$GABA_A$ receptor desensitization shapes the kinetics and plasticity of inhibitory neurotransmission

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A thesis submitted to University College London for the Degree of Doctor of Philosophy

January 2019

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Declaration

I, Martin Field, 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

$GABA_A$ receptors are anion-permeable pentameric ligand-gated ion channels. They are the primary mediators of inhibitory neurotransmission in the central nervous system. As with many other ion channels, they are known to desensitize. This is a process involving the entry into a long-lived closed state after exposure to agonist. Although studies of receptors in recombinant systems and structural work have provided a lot of information about the entry of the receptor into the desensitized state, its physiological role remains unclear. In this project I sought to further elucidate the physiological role of entry of $GABA_A$ receptors into the desensitized state using mutations in the desensitization gate previously identified by our laboratory. I initially studied these mutations in recombinant expression systems to further characterise their effects on $GABA_A$ receptor kinetics and thus demonstrated that desensitization indeed effects the kinetics of phasic- and tonic-like responses. I then confirmed these phenotypes in cultured hippocampal neurons. Treatments of neurons with GABA and allosteric modulators of $GABA_A$Rs were then used in combination with the desensitization mutants to assess whether desensitization has any long-term effects on inhibitory synapses. $GABA_A$R desensitization was found to result in a PKC-dependent long-term potentiation of inhibitory synapses.

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Impact statement

The $GABA_A$ receptor is the major inhibitory neurotransmitter receptor in the brain with responsibility for controlling neuronal excitability over short- and long-term periods. Dysfunction of these receptors is associated with many neurological diseases and as a consequence they have become targets for many clinically important drugs, including benzodiazepines such as diazepam, and general anaesthetics such as Propofol and isoflurane. These drugs along with the endogenous ligand for the receptor, GABA, and endogenous modulators such as the neurosteroids, have long been known to cause, or enhance, the opening of a channel in the receptor that allows anions to flow across the cell membrane. Such ionic currents are well established conduits for regulating neuronal excitability, usually acting to inhibit action potential firing. However, the binding of these drugs to the $GABA_A$ receptor is also known to cause the process of receptor desensitization, in which the receptor slowly enters a specific closed state defined as the desensitized state. In this project, we aimed to establish how this state impacts the physiological function of synaptic and extrasynaptic $GABA_A$ receptors.

This study has discovered how this desensitised state of the receptor affects basal inhibitory synaptic and tonic neurotransmission, in the process providing important new insight into how drugs targeting the desensitized state can affect neuronal excitability. In particular, this study shows that the desensitization only restricts GABA channel opening during synaptic forms of inhibition. By contrast, and counterintuitively, tonic GABA currents generated by exposure of the receptor to low concentrations of ambient GABA are strengthened by measured entry of the receptor into the desensitized state. This work thus highlights a potential problem for the design of drugs that

might target the desensitized state: such modulators will likely have conflicting effects on phasic and tonic inhibition, depending on the level of ambient GABA.

Another major finding from this study followed probing the long-term consequences of $GABA_AR$ desensitization for inhibitory neurotransmission and plasticity. Here we have demonstrated that, contrary to its name, entry of receptors into desensitized states actually promotes the upregulation of $GABA_ARs$ at inhibitory synapses. This novel form of long term potentiation at inhibitory synapses has numerous potential implications for the understanding of the actions of drugs that target $GABA_A$ receptors, and for processes such as learning and memory.

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Acknowledgements

I would like to thank all of the Smart lab for their constant support and during this project. Especially Trevor and Phil for all their help making my PhD happen. I would also like to thank Megan, Mamo, and Damers for all their guidance and support on getting the experiments to work.

I also can't thank my family enough for all the support they've given me these last few years, especially with all the difficulties we've faced. I am particularly grateful to George, Jack, and James for being the best brothers anyone could have.

Finally, I am also grateful to the MRC and the LMCB for funding me.

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List of abbreviations

$5\text{-}HT_3R$ – 5-hydroxytryptamine type 3 receptors

AMPA – 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid

BCA - bicinchoninic acid

BDNF – brain derived neurotrophic factor

BSA – bovine serum albumin

CNS – central nervous system

DAG - diacylglycerol

DHEAS - dehydroepiandrosterone sulfate

DIV – days *in vitro*

ECD – extracellular domain

eGFP – enhanced green fluorescent protein

GABA – γ-aminobutyric acid

$GABA_AR$ – $GABA_A$ receptor

GlyR – glycine receptor

GPCR – G-protein coupled receptor

GRK – G-protein coupled receptor kinase

HRP – horseradish peroxidase

ICD – intracellular domain

IPSC – inhibitory postsynaptic current

LTD – long-term depression

LTP – long-term potentiation

mGluR – metabotropic glutamate receptor

nAchR – nicotinic acetyl choline receptor

NMDA – N-Methyl-D-aspartate

PKC – protein kinase C

PLC – phospholipase-C

pLGIC – pentameric ligand gated ion channel

PMA – phorbol myristate acetate

PS – pregnenolone sulfate

RIPA – radioimmunoprecipitation assay buffer

RTK – receptor tyrosine kinase

SDS – sodium dodecyl sulfate

sIPSC – spontaneous inhibitory postsynaptic current

TBS – tris buffered saline

TBST – TBS with 0.1 % tween

TEMED - Tetramethylethylenediamine

THDOC – tetrahydrodeoxycorticosterone

TMD – transmembrane domain

Chapter 1: Introduction

1.1. An introduction to $GABA_A$ receptors

The nervous systems of animals consist of neurons and glial cells that are capable of generating self-propagating electrical signals known as action potentials. Such action potentials can be transduced across large distances along the plasma membranes of neurons but cannot directly cross the gaps to adjacent cells. Instead, communication between the different neurons that make up the nervous system is achieved using structures known as synapses, where an action potential in the pre-synaptic cell causes the release of a substance known as a neurotransmitter onto a cluster of receptors on the postsynaptic cell.

There is a diverse range of different neurotransmitters and neurotransmitter receptors that are expressed at the synapses of the mammalian brain. However, such receptors can be broadly defined as either excitatory or inhibitory depending on whether their activation increases or decreases the probability of the postsynaptic cell generating an action potential of its own. The focus for this thesis is on the inhibitory receptors, and in particular the $GABA_A$ receptor ($GABA_AR$). These receptors respond to γ-aminobutyric acid (GABA), one of the primary types of neurotransmitter found in the mammalian central nervous system (CNS). The receptors for GABA are divided primarily into two different families: $GABA_A$ receptors, and $GABA_B$ receptors. Although both types of GABA receptor respond to GABA and are generally considered inhibitory in their effects, the $GABA_A$ receptors are ion channels whilst $GABA_B$ receptors are G-protein coupled receptors (GPCRs). Some authors (Johnston, 1991) also distinguish between these families and another referred

to as the $GABA_C$ receptors; however, these receptors are structurally and functionally related to the $GABA_A$ receptors.

Recent structural and functional studies have increased our understanding of how $GABA_A$Rs are activated by GABA and elucidated the conformational changes that occur after GABA binds. This thesis focuses on one of these conformational states: the desensitised state, and in particular, how this conformational state shapes inhibitory neurotransmission.

1.1.1. The $GABA_A$ receptor is a pentameric ligand-gated ion channel

The $GABA_A$R is a type of protein known as an ion channel. These proteins are present in the plasma membranes of cells and, when open, provide a passage for ions to passively move across the plasma membrane according to their electrochemical gradient. In particular, the $GABA_A$Rs are members of a family of ion channels known as the pentameric ligand-gated ion channel (pLGIC) family (Barnard et al, 1987). This family has traditionally also been referred to as the Cys-loop family due to the presence of a disulphide bridge between conserved cysteines flanking one of the loops of the extracellular domain. However, the discovery of prokaryotic pLGICs (Tasneem et al, 2004) without the conserved cysteines has led to the renaming of this group to the Pro-loop family due to the observation that a proline that happens to be in the same loop is in fact the most conserved residue across the whole family (Jaiteh et al, 2016). Other notable members of this family that are found within the mammalian nervous system include the cation-permeable nicotinic acetylcholine receptors (nAChRs), and 5-hydroxytryptamine type 3 ($5\text{-}HT_3$) receptors, in addition to the anion-permeable glycine receptors (GlyR). Additional members of this family found in invertebrates and prokaryotes include GLIC from *Gloeobacter violaceus* (Bocquet et al, 2007), ELIC from

Erwinia chrysanthemi (Hilf and Dutzler, 2008), and the glutamate-gated anion channel GluCl from *Caenorhabditis elegans* (Cully et al, 1994).

α	β	γ	others
α1	β1	γ1	δ
α2	β2	γ2 (short or long splice isoforms: γ2s, γ2L)	ε
α3	β3	γ3	π
α4			θ
α5			ρ1
α6			ρ2
			ρ3

Table 1.1: GABAAR subunit families.

As pentameric ion channels, each individual $GABA_AR$ is composed of five subunits. A total of 19 different genes encoding $GABA_AR$ subunits from 8 different subunit families (α1-6, β1-3, γ1-3, δ, ε, π, θ, ρ1-3; Table 1.1) have been identified (Sieghart and Sperk, 2002). Although β (Krishek et al, 1996; Wooltorton et al, 1997), and ρ (Enz and Cutting, 1998) subunits have been observed to assemble into homo-pentamers, only for ρ containing receptors are homomers thought to be the predominant physiological form of the receptor. Most $GABA_ARs$ instead are expressed as heteropentamers, with αβγ and αβδ being the most common combinations, generally with two α subunits, two β subunits, and one γ or δ subunit (Sieghart and Sperk, 2002). These heteromeric subunit combinations all produce anion permeable channels that are responsive to GABA. However, the responses of the channel to GABA are affected by subunit composition, with the largest differences being between pentamers containing the δ and γ subunits. Those containing the γ subunit are characterised by their lower sensitivity, but higher efficacy responses; whilst those containing the δ subunit tend to have higher sensitivity to GABA, but lower efficacy (Haas and Macdonald, 1999; You and Dunn, 2007). Additionally, different subunits also confer different

pharmacological profiles to the receptors (Chua and Chebib, 2017), with the most notable difference being that only receptors containing γ subunits, along

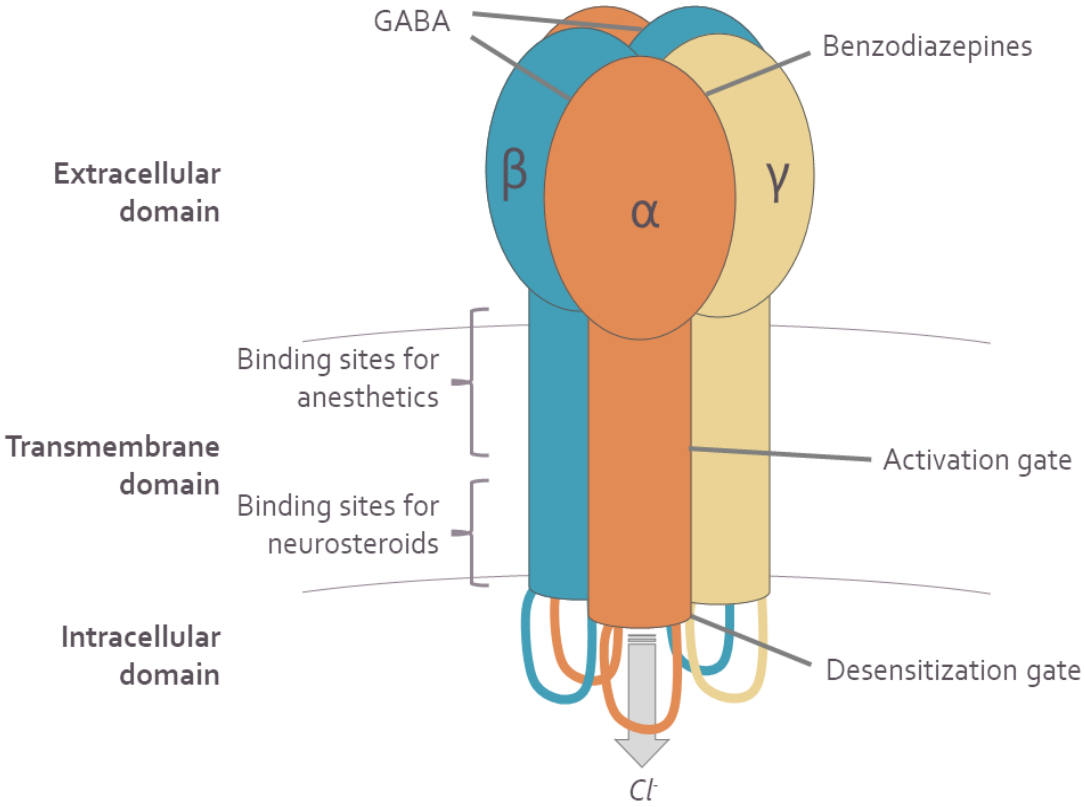

Figure 1.1. Schematic of a $GABA_AR$ demonstrating approximate locations of some drug binding sites and channel gates.

with α(1, 2, 3, or 5), are sensitive to benzodiazepines; whilst those containing δ (Shivers et al, 1989), or α4/6 (Wieland et al, 1992) are not.

The overall architecture of the Pro-loop receptors is well conserved. The five subunits of the pentamer are arranged in a ring within the membrane. Each pentameric receptor is broadly organised into three domains (Fig. 1.1). The extracellular domain consists mostly of beta-sheets and possesses binding sites for agonists at the interfaces between subunits in addition to binding sites for some modulators such as benzodiazepines. It is often glycosylated in mammalian expression systems (Chen et al, 2012; Lo et al, 2010). Beneath this is the transmembrane domain, which consists entirely of alpha-helices, four (M1-4) contributed by each subunit, with the M2 helices lining the

channel. The residues which line the pore of the transmembrane domain are often denoted using a numeric notation, with 0' being the residue at the base of M2 (usually Arg), up to 20', which is a ring of residues located at the top of the transmembrane domain (Laverty et al, 2017, Fig. 1.2). The extended loop between M3 and M4 is classified as the intracellular domain. This loop is often considered to be at least partly unstructured, although it can also form alpha helices (Puthenkalam et al, 2016). It provides sites for interactions with intracellular proteins (e.g. gephyrin) and for post-translational modifications such as phosphorylation (Chen and Olsen, 2007; Kittler and Moss, 2003; Moss and Smart, 1996).

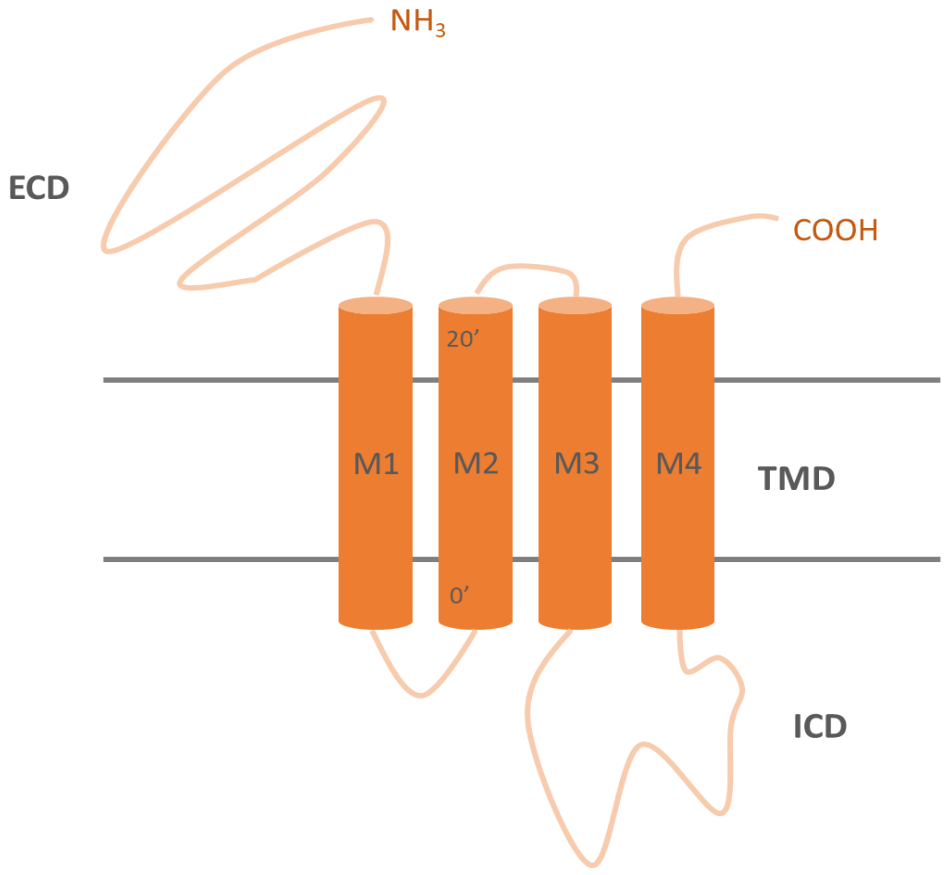

Figure 1.2. Topology of a $GABA_AR$ subunit showing the numbering system for the M2 helix

The binding of GABA to the extracellular domain of the $GABA_AR$ leads to the opening of the channel, allowing the passage of anions, primarily chloride and bicarbonate, across the cell membrane. The $GABA_AR$ possesses two GABA binding sites, located at the interfaces between the principal side (+) of beta and complementary side (-) of alpha subunits (Smith and Olsen, 1995, Fig. 1.1). Binding of agonist to these sites results in conformational changes in the extracellular domain, involving the closure of a loop referred to as loop-C over the binding site in a capping motion (Masiulis et al, 2019; Miller and Aricescu, 2014). Kinetic schemes for some pro-loop receptors include a state known as the preactivation or flip state (Gielen et al, 2012; Sivilotti, 2010), which may correspond to these initial capping structural changes (Miller and Smart, 2010). Subsequently, in a step referred to in both kinetic and structural schemes as activation, the extracellular domain undergoes further conformational changes involving an anticlockwise rotation of the subunits which are transmitted to the transmembrane domain to promote opening of the channel (Masiulis et al, 2019). This involves movements of the β1-2 loop and the Pro-loop towards the M2-M3 loop of the transmembrane domain, causing the M2 helices to move outwards (Du et al, 2015). The gate, which blocks the passage of ions in the resting state, is located approximately half-way along the M2 helix, at the 9’ position, and is opened by these conformational changes (Du et al, 2015, Fig. 1.3).

The opening of this gate allows ions to pass through the channel. The anion selectivity of Pro-loop receptors is determined by multiple charged regions along the length of the pore (Miller and Smart, 2010). The primary determinant for charge discrimination is a ring of positively charged residues at the intracellular end of the channel in the -1’ position, and the M1-M2 linker (Jensen et al, 2005). Other positively charged residues at the 20’ position,

and in the extracellular and intracellular domains also exert some influence on the selectivity of the channel (Miller and Smart, 2010; Keramidas et al, 2004; Fig 1.2).

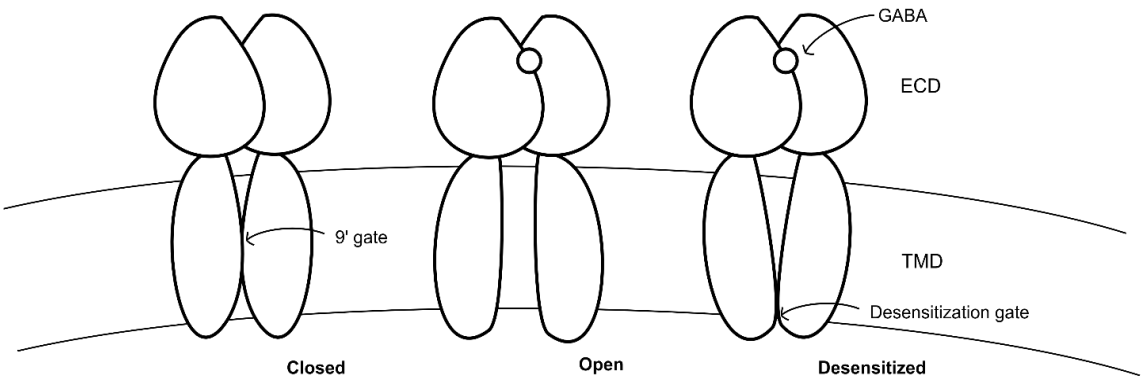

Figure 1.3. $GABA_AR$ gating: In the resting (closed) state a gate approximately half way up the TMD is closed. The binding of GABA results in conformational changes that result in the opening of this gate. GABA can also cause other conformational changes including desensitization, which involves the closure of a separate gate at the cytoplasmic end of the receptor.

Once the channel is open, anions including Cl^- and HCO_3^- simply pass through the pore along their electrochemical gradient. The binding of GABA to these receptors therefore results in an electrical signal. This allows the $GABA_ARs$ to regulate the membrane potential of the cell and, for excitable cells, to alter the probability of the cell firing.

1.1.2. The $GABA_AR$ is the main inhibitory neurotransmitter receptor in the central nervous system

Although $GABA_ARs$ are present in a variety of tissues throughout the body such as the pancreas, liver, and lungs, the most well studied and likely most important role of these receptors is to mediate inhibitory neurotransmission in the central nervous system. In the CNS, $GABA_ARs$ are found on the plasma

membranes of neurons, where they respond to the release of GABA from synaptic vesicles, producing currents which shape the excitability of the neurons.

The effect of opening of $GABA_A$Rs on a neuron in the adult CNS is usually inhibitory, meaning that they reduce the probability of the neuron firing. This is because $GABA_A$Rs are anion-selective ion channels, with the currents that pass through them being primarily carried by chloride and bicarbonate ions. In the adult central nervous system, the Nernst equilibrium potential of chloride (E_{cl^-}) tends to be either hyperpolarised compared to the resting membrane potential, or at a similar value to the resting membrane potential. The current through open $GABA_A$Rs therefore acts to either hyperpolarise the membrane potential or clamp it at a value close to the resting potential and resist further depolarisations towards the threshold for firing. This second type of inhibition, where the equilibrium potential is close to resting potential, is referred to as shunting inhibition. Shunting inhibition can also potentially occur if the equilibrium potential for chloride is slightly positive relative to the resting membrane potential but still negative to the threshold for firing an action potential. In this case the opening of the $GABA_A$Rs will depolarise the membrane, but to a subthreshold potential, and will then act to resist further depolarisation (Farrant & Kaila, 2007).

Whilst such inhibition is considered to be the predominant effect of $GABA_A$R mediated conductances in the mature central nervous systems, there are some circumstances where the equilibrium potential of chloride can shift to values sufficiently depolarised relative to the resting membrane potential for $GABA_A$Rs to excite neurons. Given that $GABA_A$Rs are primarily selective for chloride ions, and that chloride conductances are hyperpolarising when the extracellular concentration of the ion is much higher than that of the

cytoplasm, such a reversal in the polarity will occur when chloride is accumulated inside the cell. This is known to be the case early in development, when the action of a $Na^+/K^+/Cl^-$ symporter (NKCC1) results in high internal chloride concentrations and therefore makes $GABA_A$Rs excitatory (Ben-Ari et al, 2007; Rivera et al, 1999). The switch to a more negative E_{Cl} that causes signalling through $GABA_A$Rs to become inhibitory occurs when the K^+/Cl^- symporter KCC2 begins to be expressed (Ben-Ari et al, 2007; Rivera et al, 1999; Fig. 1.4.). After this switch, the equilibrium potential for chloride ions tends to be similar to the resting potential.

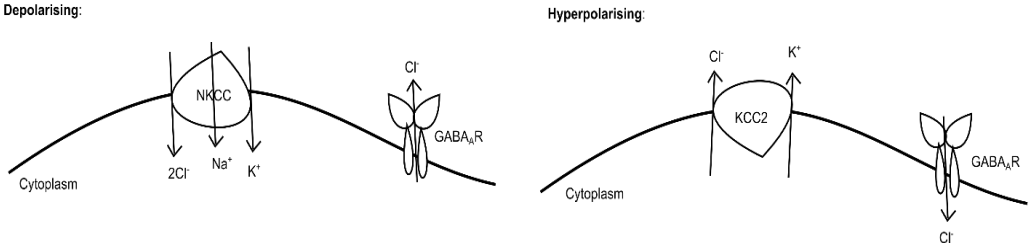

Figure 1.4. The regulation of $GABA_A$R current polarity through chloride gradients. When intracellular chloride is high (e.g. through the action of the NKCC transporter), chloride exits the cell through open $GABA_A$Rs, acting to depolarise the membrane. Conversely, when intracellular chloride is low (e.g. due to the action of KCC2 and other transporters), chloride enters the cell and thus $GABA_A$R currents are hyperpolarising.

In addition to $GABA_A$Rs, glycine receptors also inhibit neuronal firing by allowing the passage of chloride ions. Similarly, to $GABA_A$Rs, glycine receptors are anion-permeable members of the pentameric pro-loop family. These receptors respond primarily to the synaptic release of glycine rather than GABA but share many structural and functional similarities to $GABA_A$Rs (Kasaragod and Schindelin, 2018). They are typically found on neurons of the spinal cord but are also found within some other brain regions, e.g. brainstem (Gamlin et al, 2018).

The other primary source of inhibitory currents in the CNS are potassium channels. Potassium generally has a more negative equilibrium potential than resting potential and tends to inhibit firing through simply hyperpolarising the membrane. There are a variety of different types of potassium channels in the central nervous system, many of which are voltage-gated (Miller, 2000). Some are also regulated by GABA via $GABA_B$ receptors. These receptors are G-protein coupled receptors (GPCRs) that respond to GABA and tend to be inhibitory in their actions but are otherwise distinct from $GABA_A$Rs in their pharmacology and cellular localisation (Enna & Bowery, 2004; Pinard et al, 2010; Ulrich & Bettler, 2007). $GABA_B$ receptors activate tri-heteromeric G-proteins, causing their dissociation, with the Gβγ subunits subsequently activating Kir3-type potassium channels known as GIRK channels (Pinard et al, 2010, Padgett and Slesinger, 2010). The opening of these channels acts to hyperpolarise the membrane and therefore is a form of GABA-mediated inhibition. However, this form GABAergic inhibition differs from that involving $GABA_A$ receptors in two main ways. Firstly, the release of GABA from a single interneuron is generally not sufficient to activate $GABA_B$ receptors, instead summation of GABA from multiple sources or due to the blockade of GABA uptake is often required (Scanziani, 2000). Secondly, the kinetics of $GABA_B$ mediated inhibitory synaptic currents (IPSCs) tend to be slower than those mediated solely by $GABA_A$Rs (Mott et al, 1999).

Although inhibitory signalling in the CNS can involve $GABA_A$, $GABA_B$, or glycine receptors, the $GABA_A$Rs are generally considered the primary mediators of inhibition (Bowery and Smart, 2006; Chua and Chebib, 2017). This importance is underlined by their involvement in numerous neurological diseases (Möhler, 2008), and by the fact that $GABA_A$Rs are the target for

numerous widely-used drugs, such as general anaesthetics, benzodiazepines, and barbiturates (Sieghart, 2015).

1.1.3. GABAergic signalling involves both phasic and tonic responses to GABA

As would be expected for a heteromeric receptor that can be expressed in numerous different subunit combinations (Sieghart, 1995), the profiles of inhibitory neurotransmission mediated by $GABA_A$Rs vary widely across different neurons and different regions of the nervous system. The most important distinction between different types of inhibitory signalling is that between phasic and tonic inhibitory signalling (Fig. 1.5.). Phasic signalling is a

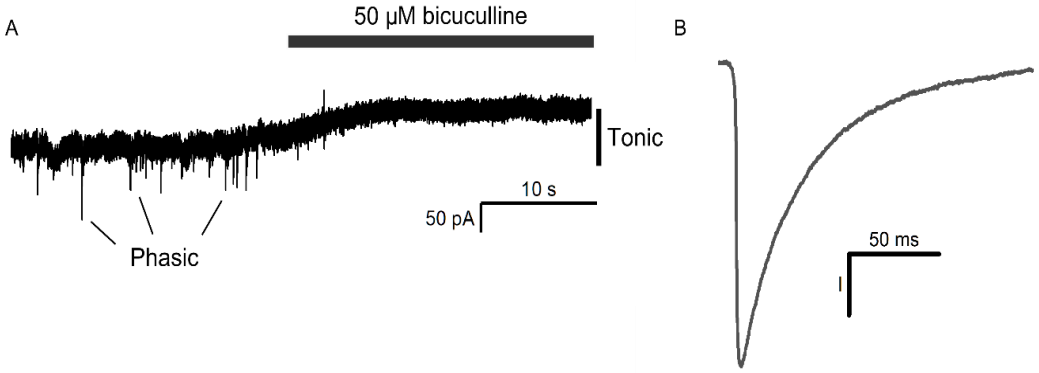

Figure 1.5. Examples of phasic and tonic inhibition, A) Representative voltage clamp recording from a cultured hippocampal neuron before and during the application of 50 µM bicuculline. Note the shift in holding current representing the block of tonic inhibition by the bicuculline. **B)** An average inhibitory post-synaptic current waveform (from recordings made at room temperature). In both panels, the inhibitory currents show a negative polarity due to the use of a high chloride internal solution.

classical form of synaptic signalling where postsynaptic receptors are transiently exposed to high concentrations of GABA after its release from presynaptic vesicles. Conversely, tonic signalling involves the persistent

response of (mostly) extrasynaptic $GABA_AR$s to low levels of ambient GABA (Farrant & Nusser, 2005).

Given that phasic signalling occurs at inhibitory synapses, it is mediated by $GABA_AR$s that are capable of clustering at the synapse. These often include the γ2 subunit, particularly in conjunction with either α1, α2, or α3 subunits, with the α1β2γ2 combination being the most prevalent (Fritschy & Panzanelli, 2014; Chua and Chebib, 2018). The clustering of these particular subunit combinations at synapses is mediated by interactions with cytoplasmic proteins, such as gephyrin, the GARLH proteins, and GABARAP (Sassoè-Pognetto et al, 2000; Tyagarajan and Fritschy, 2014; Wang et al, 1999; Yamasaki et al, 2017).

At inhibitory synapses these receptor clusters are located directly opposite presynaptic clusters of neurotransmitter-filled vesicles (Tao et al, 2018). These vesicles fuse to the membrane in response to stimulation of the presynaptic cell, causing the release of GABA into the synaptic cleft. This results in a high concentration of GABA being achieved within the synaptic cleft (Overstreet et al, 2003). However, GABA then rapidly diffuses away and is removed from the extracellular space by GABA transporters, causing the resulting GABA transient to last for approximately 1 ms (Overstreet et al, 2003).

The currents evoked by such GABA release present as events (physiologically, inhibitory post-synaptic potentials) with extremely fast rise phases (less than a millisecond; Farrant and Nusser, 2005) and long decay phases (tens – hundreds of milliseconds, depending on temperature/receptor subtypes). These decay phases largely occur after GABA has been removed from the synaptic cleft and therefore represent the response of the receptor to agonist molecules that were bound in approximately the first millisecond of

the response. This process is thus governed by the dissociation of these agonist molecules from the receptor and is generally referred to as deactivation. The γ2 subunit-containing receptors that mediate these currents have two key properties that make them ideal for mediating phasic signalling. Firstly, they are high efficacy receptors that therefore have high peak open probabilities (Nusser et al, 2001). Additionally, they exhibit low affinity for GABA, giving concentration response curves with EC_{50} values in the low micromolar range (Ahring et al, 2016). These two properties ensure that the receptors do not respond to or get desensitized by low concentrations of ambient GABA but give large responses to brief pulses of high concentrations of GABA.

