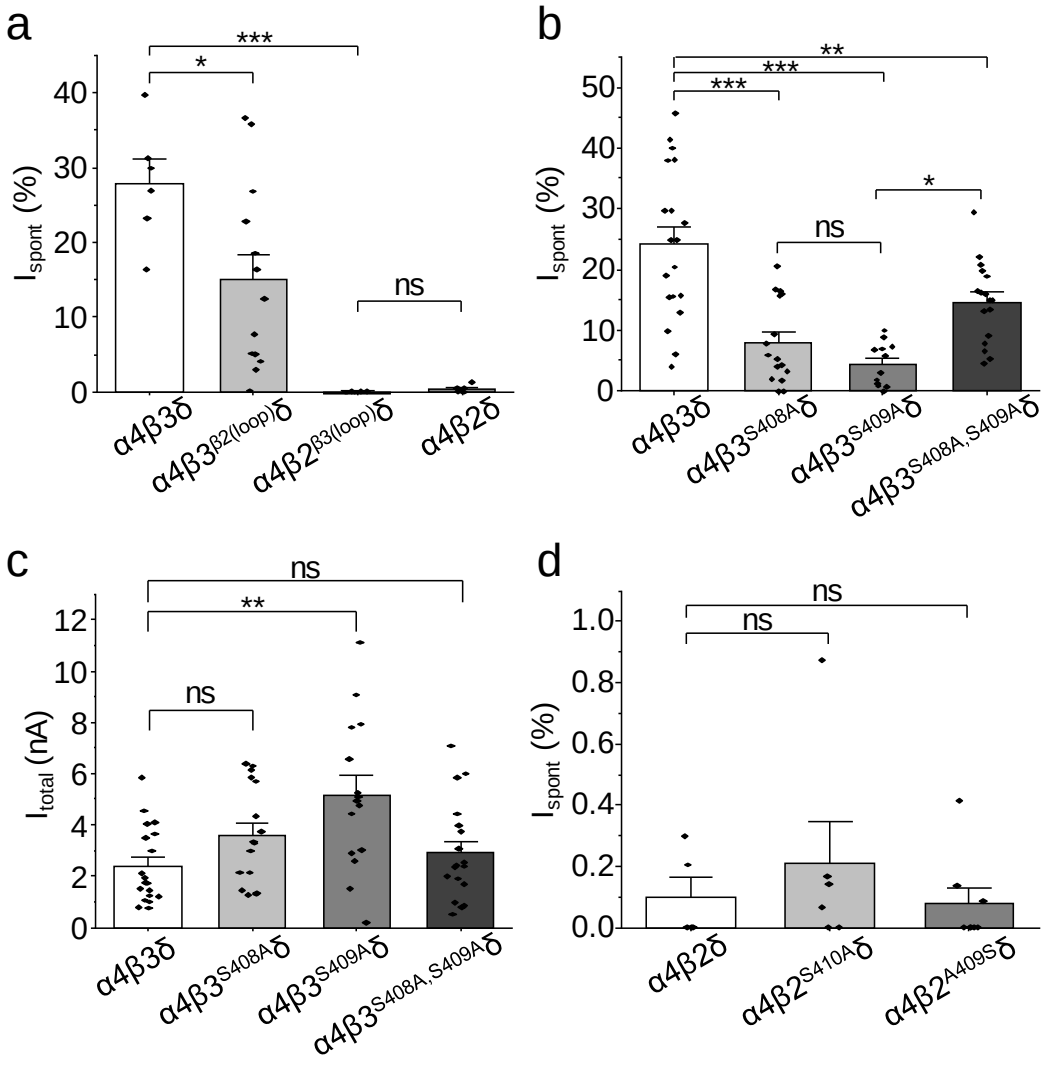

Figure 4.1: Phosphorylation of the β3 intracellular loop impacts on the spontaneous activity of the receptor. a) Calculation of I_{spont} of receptors expressing chimeric β subunits where the intracellular M3-M4 loops have been exchanged (n = 6-13; ANOVA with Tukey's post hoc test). b) Calculation of I_{spont} of receptors incorporating β3 subunits where one or both of the adjacent phosphorylation sites within the M3-M4 loop have been removed through mutagenesis (n = 12-19; ANOVA with Tukey's post hoc test). c) Measurement of the total receptor current (GABA-activated + spontaneous) passed by the phospho-null mutants (n = 15-19; Kruskal-Wallis with Dunn's post hoc test). d) Calculation of the I_{spont} of receptors incorporating mutant β2 subunits expressing manipulated phosphorylation sites within the M3-M4 loop (n = 5-8; Kruskal-Wallis with Dunn's post hoc test). For all graphs, columns and error bars represent the mean and SEM, respectively (*p<0.05; **p<0.01; ***p<0.001; ns: no significance).

Mutagenesis studies are important in order to identify possible implications of phosphorylation on receptor function. However, a more direct approach is required to ensure the effects observed are due to kinase-mediated phosphorylation and not simply a steric effect of replacing the residue with another, albeit similar, residue (Adams et al., 2015). To ensure the effects described previously were caused by genuine effects on subunit phosphorylation, we began by applying the wide-spectrum kinase inhibitor staurosporine (200 nM) to α4β3δ receptors for 2 mins and measured changes to the spontaneous holding current. During application of the DMSO control, the holding current was unchanged (baseline: 364.3 ± 52.9 pA; control: 366.2 ± 58.1 pA; n = 6), but when staurosporine was applied significant reductions were observed (baseline: 484.1 ± 82.6 pA; staurosporine: 370.1 ± 78.6 pA; n = 8; Fig. 4.2a,b). To ensure these effects were mediated by the inhibition of kinases targeting the S408 and S409 residues, a $\beta3^{S408D,S409D}$ mutant subunit was created. The negative charge of the aspartic acid mimics a phosphorylated serine residue, and so these mutants mimic a fully phosphorylated subunit (Dissmeyer & Schnittger, 2011). These receptors should therefore be unaffected by kinase inhibition, and indeed $\alpha4\beta3^{S408D,S409D}\delta$ receptors showed no change in holding current after either DMSO control (baseline: 259.5 ± 42.1 pA; control: 272.2 ± 42.0 pA; n = 10) or staurosporine application (baseline: 222.7 ± 30.7 pA; staurosporine: 214.9 ± 22.5 pA; n = 10; Fig. 4.2c). Importantly, this receptor also displayed comparable levels of spontaneous activity to wild-type α4β3δ receptors, indicating that, under basal conditions, wild-type receptors are likely to be fully phosphorylated (α4β3δ: 23.1 ± 5.5%, n = 9; $\alpha4\beta3^{S408D,S409D}\delta$: 20.9 ± 2.5%, n = 8; Fig. 4.2d).

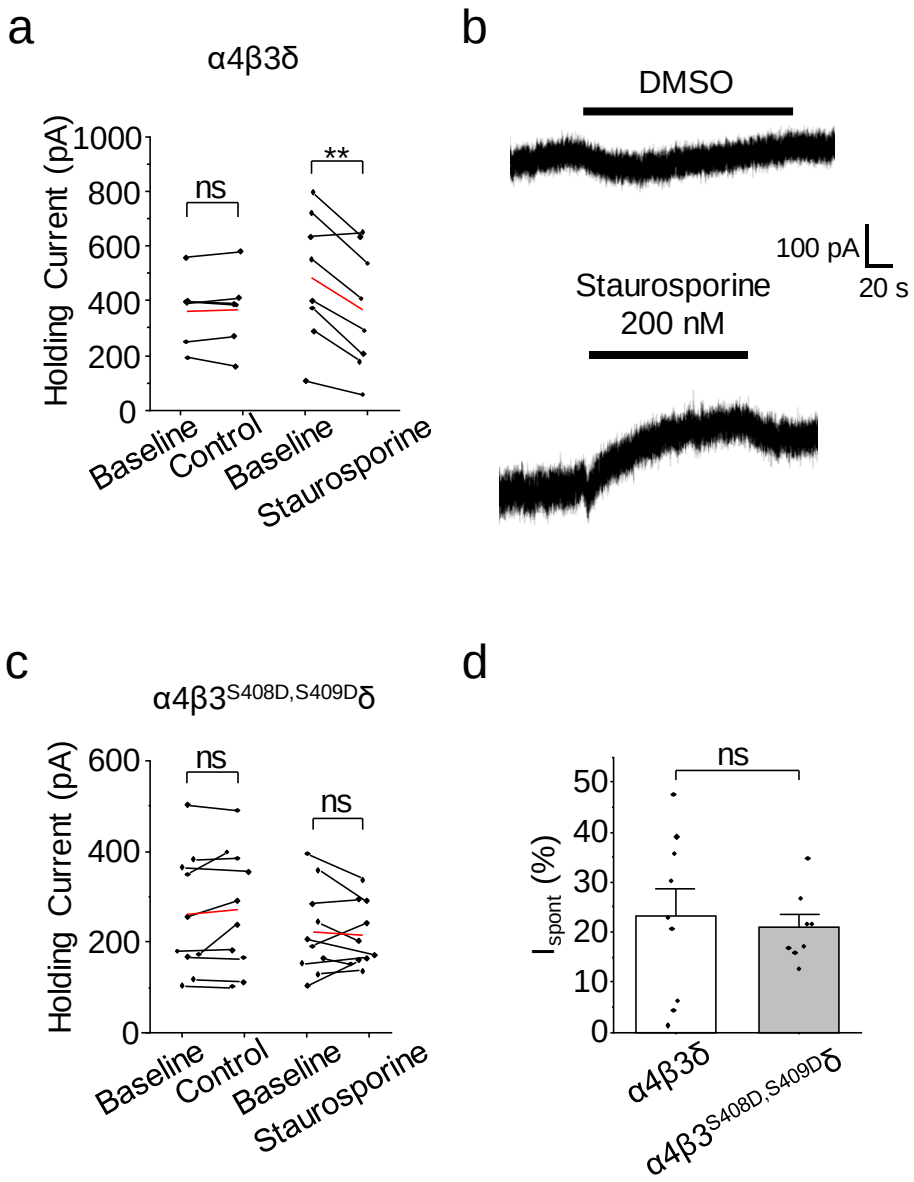

Figure 4.2: Staurosporine reduced spontaneous currents of wild-type α4β3δ receptors by inhibiting phosphorylation of S408 and S409. a) The spontaneous holding currents before and after application of either DMSO control (n = 6) or the wide-spectrum kinase inhibitor staurosporine (200 nM; n = 8), applied for 2 mins on HEK cells expressing α4β3δ receptors. The connected red line indicates the average holding current pre-application and after 2 min exposure to staurosporine (paired t-test). b) Representative recordings of α4β3δ receptors exposed to either DMSO or staurosporine. c) The spontaneous holding currents before and after application of either DMSO control (n = 10) or staurosporine (n = 10), applied for 2 mins on HEK cells expressing phosphomimetic $\alpha4\beta3^{S408D,S409D}\delta$ receptors. The connected red line indicates the average holding current pre-application and after 2 min exposure to staurosporine (paired t-test). d) Calculation of the I_{spont} of the phosphomimetic $\alpha4\beta3^{S408D,S409D}\delta$ receptor (n = 8-9). Columns and error bars represent the mean and SEM, respectively (unpaired t-test; **p<0.01; ns: no significance).

Various serine/threonine kinases are known to target S408 and S409 in β3 subunits, including PKA, PKC, PKG and CaMKII (McDonald & Moss, 1997; McDonald et al., 1998; Houston et al., 2007; Nakamura et al., 2015). To identify which protein kinases are responsible for regulating the spontaneous $GABA_A$ receptor current, selective kinase inhibitors were used. The PKA inhibitor peptide 14-22 (PKI; 1 μM) was first included in the patch electrode solution. HEK cells expressing α4β3δ receptors exposed to internal PKI displayed significantly lower (61.7% of control) spontaneous current than those exposed to control internal solution (control: 20.6 ± 2.6%, n = 8; PKI: 12.7 ± 2.5%, n = 8; Fig. 4.3a). We further investigated PKA-dependent effects on spontaneous activity by including the PKA activator 8-Br cAMP (1 mM) in the patch electrode solution. This did not have an effect on the measured spontaneous activity (control: 14.7 ± 1.8%, n = 8; 8-Br cAMP: 12.8 ± 1.5%, n = 7; Fig. 4.3b), in agreement with the previous data using the phosphomimetic $\beta3^{S408D,S409D}$ subunit which indicated α4β3δ receptors were maximally phosphorylated under basal conditions (Fig. 4.2d). To further substantiate the actions of PKA in phosphorylating the adjacent serine residues of β3, $\alpha4\beta3^{S408D,S409D}\delta$ receptors were expressed and PKI included in the patch electrode solution. Unlike with wild-type α4β3δ, there was no reduction in the measured spontaneous activity (control: 8.2 ± 2.6%, n = 10; PKI: 7.1 ± 2.3%, n = 8; Fig. 4.3c).

The selective PKC inhibitor bisindolylmaleimide-I (BIS-I, 200 nM) was the next to be trialled and included in the patch electrode solution. Inhibition of the spontaneous activity was more profound than using PKI, with receptors exposed to BIS-I displaying spontaneous activity 39.1% of control (control: 16.6 ± 1.9%, n = 8; BIS-I: 6.5 ± 2.2%, n = 7; Fig. 4.3d). To investigate the effect of PKG, the selective inhibitor KT 5823 (1 μM) was next included in the patch electrode solution and the spontaneous activity recorded. Spontaneous activity of α4β3δ receptors exposed to KT5823 was 47.5% of the spontaneous activity of receptors exposed to the control (control: 9.6 ± 1.7%, n = 9; KT 5823: 3.8 ± 0.8%, n = 9; Fig. 4.3e). These results suggest that PKA, PKC and PKG target the two adjacent phosphorylation sites to affect α4β3δ receptor-mediated spontaneous currents.

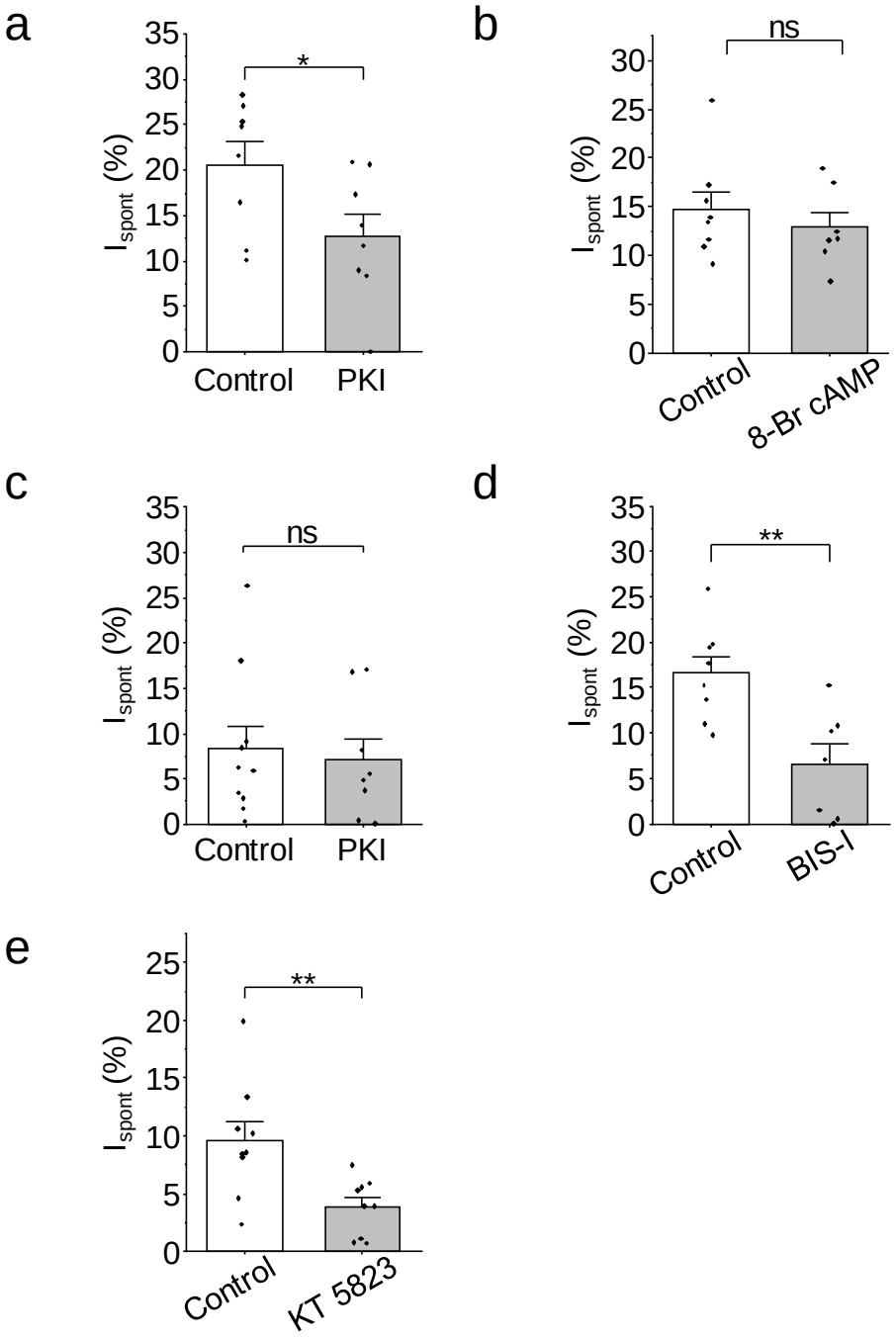

Figure 4.3: PKA, PKC and PKG modulate spontaneous activity through phosphorylation of the β3 subunit. Calculation of I_{spont} of α4β3δ (n = 8) receptors expressed in HEK cells when (a) the PKA inhibitor PKI (1 μM; n = 8) and (b) the PKA activator 8-Br cAMP (1 mM; n = 7) were included in the patch electrode solution. c) Calculation of I_{spont} for phosphomimetic α4β3^{S408D,S409D}δ receptors under control conditions (n = 10) or when PKI (1 μM) was included in the patch electrode solution (n = 8). d) Calculation of the I_{spont} of α4β3δ receptors under control conditions (n = 8) or when the PKC inhibitor BIS-I (200 nM; n = 7) was included in the patch electrode solution. e) Calculation of the I_{spont} of α4β3δ receptors under control conditions (n = 9) or when the PKG inhibitor KT 5823 (1 μM; n = 9) were included in the patch electrode solution. For all graphs, columns and error bars represent the mean and SEM, respectively (unpaired t-test for each condition; *p<0.05; **p<0.01; ns: no significance).

In addition, we examined phosphorylation by CaMKII. This has been identified as a phosphorylating kinase at the S408 and S409 residues, but also at an additional residue: S383 (McDonald & Moss, 1997; Houston et al., 2007; Saliba et al., 2012). However, ablation of this site with the $\beta3^{S383A}$ mutant subunit had no effect on the spontaneous activity of $\alpha4\beta3^{S383A}\delta$ receptors ($\alpha4\beta3\delta$: 18.6 ± 3.0%, n = 9; $\alpha4\beta3^{S383A}\delta$: 15.9 ± 2.8%, n = 11), and neither did incorporation of the selective CaMKII inhibitor KN-62 (3 μM) in the patch electrode solution (control: 9.6 ± 1.7%, n = 9; KN-62: 9.8 ± 1.7%, n = 9; Fig. 4.4a,b). While this does imply the S383 site and CaMKII activity does not modulate spontaneous activity, there are difficulties in fully analysing the effect of CaMKII in HEK cells (Houston & Smart, 2006).

Along with the S383 phosphorylation site, the α4β3δ receptor has another serine/threonine kinase phosphorylation site at the S443 residue of the α4 subunit (Abramian et al., 2010; Abramian et al., 2014). We investigated the effect of phosphorylation of this site by expression of $\alpha4^{S443A}\beta3\delta$ receptors, but these showed similar spontaneous activity to wild-type receptors ($\alpha4\beta3\delta$: 23.0 ± 3.9%, n = 12; $\alpha4^{S443A}\beta3\delta$: 20.6 ± 2.8%, n = 13; Fig. 4.4c).

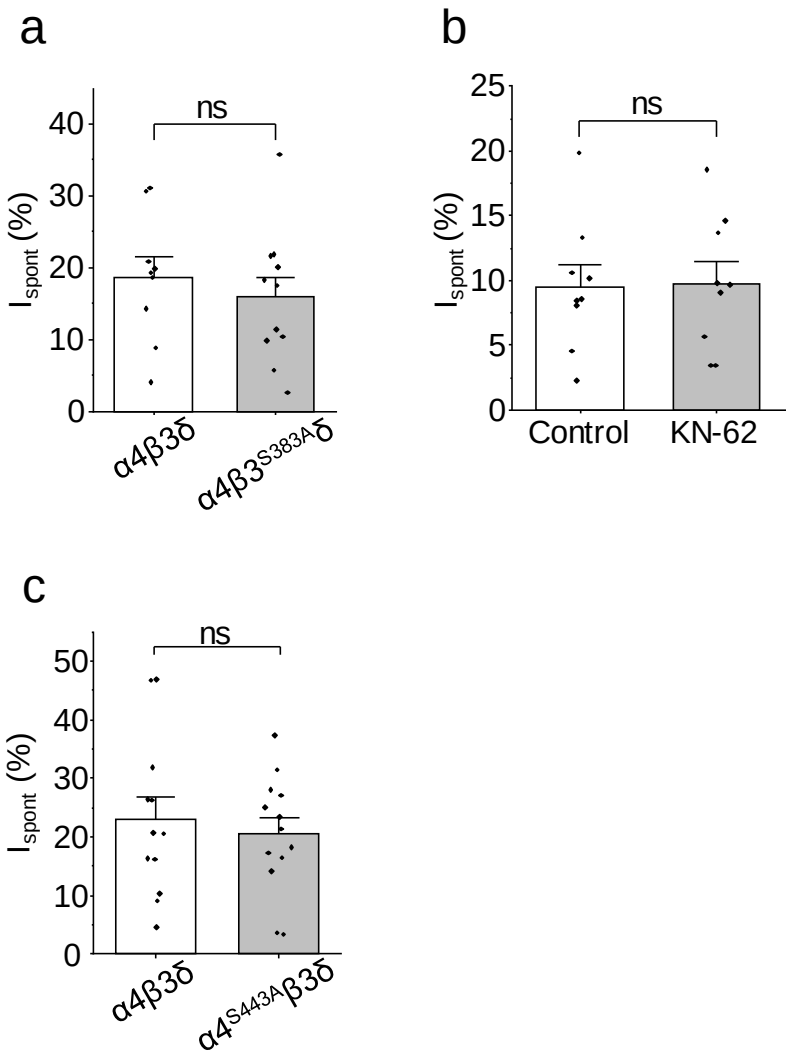

Figure 4.4: Alternative phosphorylation sites, β3^{S383} and α4^{S443}, do not impact on spontaneous activity of α4β3δ receptors. Investigation of the impact of CaMKII-mediated phosphorylation on α4β3δ receptors expressed in HEK cells by calculating the I_{spont} of (a) receptors incorporating the phospho-null β3^{S383A} subunit (n = 9-11) and (b) using the selective CaMKII inhibitor KN-62 (3 μM; n = 9). c) The calculated I_{spont} of α4β3δ receptors expressing the phospho-null α4^{S443A} subunit (n = 12-13). For all graphs, columns and error bars represent the mean and SEM, respectively (unpaired t-test for all conditions; ns: no significance).

