Characterization of α3-containing GABA$_A$ receptors modified by RNA editing

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Declaration

I, Ho Yan Cheung, 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.

(Ho Yan Cheung)

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Abstract

Editing of ribonucleic acid (RNA) is a post-transcriptional processing mechanism that increases heterogeneity of gene products. The $\alpha 3$ subunit of the $GABA_A$ receptor (Gabra3) has been shown to undergo RNA editing. This results in a change from an isoleucine (I) to a methionine (M) in the third transmembrane domain of the subunit. In the work described in this thesis I have used patch-clamp recording techniques to determine the effects of this amino acid switch on the properties of recombinant $GABA_A$ receptors. I examined both macroscopic and microscopic features of GABA-evoked currents using whole-cell, cell-attached and outside-out patch-clamp recording. Although, when expressed with β and γ subunits, both un-edited $\alpha 3(I)$ and edited $\alpha 3(M)$ subunits were functional, the edited subunit yielded lower current densities, suggesting a reduced surface expression. Analysis of current-voltage relationships showed clear voltage-dependence of whole-cell currents, with prominent outward rectification at low GABA concentrations. However, for neither $\alpha 3 \beta 2 \gamma 2L$ nor $\alpha 3 \beta 3 \gamma 2L$ receptors, was there any difference in rectification between $\alpha 3(I)$- and $\alpha 3(M)$-containing receptors. Results with a non-editable subunit suggested no confounding effect of endogenous editing. In cell-attached single-channel recordings both $\alpha 3(I)$- and $\alpha 3(M)$-containing receptors exhibited high intra-burst open probabilities and long burst lengths, with a trend toward longer burst lengths with $\alpha 3(M)$. Single-channel conductance was not affected by editing, and for both $\alpha 3(I)$- and $\alpha 3(M)$-containing receptors the current-voltage relationships were essentially linear. Rapid application of GABA to outside-out patches revealed a much slower activation of $\alpha 3(I)$-containing receptors but no marked difference in desensitization or deactivation I discuss my findings in terms of the biology of $\alpha 3$-containing $GABA_A$ receptors, the origin and postulated role(s) of $GABA_A$ receptor rectification, and the likely basis of the differences between my findings and those of other groups, which may, in part, reflect differences in the behaviour and relative prevalence of $\alpha \beta \gamma$ and $\alpha \beta$ subunit assemblies.

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List of abbreviations

Abbreviation	Meaning
5-HT$_3$	5-hydroxytryptamine
α3IA	α3-selective inverse agonist
A-to-I	adenosine-to-inosine
ACh	acetylcholine
AChBP	acetylcholine binding protein
ADAR	adenosine deaminase acting on RNA
ALS	amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	adenosine-5'-triphosphate
BZs	benzodiazepines
C	cytidine
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
Cl$^-$	chloride
[Cl$^-$]$_i$	intracellular chloride
CLC2	voltage-gated chloride channel
CNS	central nervous system
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
E_{Cl}	Cl$^-$ reversal potential
EEG	electroencephalography
E_{GABA}	reversal potential for GABA$_A$
EGFP	enhanced green fluorescent protein
EPSCs	excitatory postsynaptic currents
ER	endoplasmic reticulum
Gabra3	gene that codes for the GABA$_A$ α3 subunit
GABA	γ-aminobutyric acid
GABA$_A$R	GABA$_A$ receptor
GABA-T	GABA transaminase
GAD	glutamic acid decarboxylase

GAT	GABA transporter
GDP	guanosine diphosphate
GHK	Goldman-Hodgkin-Katz
Gly	glycine
GPCRs	G-protein-coupled receptors
GTP	guanosine triphosphate
HCC	hepatocellular carcinoma
HCO_3^-	bicarbonate
HEK	human embryonic kidney
IPSCs	inhibitory postsynaptic currents
I/M	isoleucine to methionine
I/V	current-voltage
I/V	isoleucine to valine
KCC2	potassium-chloride co-transporter
LGIC	ligand-gated ion channel
mGluR	metabotropic glutamate receptor
mIPSCs	miniature $GABA_A$ receptor-mediated synaptic currents
mRNA	messenger RNA
Na^+-K^+ pump	sodium-potassium pump
nACh	nicotinic acetylcholine
NKCC1	sodium-potassium-chloride co-transporter
NMDA	*N*-methyl-D-aspartate
nRT	thalamic reticular nucleus
PLC	phospholipase C
P_o	open probability
Q/R	glutamate to arginine
R/G	arginine to glycine
RI	rectification index
RNA	ribonucleic acid
SEM	standard error of mean
SINEs	Short Interspersed Nuclear Elements
SSADH	succinic semialdehyde dehydrogenase
STP	stiripentol

t_{crit}	critical shut time
τ_m	membrane time constant
TM	transmembrane domains
U	uracil
VDCC	voltage-dependent calcium channel
VGAT or VIAAT	vesicular GABA transporter
V_m	resting membrane potential
Y/C	tyrosine to cysteine
ZAC	zinc-activated

Chapter 1

Introduction

1.1 Signalling among neurons

1.1.1 Background

The nervous systems of vertebrates are composed of billions of electrically excitable cells arranged in complex interconnected networks. Communication between these anatomically and functionally diverse neurons is crucial to the normal functioning of the nervous system. Importantly, like other cells, neurons are isolated from their environment by a lipid bilayer – the plasma membrane. This hydrophobic membrane serves to retain cell components but also prevents the free exchange of hydrophilic or charged substances by diffusion (Hille 2001). To selectively transport substances across the plasma membrane, and allow communication with their environment and with other cells, neurons are able to package substances into membranous vesicles for transport into (endocytosis) and out of (exocytosis) the cell. Additionally, substances can translocate via membrane proteins, which act either as carriers or channels (Gouaux and Mackinnon 2005, Pavlov *et al.* 2009). Carriers bind specific solutes and undergo a series of conformational changes to transfer them across the membrane. By contrast, channels form aqueous pores that extend across the lipid bilayer allowing rapid diffusion of ions or small molecules. The passage of ionic species depends on their size and charge and can be gated or modulated by a various stimuli.

1.1.2 Membrane voltage and the action potential

Neurons take advantage of both carriers and channels to establish a difference in electrical charge across their membrane. The Na^+-K^+ pump (Na^+-K^+ ATPase) is an energy-dependent ion exchanger that transports three Na^+ ions out of the cell and two K^+ ions into the cell for each molecule of ATP hydrolysed. The K^+ ions are free to move out of the cell down their electrochemical gradient through 'leak' channels selectively permeable to K^+. This causes the inside the neuron to become more negative than the outside (reference or ground potential, 0 mV) (Koester 2000). For most neurons the 'resting' membrane potential is typically around −70 mV. By increasing (or decreasing) the permeability of the membrane to different ions, the opening (or closing) of voltage-gated and ligand-gated channels can rapidly change the membrane potential. It is these changes in membrane potential that are critical for normal signaling in excitable cells.

A key feature of neurons is their ability to generate action potentials, *via* the regenerative activation of voltage-sensitive currents (Bean 2007). These are rapid, all-or-none fluctuations in membrane potential that occur when depolarization of the membrane passes a critical threshold, typically at around −40 mV, triggering the opening of voltage-gated Na^+ channels. This often occurs first at the axon initial segment. Entry of Na^+ ions causes further depolarization and further opening of Na^+ channels. Eventually, Na^+ channels inactivate and voltage-gated K^+ channels activate, causing the membrane potential to repolarize. Action potentials propagate along the axon, toward the axon terminals where specialized contacts, termed 'synapses', are formed (usually with the dendrites) of target neurons.

1.1.3 Chemical synaptic transmission

The term synapse was first employed by the physiologist Charles Sherrington, when he proposed that if neurons were independent elements, as described by Santiago Ramón y Cajal, then information must be passed between them at 'junctions' (see (Bennett 1999). It is now recognised that,

for the most part, this information is conveyed chemically, in a way that allows rapid communication between neurons. Chemical synapses consist of a presynaptic structure packed with membranous vesicles containing neurotransmitter. These vesicles sit close to the presynaptic membrane. Action potential-mediated depolarisation produces Ca^{2+} entry via voltage-gated channels, causing vesicles to fuse with the presynaptic membrane, releasing neurotransmitter into the synaptic cleft (Delaney 2009, Kochubey *et al.* 2011).

Transmitter release into, and diffusion out of, the synaptic cleft (the ~20 nm gap between the pre- and postsynaptic membrane specialisations) is very rapid, and the transmitter time course in the synaptic cleft is typically very brief (<<1 ms) (Clements 1996, Overstreet *et al.* 2002, Rusakov *et al.* 2011). Nevertheless, neurotransmitter receptors in the postsynaptic membrane detect this brief change in neurotransmitter concentration and signal to the postsynaptic neuron. In the case of rapid synaptic communication, the postsynaptic receptors are ligand-gated ion channels, whose activation causes short-lived changes in the conductance of the postsynaptic membrane. These conductance changes generate current flow. Given the time course of the concentration of transmitter in the synaptic cleft and the kinetic properties of the postsynaptic receptors, synaptic currents typically show a rapid rising phase (10-90% rise time ~200 µs), and a slower multi-exponential decay.

1.1.4 Excitation and inhibition

Binding of a neurotransmitter molecule to a receptor triggers a postsynaptic response specific for that receptor. Put very simply, excitatory postsynaptic currents (EPSCs) and inhibitory postsynaptic currents (IPSCs) bring about changes in membrane voltage that act to increase (excitatory transmission) or decrease (inhibitory transmission) the probability of an action potential firing in the postsynaptic neuron. The difference between excitatory and inhibitory transmission is a consequence of differences in the reversal

potential E of the ionic species involved. The current (I_{syn}) generated by receptors at a synapse can be written as

$$I_{syn} = g_{syn}(t) \, [V(t) - E_{syn}]$$

where V is the membrane potential, g_{syn} the synaptic conductance and t time. The difference between the resting membrane potential and the reversal potential of the synaptic current (E_{syn}) can have either a positive or a negative sign. If receptor activation results in the postsynaptic membrane becoming more electrically positive (depolarized), this is an excitatory postsynaptic potential (EPSP). If the postsynaptic cell becomes more negative (hyperpolarized), this is an inhibitory postsynaptic potential (IPSP). Unlike the rapid conductance changes, EPSPs and IPSPs decay slowly (many ms), with durations that are determined largely by the membrane time constant, τ_m (Hille 2001). Excitation and inhibition do not depend on the properties of neurotransmitter molecule itself, or on those of the receptor, but on the transmembrane gradient of the permeant ions. Receptors that allow the entry of sodium or calcium ions are excitatory and produce a depolarization of the postsynaptic membrane, whereas receptors coupled to chloride or potassium channels are typically inhibitory and produce a hyperpolarization of the postsynaptic membrane. Importantly, shifts in the reversal potential for certain ions can lead to changes in the sign of a synapse (see section 1.3.1).

The amino acids glutamate and GABA (γ-aminobutyric acid) are among the most abundant of all neurotransmitters present within the central nervous system (CNS). The predominant mechanism for fast excitatory synaptic transmission in the mammalian CNS is the depolarisation of the postsynaptic membrane brought about by the activation of various classes of ionotropic glutamate receptors: NMDA (*N*-methyl-D-aspartate), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and kainate receptors (Traynelis *et al.* 2010). These receptors gate channels that produce mixed cation conductances, with reversal potentials of ~0 mV. The reversal potential is the potential at which the net flow of current is zero; an increase in the conductance underlying a given current will move membrane potential closer

to the reversal potential for that current. Activation of excitatory synaptic conductances will move the postsynaptic membrane potential towards and above spike threshold, triggering action potentials. Conversely, GABA-mediated inhibitory synaptic transmission principally involves the activation of conductances that reverse at membrane potentials that may be more hyperpolarized than resting membrane potential and certainly more hyperpolarized than spike threshold. Thus, activation of inhibitory synapses reduces the likelihood of action potential generation.

1.1.5 Excitation/inhibition (E/I) balance

The process by which excitatory and inhibitory synaptic input shapes action potential firing is termed synaptic integration. On a broad scale, the CNS operates with a finely tuned balance between excitatory and inhibitory signalling (Eichler and Meier 2008, Fritschy 2008). GABA and glutamate serve to regulate the excitability of virtually all neurons in the brain and have been implicated as important mediators of many critical physiological as well as pathophysiological events that underlie brain function and/or dysfunction (Eichler and Meier 2008). Information transfer in the brain requires homeostatic control of neuronal excitability and therefore a functional balance between excitatory and inhibitory systems is established during development and maintained throughout life (Tretter and Moss 2008).

The balance between excitation and inhibition in the mature nervous system is mainly dependent on the generally opposing effects of glutamate (acting on ionotropic AMPA, NMDA, and kainate receptors and metabotropic mGluR receptors) and GABA (acting on ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors; see section 1.2.3). The striking heterogeneity of $GABA_A$ receptors and the distinct distribution and pharmacology of specific subtypes (see section 1.3.3) play an important role in orchestrating neuronal inhibition and therefore contribute to the maintenance of an appropriate E/I balance. This is because the functional diversity *per se* can translate into higher-level network properties, which determine the behaviour of interconnected neurons. For

example, as will be elaborated in a later section, specific receptor subtypes have been associated with different forms of inhibition with different functional effects (Mody and Pearce 2004). Dysregulation of the E/I balance is associated with a number of nervous system disorders such as epilepsy, mental retardation and autism (Eichler and Meier 2008), as well as disturbance in homeostatic plasticity resulting from either insufficient or excessive compensatory mechanisms in response to changes in network activity.

1.2 GABAergic neurotransmission

1.2.1 Background

GABA, γ-aminobutyric acid, is a non-protein amino acid. It is an ω-amino acid, a molecule with an unsubstituted amino group and an acidic (carboxylic) group separated by an unbranched carbon chain. GABA is a four-carbon carboxylic acid with an amino group at the γ (4 position) carbon – hence γ-aminobutyric acid or 4-aminobutanoic acid. GABA was identified in mammalian brain in 1950 (Awapara *et al*. 1950, Roberts and Frankel 1950, Udenfriend 1950). In 1953, Florey observed that an unknown compound 'factor I' (where 'I' represented inhibitory action on neuronal activity) extracted from horse brain inhibited the crayfish stretch receptor when applied exogenously (Florey 1954). Florey later demonstrated that this same factor inhibited the patellar reflex in cats and thus extended the action of this factor to vertebrates (Florey and McLennan 1955). By purification of the active factor and examination of the infrared spectrum the pure crystals, Factor I was identified as γ-aminobutyric acid, and chemically synthesized GABA was shown to similarly inhibit the crayfish stretch receptor (Bazemore *et al*. 1956). These observations prompted Florey to propose that GABA was acting as an inhibitory neurotransmitter in the brain. By the early 1970s GABA had been shown to satisfy all of the criteria of a neurotransmitter and was also widely accepted as the principal inhibitory neurotransmitter in the

adult mammalian CNS (Curtis and Watkins 1960, Krnjevic and Schwartz 1967); for review (Bowery and Smart 2006).

GABA is found in many types of interneurons. In most brain areas, inhibitory synapses are formed by local circuit GABAergic interneurons, a diverse population of cells that perform a multitude of functions, which are crucial in establishing the functional balance, complexity, and computational architecture of neuronal circuits (DeFelipe *et al.* 2013, Freund and Buzsaki 1996, Somogyi *et al.* 1998).

GABA-mediated inhibition regulates synaptic integration, probability and timing of action potential generation. Furthermore, interneurons generate and maintain network oscillations, which provide temporal structures for orchestrating activities of neuronal populations (Jonas *et al.* 2004, Klausberger and Somogyi 2008, Whittington and Traub 2003). The rich variety and fine details of inhibition are achieved by diverse interneuron cell types, which display distinct morphology, physiological properties, connectivity patterns, and gene expression profiles (Somogyi and Klausberger 2005). The biophysical properties of different cell types are optimized for generating a rich array of firing patterns and synaptic dynamics to precisely control electrical signalling in neural networks. Thus, understanding the organization, function, and plasticity of GABAergic interneurons is key to discover general principles that govern how information is processed by neural circuits.

1.2.2 GABA synthesis, release and uptake

In the mammalian brain, much of the glutamate and GABA used as neurotransmitter is derived from glial storage pools of glutamine (Bak *et al.* 2006). GABA is formed via a metabolic pathway called the 'GABA shunt'. The initial step in this pathway utilizes α-ketoglutarate formed from glucose metabolism via the Krebs cycle. α-Ketoglutarate is then transaminated by α-oxoglutarate transaminase (GABA transaminase; GABA-T) and other transaminases to form glutamate, the immediate precursor of GABA. GABA

is then finally synthesized from glutamate by an enzyme L-glutamic acid decarboxylase (GAD), which decarboxylates glutamate to GABA. Like most small molecule neurotransmitters, GABA is loaded and stored in synaptic vesicles and is released to the synaptic cleft by calcium (Ca^{2+})-dependent exocytosis upon depolarization of the presynaptic membrane. Loading of vesicles depends on the action of a vesicular GABA transporter (VGAT, also known as VIAAT for vesicular inhibitory amino acid transporter). Non-vesicular forms of GABA secretion (for example, by reversed transporter action, see below) have also been described which are Ca^{2+}-independent (Belhage *et al.* 1993, Pin and Bockaert 1989). In addition, release of GABA from glial cells has been suggested to occur by several mechanisms *(Lee et al.* 2011), including direct permeation of GABA through the Best1 anion channel in response to elevation of intracellular Ca^{2+} and changes in cell volume (Lee *et al.* 2010).

Following conventional release of vesicular GABA into the synaptic cleft, GABA interacts with its receptors before the signals are terminated by the rapid diffusion of GABA away from the cleft and its subsequent uptake into presynaptic nerve terminals and/or surrounding glial cells (Schousboe and Waagepetersen 2007). The reuptake of GABA occurs via highly specific high-affinity transmembrane transporters – GABA transporter (GAT)-1, betaine/GABA transporter-1 (BGT-1), GAT-2, and GAT-3 – which are members of a large family of sodium (Na^+)-dependent neurotransmitter transporters (Borden 1996, Guastella *et al.* 1990). In both neurons and glia, GABA is finally metabolized by GABA-T, which catalyses the conversion of GABA and 2-oxoglutarate into succinic semialdehyde. Succinic semialdehyde is oxidized by succinic semialdehyde dehydrogenase (SSADH) to succinic acid, which then re-enters the Krebs cycle (**Figure 1.1**).
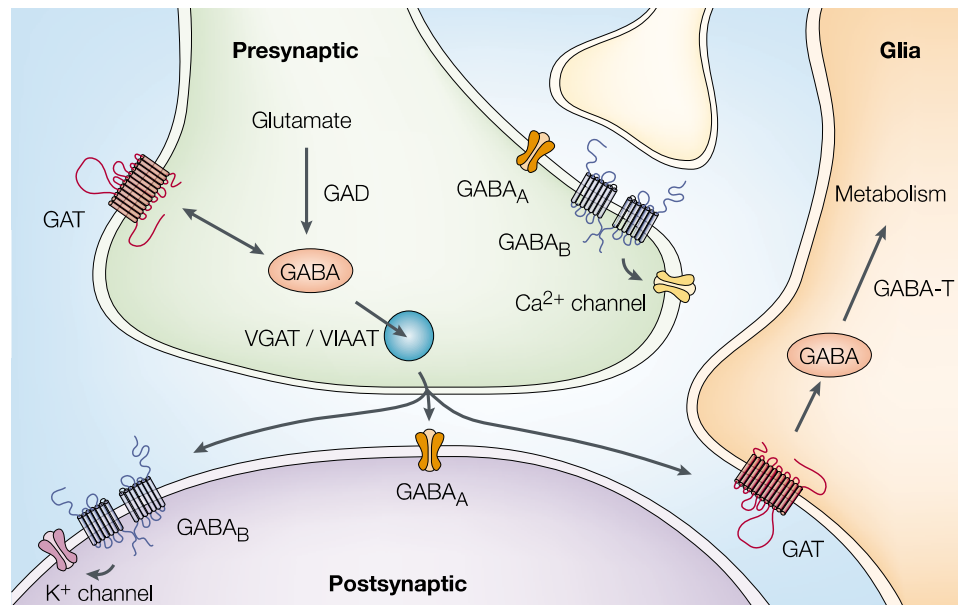
Figure 1.1 Simplified overview of a GABAergic synapse.
GABA is synthesized in inhibitory neurons from glutamate by the enzyme glutamic acid decarboxylase (GAD), and is transported into vesicles by a vesicular neurotransmitter transporter (VGAT or VIAAT). GABA can be released either vesicularly or non-vesicularly (by reversal of transporters). GABA receptors are located at pre- and postsynaptic sites. Reuptake of GABA by surrounding neurons and glia occurs via GABA transporters (GAT). Subsequently, GABA is metabolized by a transamination reaction, which is catalysed by GABA transaminase (GABA-T). Adapted from (Owens and Kriegstein 2002).

1.2.3 Actions of GABA

As indicated above, GABA acts on receptors that are either ionotropic (i.e., their activation results directly in enhanced membrane ion conductance) or metabotropic (i.e., their activation results indirectly in altered ion channel function or altered intracellular levels of second messengers, following activation of G-proteins). These two very different classes of receptors are the ionotropic $GABA_A$ (Barnard *et al.* 1998, Olsen and Sieghart 2008) and the metabotropic $GABA_B$ receptors (Bowery *et al.* 2002). This classification of vertebrate GABA receptors was initially pharmacological – based on the actions of bicuculline, a compound shown to block GABA responses in recordings from cat spinal neurons (Curtis *et al.* 1970). The term '$GABA_A$' was introduced by Hill and Bowery in 1981 (Hill and Bowery 1981) in order to distinguish the 'classical' bicuculline-sensitive ionotropic GABA receptor of vertebrates neurons (Bowery and Brown 1974, Curtis *et al.* 1970) from the newly identified bicuculline-insensitive receptor activated by (*R*)-baclofen, that was termed '$GABA_B$' (Bowery *et al.* 1980).