In contrast, tonic inhibitory currents tend to involve $GABA_AR$s containing the δ subunit in combination with either the α4 or α6 subunits, or the γ2 subunit in conjunction with the α5 subunit (Farrant and Nusser, 2005). Such inhibition is often evident in voltage-clamp recordings from neurons as a holding current that can be blocked by application of $GABA_AR$ antagonists. Some authors distinguish between tonic currents that can be blocked with competitive antagonists such as gabazine and are therefore thought to result from exposure to ambient GABA, and those that can only be blocked by non-competitive antagonists such as picrotoxin and are therefore considered to result from the spontaneous activation of the channel (McCartney et al, 2007; Wlodarczyk et al, 2013;). The magnitudes of these tonic currents can be regulated by several mechanisms including: the spill-over of GABA from the synaptic cleft (reviewed in Farrant and Nusser, 2005), exposure to allosteric modulators such as neurosteroids (Reviewed in Carver and Reddy, 2013), alterations in GABA uptake (reviewed in Farrant and Kaila, 2007), or through

modification of the $GABA_A$Rs such as phosphorylation (Bright and Smart, 2013; Modgil et al, 2017).

In contrast to the $GABA_A$R subunit combinations that mediate phasic signalling, those that mediate tonic signalling display very different kinetic profiles. GABA is generally observed to have quite low efficacy at δ-containing receptors, to the point where some authors consider it to only be a partial agonist (Ahring et al, 2016). Conversely, these receptors display higher apparent affinities for GABA (Ahring et al, 2016), allowing them to respond to the sub-micromolar ambient concentrations of GABA.

Whilst tonic inhibition is generally thought to be mediated by extrasynaptic receptors, this is not necessarily the case. Whereas synaptic localisation is required for $GABA_A$Rs to be involved in phasic signalling, in principle it is possible for tonic signalling to involve either extrasynaptic or synaptic receptors. In fact, the differential contributions of different subunits to these two types of signalling likely reflect differences in their kinetics as much as their localisation. The low affinity of γ2-containing receptors prevents ambient GABA from stimulating and desensitizing them. Conversely, the lowered efficacy of GABA at δ-containing receptors means that they have low peak-open probabilities and therefore only produce small responses to the GABA transients found in the synaptic cleft (Sun et al, 2018).

Both of these types of inhibition act to reduce the probability of neuronal firing by providing a chloride conductance. However, the differences in their kinetics allow them to mediate very different forms of signalling, with phasic signalling providing a large but transient and localised current in response to the release of GABA from the presynaptic cell, and tonic providing a persistent inhibitory tone that is regulated over much slower timescales.

1.1.4. A variety of endogenous and clinically-utilised compounds act as allosteric modulators of $GABA_A$Rs

As the primary mediators of inhibition in the CNS, $GABA_A$Rs are the targets for numerous clinically important drugs, including benzodiazepines, and general anaesthetics (Korpi et al, 2002). Many of these drugs are positive allosteric modulators of $GABA_A$Rs, and therefore act to enhance inhibition in the CNS. Such enhancements of inhibition have proven to be useful in the clinic for their sedative, anxiolytic, anti-convulsant, and general anaesthetic properties. Additionally, $GABA_A$Rs are also the targets of endogenous allosteric modulators known as neurosteroids (Belelli & Lambert, 2005). The allosteric modulation of $GABA_A$Rs is therefore important both physiologically and clinically.

Benzodiazepines and other benzodiazepine binding-site ligands such as the z-drugs are widely prescribed for conditions such as anxiety, epilepsy, and insomnia (Dell'osso and Lader, 2013). They act selectively on $GABA_A$Rs containing a γ subunit in conjunction with α1, 2, 3, or 5, binding specifically to a site in the extracellular domain at the interface between the α and γ subunits (Olsen, 2018). Most benzodiazepines potentiate the responses of the receptor to GABA when bound at this site. They are generally thought to achieve this either by enhancing the binding of GABA to the receptor, or by increasing the rate of preactivation of the receptor, a conformational change which follows binding but precedes opening (Gielen et al, 2012; Goldschen-Ohm et al, 2014; Lavoie and Tyman, 1996). By doing this, they tend to either slow the decays of inhibitory currents or enhance tonic conductances. This mechanism of enhancement of GABA-evoked currents without direct activation of the receptor, combined with the subtype selectivity of these compounds provides them with a wider therapeutic window than many other

modulators of the $GABA_A$ receptor and has thus made them safer to use in the clinic (Dell'osso and Lader, 2013).

In contrast, the general anaesthetics that act through $GABA_A$Rs are a structurally diverse group of compounds, including: propofol, etomidate, barbiturates, and inhalational anaesthetics such as isoflurane (reviewed in Garcia et al, 2010). These compounds generally act via binding to sites in the transmembrane domain and promoting the opening of $GABA_A$Rs (Reviewed in Forman and Miller, 2011; Olsen and Li, 2010; Sieghart, 2015). In contrast to benzodiazepines, general anaesthetics tend to have multiphasic effects on the receptor, acting to potentiate the actions of GABA at low concentrations, but directly opening the receptor at high concentrations, and in some cases inhibiting it at extreme concentrations (reviewed in Feng and Forman, 2017; Feng and Macdonald, 2004; Feng, 2010; Forman and Miller, 2011).

The neurosteroids are an endogenous class of modulators that act in a similar manner to the general anaesthetics by binding to the transmembrane domains of $GABA_A$Rs and regulating their opening. Some of these compounds, such as pregnanolone, and allopregnanolone act as positive modulators and therefore enhance the opening of $GABA_A$Rs. Conversely, the sulfated neurosteroids, pregnenolone sulfate, and dehydroepiandrosterone sulfate (DHEAS), act as negative allosteric modulators of $GABA_A$Rs (Sachidanandan and Bera, 2015). These compounds are produced in the brain by both neurons and glial cells (Reddy, 2011), and have been implicated in a variety of neurological conditions (Porcu et al, 2016). More recently, structural studies using chimeric receptors containing the transmembrane domains of $GABA_A$Rs fused to the extracellular domains of prokaryotic pro-loop receptors have elucidated the binding sites of pregnanolone (Miller et al, 2017), alphaxalone (Chen et al, 2018), THDOC

and pregnenolone sulfate (Laverty et al, 2017). Although the precise binding sites of each of these compounds differ, all of them were located in the bottom half of the transmembrane domain.

Despite the existence of this wide variety of drugs targeting $GABA_AR$s, the development of novel therapeutics continues. This is largely due to two key problems with the existing $GABA_AR$ modulators. The first problem is that many of the drugs discussed here have undesirable side-effects. The second is that these drugs are prone to causing tolerance and dependence.

The side effects of these drugs occur due to the wide-ranging roles of $GABA_AR$s in the CNS. They include unwanted sedation when used as anxiolytics or anti-convulsants, impairments in cognitive performance, and psychomotor impairments (reviewed in Baldwin et al, 2013). One promising step forward in the design of $GABA_AR$ modulators that are more selective in their effects comes from the discovery that benzodiazepines mediate their different actions through $GABA_AR$s containing different types of α subunit (reviewed in Sieghart and Savić, 2018). In particular, anxiolysis is mediated by the enhancement of currents through α2-containing receptors (Löw et al, 2000), whilst sedation is mediated by actions on those containing α1 subunits (Rudolph et al, 1999) and the cognitive effects through those with α5 (Collinson et al, 2006). The design of α-subunit selective modulators has thus become a priority in the development of novel drugs targeting inhibition. The only such drug class to achieve widespread use in the clinic so far are the z-drugs (Reviewed in Nutt and Stahl, 2010) In particular, zolpidem acts as an α1-selective ligand for the benzodiazepine site, producing sedative effects (Nutt and Stahl, 2010) .

Conversely, the basis of the tolerance to these drugs and the associated withdrawal symptoms is less clear. Some lines of evidence suggest that these

effects also have some basis in the non-selective effects of these drugs (Vinkers and Olivier, 2012), and thus that avoidance of effects on particular subunit combinations or neuronal pathways may prevent these issues. However, other lines of evidence seem to hint that these effects may be related to more fundamental mechanisms involved in the control of $GABA_AR$ expression or functionality, with many different modulators having been observed to evoke the downregulation of $GABA_ARs$ or to decrease their sensitivity to further modulation in both neuronal and recombinant preparations (reviewed in Gravielle, 2018). If this is indeed the case, then subtype-specific modulation may not be a sufficient strategy for avoiding problems associated with long-term use of drugs such as benzodiazepines. The basis for such long-term effects of these drugs is thus far unclear, and the current data and findings regarding it will be explored further in a subsequent section of this chapter. Nevertheless, given that long-term use of benzodiazepines is quite common in the treatment of disorders such as anxiety and epilepsy, the design of drugs that can avoid the development of tolerance and dependence will likely become a priority.

1.2. $GABA_AR$ desensitization: closing the channel?

1.2.1. The $GABA_AR$ desensitizes

The pro-loop receptors, and other ion channels, have long been known to undergo a process known as desensitization in response to prolonged exposure to agonists. This phenomenon was first described in terms of a neuromuscular block resulting from prolonged exposure of skeletal muscle preparations to various agonists of nicotinic acetylcholine receptors (Thesleff, 1955). The block was shown to be unrelated to the depolarisation caused by

these agents (Thesleff, 1955), although in some cases was affected extracellular ionic concentrations (Magazanik and Vyskocil, 1970) and was subsequently attributed to the slow entry of the acetylcholine receptors into a long-lived closed state (Katz and Thesleff, 1957; Del Castillo and Katz, 1957), or through open-channel block by the agonist (Adams, 1975).Such receptor desensitization is therefore an inherent property of the channel and does not require internalisation of the receptors or modifications such as phosphorylation. It thus occurs spontaneously in the presence of agonist and is fully reversible upon agonist removal.

Voltage-clamp studies of hippocampal neurons later showed that GABA induced currents also undergo a similar phenomenon that is not dependent on either dissipation of the chloride gradient or the development of a compensatory current and is therefore a form of receptor desensitization (Numann and Wong, 1984; Ozawa and Yuzaki, 1984). This desensitization of

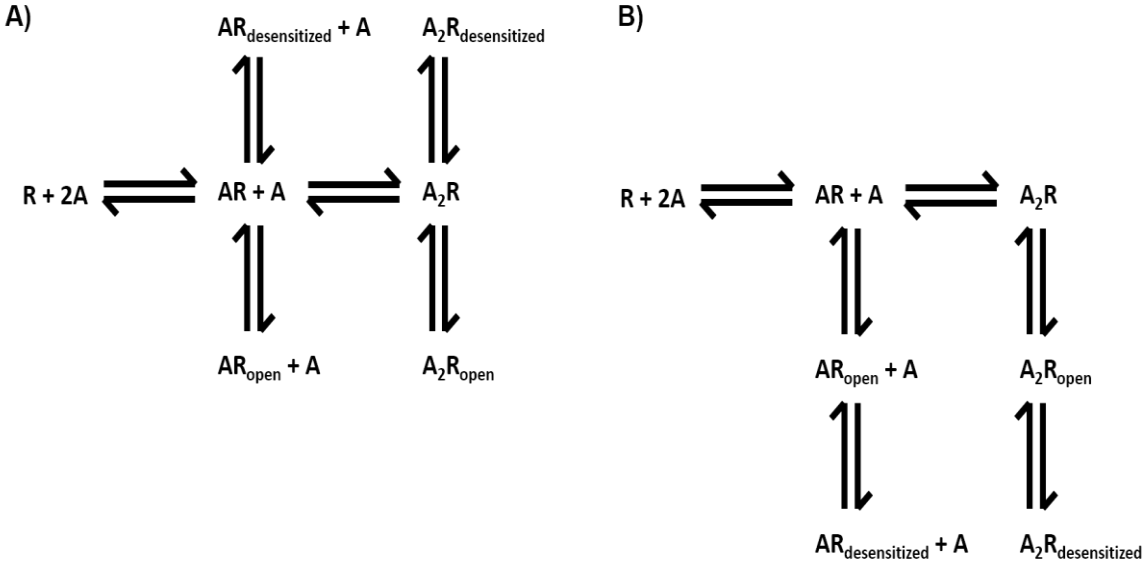

Figure 1.6. Kinetic schemes for $GABA_A$Rs incorporating desensitized states. A) Branched-type model adapted from Jones and Westbrook (1995). 'A' represents agonist molecules, 'R' represents receptors. **B)** A linear-type model for desensitization adapted from Bianchi et al (2007) and altered to include the binding of two agonist molecules.

$GABA_AR$s is also considered to result from the entry of the receptor into a long-lived, agonist-bound, closed state. The entry into this state can be modelled as preceding directly either from the open state(s) (Bianchi et al, 2007; Katz and Thesleff, 1957; Fig. 1.6B), or from a closed state(s) connected to the open state in branched kinetic models (Jones and Westbrook, 1995; Fig. 1.6A).

Recent structural studies have demonstrated the existence of a second gate in the pore of the pro-loop receptors, which appears to be closed in the presence of agonist and thus is thought to underlie the desensitization of the pro-loop receptors (Miller and Aricescu, 2014; Gielen et al, 2015; Huang et al, 2015; Laverty et al, 2017; Miller et al, 2017; Fig. 1.7A & B). This gate is located at the -2' position of M2, and for $GABA_AR$s consists of prolines from the α and γ subunits, and alanines from the β subunits. Whilst this gate is closed in a desensitised structure of a $GABA_AR$, the pore lining residues located higher up the transmembrane domain are in a conformation resembling that of the open state (Miller and Aricescu, 2014). Notably, the 9' gate is observed to be open in this structure (Miller and Aricescu, 2014), perhaps consistent with closure of the desensitization gate following from the open state of the channel. A site-directed mutagenesis study carried out at the same time as these initial structural studies confirmed the base of M2 as the location for the desensitization gate of the inhibitory pro-loop receptors (Gielen et al, 2015). This was further corroborated by the demonstration that picrotoxin, which acts as a blocker in the channel of $GABA_AR$s by binding to the -2'/2' region of M2 (Hibbs and Gouaux, 2011), can block entry into the desensitized state by physically preventing the closure of the gate (Gielen et al, 2015; Gielen and Corringer, 2018).

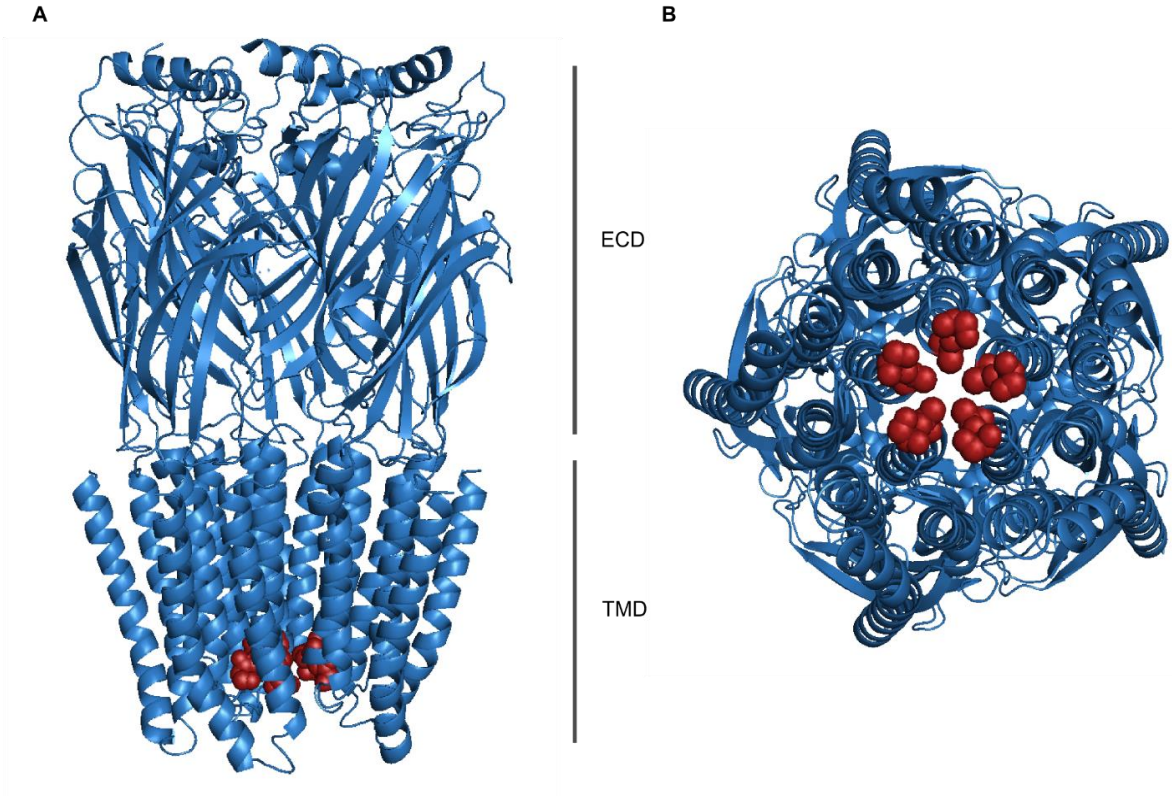

Figure 1.7. The structural basis of $GABA_AR$ desensitization. A) Crystal structure of a homomeric β3 $GABA_AR$ (adapted from Miller and Aricescu, 2014 [4COF]) in a desensitized conformation. The main subunit chains are represented in blue and two residues that form part of the desensitization gate (S248, A249, $GABA_AR$ β3 numbering) are shown in red. (ECD – extracellular domain; TMD – transmembrane domain). **B)** Same structure viewed from the cytoplasmic end of the channel to highlight the occlusion of the pore by the desensitization gate at -2'.

Whilst the structural mechanism of $GABA_AR$ desensitization is now largely elucidated, the kinetics of the process of desensitization are still somewhat controversial, with differing numbers of desensitised states and widely differing rates of macroscopic desensitization reported for $GABA_ARs$ expressed in different experimental models (reviewed in Gielen and Corringer, 2018). Some authors have interpreted these differences as representing different underlying desensitization processes (Gielen and Corringer, 2018). However, when considering these macroscopic rates, it is important to note that whilst the rates of decays of currents elicited by

prolonged exposure to agonist are generally referred to as desensitization rates, they are not equivalent to the microscopic rates of entry into the desensitized states depicted in kinetic schemes and do not even necessarily correlate to such microscopic rate constants (Bianchi et al, 2007, Keramidas and Lynch, 2013; Papke, 2010). The differing rates of macroscopic desensitization observed for different experimental systems therefore may not even represent true differences in the underlying entry into the desensitized states or their stabilities. Interestingly, the differences in the reported macroscopic desensitization rate tend to correlate with the speed of solution exchange of the system used to apply the agonist. For example, with recordings from *Xenopus* oocytes perfused with exchange rates of 100 ms – 1 s, macroscopic desensitisation time constants on the order of 1 s to 10 s are reported, whilst recordings made from outside-out patches with a theta tube used to achieve exchange rates of approximately 100 µs report desensitization time constants of the order or 1 ms – 100 ms (Gielen and Corringer, 2018). This has led to the suggestion that differences in macroscopic kinetic parameters may simply reflect the differences in the speed of agonist application rather than changes in the underlying kinetics (Papke, 2010).

Although the presence of multiple desensitized states therefore cannot be inferred solely from the analysis of macroscopic decays, they can be predicted by fitting kinetic schemes with varying numbers of desensitized states to ensembles of experimental data and examining how well the predictions of the various schemes match the experimental data (Keramidas and Lynch, 2013). Such analyses have led to predictions that pro-loop receptors possess more than one desensitized state (Bianchi and Macdonald, 2002; Elenes and Auerbach, 2002; Solt et al, 2007). However, currently the

collapse of the base of M2 into the pore remains the sole distinct structural mechanism of desensitization (Gielen and Corringer, 2018) and the structural basis for any additional desensitized states therefore remains unclear.

The desensitization of $GABA_AR$s is thus now understood on several levels, in terms of kinetics, structure, and its effects on the responses of ensembles of receptors. However, it is important to note that the general use of the word desensitization to describe both the entry of the receptor into the desensitized conformation, and the decays of currents generated by populations of receptors, results in an ambiguity given the non-equivalence of the two phenomena. Desensitization shall thus be defined in this thesis as the entry of $GABA_AR$s into the relatively stable agonist-bound closed state now known as the desensitized state. This definition therefore encompasses the structural and kinetic definitions of desensitization, but not necessarily the decays of ensemble currents, which are simply one manifestation of these microscopic processes. The question addressed in this thesis can thus be understood as how does the entry of $GABA_AR$s into this conformation impact upon inhibitory neurotransmission?

1.2.2. $GABA_AR$ desensitization is likely relevant to both phasic and tonic signalling

Phasic inhibitory signalling involves postsynaptic $GABA_AR$s responding to transient exposure to high concentrations of GABA released into the synaptic cleft by the fusion of GABA-filled vesicles to the presynaptic membrane (Overstreet et al, 2002). Conversely, tonic inhibitory signalling involves persistent responses to low levels of ambient GABA (Farrant and Nusser, 2005). $GABA_AR$s in the central nervous system are therefore unlikely to be exposed to high concentrations of GABA for prolonged periods. The macroscopic phenomenon of desensitization, where currents decay during

prolonged exposure to agonist, is therefore likely to be not of direct relevance to neuronal signalling. However, this does not imply that the entry of the channel into the desensitized state will also be irrelevant. As argued above, although such decays of ensemble currents are a clear manifestation of entry into the desensitized state, there is no reason to suppose that the conditions required to produce them are also required for entry of the receptor into the desensitized state. Kinetic schemes tend to place the desensitized state as either directly connected to the open state or to a closed agonist bound state connected to the open state (Jones and Westbrook, 1995; Bianchi et al, 2007; Katz and Thesleff, 1957). Entry into the desensitized state would therefore be predicted to be possible under most circumstances where the receptor binds to GABA and opens.

Thus, during phasic signalling, the receptors will potentially enter the desensitized state after their initial burst of opening. This has several implications. The first is that entry into the desensitized state may terminate the initial burst, and therefore regulate the deactivation of the channel. In the case of linear kinetic models, where the desensitized state is directly connected to the open state, entry to the desensitised state would directly terminate the initial burst of activation. Conversely for branched models, where the desensitized state is connected to a closed state preceding the open state, entry into the desensitized state may reduce the number of sojourns in the open state. The effect of such sojourns into the desensitized state during $GABA_AR$ deactivation has already been investigated; with entry into the desensitized state indeed being predicted to speed up the initial phase of current decay (Jones and Westbrook, 1995). However, late re-openings of the channel from the relatively long-lived agonist-bound desensitized state were also observed to slow the slowest component of the

decay, therefore actually prolonging the overall responses of the receptor to pulses of GABA (Jones and Westbrook, 1995).

Entry into the desensitized state may also constitute a post-synaptic mechanism of short-term depression during high frequency firing of inhibitory synapses (Papke et al, 2011). This would occur when channels enter the desensitized state during the response to release of GABA from the presynaptic terminal and are yet to exit the state when the next release event occurs. The amplitude of the response to the second event will therefore be reduced by an amount proportional to the number of receptors still in the desensitized state and thus relatively refractory to GABA (Papke et al, 2011). The magnitude of this effect will depend on both the lifetime of the desensitized state, and the frequency at which the inhibitory synapses are activated. Previous work examining whether this effect occurs at inhibitory synapses in neurons has given conflicting results. In some cases, $GABA_AR$ desensitization indeed appears to mediate short-term depression of the synapses (Davies et al, 1990; Overstreet et al, 2000), whilst others have reported a lack of desensitization-mediated depression (Behrends et al, 2001).

Similarly, for tonic signalling it has previously been shown that entry into the desensitised state attenuates the responses of extrasynaptic receptors to the spill-over of GABA from the synaptic cleft (Bright et al, 2011). Additionally, as tonic currents are a steady-state response to GABA, they are likely affected by the equilibrium between desensitised and open states. However, the relationship between desensitization and tonic currents will likely be complicated by the fact that such currents are evoked by non-saturating concentrations of GABA. Under saturating conditions, the steady state response will be mostly dominated by the equilibrium of open and

desensitized states. Conversely, at the concentrations of GABA that evoke tonic responses, the receptor will likely remain in the unbound state for substantial periods, meaning that the equilibrium between the bound and unbound states will also be an important determinant of current size. Desensitization will likely affect the unbinding of agonist from the receptor as the desensitized state is a stable high affinity state (Chang et al, 2002) and will therefore allow the receptor to remain agonist bound for longer periods.

Whilst most of the effects of desensitization discussed is this section have been established using kinetic modelling, and through studies of receptors in recombinant systems, very little data demonstrating which effects occur in neurons at physiological temperature is currently available. Given that mechanisms proposed here may increase or decrease GABA evoked currents, it is thus currently unclear whether entry of the receptor into the desensitized state enhances or impairs inhibitory neurotransmission. One of the primary aims of this project was therefore to quantify these phenomena in a neuronal context in order to understand the contribution of desensitization to neuronal signalling.

1.2.3. The desensitization of a variety of ion channels has metabotropic effects in addition to the direct effects on channel open probability

Although $GABA_AR$ desensitization is a process mediated primarily through agonist induced conformational changes in the receptor rather than internalisation or post translational modification, it does not necessarily follow that entry into the desensitized state does not affect such phenomena. In fact, the conformational changes associated with desensitisation are proximally located to regulate interactions with cytoplasmic proteins or post-translational

modification of the intracellular domain, given that the desensitization gate is located at the intracellular face of the plasma membrane.

Indeed, for several ion channels, entry into desensitized states has been shown, or proposed to have additional metabotropic effects aside from the direct effects on receptor open probability. If the conformational changes that occur during the desensitization process involve changes in the structure of elements at the intracellular face of the membrane, then desensitization could plausibly alter any interactions those structural elements have with intracellular proteins. This seems to be the case for AMPA receptors, where desensitization has been proposed to break interactions with the accessory protein stargazin after activation of the receptor (Constals et al, 2015). The primary consequence of the breaking of this interaction was to enhance the mobility of the receptor in the cell membrane. In this particular case, the increase in mobility subsequently counteracted the effects of receptor desensitization on the amplitudes of synaptic currents by allowing non-desensitized receptors to enter the synapse and take the place of the desensitized receptors (Constals et al, 2015). $GABA_A$Rs have also been shown to be mobile in their desensitized state, although it is unclear whether this mobility is consequence of entry into the desensitized state (de Luca et al, 2017). In contrast to excitatory synapses, in this case the mobility of the desensitized receptors seemed to act to spread them to other nearby synapses, thus allowing desensitization of the receptor to act as a heterosynaptic form of short-term plasticity (de Luca et al, 2017).

As desensitization involves conformational changes at the intracellular ends of pro-loop receptors in response to agonist binding to the extracellular domain, it could allow the channels to act as metabotropic receptors by regulating bound enzymes or modulators of second-messenger systems.

Both $GABA_AR$s and nicotinic acetylcholine receptors have indeed been proposed to have such actions on phospholipase-C (PLC) mediated calcium signalling in response to stimulation with agonists or positive allosteric modulators (Kabbani and Nichols, 2018; Nicholson et al, 2018). Stimulation of $GABA_AR$s in conditions where the Cl^- equilibrium potential is depolarised relative to the resting potential is well established to result in the accumulation of intracellular calcium (reviewed in Ben-Ari et al, 2007). However, it has recently been shown that the β subunits of $GABA_AR$s interact with PLCδ (Nicholson et al, 2018) and that this interaction decreases upon stimulation of the receptors with diazepam, resulting in the activation of the dissociated PLC and subsequent release of calcium from the endoplasmic reticulum (Nicholson et al, 2018). Conversely, the intracellular loop of α7 nicotinic acetylcholine receptors has been shown to interact with G protein α-subunits (Kabbani and Nichols, 2018). Desensitization of the receptor results in dissociation of Gαq and apparent guanine nucleotide exchange, resulting in activation of Gαq and subsequently PLC (Kabbani and Nichols, 2018).

It is worth noting that ion channels are not the only types of receptor to undergo a process of desensitization; many different types of receptor undergo some form of downregulation after exposure to their agonist. In the case of GPCRs, desensitization involves conformational changes which result in the phosphorylation of the receptor and consequent alterations in its interactions with arrestin and G-proteins (Gurevich et al, 2012), leading to subsequent internalisation of the receptors. Although this desensitization process is clearly distinct from that defined for pro-loop receptors, it is worth considering whether the entry of $GABA_AR$s into the desensitized state could be involved in more far-reaching forms of receptor regulation such as phosphorylation and cell surface-stability.

Interestingly, phosphorylation is known to regulate the desensitization and subsequent recovery of pro-loop receptors (Hinkle and Macdonald, 2003; Lee et al, 2015). In the case of the α4β2 nicotinic acetylcholine receptor (nAchR) such phosphorylation accelerates the recovery from desensitization (Lee et al, 2015). However, it is currently unclear whether the phosphorylation occurs as a response to desensitization of the receptor to release it from the desensitized state, or if phosphorylation occurs independent of desensitization and simply alters the kinetics of the receptor to allow quicker recovery. Nevertheless, it has been shown that stimulation of nAChRs by nicotine, an agonist known to promote desensitization (Picciotto et al, 2008), does indeed result in enhanced phosphorylation of receptor subunits (Wecker et al, 2010). This could perhaps result from the activation of PKC by the nAChRs through the activation of phospholipase C by desensitization of the receptor (Kabbani and Nichols, 2018), or through other as yet unidentified mechanisms.

Additionally, prolonged exposure of nAChRs to nicotine is also known to result in upregulation of the receptor (Buisson and Bertrand, 2001; Colombo et al, 2013), perhaps consistent with entry into the desensitized state acting to regulate cell surface levels. In the case of α4 subunit-containing receptors such upregulation indeed seems to be a direct result of the enhanced desensitization and subsequent PKC-dependent phosphorylation of the α4 subunit (Fenster et al, 1999; Wecker et al, 2010). However, it should be noted that several other mechanisms have also been proposed to explain such receptor upregulation, including: stabilisation of a high affinity state of the receptor by nicotine (Vallejo et al, 2005), nicotine acting as a pharmacological chaperone for nAChRs (Kuryatov et al, 2005), inhibition of proteosomal degradation of nAChRs and other synaptic proteins by nicotine (Rezvani et al,

2007), and increased frequency of nAChR-containing vesicle fusion with the plasma membrane (Richards et al, 2011). Additionally, upregulation of nAChRs in HEK293 cells independent of activation or desensitization of the receptor has also been reported (Kishi and Steinbach, 2006). The apparent conflicts between these datasets may be resolved by observations that the upregulation of nAChRs after nicotine exposure involves multiple processes rather than being the result of one unique mechanism (Govind et al, 2012; Thomsen and Mikkelsen, 2012).