4.2.2. Allosteric modulators of GABA-mediated receptor activation also affect spontaneous activity

$GABA_A$ receptors are regulated by an array of endogenous and exogenous allosteric modulators, but it is largely unknown whether they will modify the spontaneous current (Kittler & Moss, 2003; Sigel & Steinmann, 2012). Neurosteroids are important endogenous allosteric modulators of $GABA_A$ receptors, responsible for regulating inhibitory signalling mediated by extrasynaptic and synaptic receptors (Belelli et al., 2002; Wang, 2011; Carver & Reddy, 2013). We assessed modulation of spontaneous activity using two neurosteroids, THDOC and allopregnanolone, which were applied to HEK cells expressing α4β3δ receptors. Both THDOC and allopregnanolone (100 nM) significantly increased the amplitude of spontaneous currents by 74.6% and 80.1%, respectively (Fig. 4.5a,b). Neurosteroids will directly activate $GABA_A$ receptors at higher concentrations, but at 100 nM there was no activation of the non-spontaneous α4β2δ receptor (Fig. 4.5a), indicating that the potentiating effect of low neurosteroid concentration at β3-containing receptors represented an enhancement of intrinsic spontaneous activity. To ensure the neurosteroids were acting at their known binding sites on $GABA_A$ receptors, we examined the neurosteroid-insensitive α4 Q246M mutant in $\alpha4^{Q246M}\beta3\delta$ receptors (Hosie et al., 2006; Laverty et al., 2017). Abolishing neurosteroid binding to the receptor had no effect on the basal spontaneous current (α4β3δ: 13.9 ± 2.0%, n = 8; $\alpha4^{Q246M}\beta3\delta$: 12.2 ± 1.6%, n = 7; p = 0.53, unpaired t-test) , but ablated the potentiation of the spontaneous current by both THDOC and allopregnanolone (Fig. 4.5a).

We also examined neurosteroid modulation at $\alpha4\beta3^{S409A}\delta$ $GABA_A$ receptors. We have shown here that this phosphorylation mutant displays significantly impaired spontaneous activity (Fig. 4.1b), and it has been previously reported that β3 phosphorylation at this site affects neurosteroid potentiation (Adams et al., 2015). THDOC and allopregnanolone potentiated the small spontaneous currents of these receptors (68.9% and 90.2%, respectively) to a similar extent as for wild-type α4β3δ receptors, although this effect was not statistically significant for THDOC (Fig. 4.5a). This opens up the

possibility of receptor phosphorylation and neurosteroid modulation acting in concert to regulate spontaneous $GABA_A$ receptor activity.

Sulphated neurosteroids such as pregnenolone sulphate (PS) are also synthesised in the brain, but unlike their potentiating counterparts they inhibit GABA-induced currents (Akk et al., 2001; Seljeset et al., 2015; Seljeset et al., 2018). In contrast to THDOC and allopregnanolone, PS (10 µM) behaved as a negative allosteric modulator and inhibited spontaneously-active α4β3δ (n = 8), $\alpha4^{Q246M}\beta3\delta$ (n = 7) and $\alpha4\beta3^{S409A}\delta$ (n = 7) receptors by 56.1%, 54.3% and 63.2%, respectively (Fig. 4.5c,d). This inhibition was independent of the Q246 site on α4, as pregnenolone sulphate has a distinct binding site on $GABA_A$ receptors from the positive modulatory neurosteroids (Park-Chung et al., 1999; Laverty et al., 2017).

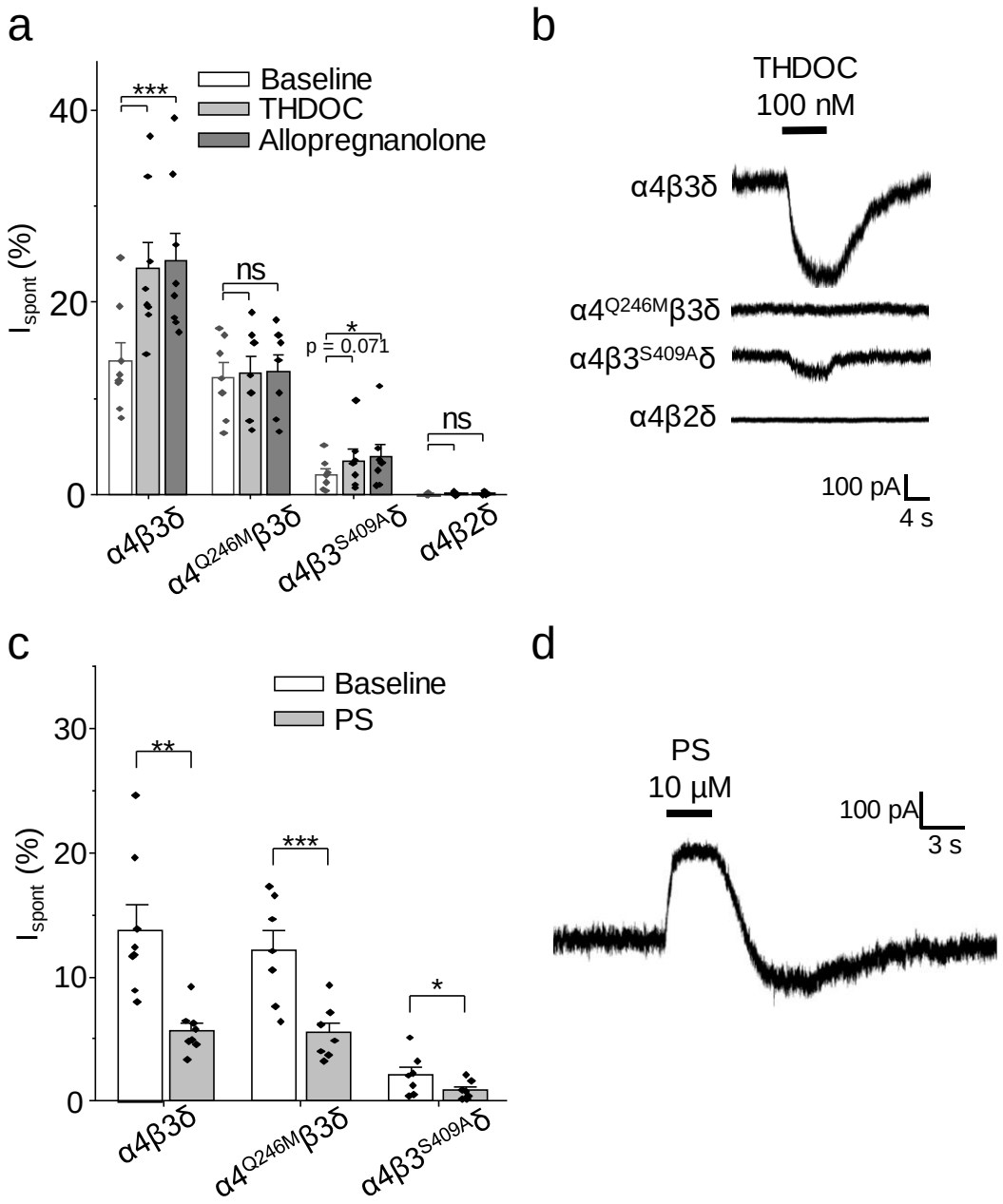

Figure 4.5: Potentiating and inhibitory neurosteroids modulate spontaneous activity. a) The effects of the neurosteroids THDOC and allopregnanolone (100 nM) on the spontaneous activity of wild-type α4β3δ (n = 8), neurosteroid-insensitive $\alpha4^{Q246M}\beta3\delta$ (n = 7), the phosphorylation mutant $\alpha4\beta3^{S409A}\delta$ (n = 7) and wild-type α4β2δ (n = 5) receptors. Significance determined by one-way repeated-measures ANOVA with Tukey's post hoc test. b) Representative recordings of THDOC (100 nM) applications to the aforementioned receptors. c) The impact of pregnenolone sulphate (10 µM) on α4β3δ (n = 8), $\alpha4^{Q246M}\beta3\delta$ (n = 7) and $\alpha4\beta3^{S409A}\delta$ (n = 7) receptors. Significance determined using paired t-tests. d) Representative recording of wild-type α4β3δ receptors during PS application. For all graphs, columns and error bars represent the mean and SEM, respectively (*p<0.05; **p<0.01; ***p<0.001; ns: no significance).

$GABA_A$ receptors are also the target for various classes of clinically important drugs, including some general anaesthetics (Garcia et al., 2010). The widely-used intravenous anaesthetic propofol is a potent positive allosteric modulator acting *via* a discrete binding site on $GABA_A$ receptors to that of neurosteroids (Yip et al., 2013). Recording from α4β3δ (n = 8), $\alpha4^{Q246M}\beta3\delta$ (n = 7) and $\alpha4\beta3^{S409A}\delta$ (n = 7) receptors revealed that propofol (1 μM) enhanced the intrinsic spontaneous activity of these receptor isoforms, potentiating spontaneous currents by 39.7%, 68.3% and 56.7%, respectively (although not statistically significant for $\alpha4\beta3^{S409A}\delta$ receptors). By contrast, this low concentration of propofol had no effect on the spontaneously-silent α4β2δ receptor (n = 5; Fig. 4.6a,b).

In contrast to the foregoing agents, benzodiazepines are more selective modulators of $GABA_A$ receptors, with effects restricted to αβγ isoforms by the presence of a specific binding site at the α^+-γ^- interface (Rudolph et al., 1999; Sigel & Ernst, 2018). To examine the role of benzodiazepines in modulating spontaneously active receptors, the partially spontaneously-active α1β3γ2L receptor was expressed in HEK cells and flurazepam (300 nM) applied. Despite the inherently low spontaneous activity of this isoform, a clear increase in the holding current of these cells was observed, increasing spontaneous activity by 103.6% (n = 7), which was not apparent for the benzodiazepine-insensitive $\alpha1^{H101R}\beta3\gamma2L$ receptor (n = 6; Fig. 4.6c,d) (Wieland et al., 1992). Previous studies have concluded that the molecular mechanisms underlying the benzodiazepine effect is to effectively increase the affinity of the receptor for the agonist, and that in the absence of agonist benzodiazepines have no effect (Twyman et al., 1989), however this contrasts with the observations described here. Our findings are in agreement with previous work that showed benzodiazepines enhance spontaneous currents of $GABA_A$ receptors (Birnir et al., 2000; Jatczak-Śliwa et al., 2018). Indeed, earlier studies examining the impact of benzodiazepines on spontaneously gating $GABA_A$ receptor mutants suggest that benzodiazepine binding at the α^+-γ^- interface lowers the energy barrier required for the receptor to gate (Campo-Soria et al., 2006). In receptors that display constitutive activity, this would translate to increased spontaneous gating. The data presented here therefore indicate that

benzodiazepines, and indeed endozepines, could partially exert their physiological effect through potentiation of the spontaneous activity of γ2-containing receptors.

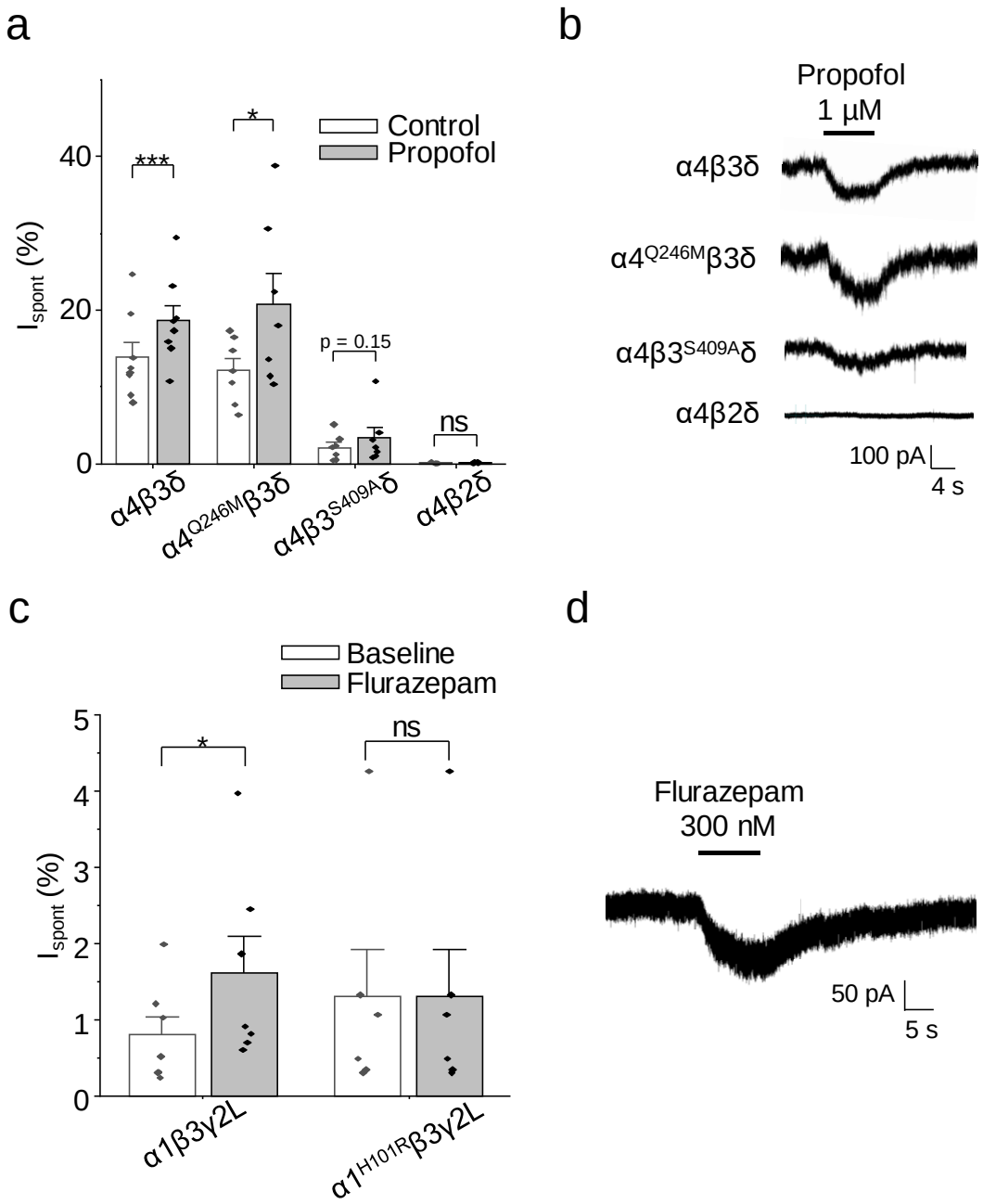

Figure 4.6: Clinically-used pharmacological compounds enhance spontaneous currents. a) The effects of the general anaesthetic propofol (1 μM) on the spontaneous activity of wild-type and mutant α4βδ receptors (n = 5-8; paired t-tests for each comparison). b) Representative traces of the aforementioned receptors exposed to 1 μM propofol. c) The effect of the benzodiazepine flurazepam (300 nM) on wild-type α1β3γ2L (n = 7; paired t-test) and benzodiazepine-insensitive $\alpha1^{H101R}\beta3\gamma2L$ (n = 6; Wilcoxon signed rank test) receptors. d) Representative recording of flurazepam (300 nM) applied to α1β3γ2L receptors. For both graphs, columns and error bars represent the mean and SEM, respectively (*p<0.05; ***p<0.001; ns: no significance).

4.3. Discussion

4.3.1. Kinase activity regulates spontaneous currents

We have demonstrated here that the magnitude of α4β3δ spontaneous activity is dependent on the phosphorylation status of two key residues within the large intracellular loop of the β3 subunit. Mutagenesis of the S408 and S409 residues to prevent their phosphorylation significantly reduced the extent of spontaneous activity. This effect was dependent on the number of phosphorylated residues, as receptors with both residues mutated did not show the same extent of impairment as single-site mutants. We have identified the kinases involved in this as PKA, PKC and PKG, all of which have been previously identified as targeting these residues on the β3 subunit (McDonald & Moss, 1997; Brandon et al., 2002b; Nakamura et al., 2015). Kinases have previously been demonstrated to have multiple effects on receptor trafficking (Kittler et al., 2005; Abramian et al., 2010; Saliba et al., 2012; Comenencia-Ortiz et al., 2014; Nakamura et al., 2015). However, by normalising our measurement of the spontaneous current to the maximum response to a saturating GABA concentration, we therefore control for alterations in surface expression caused by phosphorylation.

The impact of phosphorylation and kinase activity is in agreement with an earlier study that showed PKA-dependent enhancement of spontaneous activity through an increase in the frequency of channel opening (Tang et al., 2010). This study also showed that PKA activation in the presence of 1 μM GABA had no effect on the current. They concluded that PKA serves to modulate the spontaneous, tonic current in the absence of GABA, but that this is then superseded when GABA is present, thereby acting as a regulator during temporary reductions in ambient GABA. This study did not clearly identify the target of PKA, and we show here that PKA and other kinases mediate much of their effects on spontaneous activity through the β3 subunit.

The concept of a kinase-dependent regulation of spontaneous activity is credible in a physiological context. Spontaneous activity could be defined

as a leak conductance and not mediated by an extracellular signal, unlike conventional GABA-dependent tonic inhibition. This then represents an impairment in the ability of the individual cell and network to regulate levels of tonic current quickly and reliably to maintain a regulated level of excitability (Hartmann et al., 2008; Saliba et al., 2012; Battaglia et al., 2018). However, a dependence on the phosphorylation state gives some control to the extent of spontaneous activity. Importantly, this can be regulated through network activity (Saliba et al., 2012; Connelly et al., 2013a; Crunelli & Di Giovanni, 2014). Various signalling pathways have been implicated in altering the phosphorylation state of $GABA_A$ receptors, including receptor tyrosine kinases, dopamine receptors and $GABA_B$ receptors (Connelly et al., 2013a; Connelly et al., 2013b; Crunelli & Di Giovanni, 2014; Nakamura et al., 2015; Khatri et al., 2019). For example, dopamine receptors D1 and D2 differentially regulate GABAergic transmission in the olfactory bulb, causing a reduction in GABA responses *via* PKA-dependent effects in granule cells, whilst enhancement of responses in a PKC-dependent manner was observed in mitral cells (Brunig et al., 1999). Changes in $GABA_A$ receptor phosphorylation may therefore underlie odour detection and learning. Spontaneous $GABA_A$ receptor currents potentiated by signalling molecules could represent a novel mechanism through which network activity can be reported to these receptors in order to modulate tonic inhibition of neurons. We have shown how PKA and PKC can both phosphorylate the β3 intracellular loop, and therefore signalling molecules which alter the activity of these kinases will impact on spontaneous, tonic inhibition. Activation of G-protein-coupled receptors (GPCRs), such as the dopamine receptors, result in the activation of GTPases to alter kinase activity (Connelly et al., 2013a; Crunelli & Di Giovanni, 2014; Huang & Thathiah, 2015). The $G_{\alpha s}$ GTPase activates PKA through the generation of cAMP *via* adenylate cyclase, whilst the $G_{\alpha i/o}$ GTPase inhibits this enzyme, thereby reducing PKA activity (Hanlon & Andrew, 2015). Receptors coupled to $G_{\alpha q}$ cause the activation of phospholipase C, catalysing the formation of diacylglycerol (DAG) and inositol trisphosphate (IP_3), the latter of which increases intracellular Ca^{2+}, activating PKC, whilst DAG promotes its association to the membrane (Steinberg, 2008; Hanlon & Andrew, 2015). The activation of these GPCRs by monoamines, glutamate or GABA therefore

represents a mechanism by which phosphorylation of receptors, and subsequent spontaneous activity, is sensitive and responsive to neural network signals.

The reporting of network activity and subsequent changes to tonic inhibition represents a form of neuronal plasticity. The maintenance of a tonic inhibitory current controls the excitability of neurons (Mitchell & Silver, 2003; Chen et al., 2010; Lee & Maguire, 2014; Pavlov et al., 2014), and knock-out of the α6 subunit in mice cerebellar granule cells resulted in an upregulation of two-pore domain K^+ channels to maintain the shunting inhibition which would be lost with the loss of α6βδ receptors in these animals (Brickley et al., 2001). These neurons are therefore sensitive to changes in tonic inhibition and upregulate channels accordingly to maintain normal levels of activity (Chen et al., 2010). Phosphorylation may therefore represent a mechanism through which homeostatic plasticity regulates the level of tonic inhibition (Mody, 2005; Chen et al., 2010).

Neuronal plasticity is also exemplified by an effect of phosphorylation on the surface expression of $GABA_A$ receptors, either increasing or decreasing their number through an effect on trafficking (Kittler & Moss, 2003; Mody, 2005). Protein kinase B (PKB)-dependent phosphorylation of the S410 residue on β2 increases membrane insertion (Wang et al., 2003), and phosphorylation of β3 at S408 and S409 promotes surface expression through suppression of internalisation *via* AP2-binding (Kittler et al., 2005). However the impact of phosphorylation on tonic inhibition is far from clear, as PKC activation reduces tonic currents in dentate gyrus granule cells and dorsal lateral geniculate relay neurons (Bright & Smart, 2013b; Connelly et al., 2013a). The effect of phosphorylation on tonic inhibition is likely dependent on receptor isoform and kinase identity, as well as the regional expression of other proteins altering $GABA_A$ receptor activity (Brandon et al., 2002a; Kittler & Moss, 2003). For example, the GABA uptake transporter GAT-1 can be phosphorylated, altering its localisation and activity (Scimemi, 2014). This will then impact on GABA-mediated tonic currents. There is likely a complex interplay between kinase activity, receptor surface expression, spontaneous gating and tonic inhibition. Despite this, phosphorylation-dependent modulation of spontaneous activity

represents a novel mechanism through which neurons can maintain normal levels of excitability or alter excitability depending on the needs of the system, such as during changes in network activity or in regional/temporal absences of ambient GABA.

Another aspect of cell activity in which phosphorylation plays a role is activity-dependent plasticity. Calcium-dependent processes are found throughout homeostatic and potentiating/depressing plasticity, including plasticity of $GABA_A$ receptors and synapses utilising GABA (Gaiarsa et al., 2002; Mody, 2005). CaMKII, activated *via* Ca^{2+} influx, is known to be involved in many processes involving neuronal plasticity (Zalcman et al., 2018). CaMKII has been determined to promote phosphorylation at the β3 S408 and S409 residues through GST binding assays (McDonald & Moss, 1997), but experiments testing receptor function showed no effect of CaMKII at these residues and, instead, the effects were almost entirely mediated through phosphorylation of the S383 residue (Houston et al., 2007). We did not observe any effect on the spontaneous activity profile when mutating the CaMKII-targeted S383 residue of β3, and this was confirmed by the application of the CaMKII inhibitor KN-62, supporting the hypothesis that CaMKII activity does not impact on spontaneous currents. However, the functionality of CaMKII in HEK cells has been questioned, and instead NG-108 cells are a better expression system to use, likely due to their neuronal lineage (Houston & Smart, 2006).