The structure and function of $GABA_A$ receptors is described in the following pages (see section 1.3). $GABA_B$ receptors are functionally distinct receptors, which are coupled to G proteins (pertussis toxin-sensitive $G_{i/o}$ family). The original cloning of a $GABA_B$ receptor (Kaupmann *et al.* 1997), showed it to be a 7-transmembrane domain protein similar to mGluRs. It is now known that there are two distinct subunits ($GABA_{B1}$ and $GABA_{B2}$) both of which are required for receptor function (Jones *et al.* 1998, Kaupmann *et al.* 1998, White *et al.* 1998), with $GABA_{B1}$ involved in agonist activation and $GABA_{B2}$ involved in membrane targeting (Calver *et al.* 2001, Robbins *et al.* 2001). When GABA binds to the $GABA_B$ receptor it causes a conformational change, which allows the receptor to act as a guanine nucleotide exchange factor. The receptor can then activate an associated G-protein by exchanging its bound GDP (guanosine diphosphate) for a GTP (guanosine triphosphate). The G-protein's α subunit, together with the bound GTP, can then dissociate from the β and γ subunits. The most well established $GABA_B$ receptor actions are mediated via the $\beta\gamma$ dimer of the activated G protein. At

presynaptic sites, voltage-sensitive P/Q- and N-type Ca^{2+} channels are the predominant effectors of $GABA_B$ receptors. $GABA_B$ receptor activated $G\beta\gamma$ inhibits Ca^{2+} channel activity by slowing their current activation kinetics, which eventually results in reduced transmitter release (Dunlap and Fischbach 1981, Tatebayashi and Ogata 1992). Postsynaptically, $GABA_B$ receptor effects are mainly mediated by the family of G protein-gated inwardly rectifying K^+ channels (GIRK1-4). $G\beta\gamma$ directly binds to GIRK channels and activates them, resulting in an outward K^+ current (Gahwiler and Brown 1985, Newberry and Nicoll 1985). This hyperpolarizes the membrane and consequently inhibits neuronal activity.

This differential action is determined, in part, by the two different isoforms of $GABA_{B1}$ ($GABA_{B1a}$ and $GABA_{B1b}$). $GABA_{B1a}$ appears to target $GABA_B$ receptors to presynaptic terminals of excitatory synapses, where it modulates glutamate release, while $GABA_{B1b}$ is preferentially located in postsynaptic spines and contributes to the coupling to K^+ channels (Vigot *et al.* 2006). $GABA_{B1b}$ also underlies the inhibition voltage-gated Ca^{2+} channels in dendrites (Chalifoux and Carter 2011, Perez-Garci *et al.* 2006). In addition, activation of $GABA_B$ receptors also modulates cAMP production, by inhibiting adenylate cyclase activity (Hill 1985), leading to a wide range of actions on ion channels and proteins that are targets of the cAMP-dependent kinase (protein kinase A or C) (Bray and Mynlieff 2011, Kubota *et al.* 2003).

1.3 GABA$_A$ receptors

1.3.1 Ionic basis of action

Activation of GABA$_A$ receptors leads to increase in chloride (Cl$^-$) and bicarbonate (HCO$_3^-$) ion conductance. As the ion channels are ~4-5 times more permeable to Cl$^-$ than HCO$_3^-$ (Bormann 1988, Farrant and Kaila 2007), the reversal potential for GABA$_A$ receptor-mediated currents (E_{GABA}) is largely determined by the Cl$^-$ distribution. In turn, the effect of GABA$_A$ receptor activation is dictated (as indicated above) by the difference between the resting membrane potential and E_{GABA}. In mammalian nerve cells, Cl$^-$ is distributed unevenly across the neuronal membrane with the intracellular Cl$^-$ concentration typically being about 10-20 times lower than the extracellular Cl$^-$ concentration of approximately 140 mM (the mechanisms responsible for establishing this gradient, and its variations, are discussed below). Thus, E_{GABA} is typically slightly negative to the resting membrane voltage. Accordingly, an increase in Cl$^-$ ion conductance following GABA$_A$ receptor activation results in Cl$^-$ entry into the cell and hyperpolarization of the neuronal membrane. This leads to an increase in the depolarization required in order to generate an action potential. Irrespective of effects on membrane voltage, opening of GABA$_A$ receptor ion channels also leads to a local increase in membrane conductance that causes 'shunting' inhibition, reducing the amplitude and duration of the voltage change produced by excitatory inputs.

The situation just described is typical of many but not all mature neurons and also differs during development. This reflects differences in the regulation of intracellular chloride ([Cl$^-$]$_i$) by chloride co-transporters, exchangers, and channels. In particular, two cation-chloride co-transporters play pivotal roles in developmental changes in [Cl$^-$]$_i$ (Payne *et al.* 2003). The sodium-potassium-chloride co-transporter NKCC1 (a Cl$^-$ accumulator) is expressed in virtually all mammalian cells and is thought to play a housekeeping role in cell volume homeostasis and the common control of cytosolic ion content. NKCC1 does not use ATP but operates using electrochemical gradient for Na$^+$ and K$^+$ produced by Na$^+$-K$^+$-ATPase. In contrast, the potassium-chloride

co-transporter KCC2 is the principal transporter responsible for Cl^- extrusion from neurons. KCC2 extrudes K^+ and Cl^- using the electrochemical gradient for K^+.

At early stages of development, the Cl^- reversal potential (E_{Cl}) is more positive than the resting membrane potential (V_m) and $GABA_A$ receptor activation depolarizes the cell membrane, even causing overt excitation. This reflects the immature pattern of NKCC1 and KCC2 expression. NKCC1 is expressed relatively early in neurons (before KCC2), and this leads to a relative elevation of intracellular Cl^- (Yamada *et al.* 2004). Later in development, GABA becomes inhibitory due to the increasing expression of KCC2, leading to a negative shift in E_{Cl} (Akerman and Cline 2006, Fiumelli and Woodin 2007, Lee *et al.* 2005, Rivera *et al.* 1999) (**Figure 1.2**).
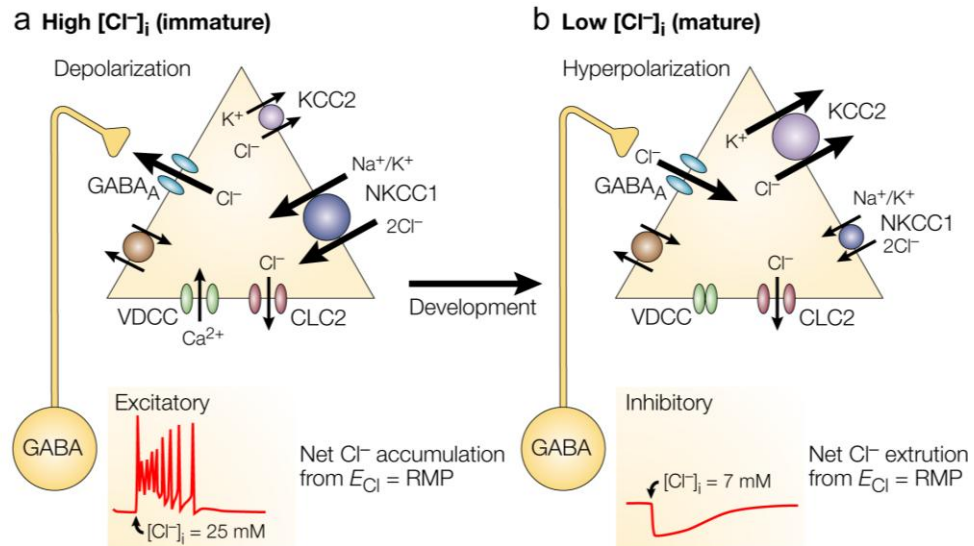
Figure 1.2 Schematic diagram showing the changes in expression of co-transporters in developing neurons.
a) NKCC1 expression predominates in immature neurons, in which the intracellular concentration of chloride ($[Cl^-]_i$) is relatively high. **b)** KCC2 expression predominates in mature neurons. Note that the activation of $GABA_A$ receptors generates an efflux of chloride leads to an excitation of immature neurons, whereas an influx of chloride causes an inhibition in adult neurons. CLC2, voltage-gated chloride channel 2; E_{Cl}, chloride reversal potential; RMP, resting membrane potential (V_{rest}); VDCC, voltage-dependent calcium channel. Adapted from (Ben-Ari 2002).

1.3.2 The GABA$_A$ receptor is a member of the ligand-gated ion channel (LGIC) superfamily

Although the difficulties of crystallizing these multi-subunit membrane proteins, have, to date, precluded the determination of their structure at atomic resolution, a great deal is known about GABA$_A$ receptors at the molecular level. In 1987, Schofield and colleagues first reported the identification and sequencing of cDNAs for the α and β subunits of the GABA$_A$ receptor (Schofield *et al.* 1987). These deduced amino acid sequences indicated that each subunit was approximately 50-60 kDa in size and had four α-helical hydrophobic membrane-spanning sequences of approximately 20-30 amino acids. Sequence similarity and conserved sequence motifs, including a Cys-disulfide separated by 13 residues in the *N*-terminus domain, led to the recognition of the ligand-gated ion channel (LGIC) superfamily or 'Cys-loop' superfamily (Ortells and Lunt 1995, Stephenson 1995). Vertebrate members of this family include serotonin (5-HT$_3$), acetylcholine (nicotinic ACh), glycine (Gly), γ-aminobutyric acid (GABA$_A$) and zinc-activated (ZAC) receptors. Cys-loop receptors gated by various neurotransmitters are also found in invertebrates, and include, for example, EXP-1 (Beg and Jorgensen 2003), MOD-1 (Ranganathan *et al.* 2000), pHCl (Schnizler *et al.* 2005), RDL (Ffrench-Constant *et al.* 1993) and GluCl (Vassilatis *et al.* 1997). Related proteins have also been identified in bacteria (Bocquet *et al.* 2007, Tasneem *et al.* 2005).

Models of predicted GABA$_A$ receptor structure have been based heavily on the electron microscopic studies of the nACh receptor isolated from the ray *Torpedo marmorata* (Unwin 2005) and crystal structures of the secreted acetylcholine binding protein (AChBP) from *Lymnaea stagnalis*, a homolog of the ligand-binding domain of the nAChR (Brejc *et al.* 2001). Important comparisons have also been drawn with crystal structures of prokaryotic pLGICs from the bacteria *Erwinia chrysanthemi* (ELIC) (Hilf and Dutzler 2008) and *Gloebacter violaceus* (GLIC) (Hilf and Dutzler 2009), and, most recently, with that of GluCl from *Caenorhabditis elegans* (Hibbs and Gouaux 2011).

1.3.3 Subunit structure and diversity

GABA$_A$ receptors are heteropentameric assemblies of subunits. Nineteen different mammalian GABA$_A$ subunit genes have been identified, and these are divided into subfamilies according to their amino acid sequence similarities (α1-6, β1-3, γ1-3, δ, ϵ, θ, π, ρ1-3) (Barnard *et al.* 1998, Fritschy and Brunig 2003, Simon *et al.* 2004). Although the individual subunits (50-60 kDa) have varying degrees of sequence identity they conform to the same tertiary architecture. This consists of a transmembrane domain with four membrane-spanning α-helices (TM1-TM4), a large extracellular N-terminal domain, a large intracellular domain between TM3 and TM4 and a short extracellular C-terminal portion (**Figure 1.3**). The intracellular loop between TM3 and TM4 contributes most of the cytoplasmic domain of these receptors and includes multiple interaction sites for putative trafficking and postsynaptic scaffold proteins as well as phosphorylation sites for diverse serine/threonine and tyrosine kinases (Smart 1997). The large extracellular domain of each pentameric receptor contains the binding site for GABA at the interfaces between adjacent α and β subunits. Binding of two GABA molecules triggers a conformational change, which results in the opening of a gate in the transmembrane region, which permits ions to flow through the channel (Goldschen-Ohm *et al.* 2011, Horenstein *et al.* 2001). For recent reviews of GABA$_A$ and Cys-loop receptor structure and function see (Cederholm *et al.* 2009, Miller and Smart 2010, Sine *et al.* 2010, Smart and Paoletti 2012, Thompson *et al.* 2010).
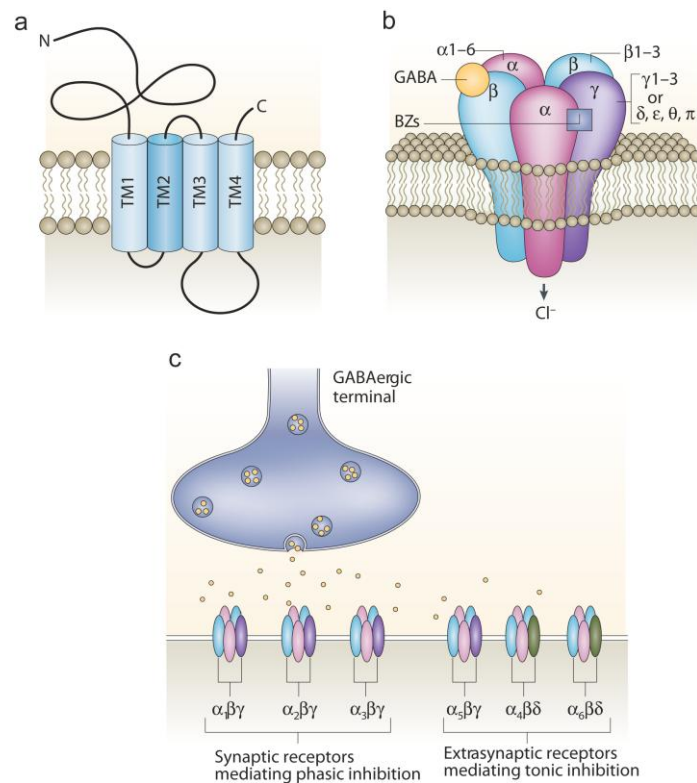
Figure 1.3 GABA$_A$ receptor structure and neuronal localization
a) GABA$_A$ receptor subunits consist of four hydrophobic transmembrane domains (TM1–4), with TM2 lining the pore of the channel. The large extracellular amino terminus is the site of GABA binding, and also contains binding sites for benzodiazepines (BZs). Each receptor subunit also contains a large intracellular domain between TM3 and TM4 that is the site for various protein interactions as well as for post-translational modifications that modulate receptor activity. **b**) Five subunits from seven subunit subfamilies (α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3) assemble to form heteropentameric Cl$^-$-permeable channels. Despite the extensive heterogeneity of the GABA$_A$R subunits, most GABA$_A$Rs expressed in the brain consist of two α subunits, two β subunits and one γ subunit; the γ subunit can be replaced by δ, ε, θ or π. Binding of the neurotransmitter GABA occurs at the interface between the α and β subunits and triggers the opening of the channel. BZ binding is also found between the α(1, 2, 3 or 5) and γ subunits and potentiates GABA-induced Cl$^-$ flux. **c**) GABA$_A$ receptors composed of α(1–3) subunits together with β and γ subunits are primarily localized at synapses, whereas α5$\beta\gamma$ receptors are located largely at extrasynaptic sites. Both these types of GABA$_A$ receptors are BZ sensitive. By contrast, receptors composed of α(4 or 6)$\beta\delta$ are BZ insensitive and localized at extrasynaptic sites. Adapted from (Jacob *et al.* 2008).

The multiplicity of $GABA_A$ receptor subunits, and their differential distribution (Kralic *et al.* 2006, Laurie *et al.* 1992b, Pirker *et al.* 2000, Sinkkonen *et al.* 2001, Wisden *et al.* 1992), suggests a large number of possible subunit combinations, and thus receptor subtype diversity. Importantly, studies of $GABA_A$ receptors expressed in *Xenopus* oocytes or cultured mammalian cell lines have demonstrated that different subunit combinations confer distinct physiological and pharmacological properties. Following the initial report of the cloning and expression of α and β subunits, it became clear that co-expression of these subunits reproduced many, but not all, of the properties of native $GABA_A$ receptors (Levitan *et al.* 1988). The notable omission was a reproducible response to benzodiazepines. This was resolved when it was found that co-expression of another subunit – the γ – was necessary for the potentiation of GABA responses by benzodiazepines (Boileau *et al.* 2002a, Pritchett *et al.* 1989). Indeed, receptors composed of $\alpha1\beta2\gamma2$ subunits are acknowledged as being the most abundant $GABA_A$ receptors in the brain (Mohler 2006, Whiting 2003), with a stoichiometry of two α subunits, two β subunits and a γ subunit (Backus *et al.* 1993).

Receptor diversity arises with the inclusion of different α, β or γ subunits, the combination of two different isoforms of the α or β subunit, or the replacement of γ subunit by the δ subunit (Barnard *et al.* 1998, Olsen and Sieghart 2008, Whiting 2003) (**Figure 1.3**). Alternative splicing further increases subunit diversity. For example, the $\gamma2$ subunit exists in two forms – $\gamma2$short ($\gamma2S$) and $\gamma2$long ($\gamma2L$) (Simon *et al.* 2004). Evidence of native receptors containing the more minor subunits – $\gamma1$, $\gamma3$, ε, θ or π – is less conclusive (see (Olsen and Sieghart 2008) for discussion), although a number of more recent studies have described such receptors (e.g. (Belujon *et al.* 2009, Esmaeili *et al.* 2009). The ρ subunits are unusual in forming homomeric assemblies with properties typical of so-called 'GABA$_C$' receptors (Johnston 2002) and are found primarily in the retina.

The subunit composition determines the GABA sensitivity and pharmacological properties of the $GABA_A$ receptor (Boileau *et al.* 2002b,

Hevers and Luddens 1998, Mohler 2006, Sieghart 1995, Smith and Olsen 1995). The functional heterogeneity of $GABA_A$ receptors is clearly demonstrated in the hippocampus, for example, where low affinity GABA responses are more strongly affected by benzodiazepines as compared with cells exhibiting high affinity responses (Schonrock and Bormann 1993).

Aside from GABA and its agonists (e.g. muscimol) or antagonist (e.g. bicuculline), $GABA_A$ receptors can be modulated by a great variety of clinically and pharmacologically important drugs including benzodiazepines, barbiturates, neurosteroids and anaesthetics (Johnston 1996, Johnston 2005, Alexander *et al.* 2011). Benzodiazepines and barbiturates are examples of widely used therapeutic agents that act as positive allosteric modulators at $GABA_A$ receptors. On the other hand, agents that reduce the action of GABA on $GABA_A$ receptors are known as negative allosteric modulators (inverse agonists); they have the opposite action to those of the classical benzodiazepines. A list of agents acting on $GABA_A$ receptors are shown in **Table 1.1**.

Mutation studies indicate the GABA sensitivity is determined by both the α and β subunits (Macdonald and Olsen 1994, Mihic *et al.* 1995). For example, a point mutation in the rat $\alpha 1$ subunit, in the putative N-terminal extracellular domain produced a marked decrease in agonist and antagonist affinities when co-expressed with $\beta 2$ and $\gamma 2$ subunits (Sigel *et al.* 1992). Two other regions in the $\beta 2$ subunit were also shown to affect functional activation by GABA. Mutations in the rat $\beta 2$ subunit between the disulfide bridge and TM1 reduced the binding affinities of GABA agonists and antagonists at the GABA site, whereas equivalent mutations in the α or γ subunits had minor effects (Amin and Weiss 1993). The mutated receptors were still able to be activated by pentobarbital, suggesting that there was no difference in the channel structure. Similarly, the TM2 determines the selectivity of β subunits for etomidate (Belelli *et al.* 1997), and a knock-in mouse for $\beta 2N265M$ shows reduced sensitivity to the anaesthetic effects of etomidate and propofol (Jurd *et al.* 2003).

Table 1.1 Agents acting on GABA$_A$ receptors. Adapted from (Alexander *et al.* 2011)

Antagonists	
Competitive	Bicuculine, gabazine (SR95531), TPMPA, *cis*- and *trans*-3-ACPBPA, Aza-THIP
Selective antagonists (at BZ site)	Flumazenil, ZK93426, L838417
Agonists	GABA, muscimol, isoguvacine, THIP, piperidine-4-sulphonic acid, isonipecotic acid, ($\pm$)-*cis*-2-CAMP, 5-MeIAA
Positive allosteric modulators	Diazepam, flunitrazepam, bretazenil, ocinaplon, L838417, Ro154513, TP003, TPA023
Inverse agonists	DMCM, Ro194603, α3IA, L655708, RY024, α5IA, MRK016, Ro4938581

1.3.4 Regulation of GABA_A receptor assembly

The fidelity of synaptic function is dependent on (i) the expression of the appropriate neurotransmitter receptor subtype at the right point in development and (ii) the targeting and trafficking of these defined receptors to the appropriate subcellular compartment.

As with other LGICs, GABA$_A$ receptor subunits are assembled in the ER. Chaperone molecules such as calnexin, BiP (immunoglobulin heavy-chain binding protein) and protein-disulfide isomerise monitor the fidelity of protein folding and assembly within the ER. On the other side of the membrane, the masking of ER retention signals during assembly ensures the cytoplasmic fidelity and transport competence of receptors. Receptor biogenesis is facilitated by the use of assembly signals that direct specific subunit interactions. It is mediated by defined motifs within the respective extracellular N-terminal domains that ensure that the appropriate subunits assemble in the correct orientation (Bollan *et al.* 2003a, Bollan *et al.* 2003b, Klausberger *et al.* 2001). Point mutations within these extraceullular domains, as reported for certain inherited forms of epilepsy, result in impaired cell-surface trafficking and/or subunit assembly, highlighting the importance of quality control mechanisms in the expression of the requisite number of assembled functional neurotransmitter receptors (Mizielinska *et al.* 2006). Unassembled subunits fail to gain transport competence and are retained in the ER, where they are degraded.

While in the ER, GABA$_A$ receptor subunits also undergo typical protein modifications, including the early stages of N-linked glycosylation (Connolly *et al.* 1996). Properly folded and assembled subunits proceed to the Glogi apparatus, where they undergo further modification such as palmitoylation and glycan trimming (Keller *et al.* 2004). With the assistance of multiple GABA$_A$ receptor-associated proteins, receptors are then trafficked to the neuronal surface. They may be inserted directly into their final subcellular location (i.e. post-, peri-, or extrasynaptic), or they may diffuse to their final location after membrane insertion (Arancibia-Carcamo and Kittler 2009,

Bogdanov *et al.* 2006, Thomson and Jovanovic 2010). Finally, $GABA_A$ receptors undergo constitutive and activity-dependent endocytosis (both clathrin-dependent and -independent), after which they are recycled to the cell surface or targeted for lysosomal degradation.

After termporal and spatial regulation of subunit expression, the functional expression of $GABA_A$ receptors at the cell surface is first controlled at the level of assembly of subunits into heteropentameric complexes. Indeed, several studies have indicated that, though all subunit combinations can form oligomers, only a subset can form pentamers (Sarto-Jackson and Sieghart 2008). This is a key distinction because pentamers are trafficked to the cell surface, but oligomers of lower molecular weight are retained in the ER and subsequently degraded. Connolly *et al.* demonstrated that forced expression of subunits in heterologous cells can lead to homomeric assemblies and complexes between α and γ or β and γ subunits that are, however, in most cases retained in the ER (Connolly *et al.* 1996). By contrast, coexpression of α and β subunits in heterologous cells resulted in formation of functional receptors that can reach the surface. It has been suggested that $\alpha\beta$ receptors may exist naturally in small numbers and contribute to tonic inhibition of neurons (Brickley *et al.* 1999). However, when α, β and $\gamma2$ subunits are coexpressed the formation of receptors containing all three types of subunits is strongly favoured over receptors composed of α and β subunits alone (Angelotti and Macdonald 1993). The fundamental role of α and β subunits in the assembly of $GABA_A$ receptors is further revealed by data from knock-out mice, which indicate that deletion of α or β subunits results in loss of corresponding receptors (Homanics *et al.* 1997, Kralic *et al.* 2002, Krasowski *et al.* 1998). By contrast, deletion of the $\gamma2$ subunit results in only a modest reduction of GABA binding sites and is therefore largely dispensable for assembly of α and β subunits (Gunther *et al.* 1995).