Similarly, to nAChRs, the macroscopic desensitization and resensitization of $GABA_A$Rs can be regulated by phosphorylation (Hinkle and Macdonald, 2003; Moss et al, 1992). Phosphorylation of the β subunits of $GABA_A$Rs has been reported to alter receptor kinetics by increasing microscopic desensitization (Hinkle and Macdonald, 2003); although others have reported similar kinetic changes that instead result from phosphorylation reducing the lifetime of the open state without affecting microscopic desensitization rates (Jones and Westbrook, 1997). However, as with the nAChRs, it is currently unclear whether entry of $GABA_A$Rs into the desensitized state acts to regulate the phosphorylation of receptor subunits or their expression levels. Nevertheless, activation of $GABA_A$Rs has previously been reported to evoke a variety of secondary effects on the channel, including increases in phosphorylation and alterations in receptor expression levels (Gravielle, 2018).

The observations that other ion channels undergo additional secondary forms of regulation, after entry into their desensitized states, raises the possibility that $GABA_A$Rs may also undergo such desensitized-state mediated regulation. As discussed above, the desensitization gate of the $GABA_A$R is located at the intracellular end of the channel, and it is therefore plausible that the conformational changes induced during the desensitization of the receptor

could regulate interactions with proteins at the intracellular face of the membrane. Given that desensitization involves the reliable transduction of conformational changes to the cytoplasmic end of the receptor upon agonist binding, it could even in principle allow the $GABA_AR$ to act as a metabotropic receptor. Such phenomena could allow receptor desensitization to have much more long-lasting consequences for inhibitory neurotransmission beyond the relatively short life-time of the desensitized state, a possibility that will be discussed further in the following section.

1.3. Synaptic plasticity: a potential role for desensitization?

1.3.1. Inhibitory synapses are plastic

Synaptic plasticity is broadly defined as a sustained alteration of the efficacy of transmission at a synapse, through either presynaptic mechanisms altering release or postsynaptic mechanisms altering the responses of the postsynaptic receptors. Plasticity has been most studied at excitatory synapses, where different forms are known to occur over different timescales. Short term plasticity involves changes in transmission efficacy that last for milliseconds to minutes, resulting from phenomena such as the accumulation of calcium at the presynaptic terminal, depletion of synaptic vesicles, and receptor desensitization (Zucker and Regehr, 2002). Conversely, long-term plasticity can take the form of the long-term potentiation (LTP) or long-term depression (LTD) of synaptic efficacy and involves changes that can be rapidly induced by neuronal activity but then subsequently maintained almost indefinitely (Citri and Malenka, 2008). LTP and LTD often involve changes in the number of receptors in the postsynaptic membrane or sustained changes in release probability (Citri and Malenka, 2008). A further form of plasticity

known as homeostatic plasticity, acts to constrain these long-term forms of plasticity, preventing positive-feedback cycles of potentiation or depression resulting in destabilisation of network activity (Turrigiano, 2012). Homeostatic plasticity is also long lasting, similar to LTP and LTD, but is differentiated from these processes by its relatively slow initiation.

The long-term plasticity of inhibitory synapses appears to take a wide variety of forms (Castillo et al, 2011; Hennequin et al, 2017; Kullmann et al, 2012; McBain & Kauer, 2009; Rozov et al, 2017). In some cases, inhibitory synapses simply appear to be regulated by similar mechanisms to those which regulate excitatory synapse plasticity. For example, the LTP of excitatory synapses on CA1 pyramidal cells induced by tetanic stimulation is known to be accompanied by an LTD of adjacent inhibitory synapses (Lu et al, 2000). This results from the activation of calcineurin by the calcium influx through NMDA receptors that occurs during such stimulation (Lu et al, 2000). Calcineurin interacts with the γ2 subunit of the $GABA_A$Rs (Wang et al, 2003), and dephosphorylates the S327 residue resulting in the dispersal of $GABA_A$R clusters (Muir et al, 2010; Nicholson et al, 2018). Although it has not been directly shown whether the firing of the inhibitory synapses regulates this plasticity, it is likely that $GABA_A$R opening will exert an indirect effect by opposing the depolarisation required to release the Mg^{2+} block of the NMDA receptors.

$GABA_A$Rs may also regulate the plasticity of inhibitory synapses by depolarising the membrane and therefore increasing cytoplasmic calcium levels. Such depolarising GABA evoked currents occur under conditions where the equilibrium potential for Cl^- is depolarised relative to the resting potential. This has been observed in neonatal hippocampal slices, where depolarisation caused by $GABA_A$Rs results in the activation of NMDA

receptors and subsequent input-specific LTD of the inhibitory synapses (McLean et al, 1996). Although the requirement for the activation of NMDA receptors seems consistent with the postsynaptic calcineurin mechanism of LTD discussed above, this particular form of neonatal LTD was subsequently attributed to a presynaptic effect (Caillard et al, 1999). Interestingly, the same protocol resulted in LTP of the inhibitory synapses when NMDA receptors were pharmacologically blocked (McLean et al, 1996). Both effects were homosynaptic and could be expressed at the same synapse (McLean et al, 1996) suggesting that activation of $GABA_A$Rs can result in different forms of plasticity depending on the coupling of the currents to calcium signalling of the postsynaptic cell.

It is likely that the currents through $GABA_A$Rs have roles to play in many forms of synaptic plasticity, given that channels such as NMDA receptors and voltage-gated calcium channels are common regulators of LTP and LTD. However, the regulation of inhibitory plasticity solely through the generation of currents that oppose those created by excitatory synapses potentially creates a problem: how can the cell distinguish between increases in inhibitory input and reductions in excitatory input? The presence of NMDA receptors at excitatory synapses arguably provides intracellular signalling pathways with a more direct ‘readout’ of the activity at specific synapses, whilst no such mechanism is currently apparent at inhibitory synapses. As argued above, the entry of the $GABA_A$Rs into the desensitized state could in principle provide such a mechanism.

1.3.2. Possible roles for $GABA_A$R desensitization in inhibitory plasticity

Receptor desensitization is of clear relevance to short-term plasticity, where entry into the desensitized state will result in short-term depression of

synaptic responses when the frequency of events is sufficiently high. However, if entry into the desensitized state can indeed regulate the phosphorylation and expression of $GABA_AR$ subunits, it could potentially be an important factor in the regulation of long-term inhibitory synapse plasticity. Entry into the desensitized state should occur reliably after the activation of inhibitory synapses and involves conformational changes at the intracellular end of the $GABA_A$ receptor (Miller and Aricescu, 2014; Huang et al, 2015). It therefore could be utilised by mechanisms governing postsynaptic inhibitory plasticity as a signal of the activation of $GABA_ARs$ at inhibitory synapses. Such a signal would be of particular importance at inhibitory synapses as they currently only appear to exert indirect control over their own plasticity, perhaps making its regulation somewhat less precise than that observed at excitatory synapses (Kullmann et al, 2012).

It is thus worth considering if receptor desensitization might have a direct role in the regulation of inhibitory synapse plasticity by causing conformational changes in the $GABA_ARs$ that alter their interactions with intracellular proteins. It should be noted here that there is in fact little reason to suppose that the entry of the receptor into the desensitized state would result in a long-term downregulation of the receptors, as the term desensitization only refers to the fact that the receptor is refractory to GABA whilst in the desensitized state. It is perhaps just as plausible that any long-term alterations in synaptic efficacy resulting from desensitization could involve the potentiation of inhibitory synapses. In fact, whilst use-dependent downregulation is a common theme in receptor pharmacology, ligand-gated ion channels provide some quite notable exceptions. The first is the long-term potentiation of excitatory synapses, in which the firing of a synapse results in a calcium

signal that subsequently strengthens the same synapse. The second is the upregulation of the surface levels of nAChRs by nicotine, as discussed above.

Whilst the role of the desensitized state in the long-term plasticity of $GABA_A$Rs has not yet been investigated, many studies have been carried out into the effects of prolonged exposure of $GABA_A$Rs to agonists or allosteric modulators (reviewed in Gravielle, 2018). Such long term (minutes – days) treatments have indeed been observed to alter the levels and functionality of both synaptic and extrasynaptic receptors, although the particular effects that have been reported are quite variable (reviewed in Gravielle, 2018).

One common theme seems to be that prolonged activation of the $GABA_A$Rs can act to downregulate the receptors (Baumgartner et al, 1994; Brady et al, 2018; Calkin and Barnes, 1994; Chaumont et al, 2013; Gouzer et al, 2014; Gravielle, 2018; Gutiérrez et al, 2014; Lyons et al, 2000; Lyons et al, 2001; Nicholson et al, 2018; Roca et al, 1990; Rusek et al, 2000). Such downregulation appears to occur over multiple time-scales, and can take multiple forms, such as: decreased synaptic clustering (Brady et al, 2018; Gouzer et al, 2014; Nicholson et al, 2018;), internalisation of receptors (Calkin and Barnes, 1993; Chaumont et al, 2013; Gutiérrez et al, 2014; Nicholson et al, 2018;), and decreases in mRNA levels (Baumgartner et al, 1994; Lyons et al, 2000; Russek et al, 2000;). In some cases, the downregulation was shown to be dependent on coupling of the GABAergic signalling to calcium signals (Brady et al, 2018; Lyons et al, 2001; Nicholson et al, 2018), perhaps via excitatory chloride currents (Brady et al, 2018; Lyons et al, 2001). Although, as discussed above, it was also recently observed that $GABA_A$Rs bind to a PLC isoform and can activate it during prolonged stimulation with agonists or benzodiazepines, leading to the activation of calcineurin and downregulation of the receptors (Nicholson et al, 2018). Given that this mechanism involves

the release of a bound protein from the intracellular loop of the receptor during receptor activation, it is plausible that it could be mediated by entry into the desensitized state, suggesting that desensitization could regulate such plasticity.

On the other hand, others have reported that prolonged exposure to GABA results in an upregulation of $GABA_AR$s (reviewed in Barnes, 1996). This particularly seems to be the case in cultured cerebellar granule neurons (Belhage et al, 1990; Elster et al, 1995; Meier et al, 1984; Kim et al, 1993), although it has also been observed in post-natal rabbit retinas (Madtes and Redburn, 1983). The cause of this upregulation was unclear, although the range of drugs that were observed to evoke it (induced by GABA, muscimol, and THIP, blocked by bicuculine; Elster et al, 1995; Kim et al, 1993; Meier et al, 1984; Madtes and Redburn, 1983) is consistent with a dependence on $GABA_AR$ activation rather than $GABA_B$-dependent signalling.

In addition to up- and down-regulation of the receptors, other studies have reported a number of other secondary effects of receptor activation, including: increased phosphorylation of receptor subunits (Abramian et al, 2014; Adams et al, 2015; Ferreri et al, 2015; Gutierrez et al, 2014), alterations in the mobility of the receptors in the plasma membrane (Gouzer et al, 2014; Lévi et al, 2015;), and changes to the pharmacological properties of the receptor (Gravielle et al, 2005). As with the alterations in the expression levels of $GABA_AR$s, it has not yet been conclusively shown whether these phenomena are due to the currents through open $GABA_AR$s or occur as a result of other processes such as the entry of the receptor into the desensitized state. Desensitization could plausibly regulate any of these phenomena. In the case of mobility, desensitization could simply act to break the interactions responsible for clustering the receptors at the synapse, as is already thought

to occur at excitatory synapses (Constals et al, 2015). With regards to increases in phosphorylation, all of the phosphorylation sites so far identified to undergo this process are known to be phosphorylated by PKC, so the activation of PLC by $GABA_A$Rs (Nicholson et al, 2018) could perhaps provide a potential explanation. Although in at least one case (Abramian et al, 2014), it was observed that the increases in $GABA_A$R subunit phosphorylation were not accompanied by increases in the phosphorylation of other PKC substrates, suggesting that the increases in subunit phosphorylation involved a mechanism downstream of activation of the kinase.

Although it is currently unclear whether the entry of the receptor into the desensitized state has any role in these long-term regulatory processes, it is conceivable and, in some cases, has previously been suggested (Friedman et al, 1996). If entry into the desensitized state indeed does have long term consequences for inhibitory neurotransmission there could be important implications for the design of drugs targeting the desensitized state. The GABA receptor modulators currently in use in the clinic are well known to have undesirable long-term effects such as withdrawal and tolerance, as discussed above. If any of these effects are attributable to entry of the receptors into the desensitized state, then positive modulators which act by destabilising the desensitized state might be advantageous in situations where long-term use is needed. This project will thus also seek to establish whether entry of $GABA_A$Rs into their desensitized state has any long-term consequences for inhibitory neurotransmission.

1.4. Summary and aims of the project

$GABA_A$Rs mediate inhibition throughout the nervous system. Upon stimulation with GABA, they activate and open an integral channel and thus

allow an anion-mediated current to pass through the membrane. However, they then enter a long-live closed state known as the desensitized state. The physiological role of this state is currently unclear, although, in principle, the receptors should reliably and regularly enter it during physiological inhibitory neurotransmission. This project thus aimed to define the physiological roles of this ubiquitous conformational state. This was achieved using mutations previously identified to alter receptor desensitization (Gielen et al, 2015) in combination with pharmacological approaches, to study the effects of desensitization during both phasic and tonic inhibitory signalling in hippocampal neurons, and to establish the effects of prolonged occupancy of this state on neuronal inhibition.

Chapter 2: Materials and methods

2.1. HEK cell culture

Human embryonic kidney cells (HEK293) were cultured in Dulbecco's modified Eagle's media (Gibco) containing 10 % v/v fetal bovine serum (heat-inactivated, Brazil, Gibco) and 100 u /ml penicillin and 100 µg/ml streptomycin (Gibco). They were maintained in a humidified 37 °C, 5 % CO_2 incubator at all times. Cells were passaged approximately twice per week. Briefly, cells were detached from the plasticware using trypsin (Trypsin - EDTA, 0.05%, Gibco). The dissociated cells were then centrifuged at 1000 rpm for 2 minutes before being resuspended in culture media at the desired density and plated.

For electrophysiology experiments, cells were plated onto 22 mm poly-L-lysine (mw: 70,000 – 150,000; Sigma) coated coverslips. Cells were transfected with the indicated $GABA_AR$ constructs (all in pRK5 vector, *Mus musculus* cDNA) and eGFP (pEGFP-C1) in a 1:1:1:1 ratio using the calcium phosphate precipitation method. Briefly, for each coverslip 4 µg of total DNA was mixed with 20 µL of 340 mM $CaCl_2$. This solution was then mixed with 24 µL 2x HBS (50 mM HEPES, 280 mM NaCl, 2.8 mM Na_2HPO_4). The solution was then added dropwise to the cells. Transfected cells were incubated in a humidified 37 ^oC/5% CO_2 incubator for 24 - 48 hrs prior to recording. Cells that were incubated for 48 hrs were washed in Hanks balanced salt solution (HBSS, Sigma) after 24 hrs to remove the transfection media. For immunoblotting, a similar protocol was used with the cells being seeded onto 6-well plates.

2.2. Generation of mutants

The constructs $\alpha1^{V296L}$ and $\gamma2L^{V262F}$ (both generated from *Mus musculus* cDNA, pRK5 vector) were generated prior to this project, as described in Gielen et al (2015). The α2 equivalent of $\alpha1^{V296L}$ ($\alpha2^{V296L}$) was generated using site-directed mutagenesis of the wild-type α2 construct (*Mus musculus* cDNA, pRK5 vector). Briefly, an inverse PCR was carried out with primers containing the mutation (forward: 5'-CTGTTCTCTGCCCTAATTGAATTTGCA-3'; reverse: 5'-AAACGCATAACAAACAGCTATAAACCAGTCC-3'). The products of the reaction were ligated with T4-ligase (Roche), and then introduced into competent *E. coli* cells (NEB 5αs, New England Biolabs). Clonal bacterial cultures were then produced, and the DNA harvested using a maxiprep kit (Qiagen, manufacturers protocol). All constructs were validated using Sanger sequencing (Source biosciences).

2.3. Outside out patch and whole-cell voltage-clamp recordings from HEK293 cells

Outside-out patches were pulled from HEK cells using thick-walled borosilicate electrodes (4-6 MΩ) filled with an internal solution consisting of: 140 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 4 mM MgATP, 10 mM BAPTA, pH 7.2. The bath was continuously perfused with Krebs solution: 140 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 5 mM HEPES, 11 mM D-glucose, pH 7.4. The outside out patches were voltage clamped at -20 mV with an Axopatch 200B (Molecular devices) and currents were recorded with a 4-pole low pass Bessel filter 5 kHz filter, and a sampling rate of 50 kHz. Drugs were applied using a glass θ-tube, pulled to a tip diameter of 50 – 100 µm, connected to a piezoelectric transducer (Burleigh Instruments). Open tip liquid junction currents were used to confirm the rate of solution exchange (10-90

%: 100 – 250 µs) and that exposures were free from oscillation artefacts. All recordings from outside-out patches were made at 21 ± 1 °C.

Decay curves were fitted within Clampfit (Molecular Devices) using the standard exponential function with two - three components. The number of components used was determined by whether additional components decreased the sum of squared errors. Weighted time constants were calculated with the formula:

$$T_w = \frac{a_1 \times t_1 + a_2 \times t_2 + a_3 \times t_3}{a_1 + a_2 + a_3}$$

Where *a* indicates the amplitude of each exponential component and *t* indicates the time constant of each component. Extents of desensitization were calculated as the percentage reduction in current from peak to steady state:

$$extent = \left(1 - \left(\frac{steady\ state}{peak}\right)\right) \times 100$$

For whole-cell recordings, cells were patched with 2 - 3 MΩ electrodes. The same internal and external solutions were used as described above in 2.2. Drug application was carried out with a Y-tube perfusion system. Recordings were made with a holding potential of -60 mV at a temperature of 21 ± 1 °C. A 5 kHz filter was applied during the recordings, and the sample rate was kept at 20 kHz. Series resistance compensation of >85 % was applied. Recordings were discarded if series resistance changed more than 25 %. All responses to drugs were normalised to the responses caused by 1 mM GABA.

GABA concentration response curves were constructed for wild-type and mutant $GABA_AR$s by sequential application of GABA concentrations for 20 seconds to HEK cells expressing recombinant receptors (individual drug

exposures were separated by 60 second wash-off periods). Data points (peak and steady state currents) were normalised to the peak response of a saturating concentration of GABA (1 mM). The data points were fitted using the Hill equation:

$$I/Imax = 1/(1 + ((EC_{50}/[A])^{n})$$

Where I and Imax represent GABA-activated current and maximal GABA current respectively. The EC_{50} is the GABA concentration that induces a half maximal response and 'n' is the Hill slope.

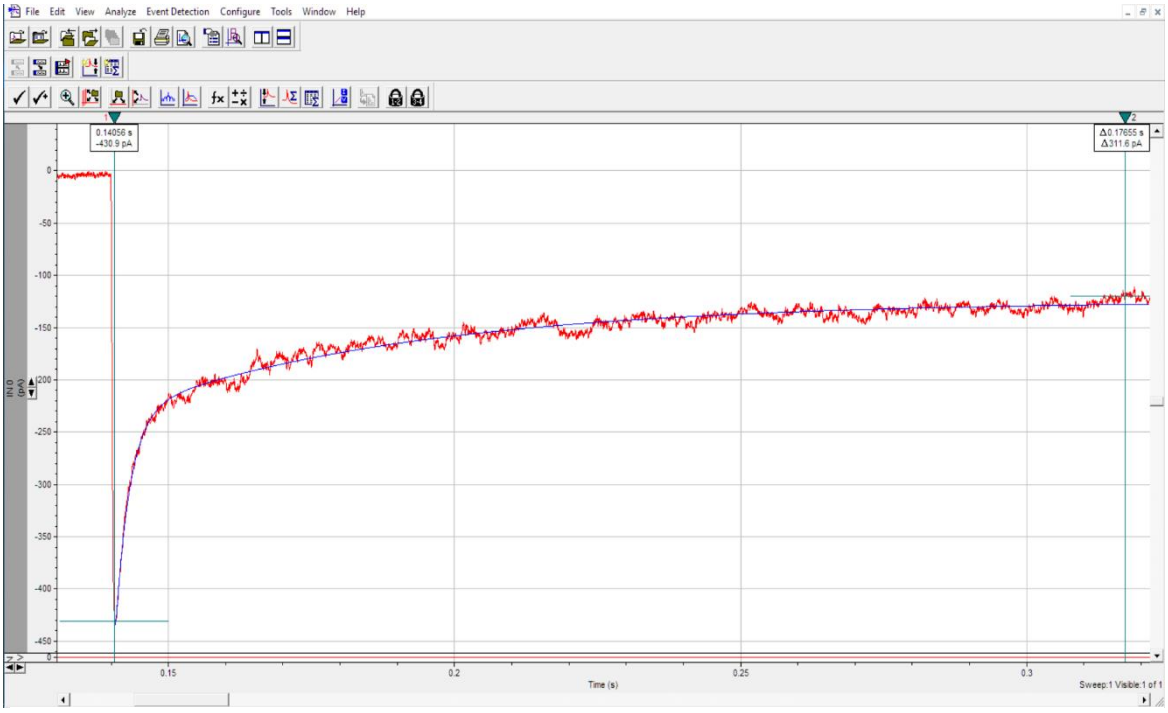

Figure 2.1. Example of a trace fitting in Clampfit. The blue line represents a tri-exponential fit of the experimental data (red)

2.4. Neuronal culture

Hippocampal cultures were prepared from E18 Sprague-Dawley rat embryos. Animals were sacrificed in accordance with the Animals (Scientific Procedures) Act 1986. The dissected hippocampi were dissociated by

trypsinization and trituration using flame polished Pasteur pipettes and the resulting cell suspension plated onto 22 mm coverslips previously coated in poly-L-ornithine (Sigma, mw 100,000 – 200,000), in a plating media consisting of: minimum essential medium (Gibco), 5 % v/v heat-inactivated horse serum (New Zealand, Gibco), 5 % v/v heat-inactivated fetal calf serum (South America, Gibco), 2 mM glutamine (Gibco), 20 mM glucose (Sigma), 100 U /100 µg /ml penicillin-G/streptomycin (Sigma). One hour after plating, the plating media was removed and replaced with maintenance media consisting of: Neurobasal-A (Gibco), 0.5x B27 supplement (Gibco), 0.5x Glutamax (Gibco). 35 mM D-glucose (sigma), 100 U/100 µg/ml penicillin-G/streptomycin. The maintenance media was topped up with 0.5 ml fresh media twice a week.

Neurons were transfected after seven days *in vitro* using Effectene (Qiagen). For this 0.4 µg of the indicated $GABA_AR$ subunit DNA (in pRK5) and 0.4 µg of eGFP DNA (pEGFP-C1) were mixed with: 100 µl EC buffer, 3.2 µl enhancer, 10 µl effectene, 600 µl maintenance media. This solution was mixed on a vortexer and added to neurons dropwise in fresh media. Two hours after the addition of the transfection media, it was removed and replaced with media harvested from the neurons prior to the transfection.

2.5. Neuronal cultures – whole-cell voltage clamp recordings

Coverslips were removed from the incubator on the indicated day of recording (between 20 - 22 days *in vitro*) and were placed into a bath continuously perfused with Krebs solution. Transfected cells were identified by their fluorescence due to the expression of eGFP. Only cells with pyramidal cell-like morphologies were recorded from. Recording electrodes pulled to

resistances of 2.5-3.5 MOhm and filled with a CsCl based internal solution (consisting of: 140 mM CsCl, 2 mM NaCl, 10 mM HEPES, 5 mM EGTA, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 2 mM Na-ATP, 0.5 mM Na-GTP, 2 mM QX-314) were used to patch cells in the whole-cell configuration. Recordings were made using an axopatch 200B amplifier. A holding potential of -60 mV was used in all neuronal experiments. A series resistance compensation of > 85 % was applied. Recordings were then made with sampling rates of 20 kHz and a 4-pole low pass Bessel filter of 5 kHz. The access resistance was assessed every 5 minutes. If it was observed to have changed by more than 25 %, the data from the intervening period was discarded. For the recording of spontaneous inhibitory post synaptic currents (sIPSCs), 2 mM kynurenic acid was included in the bath solution to block glutamate receptors. Bicuculline applications were made by inclusion in the bath solution. Temperature was controlled either by working at a controlled ambient temperature (21 °C, as measured with a thermometer and thermocouple periodically immersed in the bath solution), or by warming the bath solution with an inline heater to warm the solution to 37 °C (again, confirmed by inserting a thermocouple into the bath).

For experiments involving drug pre-treatments, the final drug concentrations were applied into the maintenance media of the cultured neurons and the dishes were subsequently returned to the incubator for the duration of the treatment (20 minutes). The coverslips were then removed and placed into the recording chamber. Krebs solution was then allowed to flow over the coverslip for 25 minutes prior to recording to allow the washout of the drug treatment. For recordings carried out 24 hours after GABA treatment, the treatment media was removed, and the cells were washed five times in

HBSS, followed by one 20 minutes wash in fresh maintenance media before being returned to media harvested from the cells prior to the treatments.

sIPSCs were identified in Clampfit (Molecular Devices) using the threshold search to identify all events within a 5 min recording. All recorded events were visually inspected. For the purposes of amplitude analysis, events were discarded if they displayed inflections on the rise phases. Events used for kinetic analysis were also discarded if they displayed any secondary peaks during the decay phase. For each cell, at least 30 events were analysed. Decay curves were fitted within Clampfit using the standard exponential function with two components. Weighted time constants were calculated with the formula:

$$T_w = \frac{a_1 \times t_1 + a_2 \times t_2}{a_1 + a_2}$$

Where *a* indicates the relative weighting of each exponential component and *t* indicates the time constant of each component. For presentation purposes, raw traces were filtered with a 1 kHz 8-pole Bessel filter.

Tonic currents were measured by adding 50 µM bicuculline to the bath solution and measuring the amplitude differential following the resulting reduction in the holding current. This was done by taking a 5 ms moving average every 100 ms, and then calculating the mean current for 20 seconds before and after bicuculline application.

2.6. Immunoblotting

Dishes containing HEK cells were taken from the incubator and placed on ice. The media was removed, and the cells were briefly washed in ice cold Tris-buffered saline (TBS). The TBS was then removed and radioimmunoprecipitation assay buffer (RIPA) buffer was placed onto the cells

(1 % NP-40, 0.5 % Na-deoxycholate, 0.1 % sodium-dodecyl sulfate (SDS), 150 mM NaCl, 50 mM Tris). The dishes containing the cells and RIPA buffer were then placed immediately into a -80 ^{o}C freezer, where they were incubated for approximately 5 minutes to freeze the cells. The plates were then removed from the freezer and thawed on ice. The cell lysates were then scrapped and placed into ice cold Eppendorf tubes. The lysates were left rotating at 4 °C for 1 hour to allow solubilisation of the membrane proteins to occur. They were subsequently spun at 13,000 rpm for 30 minutes to clear insoluble material. Bicinchoninic acid (BCA) assays (Thermo Scientific) were carried out and the protein concentrations normalised. Sample buffer was then added to the lysate (final concentrations of: 17.5 % glycerol, 5 % SDS, 250 mM tris, 5 mg/ml bromophenol blue, 4 M urea, 200 mM dithiothreitol). The samples were then incubated at room temperature for 45 minutes prior to loading onto the gels.

Poly-acrylamide gels (10 %) were prepared by mixing the following recipe: 10 % acrylamide, 375 mM bis-Tris, 0.2 % SDS, 0.15 % ammonium persulfate, 0.015% TEMED; and pouring the resulting solution into glass cassettes (Biorad). The solution was allowed to polymerise for at least 1 hour before the samples were loaded. Samples were loaded directly onto the gel along with a pre-stained protein marker (New England Biolabs). Samples were run with a constant voltage of 60 V. Sodium metabisulfite was added to the electrophoresis running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) to a final concentration of 50 mM. Proteins from the gel were then transferred to nitrocellulose membranes for 1 hour at 100 V. Blocking was then carried out in TBS 0.1 % Tween (TBST) 5% BSA for 30 minutes at room temperature.

After blocking, proteins were exposed to primary antibodies overnight at 4 ^{o}C (anti-γ2(phospho-S327): Abcam, ab73183, 1:1000). The antibodies were

diluted in the same buffer as was used for blocking. The membranes were then washed in TBST for 1 hour and then probed with secondary antibodies (HRP-conjugate anti-rabbit, 1:10000, Rockland) (in TBST 5 % BSA) for two hours at room temperature. After exposure to the secondary the membranes were washed three times in TBST (total wash time of one hour) and were then developed with luminata crescendo ECL substrate (Millipore) on an ImageQuant LAS 4000 (GE Healthcare). The membranes were then stripped in mild stripping buffer (200 mM glycine, 0.1 % SDS, 1 % Tween20, pH 2.2), and were subsequently re-probed for total γ2 $GABA_AR$ subunit levels (anti-γ2: Alomone, 1:500, AGA-005). Analysis was carried out using GelAnalyser 2010a. Lanes and bands were detected automatically, and a rolling-ball background correction was applied prior to quantification.

2.7. Statistics

Unless otherwise indicated, all data are presented as means ± standard errors. Comparisons of the means of multiple groups was carried out via a one-way ANOVA followed by a Tukey post-hoc test. In the case of experiments with two conditions, two-tailed t-test's were performed. Results with p values < 0.05 were considered to be statically significant.

Chapter 3: $GABA_AR$ desensitization mutants alter the kinetics of phasic and tonic-like GABA responses for recombinant $GABA_ARs$

3.1. Introduction

Previous work from our laboratory has identified several mutations in the desensitization gate of the $GABA_ARs$ (Gielen et al, 2015). As the aim of the overall project is to probe the roles of desensitization in synaptic signalling, we decided to explore the properties of mutations that affect desensitization inserted into subunits that are typically present at inhibitory synapses of hippocampal neurons, namely: α1, α2 and γ2L (Chua and Chebib, 2017). We also decided to use two different mutations, one selected to enhance desensitisation and another chosen to decrease it, allowing a broader assessment at the physiological level of desensitization when the two mutations are expressed in neurons. Although during the prior study of these mutations in oocytes (Gielen et al, 2015) they were often introduced into multiple subunits simultaneously, we chose to utilise mutations in individual subunits in order to minimise overexpression artefacts during neuronal expression. The mutations $α1^{V296L}$ and $γ2L^{V262F}$ (Fig. 3.1) were chosen on this basis. $α1^{V296L}$ was previously observed to decrease the extent of macroscopic desensitization of $GABA_ARs$ expressed in oocytes, whilst $γ2L^{V262F}$ substantially increased it (Gielen et al, 2015).