The concept of a CaMKII-dependent alteration in the level of spontaneous activity would be intriguing. CaMKII activation has been shown to increase the amplitude of IPSCs in a variety of areas, including spinal cord dorsal horn neurons and hippocampal CA1 neurons (Wang et al., 1995) and cerebellar granule neurons (Houston & Smart, 2006; Houston et al., 2008). It has also been shown that phosphorylation of S383 promotes the insertion of extrasynaptic receptors in the hippocampus, thereby increasing tonic inhibition (Saliba et al., 2012). Importantly, the activation of CaMKII and the receptor insertion into the membrane *via* phosphorylation of S383 were shown to be dependent on neuronal activity in a bi-directional manner (Saliba et al., 2012). Further experiments in expression systems such as NG-108 cells are required

to fully understand the impact of CaMKII on spontaneous activity (Houston & Smart, 2006). This would indicate whether CaMKII-mediated phosphorylation of the S408/409 sites occurs, and if phosphorylation of the S383 site impacts on spontaneous activity. If either of these events occur, then CaMKII-dependent phosphorylation of extrasynaptic (and indeed, synaptic) receptors would both increase membrane insertion and enhance the spontaneous activity of receptors, resulting in a potentially significant increase in tonic inhibition through both GABA-dependent and independent mechanisms.

What is the mechanism by which phosphorylation affects the level of spontaneous activity? Investigations have already revealed a possible role for the large intracellular loop of α subunits in determining the gating kinetics (Fisher, 2004), and modifications of the intracellular loop of other pentameric receptors, including $5HT_3$ and nicotinic acetylcholine receptors, have revealed changes to single channel conductance and gating (Milone et al., 1998; Akk & Steinbach, 2000; Kelley et al., 2003; Hales et al., 2006). Phosphorylation of the loop could therefore impact on spontaneous receptor kinetics. In the absence of direct kinetic models of spontaneous activity which take into account phosphorylation states, we can speculate by examining previous studies which highlight the effects of phosphorylation on receptor kinetics. Investigations of PKC-dependent phosphorylation of $GABA_A$ receptors indicate that phosphorylation of α1β2γ2 receptors decreases the slow phase of desensitisation (Leidenheimer & Chapell, 1997). Activation of PKA has also been demonstrated to affect deactivation and desensitisation kinetics of α1β1/3γ2 receptors, with an increased rate and extent of desensitisation and a decreased rate of deactivation (Hinkle & Macdonald, 2003). Interestingly, this effect on desensitisation and deactivation was only dependent on phosphorylation of the S409 residue of β3. We have shown that the singular removal of either the S408 or S409 phosphorylation sites through mutagenesis impairs spontaneous activity to a larger extent than when mutating both together. The large impact of single mutations could be linked with the movement of the $GABA_A$ receptors into various conformational states. It is unknown to what extent impaired/promoted desensitisation kinetics has on the spontaneous activity of receptors, or even if these spontaneous receptors

transition into a desensitised state as they are not bound to GABA. However, they evidently enter into an open conformation which is likely similar, if not identical, to a GABA-bound open state, as indicated by the unimpaired potentiation of these currents by modulators such as neurosteroids and benzodiazepines.

4.3.2. The role of neurosteroids in modulating spontaneous activity

We have demonstrated here the efficacy of conventional allosteric modulators to alter currents arising from the spontaneous activity of $GABA_A$ receptors. Endogenous modulators such as the neurosteroids, both potentiating and inhibitory, as well as clinically used pharmacological modulators, such as the anaesthetic propofol and benzodiazepines, exert an influence on spontaneous currents. This modulation does not occur *via* direct activation of the receptor, as there was no effect on the non-spontaneous α4β2δ receptor, and the neurosteroids and propofol only marginally increased the minor spontaneous currents of the $\alpha4\beta3^{S409A}\delta$ receptor.

We examined here the neurosteroids THDOC and allopregnanolone. These potentiating neurosteroids enhance both the frequency and duration of channel openings in the presence of GABA (Twyman & Macdonald, 1992). We can suggest that they exert similar effects on spontaneous channel activity too, although detailed analysis of single channel currents should be performed to firmly establish this. Neurosteroids bind at the interface within the transmembrane domains at the β^+-α^- interface (Laverty et al., 2017; Miller et al., 2017). This site, although partially contributed to by the β subunit, is distinct from the GKER motif within the ECD of the β3 subunit at the α^+-β^- interface; therefore, neurosteroid binding is unlikely to have direct effects on spontaneous activity *via* an effect on these residues. Instead, neurosteroid, and indeed benzodiazepine and propofol, binding is likely to result in a general enhancement of receptor gating and acts downstream of both GABA-binding and spontaneous channel activity originating in the ECD. Single channel analysis of the potentiation of both GABA-mediated and spontaneous currents

may show a difference in gating kinetics, identifying differences in the transduction pathway arising from spontaneous and GABA-mediated origins.

The effects of the potentiating neurosteroids have a significant dependence on subunit composition of the $GABA_A$ receptor (Stell et al., 2003; Smith et al., 2007; Carver & Reddy, 2013). The identity of the γ subunit impacts on the potency of neurosteroids (γ1-containing receptors are not potentiated at normal physiological concentrations) and the presence of the δ subunit significantly increases potentiation (Belelli et al., 2002; Lambert et al., 2003). The greater effect on extrasynaptic δ-containing receptors suggests an important role for neurosteroid modulation of tonic currents, of which spontaneous activity may be physiologically relevant (Brown et al., 2002).

The inhibitory neurosteroid PS blocked spontaneous currents as they do GABA-dependent currents, binding to a site distinct from that of THDOC and allopregnanolone (Akk et al., 2001; Seljeset et al., 2015; Laverty et al., 2017; Seljeset et al., 2018). Similar to the potentiating neurosteroids, PS shows a higher efficacy at extrasynaptic δ-containing receptors (Seljeset et al., 2018), indicating a greater impact on tonic currents. Interestingly, PS appears to inhibit $GABA_A$ receptors by promoting desensitisation; there is more pronounced inhibition at higher GABA concentrations, possibly indicating preferential binding of PS to desensitised receptors (Seljeset et al., 2018). As previously discussed, it is unknown whether spontaneous receptors transition into a desensitised conformation, but if this occurred they would represent preferential targets for PS to bind and stabilise the closed, desensitised state.

The modulation by both classes of neurosteroids on spontaneous tonic currents will be affected by ambient GABA concentrations and receptor subunit composition. For example, they might have a greater impact in the dentate gyrus, where there is high spontaneous activity likely mediated by α4βδ receptors (Stell et al., 2003; Wlodarczyk et al., 2013), than in CA3 and CA1 areas where the less spontaneous α5βγ2 receptor predominates in generating the tonic current (Caraiscos et al., 2004). Tonic inhibition has a role in many brain functions, and the impact of neurosteroids on these processes

has been studied (Wang, 2011; Carver & Reddy, 2013). PS inhibition of GABAergic activity is associated with improved learning and memory (Reddy & Kulkarni, 1998; Ratner et al., 2019), whilst THDOC application promotes anxiolysis and is anticonvulsant (Biagini et al., 2006; Longone et al., 2011; Reddy, 2011; Reddy & Estes, 2016). These mechanisms of action occur *via* potentiation of both synaptic and extrasynaptic $GABA_A$ receptors, the latter of which likely includes modulation of spontaneous activity (Herd et al., 2007). Intriguingly, fluctuations in neurosteroid concentrations have a direct effect on the expression of $GABA_A$ receptors associated with tonic inhibition (Maguire & Mody, 2009; Carver & Reddy, 2013; Abramian et al., 2014). Brief exposure to neurosteroids due to hormonal fluctuation upregulates δ-containing receptors, increasing tonic inhibition and resulting in anxiolysis, whilst prolonged upregulation in levels decreases δ-containing receptors, possibly reflecting a homeostatic mechanism (Hsu et al., 2003; Maguire et al., 2005; Wu et al., 2013). Changes in subunit expression will impact on the level of spontaneous activity. Therefore, neurosteroid levels have a direct impact on both short-term GABA-dependent and -independent receptor activity, and long-term levels of tonic inhibition mediated by spontaneously active $GABA_A$ receptors.

4.3.3. Interactions between phosphorylation and neuromodulators of $GABA_A$ receptors: consequences for neuronal excitability

The present study has demonstrated the ability of spontaneous currents to be modulated by phosphorylation and pharmacological mechanisms. There is considerable evidence that these two distinct modes of modulation are interlinked, and that overall $GABA_A$ receptor activity is determined, to an extent, by this interaction (Comenencia-Ortiz et al., 2014; Parakala et al., 2019).

Early studies examining phosphorylation and allosteric modulators showed greater binding of the benzodiazepine flunitrazepam after phosphorylation of the α1 subunit by CaMKII (Churn et al., 2002). Increased current amplitude in response to benzodiazepines has also been observed

after kinase activation, suggesting an impact on receptor kinetics (Leidenheimer et al., 1993). However, this effect was instead isolated to an effect of phosphorylation on receptor internalisation and trafficking (Ghansah & Weiss, 2001). Receptor phosphorylation is also intertwined with modulation by neurosteroids. A reciprocal effect of neurosteroids on phosphorylation has been observed, whereby activation of PKC-dependent phosphorylation of S443 of the α4 subunit is promoted by neurosteroids, increasing surface expression of this receptor and hence increasing neurosteroid-dependent currents (Abramian et al., 2010; Abramian et al., 2014). Similarly, PKC-dependent phosphorylation of β3 S408 and S409 is upregulated by neurosteroid activity, and this again promotes insertion into the membrane of both synaptic α1β3γ2 and α4β3δ receptors (Adams et al., 2015; Parakala et al., 2019). Phosphorylation of either of these two residues also results in greater ability of THDOC to potentiate GABA-mediated currents independent of changes to receptor expression (Adams et al., 2015). This could be related to our findings which show phosphorylation of these residues is required for maximal spontaneous activity. If phosphorylation of these residues impacts on receptor gating, then it is unsurprising that both spontaneous and neurosteroid-dependent gating is affected. We saw very little spontaneous activity when expressing $\alpha4\beta3^{S409A}\delta$ receptors, and due to this low intrinsic spontaneity the application of neurosteroids and propofol did not make significant alterations to the measured spontaneous current. However, despite the small effect, the relative amplification of spontaneous currents was no different to that of wild-type receptors. Removal of both S408 and S409 phosphorylation sites has been previously shown to significantly reduce neurosteroid potentiation of GABA-mediated currents (Adams et al., 2015). Examining the impact of neurosteroid potentiation of the spontaneous current at the phospho-null double mutant $\alpha4\beta3^{S408A,S409A}\delta$ receptor would provide further information about the dependence of neurosteroid potentiation of spontaneous activity on the phosphorylation status of the receptor.

4.4. Conclusion

We have demonstrated a clear dependence of the spontaneous current of recombinant $GABA_A$ receptors on the phosphorylation state and on neuromodulation by endogenous agents. This represents a novel avenue through which tonic inhibition can be regulated, and also illuminates further complexity in the ability of phosphorylation to regulate inhibition within the brain. The plasticity of these spontaneous currents allows these receptors to act not simply as leak currents, but as a highly regulated mechanism to control neuronal activity.

Chapter 5: The physiological and pathophysiological role of spontaneous $GABA_A$ receptor activity

5.1. Introduction

In the previous sections, recombinant receptors were used to investigate key receptor structural determinants of spontaneous activity, and explored how this spontaneous activity can be regulated by endogenous modulators. To further understand the role of spontaneous $GABA_A$ receptor activity in a native environment, the next step was to investigate such spontaneity in neuronal cultures and in acutely prepared brain slices, which retain their cytoarchitecture and wiring. This approach should provide a better understanding of the physiological role of spontaneous $GABA_A$ receptor activity.

Previous studies have identified spontaneous activity of recombinant $GABA_A$ receptors (Khrestchatisky et al., 1989; Sigel et al., 1989; Cestari et al., 1996; Wooltorton et al., 1997; Neelands et al., 1999; Tang et al., 2010). In terms of a reductionist approach, this is straight-forward, as a single receptor subtype can be expressed and recorded from in isolation of other confounding factors (Thomas & Smart, 2005). For example, neurons express a panoply of different $GABA_A$ receptor subunits (Sieghart & Sperk, 2002; Olsen & Sieghart, 2008) and so deducing the isoform responsible for spontaneous currents, and indeed distinguishing between GABA-mediated and spontaneous tonic currents, is problematic. Despite these difficulties, some earlier studies have attempted to investigate spontaneous receptor openings in both cultured neurons and brain slices (Birnir et al., 2000; McCartney et al., 2007; Wlodarczyk et al., 2013; O'Neill & Sylantyev, 2018).

Whole-cell recordings from hippocampal neurons in culture revealed significant tonic current blocked by PTX and bicuculline, a competitive antagonist with negative allosteric properties, but little effect with gabazine, which has less intrinsic negative modulatory effects and so predominantly blocks GABA-activated currents (Ueno et al., 1997; McCartney et al., 2007).

This was confirmed with single channel analysis from outside-out patches of these neurons, which showed the presence of gabazine-resistant spontaneous activity with conductance states comparable to those of GABA-activated $GABA_A$ receptors (McCartney et al., 2007).

Acute brain slices, which better recapitulate the native environment for synaptic architecture and receptor distribution, have also been investigated for spontaneous $GABA_A$ receptor activity (Wlodarczyk et al., 2013; O'Neill & Sylantyev, 2018). Cell-attached recordings of CA1 pyramidal neurons show spontaneous $GABA_A$ receptor openings which were unaffected by gabazine, but potentiated by diazepam, indicating the spontaneous opening of γ subunit-containing receptors (Birnir et al., 2000). Spontaneous receptor activity has also been observed in dentate gyrus granule cells (DGGCs), where it was estimated to account for almost all the tonic inhibition in the dentate gyrus, indicating a clear importance for spontaneous activity in regulating hippocampal activity (Wlodarczyk et al., 2013). Single channel analysis of spontaneous currents of DGGCs reveal similar conductance states to GABA-activated counterparts, and that GABA serves to increase the frequency and duration of burst activity (O'Neill & Sylantyev, 2018).

These studies are important for showing that neurons express spontaneously gating $GABA_A$ receptors. However, to date the identity of these receptors has not been determined and their role in neuronal activity is yet to be fully explored (O'Neill & Sylantyev, 2019). In this chapter, the experimental data reveal that the β3 subunit and the GKER motif are as essential for promoting spontaneous activity in a neuronal environment as they are in recombinant receptors, and that enhancement of spontaneous currents can regulate neuronal excitability.

5.2. Results

5.2.1. The β3 subunit is required for inducing spontaneous currents in hippocampal neurons in culture

Initially, hippocampal neurons in culture were investigated for the presence of spontaneous activity, and whether our findings in HEK cells, that the β3 subunit was essential for spontaneous activity, would be consistent in a neuronal setting. Gabazine and PTX were applied as probes to distinguish between GABA-activated and spontaneous currents, respectively (Ueno et al., 1997; McCartney et al., 2007; Wlodarczyk et al., 2013), although significant levels of GABA-activated tonic currents were not expected due to continual superfusion of the cells. In addition to examining these components at native receptors, the $GABA_A$ receptor subunit composition of neurons was also manipulated by cDNA transfection.

Neurons transfected with either single α4, β3 or δ subunit cDNAs were recorded from, comparing the gabazine and PTX responses to neurons transfected with control eGFP. This was repeated using neurons transfected with all three (α4, β3 and δ) subunit cDNAs. Under control conditions holding at -60 mV, small albeit non-significant *increases* in the holding current were observed when gabazine (1 μM) was applied (0.06 ± 0.03 pA/pF, n = 8; Fig. 5.1a). This unexpected result is consistent with previous reports that show partial agonist effects of gabazine in hippocampal cultures and brain slices, despite a clear competitive antagonist and negative allosteric effect in recombinant receptors and when GABA concentrations are more pronounced (Wlodarczyk et al., 2013; O'Neill & Sylantyev, 2018). In control neurons, there was a small but significant spontaneous current revealed by PTX application (0.10 ± 0.02 pA/pF, n = 8; Fig. 5.1b). PTX did not abolish spontaneous inhibitory postsynaptic currents (sIPSCs) entirely, likely due to the use-dependence of the antagonist. Similar small inward and outward currents were noted in response to gabazine and PTX application for both α4 (gabazine: 0.003 ± 0.007 pA/pF; PTX: 0.04 ± 0.01 pA/pF; n = 6) and δ (gabazine: 0.03 ± 0.01 pA/pF; PTX: 0.10 ± 0.02 pA/pF; n = 7) cDNA-transfected neurons (Fig.

5.1a,b). Intriguingly, significant holding currents were revealed in neurons transfected with all three (α4, β3 and δ) subunit cDNAs, or with β3 cDNA alone. Recording from these neurons, gabazine caused inhibition of the holding current (α4β3δ: 0.73 ± 0.1 pA/pF, n = 6; β3: 0.54 ± 0.12 pA/pF, n = 7; Fig. 5.1a), as did PTX but to a greater (2-3 fold) extent (α4β3δ: 1.83 ± 0.40 pA/pF, n = 6; β3: 1.56 ± 0.40 pA/pF, n = 7; Fig. 5.1b). The blockade of the holding current by gabazine is most likely due to its negative allosteric properties rather than competitive antagonism of ambient GABA, as similar effects were seen with recombinant α4β3δ receptors expressed in HEK cells, where ambient GABA is absent (Fig. 3.3b). The small partial agonist effect observed under control conditions was absent when the spontaneous tonic current was high. PTX application confirmed the significant level of spontaneous activity in these neurons.

Intriguingly, the level of block by both gabazine and PTX was not significantly different between neurons transfected with all three subunit cDNAs and those transfected with β3 cDNA alone. Coupled with the other observation, that transfection of either α4 or δ subunit cDNAs alone does not impact on spontaneous activity of the neuron, leads to the conclusion that the presence of the β3 subunit is the limiting factor in the expression of spontaneously active receptors. This is consistent with our findings in HEK cells, where the presence of the β3 subunit is largely responsible for spontaneous currents for all receptor subunit combinations examined. Example whole-cell recordings of gabazine and PTX application are shown in Fig. 5.1c. The slight, albeit non-significant, agonist behaviour exhibited by gabazine in native receptors has not been examined in great detail before. Application of gabazine to α4β3δ and α5β3γ2L receptors expressed in HEK cells, which are predicted to be responsible for tonic currents in hippocampal neurons (Caraiscos et al., 2004; Scimemi et al., 2005; Lee & Maguire, 2014), resulted in no agonist effect and, consistent with our previous results (Fig. 3.3b) caused blockade of spontaneous activity rather than agonism (Fig. 5.1d). It is therefore still unclear as to what causes the small increase in holding current when gabazine is applied to these neurons.

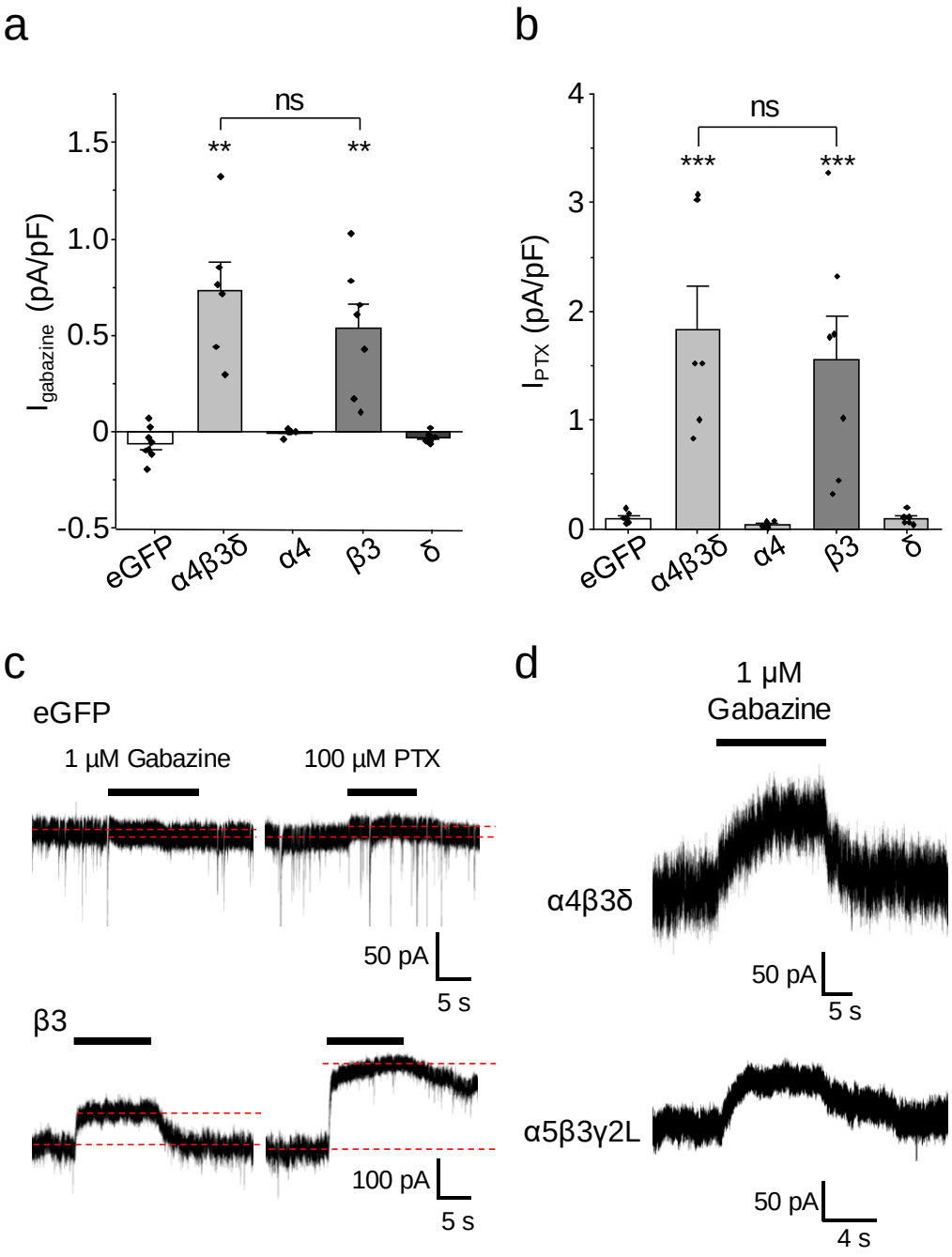

Figure 5.1: Primary hippocampal neurons display a spontaneous current which can be enhanced through transfection with β3 subunit cDNA. Hippocampal neurons in culture were transfected with the cDNA of eGFP alone (n = 8), eGFP with α4 (n = 6), β3 (n = 7) or δ (n = 7) subunit cDNAs, or eGFP with all three subunit cDNAs combined (n = 6). Application of 1μM gabazine (a) or 100 μM PTX (b) revealed changes in the spontaneous holding current density. For both graphs, columns and error bars represent the mean and SEM, respectively (Kruskal-Wallis with Dunn's post hoc test and ANOVA with Tukey's post hoc test were used for (a) and (b), respectively; **p<0.01; ***p<0.001; ns: no significance). c) Representative recordings of neurons transfected with cDNAs of either eGFP alone or with β3, and exposed to gabazine and PTX. Dashed lines were manually drawn and indicate the holding currents before and during application. d) Representative recordings of HEK cells expressing α4β3δ or α5β3γ2L receptors exposed to 1 μM gabazine.