Importantly, some disease-causing mutations appear to reduce surface expression and function by disruption the process of oligomerization (Benke *et al.* 2004, Duggan *et al.* 1991, Macdonald and Kang 2009, Mizielinska *et al.* 2006).

Overall, these trafficking events need to be precisely coordinated to ensure an appropriate number of receptors in the plasma membrane for signalling. Moreover, regulation of each of the different trafficking steps permits adjustment of the number of cell surface receptors, and thus signalling strength, according to the physiological requirements.

1.3.5 Receptor heterogeneity and diversity of inhibitory signalling

$GABA_A$ receptor subunit composition differs not only between neuronal populations, but also at different subcellular locations in individual cells. $GABA_A$ receptors clustered at synapses commonly contain α1-3, β2-3, and γ2 subunits. Such receptors may also be found extrasynaptically. Another group of receptors are typically found only (or largely) at extrasynaptic or perisynaptic sites and are composed of α4/6, β and δ subunits (Nusser *et al.* 1998, Wei *et al.* 2003) or α5$\beta\gamma$2 subunits (Brunig *et al.* 2002, Serwanski *et al.* 2006). These two groups of receptors mediate different forms of signaling – 'phasic' and 'tonic' inhibition, respectively (Belelli *et al.* 2009, Farrant and Nusser 2005, Glykys and Mody 2007, Semyanov *et al.* 2004).

Phasic GABAergic signalling is mediated by synaptic receptors that respond to rapidly rising but short-lived (< 1 ms) GABA transients (0.3-1.0 mM) that result from vesicular release. 'Synaptic' $\alpha\beta\gamma$ receptors have a relatively low affinity for GABA and activate quickly. By contrast, 'extrasynaptic' $GABA_A$ receptors have a high affinity for GABA (Karim *et al.* 2012, Mortensen *et al.* 2011), and, being distant from sites of GABA release, are exposed to 'ambient' GABA concentrations thought to be in the 10-100s of nM (Farrant and Nusser 2005, Santhakumar *et al.* 2006, Wu *et al.* 2007). Although a resistance to desensitization was thought key in enabling extrasynaptic δ-containing receptors to generate a maintained response (see (Farrant and Nusser 2005) for discussion), recent studies suggest that these receptors do in fact desensitize (Bright *et al.* 2011, Mortensen *et al.* 2010). For $\alpha\beta\gamma$-containing receptors, α subunit content is particularly influential. Thus, clear differences in agonist affinity, gating and pharmacology are seen among

recombinant $GABA_A$Rs with different α subunits (Bianchi and Macdonald 2002, Borden 1996, Bright *et al.* 2011, Gingrich *et al.* 1995, Lavoie *et al.* 1997, Macdonald and Olsen 1994, Picton and Fisher 2007, Verdoorn *et al.* 1990, Vicini 1999) and this is reflected in the different properties of IPSCs in neurons that express different α subunits or different mixtures of α subunits at their synapses (Browne *et al.* 2001, Eyre *et al.* 2012, Mozrzymas *et al.* 2007).

The involvement of functionally distinct $GABA_A$ receptor subtypes in distinct higher level phenomena has been elucidated in genetically modified mice, in which individual α subunits have been rendered insensitive to modulation by diazepam and the differential effects on drug action examined (Rudolph *et al.* 1999). Histidine-to-arginine point mutation at a conserved residue in the α1, α2, α3 or α5 subunit abolishes binding of diazepam, while the actions of GABA are preserved. Using this approach, it has been shown that the α1 subunit mediates the sedative, anterograde, amnestic and some of the anticonvulsant effects of diazepam; the α2 and α3 subunits mediate the anxiolytic and myorelaxant effects, respectively, and the α5 subunit is involved in amnestic effects as well as other aspects of learning and memory (Rudolph and Knoflach 2011, Rudolph and Mohler 2004). The α4 and α6 subunits are benzodiazepine-insensitive (Luddens *et al.* 1990, Wisden *et al.* 1991).

Interestingly, a third form of postsynaptic $GABA_A$ receptor-mediated inhibitory response that displays characteristics intermediate between phasic and tonic inhibition has also been described (Szabadics *et al.* 2007). These events have been named $GABA_{Aslow}$ inhibitory postsynaptic currents (IPSCs) and are mediated by the Ivy/Neurogliaform family of interneurons (Ivy/NG cells) (Capogna and Pearce 2011). In contrast to the more classical, phasic $GABA_{Afast}$ IPSCs, $GABA_{Aslow}$ IPSCs have almost an order of magnitude slower rise- and decay kinetics, are highly sensitive to blockade of GABA uptake and are modulated differently by benzodiazepine agonists.

The kinetics of these currents differs because they result from a long-lived, distributed, low-concentration GABA concentration profile, and are produced by receptors with different kinetic and pharmacological properties. The most compelling evidence that the receptors underlying $GABA_{Afast}$ and $GABA_{Aslow}$ are distinct in their differential sensitivity to furosemide (Pearce 1993) and other agents (Banks *et al.* 1998, Pearce 1996) that block $GABA_{Afast}$ but have little effect on the amplitude of $GABA_{Aslow}$.

Based upon the physiological and anatomical differences between the two $GABA_A$-mediated inhibitory systems, it has been suggested that their activation may have different functional consequences. The fast somatic current $GABA_{Afast}$ controls the spike output of pyramidal cells in response to summed excitation at the soma (Miles *et al.* 1996, Pearce 1993), whereas the dendritic current $GABA_{Aslow}$ acts locally, controlling the level of dendritic polarization and thereby modulating the efficacy of specific excitatory inputs in the dendrites of these cells.

Furthermore, their kinetics also suggest different roles in temporal patterning in hippocampal circuits, for example, with $GABA_{Aslow}$ underlying theta (3-8 Hz) and $GABA_{Afast}$ underlying gamma (20-80 Hz) oscillations that are thought to play separate functional roles in memory and arousal. Further characterization of the properties of these currents and other elements of cortical inhibitory circuits will improve our understanding of how these circuits contribute to specific behaviours and will permit more rational development of targeted therapeutic interventions in the brain.

Recent finding indicated that receptors containing $\alpha 5$ subunits are also located at dendritic synapses (Serwanski *et al.* 2006) and that they underlie a slow form of synaptic (phasic) inhibition in hippocampal CA1 pyramidal neurons (Prenosil *et al.* 2006, Zarnowska *et al.* 2009). By virtue of its dendritic location and slow kinetics, which match those of NMDA receptor-mediated excitation, this $GABA_{Aslow}$ inhibitory current is well suited to control synaptic plasticity and memory formation (Banks and Pearce 2000, Pearce 1993).

Recently it was recognised that ribonucleic acid (RNA) editing, more specifically adenosine-to-inosine (A-to-I) editing of pre-messenger RNA (mRNA), a widespread post-transcriptional processing event (Sakurai *et al.* 2010), was capable of further increasing GABA$_A$ receptor diversity. In the following section I describe the general principle of RNA editing and the recent identification of RNA editing of the GABA$_A$ receptor α3 subunit, which prompted the work described in this thesis.

1.4 RNA editing as a source of receptor heterogeneity

After transcription of a eukaryotic RNA molecule from deoxyribonucleic acid (DNA), the newly formed transcript undergoes a number of post-transcriptional modifications that determine the final RNA, and thus the protein product. Alternative splicing creates large-scale rearrangements of the original RNA message by removing a large block of contiguous sequence. By contrast, RNA editing allows single or multiple base insertions or deletion as well as base substitutions. These post-transcriptional processes are mechanisms for generating a diverse set of RNA and protein products from a limited number of genes (Zinshteyn and Nishikura 2009).

Following the initial discovery of protozoan RNA editing in an mRNA encoded by the kinetoplastid mitochondria of trypanosomes (Benne *et al.* 1986, Estevez and Simpson 1999), the first RNA editing example in mammals was reported in the apolipoprotein B transcript (Powell *et al.* 1987). In mammals, one type of substitutional RNA editing, characterized by site-specific base modification, was shown to modulate important physiological processes. The underlying reaction mechanism of substitutional RNA editing involves hydrolytic deamination of cytidine (C) or adenosine (A) bases to uracil (U) or inosine (I), respectively (Maas and Rich 2000). The most prevalent type of RNA editing is mediated by adenosine deaminase acting on RNA (ADAR) enzymes; ADAR converts adenosine (A) to inosine (I) in double-stranded RNA (dsRNA) substrates (**Figure 1.4**). This conversion is known as A-to-I RNA editing, and can lead to a codon change and

consequent alterations of protein-coding sequences of selected genes, resulting in a diversification of their protein functions (Zinshteyn and Nishikura 2009).

A-to-I editing is now recognised as a very widespread phenomenon (Levanon *et al.* 2004, Maas *et al.* 2011). In the past few years, bioinformatics and experimental studies have revealed tens of thousands of editing sites affecting over 1600 different genes. In human, most editing events occur predominantly in the highly abundant repetitive *Alu* elements, which comprise more than 10 % of the gemone. *Alu* is a retrotransposon, about 280 base pair long, belonging to the class of Short Interspersed Nuclear Elements (SINEs). The majority of the editing sites are located in non-coding sequences and the precise role of RNA editing in *Alu* repeats is as yet a mystery. Despite recent progress in identifying additional genes that undergo RNA editing, the total number of currently known A-to-I edited genes in mammals is still small (Bass 2002). However, the activity of the mammalian editing machinery, as measured by inosine content in mRNA fractions is much higher than expected based on the current number of known substrates (Bass 2002).
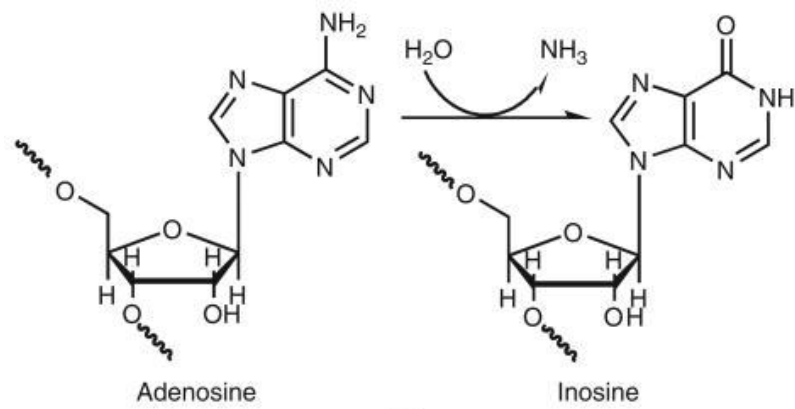
Figure 1.4 A-to-I RNA editing
Adenosine (A) is converted to inosine (I) by hydrolytic deamination. Adapted from (Zinshteyn and Nishikura 2009).

A-to-I editing affects gene expression through a number of mechanisms: as inosine is recognized as guanosine by the translation machinery, A-to-I editing can lead to modification of splice sites in introns, inducing premature termination, frame-shift, or new exon formation (Maas and Rich 2000), thus leading to a change in the primary sequence of an RNA molecule. At another level, since inosine base-pairs with cytidine, the three-dimensional structure of RNA can be altered by the addition or removal of bulges formed by mismatched base pairs (Maas *et al.* 2011) which will influence the stability of RNA molecules. Editing efficiency (the extent of conversion from A to I) varies depending on substrate, developmental timing, and location, allowing mixed populations of products to exist, and for these populations to change in response to changing conditions (Zinshteyn and Nishikura 2009).

1.4.1 Hydrolytic deamination of adenosine to inosine by ADARs

A family of different ADAR enzymes (Bass 2002) catalyses the A-to-I editing reaction. These proteins, which are conserved across many eukaryotes, contain a C-terminal catalytic domain, as well as several double-stranded RNA-binding domains (**Figure 1.5**). There are three vertebrate ADAR genes, which give rise to several ADAR proteins. The first mammalian gene identified, human *ADAR1*, was cloned following biochemical purification and microsequencing of ADAR1 protein. This led to the later identification of *ADAR2* and *ADAR3*. The ADAR1 protein has long (p150) and short (p110) isoforms, which arise from the use of alternative promoters and start codons. ADAR2 and ADAR1p110 are mainly present in the nucleus, whereas ADAR1p150, driven by an interferon-inducible promoter, is present in both nucleus and cytoplasm (Bass 2002). *ADAR1* and *ADAR2* are expressed in most tissues, whereas *ADAR3* is only found in the central nervous system. While ADAR1 and ADAR2 must form homodimers for activity, ADAR3 is monomeric. The enzymatic activity of ADAR1 or ADAR2 has been demonstrated, whereas ADAR3 shows no deaminase activity *in vitro*.
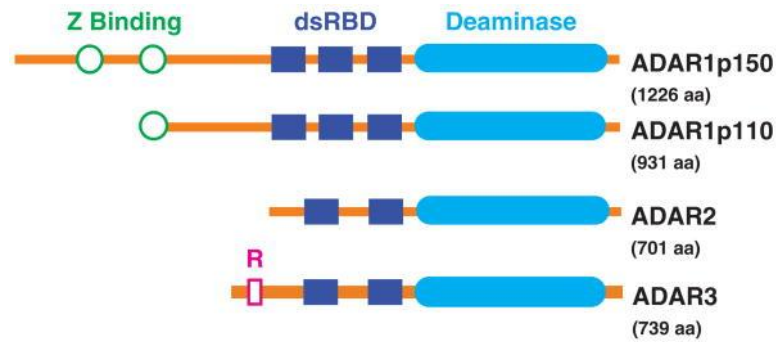
Figure 1.5 Human ADAR proteins

Three different ADAR proteins have been characterized and all of them share a common domain structure with two or three double stranded (ds) RNA-binding domains (filled rectangles) and a C terminal catalytic deaminase domain (filled ovals). ADAR1 protein is the largest of the three family members and is expressed in two major splice variants ADAR1 p150 and ADAR1 p110. ADAR1 p150 contains an extended N terminus including two zinc DNA/RNA-binding motifs (open circles). The R domain (open rectangle) of ADAR3 has been reported to bind to single-stranded RNA, but its protein function remains unknown. Adapted from (Zinshteyn and Nishikura 2009).

In recent years, several reports have been published describing the phenotypes of animals with deletions in genes encoding proteins of the ADAR family. These animal models have demonstrated the importance of editing for normal physiology and also revealed some intriguing connections to human disease phenotypes. Flies containing a homozygous deletion in their single *ADAR* gene exhibit normal morphology but have neurological phenotypes such as locomotor deficiencies, seizures, premature neurodegeneration, and altered reproductive behaviour (Farajollahi and Maas 2010). These defects all become more severe with age. In mice, the genetic inactivation of *Adar1* or *Adar2* also leads to severe phenotypes. *Adar1* knockout is embryonically lethal and causes defects in the proliferation and differentiation of blood cells during haemopoiesis (Hartner *et al.* 2009). Meanwhile, *Adar2* is essential for normal murine brain function because homozygous knockout mice develop epileptic seizures shortly after birth and die within a few weeks of age. This phenotype can be attributed to the consequences of the deficiency in editing of the mRNA for the glutamate receptor subunit GluA2 (see below), which is edited specifically by ADAR2 in neurons (Higuchi *et al.* 2000). In both *Adar1* and *Adar2* knockout mice, the loss of editing activity of one ADAR is partially compensated by the overlapping activity of the other. Homozygous *Adar3*⁻ knockout mice did not display lethal phenotype; therefore, the loss of ADAR3 appears to be compensated for by other members of the ADAR family.

1.4.2 Functional consequences of A-to-I RNA editing

Currently, most of the identified targets of A-to-I RNA editing are found in the mammalian nervous system – in ion channels and neurotransmitter receptors (Seeburg and Hartner 2003, Tan *et al.* 2009). Some notable examples include a number of glutamate-gated ion channels, the voltage-gated $K_V1.1$ potassium channel, the serotonin $5HT_{2C}$ receptor and the $GABA_A$ receptor. Recoding of these proteins by RNA editing provides a mechanism for customizing specific channel function, and dysregulation of the process may contribute to the pathogenesis of certain diseases (Tan *et al.*

2009). Furthermore, the role of ADARs in modulation of RNA editing of receptors/channels could represent a future target for the treatment of CNS dysfunction.

Glutamate-gated ion channels

The functional consequences of A-to-I editing have been studied most extensively in the GluA2 (GluR-B or GluR2) subunit of AMPA-type glutamate receptor subunit, which was among the first A-to-I editing substrates identified (Sommer *et al.* 1991). As the major excitatory neurotransmitter in the CNS, L-glutamate activates three pharmacologically and electrophysiologically distinct receptor families – NMDA (*N*-methyl-D-aspartate), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and kainate receptors. Each receptor is assembled from a subset of evolutionary related protein subunits. These channels mediate fast excitatory neurotransmission in the brain and exhibit enormous diversity in their electrophysical properties (Traynelis *et al.* 2010). This diversity comes from the heterogeneity of GluR subunits as well as from RNA splicing and RNA editing which lead to multiple isoforms of the individual GluR subunits. In particular, functional studies with recombinantly expressed wild-type and mutant AMPA receptors demonstrated that the GluA2 subunit has a profound impact on biophysical properties of the receptors – most notably, eliminating Ca^{2+} permeability. The molecular determinant for this dominant effect of GluA2 is related to a single arginine (R) residue located in the channel-pore lining segment of TM2 (Seeburg *et al.* 2001). The reduction of calcium permeability is accompanied by a loss of sensitivity to intracellular polyamines, which confer inward rectification on GluA2-lacking AMPA receptors (Bowie and Mayer 1995, Kamboj *et al.* 1995, Koh *et al.* 1995).

Five subunits of the glutamate receptor (GluA2, GluA3, GluA4, GluK1 and GluK2; originally termed GluR-B, GluR-C, GluR-D, GluR-5 and GluR-6; see (Collingridge *et al.* 2009) have been found to undergo ADAR-mediated RNA editing. Currently, a total of 4 editing sites that result in amino acid changes

have been identified, namely glutamate to arginine (Q/R), arginine to glycine (R/G), isoleucine to valine (I/V), and tyrosine to cysteine (Y/C). The Q/R editing site occurs in the AMPA receptor subunit GluA2, as well as in the kainate receptor subunits GluK1 and -2. As the name implies, editing at this site changes a glutamate (Q) to arginine (R) in the pore-lining segment of TM2 (Seeburg *et al.* 2001). This position is critical for determining the ion permeability of AMPA receptor as inclusion of the edited R form of the subunit reduces Ca^{2+} permeability. It has been shown that high Ca^{2+} permeability of AMPA receptor may adversely affect neuronal function (Whitney *et al.* 2008). Indeed, rendering GluA2 transcripts un-editable at the Q/R site causes animals to develop early-onset epilepsy and die within three weeks of birth (Seeburg and Hartner 2003). Not only the Q/R site, but also the I/V and Y/V site of GluK2 have impact on Ca^{2+} permeability (Barbon and Barlati 2011).

RNA editing was also found to occur in the coding region between TM3 and TM4 of GluA2, -3 and -4 (Barbon and Barlati 2011). At this R/G editing site, an arginine (R) is converted to a glycine (G). Alteration of this amino acid leads to faster rates of recovery from desensitization (Lomeli *et al.* 1994). The R/G site is largely unedited in the embryonic brain but editing increases after birth. In addition to regulating the electrophysiology and kinetics of the channel, editing also plays a role in other aspects of channel function including channel assembly (Greger *et al.* 2003) and exit from the endoplasmic reticulum (ER) (Greger *et al.* 2002). Editing at the Q/R site as well as at the R/G site has been shown to affect the availability of functional channels at synapses, since RNA editing diminished the assembly of the GluA2 subunit, and hence decreased the expression of the subunit at the synapse (Ma-Hogemeier *et al.* 2010). The edited GluA2(R) isoforms mainly exist as monomers in the ER, whereas unedited GluA2(Q) isoforms have a higher propensity to tetramerize and be transported to the synaptic membrane. Thus, A-to-I editing may be involved in regulating the responsiveness of the receptor as well as controlling the receptor assembly.

Voltage-gated potassium (K$^+$) channels

Voltage-gated potassium channels such as Kv1.1 play a fundamental role in neuronal excitability. These channels open upon depolarization, thereby re-establishing the resting membrane potential that is changed by, for example, the opening of sodium channels during an action potential. After opening, these voltage-gated potassium channels undergo fast inactivation which involves pore blockage by an 'inactivating particle' that is part of the *N*-terminus of the channel or by similar parts of β-subunits. The mRNA transcript of human Kv1.1 is subject to A-to-I RNA editing, resulting in change from an isoleucine (I) to valine (V) residue (Bhalla *et al.* 2004). This process occurs specifically at position 400 in the channel's sixth transmembrane segment (S6), which has been shown to line the pore and point toward the internal cavity where the inactivation particle is thought to dock (Decher *et al.* 2010). In most cases, RNA editing is directed by double-stranded RNA structures formed between residues close to the editing site and those in an intron. In contrast, the gene encoding hKv1.1 contains no introns and the editing site within the hKv1.1 mRNA is specified by an exonic hairpin structure (Bhalla *et al.* 2004). Electrophysiological studies showed that the edited hKv1.1 exhibits reduced open-channel block by exogenous drugs and endogenous highly unsaturated lipids (arachidonic acid, docosahexaenoic acid and anandamide), as well as rapid recovery from fast inactivation as compared to the wild-type channel (Bhalla *et al.* 2004, Decher *et al.* 2010). The rate of inactivation of potassium channels after depolarization is an important determinant of the firing rate of neurons and thus of the transmission of information in the nervous system (Trimmer and Rhodes 2004). It is suggested that the regulation of editing could allow the CNS to fine-tune action potentials. This regulation could be quite complex because potassium channels exist as tetramers, and each channel may contain different ratios of edited and unedited subunits. The different extent of editing of each channel could have very different effects on the final properties of channel inactivation and thus a neuron's firing pattern.