Before introducing these mutations into neurons, we sought to further examine their effects on $GABA_A$ receptor kinetics. This was considered necessary for two reasons. Firstly, some authors have previously criticised the use of the macroscopic decays of currents elicited by a saturating

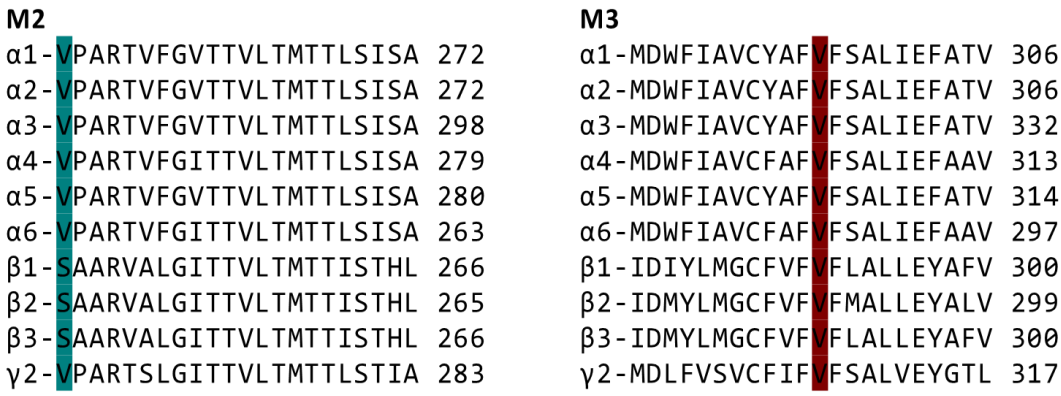

Figure 3.1. Sequence alignments of $GABA_AR$ transmembrane spans 2 & 3 displaying the positions of the residues mutated in $\alpha1^{V296L}$ (red) and $\gamma2L^{V262F}$ (blue)

concentration of agonist to measure the underlying kinetics of desensitization, particularly in the case of oocyte recordings due to the relatively slow application of drug that characterises such systems (Papke, 2010). We therefore sought a deeper understanding of the effects of these mutations on receptor kinetics in order to confirm that they are exerting their effects on the desensitization gate rather than affecting other parameters such as the opening rate of the $GABA_A$ receptor channel. Secondly, inhibitory neurotransmission rarely, if ever, involves the prolonged exposure of receptors to high concentrations of agonist. Instead, phasic inhibitory signalling involves currents elicited by non-stationary transient exposure to high concentrations of GABA (Farrant and Kaila, 2007), making the deactivation rate of the receptor, in response to fast applications of GABA, a better predictor for the effect of the mutants on synaptic signalling. Conversely, tonic signalling involves the persistent response of $GABA_ARs$ to low concentrations of ambient GABA (Farrant and Kaila, 2007). An investigation of how these mutations affect the responses of extrasynaptic-type $GABA_A$ receptors to low concentrations of GABA was therefore required in order to ascertain how they might affect tonic signalling.

An additional problem with relying on the previous measures of desensitization for $GABA_A$Rs with these mutations in oocytes, is raised by observations that the macroscopic desensitization rates of ion channels vary by several orders of magnitude between different experimental systems (Gielen and Corringer, 2018). The reasons for this variation are unclear, with some authors attributing the differences to underlying biological changes in the context of the receptor, such as phosphorylation or lipid interactions (Gielen and Corringer, 2018); whilst others attribute these changes to the drastically different drug application rates achieved for the different experimental setups (Papke, 2010). We therefore sought to confirm the previously observed 'desensitization phenotypes' of the $\alpha1^{V296L}$ and $\gamma2L^{V262F}$ mutations in an alternative experimental system.

To achieve these aims, the mutations were expressed as recombinant $GABA_A$ receptor αβγ assemblies in HEK293 cells. Outside-out patches were pulled from these cells and exposed to fast applications of GABA using a theta-tube mounted on a piezo-electric transducer. This system allows extremely rapid drug application and wash-off (Jonas, 1995), with solution exchange times of the order of 100 – 300 µs. This allowed the assessment of the effects of the mutations on phasic-like GABA signalling that would be typically experienced at inhibitory synapses. Additionally, such systems are observed to give extremely fast macroscopic desensitization rates compared to those observed when using oocytes (e.g. Papke et al, 2011). The use of this system therefore allowed us to probe whether the effects of the mutations are artefacts of the oocyte two-electrode voltage-clamp system or are indeed true $GABA_A$R desensitization mutants. Such a system also permitted an investigation of whether the mutations still substantial phenotypes have when examined on timescales more relevant to synaptic signalling.

It has previously been predicted that entry into the desensitized state and subsequent reopening acts to prolong the deactivation of $GABA_A$ receptors in response to brief pulses of GABA (Jones and Westbrook, 1995). We sought to assess this finding here by measuring the deactivation rates of the mutants in response to 1 ms pulses of GABA. To further examine the role of desensitization on these time scales, a paired-pulse drug application protocol was used to assess how the occupancy of the desensitized state changes during the responses to such pulses.

In addition to the fast-kinetic analyses, we also established concentration-response relationships for $GABA_A$ receptors expressed in HEK293 cells patched in the whole-cell configuration. Peak-current concentration-response curves were used to further probe the effects of desensitization on GABAAR kinetics ; whilst steady-state GABA concentration response curves allowed us to probe the effect of these mutations on tonic-like inhibitory GABA signalling.

Finally, paired-pulse recordings from outside-out patches were used to assess the effects of allosteric modulators on the occupancy of the desensitized state during phasic-like signalling. Some allosteric modulators of the $GABA_AR$ have previously been identified as achieving their effects on channel open probability by modulating the entry or exit rates of the receptor into the desensitized state. In particular, the neurosteroid pregnenolone sulfate, a negative allosteric modulator, has previously been shown to act via the stabilisation of the desensitized state (Shen et al, 2000; Sachidanandan and Bera, 2015; Seljeset et al, 2018). Additionally, allosteric modulators that do not directly affect the microscopic desensitization rates will likely still have effects on the occupancy of the desensitized state. This is because desensitization tends to occur after the opening of the channel (Gielen and Corringer, 2018). Therefore, positive allosteric modulators, which enhance the

open probability of the receptor (e.g. benzodiazepines and neurosteroids) will also likely enhance the occupancy of the desensitized state, whilst negative allosteric modulators will likely reduce it.

3.2. Results

3.2.1. The desensitization mutants ($\alpha1^{V296L}$ and $\gamma2L^{V262F}$) affect macroscopic desensitization profiles of recombinant $GABA_A$Rs in outside-out patches

Outside-out patches of membrane were pulled from HEK293 cells expressing α1β2γ2L $GABA_A$Rs. These patches were then exposed to a saturating concentration of GABA (10 mM) for 200 ms using a theta-tube perfusion system and the resulting currents measured by applying a holding potential of -20 mV (Fig. 3.2A). Liquid junction currents were used to assess solution exchange times prior to drug applications to ensure that the applications were free from solution interface oscillation-induced artefacts and that the 10-90 % exchange times were <250 µs. All experiments were carried out at room temperature (21 ± 1 °C). In this experimental system, the macroscopic desensitization rates were observed to be much faster than those previously observed for oocytes (Gielen et al, 2015). The extent of desensitization was measured as the percentage decrease in current from the peak response after 200 ms. The mutations appeared to have qualitatively similar effects on the extents of desensitization to those previously observed in oocytes (Gielen et al, 2015), though over different timescales. Specifically, $\alpha1^{V296L}$ decreased the mean extent of desensitisation after 200 ms from 66.8 ± 3.9 % to 34.1 ± 4.8 % (Fig. 3.2B; one-way ANOVA: $F_{(2, 26)} = 43.86$, $p = 4.67 \times 10^{-9}$; Tukey post-hoc test (wt $\alpha1^{V296L}$): p = 0.000011), whilst $\gamma2L^{V262F}$ increased it to 95.2 ± 0.7 % (Tukey test(wt $\gamma2L^{V262F}$): p = 0.00019). Conversely, the effects of the mutations on the rate of desensitisation were somewhat different to those

previously observed for the same mutations in oocytes. $\alpha1^{V296L}$ did not appear to significantly alter the rate of macroscopic desensitization (weighted time constant = 10.6 ± 3.2 ms) compared to wild type (18.0 ± 2.6 ms) (Fig. 3.2C; one-way ANOVA: $F_{(2, 25)} = 11.04$, p = 0.00036; Tukey test (wt, $\alpha1^{V296L}$): p = 0.38), whilst $\gamma2L^{V262F}$ increased the weighted time constant to 39.0 ± 6.8 ms (Fig. 3.2C; Tukey test (wt, $\gamma2L^{V262F}$), p = 0.002). This was because, in this experimental system, $\gamma2L^{V262F}$ exerted its effects almost entirely on the slower phase of desensitization, slowing it and increasing its amplitude, and in doing so enhancing its contribution to the weighted time constant (Fig. 3.3A & 3.3B).

It has been suggested that the different time components of the macroscopic desensitisation rate of the $GABA_AR$ represents the entry of the receptor into distinct desensitised conformational states (Gielen and Corringer, 2018). The

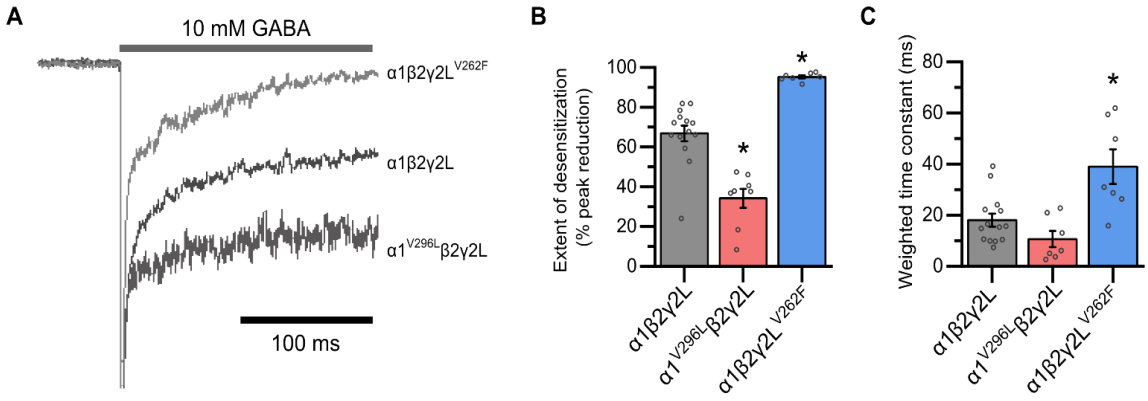

Figure 3.2. Macroscopic desensitization phenotypes of $\alpha1^{V296L}$ and $\gamma2L^{V262F}$. A) Representative currents recorded from outside-out patches pulled from cells expressing the indicated mutations. Currents were peak normalised. **B)** Extents of desensitization expressed as the percentage reduction from peak current after 200 ms. n = 14 patches for α1β2γ2L, 8 for $\alpha1^{V296L}$β2γ2L, and 7 for $\alpha1\beta2\gamma2L^{V262F}$ **C)** Weighted time constants from exponential fits of the decays. n = 14 patches for α1β2γ2L, 7 for $\alpha1^{V296L}$β2γ2L, and 7 for $\alpha1\beta2\gamma2L^{V262F}$. Error bars represent SEM, circles represent individual data points, and '*' indicates a significant difference from wild type with a p value < 0.05.

above observations would imply that the desensitization mutants are solely affecting the slow desensitised state rather than the fast one. However, the

fastest phase of the macroscopic decay may not actually be attributable to a distinct desensitised state. Interestingly, in a previously published kinetic scheme using three open states, based on single channel data, it was observed that when the desensitised states are removed from the scheme a small fast decay similar to that observed for $\alpha1^{V296L}$ still remains (Bianchi et al, 2007). This seems not to be due to the presence of any particular state acting as a desensitised state but instead occurs because of the presence of the additional open state in the single channel model.

Overall it seems that the mutations behaved similarly in this experimental system with regards to their macroscopic desensitization when compared to previous oocyte date (Gielen et al, 2015), but that the faster solution

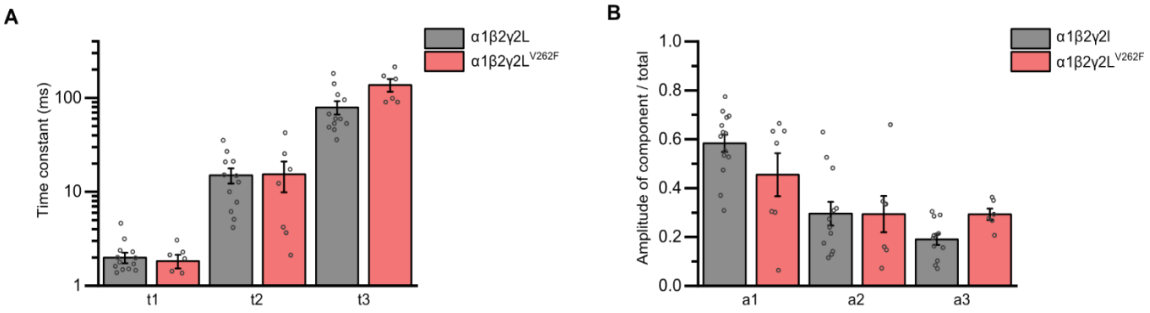

Figure 3.3. Quantitation of the individual time constants and amplitudes of the macroscopic desensitization of wild-type and $\gamma2L^{V262F}$. A) Time constants (t1-3) for each component of the exponential fits of data shown in figure 3.1. **B)** Amplitudes (a1-3) for each component (numbering of the components corresponds to that of the first panel). Means ± SEMs are shown, circles represent individual data points. N = 14, and 7 patches respectively for wild type and $\gamma2L^{V262F}$

exchange rates achieved in the present study allowed better resolution of the effects of the mutations on the different phases of macroscopic desensitization. The effects of the mutations on the extents of macroscopic desensitization are consistent with $\gamma2L^{V262F}$ acting to enhance desensitization, with little current remaining at steady state (see Fig. 3.2A), likely due to the accumulation of receptors in the desensitized state. Conversely, the decrease

in the extent of desensitization for $\alpha1^{V296L}$ suggests that it lowers the accumulation of desensitized receptors during prolonged exposure to agonist.

3.2.2. Both desensitization mutations ($\alpha1^{V296L}$ and $\gamma2L^{V262F}$) increase the rate of deactivation of the $GABA_A$ receptor

To assess the process of receptor deactivation, the α1 and γ2L mutant receptors were exposed to 1 ms pulses of 10 mM GABA. This experiment was designed to assess how these mutants might affect the kinetics of synaptic responses. The waveforms of the responses to these pulses displayed extremely rapid rises to a peak, followed by a decay phase governed by the deactivation of the receptor (Fig. 3.4A). Despite the opposing effects of $\alpha1^{V296L}$ and $\gamma2L^{V262F}$ on macroscopic desensitization, they both appeared to have almost identical effects on the deactivation kinetics of GABA responses, decreasing the mean weighted time constants from 61.7 ± 6.9 ms to 17.6 ± 2.7 ms ($\alpha1^{V296L}$) and 22.8 ± 4.4 ms ($\gamma2L^{V262F}$) respectively (Fig. 3.4B; one-way ANOVA: $F_{(2, 53)} = 20.45$, $p = 0.26x10^{-6}$; Tukey test (wt, $\alpha1^{V296L}$): $p = 0.63x10^{-6}$; Tukey (wt, $\gamma2L^{V262F}$): $p = 0.00011$). Both mutations shortened the duration of the response and therefore reduced the overall charge passed. Conversely, neither mutation appeared to affect the rise rate of the currents (Fig. 3.4C; one-way ANOVA: $F_{(2, 53)} = 0.37$, $p = 0.695$), consistent with the mutations exerting their effects through a role for the desensitized state in deactivation rather than altering the binding of agonist or opening of the receptors.

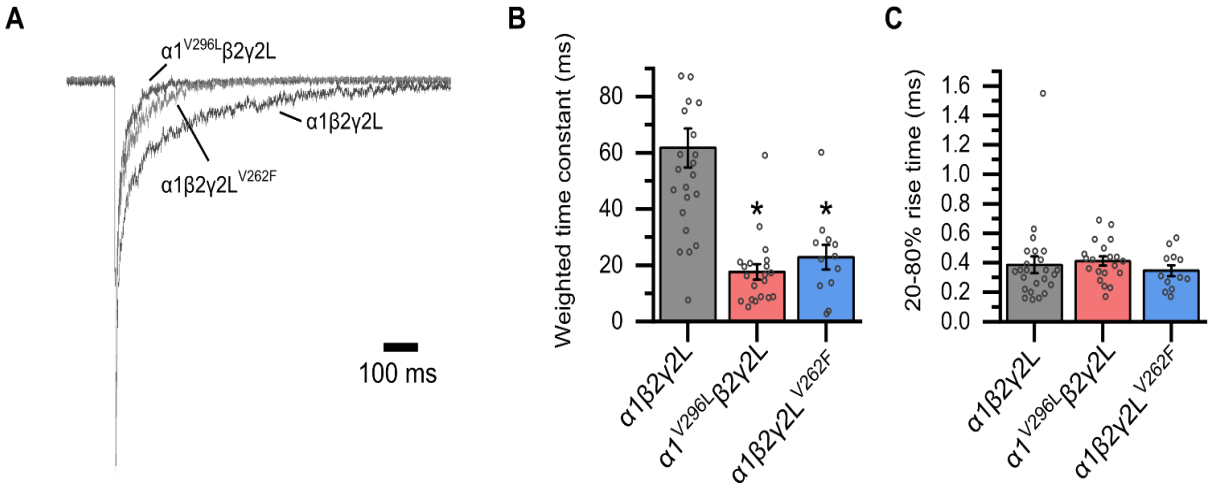

Figure 3.4. Deactivation phenotypes of α1^{V296L} and γ2L^{V262F}. A) Representative traces of currents elicited by 1 ms pulses of 10 mM GABA. All currents are peak normalised. **B)** Weighted time constants from multi-exponential fits of the deactivation of the mutant receptors. N = 24, 20, 12 patches respectively. **C)** Rise times of the responses measured as the interval between 20 % and 80 % of maximum. N = 24, 20, 12 patches respectively. Means ± SEMs are shown, circles represent individual data points. '*' represents significant differences from wild-type at $p < 0.05$ when assessed with a tukey test.

The shortening of these responses by α1^{V296L}, which appears to decrease desensitization (Fig. 3.2A), is consistent with the predictions of Jones and Westbrook (1995) who state that occupancy of the desensitized state acts to prolong the deactivation of the receptor. It seems likely that this mutation is reducing the occupancy of the desensitized state, and therefore reducing the number of receptors capable of late re-openings predicted to prolong the overall response. However, it is less clear as to why γ2L^{V262F} also shortened the lengths of the responses. Given that the effect of this mutation on deactivation was essentially identical to that of α1^{V296L}, it seems likely that it too prevented the late re-openings required for prolongation of deactivation of the receptors. One plausible explanation for this in the context of the two-gate model of desensitization (Gielen and Corringer, 2018) is that this mutation stabilises the desensitized state in such a way that by the time the

desensitization gate has re-opened the main gate of the channel has closed and the agonist has been released from the receptor. Consistent with this, it has previously been observed that the lifetime of the desensitized state can exceed the time for which the $GABA_A$ receptor remains bound to agonist (Chang et al, 2002).

3.2.3. $\alpha1^{V296L}$ prevents entry into the desensitised state, whilst $\gamma2L^{V262F}$ stabilises it

To further assess how the α1 and γ2L subunit mutations affect the occupancy of the desensitized state during the responses to brief pulses of applied GABA, and therefore how they achieve identical deactivation phenotypes, a paired-pulse protocol was used to assess how many receptors are in refractory closed states during the deactivation phase of the response (Papke et al, 2011). This protocol involved exposing the cell patches to two 1 ms duration pulses of GABA with varying intervals between the pulses (Fig. 3.5A). The amplitude of the current elicited by second GABA pulse compared to the first was plotted against the time interval between the pulses to generate curves for resensitization. For wild type receptors, the second pulse was initially depressed, but recovered after approximately one second. Conversely, the responses of $\alpha1^{V296L}$ to the second pulse of GABA were never depressed (Fig. 3.5B), even with an inter-pulse interval of 50 ms, suggesting that either it does not enter the desensitised state during these responses or that it does so only for an extremely brief time period. Conversely, $\gamma2L^{V262F}$ exhibited prolonged depression of the second pulse (Fig. 3.5B), with complete recovery taking approximately 10 seconds, suggesting that it acts to stabilise the desensitized state. However, it did not exhibit greater depression than wild type receptors at shorter time points, suggesting that it has minimal effect on entry into the desensitised state.

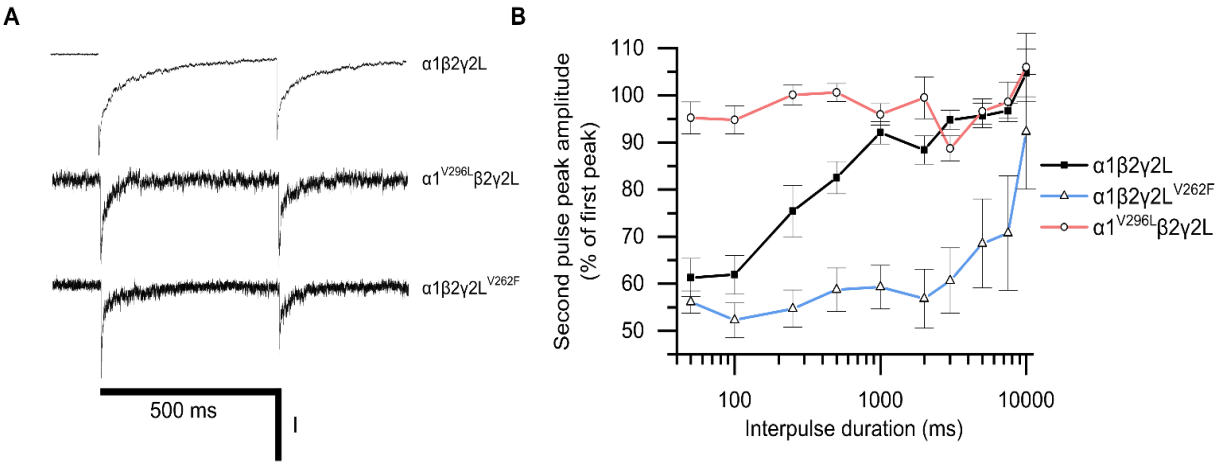

Figure 3.5. Resensitization phenotypes of α1^{V296L} and γ2L^{V262F}. A) Representative traces of currents elicited by paired 1 ms pulses of 10 mM GABA with a 500 ms interval. **B)** Magnitude of the second pulse relative to that of the first plotted against the interval between the pulses. Means ± SEM are plotted. n = 8 patches for wild type, 8 for α1^{V296L}, and 9 for γ2L^{V262F}.

These findings may provide explanations for the effect of these mutations on the deactivation rates of these currents. α1^{V296L} appears to abolish entry into the desensitized state during the response to consecutive pulses of GABA and therefore would not be expected to exhibit late re-openings during deactivation simply because there is no long-lived desensitised state from which to re-open. Conversely, if γ2L^{V262F} exerted its effects by enhancing entry into the desensitized state, it would still have been predicted to prolong the deactivation of the receptor rather than shorten it. However, it seems that this mutation is instead acting to stabilise the desensitized state, with complete recovery taking almost 10 times longer than that of wild-type receptors, with little effect on the entry rate into desensitisation. Such a profound stabilisation of the desensitized state is consistent with the hypothesis that this mutant does not exhibit late re-openings during deactivation because the desensitization gate itself does not re-open before the main gate of the ion channel has already closed. Curiously, the mutations therefore achieve identical deactivation phenotypes by opposing means.

3.2.4. Both desensitization mutants reduce the steady-state responses to low concentrations of GABA

Although the subunits studied here are primarily considered to be synaptic, γ2 will still likely contribute to tonic signalling, particularly in the context of α5 containing receptors, and α1 can also be found in the extrasynaptic space and thus may contribute to tonic currents (Sieghart and Sperk, 2002). We therefore decided to investigate the effects of these mutations on tonic-like responses to assess whether desensitization can regulate tonic currents and to further elucidate the kinetic phenotypes of these mutations. In contrast to phasic signalling, tonic inhibitory signalling involves persistent responses to low concentrations of GABA. Steady state concentration-response relationships were determined to assess the effects of the mutations on tonic-like GABA responses. To do so, HEK293 cells were recorded from in the whole cell configuration and exposed to various concentrations of GABA using a Y-tube drug application system. To assess steady state currents, each concentration was applied for 20 seconds, and both the peak, and residual currents after 20 s, measured. Whilst neither mutation caused significant changes in the peak concentration response curves (Fig. 3.6A), the effects on the steady-state concentration response curves were substantial (Fig. 3.6B), as would be expected for mutations affecting receptor desensitization. At high GABA concentrations, the desensitisation mutants had effects consistent with those previously observed with macroscopic desensitisation curves (Fig 3.2); $\alpha1^{V296L}$ caused a large increase in the steady state response whilst $\gamma2L^{V262F}$ almost completely abolished it (Fig. 3.6B). $\gamma2L^{V262F}$ also abolished steady state responses to low concentrations of GABA (Fig. 3.6B), suggesting that accumulation in the desensitized state can occur at any concentration of GABA capable of eliciting a response.

Intriguingly, α1^{V296L} *also* reduced the steady state responses to low doses of GABA, perhaps suggesting a role for the desensitized state in setting the amplitude of tonic currents (Fig. 3.6B).

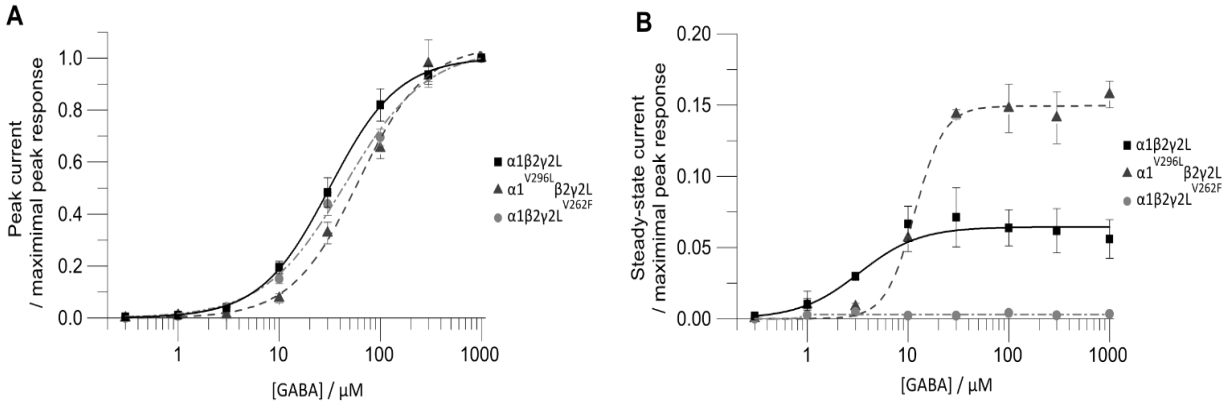

Figure 3.6. Peak and steady state concentration response curves. A) Peak response amplitude plotted against GABA concentration. All responses are normalised to the amplitude of the peak of a response to 1 mM GABA. EC_{50} values were 33.3 ± 4.0 µM, 63.8 ± 9.1 µM, and 46.4 ± 6.0 µM for α1β2γ2L, α1^{V296L}β2γ2L, α1β2γ2L^{V262F}**. B)** Curves showing how the amplitudes of the residual currents after 20 seconds of drug application vary with concentration of GABA applied. All points are also normalised to the amplitude of the peak of a response to a 1 mM concentration of GABA. EC_{50} values were 2.8 ± 0.36 µM, 11.9 ± 1.08 µM, and for α1β2γ2L, α1^{V296L}β2γ2L respectively. Mean ± SEM are plotted. Lines represent hill fits. n = 3 cells for each curve.

Both mutants will therefore likely reduce tonic currents in neurons which occur at low GABA concentrations. In the case of γ2L^{V262F}, this phenotype seems likely to be explained by the accumulation of the receptor in the desensitized state, even in response to low concentrations of GABA. However, the reduction of responses to low concentrations of GABA observed for α1^{V296L} highlights a role for the desensitized state that has not previously been predicted, i.e. the generation of currents in response to low concentrations of agonist. Although steady state currents would be predicted to be governed by the balance of desensitized and open states, this is only necessarily the case when the receptors are saturated with agonist. At low concentrations of

agonist, the steady state responses are more likely to be governed by the binding and unbinding of the agonist.

There are two plausible mechanisms by which desensitization could act to enhance tonic currents elicited by low concentrations of GABA. The first is similar to that proposed by Jones and Westbrook (1995) to explain the prolongation of deactivation by entry into the desensitized state and subsequent reopening. If receptors are indeed capable of this behaviour in response to brief pulses, they should also be capable of desensitizing and reopening when fully liganded regardless of the concentration of agonist applied. If the lifetime of the desensitized state is shorter than the expected average lifetime of the unbound state during exposure to a given concentration of GABA, then desensitization and subsequent reopening will act to enhance ‘tonic’ currents. The desensitized state, as a high affinity state from which the receptor can open, will therefore promote channel opening when ligand binding is an infrequent event by effectively slowing the release of agonist from the receptor. The second mechanism predicts that desensitization instead may increase the effective rate at which the receptor becomes fully liganded. The reason desensitization could do this is that it has previously been predicted that entry into the desensitized state can occur from monoliganded states of the receptor (e.g Jones and Westbrook, 1995). If this is the case, entry into the desensitized state could act to increase the lifetimes of such monoliganded states by virtue of its properties as a high-affinity long-lived state of the receptor. Doing so would increase the probability that at low GABA concentrations monoliganded states would procood to become fully liganded and therefore enhance the open probability of the receptor under conditions where monoliganded states of the receptor

are common and the subsequent binding of a second agonist molecule occurs infrequently.

3.2.5. The equivalent α2 mutation, and same γ2L mutation display similar kinetic phenotypes in α2β2γ2L containing receptors

This project aims to investigate the effects of these mutations in hippocampal cultures generated from embryonic brains; the α2 subunit is known to be more dominant in the developing hippocampus (Fritschy et al, 1994) and therefore may even be the dominant synaptic α subunit in such preparations. Previous work has shown that this is indeed the case for dissociated hippocampal cultures, where α2 is the more dominant synaptic α subunit on pyramidal neurons, whilst α1 shows more limited expression principally in interneurons (Brünig et al, 2002; Mangan et al, 2005). We therefore sought to determine whether the mutations display similar phenotypes in the context of pentamers containing the α2 subunit.