When transfecting neurons with β3 cDNA alone, we may be inducing overexpression of the subunit. Several experiments were performed to determine if these β3 subunits were being incorporated into triheteromeric $GABA_A$ receptors, rather than forming non-physiological homomers and thus contributing to spontaneous activity (Wooltorton et al., 1997). Firstly, transfected cells were exposed to 1 μM GABA, which induced significantly larger whole-cell currents in β3-transfected cells compared to eGFP controls (27.4 ± 4.3 pA/pF, n = 11; and 14.1 ± 1.5 pA/pF, n = 10, respectively; Fig. 5.2a). As β3 homomeric receptors lack an orthosteric binding site (Cestari et al., 1996; Wooltorton et al., 1997), this indicates that enhancing the β3 subunit expression promotes the formation of heteromeric receptors. However, this does not discount a subpopulation of homomeric receptors. The lack of an orthosteric binding site additionally results in these receptors being unaffected by the negative allosteric effects of gabazine, but are still blocked by PTX binding (Fig. 3.3b). If a population of homomeric β3 receptors were present and contributing to the spontaneous current, we would therefore expect the gabazine/PTX inhibition ratio to be significantly lower for β3-transfected neurons compared to neurons transfected with all three subunits. However, there was no significant difference in this ratio between neurons transfected with α4, β3 and δ subunit cDNAs, and those transfected with just β3 (0.42 ± 0.07, n = 7; and 0.37 ± 0.04, n = 7, respectively; Fig. 5.2b). Moreover, there was also no significant difference in the inhibition ratio between neurons and HEK cells expressing α4β3δ receptors (0.33 ± 0.04, n = 6), suggesting that ambient GABA does not contribute to the gabazine-sensitive tonic current in these neurons.

To further investigate whether these tonic currents were caused either by intrinsic spontaneous $GABA_A$ receptor activity or receptor activation by ambient GABA, cultured neurons were incubated for 1-2 hrs with 500 nM concanamycin A. This inhibitor of vesicular H^+-ATPase will prevent normal vesicular refiling with neurotransmitter at axon terminals (Muroi et al., 1993). Treating neurons with concanamycin-A caused a cessation of sIPSCs (Fig. 5.2d), indicating an absence of synaptic GABA release, but no difference in the amplitude of the PTX-sensitive current of concanamycin-treated neurons

(0.33 ± 0.11 pA/pF, n = 8) compared with neurons treated with a DMSO control (0.22 ± 0.08, n = 6; Fig. 5.2c). This was also true for neurons transfected with β3 cDNA (1.73 ± 0.30, n = 6; and 1.51 ± 0.11 pA/pF, n = 6 under control and concanamycin-treated conditions, respectively; Fig. 5.2c,e). Since ambient GABA concentration depends, in part, upon synaptic activity (Bright et al., 2007), the preservation of the tonic current in the absence of vesicular GABA release suggests the predominance of spontaneous $GABA_A$ receptor opening in these neurons.

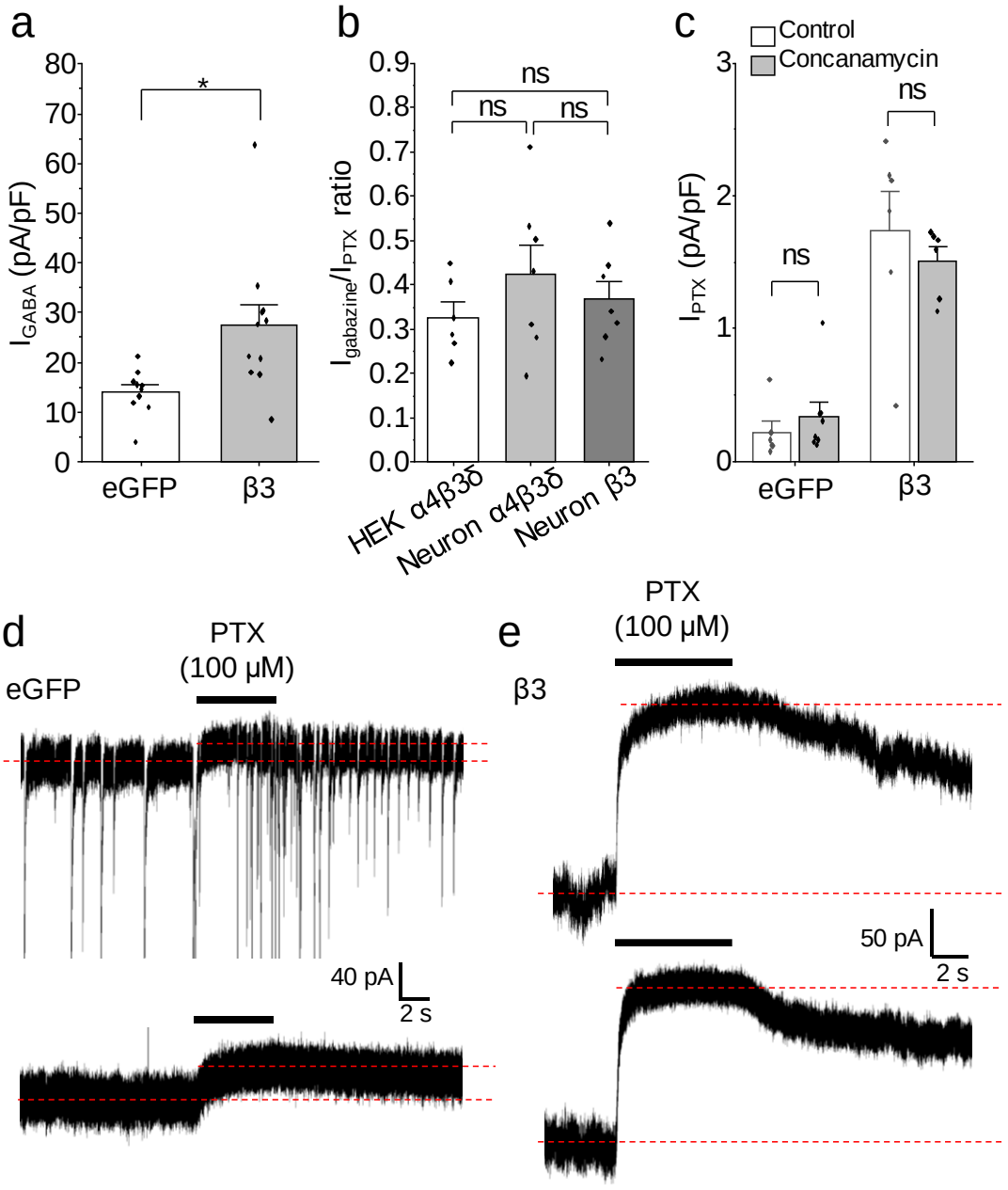

Figure 5.2: Hippocampal neurons transfected with β3 subunit cDNA predominantly form spontaneously active heteromeric GABA$_A$ receptors. a) Current density response to 1 μM GABA in neurons transfected with β3 cDNA (n = 11) compared to control (n = 10; unpaired t-test). b) The ratio of the inhibitory response to gabazine (1 μM) and PTX (100 μM) of neurons transfected with α4, β3 and δ cDNAs (n = 7) or with β3 alone (n = 7), and in HEK cells expressing α4β3δ receptors (n = 6; ANOVA with Tukey's post hoc test). c) Current density of hippocampal neurons transfected with eGFP alone (n = 6-8) or with β3 (n = 6) cDNA were incubated with concanamycin A (500 nM) for 1-2 hrs to prevent vesicular replenishment and subsequent synaptic neurotransmitter release (Wilcoxon signed-rank test and unpaired t-test used for eGFP and β3 conditions, respectively). Representative recordings of eGFP- (d) and β3-transfected neurons (e) incubated in control (upper panels) or concanamycin (lower panels) during PTX application. IPSCs in (d) are cropped for clarity. Dashed lines were manually drawn and indicate the holding currents before and during application. For all graphs, columns and error bars represent the mean and SEM, respectively (*p<0.05; ns: no significance).

Previous experiments demonstrated the importance of the GKER motif (Fig. 3.4b) and the phosphorylation state of the intracellular loop (Fig. 4.1b) in the β3 subunit for determining receptor spontaneous activity in HEK cells. The importance of both these aspects was investigated in a neuronal setting by expressing the β3 mutants $\beta3^{DNTK}$ and $\beta3^{S409A}$ in neurons. Assembly of either mutant subunit impaired spontaneous activity in $\alpha4\beta3^{DNTK}\delta$ and $\alpha4\beta3^{S409A}\delta$ receptors expressed in HEK cells. We predicted a similar impairment to spontaneous activity in neurons. Expressing $\beta3^{DNTK}$ in neurons resulted in a slight (but non-significant) increase in the level of spontaneous activity (0.44 ± 0.10 pA/pF, n = 7) compared to eGFP controls (0.12 ± 0.03 pA/pF, n = 9), and significantly less than achieved by expressing wild-type β3 (2.46 ± 0.34 pA/pF, n = 8; Fig. 5.3a). Expressing $\beta3^{S409A}$, however, showed larger spontaneous activity (2.73 ± 0.39 pA/pF, n = 7) than control eGFP, and no different from wild-type β3 (Fig. 5.3a).

To investigate further, the sIPSC amplitude and frequency of eGFP-, β3- and $\beta3^{S409A}$-expressing neurons was examined, as this may give an indication as to changes in receptor expression or neuronal activity to explain the high spontaneous current conferred by transfection of the $\beta3^{S409A}$ subunit. The sIPSC amplitude of β3-expressing neurons was smaller, but not significantly so, than either eGFP or $\beta3^{S409A}$-transfected neurons (eGFP: 165.7 ± 27.8 pA, n = 6; β3: 107.2 ± 29.0 pA, n = 6; $\beta3^{S409A}$: 186.9 ± 25.1 pA, n = 5; Fig. 5.3b). The frequency of sIPSCs showed a significant (7-8 fold) reduction in β3-, but not $\beta3^{S409A}$-transfected neurons (eGFP: 0.81 ± 0.19 Hz, n = 6; β3: 0.11 ± 0.02 Hz, n = 5; $\beta3^{S409A}$: 0.75 ± 0.15 Hz, n = 5; Fig. 5.3c). To ensure the higher sIPSC frequency in $\beta3^{S409A}$-transfected neurons did not lead to ambient GABA causing a tonic effect and thus providing false estimates of spontaneous activity, we measured the gabazine/PTX ratio. However, in $\beta3^{S409A}$-transfected neurons, this was not significantly different from wild-type β3 (0.37 ± 0.07, n = 7; and 0.31 ± 0.03, n = 8, respectively; Fig. 5.3d). Example recordings of eGFP-, β3- and $\beta3^{S409A}$-transfected neurons are shown in Fig. 5.3e.

The unexpected ability of the $\beta3^{S409A}$ subunit to promote spontaneous current in transfected neurons to the same extent as wild-type β3 could

perhaps be explained by a change in trafficking, with mutant subunits being expressed to a higher degree throughout the neuron (as indicated by expression in HEK cells; Fig. 4.1c). Spontaneous tonic current, although impaired at the level of the single receptor, may be compensated for by the presence of greater total receptor number.

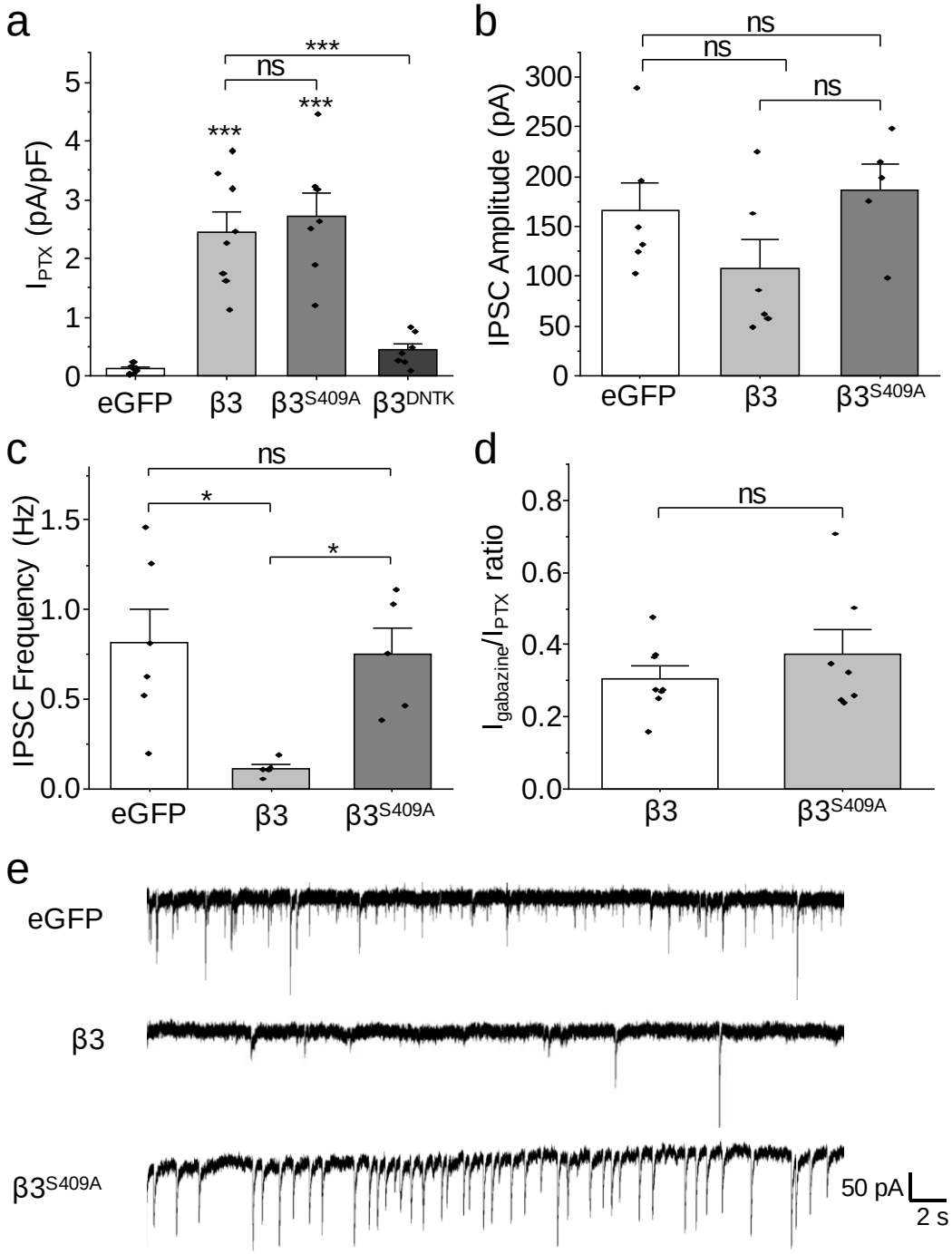

Figure 5.3: Spontaneous activity in neurons is supported by the GKER motif in β3 subunits, but phosphorylation is less important due to other changes in receptor activity. a) Spontaneous currents, determined by PTX (100 μM) application to hippocampal neurons transfected with cDNAs for wild-type and mutant β3 subunits which displayed impaired spontaneous properties in recombinant receptors (n = 7-9). Prolonged (5-10 mins) recordings (n = 5-6) from these neurons allowed the calculation of the mean sIPSC amplitude (b) and frequency (c). ANOVA with Tukey's post hoc tests used for comparisons in (a), (b) and (c). d) The ratio of inhibition by gabazine (1 μM) and PTX (100 μM) of neurons expressing either β3 (n = 8) or $β3^{S409A}$ (n = 7; unpaired t-test). e) Representative recordings from neurons transfected with either eGFP cDNA alone or with β3 or $β3^{S409A}$ cDNAs. For all graphs, columns and error bars represent the mean and SEM, respectively (*p<0.05; ***p<0.001; ns: no significance).

The importance of β3 expression in promoting spontaneous current in both heterologous expression systems and in cultured neurons seems clear. However, the previous experiments have relied on transfection with another subunit. To corroborate our findings, we adopted a different approach based on 'subunit knockdown'. The role of native β3 subunits in generating spontaneous tonic currents was investigated by transfecting neurons with β3-selective short hairpin RNAs (shRNAs) to reduce β3 subunit expression (Paddison et al., 2002; Li et al., 2005). Knock-down of native β3 subunits was confirmed using immunocytochemistry in conjunction with confocal microscopy. Neurons were permeabilised and native β3 subunits were labelled with a β3-selective antibody recognising epitopes within the intracellular loop. This indicated a reduction to 64.1 ± 7.4% β3 expression levels in neurons transfected with shRNA compared to those transfected with a scrambled control (n = 23 and 26, respectively; Fig. 5.4a,b). This targeted knock-down of β3 was accompanied by a 52% reduction in spontaneous current density compared with neurons transfected with the scrambled control (0.12 ± 0.03 pA/pF, n = 7; and 0.25 ± 0.03 pA/pF, n = 8, respectively; Fig. 5.4c). The residual spontaneous holding current is likely to reflect incomplete knock-down of the β3 subunit. Intriguingly, the sIPSC amplitude was unchanged, indicating that β3-containing receptors contribute little to synaptic inhibition in these neurons (134.9 ± 10.5 pA, n = 9; and 135.7 ± 22.2 pA, n = 7 for scrambled and shRNA conditions, respectively; Fig. 5.4d). There was also a slight, albeit non-significant, increase in the IPSC frequency when cells were transfected with shRNA (scrambled: 0.61 ± 0.14 Hz, n = 9; shRNA: 1.08 ± 0.29, n = 8; unpaired t-test, p = 0.18). This is in contrast to the dramatic reduction in sIPSC frequency when neurons were transfected with β3 subunit-containing receptors (Fig. 5.3c). This further suggests that β3-containing $GABA_A$ receptors may be involved in determining IPSC frequency of hippocampal neurons in culture.

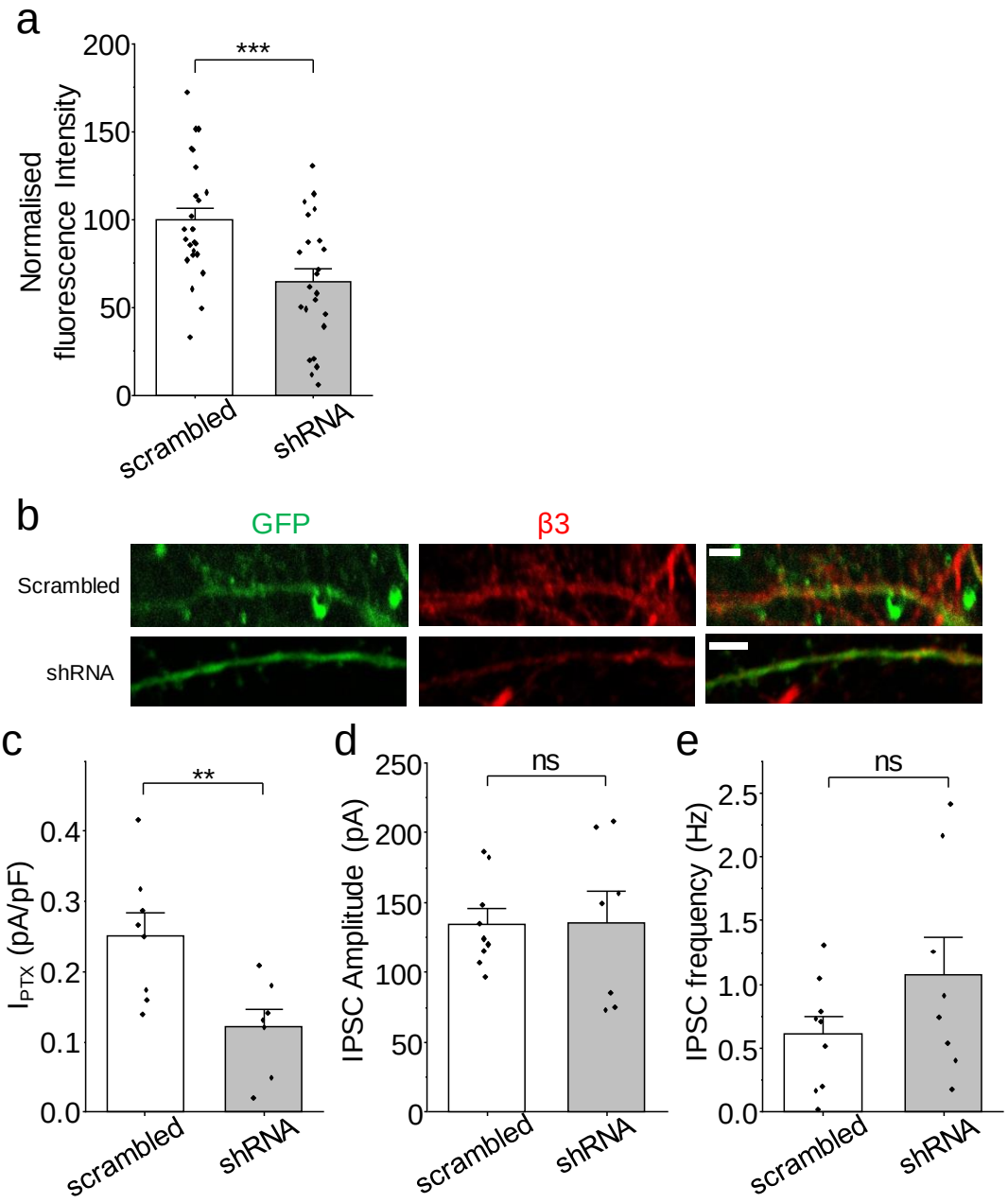

Figure 5.4: shRNA-mediated knockdown of β3 reduced spontaneous tonic current without affecting sIPSC amplitude. Hippocampal neurons were transfected with two shRNAs selective for rat β3, or a scrambled control. Cells were identified from GFP expression encoded within the shRNA vector. a) Neurons from 3 cultures were fixed, permeabilised and stained with selective anti-β3 antibodies and the level of subunit expression determined from the fluorescence intensity. Mean intensity was recorded from 2-4 proximal dendrites and averaged per cell (n = 23-26) and normalised to the average intensity of the scrambled control per neuronal culture. b) Representative images taken from proximal dendrites of neurons transfected with either scrambled control or shRNA. The scale bars represent 5 µm. c) Spontaneous tonic currents recorded from scrambled control- (n = 7) and shRNA-expressing (n = 8) neurons, determined by PTX (100 µM). d) Average amplitude and (e) frequency of sIPSCs recorded from neurons transfected with scrambled control (n = 9) or shRNA (n = 7-8). For all graphs, columns and error bars represent the mean and SEM, respectively (unpaired t-tests used for all comparisons; **p<0.01; ***p<0.001; ns: no significance).