5-HT2C (serotonin) receptors

5-HT receptors (with the exception of the ionotropic 5-HT3 class) are seven transmembrane-spanning receptors, which transmit extracellular signals to the cell through their interaction with G proteins (G-protein-coupled receptors; GPCRs). To date, 14 5-HT receptors have been identified, and classified into seven subfamilies based on sequence, pharmacological properties and signal transduction. The 5-HT2 subfamily of receptors activates the phospholipase C (PLC) signalling pathway, while most of other subfamilies modulate adenylate cyclase signalling pathways. Among the various receptors, the 5-HT2C receptor is the only one to undergo A-to-I type RNA editing, by which the receptor activity can be dynamically regulated. Five editing sites (A to E) have been identified that result in distinct amino acid changes in the putative second intracellular loop of the receptor, a region known to be important for G protein coupling (Gaborik *et al.* 2003). RNA isolated from brain showed that the editing events occur in various combinations that can generate up to 24 protein isoforms (Burns *et al.* 1997), potentially adding immense diversity to the function of the receptor *in vivo*. Functional studies revealed that the unedited receptor displayed a greater ability to stimulate phospholipase C than the fully edited version, possibly because the edited receptors couple less efficiently to the G protein (Olaghere da Silva *et al.* 2010). Further studies suggested that the unedited isoforms have a higher level of constitutive activity because of a greater tendency to isomerize to an active conformation that can couple to the G protein. Editing of the 5-HT2C receptor yields transcripts that not only have distinct functional properties (affecting agonist potency and G-protein coupling), but also differ in their pattern of CNS expression (Berg *et al.* 2008). Differently distributed isoforms are believed to have distinct physiological and pathophysiological functions in those regions in which they are expressed.

In summary, RNA editing of several evolutionary-conserved recoding sites is known to be of critical importance to proper cell development and function (Streit and Decher 2011). The power of RNA editing in generating protein diversity lies in the fact that (usually) both the edited and unedited versions of

the RNA and/or protein co-exist in the same cells, and the ratio between the unedited and various edited variants can be regulated in a cell-type specific or time-dependent manner. In fact, the GluA2 Q/R site is the only known transcript edited to nearly 100% at all developmental stages.

It is not surprising that neuronal tissues show high RNA editing activity and that many recoding A-to-I editing events affect brain-specific genes (Mehler and Mattick 2007). Thus, highly complex systems and their complex physiology and behaviour might strongly rely on epigenetic sources of variation, such as A-to-I editing. Accordingly, defects or misregulation in RNA editing might cause or accompany disturbances in higher order function more frequently than they disturb basic physiological processes (Nishikura 2010). Indeed, the deficiency or misregulation of A-to-I RNA editing has been implicated in the aetiology of neurological and psychiatric diseases in mammals, such as epilepsy, amyotrophic lateral sclerosis (ALS), and depression (Mehler and Mattick 2007). In this respect, it is interesting to note that behavioural differences between mouse strains correlate with distinct RNA editing profiles, and that several animal models of editing misregulation display behavioural abnormalities. One example is the 5-HT2C receptor, which has established roles in emotion, locomotion, appetite, metabolic rate control, depression, schizophrenia and drug addiction (Nishikura 2010). In human patients with schizophrenia and depression, changes in 5-HT2C editing patterns are apparent and, intriguingly, the treatment of mice with a serotonin uptake inhibitor is accompanied by converse alterations in editing. Misediting is also observed in some cases of schizophrenia (Iwamoto *et al.* 2009). Mice that express only the fully-edited version of the serotonin receptor (5HT2C-VSV) display increased metabolism, hyperphagia and growth retardation. Another example comes from the GluA2 receptor, where under-editing of the Q/R site has been proposed to be responsible for motor neuron death in ALS (Hideyama *et al.* 2010), a disease associated with progressive symptoms of muscle weakness, muscle atrophy and spasticity.

1.5 α3-containing GABA$_A$ receptors and A-to-I editing of Gabra3

When this work was started, little was known about the possible RNA editing of GABA$_A$ receptor subunits. The work described in this thesis was prompted by the finding of Marie Öhman (Stockholm University) that the α3 subunit of the GABA$_A$ receptor (Gabra3) was subject to A-to-I editing (Ohlson *et al.* 2007).

1.5.1 The distribution and functional roles of α3-containing GABA$_A$ receptors

GABA$_A$ receptors containing the α3 subunit represent approximately 10-15% of the total GABA$_A$ receptors in adult brain (McKernan and Whiting 1996). *In situ* hybridization and immunohistochemical studies showed that α3 subunits are expressed in the glomerular and external plexiform layers of the olfactory bulb, the inner layers of the cerebral cortex, the reticular thalamic nucleus, the zonal and superficial layers of the superior colliculus, the amygdala, the basal forebrain, the hypothalamus and the brainstem (Fritschy and Mohler 1995, Pirker *et al.* 2000, Wisden *et al.* 1992, Wisden *et al.* 1988). α3-containing GABA$_A$ receptors are therefore likely to contribute to GABAergic control over a broad range of behavioural and cognitive states.

Three facets of this expression pattern have received particular attention. Firstly, the strong expression of α3 in the thalamic reticular nucleus (nRT), together with expression layer VI of the neocortex, has led to consideration of the role of α3-containing receptors in the function of thalamo-cortical circuits. These depend critically on the GABAergic control of reciprocal synaptic loops between thalamic relay nuclei, the nRT and the neocortex, and give rise to specific oscillatory activities that underlie electroencephalography (EEG) rhythms (Llinas and Steriade 2006). Abnormal activity of the thalamo-cortical network can lead to the onset of spike-wave discharges that are EEG hallmarks of absence seizures. Genetically epilepsy-prone rats (WAG/ Rij) – which exhibit a specific loss of

α3-containing GABA$_A$ receptors in the nRT – display absence-like seizures (Liu *et al.* 2007). Interestingly, mice with a global knockout of α3 do not exhibit an epilepsy phenotype (Winsky-Sommerer *et al.* 2008, Yee *et al.* 2005), possibly reflecting the compensatory gain in IPSCs seen in the nRT (Schofield *et al.* 2009, Winsky-Sommerer *et al.* 2008).

Secondly, the predominant expression of α3 in basal forebrain cholinergic neurons and in monoaminergic neurons of the brain stem (including the substantia nigra, ventral tegmental area, raphe nuclei and locus coeruleus) (Corteen *et al.* 2011, Rodriguez-Pallares *et al.* 2001) suggests that α3-containing receptors mediate the primary GABAergic inhibition of the dopaminergic, serotoninergic and noradrenergic systems. Accordingly, α3-containing GABA$_A$ receptors are considered as a pharmacological target for the treatment of mental illnesses such as schizophrenia and affective disorders. While α3 knockout mice do not exhibit an epilepsy phenotype, they are hyperactive and exhibit a sensory-motor deficit possibly related to over activity of dopaminergic neurons (Fiorelli *et al.* 2008, Yee *et al.* 2005).

Finally, the pronounced expression of α3 in the amygdala, a brain region intimately involved the generation of fear and anxiety (Davis 1992), suggests a possible role of α3-containing GABA$_A$ receptors in anxiety. This is a somewhat controversial area (see (Smith and Rudolph 2012). While pharmacological studies have suggested that the α3-selective agonist TP003 (Dias *et al.* 2005) and inverse agonist α3IA (Atack *et al.* 2005) can be anxiolytic and anxiogenic, respectively, studies of mice expressing α3 subunits with a histidine-to-arginine substitution (3H126R) making them insensitive to diazepam have suggested no role of α3-containing receptors in the sedative or anxiolytic effects of diazepam (Crestani *et al.* 2001, Low *et al.* 2000). Interestingly, a recent study has shown that in the basolateral amygdala, in marked contrast to the nRT, α3-containing GABA$_A$ receptors are predominantly extrasynaptic, and contribute to a robust tonic current in principal cells. Moreover TP003 was shown to increase the tonic current in

(and reduce the excitability of) principal cells of wild-type mice but have no effect in cells from $\alpha3$ knockout mice (Marowsky *et al.* 2012).

1.5.2   Developmentally regulated expression of the $\alpha3$ subunit

Developmental alterations in GABA$_A$ receptor subunit expression are more significant among α subunits than among the other subunits, and one of the most notable events is the developmental decrease in $\alpha2/\alpha3$ expression and increase in $\alpha1$ expression (Fritschy *et al.* 1994, Laurie *et al.* 1992b). In rat, the $\alpha3$ subunit is the predominant α subunit at birth but declines around postnatal day 10-12, whereas the $\alpha1$ subunit increases with development, becoming the dominant subunit in adult (Pirker *et al.* 2000).

Although multiple pre- and postsynaptic factors can influence the duration of synaptic currents (e.g. (Eyre *et al.* 2012, Sauer and Bartos 2011), $\alpha3$-containing receptors are seen to underlie relatively prolonged IPSCs, while $\alpha1$-containing receptors underlie relatively short-lasting IPSCs (Bosman *et al.* 2005, Eyre *et al.* 2012, Mozrzymas *et al.* 2007, Okada *et al.* 2000, Vicini *et al.* 2001). Thus, the developmental shift from $\alpha3$ to $\alpha1$ subunit expression is associated with speeding of synaptic currents (Bosman *et al.* 2002, Heinen *et al.* 2004, Ortinski *et al.* 2004).

1.5.3 Pharmacology and pathology of $\alpha3$-containing GABA$_A$ receptors

With the general aim of dissociating the anxiolytic actions of classical benzodiazepines from their sedative or addictive effects, there has been a continued search for GABA$_A$ receptor subtype-selective compounds (Rudolph and Knoflach 2011). In the course of this work, several $\alpha3$-selective drugs have been identified, notably the $\alpha3$-selective agonist TP003 (Dias *et al.* 2005) and the $\alpha3$-selective inverse agonist $\alpha3$IA (Atack *et al.* 2005) (see section 1.5.1). Because of the restricted expression pattern of $\alpha3$-containing GABA$_A$ receptors in the adult brain, it has been suggested that drugs

targeting these receptors may provide selective anxiolysis or be of benefit in treating cognitive impairments of schizophrenia.

Additionally, stiripentol (STP), a novel antiepileptic drug that is structurally unrelated to other anticonvulsants (Trojnar *et al.* 2005), acts as a positive allosteric modulator of GABA$_A$ receptors and has its greatest effect on receptors containing the α3 subunit (Fisher 2009). A preferential action at α3-containing receptors provides a plausible mechanism for the observation that STP is very effective in treating childhood seizures (as the α3 subunit is highly expressed in the immature, developing brain) (Fisher 2011).

Drugs acting at α3-containing GABA$_A$ receptors may also be useful for other therapies, such as treatment of neuropathic and inflammatory pain (Munro *et al.* 2009). Studies of mutant mice suggested that the α3-containing GABA$_A$ receptors may be responsible for the antihyperalgesic effect of spinally applied benzodiazepines (Knabl *et al.* 2009). Of note, pronounced analgesia was achieved by specifically targeting spinal GABA$_A$ receptors containing α2 and/or α3 subunits (Knabl *et al.* 2008, Rudolph and Knoflach 2011).

Intriguingly, although GABA functions as an inhibitory neurotransmitter in the mature CNS, abnormal levels of gene and protein expression of GABA$_A$ receptor subunits have been detected in certain malignant tumours. This suggests that GABAergic system may play a role in the pathogenesis and development of such tumours. In particular, the α3 subunit has been shown to be overexpressed in Hepatocellular carcinoma (HCC) cells, and it has been suggested that GABA may stimulate HCC cell proliferation through α3-containing receptors (Liu *et al.* 2008).

1.5.4   RNA editing of the α3 subunit

Co-immunoprecipitation of native ADAR2-mRNA complexes extracted from mouse brain using anti-ADAR2 antibodies identified a novel site of A-to-I editing within exon 9 of *Gabra3*, the gene that codes for the GABA$_A$ α3

subunit (**Figure 1.6**; (Ohlson *et al.* 2007). At this position, the AUA codon is changed to an AUI codon and read as AUG. As a result, an amino acid change occurs from isoleucine (I) to methionine (M). The editing complementary sequence is located 15 bases upstream of the edited site in a short predicted stem loop of 54 nucleotides within exon 9. Mutagenesis analyses revealed that a mini duplex structure (shorter than 10 base pairs with one A-C mismatch and a capped by a tetraloop) is required for efficient RNA editing. This is the shortest loop stem known to be edited efficiently by ADAR (Tian *et al.* 2011).

The extent of α3 editing was found to be developmentally regulated, and to vary in different brain regions. Quantitative analyses of *Gabra3* mRNA levels showed that editing is gradually increased from ~15% at embryonic day (E)15 to a maximal level of 85% at P7, which then persists into adulthood (Rula *et al.* 2008). A similar editing pattern was later observed in developing chick retina, in which the frequency of editing increased from 15% at E6.5 to 95% in the adult (Ring *et al.* 2010).

The I/M site was found to be located toward the extracellular end of the third transmembrane domain (TM3) of the subunit (**Figure 1.7**). The edited residue is believed to lie within a region of the helix that faces the TM2 domain, and which is structurally adjacent to residues that can cross-link to TM2, and may influence conformational stability of the subunit (Jansen and Akabas 2006). GABA binding is known to cause changes in the secondary structure of TM3, and residues within this region contribute to a binding pocket for volatile anaesthetics and ethanol (Bonin and Orser 2008). Although the change from isoleucine to methionine maintains the highly hydrophobic nature of the region, it will alter the length of the side chain and could thus affect receptor properties.

Of note, all prior electrophysiological studies with recombinant GABA$_A$ receptor α3 subunits used the edited form, as this was the form originally identified by cloning (Levitan *et al.* 1988). In the following chapters, I describe the results of experiments in which I set out to examine the effects of A-to-I

editing on the basic function (Chapter 3), single-channel properties (Chapter 4) and kinetic features (Chapter 5) of α3-containing GABA$_A$ receptors.
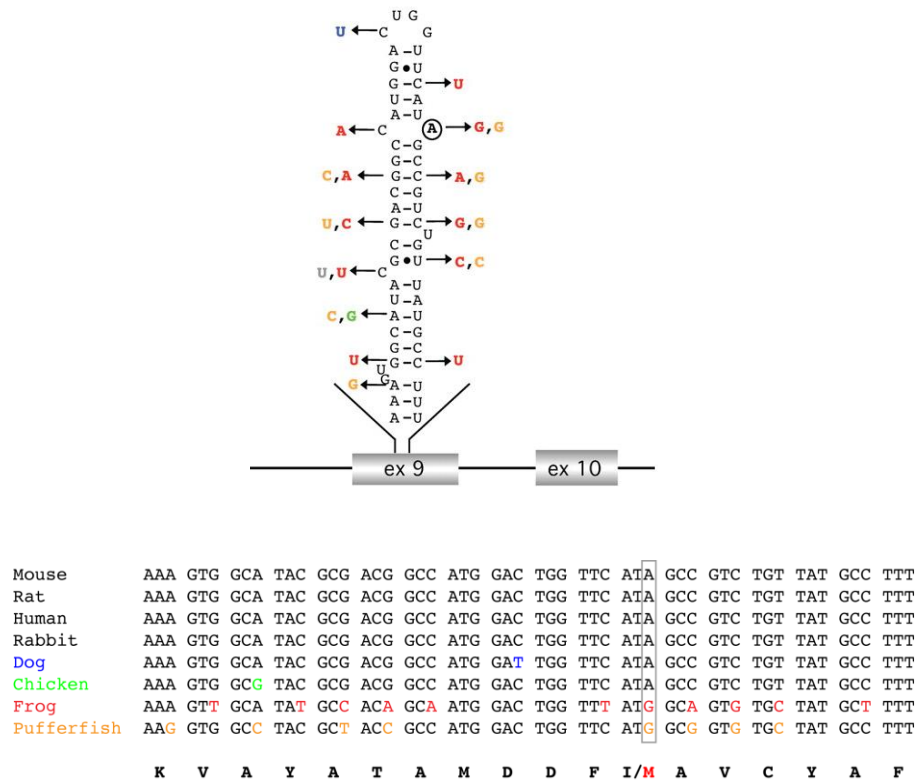
Figure 1.6 Conserved editing of the Gabra3 transcript
The upper panel shows the predicted stem-loop structure of the Gabra3 transcript at the I/M site in mouse (mGabra3) exon 9. The edited adenosine is circled. The arrows indicate nucleotide substitutions in Gabra3 of other species, as shown below. The editing site is boxed in grey. The amino acid sequence is shown at the very bottom with the I/M switch indicated in red. Adapted from (Daniel *et al.* 2011).

```
                                 ...M2                          M3...
        alpha1  298    SISARNSLPKVAYATAMDWFIAVCYAFVF
        alpha2  297    SISARNSLPKVAYATAMDWFIAVCYAFVF
        alpha3  322    SISARNSLPKVAYATAMDWFIAVCYAFVF
 edited alpha3  322    SISARNSLPKVAYATAMDWFMAVCYAFVF
        alpha4  303    SISARHSLPKVSYATAMDWFIAVCFAFVF
        alpha5  304    SISARNSLPKVAYATAMDWFIAVCYAFVF
        alpha6  287    SISARHSLPKVSYATAMDWFIAVCFAFVF

        beta1   290    STHLRETLPKIPYVKAIDIYLMGCFVFVF
        beta2   289    NTHLRETLPKIPYVKAIDMYLMGCFVFVF
        beta3   289    NTHLRETLPKIPYVKAIDMYLMGCFVFVF

        gamma1  317    STIARKSLPKVSYVTAMDLFVSVCFIFVF
        gamma2  318    STIARKSLPKVSYVTAMDLFVSVCFIFVF
        gamma3  300    STIARKSLPRVSYVTAMDLFVTVCFLFVF
```

Isoleucine (Ile) **I**

pKa 2.26

pKa 9.60

Methionine (Met) **M**

pKa 2.16

pKa 9.08
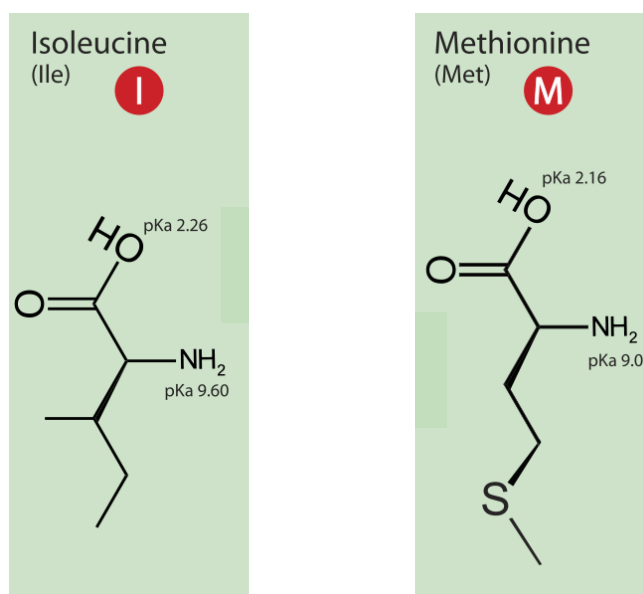
Figure 1.7 Alignment of the TM2-TM3 linkers regions for rat GABA$_A$ receptor subunits
The grey boxes at the top indicate the ends of transmembrane regions (Jansen and Akabas 2006, Wilkins et al 2005). The isoleucine to methionine alteration in the edited α3 sequence is shown in red. Shown at the bottom are the structures of isoleucine (I) and methionine (M) residues.

Chapter 2

Materials and Methods

2.1 Recombinant GABA$_A$ Receptors

Rat $\alpha1$, $\alpha3$, $\alpha6$, $\beta2$, $\beta3$, $\gamma2L$ and δ GABA$_A$R subunit complementary DNAs (cDNAs) were individually sub cloned into the mammalian expression vector pRK5 (Invitrogen, Paisley, UK) (**Figure 2.1**). The pRK5 vector contains a powerful cytomegalovirus (pCMV) promoter and is designed for high-level expression of cloned genes in cultured mammalian cells (Guo *et al.* 1995). pCMV promoters have been used to express different cDNAs and genes, including receptors, transcription factors, G-proteins and viral proteins. The pRK5 vectors have been used to transfect a wide range of cell lines. Gabra-1, Gabra-6, Gabrb-3 and Gabrd were kind gifts from Hartmut Lüddens (Johannes Gutenberg-University, Mainz, Germany). Wild-type Gabra-3 cDNA ('un-edited'; ra3wt.pRK5) was generated by mutating a guanosine into adenosine at the I/M site of Gabra-3 gene sequence. All $\alpha3$ cDNAs were kind gifts from Marie Öhman (Stockholm University, Stockholm, Sweden) (**Table 2.1**).
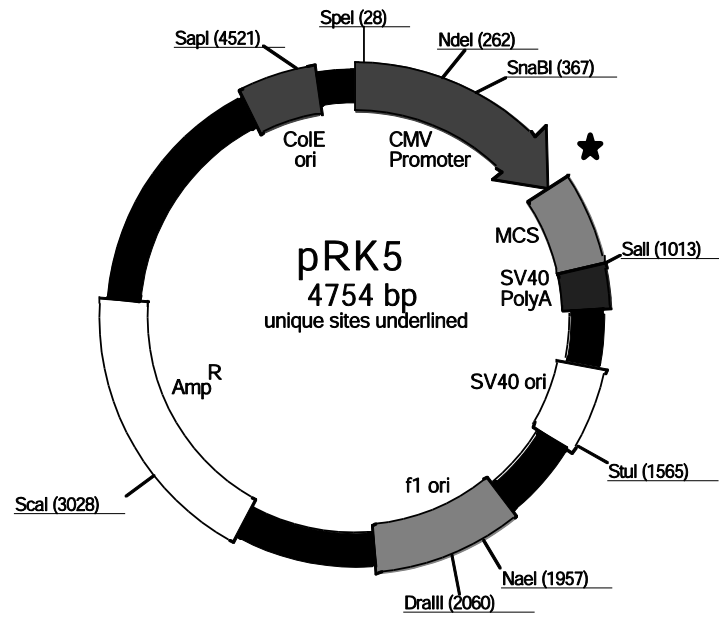
Figure 2.1 Basic structure of the expression vector pRK5
The pRK5 vector contains the following sequences specific for its functions: a pCMV promoter domain from the major immediate-early region of the human cytomegalovirus; a multiple cloning region (MCS) including the restriction sites for (from 5' to 3') XbaI, PstI, NotI, EcoRII, and HindIII; SV40 polyadenylation (SV40 PolyA) signals for RNA processing in mammalian cells; a bacteriophage f1 origin (f1 ori) of replication for production of single-stranded plasmid DNA; and an amplicillin-resistant (Amp^R) gene for amplification in *E. coli* bacterial strains. The gene of interest was inserted as indicated (star).