To do this, recordings were performed from outside-out patches pulled from HEK cells expressing α2β2γ2L $GABA_A$Rs. The same $\gamma2L^{V262F}$ construct used, whilst $\alpha1^{V296L}$ was substituted with its α2 equivalent, $\alpha2^{V296L}$. Macroscopic desensitization curves were generated by exposing the patches to 10 mM GABA for 200 ms (Fig. 3.7A). The $\alpha2^{V296L}$ and $\gamma2L^{V262F}$ desensitisation mutations caused similar effects on the extent of desensitization to those previously observed for α1-containing pentamers, with $\alpha2^{V296L}$ causing a large decrease in the mean extent of desensitization from 55.6 ± 5.9 % to 11.7 ± 4.3 %, whilst $\gamma2L^{V262F}$ increased it to 82.7 ± 2.4 % (Fig. 3.7B) (one-way ANOVA: $F_{(2, 17)} = 92.45414$, $p = 7.33 \times 10^{-10}$; Tukey test(wt, $\alpha2^{V296L}$):

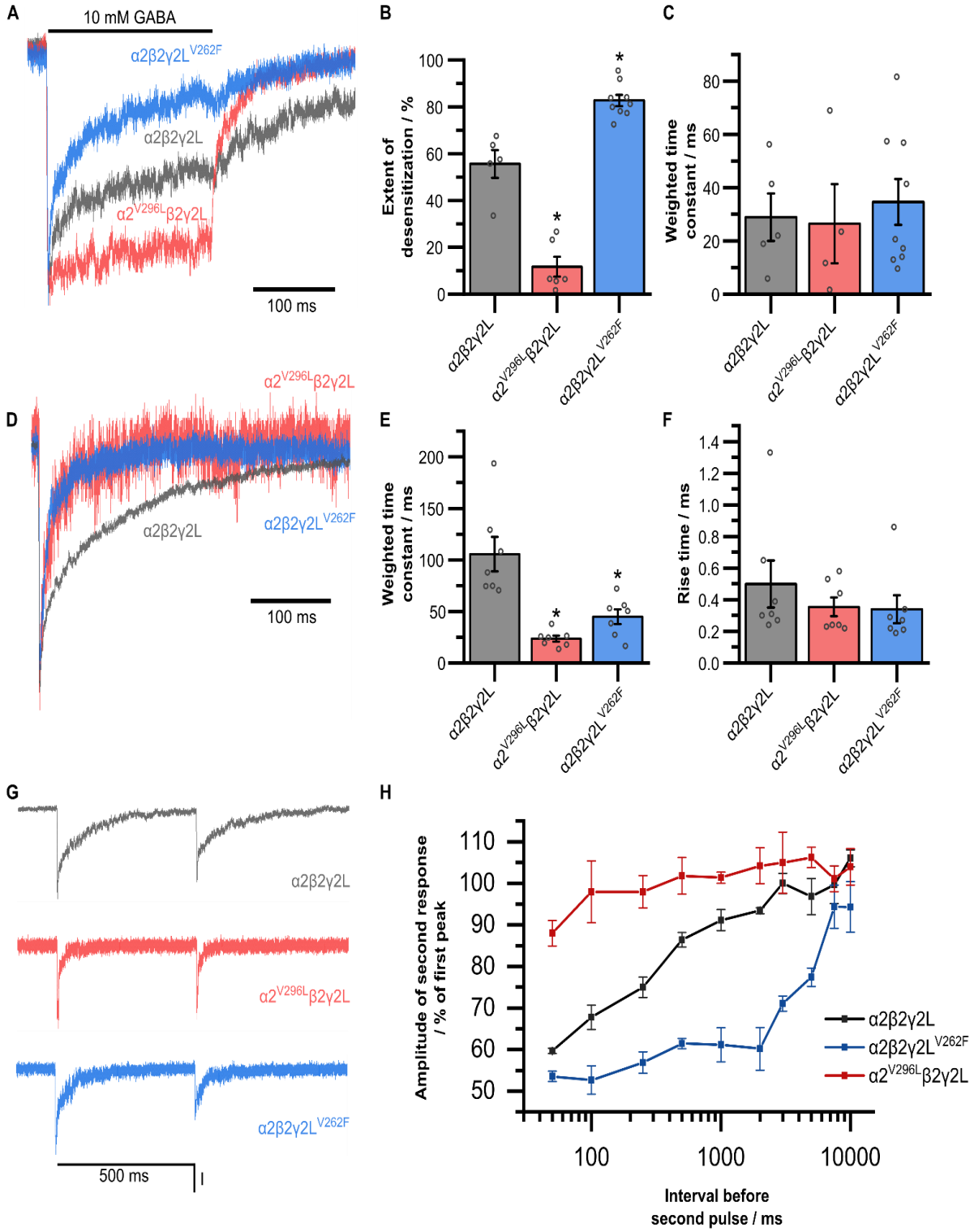

Figure 3.7. Kinetic phenotypes of α2^{V296L} and γ2L^{V262F} in α2-containing receptors. **A)** Average current traces displaying the macroscopic desensitization elicited by 200 ms exposure to 10 mM GABA. All currents are peak-normalised. **B)** Extents of macroscopic desensitization expressed as the percentage reduction in current from peak to steady-state. n = 5, 6, and 9 patches respectively. **C)** Weighted time constants from bi-exponential fits of the macroscopic desensitization. n = 5, 4, and 9 patches respectively. **D)** Average

current traces of the deactivation of the receptors after exposure to 10 mM GABA for 1 ms. All currents were peak normalised. **E)** Weighted time constants of bi-exponential fits of the deactivation of the receptors. n = 7 patches for each. **F)** Rise times of the responses to 1 ms pulses of 10 mM GABA, measured as the time taken for the current to rise from 20 % to 80 % of the maximum. n = 7 patches for each. '*' represents significant differences from wild type at $p < 0.05$. **G)** Representative traces of the responses to paired-pulses of 10 mM GABA with an inter-pulse interval of 500 ms. **H)** Resensitization of the receptors after a 1 ms pulse of 10 mM GABA. Amplitude of the response to the second pulse relative to that of the first is plotted against the interval between the pulses. n = 4 patches for each.

$p = 0.0000034$; Tukey test(wt, $\gamma2L^{V262F}$): $p = 0.00038$). Interestingly, in the context of the α2-containing pentamers, no significant differences in the rates of the decay were observed (Fig. 3.7C) (one-way ANOVA: $F_{(2,15)} = 0.18$, $p = 0.84$).

The effects of the mutations on deactivation were also assessed by exposing the patches to 10 mM GABA for 1 ms. Again, both mutations caused a substantial increase in the rates of deactivation, with $\alpha2^{V296L}$ decreasing the weighted time constant from 105.5 ± 16.7 ms to 23.6 ms, and $\gamma2L^{V262F}$ to 44.9 ± 7.1 ms (Fig. 3.7D, Fig. 3.7E; one-way ANOVA: $F_{(2, 18)} = 15.95$, $p = 0.00010$; Tukey test(wt, $\alpha2^{V296L}$): $p = 0.00010$; Tukey test(wt, $\gamma2L^{V262F}$): $p = 0.0022$). This suggests that, as for α1 containing pentamers, α2-containing receptors also desensitize in response to 1 ms pulses of 10 mM GABA and subsequently reopen. Both mutations again block these late re-openings of the channel, presumably by the same mechanisms as discussed above for α1 subunit-containing receptors. No significant effects were observed on the rise time of these responses (Fig. 3.7F) (one-way ANOVA: $F_{(2,18)} = 0.69$, $p = 0.51$), again confirming that these mutations do not affect the activation of the

receptor, implying that the macroscopic desensitization phenotypes are not due to alterations in the speed of agonist-binding, or receptor opening.

Finally, a paired-pulse drug application protocol was again used to probe how these mutations alter the occupancy of the desensitized state during responses to 1 ms pulses of GABA (Fig. 3.7G). The mutations displayed similar phenotypes to those observed for α1-containing pentamers, with $\alpha2^{V296L}$ reducing the entry into the desensitized state and $\gamma2L^{V262F}$ acting to stabilise it (Fig. 3.7H).

3.2.6. Allosteric modulators of the $GABA_A$ receptor alter the occupancy of the desensitized state

Allosteric modulators of $GABA_A$Rs are commonly used experimentally to probe $GABA_A$R function, and in the clinic as sedatives, general anaesthetics, anxiolytics, and anti-convulsants (Sieghart, 2015). We therefore decided to probe how such modulators might alter the occupancy of the desensitized state of the $GABA_A$R at inhibitory synapses. Some allosteric modulators of ion channels are known to directly exert their effects by binding to the desensitized form of the receptor and affecting its stability, or by directly altering the ability of the receptor to enter into desensitized conformations. For example, cyclothiazide acts as a positive allosteric modulator of AMPA receptors by impairing entry into the desensitized state (Fucile et al, 2006). Some modulators of the $GABA_A$R are also thought to exert their actions through the desensitized state, such as pregnenolone sulfate (discussed above). Structural studies have also shown that neurosteroids that potentiate the $GABA_A$R bind to the transmembrane domain of the $GABA_A$R α and β subunits close to the desensitization gate and could conceivably exert their effects by impairing its closure (Laverty et al 2017, Miller et al, 2017). Such is the case for 3α,5β-pregnanolone which was proposed, based on structural

data, to exert its action as a positive allosteric modulator by destabilising the desensitized state (Miller et al, 2017). However, further functional studies will be required to validate this claim, especially as the limited electrophysiological data shown in the same paper demonstrate that pregnanolone enhances peak currents with little effect on the steady-state. Such an effect on current profiles seems inconsistent with a destabilisation of the desensitized state, suggesting that the potentiation may be mediated via another mechanism

Nevertheless, allosteric modulators that do not act directly on the desensitized state are still capable of indirectly altering its occupancy. Such alterations to the desensitized state occupancy will be analogous to how positive allosteric modulators can increase open probability without directly affecting the microscopic opening rates or open state exit rates within a kinetic scheme. For instance, benzodiazepines are generally considered to act via increasing either the microscopic binding or preactivation rates (Gielen et al, 2012), and therefore enhance open state occupancy without directly altering the open state entry or exit rates. Given that entry into the desensitized state tends to occur either directly from the open state, or from an intermediate closed state located close to it in the kinetic scheme, it seems likely that promotion of the open state will also result in promotion of the desensitized state. Positive allosteric modulators will therefore tend to enhance the occupancy of the desensitized state whilst negative ones will decrease it. Alternatively, drugs such as pregnenolone sulfate, that act by directly altering the stability or accessibility of the desensitized state (Shen et al, 2000; Sachidanandan and Bera, 2015; Seljeset et al, 2018), will instead tend to display a negative correlation between open and desensitized state occupancies and therefore decrease open probability whilst increasing the occupancy of the desensitized state.

To test these hypotheses, outside out-patches pulled from HEK293 cells expressing α1β2γ2L were exposed to paired 1 ms pulses of 10 mM GABA in the presence of either the positive allosteric modulator etomidate (5 μM), or the negative allosteric modulator pregnenolone sulfate (10 μM). Again, the size of the response to the second pulse relative to that of the first was used as a measure of desensitized state occupancy.

In both cases, the allosteric modulators appeared to shift the resensitization curves to the right (Fig. 3.8A, 3.8B), indicating that they enhanced the occupancy of the desensitized state. Pregnenolone sulfate achieved this without substantially affecting the kinetics of the decays themselves (Fig. 3.8C). This is consistent with the pregnenolone sulfate acting via the desensitized state of the receptor. Conversely, the positive allosteric modulator etomidate slowed the decays, increasing the mean weighted time constant from 77.8 ± 4.2 ms to 416.1 ms (Fig. 3.8C; one-way ANOVA: $F(2,9) = 55.9$, $p = 0.000049$; Tukey test(vehicle, etomidate): $p = 0.000067$), displaying an extremely slow tail-like phase of deactivation, consistent with its action as a positive allosteric modulator.

These results suggest that positive allosteric modulators can indeed enhance the occupancy of the desensitized state. They may therefore, in certain conditions be considered to be acting as negative allosteric modulators. However, it is important to note that this will only be true in a very specific sense. Indeed, for etomidate, it was only the peak current that was depressed; the slowed decays exhibited in its presence will likely compensate for the reduced peak amplitude, in terms of overall charge transfer during the synaptic event. Furthermore, this experiment does not take into account that in a physiological context, positive allosteric modulators will also likely enhance responses to ambient GABA, thus generating or enhancing tonic

currents. Nevertheless, it is possible that positive allosteric modulators could act to depress the peaks of synaptic currents, elicited through desensitizing receptors, when the frequency of presynaptic release is sufficiently high.

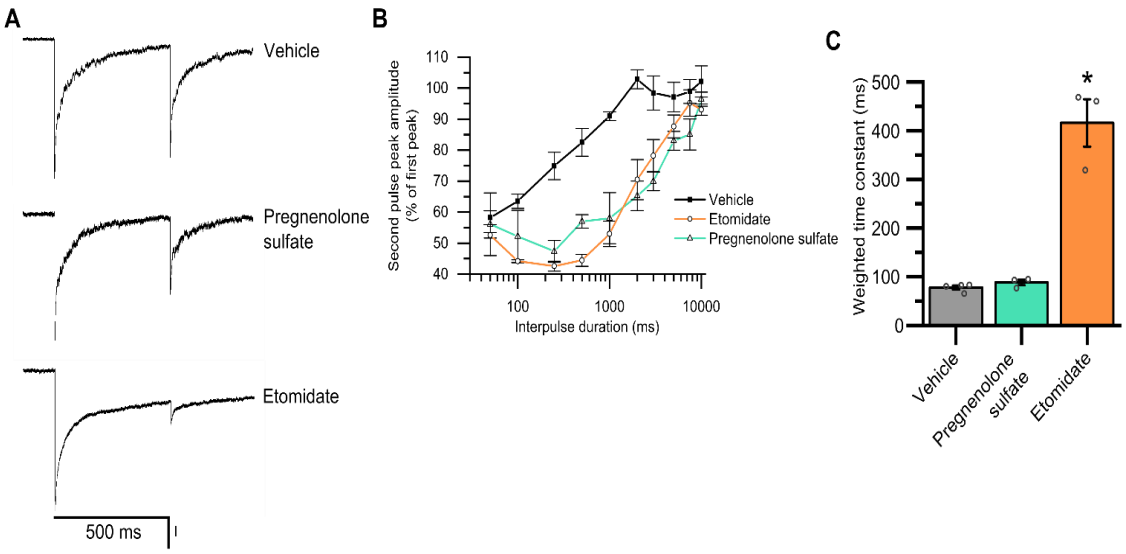

Figure 3.8. Allosteric modulators alter the occupancy of the desensitized state during phasic-like responses. **A)** Representative current traces of the responses of α1β2γ2L $GABA_A$Rs to paired pulses of 10 mM GABA applied for 1 ms in the presence of either vehicle, pregnenolone sulfate (10 μM), or etomidate (5 μM). **B)** Resensitization of $GABA_A$Rs in the presence of the indicated modulators. The amplitude of the response to the second pulse as percentage of the size of the first peak is plotted against the interval between the pulses. **C)** Weighted time constants of exponential fits of the decays elicited by a 1 ms pulse in the presence of the indicated allosteric modulator. N = 4, 3, and 3 patches respectively. '*' represents a significant difference from vehicle treated patches at $p < 0.05$.

Furthermore, if desensitization indeed has any roles in metabotropic signalling, or in the regulation of the modification of the receptors (as discussed in the introduction), it is likely that these modulators will enhance its effects.

3.3. Discussion

In order to study how the desensitization of the $GABA_AR$ affects inhibitory neurotransmission, it was important to first identify tools that could be used to alter the characteristics of receptor desensitization. The mutations outlined in the study by Gielen et al (2015), were obvious candidates for the manipulation of desensitization in neurons because for recombinant $GABA_ARs$ expressed in oocytes, two mutations, $\alpha1^{V296L}$ and $\gamma2L^{V262F}$ significantly decreased and increased, respectively, the extent of apparent receptor desensitisation. However, some studies have previously raised issues with the type of assay that was primarily used to assess these mutations, i.e. the prolonged exposure of receptors to high concentrations of agonist (Papke, 2010; Bianchi et al, 2007). As a result, further examination of the kinetic effects of these mutants was required in order to determine whether they actually exert their effects via alterations of the microscopic desensitization rates. The overall conclusion is that it seems likely to be the case for $\alpha1^{V296L}$, $\alpha2^{V296L}$ and $\gamma2L^{V262F}$. It is possible that a mutation such as $\alpha1^{V296L}$, which reduces the extent of macroscopic desensitization, may simply do so by depressing the peak GABA response towards the level of the steady-state current, rather than raising the steady-state current towards the peak value as would be expected for a true desensitization-ablating mutant. However, this does not seem to be the case here, as this mutant did not alter either the rise times of responses, or the peak concentration-response curve, suggesting that this mutation has little effect on the ability of the receptor to reach its peak response. These observations, combined with the results of the paired-pulse studies, indicate that the α^{V296L} mutation acts to decrease entry into the desensitized state. Conversely, it seems likely that $\gamma2L^{V262F}$ acts largely to stabilise the desensitized state as it enhanced the extent of

macroscopic desensitization, thus prolonging the suppression observed in paired-pulse experiments without increasing the initial suppression of the second GABA response.

In addition to confirming that these mutations are indeed true desensitization mutants, we also demonstrated that they alter synaptic-like responses to GABA. Curiously, despite having opposing effects on desensitization, *both* mutations appeared to increase the deactivation rate of phasic-like responses and also decrease tonic-like steady state responses to GABA. It may therefore be that both mutations exhibit similar phenotypes when expressed in neurons. Such a result would likely indicate that desensitization is in fact optimised for both forms of signalling. This would have interesting implications for attempts to design drugs that directly act via the desensitized state, as substantial perturbation of the stability or accessibility of this state, in either direction, may simply result in an overall impairment of GABA channel function.

However, it should be noted that whilst the techniques used in this study more closely resemble physiological forms of inhibition than those used previously to assess these mutants, they are still not truly representative of neuronal signalling. Differences in the phosphorylation state of the receptors, membrane lipids, subunit composition, and receptor clustering with receptor-associated molecules may all alter the nature of $GABA_AR$s desensitization at inhibitory synapses (Gielen and Corringer, 2018). We have already demonstrated here that the phenotypes of these mutations do not vary substantially upon exchanging the α1 subunits for α2 subunits. However, one variable in particular will almost certainly radically change the kinetics of neuronal $GABA_AR$ responses under physiological conditions: temperature. All the above assays were carried out at approximately 21 °C. However, these

receptors exist at 37 °C under physiological conditions in man. The relationship between the deactivation rate of the receptor and temperature is quantified by a parameter known as the Q_{10} value. This value is defined as the fold change in rate for each 10 °C increase in temperature. For the deactivation of the $GABA_AR$ this value is approximately 3 (Dixon et al, 2017). The rates of deactivation of IPSCs under physiological conditions should therefore be approximately 5.8 times faster than those observed here. Unfortunately, technical difficulties in utilising the theta-tube perfusion system at higher temperatures precluded assessment of the effects of these mutations at physiological temperature.

Finally, we have demonstrated that allosteric modulators can alter the occupancy of the desensitized state during phasic responses. For etomidate, this effect is likely to be indirect, with the effect on the desensitized state occupancy simply being a consequence of the effects of the drug on open channel probability. This however, is not the case for pregnenolone sulfate, which, as previously demonstrated (Shen et al, 2000; Sachidanandan and Bera, 2015; Seljeset et al, 2018), achieves its effects by enhancing the stability of the desensitized state.

3.4. Conclusions

- γ2L^{V262F} enhances the accumulation of $GABA_A$Rs in the desensitized state during prolonged exposure to GABA, whilst α1^{V296L} decreases it.
- Both α1^{V296L} and γ2L^{V262F} prevent late re-opening of the ion channel during deactivation.
- α1^{V296L} reduces the entry rate into the desensitised state.
- γ2L^{V262F} does not affect the entry into the desensitized state but acts to stabilise it.
- In order for late re-openings of $GABA_A$Rs to occur during deactivation, exit from the desensitized state must be sufficiently rapid, consistent with the two-gate model of pro-loop receptor desensitization.
- Excessive accumulation of the receptor in the desensitized state caused by γ2L^{V262F} prevents maintenance of a steady state conductance in response to any concentration of GABA.
- α1^{V296L} reduces the steady state responses of the receptors to low concentrations of GABA, suggesting that some level of entry into the desensitized state is required for tonic signalling.
- Positive allosteric modulators can enhance the occupancy of the desensitized state during phasic-like signalling.
- Pregnenolone sulfate is a negative allosteric modulator that acts by stabilising the desensitized state of the receptor.

Chapter 4: $GABA_AR$ desensitization mutants alter the kinetics of phasic and tonic inhibition in neurons

4.1. Introduction

To assess the role of $GABA_AR$ desensitization in neurons, the mutants $\alpha2^{V296L}$ and $\gamma2L^{V262F}$ were introduced into dissociated hippocampal cultures derived from E18 rat embryos. Such preparations are well established models of neuronal networks (Nault and De Koninck, 2009). Dissociated hippocampal cultures tend to contain a mixture of pyramidal cells, inhibitory interneurons, and granule cells. Synaptogenesis is generally observed to occur within two weeks of culturing the cells (Basarsky et al, 1994; Grabrucker et al, 2009). Additional maturation steps may occur at inhibitory synapses during and after this period, including alterations to the $GABA_AR$ subunits present at the synapses (Swanwick et al, 2006; Succol et al, 2012), and shifts in the equilibrium potential for chloride ions from depolarised to hyperpolarised potentials (Obrietan and van den Pol, 1995; Owens et al, 1996; Succol et al, 2012; Brady et al, 2018). Experiments were therefore carried out on 3-week-old cultures in an attempt to record phenotypes which more closely resemble those of mature neurons.

Once such cultures have been established, they display both phasic and tonic forms of inhibition. Phasic inhibition in such a preparation may involve either α1βγ2 or α2βγ2 $GABA_AR$ subunit combinations (Mangan et al, 2005). Although α1 is the more dominant subunit in the adult hippocampus, α2 is more dominant in embryonic hippocampi (Fritschy et al, 1994). Given that these neurons were dissociated at a developmental point where α2 is more prevalent, and subsequently in culture during the period where the subtype

dominance might be expected to change, it is unclear which α subtype will be the dominant one at synapses of these cultures. Previous work has suggested that after two weeks of culturing, α2 is the primary synaptic subtype, but is replaced by α1 by the third week (Swanwick et al, 2006). Conversely, others have reported that α2 is still dominant after three weeks in culture (Brünig et al, 2002). The $\alpha2^{V296L}$ mutation was therefore used for all neuronal experiments, and overexpression of the wild-type subunit was used as a control to detect artefacts resulting from any consequent alterations in receptor subunit expression patterns. The γ2 subunit is commonly found in $GABA_AR$ clusters at inhibitory synapses throughout the central nervous system (Chua and Chebib, 2017), and is therefore likely to be involved in the production of inhibitory synaptic currents in these cultures.

The effects of the $\alpha2^{V296L}$ and $\gamma2L^{V262F}$ mutations on phasic inhibition were assessed by transfecting them into the dissociated hippocampal cultures at 7 days *in vitro* (DIV), followed by recording of sIPSCs at 20-22 DIV. The mutations were predicted to alter sIPSCs in two different ways. Firstly, they will likely alter sIPSC decays in the same way that they altered the deactivation of $GABA_ARs$ in outside-out patches from transfected HEK293 cells (chapter 3). The outside-out patch deactivation experiments were designed to resemble the exposure of synaptic receptors to GABA released from presynaptic vesicles, and therefore both mutations are expected to speed up the decays of the sIPSCs. This phenotype may however be altered in a neuronal environment if the kinetics of desensitization are altered at the synapse, perhaps by interactions with synaptic scaffolding proteins. Such an effect has previously been reported for AMPA receptors, which have their desensitization reduced by the scaffolding protein stargazin (Constals et al, 2015). Interestingly, the kinetics of $GABA_ARs$ have previously been reported

to be altered through interactions with other synaptic proteins and consequent receptor clustering (Chen et al, 2000; Petrini et al, 2003); although it is not clear if the interactions altered the microscopic desensitization rates of the receptor.

A further consideration to be made when assessing how physiologically relevant these deactivation phenotypes are, is that the temperature at which recordings are made will drastically alter the speed of deactivation of the responses. Increases in temperature have previously been reported to increase the rate of $GABA_A$ receptor deactivation with a Q_{10} (the fold change after a 10 °C increase in temperature) of approximately 3 (Dixon et al, 2017). Given that the studies of deactivation made during this project, and those used to predict the role of desensitization in prolonging the deactivation of the receptors (Jones and Westbrook, 1995), were carried out at room temperature, it is currently unclear whether desensitization will affect the kinetics of sIPSCs in the same manner under physiological conditions. The decays of sIPSCs were therefore measured at both room temperature and at 37 °C in order to assess the physiological relevance of desensitization to the shapes of sIPSCs.

The second potential manner by which desensitization could alter sIPSC profiles is by changing the peak amplitudes of GABA synaptic currents. Desensitization of the receptors could act to decrease sIPSC amplitudes by causing short-term depression during high frequency signalling (Papke et al, 2011). The mutant $\gamma2L^{V262F}$ would be expected to enhance such short-term depression, both increasing the magnitude of the effect and causing it to become increasingly apparent at lower release frequencies. Conversely, $\alpha2^{V296L}$ would be expected to abolish this effect since it seems to prevent entry into the desensitized state during phasic-like responses (see chapter 3).

On the basis of the paired-pulse recordings made from outside-out patches (chapter 3.2.5), this will likely only become a substantial effect for wild type receptors if the firing rates of individual synapses exceeds 1 Hz (see Fig. 3.7). The firing rates of inhibitory interneurons are very often observed to exceed this value (Gravielle, 2018). However, the firing rates of both excitatory and inhibitory neurons will likely be substantially depressed during the conditions used for recording in this study due to the complete blockade of excitatory neurotransmission used to isolate GABAergic events, thus making it unclear if the effects of the mutations on amplitudes recorded under these conditions are representative of the physiological roles of desensitization.

Tonic inhibition primarily involves extrasynaptic receptors. In the hippocampus, these receptors usually include either the α5 or δ subunits (Brünig et al, 2002; Mangan et al, 2005; Swanwick et al, 2006). α5-containing $GABA_AR$ pentamers usually also contain the β3 and γ2 subunits (Olsen and Sieghart, 2008). Overexpression of $\gamma2L^{V262F}$ may therefore alter tonic currents. Given that the effects of this mutant were shown in the previous chapter to be independent of the α subunit present, it seems likely that it will exert a similar effect on desensitization of α5-containing receptors and therefore act to reduce tonic currents. Conversely, α1 and α2 are not considered to be primary mediators of tonic currents in hippocampal neurons. Nevertheless, these α subunits can, in principle, contribute to tonic conductances and may in fact do so when overexpressed. The effects of overexpression of $\alpha2^{V296L}$ on tonic current was therefore also assessed.

4.2. Results

4.2.1. Desensitization mutants α1^{V296L} and γ2L^{V262F} enhance sIPSC decays at room temperature

To assess the effects of desensitisation on neuronal $GABA_A$Rs and inhibitory neurotransmission, the α2^{V296L} and γ2L^{V262F} mutants were transfected into cultured hippocampal neurons, which were subsequently recorded from at DIV 21. sIPSCs were recorded, initially, at room temperature (21 °C) and the average waveforms of the synaptic events were calculated for each cell (Fig. 4.1A) and subsequently fitted with two-component exponential decays. Overexpression of either wild type subunit did not result in any different kinetic phenotypes compared to those for mock-transfected cells (Fig. 4.1B). By contrast, both α2^{V296L} and γ2L^{V262F} significantly increased the sIPSC decay rates, decreasing the mean weighted time constants from 46.4 ± 2.0 ms to 34.3 ± 1.4 ms and 30.9 ± 2.3 ms respectively (Fig. 4.1B) (one-way ANOVA: $F_{(4, 56)} = 7.32$, $p = 0.00081$; Tukey test(mock, α2^{V296L}): $p = 0.018$; Tukey test(mock, γ2L^{V262F}): $p = 0.0017$), consistent with the previous observations made for receptors in outside-out patches from HEK cells in Chapter 3. As explained previously for the recombinant $GABA_A$R data, this likely results from the absence of late reopenings during the deactivation of these mutants. Specifically, α2^{V296L} acts to prevent entry of the receptors into the desensitized state therefore allowing unbinding and deactivation to proceed more rapidly, whilst γ2L^{V262F} traps the receptor in the desensitized state and therefore allows the activation gate of the channel to close before the desensitized gate reopens.

However, the precise effects of the mutations on the deactivation profiles of sIPSCs differ somewhat from their effects on the deactivation of recombinant receptors in outside-out patches (section 3.2.5). In particular, the shifts

observed for sIPSC decays appeared to be smaller than those observed in outside-out patches.

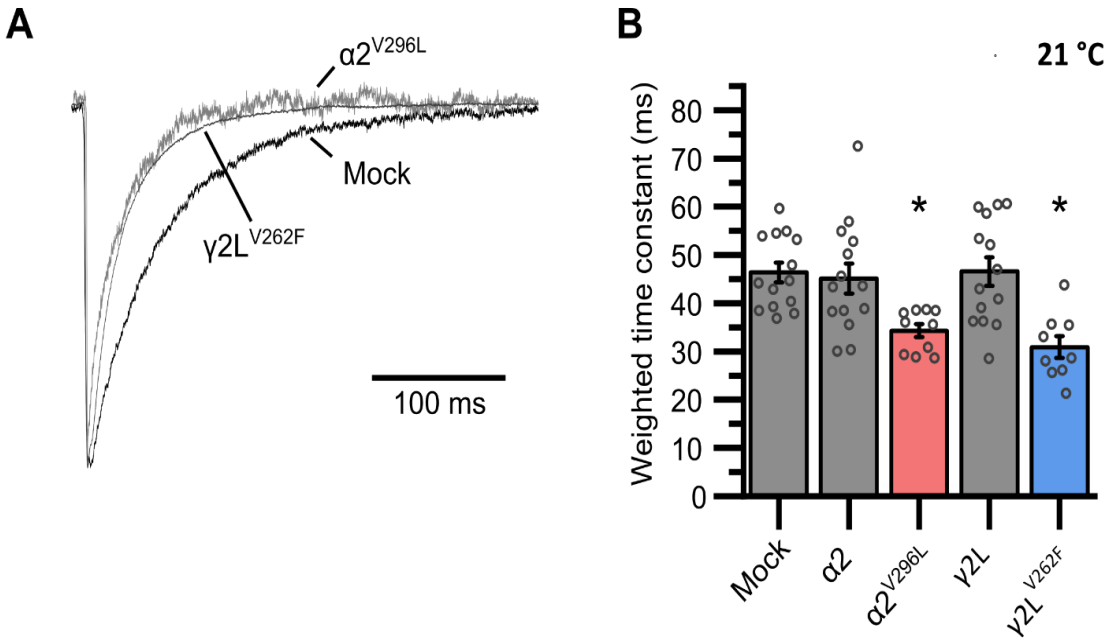

Figure 4.1. Effects of α2^{V296L} and γ2L^{V262F} on the kinetics of sIPSCs at 21 °C. A) Representative average waveforms of sIPSCs recorded from cultured hippocampal neurons transfected with the indicated construct. All traces are peak normalised. **B)** Weighted time constants of exponential fits of average sIPSC waveforms recorded from cells transfected with the indicated constructs. n = 14, 14, 11, 14, 9 neurons, from >3 cultures, respectively for each condition. '*' represents a significant Tukey test compared to mock transfected neurons at $p < 0.05$.