5.2.2. Enhancing spontaneous receptor activity reduces hippocampal neuronal excitability

We have so far discussed the importance of the β3 subunit in mediating spontaneous tonic currents in primary hippocampal cultures. Robust tonic currents in these neurons have been demonstrated, but the impact they have on excitability has not been addressed. To this end, current-clamp recording was performed on neurons transfected with β3 subunit cDNAs which were either, in heteromeric assemblies, non-spontaneous ($\beta3^{DNTK}$) or highly spontaneous ($\beta3^{K279T}$). We injected a set of constant current steps (-100 to 500 pA in 20 pA increments) and measured the frequency of action potentials elicited until they became unresolvable. To ensure parity between neurons, current was continuously injected into neurons to ensure a stable membrane potential of -70 mV. The rheobase, the minimum depolarising current required to elicit an action potential, was also measured.

Transfection with the non-spontaneous $\beta3^{DNTK}$ cDNA resulted in a rheobase not significantly different from control eGFP (37.1 ± 8.1 pA, n = 7; and 30.9 ± 4.1 pA, n = 11, respectively; Fig. 5.5a). Neurons transfected with wild-type β3 cDNA, however, required significantly larger current injection before an action potential was generated (66.0 ± 6.7 pA, n = 10). Expressing the highly spontaneous $\beta3^{K279T}$ resulted in an almost complete lack of excitability of these transfected neurons. From 8 cells trialled, only one could be induced to fire action potentials, and only with a substantial current injection (220 pA). The input-output curves are shown in Fig. 5.5b, illustrating the significant rightward shift of β3-transfected neurons compared to eGFP- and $\beta3^{DNTK}$-transfected neurons, and the almost silent $\beta3^{K279T}$-transfected neurons. Representative recordings of electrotonic potentials during current steps are shown in Fig. 5.5c. To ensure these differences in neuronal excitability were due to changes in spontaneous receptor activity, the membrane resistance for each cell was calculated from a 10 mV hyperpolarising voltage step. Neurons transfected with eGFP and $\beta3^{DNTK}$ cDNA showed near-identical resistance (eGFP: 506 ± 58 MΩ, n = 11; $\beta3^{DNTK}$: 507 ± 54 MΩ, n = 7; p = 1) indicating that expression of the non-spontaneous

$\beta3^{DNTK}$ variant did not alter resting conductivity. Transfection of wild-type β3 cDNA significantly reduced the membrane resistance compared to eGFP controls (315 ± 60 MΩ, n = 10; $p < 0.01$), as did expression of the highly spontaneous $\beta3^{K279T}$ variant to a much greater degree (49 ± 30 MΩ, n = 8; $p < 0.001$). These results correlate with the extent of the shift of the input-output curves, and show that expression of β3 and of spontaneously active $GABA_A$ receptors can substantially reduce neuronal excitability and action potential firing by increasing the membrane conductance.

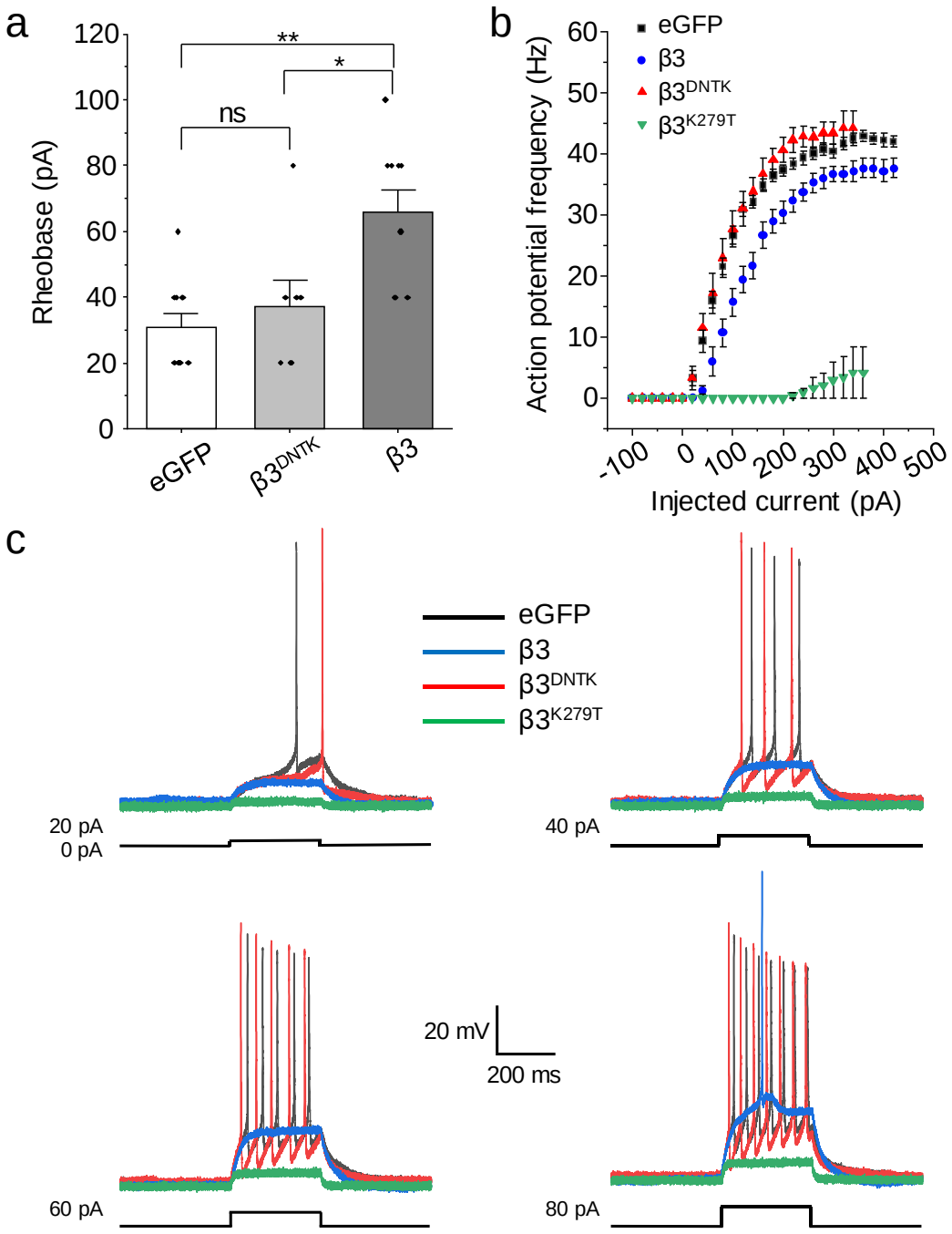

Figure 5.5: Increased expression of spontaneous β3-containing receptors reduced neuronal excitability. a) Rheobase measurements of hippocampal neurons transfected with eGFP (n = 11), β3 (n = 10) or $β3^{DNTK}$ (n = 7) cDNAs. Expression of the highly spontaneous $β3^{K279T}$ resulted in 7 of 8 neurons being silent and unable to elicit action potentials. The one excitable cell had a rheobase of 220 pA (not shown). Columns and error bars represent the mean and SEM, respectively (Kruskal-Wallis with Dunn's post hoc test; *p<0.05; **p<0.01; ns: no significance). b) Input-output curves of hippocampal neurons transfected with eGFP, β3, $β3^{DNTK}$ or $β3^{K279T}$ cDNAs. c) Representative electrotonic potential recordings from hippocampal neurons expressing eGFP, β3, $β3^{DNTK}$, or $β3^{K279T}$ during injection of various depolarising current step.

5.2.3. Spontaneous $GABA_A$ receptor activity varies between brain regions

Studies of spontaneously active receptors have predominantly used recordings from recombinant receptors or from cultured neurons (McCartney et al., 2007; Tang et al., 2010), but few studies have been performed in acute brain slices (Wlodarczyk et al., 2013; O'Neill & Sylantyev, 2018). Whether we could resolve a spontaneous component of tonic inhibitory current in native neurons within young (P21-28) rat brain slices was addressed by recording from DGGCs and dorsal lateral geniculate nucleus (dLGN) thalamic relay neurons, both of which are known to express δ subunit-containing $GABA_A$ receptors (Pirker et al., 2000; Stell et al., 2003; Herd et al., 2009; Lee & Maguire, 2014). Gabazine (20 μM) was used to block GABA binding and reveal the GABA-mediated component of the tonic current, and any residual current that was blocked by PTX (100 μM) was designated as a spontaneous component. PTX is also an allosteric inhibitor of glycine receptors, which are reported to be present in certain brain areas and may contribute to tonic inhibition, however previous reports using the antagonist strychnine suggest these receptors do not contribute to the tonic current in DGGCs (Wlodarczyk et al., 2013). Few studies have examined the role of glycine receptors in the dLGN, although there is evidence for synaptic glycine receptors in the ventrobasal thalamus. However, this study reported no change in the membrane resistance when strychnine was applied, indicating no effect on tonic inhibitory currents (Ghavanini et al., 2005).

In DGGCs, slight, but non-significant, increases in holding currents were observed when gabazine was applied (0.01 ± 0.01 pA/pF, n = 9), indicating a small agonist effect, consistent with earlier recordings from cultured hippocampal neurons (Fig. 5.1a) and as previously reported (Wlodarczyk et al., 2013), followed by a large reduction in holding current when PTX was applied (0.25 ± 0.05 pA/pF; Fig. 5.6a). By contrast, thalamic relay neurons had tonic currents which could be blocked by gabazine (0.05 ± 0.02 pA/pF, n = 10), but also displayed a small residual level of current blocked by PTX (0.04 ± 0.02 pA/pF, for a total tonic current of 0.1 ± 0.02 pA/pF, n = 10; Fig. 5.6b). These results indicate that the tonic current in DGGCs is primarily

mediated by spontaneous $GABA_A$ receptor activity, consistent with an earlier study (Wlodarczyk et al., 2013), whereas in dLGN thalamic relay neurons, the tonic current is less reliant on spontaneous gating. This difference could reflect differential receptor isoform expression, variation in levels of extracellular GABA and endogenous modulators, such as neurosteroids, and also variation in levels of protein kinase activity (Bright & Smart, 2013a; Lee & Maguire, 2014).

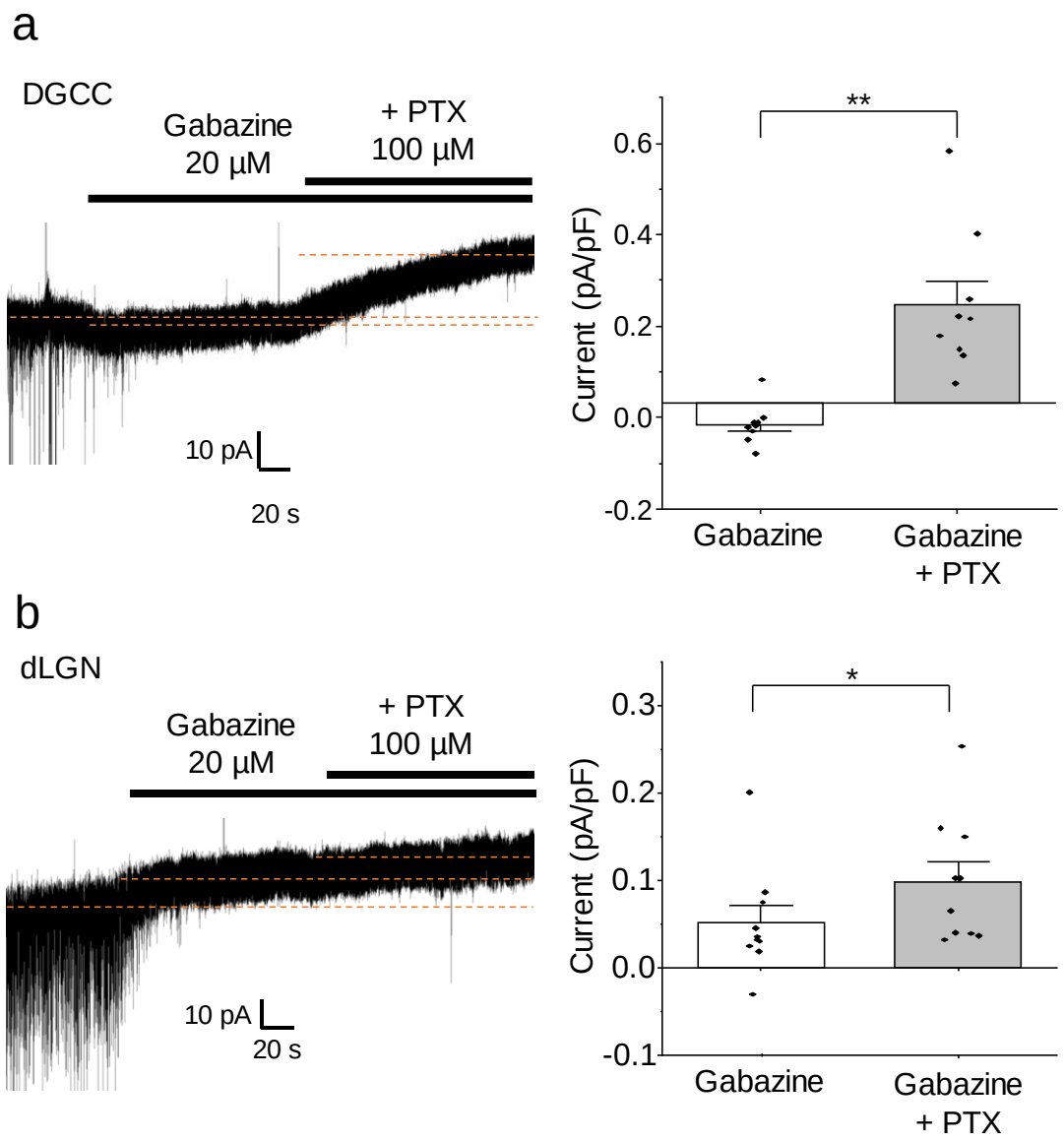

Figure 5.6: Spontaneous $GABA_A$ receptors are differentially responsible for tonic inhibition in hippocampal and thalamic neurons. a) Representative current recording from a dentate gyrus granule cell in an acute hippocampal brain slice (left) showing changes to baseline current when gabazine (20 µM) and PTX (100 µM) were applied. Holding current changes are shown in the summary bar chart (right; n = 9). b) Representative current recording from a relay neuron of the dorsal lateral geniculate nucleus in an acute thalamic brain slice (left). Current amplitude changes in response to antagonist application are shown in the summary bar chart (right; n = 10). For both graphs, columns and error bars represent the mean and SEM, respectively (paired t-test; *p<0.05; ***p<0.001).

5.2.4. Mutant β3 subunits with links to epilepsy confer altered spontaneous activity

To examine whether aberrant spontaneous $GABA_A$ receptor activity might play a role in a pathological context, we searched for mutations of the β3 subunit that are linked with a disease phenotype (Møller et al., 2017). We prioritised mutations associated with epilepsy, as many single point mutations in $GABA_A$ receptors have been identified as genetic determinants for this disease. We examined two mutations in the β3 ECD: L170R and T185I. The L170R mutation has been associated with early onset (< 9 months) partial seizures and severe intellectual disability (Zhang et al., 2015), and the T185I mutation displays a similar phenotype, resulting in Early Onset Epileptic Encephalopathy with severe intellectual disability (Møller et al., 2017). The receptor location of each mutation is shown in the amino acid sequences and $GABA_A$ receptor structural models in Fig. 5.7a,b. The L170 residue is located within the Cys-loop, a key structure of the ECD involved in the gating process after agonist binding (Cederholm et al., 2009; Miller & Smart, 2010; Thompson et al., 2010). The T185 residue is present in binding loop B, therefore involved in the agonist binding and transduction process (Miller & Smart, 2010; Lynagh & Pless, 2014).

The $\beta3^{L170R}$ mutant has previously been examined using recombinant $\alpha1\beta3^{L170R}\gamma2L$ receptors, where it was shown to have impaired expression levels, a slight leftward shift of the GABA concentration-response curve, and enhanced gating (Hernandez et al., 2017). The $\beta3^{T185I}$ mutant has not yet been examined. We chose to examine these mutant subunits expressed in α4β3δ receptors in order to examine the impact of the mutations on spontaneous activity and tonic current. The mutant β3 subunits were expressed with an N-terminal myc tag as $\alpha4\beta3^{L170R\text{-}myc}\delta$ and $\alpha4\beta3^{T185I\text{-}myc}\delta$ receptors in HEK cells, and immunocytochemistry was performed using primary antibodies selective for the myc epitope (Fig. 5.7c,d). Using confocal microscopy, significantly reduced surface expression for both of these mutant subunits was observed ($\alpha4\beta3^{L170R\text{-}myc}\delta$: 47.3 ± 6.6%, n = 26; $\alpha4\beta3^{T185I\text{-}myc}\delta$: 39.2 ± 5.7%, n = 31)

compared to $\alpha4\beta3^{myc}\delta$ (n = 28). This result was expected for $\alpha4\beta3^{L170R}\delta$ based on studies on the $\alpha1\beta3^{L170R}\gamma2L$ receptor (Hernandez et al., 2017).

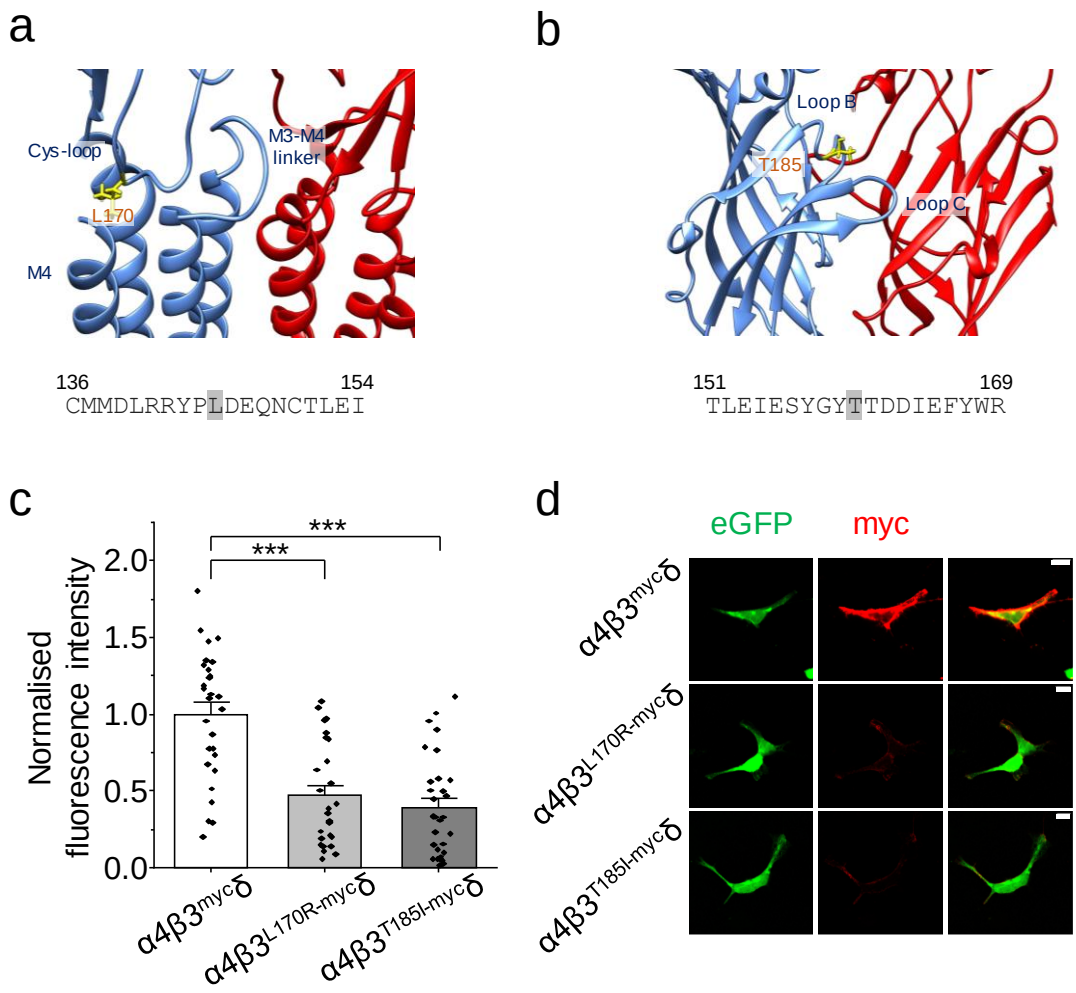

Figure 5.7: Mutant $\alpha4\beta3^{L170R}\delta$ and $\alpha4\beta3^{T185I}\delta$ receptors display reduced surface expression. The α4β3δ receptor homology model with the location of the L170 (a) and T185 (b) residues. Both residues and their side chains are shown in yellow and are located in the ECD of the β3 subunit (in blue). The α subunit is shown in red. c) Quantification of cell surface fluorescence intensity of $\alpha4\beta3^{myc}\delta$ (n = 28), $\alpha4\beta3^{L170R\text{-}myc}\delta$ (n = 26) and $\alpha4\beta3^{T185I\text{-}myc}\delta$ (n = 31) receptors, determined by anti-myc antibodies and secondary antibodies conjugated to Alexa Fluor 555. Fluorescence intensity of each cell was normalised to the average intensity of $\alpha4\beta3^{myc}\delta$ for that preparation. Columns and error bars represent the mean and SEM, respectively (ANOVA with Tukey's post hoc test; ***p<0.001). d) Representative confocal images of HEK cells expressing $\alpha4\beta3^{myc}\delta$, $\alpha4\beta3^{L170R\text{-}myc}\delta$ and $\alpha4\beta3^{T185I\text{-}myc}\delta$. Cells were transfected with eGFP for identification. Scale bars represent 10 μm.