Subunit	Species	Gene	Source	Gene Accession No.
α1	rat	Gabra-1	HL	NM_183326.2
α3	rat	Gabra-3	MO	NM_017069.2
α6	rat	Gabra-6	HL	NM_021841.1
β2	rat	Gabrb-2	MO	NM_012957.2
β3	rat	Gabrb-3	HL	NM_017065.1
γ2L	rat	Gabrg-2L	MO	NM_183327.1
δ	rat	Gabrd	HL	NM_017289.1

Table 2.1 Complementary DNAs used in this study, and their origin
Table shows the different cDNAs used with their gene name and accession
number. (HL, Hartmut Lüddens; MO, Marie Öhman).

2.2 Molecular Biology

2.2.1 PCR mutagenesis

In order to render the $\alpha3$ cDNA immune to endogenous RNA editing, a non-editable $\alpha3$ cDNA construct was generated by site-direct mutagenesis. It has previously been shown that changing a nucleotide from C to G at the site opposite the editing I/M site can have a dramatic negative effect on editing (Wong *et al.* 2001). Therefore I created a point mutation in which the C was changed to a G opposing the I/M editing site in the stem-loop structure of Gabra3 to make it non-editable. The mutated subunit was constructed by polymerase chain reaction (PCR) amplification using the uneditable subunit (I) as a template. PCR primers were used to construct a point mutation within the subunit by the gene splicing by overlap extension technique (Ho *et al.* 1989). The oligonucleotides was amplified by DNA polymerase Phusion® HF Master Mix (Thermo Scientific, UK) and the primers for the PCR reaction were Fwd:(5'-3') [Phos]GATGGACTGGTTCATAGC and Rev(5'-3') [Phos]GCCGTCGCGTATGCCA. The identity of purified DNA was confirmed by restriction enzyme analysis. Restriction enzyme (DpnI; New England Biolabs, Ipswich, MA) was added to the PCR product and digested over 30 min at 37 °C. The resulting fragment was identified by gel electrophoresis using a 1% agarose gel and ethidium bromide as a label. Gel tanks and power packs were from Bio-Rad Laboratories Ltd. (Hemel Hempstead UK). The desired fragment was then extracted and purified using the PureLink™ Quick gel extraction kit (Roche Molecular Biochemicals, Mannheim, Germany). The purified DNA fragements were ligated into the plasmid using the rapid DNA ligation kit (Roche Molecular Biochemicals, Burgess Hill, UK). The mutated subunit was verified by sequencing (see below).

2.2.2 Cell transformation and DNA amplification

DNAs for transfection were produced by transformation of plasmid DNA into bacteria. An aliquot from the mutagenesis reaction was heat shock

transformed into NovaBlue GigaSingles competent cells (Novagen, Nottingham, UK) according to the manufacturer's protocol. NovaBlue is a K-12 *E. coli* strain with very high transfection efficiency. All transformations were grown in Lysogeny broth (LB) medium (10 g l^{-1} MgCl, 5 g l^{-1} yeast extract, 10 g l^{-1} tryptone; autoclaved for 20 min at 121°C) supplemented with 50 μg ml^{-1} ampicillin. Isolation and expansion of plasmid DNA was performed using MaxiPreps or MiniPreps from Sigma. Final DNA concentrations were measured using Jenway Genova DNA Life Science Analyser (Bibby Scientific Limited, Stone, UK).

2.2.3 Sequence analysis

The editing site in α3 and the mutant were verified using PCR and direct sequencing. PCR amplification was performed with specific sense and anti-sense primers designed from GenBank sequences (Gabra3, NM_017069) to generate amplicons corresponding to the entire region of the predicted I/M site duplex within Gabra3 transcripts. Gabra3 seq443 Fwd CTCCAGATACCTTCTTCC; Gabra3 seq511 Rev GCTTGTTGGGTGTGG; and Gabra3 seq941 Fwd CTGTTCTCACCATGACC. PCRs were performed using BigDye Terminator v1.1 Sequencing Kit (Applied Biosystems, Warrington, UK). The PCR products were then precipitated and sent to the Core Molecular Biology Facility (UCL) where sequencing analysis was performed by an automated capillary DNA sequencer (ABI Applied Biosystems 3100-Avant Genetic Analyzer; Applied Biosystems, Warrington, UK). The sequencing results were checked and verified by using the sequence alignment editor Bioedit (Ibis Biosciences, Carlsbad, CA) (**Figure 2.2**).
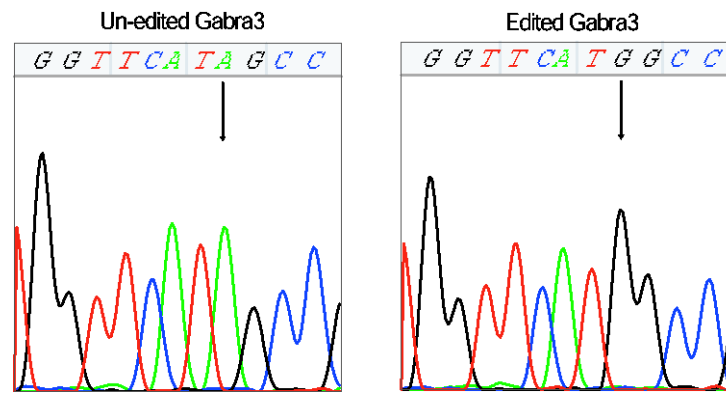
Figure 2.2 Verification of the editing site of Gabra3
Chromatogram traces showing the single amino acid change from adenine to guanine in the unedited (left) and edited (right) Gabra3 sequence.

2.3 Heterologous expression and cell culture

Heterologous expression of transmembrane receptors is commonly done in *Xenopus* oocytes or a mammalian cell line such as human embryonic kidney 293 (HEK293) cells (Thomas and Smart 2005). In the present study, recombinant $GABA_A$ receptors were expressed in tsA201 cells. The tsA201 cell line is a transformed HEK293 cell line stably transfected with the SV40 large tumor (T) antigen; it functions similarly to the HEK293 cell line, except that it is capable of a much higher protein expression level (Sheets *et al.* 1996). The large T antigen promotes replication of expression vectors containing the SV40 origin, producing amplification of the expression vector. It has been used in a variety of functional expression assays and has been reported to produce high levels of recombinant proteins (Margolskee *et al.* 1993).

Cells were grown in Dulbecco's Modified Eagle Medium (Sigma-Aldrich Co. Ltd. Gillingham, UK) supplemented with 10% fetal bovine serum (GIBCO; Invitrogen Ltd., Paisley, UK), 100 U ml^{-1} penicillin (Sigma-Aldrich Ltd., Poole, UK) and 0.1 mg ml^{-1} streptomycin (Sigma) at 37°C under 5% CO_2. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cDNAs encoding the specified α, β, and γ/δ subunits were transfected at a ratio of 1:1:10 (2.4 μg DNA per 16 mm dish for electrophysiological studies). Ten times the amount of $\gamma2$ cDNA was used in order to minimize the possibility of expressing di-heteromeric $\alpha3$:$\beta2$ instead of tri-heteromeric $\alpha3$:$\beta2$:$\gamma2$ $GABA_A$ receptors (Boileau *et al.* 2002a). In addition, cells were co-transfected with enhanced green fluorescent protein (pEGFP) to allow easy recognition of transfected cells (0.5 μg for transfection). Exponentially growing cells were dispersed with trypsin, and plated on polylysine-coated 12 mm glass coverslips (Fisher Scientific, Pittsburgh, PA, USA) 18-24 hours following transfection, and used for patch-clamp recording 48-72 hours later. The transfection efficacy of GFP was 50-55%.

2.4 Electrophysiological recording

Cells were viewed using a fixed stage upright microscope (BX51WI; Olympus) with 20x/0.50 or 40x/0.8 water immersion objectives. For identification of EGFP-positive cells, cultures were visualized with UV illumination (Olympus USH-103OL 100 W mercury discharge lamp and U-RFL-T power supply) using a GFP Fluoresence Cube Set (Exciter HQ470/40x, Dichroic Q495LP, Emitter HQ525/50m; Chroma, Rockingham, VT, USA). The cells were imaged with a CCD camera (Hamamatsu C3077-71; Hamamatsu Photonics UK Ltd., Welwyn Garden City, UK) and displayed on a BandW video monitor (Neovo X-15A; AG Neovo Technology B.V., Wendover, UK) (see **Figure 2.3**).

All patch-clamp recordings were made at room temperature (22-24°C) from EGFP-positive cells. Recordings were made using whole-cell and single-channel patch-clamp methods (Hamill et al 1981, Neher and Sakmann 1976). Patch pipettes were fabricated from thick-walled borosilicate glass (1.5 mm o.d., 0.86 mm i.d.; GC-150F; Harvard Apparatus Ltd, UK) on a two-stage vertical puller (PC-10; Narishige International Ltd., London, UK). Stray capacitance was reduced by coating the tips of the electrodes with Sylgard 184 silicone elastomer (Dow Corning, Midland, MI, USA) that was cured in a homemade heated coil. To improve seal formation, the pipettes were fire polished to a final resistance of approximately 7-10 MΩ (MF-83 microforge; Narishige International Ltd., London, UK). Cells were perfused with an 'external' solution containing (in mM): 145 NaCl, 2.4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 HEPES, the pH was adjusted to 7.3 with NaOH (**Table 2.2**)
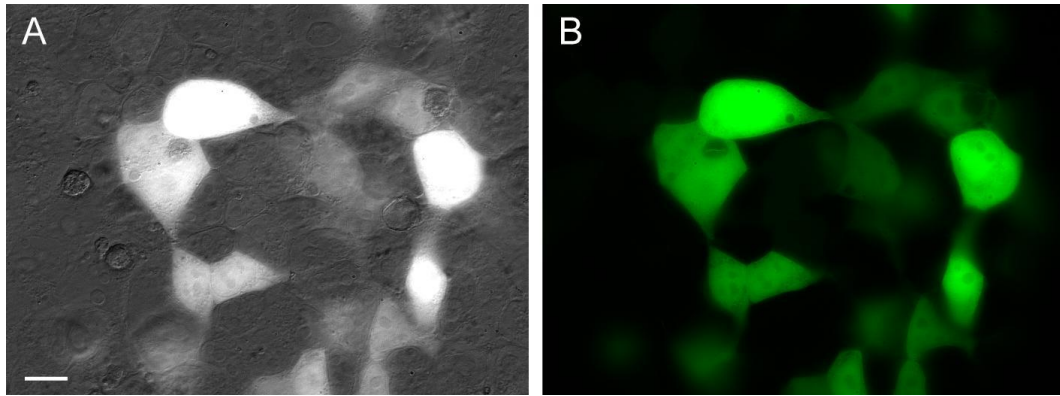
Figure 2.3 Identification of EGFP-positive cells
tsA201 cells were transfected with cDNAs for α3 and β2 GABA$_A$ receptor subunits and EGFP. For production of this high-magnification image, the cells were viewed with an Olympus BX51WI microscope with a 60x/0.90 water immersion objective and Rolera-XR camera (QImaging, Surrey BC, Canada). UV illumination 472 nm (15 nm bandwidth) was provided by a Polychrome V monochromator (Till) and emission collected at 525 nm (Dichroic Q495LP and Emitter HQ525/50m; Chroma, Rockingham, VT). (**A**) Image obtained with simultaneous transmitted light and reflected illumination. (**B**) Same field as A, but with illumination at 472 nm only. Note, in B, the greyscale image has been altered using a green lookup table to provide an appearance matching that seen down the microscope eyepieces. Scale bar indicates 10 μm.

2.4.1 Whole-cell recording

For whole-cell experiments, pipettes were back-filled with an 'internal' solution containing (in mM): 145 CsCl, 2.5 NaCl, 4 Mg-ATP, 1 EGTA-Cs and 10 HEPES (pH 7.3 with CsOH) (**Table 2.3**). The 'internal' solution was filtered through a 0.22 μm filter (Minisart RC4; Sartorius Stedium Biotech, Germany) before use and applied using a plastic/fused silica 28 gauge needle (MicroFil; WPI Inc., Sarasota, FL, USA). Filling of the electrode was facilitated by the presence of an internal filament allowing the solution to be rapidly drawn down to the electrode tip by capillary action.

The electrode was mounted in a DB-P-1.5G electrode holder (G23 Instruments, Chatham Maritime, UK) connected to the CV 201 A headstage of an Axon 200A patch-clamp amplifier (Molecular Devices, Sunnyvale, CA, USA). The headstage was mounted on a PatchStar motorised micromanipulator (Scientifica, Uckfield, UK). Stability of electrodes during patch-clamp recording is highly desirable but movement often occurs during changes in pipette pressure. This is typically most problematic when applying suction in order to rupture the patch of membrane beneath a cell-attached electrode to enable whole-cell recording (see below). The holder minimised such movement and provided improved mechanical stability by supporting the electrode at two points with rubber O-rings.

To avoid 'dirt' on the pipette tip that might prevent seal formation, positive pressure was applied to the pipette before placing it in the bath solution. The pipette was moved above the target cell and any voltage offset was zeroed. The pipette resistance was monitored from the current response to a brief −5 mV command step applied at 50 Hz. The electrode tip was placed above the target cell and slowly lowered. Positive pressure was removed once the electrode touched the cell membrane – as judged from the oscilloscope (a reduction in the current step i.e. an increase in electrode resistance) or from the visible formation of a 'dimple' in the cell surface. Gentle suction was then applied to form a 'giga-seal' between the electrode glass and the cell membrane (Suchyna et al 2009). The holding potential was then set at −60 mV. Further suction was applied to rupture the membrane and move from

the cell-attached configuration to the whole-cell condition (**Figure 2.4**). The adequacy of the 'break through' and the whole-cell recording was judged from the appearance of the capacity transients resulting from the -5 mV command steps. Initially these transients were nulled using the series resistance and whole-cell capacitance dials of the amplifier, from which values of series resistance and input capacitance were obtained. Typically, the series resistance was ranged from 11-30 MΩ and capacitance was in the range of 7-25 pF (the average series resistance for $\alpha3(I)\beta2\gamma2L$ and $\alpha3(M)\beta2\gamma2L$ was 17.2 and 22.9 MΩ respectively). Using the 'correction' circuit of the amplifier, the series resistance was compensated by 60-65% (with 10 μs lag). It is important to control for series resistance because the current that flows through the headstage into the cell must cross the resistance of the pipette tip, which is usually increased from intracellular debris that becomes lodged within the tip upon seal formation and membrane breakthrough. This resistance in the pipette tip is in series with the membrane resistance, and therefore creates a voltage divider. Series resistance is the sum of all of the resistances between the input of the patch clamp amplifier and the cell membrane. Series resistance adds two types of errors in whole-cell voltage clamp recording: 1) it introduces a voltage error, causing the cell membrane voltage (V_m) to deviate from the desired clamping voltage whenever ionic current flows. This voltage error is often called an "IR" drop since it is given by Ohm's law; 2) it lowers the temporal resolution of the voltage clamp, often to the extent that rapid physiologic processes cannot be accurately measured. Hence, series resistance compensation becomes important either when the current (I_m) is large or when rapid changes of V_m are necessary.

Cells showing an unstable holding current were rejected. Whole-cell currents were filtered at 1 kHz and sampled at 2 kHz (pCLAMP 10 software and Digidata 1440A analogue-to-digital converter; Molecular Devices).

2.4.2 Outside-out patch recording

Outside-out patches were obtained by entering the whole-cell configuration, then slowly pulling the pipette away from the cell, allowing a tube of membrane to extend out from the cell. When the pipette was pulled sufficiently far, the connecting strand of membrane detached from the cell and reformed as a patch of membrane on the end of the pipette, with the external side of the membrane facing the bath solution (**Figure 2.4**). The patch electrode was then carefully moved close to the barrel of the theta-glass application tool containing the control (external) solution (see section 2.5). The holding potential was set at -70 mV. Macroscopic patch currents were filtered at 5 kHz and sampled at 50 kHz.
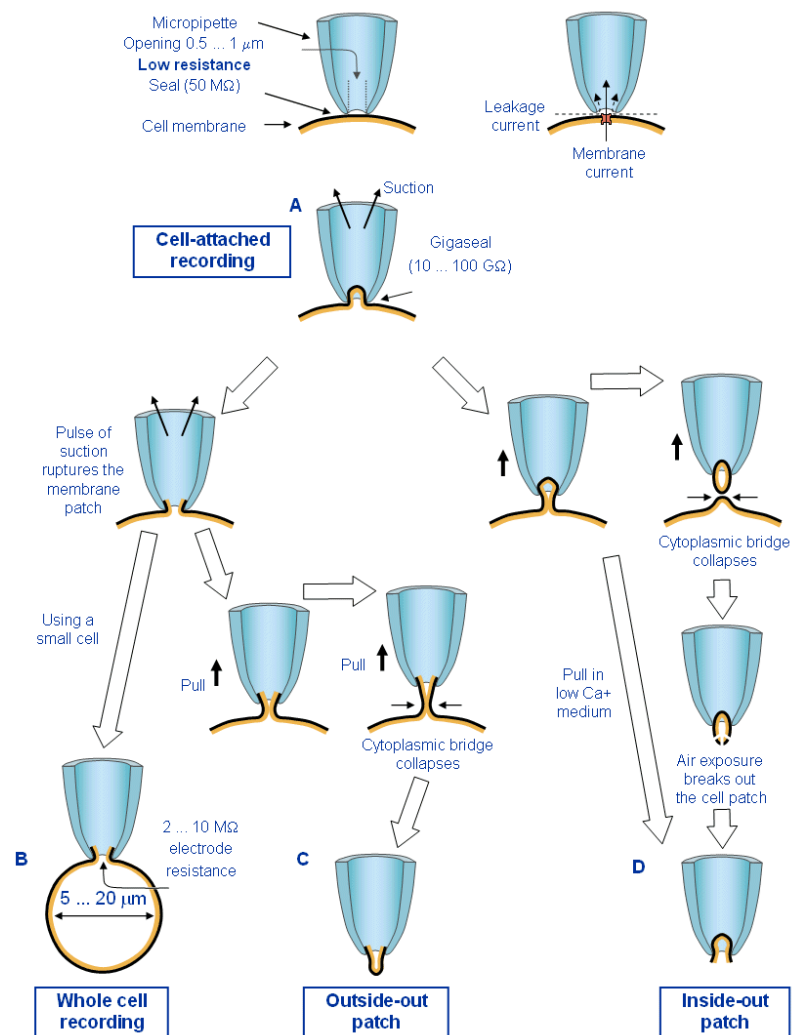
Figure 2.4 Patch-clamp configurations
An illustration showing various patch-clamp configurations, and their relationship to one another. In this thesis, recordings are presented from cell-attached (**A**), whole-cell (**B**) and outside-out (**C**) configurations. From (Malmivuo and Plonsey 1995).

2.5 Agonist application

During whole-cell recordings, GABA was applied either by bath application or, more locally, via a double-barrelled application tool, made from theta-glass (2 mm o.d.; Hilgenberg GmbH) pulled to a tip opening of ~200 μm mounted on a second PatchStar micromanipulator and positioned ~150 μm from the recorded cell. Rapid application was achieved by switching the control solution off at the same time as opening the GABA-containing line.

In the case of outside-out patch recordings (see section 2.4.2), ultra-fast application of GABA was achieved by mounting the theta-glass application tool on a piezoelectric translator (P-265.00; Physik Instrumente Ltd., Cranfield, UK) that rapidly moved in response to application of high voltage (1100 V). In this arrangement, control and agonist solutions flowed continuously through the two barrels and solution exchange occurred when movement of the translator was triggered by the voltage step (Colquhoun *et al.* 1992). The command step (provided by the pCLAMP 10 software) used to drive the high voltage piezo controller (E-508 PICA Piezo Amplifier Module; Physik Instrumente) was first filtered at ~360 kHz (VBF/3 Variable Filter; Kemo Ltd., Beckenham, UK). This ensured smooth movement of the translator without excessive mechanical oscillation. To enable visualization of the solution interface and allow measurement of solution exchange, 2.5 mg ml^{-1} sucrose was added to the agonist solution and the control solution was diluted by 5% with deionised water. The fluid interface between the control solution and GABA-containing solution was driven rapidly across the patch. The length of the command step determined the duration of GABA exposure. The adequacy of solution exchange was monitored at the end of each recording by blowing out the patch and stepping the external solution across the open electrode tip to measure a liquid junction current. The 10-90% rise times for solution exchange were consistently less than 400 μs. In all cases (bath-, local- or ultra-fast application) solution flow was gravity fed.

2.6 Single-channel recording

The cell-attached configuration was used to record single-channel currents activated by a range of GABA concentrations (10 μM – 10 mM). Pipettes were filled with standard 'external' solution with the appropriate GABA concentration added to the solution. The pipette solution was filtered through a 0.22 μm filter before use. The cell-attached configuration is simply the situation following giga-seal formation between the pipette and the membrane (**Figure 2.4**). The tight seal reduces recording noise providing a high signal-to-noise ratio. The advantage of cell-attached configuration is that the cell remains intact, allowing the recording of single-channel currents with normal intracellular ionic and biochemical conditions. Single-channel currents were recorded at pipette potentials between +60 and −100 mV. Currents were amplified with an Axopatch 200A amplifier and recorded on digital audio tape (DTR-1204; BioLogic, Claix, France; DC to 20 kHz) with the amplifier filter (4-pole low pass Bessel type) set at 10 kHz. Data were then replayed from tape, filtered at 2 kHz and digitized at 20 kHz (pCLAMP 8 and Digidata 1200 analogue-to-digital converter; Molecular Devices) for further analysis.

2.7 Data analysis

Analysis of whole-cell currents, macroscopic patch currents and single-channel currents waveforms and all curve fitting was performed with IGOR Pro 5.05 (Wavemetrics Inc., Lake Oswego, OR) using the NeuroMatic suite of analysis functions written by Dr. Jason Rothman (www.neuromatic.thinkrandom.com). Additional single-channel analysis was performed using QuB (ver. 2.0.0.7, http://www.qub.buffalo.edu/). Details of specific analyses are presented in the respective Results chapters.

2.8 Statistical analysis

All results are expressed as mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed using Prism 3.0 (GraphPad Software Inc., San Diego, CA, USA) or R 2.13.1 (the R Foundation for Statistical Computing; http://www.r-project.org/). To compare sample means, unpaired, two-tailed *t*-tests were used (Welch's test – no assumption of equal variance). Comparisons involving more than two groups were performed using one- or two-way ANOVA with Tukey's Multiple Comparison- or Bonferonni-corrected post tests. A critical value of $P < 0.05$ was used to define statistical significance.

2.9 Solutions and drugs

All chemicals mentioned above were purchased from Sigma. The external and internal solutions were prepared with deionised water (**Table 2.2** and **Table 2.3**). A 10 mM stock of GABA was made fresh on the day of experiment and then diluted to the required concentrations with external solution.