There are several possible explanations for these differences. Firstly, the presence of endogenous wild type subunits may act to dampen the phenotypes of these mutations. Secondly, the kinetics of synaptic events recorded from neurons in the whole-cell configuration are known to be distorted by effects such as space-clamp and cable filtering (Soltesz et al, 1995). It is possible that such issues may explain some of the differences between recordings from outside-out patches and from neurons, but it is unclear if such effects will account for the entirety of the differences. Finally, it

could be that desensitization of neuronal $GABA_AR$s is altered at inhibitory synapses compared with that observed for outside-out patches. Given that the phenotypes are smaller in neurons, this would suggest that desensitization is potentially suppressed at inhibitory synapses, perhaps by a similar mechanism to how stargazin mitigates the desensitization of AMPA receptors (Priel et al, 2005). Consistent with this, de-clustering of $GABA_AR$s with nocodazole or other treatments designed to disrupt the cytoskeleton, has previously been observed to increase the rate of the onset of $GABA_AR$ desensitization (Chen et al, 2000; Petrini et al, 2003).

As predicted, at room temperature both mutations speed up the decays of sIPSCs relative to mock transfected neurons. As discussed in the previous chapter, this is consistent with previous reports that desensitization acts to prolong the decays of synaptic currents (Jones and Westbrook, 1995). However, as noted before (chapter 3), the phenotype of V262F suggests that too much desensitization will simply terminate synaptic responses by keeping the receptor shut until after the activation gate has closed.

4.2.2. At physiological temperature, $\alpha2^{V296L}$ slows the decay of sIPSCs whilst $\gamma2L^{V262F}$ causes an increase

As with most protein kinetics, the deactivation rates of the $GABA_AR$ are known to increase with temperature. sIPSC kinetics would therefore be expected to be substantially faster at physiological temperatures. The previous experiment was therefore repeated with the recording chamber heated to 37 °C. As predicted, the increased temperature resulted in faster rates of sIPSC deactivation (Fig. 4.2A, 4.2B).

Intriguingly, whilst $\gamma2L^{V262F}$ continued to speed up the sIPSC decays at this temperature, decreasing the weighted time constants from 6.7 ± 0.4 ms to 4.5

± 0.2 ms (Fig. 4.2B; one-way ANOVA: $F_{(4, 78)} = 12.56$, $p = 6.1 \times 10^{-8}$; Tukey test(mock, $\gamma2I^{V262F}$): p = 0.0019), $\alpha2^{V296L}$ instead slowed the decays, increasing the weighted time constant to 8.5 ± 0.6 ms (Fig. 4.2B; Tukey test(mock, $\alpha2^{V296L}$): p = 0.017). Such a slowing of deactivation by a mutation that acts to prevent entry into the desensitized state is inconsistent with the hypothesis that entry into the desensitized state and subsequent channel reopening prolongs synaptic responses (Jones and Westbrook, 1995). This observation would instead suggest that at physiological temperatures, desensitization acts to terminate the responses of $GABA_A$Rs to brief pulses of GABA.

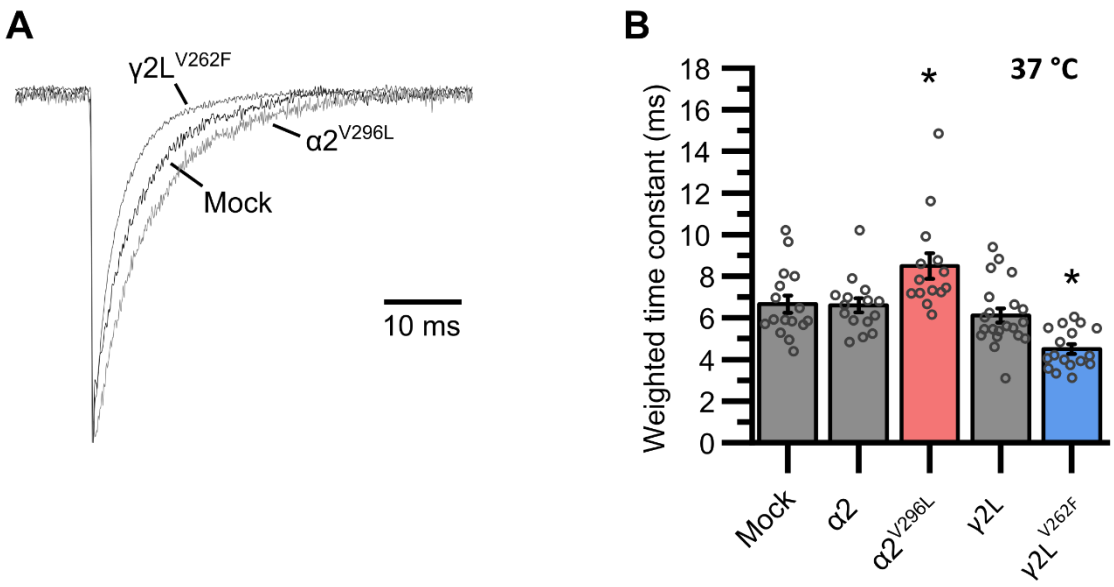

Figure 4.2. Effects of $\alpha2^{V296L}$ and $\gamma2L^{V262F}$ on the kinetics of sIPSCs at 37 °C. A) Representative average waveforms of sIPSCs recorded from cultured hippocampal neurons transfected with the indicated construct. All traces are peak normalised. **B)** Weighted time constants of exponential fits of average sIPSC waveforms recorded from cells transfected with the indicated constructs. n = 16, 15, 14, 21, and 17 neurons respectively for each condition. '*' represents a significant Tukey test compared to mock transfected neurons at $p < 0.05$.

In the scenario above, it is less clear why $\gamma2L^{V262F}$ acts to speed up IPSC decays. If the effect of the mutation is purely to stabilise the desensitized

state without enhancing the rate of entry into the desensitized state, as appeared to be the case for in outside-out patches at room temperature (Chapter 3), it might be expected to display no phenotype under conditions where reopening after desensitization does not occur. It therefore may be that some limited reopening from the desensitized state does actually occur under these conditions, but only after brief sojourns in the desensitized state. Such reopenings would be completely eliminated by γ2L^{V262F}. Alternatively, it may be that this mutation enhances the rate of entry of the receptors into the desensitized state at 37 °C. Nevertheless, the dominant effect of entry into the desensitized state would seem to be to terminate the initial opening burst.

Overall, it would seem that the observations made by Jones and Westbrook that the desensitization of the $GABA_AR$ prolongs its responses to brief applications of GABA are perhaps artefacts of experiments carried out at room temperature. At physiological temperatures it seems that the primary effect of desensitization is instead to terminate synaptic responses, perhaps because unbinding of agonist from the receptor and closure of the main gate is so quick under these conditions that reopening cannot occur following re-sensitisation. Such interpretations may not be consistent with existing kinetic models, as most would predict that reopening is equally probable regardless of the desensitized state lifetime. However, these results in the current study might be consistent with the dual-gate model of desensitization (see Gielen and Corringer, 2018), which has clear support from structural data but to our knowledge is yet to be incorporated into kinetic models. Some independence of the desensitization and primary activation gates would perhaps allow a kinetic model to account for these results.

4.2.3. γ2L^{V262F} increases the sIPSC amplitude

The other direct effect of desensitization on IPSCs would be to decrease the peak open probability of GABA synaptic responses during high-frequency signalling by acting as a postsynaptic mechanism of short-term depression (Papke et al, 2011). If firing frequencies at individual inhibitory synapses are sufficiently high such that complete exit from the desensitized state cannot occur in the periods between epochs of presynaptic GABA release, then synaptic amplitudes will decrease due to the accumulation of receptors in the desensitized state. This effect should be enhanced by γ2L^{V262F}, which for recombinant $GABA_A$Rs took up to ten seconds to re-sensitize after being exposed to 1 ms pulses of GABA in outside-out patches (Fig. 3.7). Conversely, α2^{V296L} will likely abolish this effect given that this mutation was observed to reduce entry into the desensitized state after brief agonist exposures (Fig. 3.7).

The amplitudes and frequencies of the sIPSCs of the transfected hippocampal neurons at 21 °C were measured to determine if such short-term depression substantially alters the average amplitudes of synaptic currents in these neurons. Overexpression of the wild type subunits or α2^{V296L} did not affect the amplitudes of the synaptic currents (Fig. 4.3A, 4.3B, 4.3C; one-way ANOVA: $F_{(4, 57)} = 5.67$, $p = 0.00064$; Tukey test(mock, α2^{V296L}): $p = 0.99936$). However, γ2L^{V262F} caused a large increase in amplitudes, from 65.8 ± 9.4 pA to 121.1 ± 12.1 pA (Fig. 4.3A, 4.3B, 4.3C; Tukey test(mock, γ2I^{V262F}): $p = 0.0022$), contrary to what would be predicted if desensitization was acting to cause short-term depression at inhibitory synapses. However, it is unlikely that short-term depression effects would exert substantial effects in these data as the frequencies of the sIPSCs are simply too low (Fig. 4.3D). The mean frequencies of most cells were close to, or lower than one Hz (Fig.

4.3D). Although this is similar to the frequency suggested by the paired-pulse experiments to be required for short-term depression (Chapter 3), it is important to note that for desensitization to have a substantial impact it would have to be achieved at each individual synapse rather than for the whole cell. If the mean sIPSC frequencies for each cell were divided equally among even an extremely conservative number of synapses per cell, the resulting synaptic frequencies would simply be far too low for entry into the desensitized state to result in substantial short-term depression under these conditions.

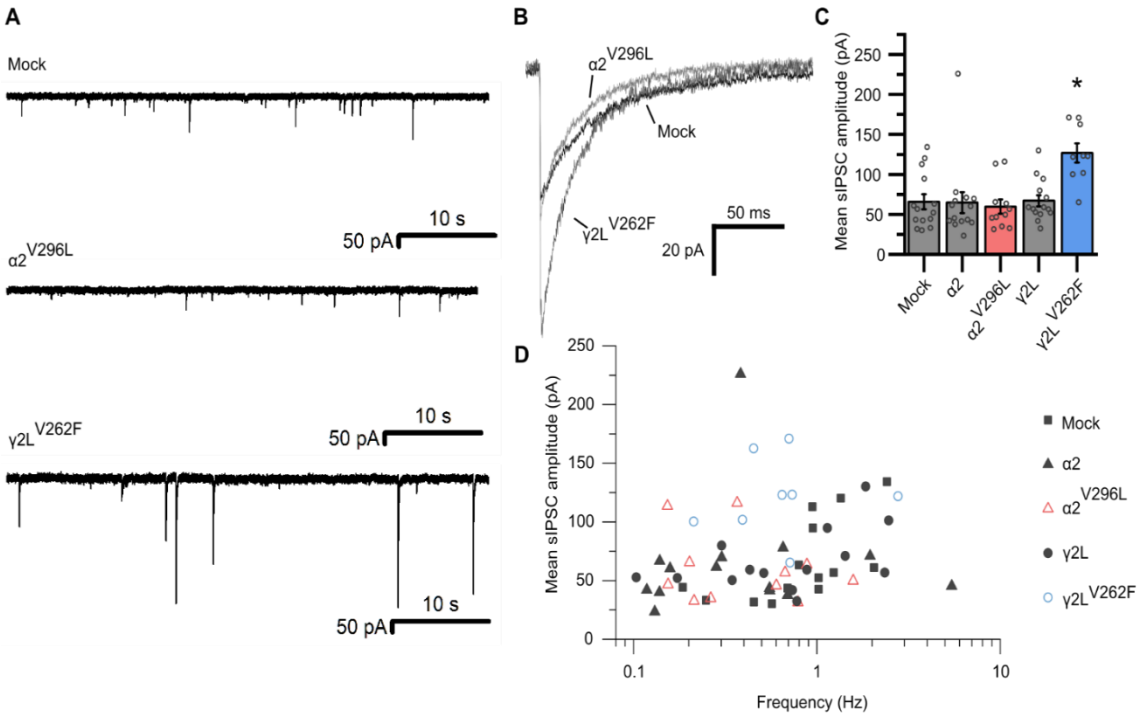

Figure 4.3. Effects of α2^{V296L} and γ2L^{V262F} on the amplitudes of hippocampal sIPSCs at 21 °C. A) Representative sIPSC traces recorded from cultured hippocampal neurons transfected with the indicated constructs. **B)** Representative average sIPSC waveforms generated from recordings made from neurons transfected with the indicated constructs. **C)** Mean amplitudes of sIPSCs. **D)** Plot of mean sIPSC amplitudes against mean frequency for each neuron. n = 14, 14, 11, 14, and 9 neurons respectively for each construct. '*' represents a significant Tukey test compared to mock transfected neurons at $p < 0.05$.

The increase in amplitudes observed for γ2L^{V262F} would not be predicted on the basis of the kinetic effects of the mutation. Entry into the desensitized state is generally predicted to occur after the initial opening burst of the receptor and therefore the stabilisation of this state should not result in an increase in peak open probability. Additionally, this mutation was not previously observed to result in enhanced currents in recombinant systems (Gielen et al, 2015), consistent with this effect not being due to enhanced peak open probability or single channel conductance. It therefore seems likely that the inhibitory synapses of neurons transfected with this mutant contain greater numbers of receptors. This effect could reflect either a role for desensitization in controlling either the expression or clustering of $GABA_A$ receptors, or a compensatory effect for the reduced current passed through this mutant due to its direct kinetic effects. These hypotheses will be explored further in the subsequent chapter.

Recordings were also carried out at 37 °C in order to gauge whether these phenotypes are still observed at physiological temperature. Indeed, the effects of these mutants on IPSC amplitudes remained the same, with only γ2L^{V262F} significantly increasing sIPSCs, from 133.6 ± 8.8 pA to 211.1 ± 25.0 pA, whilst transfection with wild type subunits or α2^{V296L} had no effect (Fig. 4.4A, 4.4B, 4.4C; one-way ANOVA: $F_{(4, 78)} = 6.67$, $p = 0.00011$; Tukey test (mock, α2^{V296L}): $p = 0.957$; Tukey test(mock, γ2l^{V262F}): $p = 0.0022$). The frequencies of the sIPSCs were generally greater but more variable at this temperature (Fig. 4.4D). However, whilst some cells achieved higher frequencies than those observed at room temperature, there did not appear to be any correlation between IPSC amplitudes and frequency (Fig. 4.4D), again suggesting that the frequencies at individual synapses are too low for short term depression to contribute substantially to the regulation of sIPSC

amplitudes under these conditions. The lack of temperature-dependence of the effects on synaptic amplitudes perhaps lends some support to the hypothesis that γ2L^{V262F} is increasing the number of receptors at the synapse, given the temperature dependence of the other kinetic effects of desensitization on sIPSCs.

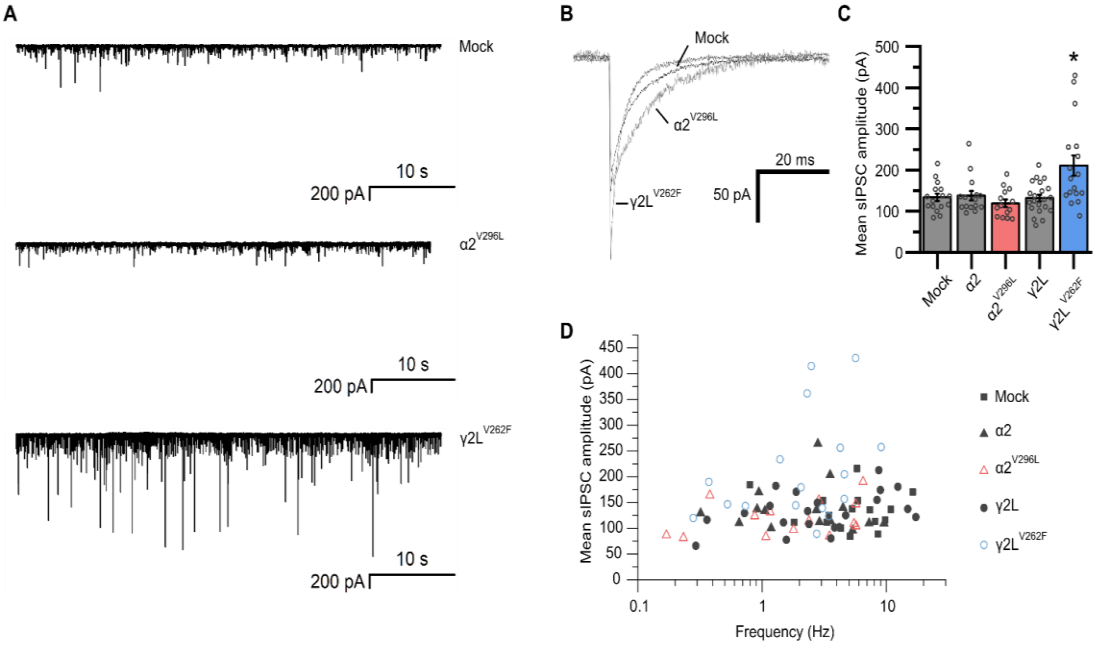

Figure 4.4. Effects of α2V296L and γ2LV262F on the amplitudes of hippocampal sIPSCs at 37 °C. A) Representative sIPSC traces recorded from cultured hippocampal neurons transfected with the indicated constructs. B) Representative average sIPSC waveforms generated from recordings made from neurons transfected with the indicated constructs. C) Mean amplitudes of sIPSCs. D) Plot of mean sIPSC amplitudes against mean frequency for each neuron. n = 16, 15, 14, 21, and 17 neurons for each condition respectively. '*' represents a significant Tukey test compared to mock transfected neurons at $p < 0.05$.

4.2.4. Both desensitization mutants decrease tonic current amplitude at ambient and physiological temperature

In addition to phasic signalling, tonic signalling was probed by exposing the patched hippocampal cells to bicuculline and measuring the resulting reduction in the baseline current. These currents result from the activation of extrasynaptic $GABA_A$Rs to low ambient concentrations of GABA. Although these extrasynaptic $GABA_A$Rs often contain the δ subunit, the γ2L subunit also contributes to tonic currents in neurons (Eugène et al, 2007). The tonic currents were initially measured at 21 °C. As previously predicted on the basis of concentration-response curves, both $\alpha2^{V296L}$ and $\gamma2L^{V262F}$ reduced the tonic currents, from 37.6 ± 8.2 pA to 13.8 ± 2.8 pA and 5.9 ± 3.5 pA respectively (Fig. 4.5A, 4.5B; one-way ANOVA: $F_{(4, 29)} = 7.60$, $p = 0.00026$; Tukey test(mock, $\alpha2^{V296L}$): $p = 0.018$; Tukey test(mock, $\gamma2L^{V262F}$): $p = 0.0031$), with $\gamma2L^{V262F}$ appearing to do this to a larger degree than $\alpha2^{V296L}$. When the experiment was repeated at physiological temperature (37 °C), almost identical effects were observed (Fig. 4.5C, 4.5D; one-way ANOVA: $F_{(4, 24)} = 15.9$, $p = 0.0000018$; Tukey test(mock, $\alpha2^{V296L}$): $p = 0.005$; Tukey test(mock, $\gamma2I^{V262F}$): $p = 0.00017$).

As previously discussed, the reduced steady-state responses to GABA observed for receptors containing $\gamma2L^{V262F}$ is likely due to the accumulation of the receptors in the desensitized state, even at low concentrations of GABA (Chapter 3, Fig. 3.6). This occurs due to the long-term stabilisation of the desensitized state by this mutation. Thus, even under conditions where entry into the desensitized state is infrequent, simply due to the low rate of agonist

binding at low concentrations, this mutant can still accumulate in the desensitized state due to its relatively long lifetime.

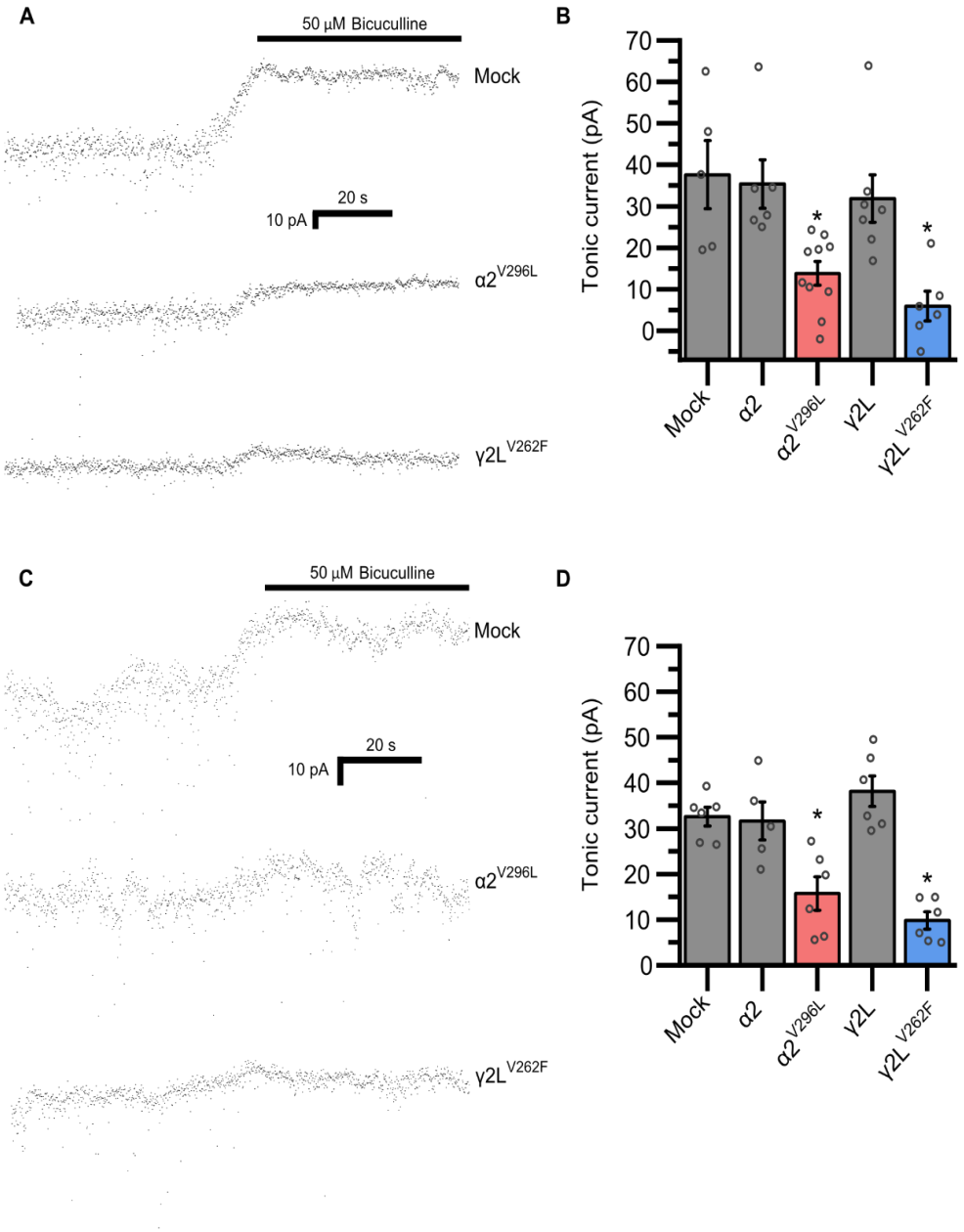

Figure 4.5. Effects of $\alpha2^{V296L}$ and $\gamma2L^{V262F}$ on the amplitudes of hippocampal tonic $GABA_AR$ currents. A) Representative current traces of the responses of neurons to 50 µM bicuculline at 21 °C. Dots represent 5 ms moving averages of the current taken every 100 ms. **B)** Mean tonic currents recorded from neurons transfected with the indicated constructs (21 °C). n = 5, 6, 10, 7, and 6 neurons respectively. **C)** Representative current traces of the bicuculline responses at 37 °C. **D)** Mean tonic currents recorded at 37 °C. '*' represents a significant Tukey test compared to mock transfected neurons at $p < 0.05$. n = 6, 5, 6, 6, and 6 neurons respectively.

Conversely, receptors containing $\alpha2^{V296L}$ exhibited reduced steady state responses to low concentrations of GABA, perhaps due to the role of the desensitized state as a high affinity state either promoting the retention of the bound agonist, or by enhancing the ability of the receptor to precede to a fully-liganded state. The observed invariance of the tonic phenotypes with temperature is perhaps inconsistent with the first hypothesis. This is because it was proposed as an extension of the hypothesis used to explain the prolongation of IPSCs proposed by Jones and Westbrook (1995). Given that the effect on phasic signalling showed a clear temperature dependence to the point where switching from room temperature to physiological temperature actually reversed the direction of the effect, the complete lack of an effect of temperature on the tonic phenotypes would perhaps rule out this hypothesis.

The observation that $\gamma2L^{V262F}$ resulted in a greater reduction in tonic current than $\alpha2^{V296L}$ has two potential explanations. Firstly, $\alpha2^{V296L}$ was observed to exhibit steady state responses to GABA even at high concentrations of GABA, whilst $\gamma2L^{V262F}$ appeared incapable of supporting steady state responses to any concentration of GABA. It therefore could be that the ambient tonic concentration of GABA is simply sufficiently high to induce a partial steady state response from $\alpha2^{V296L}$. Conversely, this effect may also be explained by the differential contribution of the receptor subtypes to tonic conductances. Although γ2 contributes to tonic currents in hippocampal neurons, it usually does so in receptors containing the α5 subunit. Conversely, α2 is not thought to make substantial contributions to tonic currents in combination with either the γ or δ subunits. Its contribution may have been enhanced in these experiments due to its overexpression. That

said, the overexpression of wild type α2 did not appear to cause any significant change in tonic currents by itself.

On the other hand, these phenotypes may be explained by alterations in the number of $GABA_A$Rs at the surface of the cell. However, it is difficult to determine if this was the case based on this data alone, given that both mutants were expected to support little or no tonic current. Nevertheless, the fact that $\gamma2L^{V262F}$ may have enhanced the numbers of receptors at inhibitory synapses whilst $\alpha2^{V296L}$ did not, would rule out a substantial deficit in cell surface expression of $GABA_A$Rs incorporating either mutation.

4.3. Discussion

The primary predictions for the role of desensitization in phasic inhibition, prior to the experiments in this chapter, were that desensitization would act to prolong synaptic responses (Jones and Westbrook, 1995), and that it would act to depress synaptic responses during high frequency signalling (Papke et al, 2011). The first of these predictions is questionable given the data presented here; whilst entry into the desensitized state may prolong IPSCs at room temperature, it in fact seems to rapidly terminate IPSCs under physiological conditions. However, these data are not sufficient to validate or dismiss the second hypothesis. Whilst desensitization clearly did not significantly depress synaptic responses in this data set, the IPSC frequencies were most likely simply too low to observe such an effect. The data gained from outside-out patches presented in chapter 3 would suggest that substantial depression will perhaps be observed at more physiological firing rates (e.g. >50 Hz for fast spiking interneurons: Wierenga et al, 2010). Whether such levels of depression will be observed at inhibitory synapses is not clear though. Previous studies have reported a lack of such

desensitization-induced short-term depression (Mellor and Randall, 2001). Given that this effect represents a fundamental receptor property, it should occur regardless of whether the receptors are in synapses or outside-out patches. Given this premise, it raises the question as to whether desensitization is in fact suppressed to some extent in neurons, possibly through receptor-protein interactions at the synapse or post-translational modifications specific to neurons. Consistent with this idea, the kinetic phenotypes of the mutations also appeared to be much smaller than would have been anticipated based on the previous data (Chapter 3) from outside-out patches. A reduction in the extent of desensitization of $GABA_A$Rs due to receptor clustering has been reported (Chen et al, 2000; Petrini et al, 2003; Petrini et al, 2004; Marchionni et al, 2009). However, the effects reported in these studies are quite small and probably insufficient to account for a complete loss of short term depression induced by desensitization. It may be that the complexity of proteome interactions at the synapse act to shape the desensitization of receptors even further than has previously been reported.

Nevertheless, the effects of these mutations on tonic signalling were at least as strong as expected; consistent with the relative suppression of the phenotypes at synapses being a result of clustering dependent effects or receptor-protein interactions. Both mutations were observed to decrease tonic currents, as was previously predicted on the basis of steady-state GABA concentration-response curves (Chapter 3). The effect of the γ2L mutation was stronger than that in the α2 subunit. This is likely explained by the relative contributions of these subunits to tonic currents. γ2L is well established to contribute to tonic currents in hippocampal neurons, particularly in combination with α5 subunits. Conversely, α2 is generally considered to be localised to synaptic clusters and to contribute primarily to

phasic signalling. However, this does not exclude it from contributing to tonic signalling. Additionally, its presence in extrasynaptic receptor populations may have been enhanced due to its overexpression in this study; although overexpression of the wt α2 subunit was not observed to affect tonic currents.

One effect observed here that was not predicted on the basis of the prior recombinant $GABA_AR$ work, or indeed any known model of receptor desensitization, was that expression of a mutation that enhances desensitization results in significantly enhanced sIPSC amplitudes. This seems likely to represent an increased number of receptors at the synapse. However, the basis for this increase in number is unclear. It seems unlikely to have been a straightforward overexpression artefact given that overexpression of the wild type subunit did not result in any similar phenotype. However, it is possible that this enhancement reflects a compensatory effect, as these cells had been expressing this mutant for a total of 14 days in culture prior to recording during a period of synaptic development. The magnitude of the charge passing through these receptors during synaptic events would be reduced due to their altered kinetics; as would the amount passing through tonically active receptors. The upregulation of synaptic receptors may therefore have represented a cellular response to reduced chloride currents. Alternatively, these results could represent a role for desensitization in the regulation of receptor number at synapses. This possibility will be further explored in the following chapter.

4.4. Conclusions

- At room temperature both $\alpha2^{V296L}$ and $\gamma2L^{V262F}$ increased the decay rates of sIPSCs, consistent with the hypothesis that desensitization prolongs synaptic responses
- At physiological temperatures however, the physiological role of desensitization instead appears to rapidly terminate IPSCs.
- Short-term depression resulting from accumulation of the receptor in the desensitized state was not observed, probably due to the low frequencies of sIPSCs observed under the conditions of recording.
- $\gamma2L^{V262F}$ enhances the amplitudes of synaptic GABA currents; this is possibly due to an increase in the number of receptors at the synapse. Whether this is a direct effect of the enhanced desensitization of these receptors is unclear on the basis of this data alone.
- Increased desensitization can suppress tonic currents; but some level of desensitization appears to be required to sustain them, perhaps related to the high affinity of the desensitized state for GABA.