However, when recording total currents from these cells ($I_{GABA\text{-}MAX}$ + I_{PTX}), there were significantly larger currents recorded from α4β3^{L170R}δ receptors (4.70 ± 0.48 nA, n = 6), and no difference for α4β3^{T185I}δ receptors (2.78 ± 0.55 nA, n = 6), compared to wild-type α4β3δ receptors (2.33 ± 0.64 nA, n = 6; Fig. 5.8a,b). These results are inconsistent with the mutants showing reduced surface expression. The desensitisation kinetics for these receptors were also measured using 1 mM GABA applied for 30 s. The weighted tau (τ_w) was calculated (Fig. 5.8c,d) , as was the extent of desensitisation, represented as a percentage of the maximum GABA-activated current (Fig. 5.8c,e). Significantly faster desensitisation of the α4β3^{L170R}δ receptor compared to wild-type was observed (2.92 ± 0.45 s, n = 9; and 6.30 ± 0.35 s, n = 8, respectively) but no difference in the rate of desensitisation of the α4β3^{T185I}δ receptor (6.62 ± 0.70 s, n = 7). However, both mutant receptors displayed significantly greater desensitisation extents than wild-type, with the α4β3^{L170R}δ receptor desensitising to the greatest degree (α4β3δ: 73.2 ± 2.7%, n = 9; α4β3^{L170R}δ: 93.2 ± 0.9%, n = 8; α4β3^{T185I}δ: 83.4 ± 0.7%, n = 7). HEK cells were voltage-clamped at -20 mV for these experiments. The greater extent and rate of desensitisation is in agreement with previous research on the synaptic α1β3^{L170R}γ2L receptor (Hernandez et al., 2017). Despite the incorporation of the δ subunit, which significantly reduces the extent of desensitisation of extrasynaptic receptors (Haas & Macdonald, 1999; Brown et al., 2002), receptors incorporating these mutant subunits will be more likely to exist in a desensitised state, potentially impacting on tonic inhibition. This may also impact on spontaneous activity by preventing a significant proportion of receptors from gating spontaneously in areas of the brain reliant on both GABA-activated and GABA-independent channel opening.

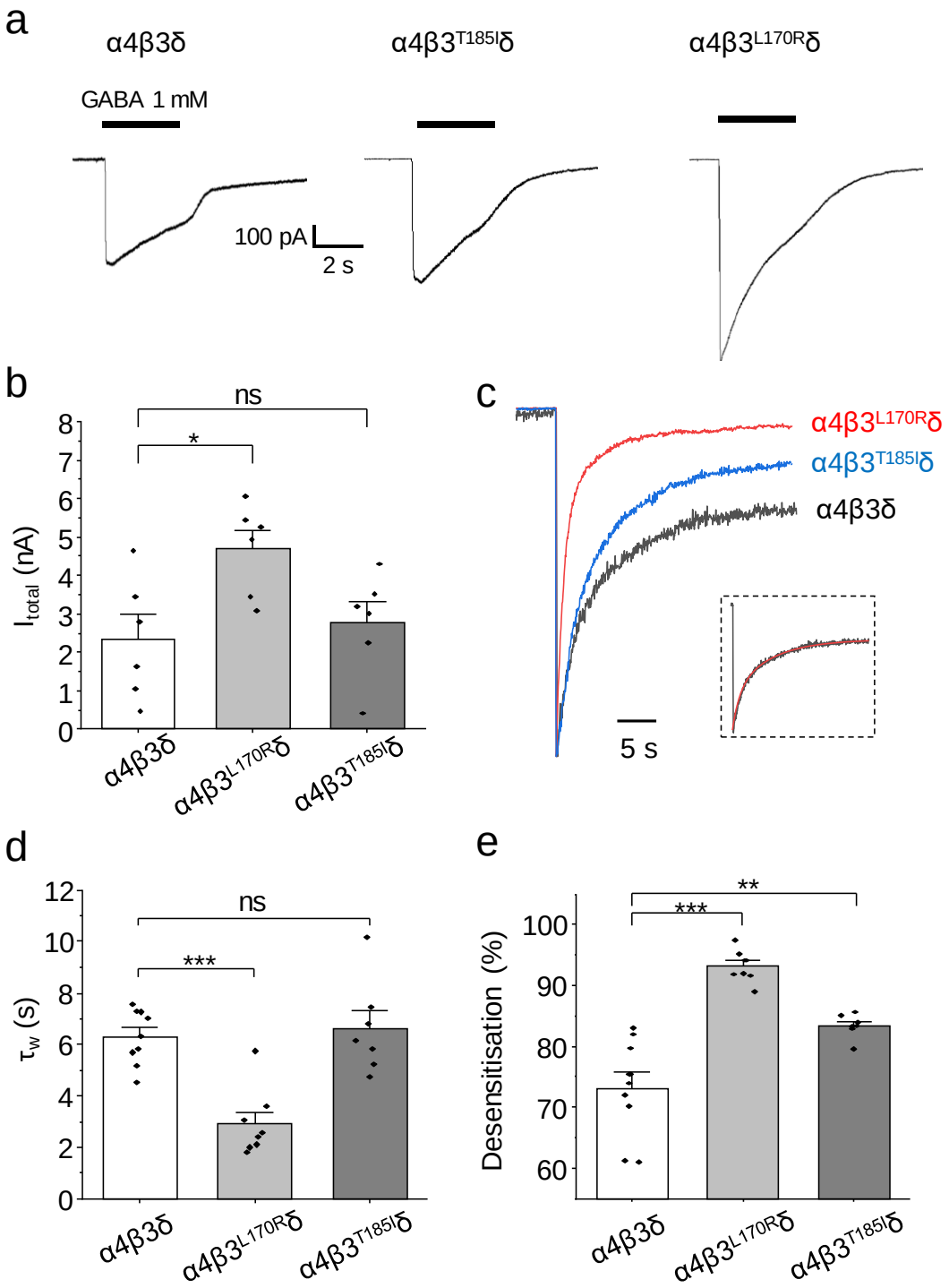

Figure 5.8: Both epilepsy mutants impact on total receptor currents and desensitisation kinetics. a) Representative recordings of α4β3δ, α4β3^{L170R}δ and α4β3^{T185I}δ receptors during 1 mM GABA application. b) The total (spontaneous and GABA-activated) current elicited from receptors (n = 6). c) Normalised current responses to 1 mM GABA applied for 30 s. Inset shows the fitted two-term exponential curve superimposed on the wild-type recording. d) The rate of desensitisation, represented as the weighted tau (τ_w) calculated for each receptor variant (n = 7-9). e) The extent of desensitisation, expressed as a percentage of the maximum GABA-activated current (n = 7-9). For all graphs, columns and error bars represent the mean and SEM, respectively (ANOVA with Tukey's post hoc test for all comparisons; *p<0.05; **p<0.01; ***p<0.001; ns: no significance).

To further understand these results, we investigated the efficacy of THIP (3 mM) at these receptors to ascertain whether changes in gating efficiency could explain the increased currents. THIP has a greater efficacy than GABA at δ-containing receptors (Wafford et al., 1996; Brown et al., 2002; Storustovu & Ebert, 2006; Mortensen et al., 2010), and evoked significantly larger currents than saturating concentrations of GABA (1 mM; response was defined as 100%) at wild-type α4β3δ (149.2 ± 10.3%, n = 5) and $\alpha4\beta3^{T185I}\delta$ (135.9 ± 5.4%, n = 6) receptors, but not at $\alpha4\beta3^{L170R}\delta$ receptors (92.6 ± 1.1%, n = 6), suggesting that this latter point mutation results in GABA acting as a more efficacious agonist (Fig. 5.9a). An alternative explanation is that THIP itself is less efficacious, but this is not supported by the increased current recorded from receptors despite reduced surface expression. Further evidence for enhanced gating of this receptor was provided by GABA concentration-response curves in the presence of the potentiating neurosteroid THDOC (Hosie et al., 2009; Wang, 2011). For wild-type α4β3δ receptors, the partial agonism of GABA is demonstrated by the ability of THDOC (100 nM) to potentiate agonist-evoked currents at all GABA concentrations, including the maximal GABA concentration, (138.0 ± 0.07%, n = 7; peak GABA response in the absence of THDOC was defined as 100%). By contrast, for $\alpha4\beta3^{L170R}\delta$ receptors, THDOC did not potentiate currents evoked by GABA (at maximal GABA, 106.0 ± 0.03%, n = 6). Interestingly, the $\alpha4\beta3^{T185I}\delta$ receptor showed significantly lower THDOC enhancements of maximal GABA-activated currents than wild-type receptors (118.6 ± 0.04%, n = 7; Fig. 5.9b,c), possibly indicating a slightly enhanced efficacy of these mutant receptors. Alternatively, THDOC may have reduced activity at these receptors, although this is unlikely considering the location of the neurosteroid binding site in the transmembrane domains, far removed from T185 residue in the ligand-binding domain. A slight increase in gating efficiency is consistent with the similar levels of total current in transfected HEK cells (Fig. 5.8b) despite apparent reduced surface expression (Fig. 5.7c). Further evidence for the enhanced gating of GABA at $\alpha4\beta3^{L170R}\delta$ and $\alpha4\beta3^{T185I}\delta$ receptors is provided through a slight leftward shift of the GABA concentration-response curves compared to wild-type α4β3δ receptors (Fig. 5.9d), as determined from the EC_{50} analysis (α4β3δ: 0.54 ± 0.13 μM, n = 7; $\alpha4\beta3^{L170R}\delta$: 0.20 ± 0.06 μM,

n = 7; $\alpha4\beta3^{T185I}\delta$: 0.14 ± 0.02 μM, n = 6; $p < 0.05$ for both mutants compared to wild-type). HEK cells were voltage-clamped at -20 mV for these experiments. Based on these data, it is possible to conclude that GABA acts as a more efficacious agonist at the $\alpha4\beta3^{L170R}\delta$ receptor than at wild-type receptors, and that the $\alpha4\beta3^{T185I}\delta$ may show slight enhancements in efficacy.

We then examined the spontaneous activity of these receptors. Due to apparent variances in GABA efficacy between the receptors, we examined the amplitude of the spontaneous holding current through application of PTX (100 μM) rather than normalising to the total GABAergic current (Fig. 5.9e). Although the amplitude of I_{PTX} was not statistically different between the mutant and wild-type receptors, the $\alpha4\beta3^{L170R}\delta$ receptors displayed higher levels of spontaneous current than wild-type receptors (907.3 ± 178.1 pA, n = 9; and 325.9 ± 63.8 pA, n = 7; p = 0.2, respectively). By comparison, the $\alpha4\beta3^{T185I}\delta$ receptor showed negligible levels of spontaneous current (38.5 ± 13.6 pA, n = 6; p = 0.1).

These two mutants may therefore have complex effects on tonic inhibition. They both display reduced surface expression, but also display higher efficacy in response to GABA. The L170R mutation appears to significantly enhance agonist gating efficiency, with a concurrent effect on spontaneous gating. The T185I mutant, however, may have slightly higher agonist gating efficiency, but displays an impairment of spontaneous activity. The different mutations therefore likely have complex effects on receptor kinetics.

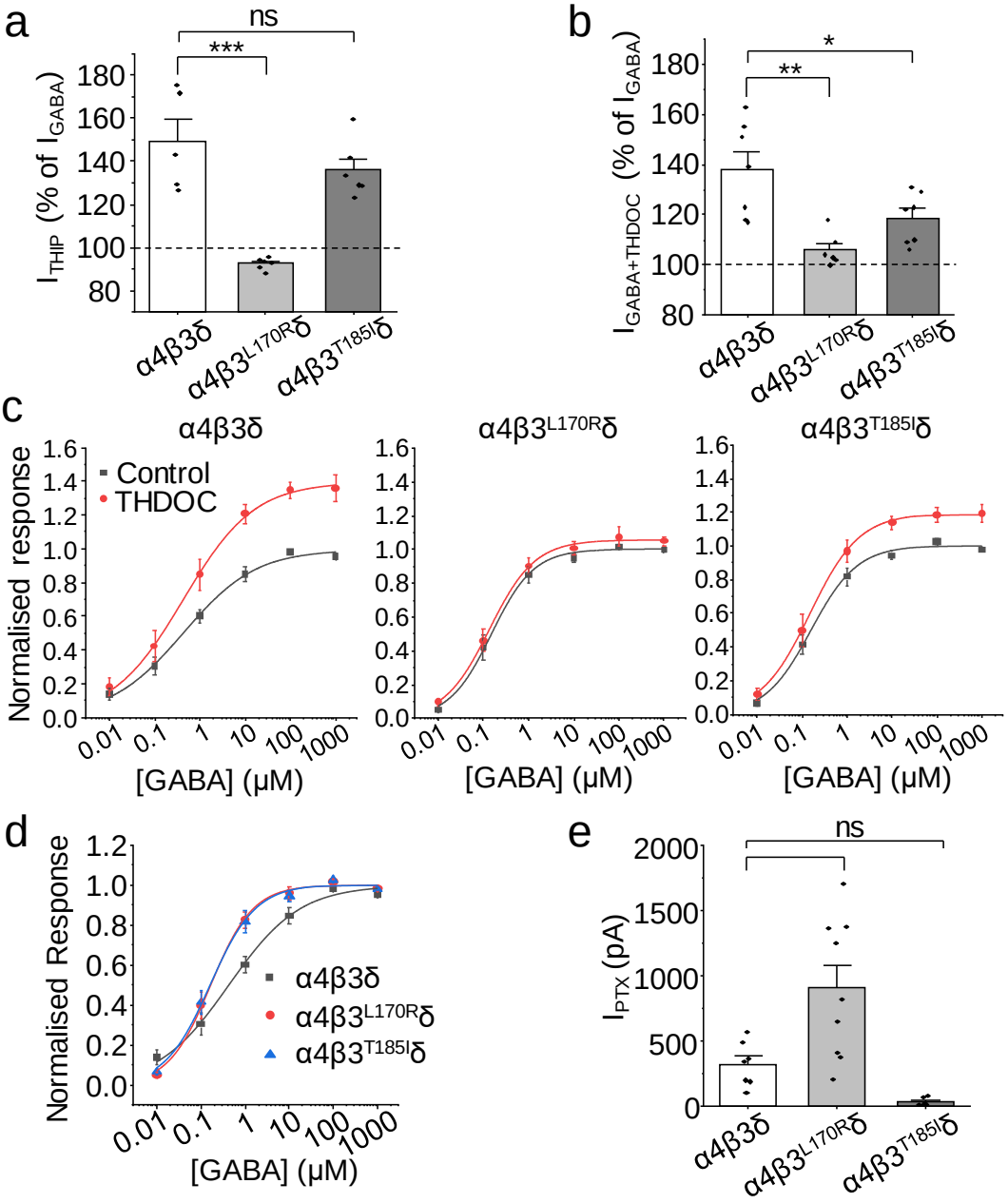

Figure 5.9: Mutant α4β3^{L170R}δ and α4β3^{T185I}δ receptors show changes to GABA efficacy, potency and spontaneous currents. a) The response of wild-type and mutant receptors to a saturating (3 mM) THIP concentration, expressed as a percentage of the response to a saturating (1 mM) GABA concentration (n = 5-6). b) The response of wild-type and mutant receptors to co-application of GABA (1 mM) and THDOC (100 nM), expressed as a percentage of the response to GABA (1 mM) alone (n = 6-7). c) GABA concentration-response curves for wild-type and mutant receptors, either alone or with co-applied THDOC (100 nM). d) GABA concentration-response curves comparing wild-type and mutant receptors. EC_{50} values for wild-type (0.54 ± 0.13 μM, n = 7) receptors were significantly larger than for α4β3^{L170R}δ (0.20 ± 0.06 μM; n = 7; $p<0.05$) and α4β3^{T185I}δ (0.14 ± 0.02 μM; n = 6; $p<0.05$) receptors. e) Spontaneous activity, measured by PTX (100 μM) application, of wild-type and mutant receptors (n = 6-9). For all graphs, columns and error bars represent the mean and SEM, respectively (ANOVA with Tukey's post hoc test was used for comparisons in (a), (b) and (d), Kruskal-Wallis with Dunn's post hoc test was used for comparisons in (e); *$p<0.05$; **$p<0.01$; ***$p<0.001$; ns: no significance).

5.3. Discussion

5.3.1. The β3 subunit is important for spontaneous $GABA_A$ receptor activity in neurons

We have previously demonstrated the importance of subunit composition on the ability of recombinant $GABA_A$ receptors to gate spontaneously. Expression of the β3 subunit is essential for spontaneous activity, but maximum spontaneity is only achieved by the co-expression of α4/6 and δ subunits, which are associated with extrasynaptic $GABA_A$ receptors. In cultures of hippocampal neurons, we have shown that the level of β3 subunit expression is the predominant determinant of spontaneous activity, as increased expression of α4 or δ subunits had no impact on the level of spontaneity, whereas β3 over-expression significantly drove the formation of spontaneously active receptors. Moreover, native receptors relied on the β3 subunit for promoting spontaneous activity, as shRNA-mediated knockdown of the β3 subunit caused a concurrent reduction in spontaneous currents. The activity of the β3 subunit was, as in recombinant receptors, dependent on the GKER motif, as expression of the non-spontaneous $β3^{DNTK}$ had no effect on the level of spontaneous current.

The expression level of the β3 subunit therefore determines the amplitude of spontaneous currents within neurons. However, the total level of spontaneous activity will heavily depend on the identity of the other subunits in the pentameric complex. If β3 is predominantly expressed as extrasynaptic (α4/6β3δ) over synaptic receptors (α1-3β3γ2), then this will drive significantly more spontaneous activity. There is, however, evidence to suggest that γ-containing receptors do make significant contributions to spontaneous tonic currents, as benzodiazepine application has been shown to enhance spontaneous activity in hippocampal cultures and slices (Birnir et al., 2000; McCartney et al., 2007). Evidence suggests that the β3 subunit is the predominant β isoform in $GABA_A$ receptors involved in synaptic transmission in hippocampal CA1 neurons (Kasugai et al., 2010; Nguyen & Nicoll, 2018). Its presence in extrasynaptic receptors remains to be fully established, though

perhaps over 50% of the total complement of β3 subunits is expressed outside synaptic areas in hippocampal CA1 neurons (Kasugai et al., 2010). In DGGCs, extrasynaptic $GABA_A$ receptors may predominantly incorporate the β2 subunit, with β3 expressed mainly in synaptic receptors (Herd et al., 2008; Hoestgaard-Jensen et al., 2014). However, Herd et al. suggest that extrasynaptic α5β3γ receptors do contribute to tonic currents. Thus, it appears that β3 expression and incorporation into distinct receptor isoforms in the hippocampus may be dependent upon neuronal identity. Further studies on the subcellular distribution of β3 and the subunits accompanying it in the receptor complex may give an indication as to the strength of spontaneous activity and its contribution to tonic inhibition in various brain areas.

5.3.2. The contribution of spontaneous currents to tonic inhibition varies between brain regions in vivo

The extent to which spontaneous activity contributes towards tonic inhibition varies between brain regions. We have demonstrated here distinct differences between the tonic currents expressed in DGGCs and dLGN relay neurons, with the former showing no gabazine-sensitive, and so no GABA-mediated, tonic inhibition and the latter showing a significant component of tonic current mediated by GABA-activated receptors. The complete lack of gabazine block, and hence dependence on spontaneous activity for maintaining the tonic current in DGGCs *ex vivo,* is consistent with previous findings suggesting that, under *in vivo* concentrations of ambient GABA, the tonic current is almost entirely dependent on spontaneous activity (Wlodarczyk et al., 2013). By contrast, tonic currents in dLGN relay neurons have been shown to be heavily reliant on vesicular release of GABA and are significantly reduced when synaptic GABA release is impaired (Bright et al., 2007).

The causes of these different levels of spontaneous currents are potentially manifold. Both neuron subtypes in the dentate gyrus and dLGN express α4βδ receptors, and this is the predominant receptor isoform responsible for tonic inhibition in these brain areas (Stell et al., 2003; Cope et

al., 2005; Lee & Maguire, 2014). As has already been discussed, spontaneous activity is dependent on β3 expression and the expression levels of the α4βδ receptor isoform. DGGCs may therefore show high β3 expression in total (α1β3γ2, α5β3γ2 *and* α4β3δ receptors), or have a high proportion of extrasynaptic α4β3δ receptors, although this latter point is confounded by the suggestion of a preferential synaptic localisation for β3 subunits (Herd et al., 2008; Nguyen & Nicoll, 2018) and the observation that the α4β1/3δ-selective agonist 4,5,6,7-tetrahydroisothiazolo[5,4-c]pyridin-3-ol (Thio-THIP) had minimal effects on DGGCs (Hoestgaard-Jensen et al., 2014). Alongside the receptor isoform, variability in the dependence on spontaneous activity for generating tonic currents could be explained by different ambient GABA concentrations or receptor phosphorylation states. Higher ambient GABA concentrations in the thalamus would lead to more extrasynaptic receptors being ligand-activated and hence having a lower reliance on spontaneous activity for tonic inhibition (Bright et al., 2007). Differences in kinase and phosphatase activities could also lead to differing phosphorylation states of $GABA_A$ receptors, which will impact on spontaneous receptor activity (Tang et al., 2010; Nakamura et al., 2015) as shown in the present study.

Using the different spontaneity profiles of recombinant $GABA_A$ receptors allows us to speculate on the contribution of spontaneous activity to tonic current in other brain areas. Significant extrasynaptic β3 expression in hippocampal CA1 neurons exists (Kasugai et al., 2010), but this area is mostly reliant on α5βγ2 receptors for tonic inhibition, which have a similar spontaneous activity profile to synaptic α1β3γ2 receptors (Caraiscos et al., 2004), and also on glycine receptors (McCracken et al., 2017). Tonic inhibition in CA1 neurons may therefore be more highly affected by temporal fluctuations in extrasynaptic neurotransmitter concentrations, and indeed tonic inhibition in CA1 is at least partially dependent on vesicular GABA release (Glykys & Mody, 2007). However, there is likely to still be a significant spontaneous component to the tonic inhibition in these neurons, as spontaneous currents could be potentiated by benzodiazepines, which do not affect δ-containing receptors (Birnir et al., 2000; McCartney et al., 2007). In addition, CA1 neurons do express extrasynaptic α4βδ receptors which partially contribute to tonic

currents (Glykys et al., 2008). The cerebellum expresses α6βδ receptors which are responsible for the majority of tonic inhibition (Brickley et al., 2001; Ye et al., 2013), however, as with all other brain areas, the identification of β2 compared to β3 subunit localisation is not clearly established. Analysing mRNA levels and immunoreactivity of subunits may provide details as to the extent of spontaneous activity through β3 expression. In dentate gyrus, CA1 and CA3 regions, there is significant β3 immunoreactivity and mRNA, with minimal β2 (although high levels of β1 have been measured) (Pirker et al., 2000; Korpi et al., 2002; Stefanits et al., 2018). By contrast, thalamic β3 immunoreactivity and mRNA levels are very low, but show significantly higher β2 levels, potentially explaining why these neurons rely more on GABA-mediated tonic inhibition (Wisden et al., 1992; Pirker et al., 2000; Korpi et al., 2002; Belelli et al., 2005). By extending this comparison to other brain areas we might be able to estimate the extent of spontaneous activity. There is significant β3 mRNA and subunit expression in areas of the basal ganglia and striatum, of note in the nucleus accumbens and caudate/putamen, which also appear to express very low levels of β1 and β2 (Wisden et al., 1992; Pirker et al., 2000; Hortnagl et al., 2013; Waldvogel & Faull, 2015). The cerebral cortex shows significant β3 levels, but also high levels of β1/2 (Pirker et al., 2000; Petri et al., 2003; Hortnagl et al., 2013). The cerebellum shows high levels of both β2 and β3 in the granule cell layer, but almost no β3 in the molecular layer (Pirker et al., 2000; Hortnagl et al., 2013). This could indicate that there is significant spontaneous activity in granule cells and Golgi cells of the cerebellar granule cell layer, but very little in Purkinje cells and interneurons found in the outer molecular layer.