Chemical	mM
NaCl	145
KCl	2.4
CaCl$_2$	1
MgCl$_2$	1
Glucose	10
HEPES	10

Table 2.2 Composition of 'external' solution for tsA201 cells
In single-channel experiments, various concentrations of GABA were added as required. The pH of the solution was adjusted to 7.3 with 1M NaOH.

Chemical	mM
CsCl	145
NaCl	2.5
Mg-ATP	4
EGTA-Cs	1
HEPES	10

Table 2.3 Composition of 'internal' solution for tsA201 cells
The pH of the solution was adjusted to 7.3 with 1M CsOH.

Chapter 3

Whole-cell currents from α3-containing GABA$_A$ receptors modified by RNA editing

RNA editing is recognised as a mechanism capable of increasing receptor diversity as well as regulating spatiotemporal receptor expression. When this work was started little was known about the role of RNA editing in regard of the GABA$_A$ receptor. A-to-I editing of Gabra3 (the gene coding for the α3 subunit of the GABA$_A$ receptor) had recently been shown to cause an isoleucine residue in the third transmembrane domain (TM3) to be replaced with a methionine (Ohlson *et al.* 2007).

In order to determine the effect of RNA editing on receptor function, I first performed whole-cell patch-clamp recordings to characterise the macroscopic properties of recombinant α3β2γ2L GABA$_A$ receptors containing either the edited (M) or unedited (I) forms of the α3 subunit.

3.1 Unedited α3(I)β2γ2L GABA$_A$ receptors are functional

Since all early electrophysiological studies with recombinant α3 subunits used the edited (M) isoform – as this was the form originally identified by cloning – it was of interest to determine whether receptors containing the α3 GABA$_A$ in its unedited (I) isoform were functional. To this end, tsA201 cells were transiently co-transfected with cDNAs of either the unedited (I) or edited (M) isoform of the α3 subunit of the GABA$_A$ receptor together with the β2 and γ2L subunit. Initially, GABA was bath applied (~0.3 ml min^{-1}) by a gravity-fed delivery system. GABA-induced currents were observed in cells expressing the unedited α3(I)β2γ2L GABA$_A$ receptors, indicating that GABA$_A$ receptors formed from this subunit are indeed functional.

With 3 μM GABA, the mean current densities (at -100 mV) for the unedited $\alpha3(I)\beta2\gamma2L$ and edited $\alpha3(M)\beta2\gamma2L$ GABA$_A$ receptors were 109.8 ± 29.5 pA/pF ($n = 12$) and 168.1 ± 62.5 pA/pF ($n = 13$), respectively ($P = 0.41$, unpaired t-test). With 200 μM GABA, the corresponding values were 130.5 ± 43.1 pA/F ($n = 11$) and 122.9 ± 30.5 pA/pF ($n = 14$) ($P = 0.88$, unpaired t-test). With 1 mM GABA the mean current densities for the edited and unedited receptors were 161.3 ± 40.6 ($n = 13$) and 141.9 ± 47.9 ($n = 16$) ($P = 0.77$, unpaired t-test). For all concentrations (with two independent transfections for each isoform per concentration), a large variability in current density was apparent. The series resistance was similar across the six conditions (mean 17-24 MΩ) and the variability in response remained following correction for the voltage-drop across the series resistance. The lack of clear concentration-dependence most likely reflected the fact that GABA was bath applied and the currents were therefore steady-state desensitized currents.

Bath application of GABA was employed for experiments where voltage steps or ramps were used to generate current-voltage (I/V) plots (see sections 3.2-3.7). However, to determine the GABA response amplitude more precisely (avoiding extensive desensitization) a modified agonist delivery system was used in which GABA was applied locally *via* a borosilicate glass theta-tube. The application tool had control bath solution connected to one barrel and GABA-containing solution connected to the other. The opening of the tool (~200 μm in diameter) was placed directly in front of the recorded cell and the control flow switched off at the same time as the GABA-containing solution flow was switched on. Representative currents evoked using this approach are shown in **Figure 3.1**. Using this rapid application method, with 3 μM GABA, the mean peak current densities (at -60 mV) for the unedited $\alpha3(I)\beta2\gamma2L$ and edited $\alpha3(M)\beta2\gamma2L$ GABA$_A$ receptors were 118.1 ± 25.8 pA/pF ($n = 12$) and 170.4 ± 74.4 pA/pF ($n = 6$), respectively ($P = 0.42$). For 1 mM GABA, the corresponding values were 2089.9 ± 488.6 pA/pF ($n = 7$) and 582.7 ± 185.0 pA/pF ($n = 6$) ($P = 0.021$). **Figure 3.2** shows a concentration-response relationship generated from this

data. Although incomplete, this strongly suggests a difference between the $\alpha3$(I)- and $\alpha3$(M)-containing receptors, with the edited (M) form exhibiting either a reduced surface expression, a reduced single-channel conductance, a reduced open probability, or some combination of these.

α3(I)β2γ2L   α3(M)β2γ2L

3 μM GABA   3 μM GABA

10 pA
1 s

1 mM GABA   1 mM GABA

500 pA
1 s
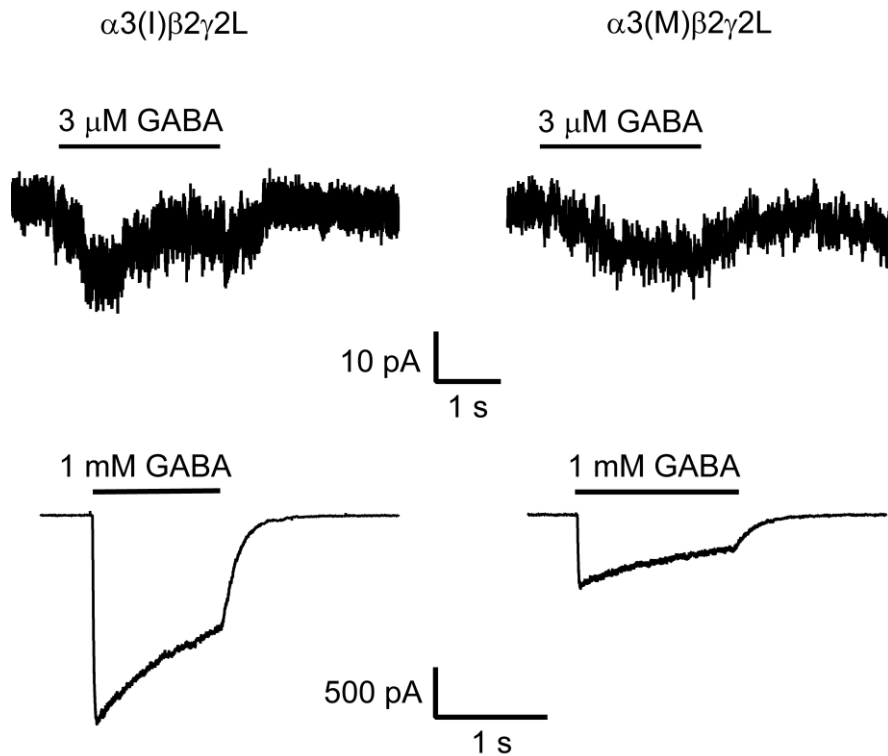
Figure 3.1 GABA$_A$ receptors formed from unedited (I) α3 subunits are functional.
tsA201 cells were transfected with either the unedited (I) or the edited (M) isoform of the α3 subunit of the GABA$_A$ receptor together with β2 and γ2L subunit cDNAs. Cells were held at −70mV and GABA was applied *via* the theta glass application tool. For illustration, currents were filtered at 1 kHz.
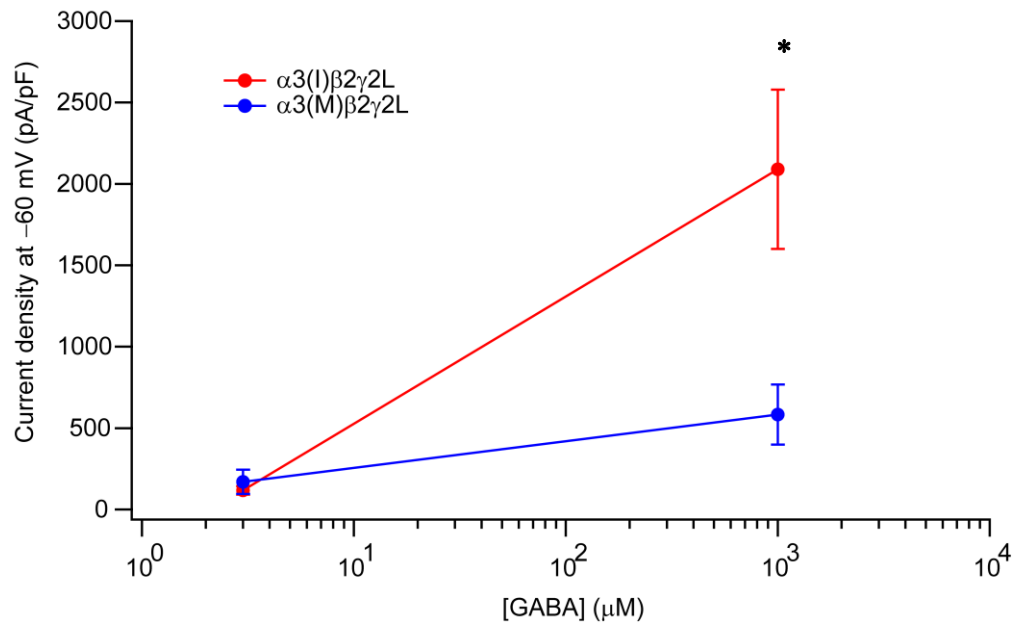
Figure 3.2 Responses of $\alpha3$ subunit-containing $GABA_A$ receptor to different concentrations of GABA.
Current amplitudes were compared between the unedited (I) and edited (M) isoforms at various GABA concentrations and are shown as mean $\pm$ SEM (n = 6-12). $*$ indicates $P < 0.05$ when compared with $\alpha3$(M)$\beta2\gamma2$L (unpaired t-test).

3.2 Does editing of Gabra3 affect GABA$_A$R rectification?

I next chose to investigate the functional consequences of Gabra3 editing on the voltage-dependence of GABA-evoked currents. Voltage steps were applied in the presence of GABA to cells expressing α3β2γ2L GABA$_A$ receptors (**Figure 3.3**). The holding voltage was stepped from −100 to +60 mV in +10 mV increments. I/Vs of GABA-evoked currents were derived by subtracting control currents from those obtained in the presence of GABA. 'Instantaneous' and 'steady-state' currents were measured in the first 20 ms (instantaneous) and the last 20 ms (steady-state) of each 300 ms voltage step. For the example shown in **Figure 3.3**, for edited α3(M)β2γ2L GABA$_A$ receptors in the presence of 200 μM GABA, the instantaneous I/V showed slight outward rectification while the steady-state I/V appeared essentially linear.

The extent of rectification was quantified by measuring the rectification index (RI). This was calculated as the ratio of the slope conductances underlying the GABA$_A$ receptor-mediated current at positive (+40 to +60 mV) and negative (−40 to −60 mV) voltages. For the example shown in **Figure 3.3**, the RI of the instantaneous response was 1.36.

I first examined the voltage-dependence of GABA-evoked currents using bath application of 200 μM GABA. For instantaneous currents, the mean RI of unedited α3(I)β2γ2L receptors was 3.15 ± 0.44 (*n* = 5) and with edited α3(M)β2γ2L GABA$_A$ receptors it was 1.67 ± 0.30 (*n* = 7) (*P* = 0.12, unpaired *t*-test). For steady-state currents the corresponding values were 3.96 ± 1.19 and 2.05 ± 0.40 (*n* = 5 and 7) (*P* = 0.08, unpaired *t*-test). Given the apparent trend toward increased outward rectification of the unedited α3(I)β2γ2L receptors, I examined this issue further, using both lower (3 μM) and higher (1 mM) GABA concentrations.
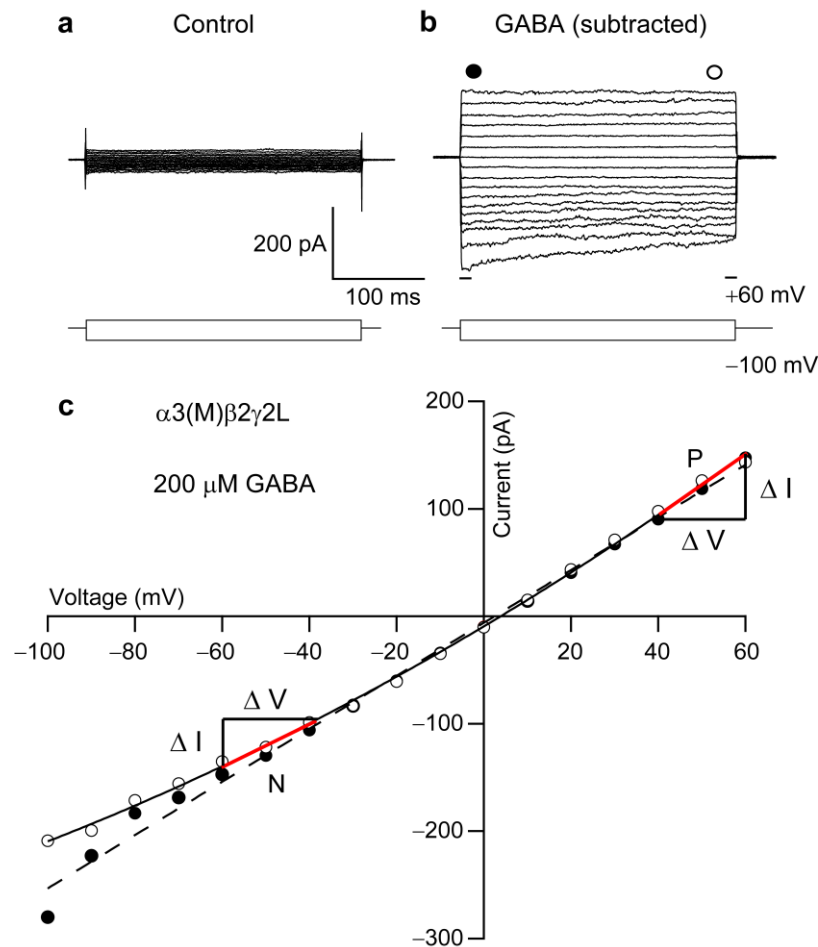
Figure 3.3 Representative net instantaneous and steady-state I/V relationships generated using voltage steps.
Representative responses from a tsA201 cell expressing edited α3(M)β2γ2L GABA$_A$ receptors. Voltage steps (−100 to +60 mV in +10 mV increments, 300 ms) were applied before (**a**) and during application of 200 μM GABA. The average control traces were subtracted from the traces obtained in the presence of GABA to yield the subtracted GABA traces as shown in (**b**). The net instantaneous (•) and steady-state currents (○) I/V relationships are shown in (**c**). Instantaneous currents were measured at the first 20 ms of the subtracted GABA currents, whereas steady-state currents were measured at the last 20 ms of the subtracted GABA currents (indicated by bars). Rectification index (RI) was calculated as a ratio of the slope conductances at positive (+40/60, 'P') and negative (−40/60, 'N') voltages.

Instantaneous and steady-state I/V relationships for $\alpha3(I)\beta2\gamma2L$ and $\alpha3(M)\beta2\gamma2L$ GABA$_A$ receptors at different GABA concentrations are shown in **Figures 3.4** and **3.5**. Together, these experiments suggested that rectification was not affected by $\alpha3$ editing, as the instantaneous and steady-state I/V relationships of both isoforms appeared identical, regardless of the GABA concentration (**Figure 3.4a** and **b; Figure 3.5a** and **b**). With a low (3 μM) GABA concentration, the mean RI of the instantaneous current was 3.49 $\pm$ 0.70 and 3.35 $\pm$ 0.67 for unedited and edited GABA$_A$ receptors, respectively (n = 8 and 7; P = 0.90, unpaired t-test). For the steady-state current, the corresponding values were 3.36 $\pm$ 0.64 and 2.93 $\pm$ 0.40 (n = 8 and 7) (P = 0.59, unpaired t-test) (**Figure 3.4c**). A similar pattern was obtained with 1 mM GABA (**Figure 3.5c**). For instantaneous current, the mean RI for unedited $\alpha3(I)\beta2\gamma2L$ and edited $\alpha3(M)\beta2\gamma2L$ GABA$_A$ receptors was 2.22 $\pm$ 0.28 and 2.75 $\pm$ 0.73 (n = 8 and 6) (P = 0.49, unpaired t-test). For steady-state current, the corresponding RI values were 3.98 $\pm$ 0.84 and 3.99 $\pm$ 1.11 (n = 8 and 6) (P = 0.10, unpaired t-test).
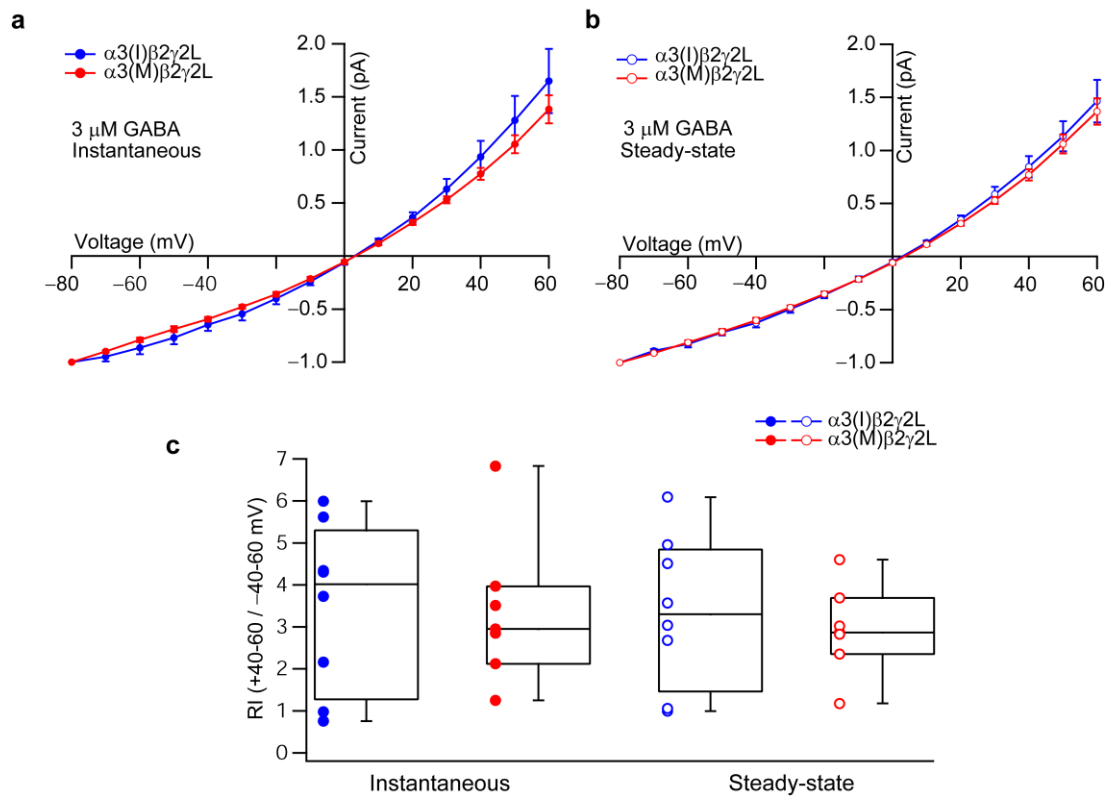
Figure 3.4 α3β2γ2L GABA$_A$ receptors containing unedited (I) or edited (M) subunits display similar rectification in response to 3 μM GABA.
Normalised I/V relationships of unedited (I) and edited (M) α3β2γ2L GABA$_A$ receptors obtained with 3 μM GABA. Average voltage step-generated currents were normalised (–80 mV) and plotted against command voltages. Instantaneous (**a**) and steady-state (**b**) I/V relationships of both isoforms display similar RIs. Data are presented as mean and standard error of mean (SEM; *n* = 7-8). (**c**) Boxplots of instantaneous and steady-state RIs for unedited (I) and edited (M) α3β2γ2L GABA$_A$ receptors. Boxes show 25-75th percentiles, whiskers show 10-90th percentiles and horizontal bars show medians. Symbols denote individual data points.
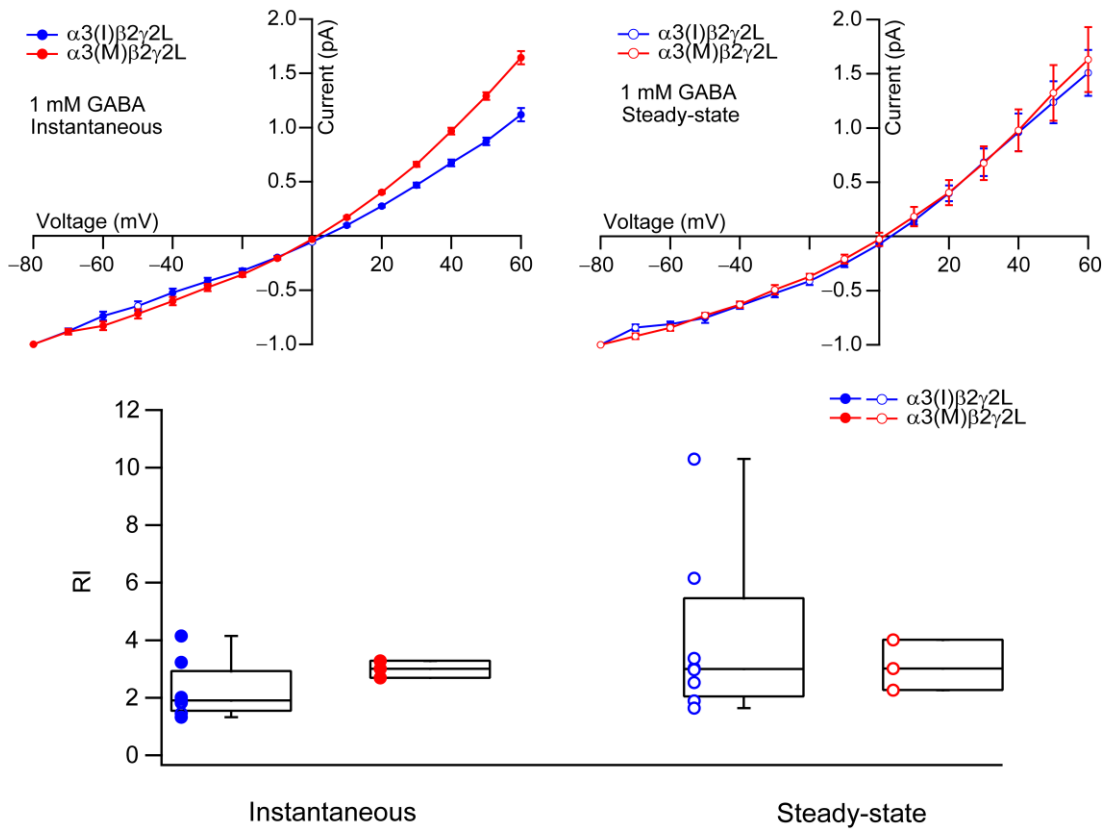
Figure 3.5 $\alpha3\beta2\gamma2L$ receptors containing unedited (I) or edited (M) subunits display similar rectification properties with 1 mM GABA.
Instantaneous (**a**) and steady-state (**b**) I/V relationships of unedited (I) and edited (M) $\alpha3\beta2\gamma2L$ GABA$_A$ receptors obtained with 1 mM GABA. Average voltage step-generated currents were normalised (–80 mV) and plotted against command voltages. Instantaneous (**a**) and steady-state (**b**) I/V relationships of both isoforms display similar RIs. Data are presented as mean and standard error of mean (SEM; n = 6-8). (**c**) Boxplots of instantaneous and steady-state RIs for unedited (I) and edited (M) $\alpha3\beta2\gamma2L$ GABA$_A$ receptors (as described in **Figure 3.4**).