Chapter 5: Desensitization of the $GABA_AR$ results in a long-term potentiation of inhibitory synapses

5.1. Introduction

The activation of $GABA_ARs$ by either agonists or positive allosteric modulators has previously been observed to result in a variety of secondary effects on the receptor in addition to modulating the opening of the channel (Gravielle, 2018). These effects include: the enhancement of the phosphorylation of receptor subunits (Gutierrez et al, 2014; Adams et al, 2015; Abramian et al, 2014; Ferreri et al, 2015), alterations in the mobility of receptors (Gouzer et al, 2014; Lévi et al, 2015), changes to the pharmacology of the receptors (Gravielle et al, 2005), the stimulation of metabotropic signalling cascades (Nicholson et al, 2018), and alterations in the expression of receptor subunits (Abramian et al, 2014; Gutiérrez et al, 2014; Modgil et al, 2017; Nicholson et al, 2018). In some cases, these effects may result from the chloride currents that pass through open GABA channels. These currents, either depolarising or hyperpolarising, can regulate calcium influx through either voltage-gated calcium channels, or NMDA receptors, therefore providing an indirect regulation of metabotropic forms of signalling. However, it is also possible that these effects result from entry of the receptor into the desensitized state. Given that most of these effects likely involve interactions with intracellular proteins, the desensitization gate at the base of the TMD is ideally positioned to regulate these interactions. The intracellular domain between M3 and M4, which mediates most of the interactions of the $GABA_AR$ with intracellular proteins and is the site of many posttranslational modifications of the receptor (Chen and Olsen, 2007; Nakamura et al, 2015), has not been studied structurally. It is therefore unclear if it undergoes conformational changes

during gating and desensitization of the receptor. However, it is plausible that any agonist-induced changes in its conformational state may occur during desensitization of the receptor, given that desensitization involves conformational changes in the regions of the receptor located adjacent to the large intracellular domain.

Consistent with this, similar effects at other ion channels have been attributed to desensitization-induced conformational changes. For example: the activation of G-protein dependent signalling by α7 nicotinic acetylcholine receptors (Kabbani and Nichols, 2018), the upregulation of nicotinic acetylcholine receptor expression by nicotine (Wonnacott, 1990; Melroy-Greif et al, 2016), and the enhanced mobility of AMPA receptors after stimulation with glutamate (Constals et al, 2015).

The desensitization mutants used in this study provide an ideal model for studying whether any of these secondary effects of $GABA_AR$ activation are indeed dependent on desensitization. Indeed, it is possible that the enhanced synaptic amplitudes observed for V262F are already an indicator of such secondary effects of desensitization on channel function. If such effects are dependent on receptor desensitization, it should be possible to better understand their mechanism and to predict when they occur and how drug treatments may affect them. With this in mind we examined whether acute desensitization of $GABA_ARs$ has any long-lasting effects on inhibitory synaptic currents. This was addressed by exposing neurons to various treatments that would be expected to desensitize $GABA_ARs$, and subsequently recording sIPSCs. The mutant $\alpha2^{V296L}$ was used to confirm the desensitization dependence of these effects by removing its influence.

As the activation of $GABA_ARs$ has previously been reported to enhance the PKC-dependent phosphorylation of sites on $GABA_AR$ subunits (Gutierrez et

al, 2014), the effects of desensitization on the γ2L^{S327}site were also assessed. This is a major site for phosphorylation of γ2S and γ2L subunits by PKC (Krishek et al, 1994; reviewed in Nakamura et al, 2015). The mechanism by which $GABA_AR$ activation regulates the level of phosphorylation is complex. There are two potential mechanisms by which this phenomenon could occur. Firstly, $GABA_AR$ activation could lead to activation of the kinase. Recently, it has been shown that $GABA_ARs$ directly interact with PLC and can activate it during prolonged stimulation (Nicholson et al, 2018); although the activation of PLC led to calcineurin activation and subsequent dephosphorylation of $GABA_ARs$ in that study, it is possible that the production of diacylglycerol (DAG) by PLC could lead to the activation of PKC. Secondly, it is possible that $GABA_AR$ activation simply facilitates phosphorylation of $GABA_AR$ subunits by increasing the affinity of the receptors for the kinase, perhaps in a manner analogous to how GPCR activation leads to phosphorylation by G-protein coupled receptor kinases (GRKs) (Gurevich et al, 2012). It is possible that desensitization, or other conformational changes, after $GABA_AR$ activation may expose phosphorylation sites in the M3-M4 intracellular domain, either by altering the interactions with scaffolding proteins around the phosphorylation sites or through conformational changes that reveal these sites. We therefore investigated whether $GABA_AR$ desensitization activates PKC or simply facilitates the phosphorylation of $GABA_ARs$ by PKC.

5.2. Results

5.2.1. Acute desensitization of $GABA_ARs$ by prolonged agonist application causes LTP of inhibitory synapses via a PKC-dependent mechanism

In order to assess whether the desensitization of $GABA_ARs$ has long-term effects on inhibitory synapses, dissociated hippocampal cultures were treated

with 1 mM GABA for 20 min prior to recording. A similar protocol has previously been observed to result in increased phosphorylation of γ2 subunits and a long-lasting uncoupling of the benzodiazepine and GABA binding sites in primary cultures of cortical neurons (Gutiérrez et al, 2014). Such a treatment should, in principle, result in desensitization of both synaptic and extrasynaptic receptors, accompanied by a large chloride conductance through open receptors.

The treatment of cultured neurons with GABA was carried out whilst the cells were still in maintenance cell culture media in the incubator prior to recording. For neuronal cultures under such conditions it is not clear what the value of E_{Cl} will be, and therefore whether the subsequent opening of $GABA_A$Rs will be inhibitory or excitatory. In order to address this question, cell-attached voltage-clamp recordings were carried out from these cultures in maintenance media (at 37 °C, bubbled with 5 % CO_2). In these recordingscurrent waveforms reflecting actions potentials in the patched cells were observed. In all four cells recorded from, robust spontaneous firing was observed (Fig. 5.1). However, the application of 1 mM GABA completely silenced the firing of the neurons almost instantaneously (Fig. 5.1), demonstrating that the activation of $GABA_A$Rs is indeed inhibitory under these conditions.

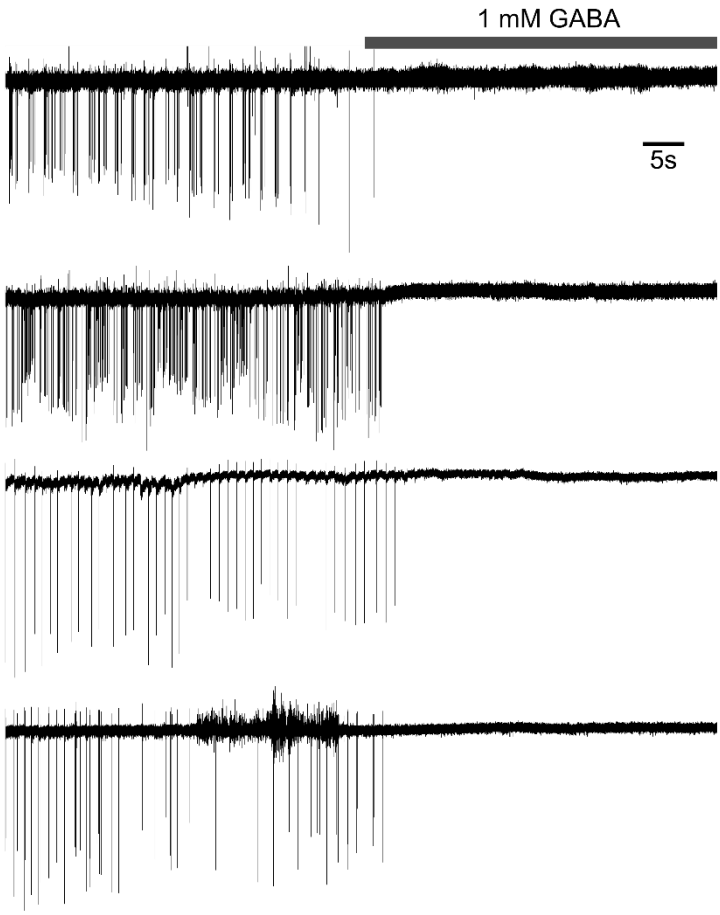

Figure 5.1. Effect of GABA on the spike firing of neurons under the same conditions used for pre-treatments in the following figures. Traces of spikes recorded from four cultured hippocampal neurons before and after the application of 1 mM GABA. Recordings were made under conditions equivalent to those under which the cells were cultured (37 °C, 5 % CO_2, bathed in maintenance media). Patch electrodes contained a HEPES buffered version of the maintenance media. The patch potential was clamped at 0 mV.

To assess the effects of GABA treatment on inhibitory synapses, recordings were made of sIPSCs using the same protocols as described in the previous chapter. Prior to the patching of the cells, the coverslips were placed into the recording chamber and washed with continuous perfusion of Krebs solution for 25 minutes to remove the exogenous GABA. After this period of time, the holding currents and frequencies of synaptic events were observed to be stable (Fig. 5.2A), suggesting wash off was largely complete at this point.

Pre-treatment with 1 mM GABA for 20 minutes prior to recording for resulted in a large increase in the average amplitude of sIPSCs, from 55.7 ± 3.8 pA to 132.2 ± 16.7 pA (Fig. 5.2A, 5.2B, 5.2C; one-way ANOVA: $F_{(3, 31)} = 13.85$, $p = 6.6 \times 10^{-6}$; Tukey test (mock, GABA): $p = 1.96 \times 10^{-5}$). Analysis of the sIPSCs showed that pre-treatment with GABA had no effect on the kinetics of the synaptic currents (Fig. 5.2B, 5.2D; test: $t_{(15)} = 15$, $p = 0.74$). No significant changes in frequency were observed either (Fig. 5.2E; one-way ANOVA: $F_{(3, 31)} = 1.329$, $p = 0.282$). Pre-treatment with GABA therefore results in a potentiation of inhibitory synapses similar to that observed after the expression of $\gamma2L^{V262F}$. This phenomenon will henceforth be referred to as LTP_{GABA}.

To assess whether this effect was dependent on the desensitization of the receptor, the pre-treatment protocol was repeated on neurons transfected with $\alpha2^{V296L}$ in order to impair the ability of synaptic $GABA_A$ receptors to enter the desensitized state during the GABA treatment. The expression of $\alpha2^{V296L}$ almost completely abolished the effect of the GABA pre-treatment, with sIPSCs recorded after the treatment having essentially identical amplitudes (57 ± 9.9 pA) to those of vehicle-treated cells (Fig. 5.2C; Tukey test(mock, $\alpha2^{V296L}$ + GABA): $p = 0.9997$). Given that $\alpha2^{V296L}$ was not previously observed to reduce the amplitudes of sIPSCs in the absence of any treatment (chapter 4), it seems likely that this effect was due to a block of the GABA-induced enhancement.

Given that other long-term effects of the treatment of neurons with GABA have previously been reported to be dependent on PKC and the phosphorylation of γ2 subunits (Gutiérrez et al, 2014), the GABA pre-treatment was repeated in the presence of a blocker of PKC (bisindolylmaleimide, 500 nM). This too completely blocked the effect of the

GABA pre-treatments on synaptic amplitudes (48.6 ± 5 pA) for untransfected neurons (Fig. 5.2C; Tukey test (mock, GABA + bisindolylmaleimide): p = 0.974), demonstrating that PKC is required for LTP_{GABA}. It is unclear on the basis of this data alone though, whether the GABA treatment causes activation of PKC, or whether this simply reflects a requirement for basal levels of PKC activity.

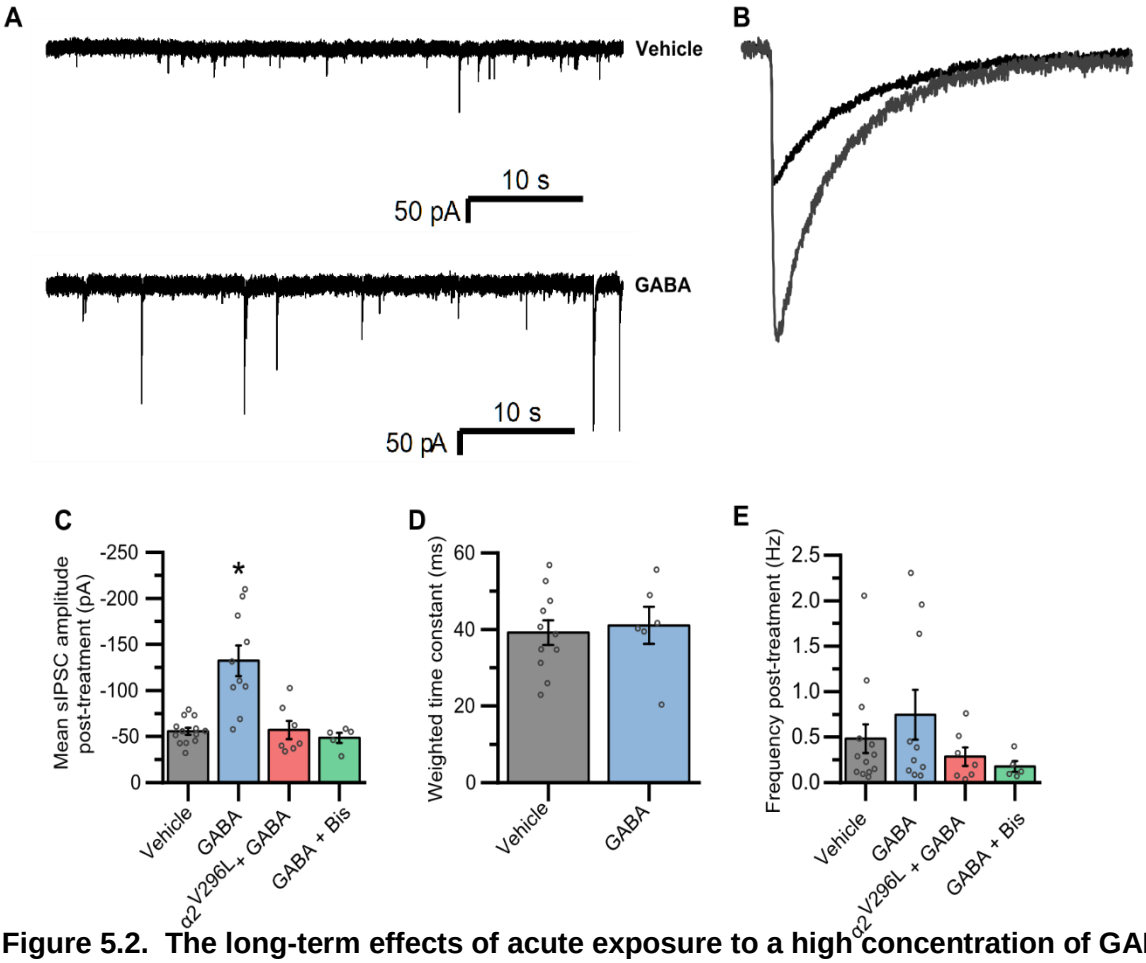

Figure 5.2. The long-term effects of acute exposure to a high concentration of GABA on the amplitudes of sIPSCs. A) Representative sIPSC traces recorded from neurons treated with either GABA (1 mM) or vehicle for 20 minutes prior to recording. **B)** Representative average sIPSC waveforms. **C)** Mean sIPSC amplitudes for neurons recorded after the indicated pre-treatments (Bis indicates co-application of 500 nM bisindolylmalemide). n = 13, 10, 7, and 5 neurons for each condition respectively. '*' represents a significant Tukey test compared to vehicle treated neurons at $p < 0.05$. **D)** Weighted time constants of exponential fits of the sIPSC decays. **E)** Frequencies of sIPSCs recorded post-treatment. **F)** Average sIPSC amplitudes recorded 24 hours after pre-treatment. n = 4, and 5 neurons respectively.

The potentiation of inhibitory GABA currents was observed immediately after the pre-treatment of GABA had been washed off. However, from the Gutierrez et al (2014) study involving a similar treatment with GABA, effects on receptor phosphorylation and benzodiazepine binding were observed 48 hours after treatment. It is possible that the potentiation observed here lasts for a similar timespan. Recordings were therefore repeated 24 hrs after the treatment with GABA in order to assess whether the observed potentiation of inhibitory synapses is also a long-lasting effect. The GABA treatment was carried out for 20 min as before but was then washed out with HBSS and fresh maintenance media, and the cells returned to the incubator for 24 hours prior to recording. The sIPSCs were then recorded and found to have increased amplitudes (98.7 ± 7.9 pA) 24 hours after treatment with GABA relative to those of vehicle treated cells (55.5 ± 2.0 pA) (Fig. 5.2F; t test: $t_{(7)}$ = 4.75, p = 0.0021). The amplitude increase was slightly smaller than that observed immediately after treatment but still significant, thus confirming that this effect indeed represents a long-term potentiation of inhibitory synapses.

Treatment of neurons with GABA therefore results in the long-term potentiation of inhibitory synapses in a PKC and receptor desensitization-dependent manner. Such an increase in the amplitudes of synaptic responses in the absence of any obvious kinetic changes would be consistent with an increase in the number of receptors at the synapses, as was proposed for receptors containing γ2L^{V262F} subunits (chapter 4). Alternatively, this could be a presynaptic effect. However, this seems unlikely for several reasons. Firstly, although a slight increase in frequencies was observed after treatment with GABA, this was not significant. Secondly, an increase in the peak concentrations of GABA achieved in the synaptic cleft is unlikely to cause such a large increase in amplitude, as the concentrations resulting from

transmitter release are regarded as near saturating (Overstreet et al, 2002), which for synaptic receptor isoforms results in peak open probabilities greater than 50 % (Goldschen-Ohm et al, 2010). Thirdly, the effect appeared to be completely blocked by $\alpha2^{V296L}$, which mitigates receptor desensitisation. Given that the transfection efficiency of the Effectene-based method in neuronal cultures is extremely low (<1% of cells express GFP at the point of recording), and that recordings were made from neurons without any obvious transfection of adjacent cells, it seems that desensitization of the postsynaptic receptors is required for this potentiation to occur, as this mutation will likely only have been present on the postsynaptic cell. A presynaptic mechanism would therefore require a retrograde signalling pathway downstream of postsynaptic receptor desensitization.

Additionally, as the sIPSCs were recorded in the whole cell configuration with a high intracellular chloride, it seems unlikely that this increase in amplitude results from alterations in the E_{Cl^-} as this parameter should have been controlled during the patch clamp recordings. Furthermore, given that $\alpha2^{V296L}$ blocked the effect despite enhancing the steady state currents through $GABA_A$Rs in response to high concentrations of GABA (Chapter 3, Fig. 3.6B), it seems unlikely that the effect of GABA was due to its effects on the excitability of the cell as the chloride current should have been enhanced. Finally, it is unclear on the basis of this experiment alone whether GABA is acting solely via $GABA_A$Rs or also through $GABA_B$ receptors. However, the observation that the effect could be completely blocked by $\alpha2^{V296L}$ suggests that even if $GABA_B$ activation drives this effect, $GABA_A$ receptor desensitization is required in some way to facilitate it.

This result of LTP_{GABA} appears to be contrary to some studies that demonstrate that treatment with $GABA_A$R agonists results in reductions in the

amplitudes of inhibitory synaptic currents. For instance, Brady et al (2018) showed that treatment of cultured cortical neurons with muscimol decreases the number of γ2 containing $GABA_A$Rs at inhibitory synapses and a consequent decrease in the amplitude of mIPSCs. However, in this study it was noted that the muscimol treatments resulted in depolarisation and subsequent increases in intracellular calcium. The alterations of the inhibitory synapses were attributed to these depolarisation-induced calcium signals. It is possible that a depolarisation-dependent LTD as observed in that study may have masked the non-depolarisation dependent LTP reported here. Consistent with this, a previous study demonstrating LTD at inhibitory synapses resulting from depolarising currents through $GABA_A$Rs, also reported an LTP from the same treatment when the coupling of the depolarising $GABA_A$R currents to the calcium signalling required for LTD was blocked by selective NMDA receptor antagonists (McLean et al, 1996).

Another recent study has also shown down-regulation of $GABA_A$Rs at inhibitory synapses after treatment with agonists or benzodiazepines (Nicholson et al, 2018). However, in this study much longer exposures to the drugs were used to elicit the changes, with reductions in the sizes of synaptic $GABA_A$R clusters only occurring after at least 48 hours after treatment.

5.2.2. LTP of inhibitory synapses is dependent on the phosphorylation of γ2L^{S327}

Given that similar treatments of cortical neurons with GABA result in increased phosphorylation of the γ2 subunit (Gutiérrez et al, 2014), a known substrate for PKC (Nakamura et al, 2015), LTP_{GABA} may involve the phosphorylation of $GABA_A$R subunits. The clustering of $GABA_A$Rs at inhibitory synapses has also been reported to be dependent on phosphorylation of γ2^{S327} (Wang et al, 2003; Muir et al, 2010; Nicholson et al, 2018;), consistent

with the potentiation of sIPSCs observed here. We therefore recorded sIPSCs from neurons transfected with the phosphorylation site mutant γ2L^{S327A} that had also been pre-treated for 20 mins with 1 mM GABA. The amplitudes of the sIPSCs recorded from neurons expressing γ2L^{S327A} were not significantly enhanced (75.7 ± 6.3 pA) relative to vehicle-treated neurons (56.8 ± 3.0 pA), suggesting that the potentiation of inhibitory synapses indeed requires the phosphorylation of this site in γ2L, perhaps coincident with increased receptor clustering (Fig. 5.3A, 5.3B; one-way ANOVA: $F(2,28) = 39.48$, $p = 0.71 \times 10^{-8}$; Tukey test (mock, γ2L^{S327A} + GABA): $p = 0.07$).

To assess whether the phosphorylation of this site is regulated by $GABA_AR$

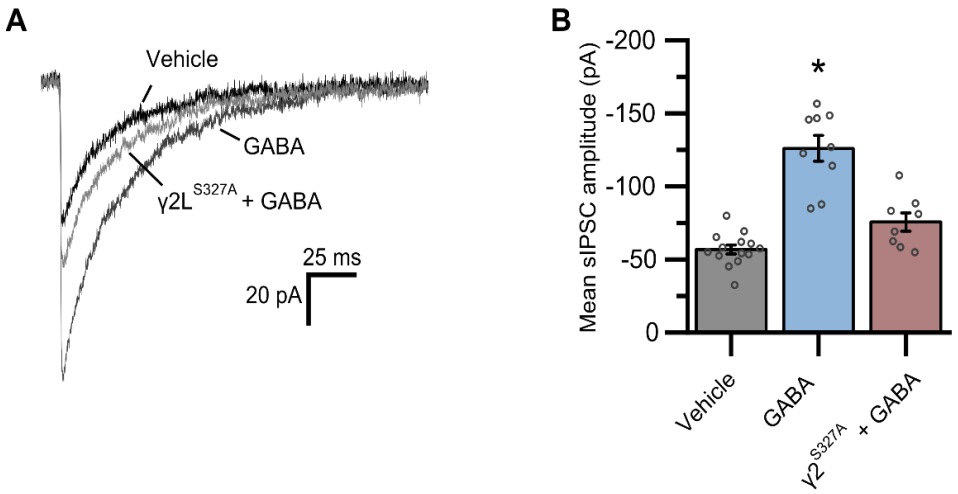

Figure 5.3. The effect of expression of γ2L^{S327A} on the potentiation of sIPSCs by GABA. A) Representative average sIPSC waveforms recorded post GABA treatment from neurons that were either mock transfected or transfected with γ2L^{S327A}. **B)** Average sIPSC amplitudes. n = 14, 9, 8 neurons respectively for each condition. '*' represents a significant Tukey test compared to vehicle treated neurons at $p < 0.05$.

desensitization, immunoblots were carried out on lysates from HEK cells transfected with α1β2γ2L $GABA_A$ receptors treated with 1 mM GABA for 20 min prior to lysis. PKC-dependent phosphorylation of this site has previously been reported in HEK cells (Krishek et al, 1994). Additionally, enhanced

phosphorylation of other $GABA_AR$ subunits caused by neurosteroids which act as modulators of $GABA_ARs$ has also been shown to occur in HEK cells, similar to the effects of neurosteroids on $GABA_AR$ receptors in neurons (Adams et al, 2015). This suggests that the mechanism by which $GABA_AR$ activation causes enhanced phosphorylation of receptor subunits is conserved in these cells. Lysates from these cells were therefore blotted for the presence of phosphorylated S327 following 20 minute GABA exposure. The levels of S327 phosphorylation were normalised to the total expression levels of γ2 subunits in the samples. Treatment of the cells containing wildtype receptor with 1 mM GABA resulted in a small increase in the levels of S327 phosphorylation (1.4 ± 0.1 fold increase compared to vehicle) (Fig. 5.4A, 5.4B), consistent with the previously observed increases in γ2 phosphorylation in neurons after a similar treatment (Gutiérrez et al, 2014). When the experiment was repeated with $γ2L^{V262F}$, the enhancement in phosphorylation was significantly larger (2.1 ± 0.2 fold increase; Fig. 5.4A, 5.4B; one-way ANOVA: $F_{(2, 18)} = 14.19$, $p = 0.0001998$; Tukey test(wt, $γ2L^{V262F}$): $p = 0.042$). This is consistent with γ2 phosphorylation being dependent on $GABA_AR$ desensitization, as this mutant tends to accumulate in the desensitized state and displayed essentially no steady-state current in response to prolonged exposure to GABA (Chapter 3, Fig. 3.6). Furthermore, $α1^{V296L}$, which alleviates desensitisation blocked the enhancement of phosphorylation (0.8 ± 0.1 fold change; Fig. 5.4A, 5.4B; Tukey test (wt, $α1^{V296L}$): $p = 0.038$), confirming that this effect on phosphorylation relied on desensitization of the $GABA_A$ receptors. Interestingly though, $α1^{V296L}$ not only blocked the increase in phosphorylation but also caused a slight decrease post-GABA treatment. This could perhaps be a result of the large chloride currents which are observed to pass through this mutant in response to

prolonged application of GABA but is unclear as to why this would result in a dephosphorylation in these cells.

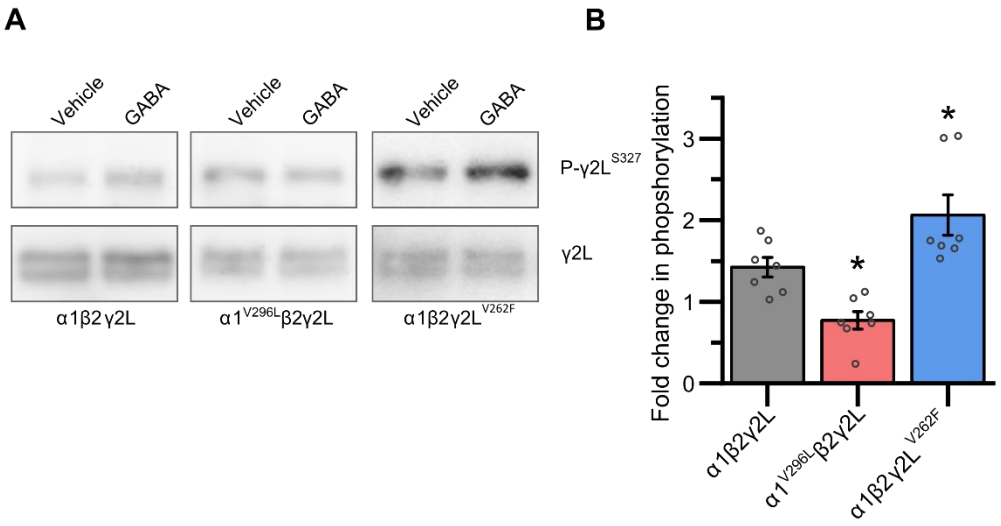

Figure 5.4. GABA-induced phosphorylation of γ2L is dependent on receptor desensitization. A) Immunoblots of lysates of HEK cells transfected with the indicated receptor construct and treated with vehicle or GABA, probed for phosphorylated γ2^{S327}, and total γ2 levels **B)** Fold change in phosphorylation of γ2^{S327} after treatment with GABA. n = 7 lysates for each condition. '*' represents a significant Tukey test result when compared to wild type at $p < 0.05$.

5.2.3. The activation and desensitization of $GABA_A$Rs is required downstream of PKC activation to produce LTP

The mechanism by which $GABA_A$R activation results in enhanced phosphorylation of receptor subunits has not previously been elucidated. It could result from either an increase in the activity of the kinase (PKC), an increased affinity of the kinase for the receptor, or a decrease in the activity of a phosphatase (likely to be calcineurin at S327; Wang et al, 2003). Stimulation of $GABA_A$Rs could result in activation of PKC via the activation of PLC (Nicholson et al, 2018). Recently, it was shown that $GABA_A$Rs interact

with PLC and stimulate the enzyme upon activation of the receptor (Nicholson et al, 2018). Although in this study, the activation of PLC was observed to result in enhanced calcineurin activation and subsequent dephosphorylation of S327, PLC could also activate PKC and therefore enhance the phosphorylation of these consensus sites.

Alternatively, phosphorylation of the receptor could be enhanced by a mechanism analogous to that used to regulate the phosphorylation of GPCRs by GRKs after activation (Gurevich et al, 2012). It could be that receptor desensitization results in conformational changes that enhance the accessibility of the phosphorylation site to an already active kinase. This could occur through the breaking of interactions that mask the phosphorylation site, or simply through conformational changes in the M3-M4 intracellular domain exposing the phosphorylation site.

Finally, $GABA_AR$ activation could reduce the activity of calcineurin by hyperpolarising the membrane and thus reducing calcium influx through NMDA receptors or voltage-gated calcium channels. However, this seems unlikely in this case, given that $\alpha1^{V296L}$ blocks the effect whilst $\gamma2^{V262F}$ enhances it, suggesting that the effect is more-likely to be dependent on conformational changes of the receptor than chloride currents passing through the receptor.

Therefore, it seems likely that the enhanced γ2L subunit phosphorylation observed after GABA treatment results either from activation of PKC, or facilitation of phosphorylation of the receptor by activated PKC. To distinguish between these two hypotheses, cultured neurons were treated with the phorbol ester, phorbol 12-myristate 13-acetate (PMA, 500 nM, 20 minute incubation prior to recording) in order to directly activate PKC. As a result, IPSC amplitudes of the PMA-treated neurons were enhanced (104.7 ± 12.5

pA) compared to those of vehicle treated neurons (60.3 ± 8.3 pA) (Fig. 5.5A, 5.5B, 5.5C; one-way ANOVA: $F_{(3,25)} = 5.844$, $p = 0.0036$; Tukey test (mock, PMA): $p = 0.022$). The amplitude increases appeared to be of a similar magnitude to that evoked by GABA pre-treatments. This treatment was then repeated in the presence of picrotoxin, a $GABA_AR$ channel blocker, to block the activation of $GABA_AR$ by endogenous GABA during the treatment. The addition of picrotoxin completely blocked the PMA-induced increase in sIPSC amplitudes (55.1 ± 8.5 pA) (Fig. 5.5C; Tukey test (mock, PMA + Ptx): $p = 0.987$). This suggests that the activation of PKC alone is not sufficient for LTP_{GABA}; $GABA_ARs$ must also be active during the treatment. The potentiation by PMA was also blocked by expression of $\alpha2^{V296L}$ (57.7 ± 6.6 pA) (Fig. 5.5C; Tukey test (mock, $\alpha2^{V296L}$ + PMA): $p = 0.9988$), suggesting that $GABA_AR$ desensitization was still required for LTP_{GABA} even when PKC was already activated. It therefore seems that the potentiation of the inhibitory synapses by prolonged GABA treatments does not result from the activation of PKC by receptor desensitization. Instead, it is likely that the desensitization of the receptors allows them to be phosphorylated by an already active PKC. Thus, LTP_{GABA} requires coincident $GABA_AR$ desensitization and the activation of PKC. In hippocampal neurons, there are several pathways which act through Gαq, and therefore PLC, that could potentially provide a basal level of PKC activity in the absence of any pharmacological manipulation. Similarly, the firing of inhibitory interneurons should provide a basal level of $GABA_AR$ desensitization. Therefore, the enhancement of either PKC activation or $GABA_AR$ desensitization alone is sufficient to result in the LTP_{GABA}, but both are required, evident by the observation that blockade of $GABA_AR$ desensitization prevents the induction of LTP_{GABA} by enhanced PKC activity and vice-versa.