We therefore have two predominant determinants of spontaneous activity; the presence of the β3 subunit, and the identity of the co-assembled subunits. Our data suggests that high β3 levels expressed as α4/6β3δ receptors show the highest level of spontaneity, but that there could be appreciable spontaneous tonic currents arising from α1-3β3γ2 and α5β3γ2 receptors. When β3 expression is low and β1/2 predominates, very low spontaneous activity will likely be observed. This does not mean there is no tonic current, as shown in our recordings from the thalamus, but that there is

more dependence on extracellular GABA, which may have important consequences for information processing.

5.3.3. Spontaneous current may contribute to the impact of tonic inhibition on neuronal excitability and information processing

Tonic inhibition has a variety of effects on the excitability of neurons and the input-output (i/o) relationship, and has been extensively investigated using *in silico* modelling and *in vitro* techniques (Silver, 2010). Alterations to the i/o relationship can be additive (left/right shifts in curve offset and alteration of the rheobase) or multiplicative (increase/decrease the gain of the curve).

The i/o curves constructed here of neurons expressing greater spontaneous tonic current displayed additive and multiplicative shifts, representing a change to both the rheobase and the gain. Significant spontaneous activity could therefore impact upon computational processing of neurons. This effect is probably complex and may rely on synaptic activity; shunting inhibition was demonstrated to reduce the gain of cerebellar granule cells, but only in the presence of varying synaptic-type conductance waveforms causing fluctuations in basal membrane voltage (Mitchell & Silver, 2003; Prescott & De Koninck, 2003). This effect of tonic $GABA_A$ receptor activation will have consequences for network activity depending on the identity of the neuron. Principal, excitatory neurons expressing these currents will likely have their output reduced. Indeed, excitatory hippocampal CA1 neurons express outwardly rectifying $GABA_A$ receptors mediating tonic inhibition, whose activation results in a rightward shift of the i/o curve, rather than affecting gain (Pavlov et al., 2009). Interesting results occur when examining the impact of tonic inhibition on interneuron activity. In CA3 interneurons, tonic inhibition depolarises neurons, an excitatory process, which also reduces EPSP amplitude through shunting inhibition. This has the dual effect of reducing the dependence of firing on external excitatory synaptic input, but also driving firing through depolarisation, synchronising network activity (Song et al., 2011; Pavlov et al., 2014). As tonic inhibition increases,

shunting inhibition begins to predominate, and reduces firing. This also varies between interneuron classes, likely dependent on variations in the chloride gradient and functional properties. Indeed, modelling experiments and recordings from different classes of cortical interneurons suggest that tonic inhibition reduces the gain in fast-spiking parvalbumin interneurons, but increases it in the non-fast-spiking somatostatin interneurons (Bryson et al., 2020).

There is clearly a complex interaction between tonic inhibition, firing rate and neuronal identity, with a variety of effects on network activity depending on the excitatory/inhibitory output of the neurons expressing the tonic inhibitory currents. Spontaneous activity may contribute to both the shunting and depolarising properties in these neurons, controlling network firing patterns within the hippocampus and cortex. The modulation of these spontaneous currents by phosphorylation and neurosteroids (ch. 3) represents a GABA-independent mechanism by which signalling molecules may modulate network activity.

5.3.4. A role for spontaneous activity in pathology

The importance of β3 expression for normal brain function is underscored by β3 subunit knock-out studies; which showed high neonatal mortality, epileptic phenotypes and severe behavioural impairments (Homanics et al., 1997; DeLorey et al., 1998). In contrast, β2 knock-out mice displayed reduced receptor expression levels, but relatively few behavioural deficits (Sur et al., 2001). Alteration in β3 expression or function therefore impacts on brain function and gives rise to significant pathology. We studied two mutations in the β3 subunit associated with severe epileptic phenotypes and significant behavioural and cognitive impairments, and which also show altered spontaneous activity (Zhang et al., 2015; Møller et al., 2017). The $\beta3^{L170R}$ subunit, when expressed as $\alpha4\beta3^{L170R}\delta$ receptors, shows increased levels of spontaneous current through enhanced receptor gating despite evidence for reduced surface expression (Hernandez et al., 2017). By

contrast, the $\alpha4\beta3^{T185I}\delta$ receptor shows severely impaired levels of spontaneous activity alongside reduced cell surface expression.

The L170 residue is located within the large Cys-loop between β sheets 6-7, and is in close proximity to M4 of the β3 subunit (Miller & Smart, 2010; Laverty et al., 2019). Previous experiments involving $\alpha1\beta3^{L170R}\gamma2L$ receptors expressed in HEK cells identified impairments in expression and increased GABA potency linked to an enhanced ability of the receptor to gate, but did not examine the spontaneous properties of the receptor (Hernandez et al., 2017). The Cys-loop is heavily involved in the transduction of ligand-binding to channel opening and alteration to this site is therefore likely to impact on gating (Cederholm et al., 2009; Thompson et al., 2010). The increased gating efficiency observed previously appears more pronounced in δ-containing receptors, as GABA appears to act with both enhanced potency and efficacy at these receptors. Indeed, both the lack of a 'super-agonist' effect with THIP and the inability of THDOC to potentiate saturating GABA-gated currents indicate that GABA may act as a full agonist at these mutant receptors. Using our method to quantify spontaneous current (applying saturating GABA and PTX sequentially) is therefore not valid when comparing with wild-type receptors, at which GABA is a partial agonist. When we instead analysed absolute holding current shifts with PTX, there was a higher level of basal spontaneous activity despite evidence for reduced cell surface expression. Alongside the enhanced potency of GABA, manifesting as a leftward shift in the concentration-response curve, this provides further evidence for the link between spontaneous activity and ligand-dependent gating, and that residues in the Cys-loop can alter the ability of receptors to gate spontaneously.

The functional effects of the T185I mutation have not been studied previously. This residue is located within loop B of the β subunit, and so is likely involved in GABA-binding and transduction (Miller & Smart, 2010; Lynagh & Pless, 2014; Laverty et al., 2019; Masiulis et al., 2019). The effect on spontaneous activity was substantial at receptors incorporating this mutant subunit, with almost complete absence of constitutive activity. Our concentration-response curves indicate, as with the L170R mutation, an increased potency of GABA at α4β3δ receptors. This is consistent with its

location at the GABA-binding site, where it is likely to enhance either the affinity of GABA for the binding site, or the transduction of ligand-binding to the opening of the channel activation gate (Colquhoun, 1998). However, it is hard to reconcile this with the previous evidence for a link between GABA potency and spontaneous activity. One explanation could lie in the mutation enhancing the efficacy of agonists and so promoting the shift into an agonist-bound open conformation, manifesting as a leftward-shift in the GABA concentration-response curve (Colquhoun, 1998), but inhibiting the transition into a constitutive open state. This would indicate that residues in the GABA-binding domain can directly alter spontaneous gating properties, as previous mutagenesis studies have shown (Boileau et al., 2002). This is also consistent with the inhibition of spontaneous activity using orthosteric antagonists such as gabazine and bicuculline (Ueno et al., 1997; McCartney et al., 2007). These results suggest that domains within subunits can have differential effects on GABA-gated and spontaneous activity.

The impact of the altered spontaneity of these receptors on the epileptic phenotype is difficult to dissect from other changes in receptor function, and will likely vary between brain regions. The L170R mutant possibly has enhanced tonic inhibition through a combination of enhanced spontaneous activity and increased GABA potency, but this is tempered by an increased rate and extent of desensitisation and reduced cell surface expression, as described previously (Hernandez et al., 2017). Additionally, the loss of neurosteroid ability to modulate the tonic current could result in further dysregulation of neural network activity (Carver & Reddy, 2013; Reddy, 2013). The T185I mutant has enhanced GABA potency but significant impairments in spontaneity. In DGGCs, this will have a significant impact on tonic inhibition due to the high dependency on spontaneous activity, whereas in relay neurons of the dLGN this effect might be lessened due to the lower reliance on spontaneous activity (Bright et al., 2007; Wlodarczyk et al., 2013). As noted for the effect of spontaneous activity on the level of tonic inhibition *in vivo*, the impact of these mutations will have a greater effect on brain areas where β3 is significantly more expressed than β2, such as DGGCs and striatal neurons (Pirker et al., 2000). The cerebellum shows expression of both β3 and β2, and

so compensation of certain tonic currents could occur to maintain extrasynaptic inhibition (Pirker et al., 2000; Hortnagl et al., 2013).

Epilepsy can arise from events other than point mutations to $GABA_A$ receptor subunits, for example through brain injury or alcohol withdrawal (Hillbom et al., 2003; Ding et al., 2016). Numerous experiments have been performed in animal models of epilepsy which suggest pronounced changes to tonic inhibition during epileptogenesis, the period between an initial seizure and the onset of the chronic epileptic phase, characterised by extensive network remodelling and altered neuronal activity (Peng et al., 2004; Walker & Kullmann, 2012; Pavlov & Walker, 2013). Temporal lobe epilepsy (TLE) can be induced experimentally through a variety of mechanisms, including kainic acid or pilocarpine injections (Levesque et al., 2016). After induction, there is an initial seizure followed by a period devoid of seizures. During this 'latent phase', various changes occur in the hippocampal regions, including neuronal death and network rewiring (Dingledine et al., 2014; Alexander et al., 2016). Mossy fibres, the axons of DGGCs that normally project to mossy cells and CA3 pyramidal neurons, instead form collateral synapses within the dentate gyrus molecular layer, resulting in feedback excitation (Bains et al., 1999; Scharfman et al., 2003) hence promoting enhanced excitability. There is also an internalisation of synaptic $GABA_A$ receptors, thereby impairing inhibitory transmission (Goodkin et al., 2005; Terunuma et al., 2008; Mele et al., 2019). This epileptogenic phase eventually leads to recurrent seizures (the chronic epileptic stage). A pilocarpine model of TLE examined changes in $GABA_A$ receptor subunit expression during epileptogenesis and chronic stages. They identified reductions in δ subunit expression in the dentate gyrus molecular layer during the latent and chronic periods, with an upregulation of α4 and γ2 expression during the chronic stage (Peng et al., 2004), although this is not consistent between studies (Goodkin et al., 2008). Alterations to $GABA_A$ receptor subunits associated with tonic inhibition in CA1 also occur, with reduced expression of the α5 subunit (Houser & Esclapez, 2003). The reliance on spontaneous $GABA_A$ receptor activity for tonic inhibitory currents in the dentate gyrus may result in this brain area being particularly susceptible to alterations in tonic inhibition through changes to subunit expression, in

particular β3. Intriguingly, this reduced expression of extrasynaptic-type subunits associated with tonic inhibition does not impact on tonic currents, with intact tonic inhibition recorded in DGGCs (Zhang et al., 2007). This could be due to the formation of perisynaptic α4βγ2 receptors responding to GABA spillover (Scimemi et al., 2005). However it could be that tonic inhibition, at least partially, is maintained by β3-mediated spontaneous activity. The maintenance of a tonic current in the hippocampus likely regulates excitability at sub-threshold levels for seizure generation, but during periods of intense neuronal activity, the loss of synaptic feed-forward and feedback inhibition, and hence fast compensatory changes, results in seizure generation (Walker & Kullmann, 2012).

In contrast, absence seizures originating in thalamocortical neurons have been associated with excess ambient GABA and raised tonic inhibition (Cope et al., 2009; Crunelli et al., 2012). Absence epilepsy is characterised by synchronous spike-wave discharges and burst firing of thalamic neurons, resulting in a brief loss of consciousness (Blumenfeld, 2005). This synchronised activity can be induced by preventing the uptake of GABA through GAT-1 inhibition, thereby increasing ambient GABA concentrations and recruiting extrasynaptic $GABA_A$ receptors (Cope et al., 2009). Although $GABA_B$ receptors have been implicated in absence seizures, and the application of the $GABA_B$ receptor agonist γ-hydroxybutyrate (GHB) induces synchronised activity (Snead, 1991), antisense knockdown of the $GABA_A$ receptor δ subunit significantly reduced seizure events (Cope et al., 2009). Tonic inhibition in the thalamus is thought to hyperpolarise thalamic relay neurons, promoting the de-inactivation of T-type Ca^{2+} channels, promoting burst firing (Huguenard & Prince, 1992; Rajakulendran & Hanna, 2016). The significant reliance on maintaining strict concentrations of ambient GABA in the thalamus may provide a physiological explanation for the lack of spontaneous activity observed in our thalamic recordings. A significant tonic current, mediated through expression of β3-containing spontaneously active $GABA_A$ receptors, could potentially promote the abnormal synchronous firing rate of relay neurons.

5.4. Conclusion

We have demonstrated spontaneous activity of $GABA_A$ receptors in neuronal tonic currents. The presence of the β3 subunit was required to promote spontaneous activity in hippocampal neurons, and the tonic inhibition generated was sufficient to significantly reduce neuronal excitability. This reliance on the presence of the β3 subunit is consistent with slice recordings from DGGCs, which show significant β3 expression and spontaneous activity, and relay neurons of the dLGN, which show little β3 expression and low spontaneous currents.

Chapter 6: General Discussion

Inhibition in the brain is provided transiently and intensively by synaptic currents and continuously, at a lower level, by tonic currents (Mody, 2001; Luscher & Keller, 2004; Farrant & Nusser, 2005; Brickley & Mody, 2012; Cherubini, 2012). In this study we have examined a relatively less explored area of tonic inhibition, that of spontaneously active $GABA_A$ receptors. This property of $GABA_A$ receptors has been reported previously (Wooltorton et al., 1997; Neelands et al., 1999; McCartney et al., 2007; Tang et al., 2010; Wlodarczyk et al., 2013) but here we have demonstrated how the extent of spontaneity is defined by the receptor subunit composition, post-translational modification, and by allosteric modulation. Furthermore, under physiological conditions, receptor spontaneity will impact upon neuronal excitability *via* its contribution to tonic current in multiple brain areas.

To investigate the mechanisms underlying spontaneity, we quantified the spontaneous holding current as a percentage of maximum receptor activity. To do so, a saturating concentration of GABA was used to determine total whole-cell $GABA_A$ receptor activity, and then a saturating concentration of the non-competitive antagonist PTX was applied to block all constitutively-active receptors. Expression of recombinant receptors in HEK cells guaranteed the absence of ambient GABA (Thomas & Smart, 2005), ensuring that recorded currents resulted entirely from spontaneous receptor activity. This method relied on several assumptions. Firstly, that the single channel conductance of the spontaneous channels was the same as for GABA-activated channels. This seems a plausible assumption based on prior studies from several groups showing that application of GABA serves to increase open frequency and duration of spontaneous openings rather than to increase channel conductance (Tang et al., 2010; O'Neill & Sylantyev, 2018). It also assumes the presence of a homogenous population of receptors in our heterologous expression system. $GABA_A$ receptor subunits can form a number of different receptor subunit configurations, including αβ heteromers and β homomers (Wooltorton et al., 1997; Brickley et al., 1999; Mortensen & Smart, 2006). A number of control experiments were performed to ensure we were

recording from predominantly triheteromeric, pentameric receptors. Expressing all three subunits together produced much larger expression levels indicated by significantly larger agonist responses and spontaneous currents. The competitive antagonist gabazine also behaved as a negative allosteric modulator, reducing spontaneous currents when all three subunits were expressed, but not when β3 subunits were expressed alone as these homomers lack the requisite orthosteric binding site. We are therefore confident the spontaneous currents that were recorded are derived from triheteropentameric receptor isoforms in recombinant cells, and when subunits were expressed in native neurons.

6.1. Structural determinants of spontaneous activity: targets for pharmacology?

6.1.1. Spontaneous activity is dependent on receptor subunit composition

We have demonstrated a clear reliance on the presence of the β3 subunit within the receptor complex for $GABA_A$ receptors to gate spontaneously. For every combination of receptor trialled, exchanging the β3 subunit for β2 was sufficient to almost completely remove constitutive activity, and this also holds for substitution of β3 with the β1 subunit. In addition to this, the presence of subunits associated with extrasynaptic receptors, α4/6 and δ, also promote the spontaneous gating of these $GABA_A$ receptors. Importantly, the requirement of these subunits for the highest spontaneous effect was determined by taking into account the fact that GABA acts as a partial agonist at δ-containing receptors but a full agonist at γ-containing receptors (Brown et al., 2002; Mortensen et al., 2010). Using GABA to measure peak receptor expression as a tool to compare between these two groups of receptors is unlikely to provide comparative levels of spontaneity, however this was overcome by using the agonist THIP, which shows super-agonist properties at δ-containing receptors and which still identified larger spontaneity at α4β3δ receptors (Krogsgaard-Larsen et al., 2004; Mortensen et al., 2010). It was

important to normalise to a peak GABA response to overcome differences in expression levels. Phosphorylation is known to impact on surface expression levels of both recombinant and native $GABA_A$ receptors (Kittler & Moss, 2003; Luscher & Keller, 2004; Kittler et al., 2005; Saliba et al., 2012; Nakamura et al., 2015). Despite observing larger GABA-mediated currents with some of the phosphorylation mutants, notably the single $\alpha4\beta3^{S408A}\delta$ and $\alpha4\beta3^{S409A}\delta$ recombinant receptors, we could reliably demonstrate that these mutants showed proportionally less spontaneous activity.

The propensity for extrasynaptic-type receptors to gate constitutively is consistent with their role in generating tonic inhibition (Brickley et al., 1996; Mody, 2001; Stell et al., 2003; Brickley & Mody, 2012). This continuing receptor activity has dual effects on neuronal excitability; a persistent hyperpolarising effect on the neuron as the membrane potential is drawn towards the reversal potential for $GABA_A$ receptors (E_{GABA}), as well as shunting inhibition caused by increased membrane conductance. This affects the amplitude and duration of membrane voltage changes and the extent with which voltages attenuate with distance as they travel through the dendritic network (Mitchell & Silver, 2003; Semyanov et al., 2004; Mann & Paulsen, 2007; Silver, 2010). Interestingly, depending on the resting membrane potential and the relative concentration of ions across the neuronal membrane, $GABA_A$ receptor activity can be excitatory (Ben-Ari, 2002; Raimondo et al., 2012). Indeed this is the case early in development and in some mature neurons (Rivera et al., 1999; Gulledge & Stuart, 2003). Nevertheless, shunting inhibition is always inhibitory as it represents an increase in membrane conductance (Silver, 2010). Spontaneous activity in these different contexts may therefore have important but quite different consequences for neuronal activity and neurodevelopment.

The role of synaptic-type receptors in maintaining spontaneous tonic activity may also have an impact, and indeed spontaneous currents in neurons are potentiated by benzodiazepines, indicating γ-containing receptors contribute to the spontaneous tonic current (Birnir et al., 2000). We have demonstrated low levels of spontaneity of $\alpha1\beta3\gamma2$ receptors, but it is conceivable that these receptors show some levels of spontaneous activity

that could represent local shunting inhibition and local hyperpolarising effects at inhibitory synapses, potentially impacting on IPSP amplitude and waveform (O'Neill & Sylantyev, 2018). Indeed, significant β3 expression at synapses in DGGCs indicates significant contribution to IPSC generation with potential ramifications for tonic inhibition and local shunting effects (Herd et al., 2008). Alongside potential impacts by receptors localised at synapses, α5βγ2 receptors are found at extrasynaptic sites within the hippocampus and are responsible for the majority of tonic inhibition in the CA3 and CA1 areas (Caraiscos et al., 2004). There is evidence for a large population of β3-containing receptors in extrasynaptic areas of hippocampal pyramidal neurons, many of which likely correspond to these γ-containing receptors (Kasugai et al., 2010). The spontaneous activity of these receptors, despite being lower than for δ-containing receptors, could still play a role in tonic inhibition.

6.1.2. Molecular determinants of spontaneous activity are present at subunit interfaces

We have identified the GKER motif of the β3 subunit as being critical for the generation of spontaneous activity in both recombinant receptors and cultured neurons. This motif was originally described as being necessary for the formation of homomeric β3 subunits and for the surface expression of βγ heteromers (Taylor et al., 1999). As β3 homomeric receptors are spontaneously active, we performed numerous control experiments to show that these residues were required for intrinsic spontaneity of heteromeric receptors and not creating a sub-population of spontaneously-active homomeric receptors. In heteromeric receptors, the GKER motif is located at the α^{+}-β^{-} and δ^{+}-β^{-} subunit interfaces, and therefore not at the orthosteric agonist-binding β^{+}-α^{-} interfaces (Taylor et al., 1999; Miller & Aricescu, 2014; Laverty et al., 2019). The motif could be involved in down-stream conformational changes during ligand-binding, explaining the enhanced potency of GABA at receptors which show higher levels of spontaneous activity. This is in accordance with studies performed on the β2 subunit, which

identified this area to be involved in receptor gating, and also showed mutations could induce spontaneous currents (Williams et al., 2010). However, we have also shown how the other subunits within the pentamer can affect spontaneous activity, and have identified residues in the ECD of α4/6 and δ subunits which mediate this, as replacement with synaptic-type α1 and γ2 residues reduced the level of spontaneity. The GKER motif is likely to interact with these residues across the subunit interface, as indicated by its requirement for the assembly of certain receptor isoforms (Taylor et al., 1999).

Subunit interfaces, both extracellular and within transmembrane domains, are important sites of ligand binding and modification of receptor function (Fig. 1.6) (Ramerstorfer et al., 2011; Sieghart et al., 2012; Laverty et al., 2017; Olsen, 2018; Masiulis et al., 2019). The gating process of the receptor involves a twisting of the subunits and closer association of subunit ECDs, resulting in a widening of the channel pore (Masiulis et al., 2019). This process is enhanced by the binding of orthosteric agonists at the β^+-α^- interfaces, and is also potentially how benzodiazepines are involved in promoting receptor activity at the α^+-γ^- interface (Campo-Soria et al., 2006; Olsen, 2018). We propose that the presence of the GKER motif at the α^+-β^- and δ^+-β^- interfaces allows closer association with the adjacent subunit than DNTK residues in β2, and that this promotes receptor activation. This may be supplemented by intra-subunit effects of the GKER motif within the β3 subunit, and intra- and inter-subunit interactions within both α4 and δ subunits, consistent with α4β3δ receptors being more able to open both spontaneously and in the presence of GABA than α1β3γ2 receptors (Brown et al., 2002). These subunit interfaces therefore represent novel therapeutic targets to impact on receptor gating. This is already evident from known agonist and modulator binding sites. The orthosteric β^+-α^- interface is already the site of action for multiple agonists and antagonists, e.g. THIP, gabazine and bicuculine, as is the benzodiazepine binding site at the α^+-γ^- interface (Laverty et al., 2019; Masiulis et al., 2019). Benzodiazepines are known to bind with low-affinity at the α^+-β^- site and this can potentiate $GABA_A$ receptor currents (Ramerstorfer et al., 2011; Sigel & Ernst, 2018). Due to the importance of the GKER motif, it is likely that molecules binding to this site could potentiate or

inhibit the spontaneous activity (Ramerstorfer et al., 2011; Sieghart et al., 2012; Varagic et al., 2013a; Varagic et al., 2013b). As this interface is homologous to the orthosteric and benzodiazepine binding sites, there should be suitable binding pockets which could be explored, opening up the possibility of selectivity for specific α subunits and targeted therapy. This could be useful as an alternative to benzodiazepines in the treatment of epilepsy, as these have limited uses due to tolerance caused by rapid benzodiazepine-dependent internalisation of γ-containing receptors (Bateson, 2002; Naylor et al., 2005; Ochoa & Kilgo, 2016).