3.3 Rectification of $\alpha3\beta2\gamma2L$ receptors is concentration-dependent but is unaffected by Gabra3 editing

Given the ill-controlled desensitization likely when GABA was applied via the bath, I next employed the theta-glass application tool to generate briefer GABA responses, during which the voltage-dependence was assessed using voltage ramps. The experimental procedure is illustrated in **Figure 3.6**. Voltage ramps (major limb from -100 to $+60$ mV at 53 mV s^{-1}) were applied before, during and after GABA application (**Figure 3.6a** and **b**). The average of the 'before' and after' responses was subtracted from that obtained in the presence of GABA to yield the GABA-mediated response (**Figure 3.6c**). This was then plotted as an I/V relationship and the RI determined from the slope conductance at negative and positive voltages (**Figure 3.6d**). **Figure 3.7a** and **b** show raw I/Vs from $\alpha3(I)\beta2\gamma2L$ ($n = 12$) and $\alpha3(M)\beta2\gamma2L$ GABA$_A$ receptors ($n = 12$) generated in response to 3 μM GABA. The I/Vs were normalised (to the current at -80 mV) and averaged (**Figure 3.7c** and **d**). The global average I/Vs from both $\alpha3(I)$- and $\alpha3(M)$-containing receptors exhibited similar outward rectification. This is seen more clearly in **Figure 3.8a**, where the I/Vs are superimposed. **Figure 3.8b** shows corresponding global average I/Vs obtained with 1 mM GABA. The RI values for both conditions are plotted in **Figure 3.8c**. The I/V relationships of both isoforms displayed outward rectifying I/V relationships with 3 μM GABA but linear relationships with 1 mM GABA. With 3 μM GABA, the average RI of unedited $\alpha3(I)\beta2\gamma2L$ and edited $\alpha3(M)\beta2\gamma2L$ receptors was 3.88 ± 0.31 and 3.54 ± 0.34. With 1 mM GABA, corresponding values were 1.00 ± 0.13 and 1.14 ± 0.16 ($n = 7$ and 12). A two-way ANOVA was conducted to compare the effect of editing status and GABA concentration on RI. Although there was a significant effect of GABA concentration ($F_{1, 39} = 93.88$, $P < 0.0001$), editing of the $\alpha3$ subunit had no significant effect on RI ($F_{1, 39} = 0.14$, $P = 0.71$).
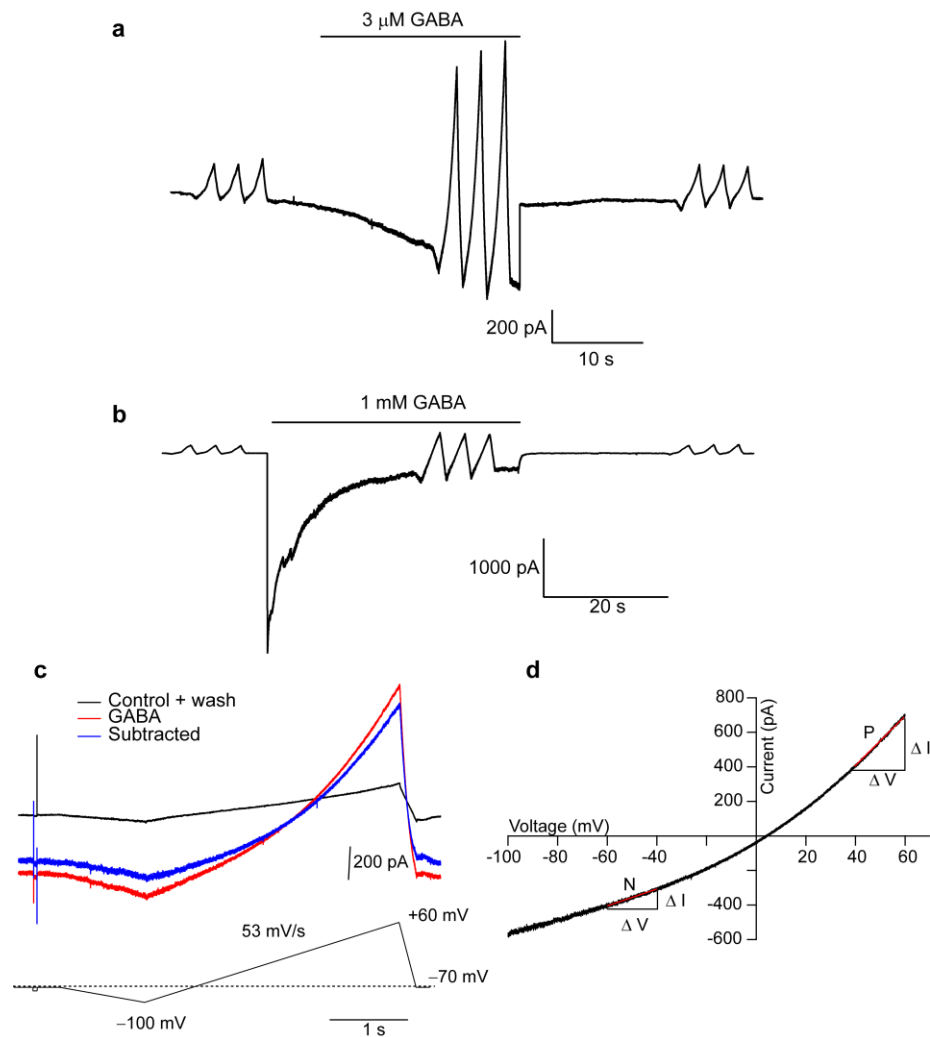
Figure 3.6 Experimental protocol for whole-cell ramp experiments.
Representative traces showing GABA-activated whole-cell responses (3 μM
(**a**) or 1 mM (**b**) GABA) obtained from tsA201 cells expressing the edited (M)
α3 GABA$_A$ receptors. GABA was locally applied via theta-borosilicate glass
capillaries placed close to the recorded cell. (**c**) Slow (53 mV s^{-1}) voltage
ramps from -100 mV to $+60$ mV applied before, during and after 1mM GABA
application. Three ramp trials were then averaged in the presence (red) and
absence (black) of GABA, and subtraction of the two gave the current shown
in blue. (**d**) The net I/V relationship obtained from **c** in response to 1mM
GABA. RI was calculated as a ratio of the slope conductances at positive
($+40/60$, 'P') and negative ($-40/60$, 'N') voltages.
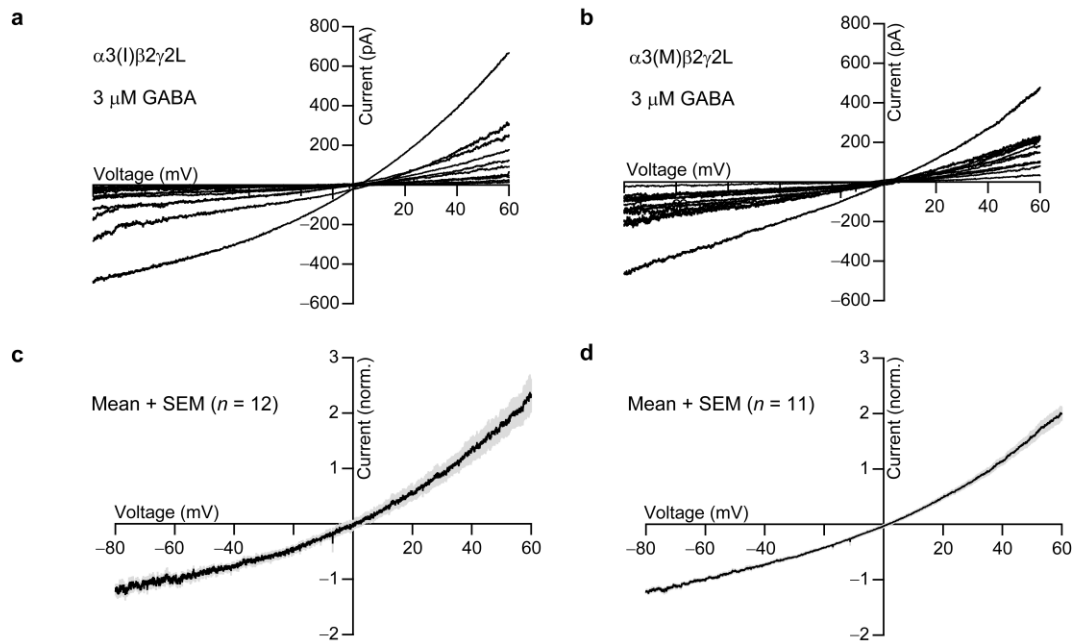
Figure 3.7 Ramp-generated I/V relationships for recombinant α3 GABA$_A$ receptors.
(**a**) and (**b**) show raw data from unedited (I) and edited (M) α3 GABA$_A$ receptors in response to 3 μM GABA. (**c**) and (**d**) show corresponding normalised (–60 mV) I/V relationships. The global average traces (*n* = 12 and 11) are shown together with their associated SEMs (shaded).
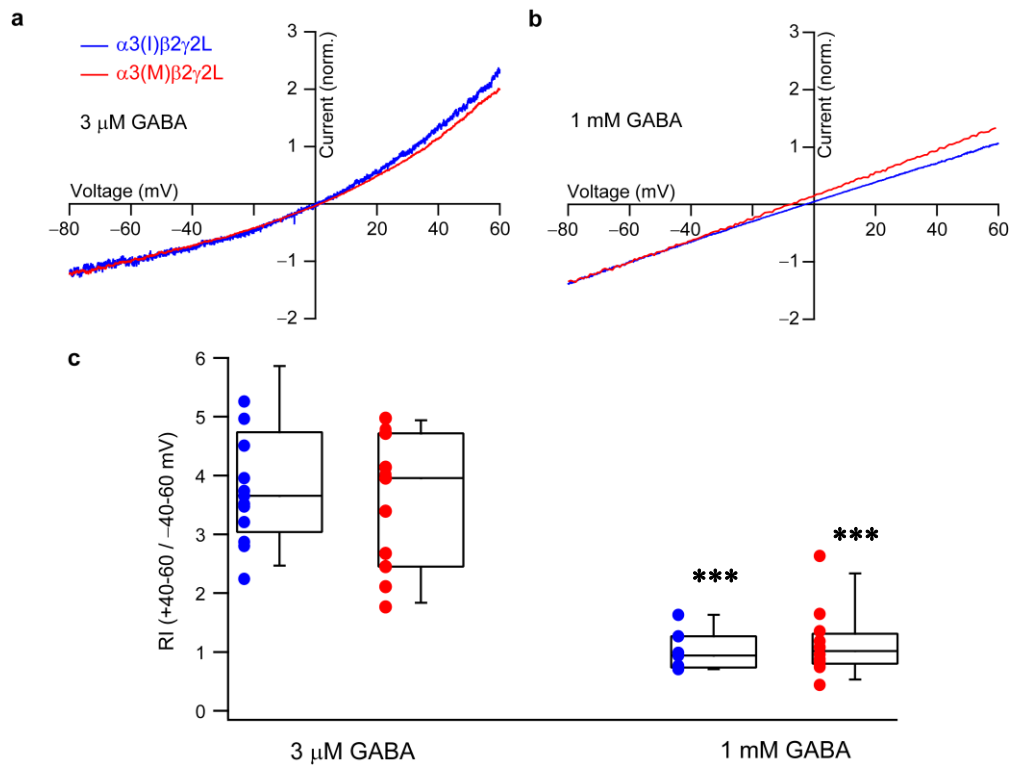
Figure 3.8 In whole-cell ramp experiments, rectification is concentration-dependent but not affected by α3 editing.
Global averaged normalised (–60 mV) I/V relationships of unedited (I) and edited (M) α3β2γ2L GABA$_A$ receptors obtained with 3 μM (**a**) or 1 mM (**b**) GABA. Average currents were normalised and plotted against command voltages. The global averaged I/V relationships of both isoforms display a linear relationship with 1 mM GABA (n = 7 and 12) as compared to outward rectification with 3 μM GABA (n = 12 and 11). (**c**) Boxplots showing the pooled data (for details see **Figure 3.3**). ∗∗∗ Indicates $P < 0.0001$ when compared with 3 μM GABA.

3.4 Similar behaviour of α3(I)- and α3(M)-containing receptors with different ramp protocols

During the course of these experiments, a first report describing functional effects of α3 editing appeared (Rula *et al.* 2008). Consistent with my data (**Figure 3.2**), these authors observed a decreased peak current amplitude in response to 1 mM GABA with edited α3(M)-containing receptors expressed in HEK293T cells. However, they also reported differences in the voltage-dependence of the GABA responses. Specifically, they observed strong outward rectification with α3(I)-containing receptors (RI = 3.1; ratio of conductance between +30 and +40 mV to the conductance between −30 and −40 mV), compared with more linear currents from edited α3(M)-containing receptors (RI = 1.7).

My failure to observe an effect of editing on rectification could reflect differences in the experimental protocol used. Two differences in the experimental protocol were the ramp speed (53 mV s^{-1} in the present study, compared to 100 mV s^{-1} in Rula *et al.*) and the voltage range over which rectification was calculated. As rectification of the macroscopic responses may reflect voltage-dependent changes in gating kinetics, it is possible that the expression or magnitude of rectification could depend on the speed of the imposed voltage changes. Therefore, I performed additional experiments using the faster ramp speed and a modified calculation of RI. **Figure 3.9** shows I/Vs obtained at different ramp speeds. The I/V relationships of the two isoforms were almost identical, regardless of the ramp speed. For this comparison, I also calculated RI values as described by Rula *et al.* With the slower ramp speed (53 mV s^{-1}), the RI of unedited α3(I)β2γ2L (3.59 ± 0.63, *n* = 12) was not significantly different from that of edited α3(M)β2γ2L (2.70 ± 0.27, *n* = 12, *P* = 0.20, unpaired *t*-test). Similarly, at the faster ramp speed (100 mV s^{-1}) there was no difference in RI values (2.94 ± 0.72 and 2.65 ± 0.58, *n* = 6 and 7 respectively; *P* = 0.76, unpaired *t*-test). No significant effect of ramp speed on RI was observed ($F_{1,\,39}$ = 0.37, *P* = 0.54, two-way ANOVA). These findings support my previous observation that rectification is not affected by Gabra3 editing.
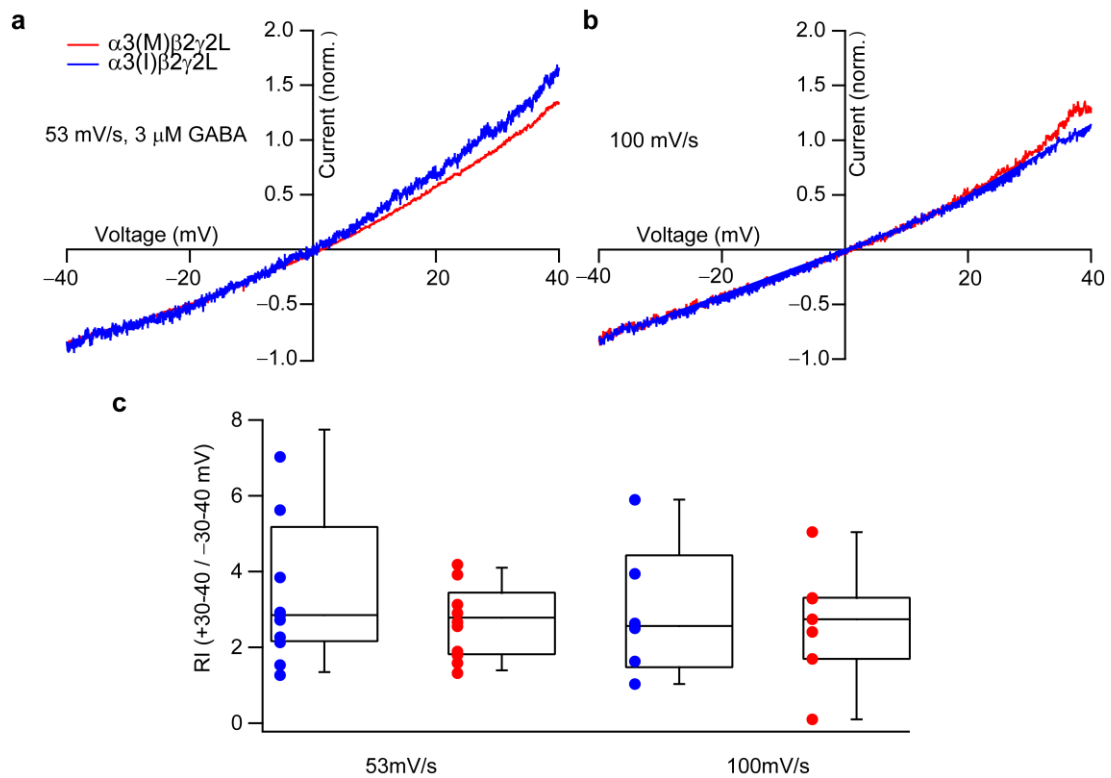
Figure 3.9 Rectification is not affected by changes in ramp speed.
Global average normalised I/V relationships of unedited (I) and edited (M) $\alpha3\beta2\gamma2L$ GABA$_A$ receptors obtained with (**a**) slow (both n = 12) or (**b**) fast ramp speed (n = 6 and 7). Average currents were normalised and plotted against command voltages. The I/V relationships of both isoforms display similar I/V relationships regardless of ramp speed. (**c**) Boxplots show the pooled data. Boxes show 25-75th percentiles, whiskers show 10-90th percentiles and horizontal bars show medians. Symbols denote individual data points.

3.5 Rectification differs between β3- and β2-containing receptors

A more obvious difference between my experiments and those of Rula *et al.* is the choice of β subunit; whereas I used β2, Rula *et al.* used β3. In fact, using the β3 subunit may be more appropriate, as β3 and γ2L subunits have been shown to be present in the same brain regions as the α3 subunit, in both mature and developing animals (Laurie *et al.* 1992a, Pirker *et al.* 2000). Accordingly, I repeated my experiments using the β3 subunit.

tsA201 cells were co-transfected with plasmids containing rat β3, γ2L and either unedited (I) or edited (M) α3 subunit cDNAs. Expression of functional receptors was first assessed with bath application of 3 μM GABA. At −60 mV I obtained large whole-cell currents with both unedited α3(I)β3γ2L receptors (-1623 ± 355 pA; $n = 6$) and edited α3(M)β3γ2L receptors (-824 ± 341 pA; $n = 5$). These current amplitudes were significantly greater than those obtained with the corresponding β2-containing receptors (both $P < 0.0001$, unpaired *t*-test; **Figure 3.10**). Although Gabra3 editing appeared to have an effect on the size of currents from β3-containing receptors, the difference was not statistically different ($P = 0.62$, unpaired *t*-test).

The degree of rectification was dependent on subunit composition. As shown in **Figure 3.11**, currents recorded from cells expressing α3β3γ2L GABA$_A$ receptors showed some outward rectification, but this was significantly less than seen in cells expressing α3β2γ2L receptors. For the β3-containing receptors, the RI was 1.49 ± 0.15 ($n = 6$) with the unedited α3(I) subunit and 1.45 ± 0.45 ($n = 5$) with the edited α3(M) subunit.

I also examined the behaviour of receptors formed from α3β3 subunits only. The RI of unedited α3(I)β3 ($n = 7$) and edited α3(M)β3 ($n = 7$) receptors was 1.15 ± 0.11 and 2.55 ± 0.39, respectively. As shown in **Figure 3.11**, this difference was statistically significant ($P < 0.05$). Together, these results indicate that α3-containing GABA$_A$ receptors with different subunit

compositions can be differentially sensitive to changes in membrane potential.
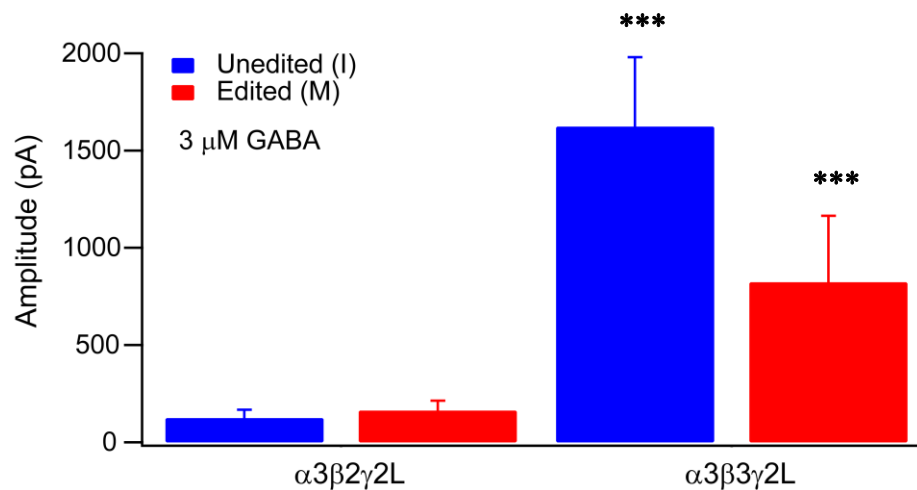
Figure 3.10 Incorporation of different β subunits affects the GABA response.