Interestingly, pre-treatment with PMA also resulted in a large increase in sIPSC frequency, from 0.2 ± 0.06 Hz to 2.9 ± 0.6 Hz (Fig. 5.5D; one-way ANOVA: $F_{(3,25)} = 6.36$, $p = 0.0024$; Tukey test (mock, PMA): $p = 0.00717$), an effect partially blocked by picrotoxin (Fig. 5.5D;Tukey test (mock, PMA + Ptx): $p = 0.775$), but not by $\alpha2^{V296L}$ (Tukey test (mock, $\alpha2^{V296L}$ + PMA): $p = 0.0128$). This is therefore likely to involve a distinct presynaptic action of PKC on GABA release as reported previously (Pitler and Alger, 1994; Capogna et al, 1995).

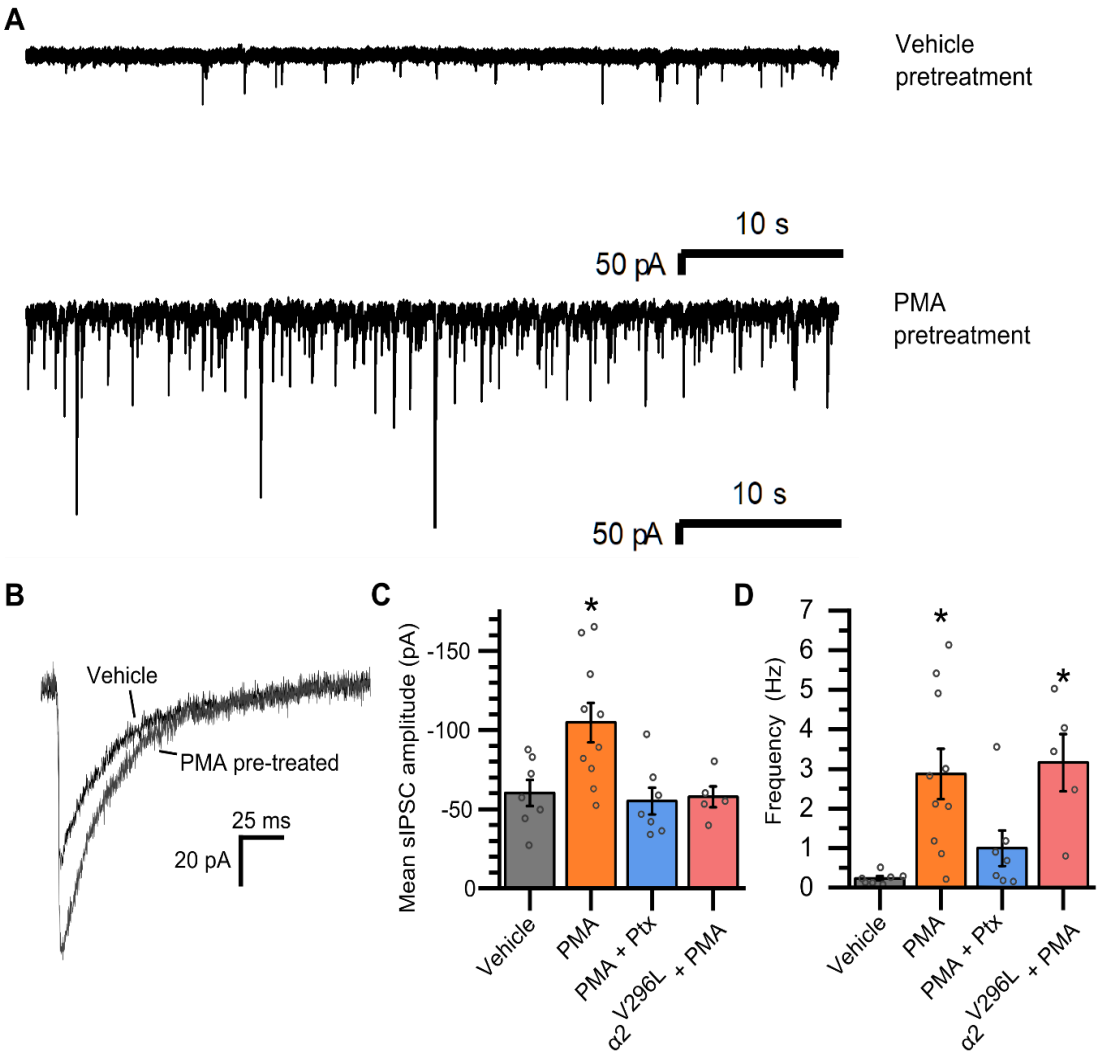

Figure 5.5. PKC dependent potentiation of sIPSC amplitudes require $GABA_AR$ activation and desensitization. A) Representative traces of sIPSCs recorded from neurons after treatment with either PMA (200 nM) or vehicle. **B)** Representative average sIPSC waveforms generated from recordings of cells after treatment with PMA or vehicle. **C)** Mean sIPSC amplitudes recorded from neurons after the indicated treatments. Ptx indicates coapplication of 100 µM picrotoxin. **D)** Frequencies of sIPSCs recorded from neurons after the indicated treatments. n = 7, 10, 7, and 5 neurons respectively for each condition. '*' represents a significant Tukey test compared to vehicle neurons at $p < 0.05$.

5.2.4. Allosteric modulators that enhance the occupancy of the desensitized state can induce LTP_{GABA}

As was argued in the introduction to this thesis, prolonged exposure of synaptic $GABA_AR$s to high concentrations of GABA is unlikely to occur under physiological conditions. However, as demonstrated in the previous chapters, occupancy of the desensitized state can and does occur during physiological forms of signalling. Nevertheless, it is clear from the results presented here that the basal levels of $GABA_AR$ activation that occur in these cultures do not result in sufficient desensitization to cause a maximal LTP_{GABA}, since further synaptic potentiation is possible by exposure to exogenous GABA. Therefore LTP_{GABA} will likely occur when the occupancy of the desensitized state is enhanced. This may occur simply through high intensity firing of the inhibitory interneurons resulting in enhanced GABA release at synapses. Alternatively, as was demonstrated in chapter 3, allosteric modulation of the channel either by endogenous neurosteroids, or by clinically used modulators of the $GABA_AR$, enhances occupancy of the desensitized state that could result in LTP_{GABA}.

In order to test this hypothesis, neurons were treated with the two allosteric modulators previously demonstrated to enhance the occupancy of the desensitized state during phasic signalling (chapter 3.2.7): etomidate and pregnenolone sulfate. Whilst etomidate is a positive allosteric modulator of the $GABA_AR$ and pregnenolone sulfate is a negative allosteric modulator, both promote occupancy of the desensitized state of the receptor (Fig. 3.8) and would therefore be expected to result in LTP_{GABA}. The pre-treatment of neurons with these modulators was carried out in the same way as those with GABA, a 20 min treatment followed by transfer of the coverslip to the recording chamber with a subsequent 25 min washout prior to recording.

sIPSCs were then recorded from the neurons as before. Neither treatment appeared to alter the kinetics of the sIPSCs (Fig. 5.6B, 5.6D; one-way ANOVA: $F_{(2,28)} = 0.00413$, $p = 0.996$), consistent with the drugs having washed off prior to recording, as judged by the absence of an etomidate effect which previously caused a marked slowing of receptor deactivation when acutely applied to outside-out patches. As predicted, both treatments resulted in the enhancement of the amplitudes of the sIPSCs, from 75.4 ± 4.8 pA to 121.8 ± 13.2 pA (pregnenolone sulfate) and 126.1 ± 11.8 pA (etomidate) (Fig. 5.6A, 5.6B, 5.6C; one-way ANOVA: $F_{(2, 29)} = 7.194$, $p = 0.0029$; Tukey test (mock, PS): $p = 0.00992$; Tukey test (mock, etomidate): $p = 0.00586$). The enhancements are of similar magnitude to those observed after treatment with GABA or PMA. The enhanced occupancy of the desensitized state resulting from the application of allosteric modulators to $GABA_A$ receptors therefore appears to be sufficient to induce LTP_{GABA}.

These results further confirm the requirement of receptor desensitization for LTP_{GABA}. Given that etomidate is a positive allosteric modulator whilst pregnenolone sulfate is a negative allosteric modulator, they will have opposite effects on the chloride currents through the $GABA_A$Rs, with etomidate enhancing and pregnenolone sulfate inhibiting. Thus, if LTP_{GABA} is dependent on the chloride conductances of open $GABA_A$Rs or the resulting changes in the excitability of the postsynaptic cell, these modulators should have had opposing effects on the sIPSC amplitudes. However, both have almost identical effects on the occupancy of the desensitized state, consistent with the mechanism of LTP_{GABA} induction being desensitization dependent.

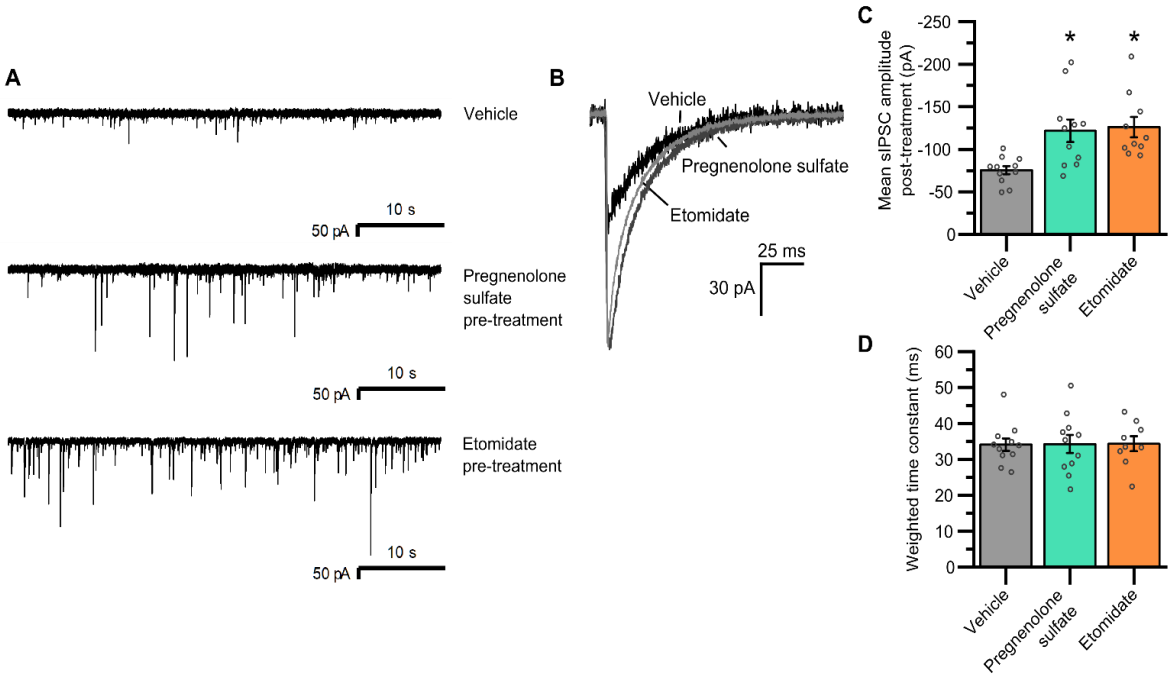

Figure 5.6. Pre-treatment with etomidate or pregnenolone sulfate results in a potentiation of sIPSCs. A) Representative traces of sIPSCs recorded from cultured hippocampal neurons after treatment with either vehicle, pregnenolone sulfate (1 µM), or etomidate (5 µM). **B)** Representative average sIPSC waveforms from cells treated with the indicated modulator. **C)** Mean amplitudes of sIPSCs recorded from cells after treatment with the indicated modulator. n = 11, 11, and 10 cells for each condition. **D)** Weighted time constants of exponential fits of the sIPSC decays. '*' represents a significant Tukey test compared to mock transfected neurons at $p < 0.05$.

5.3. Discussion

Overall, LTP_{GABA} involves the specific recognition of desensitized receptors by PKC, resulting in their phosphorylation and subsequent potentiation of inhibitory synapses. Although the activation of PKC has previously been reported to have a variety of effects on $GABA_AR$s (Nakamura et al, 2015), the potentiation of inhibitory synapses by PKC has previously been reported (Poisbeau et al 1999; Jovanovic et al, 2004; Bannai et al, 2015). In particular, the phosphorylation of $\gamma2L^{S327}$ has been correlated to the synaptic clustering

of the $GABA_AR$ (Muir et al, 2010; Nicholson et al, 2018; Wang et al, 2003). Furthermore, the enhancement of the phosphorylation of PKC-dependent phosphorylation sites on $GABA_ARs$ after activation of $GABA_ARs$, with either agonists or positive allosteric modulators, has also been reported (Gutiérrez et al, 2014; Adams et al, 2015; Abramian et al, 2014). These previous results are extended here by the demonstration that such enhancements of phosphorylation are dependent on entry into the desensitized state, and that they have long-term consequences for synaptic signalling.

The entry of $GABA_ARs$ into the desensitized state occurs during both phasic and tonic signalling. Aside from regulating the amount of current flowing through the receptor, this conformational change also appears to act as a signal of $GABA_AR$ activation to intracellular signalling pathways. Although $GABA_AR$ activation results in chloride currents that can indirectly regulate metabotropic forms of signalling by reducing the opening of voltage-gated calcium channels and NMDA receptors, such a form of regulation will be relatively non-localised, and non-specific in its regulation of inhibitory synapses. Conversely, the entry of $GABA_ARs$ into the desensitized state may allow kinases to specifically recognise receptors that were recently active, allowing a much finer control of inhibitory synaptic plasticity, perhaps even facilitating homosynaptic or spike-timing dependent forms of inhibitory plasticity.

Such a long-term form of plasticity may also be of importance to clinical drugs acting at $GABA_ARs$, given allosteric modulation of these receptors appears to be sufficient to stimulate LTP_{GABA}. Drugs such as anaesthetics and benzodiazepines are likely to enhance the occupancy of desensitized conformations as well as the open states. The enhanced phosphorylation and subsequent potentiation observed here may therefore occur during

anaesthesia, or in the brains of patients given benzodiazepines such as diazepam or zolpidem. Such a potentiation may simply form part of the initial upregulation of GABAergic signalling observed after the application of such drugs. However, treatments similar to those used here to evoke LTP_{GABA} have also been reported previously to result in benzodiazepine uncoupling, a phenomenon associated with drug tolerance (Gutiérrez et al, 2014). It is unclear if this suggests that LTP_{GABA} and benzodiazepine uncoupling are induced simultaneously or if they are alternative outcomes of similar treatments.

5.4. Conclusions

- Desensitization of $GABA_AR$s results in long-term potentiation of inhibitory synapses we have termed LTP_{GABA}
- LTP_{GABA} involves PKC-dependent phosphorylation of $\gamma2L^{S327}$
- The enhanced phosphorylation of $\gamma2L^{S327A}$ likely results from an increase in the accessibility of the consensus site to an active PKC rather than the activation of PKC by $GABA_AR$ desensitization
- Positive and negative allosteric modulators can cause LTP_{GABA} if they enhance the occupancy of the desensitized state

Chapter 6: Discussion and future directions

6.1. Summary

Desensitization is well established to regulate the amount of charge passed by $GABA_ARs$ in recombinant cell systems exposed to high concentrations of agonist for prolonged periods. Its structural basis is also now well understood. However, how the results of such experiments relate to the phasic and tonic forms of inhibitory neurotransmission in a physiological context has previously been unclear. In this project, we have demonstrated that the entry into the conformational state that underlies such macroscopic desensitization indeed regulates the levels of both tonic and phasic signalling. Entry into the desensitized state was found to limit the amount of current passed during synaptic inhibitory events but was required to maintain tonic currents. Additionally, we have discovered a novel form of inhibitory synaptic LTP that appears to be initiated by the entry of the receptors into the desensitized state.

6.2. $GABA_AR$ desensitization terminates synaptic events

Although it was previously predicted that desensitization acts to prolong inhibitory synaptic currents (Jones and Westbrook, 1995), we have demonstrated here that such results are likely to be artifacts of working at room temperature and that one important purpose of $GABA_AR$ desensitization is in fact to increase the decay rates of synaptic responses at physiological temperatures. It is not surprising that temperature alters kinetics; the deactivation of $GABA_ARs$ is known to have a Q_{10} (i.e. fold change in rate for a 10°C increase in temperature) of approximately 3 (Dixon et al, 2017). The

increase in deactivation rates between 21 °C and 37 °C was therefore to be expected. However, the temperature dependence of the phenotypes measured here was more difficult to predict, as Q_{10} values are only approximations of macroscopic behaviour and the effects of temperature are not necessarily uniformly distributed amongst the underlying microscopic rate constants.

By terminating synaptic responses, entry into the desensitized state may make the profile of the IPSP more robust to variation in the amount of GABA released. During an IPSP the postsynaptic receptors are exposed initially to high concentrations of GABA leading to a high peak open probability. However, the GABA is then rapidly removed from the synaptic cleft, resulting in receptor deactivation. If no entry into the desensitized state occurs during this period, then each deactivated receptor will immediately be available to respond to subsequent GABA applications. However, by entering into the desensitized state, they will mostly remain refractory to GABA until the response is largely over.

Additionally, desensitization likely acts as a postsynaptic mechanism of short-term depression. Although this effect was not observed in neurons during this study, as argued above this was probably due to the low frequencies of inhibitory events under the conditions recorded in. Indeed, the resensitization data from outside-out patches presented here would certainly suggest that desensitization will act as a form of short-term depression at the physiological firing rates of inhibitory interneurons.

Indeed, it has recently been reported that synaptic receptors can be desensitized by exposure to high local concentrations of GABA, resembling the result of repeated firing of interneurons, achieved by repetitive photo-uncaging of GABA (De Luca et al, 2017). This study also reported that this

desensitization spreads to adjacent synapses through the lateral diffusion of desensitized receptors from the over-stimulated inhibitory synapse. This may actually provide an alternative explanation as to why this short-term depression effect is perhaps limited at synapses. AMPA receptors have also been found to move out of the synapse when desensitized (Constals et al, 2015). However, they are subsequently replaced with non-desensitized receptors, thus preventing short-term depression from occurring (Constals et al, 2015). If such a phenomenon also occurs at inhibitory synapses, the ability of desensitization to produce short term depressions may be limited to situations where a large proportion of receptors in the membrane are simultaneously desensitized, as opposed to occurring when a single inhibitory synapse is rapidly and repetitively, activated. Therefore, whilst entry into the desensitized state affects the shape of IPSCs, it is less clear if it determines their amplitudes during basal levels of signalling.

Although a role for desensitization in terminating synaptic responses seems intuitive, as discussed above this was not actually the anticipated result. The demonstration here that desensitization acts to reduce the amount of charge transfer during synaptic events is in fact somewhat novel. This finding thus may aid in the design of drugs targeting the desensitized state by demonstrating that reducing desensitization will increase the amount of charge passed during phasic signalling, albeit only when considering direct kinetic effects.

6.3. The role of desensitization in tonic inhibition

In contrast to the results for phasic signalling, entry of receptors into the desensitized state, to some extent, seemed to be required to maintain tonic

currents. As discussed above, it is likely that this effect relates to the stabilisation of agonist-bound states by the desensitized states.

Despite this finding, desensitization can also reduce steady-state currents, and therefore tonic signalling, under some circumstances. Such a reduction of tonic currents can occur during responses to low concentrations of GABA if desensitization is enhanced, as was achieved in this study with the $\gamma2^{V262F}$ mutation. A similar effect could be achieved with an allosteric modulator that enhances desensitization, such as pregnenolone sulfate (Shen et al, 2000; Sachidanandan and Bera, 2015; Seljeset et al, 2018). Normal levels of desensitization are also known to restrain tonic currents when ambient GABA levels are elevated due to its spillover from the synaptic cleft (Bright et al, 2011).

The observation during this project that both reducing and enhancing $GABA_AR$ desensitization can reduce tonic signalling confirms this dual role and perhaps suggests that the kinetics of desensitization are in fact optimized to facilitate tonic signalling. This finding also raises a conundrum for the design of modulators that act on the desensitized state: can tonic signalling be enhanced by such a modulator, or are the kinetics of desensitization so tightly optimised that any perturbation will be detrimental? Further work, using drugs or mutations with more subtle effects on desensitization will be required to probe this.

6.4. Entry of $GABA_ARs$ into the desensitized state leads to potentiation of inhibitory synapses

One phenotype that certainly had not been previously predicted was that entry of the receptors into the desensitized state would act to potentiate

synaptic responses. This appears to be achieved not through a direct kinetic effect, but rather seems to involve the modification of receptors and perhaps an increase in their number expressed at inhibitory synapses. This potentiation required: receptor activation, desensitization, the activity of PKC, and the phosphorylation of GABA receptor γ2 subunits.

In this role, desensitization appears to be acting as a signal of receptor activation to signalling pathways in the cytoplasm. Entry into the desensitized state increases phosphorylation of $\gamma2^{S327}$ by PKC, via a mechanism acting downstream of the activation of the kinase. It is possible that this phosphorylation site is perhaps difficult to access at the synapse in the absence of GABA, and that desensitization somehow acts to reveal it to the kinase or increases the rate of phosphorylation once the kinase has bound. Such a signal of $GABA_AR$ activity to the cytoplasm will likely be of particular importance at inhibitory synapses given that the only other way in which they can influence cytoplasmic signalling is through the indirect effects of Cl^- currents on calcium signalling. This is in contrast to excitatory synapses, which have long been known to possess NMDA receptors that act as coincidence detectors for pre- and postsynaptic activity and are capable of generating calcium influxes that can directly regulate plasticity. The use of the desensitized state to identify recently active $GABA_ARs$ for phosphorylation perhaps provides inhibitory synapses with a form of regulation that would make phenomena such as synapse specificity or spike-timing dependence possible.

The enhanced desensitization required to induce LTP_{GABA} may result simply from the high frequency firing of inhibitory interneurons (Gravielle, 2018). Additionally, the observation that allosteric modulators can enhance the occupancy of the desensitized state sufficiently to trigger this form of plasticity

perhaps has important implications in the clinical use of $GABA_AR$ modulators. Etomidate was predicted to enhance the occupancy of the desensitized state simply because it enhances the opening of the receptor and entry into the desensitized state follows opening. It would therefore be predicted that many other clinically-relevant and endogenous modulators might share this property and potentially induce LTP_{GABA}.

However, desensitization was not the only requirement for successful LTP_{GABA} induction, PKC activity was also needed. Although the actions of PKC on $GABA_ARs$ have been somewhat controversial (reviewed in Nakamura et al, 2015), such a role in enhancing phasic signalling has previously been observed (Poisbeau et al 1999; Jovanovic et al, 2004; Bannai et al, 2015). Phosphorylation of $\gamma2^{S327}$ in particular seems to correlate with the synaptic clustering of $GABA_ARs$, with its dephosphorylation having been shown to result in LTD of inhibitory synapses by dispersal of $GABA_ARs$ (Muir et al, 2010; Nicholson et al, 2018; Wang et al, 2003). This would perhaps suggest that LTP_{GABA} results from the converse effect: increased phosphorylation of S327 and consequent increases in $GABA_AR$ clustering at synapses. Interestingly, glycine receptors have also been reported to undergo a PKC- and receptor activation-dependent form of clustering in spinal cord neurons (Nakahata et al, 2017). Further work will be required to establish if such increased clustering indeed occurs under the conditions reported here.

PKC might be activated by $GABA_ARs$, through their direct regulation of PLC (Nicholson et al, 2018), however this did not seem to be sufficient in this study. However, there are many other signalling pathways that are known to regulate PKC in neurons, and it is plausible that the induction of LTP_{GABA} involves a form of coincidence detection, requiring the activation of one or more of these pathways through additional forms of signalling.

One pathway well established to regulate PKC activity in neurons involves signalling through metabotropic glutamate receptors (mGluRs). These receptors form a family of GPCRs found at pre- and post-synaptic membranes that are activated by glutamate (Reviewed in Niswender and Conn, 2010). Metabotropic GluRs classed as Group I (comprising mGluR1 and 5) signal through Gq to activate PLC and therefore PKC (Niswender and Conn, 2010), and the stimulation of these receptors has indeed been observed to result in the stabilisation of $GABA_A$Rs at inhibitory synapses (Bannai et al, 2015). Additionally, mGluRs are known to be involved in the regulation of excitatory plasticity (e.g. Jones, 2017; Upreti et al, 2013; Simonyi et al, 2005; Gladding et al, 2009; Riedel et al, 2003; Bortolotto et al, 1999; Niswender and Conn, 2010).

However, PKC is also known to be under numerous other forms of regulation in the brain. The activation of classical PKCs tends to result from the activation of PLC by GPCRs or receptor tyrosine kinases (RTKs, reviewed in Kadamur and Ross, 2013). The GPCRs that may activate PKC in neurons include mGluRs, and also muscarinic acetylcholine receptors (mAChR), D1 dopamine receptors, and 5-HT_2 serotonin receptors. Indeed, some of these receptors are known to regulate $GABA_A$Rs (mAChRs – Domínguez et al, 2016; 5-HT_2Rs – Yan, 2002). Conversely RTKs in hippocampal neurons are more typically activated by neurotrophins (Skaper, 2012). BDNF in particular has previously been shown to be capable of regulating $GABA_A$Rs through not only PKC, but also other mechanisms depending on the duration of BDNF application (Jovanovic et al, 2004; Porcher et al, 2018). In addition to the DAG produced by PLC, the classical PKCs tend to be regulated by Ca^{2+}. Although Ca^{2+} will likely be released through IP_3Rs after the activation of PLC,

it is also possible that voltage-gated Ca^{2+} channels and NMDA receptors could contribute to internal Ca2+ levels.

The presence of so many potential mechanisms of PKC regulation in neurons, combined with the apparent requirement for $GABA_AR$ desensitization, suggests that the expression of LTP_{GABA} will likely be dependent on a multitude of factors. Moreover, observations that the dephosphorylation of $\gamma2^{S327}$ in response to excitatory currents results in the LTD of inhibitory synapses (Muir et al, 2010) provides an additional level of regulation. Such dephosphorylation has been shown to occur due to the activation of calcineurin by calcium influx through NMDA receptors (Muir et al, 2010). Excitatory activity will therefore likely act to oppose or reverse LTP_{GABA}.

However, it seems unlikely that this will be the only mechanism used to constrain LTP_{GABA}. The presence of homeostatic plasticity at excitatory synapses was first predicted based on the notion that the existing long-term forms of potentiation would potentially lead to positive feedback cycles of potentiation, given that they are initiated by calcium signals and subsequently lead to enhanced calcium influx via NMDA receptors. A similar phenomenon could in principle occur with LTP_{GABA}. It results in enhanced inhibition, and is downregulated after calcium influxes through NMDA receptors. However, the increased inhibition would in principle reduce such influxes and thus lead to further increases in the levels of γ2 subunit phosphorylation, therefore potentially creating a feedback cycle. Further investigation will thus be required to establish if a homeostatic mechanism exists to constrain this form of plasticity.

The molecular requirements for LTP_{GABA} thus appear to be high levels of $GABA_AR$ desensitization and PKC activity, combined with low levels of

calcineurin activity. However, further work will be required to establish how these requirements are met during physiological neuronal signalling. The $GABA_AR$ desensitization will likely result from enhanced firing of interneurons or the presence of neurosteroids and lowered excitatory input-dependent calcium influxes will likely be required to lower calcineurin activity. Conversely, the activation of PKC could result from a diverse group of signalling pathways. Uncovering which of these pathways actually contribute to the regulation of LTP_{GABA} in neurons will likely be an important future step in understanding when LTP_{GABA} might be triggered in a physiological context.

6.5. Overall conclusions regarding the effects of $GABA_AR$ desensitization on neuronal excitability

In this project, desensitization of the $GABA_ARs$ was shown to have multiple effects. On one hand it acted to limit the duration of inhibitory synaptic currents whilst concurrently and indirectly increasing their amplitude. On the other hand, it acted to enhance tonic currents, but could limit them if the desensitized state was excessively stabilised by the $\gamma2L^{V262F}$ mutation. Furthermore, desensitization could, in principle, act as a postsynaptic mechanism of short-term depression at inhibitory synapses. Whilst this particular effect was not observed in this study, this was most likely due to the low frequency of inhibitory events between cultured neurons. The blockade of excitatory currents used to isolate the inhibitory events for recording almost certainly acted to substantially reduce the firing rates of the interneurons present in the culture. Nevertheless, the data gathered from outside-out patches suggests that such a short-term depression effect would occur at physiological firing rates.

This generic picture of the roles for $GABA_A$ receptor desensitisation is further complicated by the discovery of LTP_{GABA}, which is apparently initiated by the entry of $GABA_A$Rs into the desensitized state. Given that allosteric modulators were capable of initiating this form of plasticity, it is plausible that many drugs used in the clinic could also affect its regulation. It is perhaps possible that this form of potentiation in fact already contributes to the general potentiation of inhibition by drugs such as anaesthetics and benzodiazepines, although further work will be required to establish this. It is also interesting to consider whether desensitization also has any role to play in the development of tolerance to these drugs. Indeed, treatments similar to those used here to induce LTP_{GABA} have also been observed to result in what is defined as 'benzodiazepine uncoupling' (Gravielle et al, 2005; Gutiérrez et al, 2014), a phenomenon where benzodiazepines and GABA are less able to potentiate each other's binding. It should be noted that although benzodiazepine uncoupling represents a reduction in the sensitivity of the receptors to these modulators, it does not necessarily require a general downregulation of the receptors, and in fact is often observed to occur in the absence of any change in $GABA_A$R surface levels or numbers of GABA binding sites (Gravielle et al, 2005; Gutiérrez et al, 2014; Vlainić et al, 2010). Further work will be required to establish if such uncoupling occurs concurrent with $LTP_{GABA,}$ or is simply an alternative outcome of such treatments in different circumstances.

It is thus difficult to determine with certainty what effect $GABA_A$R desensitization or its manipulation will have on the overall excitability of the cell, even before accounting for network effects. Drugs designed to target the desensitized state will therefore likely have complex effects, depending on the cells they are affecting and the activation rates of inhibitory synapses. Whilst destabilising the desensitized state might, from a naïve standpoint, have been

a tempting avenue for the design of novel sedatives or anxiolytics, the data presented here show that such a drug may reduce tonic currents or impair plasticity at inhibitory synapses. Further work on animals either engineered with these mutations or treated with such a drug affecting desensitisation will be required to establish which effects are the most dominant.

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