6.1.3. Modulation of spontaneous activity

We have demonstrated how spontaneous currents of recombinant receptors are affected in similar fashion to GABA-dependent currents by pharmacological manipulation, including benzodiazepine and neurosteroid potentiation, as well as inhibition through antagonists such as pregnenolone sulfate. The spontaneous current can also be modulated through phosphorylation of the β3 subunit, again identifying this subunit as key for spontaneous activity. As with GABA-mediated activity, neurosteroid potentiation and phosphorylation are likely to interlink to regulate spontaneous tonic currents.

Neurosteroid potentiation of spontaneous activity would provide a mechanism through which tonic inhibition could be maintained during low levels of GABA, either temporally during less intense activity resulting in reduced spillover from synapses, or regionally in which there is little GABA-activated tonic inhibition (Rossi & Hamann, 1998; Bright et al., 2007; Wlodarczyk et al., 2013). Neurosteroid potentiation of tonic currents in the dentate gyrus has been shown to be almost entirely dependent on the presence of δ-containing receptors (Stell et al., 2003), and this coincides with these receptors being more spontaneously active (Brown et al., 2002; Carver & Reddy, 2013). Considering the evidence that almost all tonic inhibition in these neurons occurs through spontaneous activity, neurosteroids likely play

an important role in mediating tonic inhibition through regulation of spontaneous currents, and hence in controlling hippocampal network activity (Wlodarczyk et al., 2013).

Neurosteroids play an important role in the regulation of tonic inhibition in the brain, the significance of which is underscored by experiments showing how modulation of neurosteroid levels in the oestrous cycle result in increased expression levels of the δ subunit (Reddy, 2010; Wu et al., 2013). This is accompanied by reduced seizure susceptibility during high neurosteroid levels, indicating the importance of these compounds in preventing pathological excitability (Maguire et al., 2005). Indeed, catamenial epilepsy is associated with fluctuations of neurosteroid levels during the menstrual cycle, affecting regulation of tonic inhibition mediated by δ-containing $GABA_A$ receptors, of which a spontaneously gating population will likely exist (Reddy, 2016; Clossen & Reddy, 2017). The regulation of spontaneous activity by neurosteroids is likely to be relevant when considering temporal lobe epilepsy, characterised by heightened levels of excitability in the dentate gyrus, an area which displays tonic inhibition highly dependent on spontaneous $GABA_A$ receptor activity (Toader et al., 2013; Wlodarczyk et al., 2013; Scharfman, 2019). High spontaneous activity would ensure high tonic current despite fluctuations in GABA concentration, and maintain appropriate excitability levels in these brain areas. Synthetic neurosteroids would therefore be an appropriate tool for treating pathology involving enhanced excitability (Biagini et al., 2010). They are reasonably non-selective and could potentiate synaptic and extrasynaptic $GABA_A$ receptors. Selectivity for certain subtypes may be possible, as they bind at the β^+-α^- interface within the transmembrane domains (Hosie et al., 2009; Laverty et al., 2017; Miller et al., 2017). Selectivity for β3 subunit interfaces may preferentially lead to the potentiation of spontaneous receptor activity and the reduction of off-target effects.

In addition to modulation by neurosteroids, we have also shown kinase-dependent modulation of spontaneous activity. This was dependent upon the β3 subunit, providing further evidence of this subunit's importance in mediating spontaneous activity. The phosphorylation sites S408 and S409 within the M3-M4 large intracellular loop are critical for modulating spontaneous activity, as

partial or absent phosphorylation significantly reduced spontaneous currents. Single channel recordings showed that PKA activation in HEK cells expressing α4β3δ receptors prolongs open channel burst duration and frequency (Tang et al., 2010). The reduced spontaneous activity seen with either mutation of the phosphorylation sites or with inhibition of kinases therefore likely represents similar properties, resulting in an overall reduced open probability of the receptor. Whether this is linked to GABA potency is unknown, but we have shown evidence for a correlation between GABA-mediated activity and spontaneity (Anstee et al., 2013; Patel et al., 2014; Akk et al., 2018). The role of the intracellular loop and phosphorylation therein should be further investigated to examine its role in spontaneous receptor kinetics, and whether these effects were caused by direct changes in gating kinetics (Milone et al., 1998; Akk & Steinbach, 2000; Kelley et al., 2003; Fisher, 2004).

Phosphorylation has multiple effects on receptors, most notably their surface expression and synaptic localisation (Kittler et al., 2005; Mukherjee et al., 2011; Saliba et al., 2012). The complexity of the role of phosphorylation was shown through transfection of neurons with the $\beta3^{S409A}$ subunit, which showed no difference in the level of spontaneity compared with neurons transfected with wild-type. The experiments performed in cultured neurons did not use the methodology used in HEK cells, whereby the PTX-sensitive spontaneous current was normalised to the total $GABA_A$ receptor current. Instead, we measured the I_{PTX} spontaneous current only, normalised to cell capacitance. Transfection of the $\beta3^{S409A}$ subunit could therefore have significantly increased the formation and surface expression of $GABA_A$ receptors, as it most likely did in recombinant receptors, but due to compromised spontaneity of individual receptors no difference in the macroscopic whole-cell spontaneous current was detected. The effect of phosphorylation of these S408 and S409 residues on receptor trafficking is well-documented and increases the complexity of the effects of phosphorylation on spontaneous activity (Comenencia-Ortiz et al., 2014; Nakamura et al., 2015). The activation of PKA in HEK 293 cells enhanced receptor expression levels through reductions in receptor internalisation (McDonald et al., 1998; Kittler et al., 2005). PKC has been shown to both

enhance and decrease receptor expression levels depending upon experimental design, expression system and PKC isoforms (Krishek et al., 1994; Abramian et al., 2010; Bright & Smart, 2013a; Nakamura et al., 2015). Any activation of kinases targeting the S408 and S409 residues on the β3 subunit will impact on spontaneous activity, but the overall effect will also depend on changes to expression levels of the receptor.

The S383 phosphorylation site on the β3 subunit is phosphorylated by CaMKII (Houston et al., 2007; Houston et al., 2009). This kinase is essential for activity-dependent plasticity changes in neurons, and has been shown to enhance insertion of $GABA_A$ receptors at interneuron-Purkinje cell inhibitory synapses within the cerebellum to enhance postsynaptic currents (He et al., 2015) and to enhance insertion of extrasynaptic receptors into hippocampal neuronal membrane to increase tonic inhibition, thereby acting as a mechanism for regulating neuronal excitability (Saliba et al., 2012). High-frequency stimulation has also been shown to induce long-term potentiation of GABAergic synapses through phosphorylation of the S383 residue by CaMKII, resulting in enhanced levels of β3-containing receptors at synapses in a gephyrin-dependent manner (Petrini et al., 2014). We observed no effect of phosphorylation at this site or any impact of inhibiting CaMKII. This could imply that CaMKII phosphorylation of S383 has no impact upon spontaneous receptor activity. However, there is difficulty in examining the full impact of CaMKII in HEK cells due to apparent differences in kinase actions, and this should therefore be further investigated in more neuronal expression systems (Houston & Smart, 2006). It would be surprising for CaMKII to not regulate spontaneity in some way, as tonic inhibition is plastic (Brickley et al., 1996; Mody, 2005) and the reporting of network activity through Ca^{2+} influx and CaMKII activation may be an important regulator of spontaneous activity in areas of the brain which have little GABA-mediated tonic inhibition, such as DGGCs (Wlodarczyk et al., 2013). However, as already discussed, there may not be a direct impact on the spontaneous gating of individual receptors, but CaMKII activation and phosphorylation of S383 of the β3 subunit does facilitate insertion of these receptors into the membrane, thereby representing an indirect increase in the amplitude of spontaneous current (Saliba et al.,

2012). The impact of CaMKII on spontaneous activity varies between brain regions, as CaMKII-mediated insertion of receptors during synaptic plasticity within the cerebellum preferentially inserts synaptic β2-containing $GABA_A$ receptors, which will likely not contribute to the spontaneous, tonic current (He et al., 2015).

Based on our data and previous findings, there is likely an intimate link between spontaneous activity, phosphorylation and neurosteroid potentiation (Abramian et al., 2014; Comenencia-Ortiz et al., 2014; Adams et al., 2015). The more receptors present on the cell-surface, the greater the impact of modulation by neurosteroids, benzodiazepines, endozepines and other regulators of spontaneous activity. Additionally, the extent of phosphorylation can be modulated by neurosteroid activity, as demonstrated by increased phosphorylation of the α4 S443 residue in the presence of neurosteroids, enhancing tonic currents through changes in expression levels (Abramian et al., 2010; Abramian et al., 2014). This effect is further shown through investigation of phosphorylation of the S408 and S409 residues of β3, which act to enhance surface expression levels, but also have a direct impact on the level of potentiation by neurosteroids (McDonald et al., 1998; Adams et al., 2015). How phosphorylation of these sites impacts on potentiation by neurosteroids may be linked to their role in determining spontaneity of the receptor, and is worthy of future study.

6.2. Spontaneous activity *in vivo*

6.2.1. Spontaneous activity impacts on tonic inhibition differentially throughout the brain

We have shown extensively *in vitro* that certain isoforms of the $GABA_A$ receptor show spontaneous activity, and that this can be affected through pharmacological manipulation and through endogenous modulators. We have also shown that these concepts hold for hippocampal neurons in culture, and then began to show the reliance on spontaneous activity for tonic inhibition of

different brain areas in *ex vivo* slice recordings. The tonic current in DGGCs appeared to largely depend upon spontaneous activity, whilst thalamic relay neurons did not show as great a reliance on spontaneous activity to maintain tonic inhibition. Using our results from recombinant receptors, we can begin to build a picture of what occurs *in vivo* to determine and regulate spontaneous activity.

The presence of the β3 subunit is integral to spontaneous activity *in vitro*, and this likely holds true for spontaneous activity in the intact brain. Areas which display higher expression levels of the β3 subunit will have a greater spontaneous component to tonic inhibition, as demonstrated by recording from DGGCs (high β3) and thalamic relay neurons (low β3) (Wisden et al., 1992; Pirker et al., 2000; Korpi et al., 2002; Belelli et al., 2005; Stefanits et al., 2018). This could be used to estimate the impact of spontaneous activity in other brain areas, including striatum, cerebellum and neocortex. The overall strength of the spontaneous activity will also be determined by the expression of extrasynaptic-type subunits α4/6 and δ. Hippocampal CA3 and CA1 neurons typically rely on α5βγ2 receptors over α4βδ receptors, and so spontaneous tonic inhibition in this area will likely be less evident (Caraiscos et al., 2004). It is possible that synaptic-type αβ3γ2 receptors will contribute to the spontaneous tonic current, but this will be overshadowed by any α4β3δ contribution depending on the relative receptor expression levels and the phosphorylation state. In neurons which express multiple β subunits, preferential localisation of β3 at synaptic or extrasynaptic sites will impact on the level of spontaneous activity. The overall spontaneity of $GABA_A$ receptors in different brain regions will therefore depend on the ratio of β3 expression to β1/2, and subcellular localisation.

Due to the effects of phosphorylation on surface expression, neurosteroid modulation and a direct effect on spontaneous activity, it is important to investigate monoamine and cholinergic connectivity and its role in defining spontaneous $GABA_A$ receptor activity. Dopamine, 5-HT, noradrenaline and acetylcholine have diffuse projections across the brain (Chandler et al., 2013; Jacob & Nienborg, 2018) that activate GPCRs, resulting in intracellular cascades activating or inhibiting protein kinases (Crunelli & Di

Giovanni, 2014; Huang & Thathiah, 2015). This is also true for the more ubiquitous neurotransmitters glutamate and GABA, acting *via* metabotropic glutamate (mGluRs) and $GABA_B$ receptors (Connelly et al., 2013a; Connelly et al., 2013b; Huang & Thathiah, 2015).

The striatum is involved in motor behaviour and is predominantly composed of GABAergic medium spiny neurons (MSNs) (Kreitzer & Malenka, 2008; Macpherson et al., 2014; Cox & Witten, 2019). Expression studies show high levels of β3 in this area (Wisden et al., 1992; Pirker et al., 2000; Hortnagl et al., 2013; Waldvogel & Faull, 2015). Two projection pathways exist to mediate movement, the direct and indirect pathways (Kreitzer & Malenka, 2008; Macpherson et al., 2014). The former is controlled by neurons expressing dopaminergic D1 receptors, and the latter by neurons expressing D2 receptors (Surmeier et al., 2007). These dopamine receptors increase (D1) or decrease (D2) cAMP production, and therefore PKA activity (Huang & Thathiah, 2015). In mouse brains, D1-positive neurons have a larger tonic inhibitory current than D2-positive neurons, possibly promoted by the expression of PKA-dependent spontaneous activity (Santhakumar et al., 2010). In other experimental conditions, D2-positive neurons show greater tonic currents, explained by a developmental switch upregulating α4 and δ subunits (Ade et al., 2008; Lee & Maguire, 2014). Intriguingly, application of etomidate, a selective β2/3 modulator, produced currents in both D1- and D2-positive neurons which could be enhanced by PKA application or modulation of dopaminergic responses. Due to the low expression levels of β2 in this brain area, this suggests a key role for phosphorylation of the β3 subunit in regulating tonic inhibition (Janssen et al., 2009). The importance of the β3 subunit for tonic inhibition of MSNs was confirmed using conditional β3 knock-out studies, in which tonic inhibition was severely impaired and neuronal excitability enhanced (Janssen et al., 2011). These studies are therefore in agreement with ours which suggest a key role for both phosphorylation and the β3 subunit in regulating tonic inhibition. A role for dopaminergic regulation of tonic currents was also shown in hippocampal slices, whereby activation of D1 receptors resulted in phosphorylation of the S408 and S409 residues and greater GABAergic currents (Terunuma et al., 2004). The impact of

dopaminergic transmission on the phosphorylation state and spontaneous activity of $GABA_A$ receptors should be further investigated to establish how tonic inhibition can be regulated.

The downstream effects of GPCR activation on tonic inhibition are not always clear and can vary between brain areas (Connelly et al., 2013a). For example, activation of $GABA_B$ receptors enhanced extrasynaptic currents in the thalamus, dentate gyrus and cerebellum, which could be mimicked by inhibiting PKA activity. This contrasts with the studies described in the striatum (Connelly et al., 2013b; Tao et al., 2013). Dissociating the mechanistic impact of kinase modification will always be difficult in a neuronal environment due to the large number of interacting proteins, expression of which could result in bi-directional effects on $GABA_A$ receptor activity. Even at the most basic level, alterations in the phosphorylation state of the β3 subunit will affect receptor trafficking and surface expression, as well as impact on spontaneous activity of the receptor (Kittler & Moss, 2003; Tang et al., 2010). The overall impact of phosphorylation on spontaneous activity therefore will depend on co-expression of other proteins and will ultimately vary between different brain regions.

In summary, tonic inhibition in the brain is controlled by a variety of mechanisms, affecting both GABA-dependent and spontaneous tonic currents. In areas of the brain where ambient GABA is the predominant source of tonic inhibition, for example the thalamus, increases to synaptic activity, glial release of GABA, and uptake mechanisms will all impact on tonic current (Pirker et al., 2000; Semyanov et al., 2003; Cope et al., 2005; Bright et al., 2007; Lee et al., 2010). In areas where spontaneous activity predominates, these mechanisms may still impact on tonic inhibition, but the spontaneous aspect would effectively decouple tonic current from synaptic activity, although the presence of ambient GABA may enhance a baseline tonic current. The activity of second-messenger receptors, such as GPCRs and receptor tyrosine kinases, will impact on both forms of tonic current (Connelly et al., 2013a). The phosphorylation state of receptors will alter surface expression and possibly synaptic and extrasynaptic receptor localisation, thereby making receptors more or less available to respond to ambient GABA (Kittler & Moss, 2003;

Nakamura et al., 2015). The spontaneous activity will also be enhanced when β3-containing receptors are more numerous on the membrane, but the spontaneous current will also be directly affected by the phosphorylation state of the receptor. The presence of GPCRs also provides a more subtle activity dependence upon spontaneous tonic current, as postsynaptic or extrasynaptic mGluRs and $GABA_B$ receptors will alter the phosphorylation state of receptors (Connelly et al., 2013a; Connelly et al., 2013b; Huang & Thathiah, 2015). Overall, when accounting for the presence of spontaneously active $GABA_A$ receptors, the final extent of tonic inhibition is determined by a variety of factors that may differ substantially between brain areas.

6.2.2. Spontaneous activity in pathology

We have shown how the $\beta3^{T185I}$ mutation can have a direct impact on the spontaneous activity of receptors, which could well contribute to the epileptic phenotype of patients by preventing sufficient tonic inhibition (Walker & Kullmann, 2012). We have also shown how the $\beta3^{L170R}$ mutant has enhanced efficacy, GABA potency and spontaneous current, albeit with reduced surface expression. This will ultimately result in dysregulated tonic inhibition, particularly in regard to the lack of modulation by neurosteroids (Biagini et al., 2010; Biagini et al., 2013). We discussed the role of tonic inhibition and the role spontaneous activity may play in Ch. 5, but point mutations and polymorphisms in the β3 subunit have been noted for many disorders other than epilepsy, including autism (Buxbaum et al., 2002; Møller et al., 2017), and alterations in tonic inhibition have been noted in multiple pathological states, including epilepsy, ischaemia and mood disorders (Brickley & Mody, 2012). We have demonstrated how the level of receptor spontaneity can be controlled through neurosteroid and kinase activity, including a high dependence on subunit expression. Disruptions to any of these factors would therefore impact on spontaneous activity and tonic currents in the brain, possibly contributing to pathological phenotypes.

$GABA_A$ receptor activity has neuroprotective properties, preventing excess excitability and excitotoxicity under various conditions (Brickley & Mody, 2012; Wu & Sun, 2015). Huntington's disease is characterised by the loss of striatal medium spiny neurons from excitotoxic stress, resulting in the movement disorders which are the hallmarks of the disease (Estrada Sanchez et al., 2008). Adult D1-positive MSNs, which display higher levels of tonic inhibition, are more protected from excitotoxic damage, whilst mice lacking the δ subunit show increased cell loss (Santhakumar et al., 2010). Enhancement of inhibitory signalling at large aspiny neurons of the striatum occurs after transient cerebral ischaemia, potentially explaining why these neurons have a greater survival chance (Li et al., 2009). This effect was dependent on presynaptic GABA release and, even though the role of tonic inhibition is unclear, the impact of $GABA_A$ receptors on cell survival after excitotoxic damage is evident. $GABA_A$ receptor α1 and β2 subunit expression levels in the CA1, CA3 and dentate gyrus decreased immediately after transient forebrain ischaemia, but showed a gradual recovery in CA3 and dentate areas over time. In the CA1 area, however, expression did not recover and this area, unlike CA3 and dentate, showed cell death (Li et al., 1993). This could explain the loss of IPSCs in CA1 pyramidal neurons after induction of ischaemia, and this study also noted an increase in the membrane resistance, indicating reductions in tonic inhibition (Xu & Pulsinelli, 1994). Loss of subunit expression may occur for both synaptic and extrasynaptic subunits, and a loss in tonic or spontaneous activity may therefore promote ischaemic cell death.

The effect of phasic and tonic inhibition on cell survival varies between brain regions, and may actually prevent functional recovery after stroke. Enhanced tonic inhibition in the peri-infarct zone reduced the plastic changes required to rewire circuits and to recover function, and reducing tonic inhibition with α5-selective negative allosteric modulators or lowering extrasynaptic subunit expression improved recovery after stroke (Clarkson et al., 2010). Hence, tonic inhibition may be generally neuroprotective, but it may also prevent recovery by dampening excitability and plasticity. Homeostatic changes to tonic inhibition are evident to maintain normal levels of activity (Brickley et al., 2001; Carver & Reddy, 2013), and it is feasible that changes

to spontaneously active receptor numbers or their subunit composition could be altered as part of these plastic changes to promote cell viability or to maintain normal function. If these processes fail, or are not refined, it could lead to dysfunction.

Alterations in $GABA_A$ receptor phosphorylation are also known to occur in various pathologies (Brickley & Mody, 2012; Nakamura et al., 2015; Mele et al., 2019). Status epilepticus results in a reduction in the phosphorylation state of the S408 and S409 residues of the β3 subunit, resulting in endocytosis of these receptors (Kittler et al., 2005; Terunuma et al., 2008). There is also an NMDA receptor-dependent mechanism of $GABA_A$ receptor dephosphorylation mediated by the Ca^{2+}-dependent phosphatase calcineurin, which is activated during high frequency stimulation associated with epilepsy (Wang et al., 2009). The loss of these receptors will not only impact on synaptic currents but also possibly on spontaneous tonic currents. Dephosphorylation of these two residues has also been observed during ischaemic experiments, and contributes to cell death (Mele et al., 2014). In models of autism spectrum disorder, reduced surface expression of the β3 subunit has been observed, along with reduced expression of the K^+-Cl^- cotransporter KCC2, indicating not only reduced GABAergic currents (and likely spontaneous activity), but changes to the chloride gradient and the reversal potential E_{GABA} (Li et al., 2017).

As with normal physiological states, dissociating the impact of phosphorylation/dephosphorylation on receptor trafficking and expression from direct effects on receptor spontaneous activity is difficult. However, as dephosphorylation is associated with internalisation of these receptors, it is likely that this would result in reduced synaptic and tonic currents through reduced expression, but also through reduced spontaneous activity of the remaining receptors.

In summary, it is probable that alterations to spontaneous activity occur during pathological states, either through changes to surface expression of receptors, or to the actions of neuromodulators such as neurosteroids, kinases and phosphatases which alter the phosphorylation state of $GABA_A$ receptors.

The impact of such alterations will depend on the reliance of a brain area upon spontaneous activity for the provision of tonic inhibition.

6.3. Concluding remarks

Spontaneous activity of the $GABA_A$ receptor represents a relatively unexplored facet of tonic inhibition. In this body of work, we have shown how the spontaneous activity of $GABA_A$ receptors is determined by subunit composition through molecular determinants; likely making essential intra- and inter-subunit interactions to promote constitutive opening of receptors. We have also demonstrated how these spontaneous currents are dependent on the phosphorylation state of the receptor, and this likely represents a mechanism to fine-tune tonic inhibition through membrane expression, as well as spontaneous activity. There is a significant requirement for the β3 subunit for spontaneous activity in neurons *in vitro*, validating our work in recombinant receptors. Selective targeting of spontaneous activity, particularly *in vivo*, would provide a novel therapeutic approach for selectively altering tonic inhibition in specified areas of the brain.

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