The current amplitude depended on the β subunit incorporated in the α3-containing GABA$_A$ receptors. GABA (3 μM) reliably evoked currents from cells expressing either unedited α3(I) or edited α3(M) receptors. The peak current amplitudes recorded from the α3β3γ2L receptors (n = 6 and 5 for α3(I)- and α3(M)-containing receptors, respectively) were significantly larger than from α3β2γ2L GABA$_A$ receptors (n = 12 for both isoforms). Current amplitudes are shown as mean ± SEM. ∗∗∗ indicates $P < 0.001$ when compared with α3β2γ2L (unpaired t-tests).
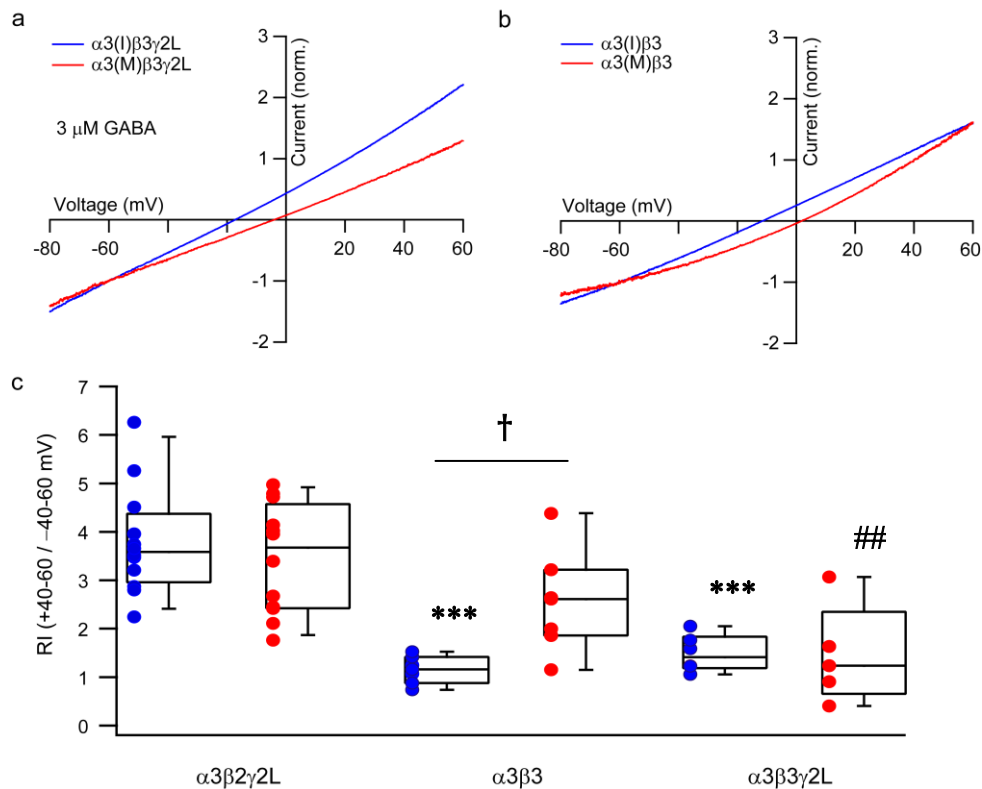
Figure 3.11 Incorporation of a different β subunit affects rectification.
Global average normalised I/V relationships of unedited (I) and edited (M)
α3β3γ2L (**a**) and α3β3 (**b**) GABA$_A$ receptors in response to 3 μM GABA
(normalised to value at –60 mV). (**c**) Boxplots showing the pooled data (for
details see **Figure 3.3**). A one-way ANOVA was conducted to compare the
effects among the different subunit combinations. Overall, there were
significant differences in RI ($F_{5, 43}$ = 12.96, P < 0.0001). Tukey's Multiple
Comparison Tests showed that α3β3-containing receptors differed from
α3β2-containing receptors (✱✱✱ P < 0.001 compared to α3(I)β2γ2L, ## P <
0.01 compared to α3(M)β2γ2L). A two-way ANOVA was conducted to
compare the effect of α3 editing status on the different receptor assemblies.
Overall, there was a significant effect of αβγ subunit composition ($F_{2, 43}$ =
22.93, P < 0.0001) but no significant effect of editing state ($F_{1, 43}$ = 1.41, P =
0.21). However, there was a significant interaction between the two main
effects, indicating that editing of the α3 subunit did not have the same effect
on all receptor types examined   ($F_{2, 43}$ = 3.44, P = 0.041). Pairwise
comparisons showed that the α3 editing state had a significant effect only on
the αβ dimers († P < 0.05).

3.6 Absence of endogenous Gabra3 editing

The significant difference I observed between the rectification of $\alpha3(I)\beta3$ and $\alpha3(M)\beta3$ receptors suggested that endogenous editing of the Gabra3 transcripts was not a confounding issue in my recordings, artificially obscuring a genuine difference between $\alpha3(I)\beta3\gamma2L$ and $\alpha3(M)\beta3\gamma2L$ receptors. Nevertheless, to control for the possibility that my observations with tri-heteromeric receptors may be have been affected by conversion of the transfected $\alpha3(I)$ to $\alpha3(M)$ by endogenous ADAR, I performed additional experiments with an 'non-editable' form of the subunit. Non-editable Gabra3 cDNA (with the cytidine at the A:C mismatch editing site converted to guanosine; **Fig. 1.6**) was made by site-directed mutagenesis. The mutant cDNA was then transiently transfected into tsA201 cells and the whole-cell ramp experiments were repeated. As shown in **Figure 3.12a**, with 3 μM GABA, the I/V relationship of the non-editable $\alpha3(I_{NE})\beta3\gamma2L$ isoform was similar to those of the other two $\alpha3\beta3\gamma2L$ GABA$_A$ receptor isoforms. The RI was 1.76 ± 0.28 ($n = 10$), which was not significantly different from the RIs of unedited $\alpha3(I)\beta3\gamma2L$ (1.49 ± 0.15; $n = 6$) and edited $\alpha3(M)\beta3\gamma2L$ (1.45 ± 0.45; $n = 5$) ($P = 0.72$, one way ANOVA) (**Figure 3.12b**).
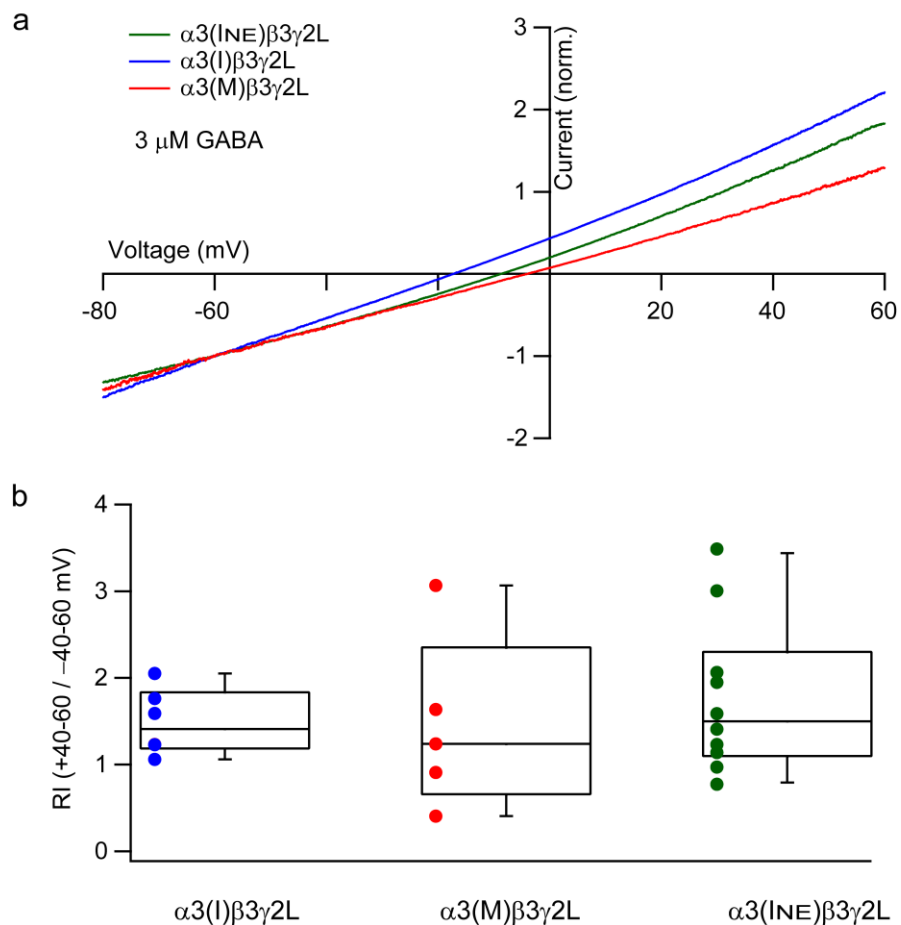
Figure 3.12 The unedited α3(I) subunit is not modified endogenously in the heterologous expression system.
Non-editable (G) Gabra3 cDNA was formed by PCR-direct mutagenesis and transiently transfected into tsA201 cells. **a**) Global average normalised I/V relationships of unedited α3(I)β3γ2L (n = 5), edited (n = 5) and non-editable α3(G)β3γ2L GABA$_A$ receptors (n = 10) exhibit similar modest outward rectification. **b**) Boxplots showing the corresponding RIs. No significant difference in RI between the unedited (I) and non-editable (I$_{NE}$) α3β3γ2L GABA$_A$ receptors, suggesting that endogenous editing of the Gabra3 transcripts was not obscuring a difference in behaviour.

3.7 Comparison with receptors containing other α subunits

As discussed in Chapter 1, the GABAergic system undergoes profound developmental changes – GABA$_A$ receptors exist as different subtype variants showing unique functional properties and defined spatio-temporal expression pattern at different stages of development (Ben-Ari *et al.* 2007). In the cerebellum, for example, immature GABAergic synapses express α3-subunit containing GABA$_A$ receptors that are progressively replaced by α1 subunit-containing receptors at mature synapses (Barberis *et al.* 2007, Ortinski *et al.* 2004). It has been suggested that long-lasting α3-mediated currents promote trophic actions in immature neurons, whereas fast α1-mediated hyperpolarizing IPSCs contribute to increase the network temporal resolution in mature neurons (Represa and Ben-Ari 2005). Similarly, in the cerebellum again, tonic GABA$_A$ receptor-mediated currents in granule cells have been shown to increase over development due to the progressive expression of the α6 and δ subunits (Brickley *et al.* 1996, Brickley *et al.* 2001).

The I/V relationship of tonically active GABA$_A$Rs in hippocampal pyramidal cells – likely mediated by α5 subunit-containing GABA$_A$Rs (Caraiscos *et al.* 2004, Glykys and Mody 2007, Prenosil *et al.* 2006, Scimemi *et al.* 2005) – exhibits strong outward rectification, particularly at low GABA concentrations (Pavlov *et al.*, 2009). Such rectification has been suggested to be of importance at it would determine the voltage range over which these tonically active receptors would influence neuronal excitability (Pavlov *et al.*, 2009). I observed marked outward rectification with α3$\beta\gamma$ GABA$_A$ receptors (particularly α3β2γ2L receptors) when activated by a low concentration of GABA. Thus, it was particularly interesting to note that a recent study by Marowsky *et al.* suggested that in the basolateral amygdala α3-containing GABA$_A$ receptors are predominantly extrasynaptic, and contribute to a robust tonic current in principal cells (Marowsky *et al.* 2012). To investigate whether outward rectification may be a common feature of α-subunit containing GABA$_A$ receptors that participate in tonic as opposed to phasic signalling, I

compared the properties of α3 subunit-containing receptors with those of α1- and α6-containing receptors.

Currents were recorded from α1β2γ2L, α6β2γ2L and α6β3δ receptors transiently expressed in tsA201 cells. As illustrated in **Figure 3.13a**, the α1β2γ2L receptor currents displayed minimal desensitization during a prolonged (approximately 6 s) application of GABA and deactivated rapidly upon removal of GABA. In contrast, the α6β2γ2L currents desensitized more rapidly and more extensively (**Figure 3.13b**). A similar behaviour was seen with α6β3δ currents. The desensitization and deactivation rates of these receptors were not quantified due to the limitations of the whole-cell application.

As shown in **Figure 3.14**, the currents from the different α-containing receptors showed different voltage-dependence. In the presence of 3 μM GABA, the α3 subunit-containing (α3(M)β2γ2L) receptors exhibited the greatest extent of outward rectification with RI 3.54 ± 0.34 (n = 12) followed by the α1β2γ2L receptors (RI = 2.13 ± 0.38, n = 6). By contrast, α6β2γ2L showed, if anything, a slight inward rectification (RI = 0.76 ± 0.50, n = 4), as did α6β3δ receptors (RI = 0.89 ± 0.40, n = 6). One-sample t-tests showed that the RI of the α1- and α3-containing receptors was significantly different from 1 (P = 0.031 and P < 0.0001, respectively), whereas the RI of the two α6-containing receptors was not different from 1 (both P = 0.67). Thus, for this (admittedly limited) range of receptors, when activated by 3 μM GABA, there did not appear to be an obvious link between the degree of outward rectification and recognised or putative roles of the receptor types in mediating tonic as opposed to phasic signalling.
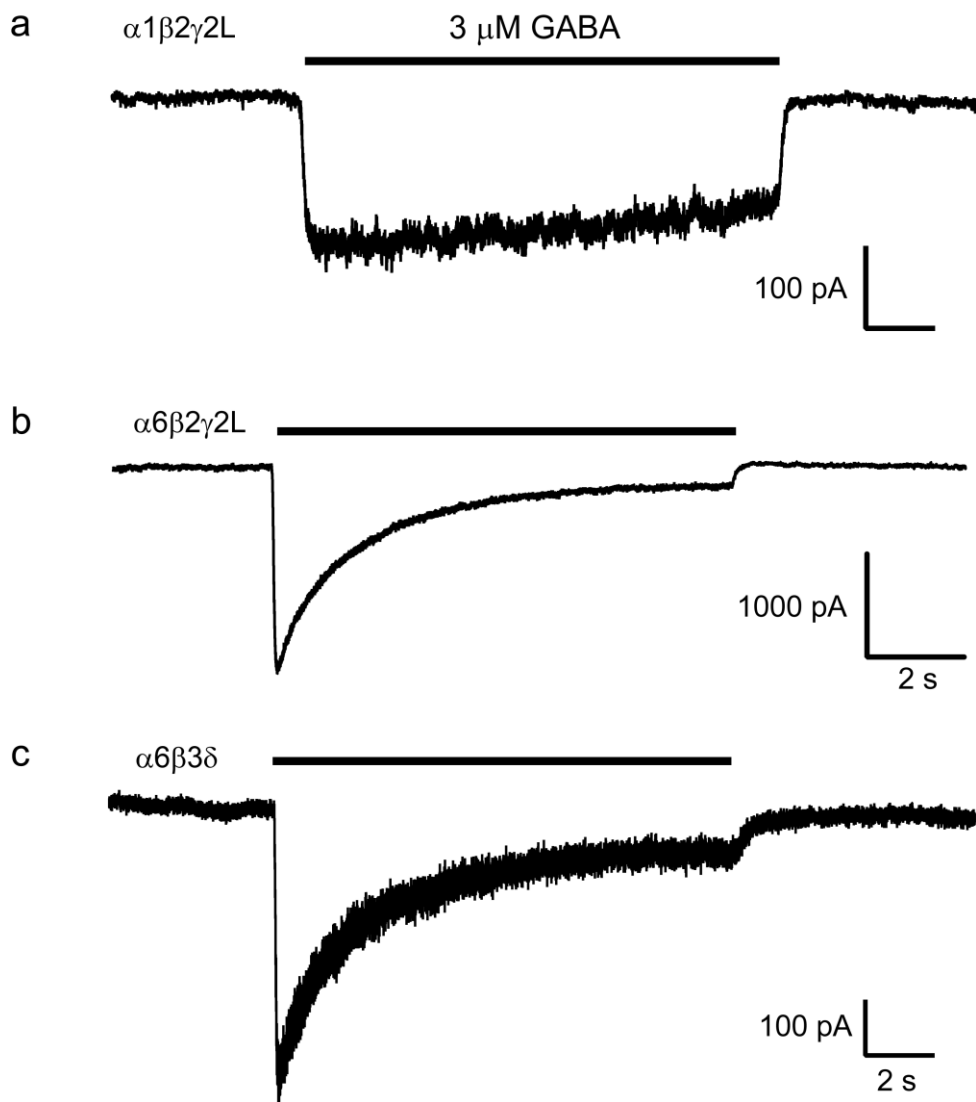
Figure 3.13 Whole-cell currents from α1 and α6-containing receptors.
Representative currents evoked by local application of GABA (3 μM; solid bars) at –60 mV. **a**) Little macroscopic desensitization was apparent for α1β2γ2L currents, and deactivation was rapid following removal of GABA. **b**) Currents from α6β2γ2L receptors desensitized extensively under identical conditions. **d**) A similar kinetic pattern was seen with currents from for α6β3δ receptors.
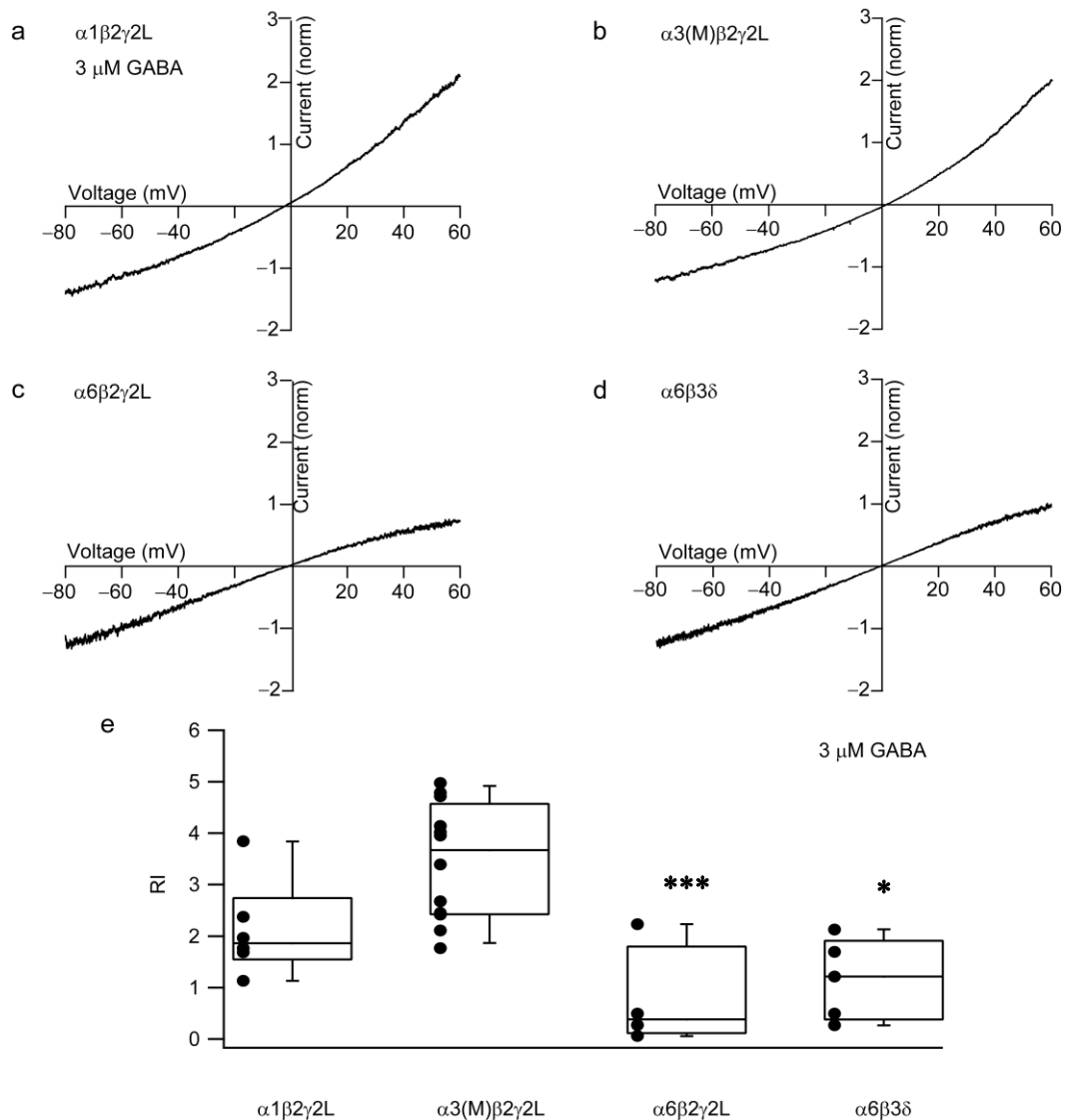
Figure 3.14 Differences in rectification between α1-, α3- and α6-containing receptors.

Whole-cell currents were recorded from tsA201 cells transfected with cDNAs encoding **a)** α1β2γ2L (n = 6), **b)** α3(M)β2γ2L (n = 12; from **Fig. 3.8a**), **c)** α6β2γ2L (n = 4) and **d)** α6β3δ receptors (n = 5) in response to 3 μM GABA and global average I/Vs were generated following ramp changes in membrane voltage. **e)** Boxplots comparing the RIs of these receptors. A one-way ANOVA was conducted to compare RI among the different subunit combinations. Overall, there were significant differences in RI ($F_{3, 26}$ = 10.27, P = 0.0002). Tukey's Multiple Comparison Tests showed that α3(M)β2γ2L receptors differed from α6β2γ2L receptors (*** P < 0.001) and from α6β3δ receptors (* P < 0.01). There was no significant difference between the two α6-containing receptors (both P > 0.05).

3.8 Discussion

RNA editing of the $\alpha 3$ subunit-containing GABA$_A$ receptors has recently been identified and proposed as an additional mechanism capable of increasing receptor diversity. As the original cloning identified the edited form (Ohlson *et al.* 2007, Schofield *et al.* 1987) the properties of the unedited form remained unknown. My initial results showed that the unedited subunit could form functional receptors, however the reduced whole-cell current with 1 mM GABA suggested that function or expression may differ. This observation is consistent with the recent findings of Daniel *et al.* (Daniel *et al.* 2011), who reported that I/M editing reduced the number of cell-surface and total number of $\alpha 3$ subunits. It was shown that RNA editing of the Gabra3 transcript increased concurrent with the decrease in $\alpha 3$ protein levels during development. The $\alpha 3$(M) internalized more efficiently than $\alpha 3$(I), and the reduced $\alpha 3$(M) surface level was suggested to reflect to an enhanced lysosomal degradation, indicating that $\alpha 3$(I) is more stable and therefore recycles to the cell surface to a higher extent than $\alpha 3$(M). However, the study of Daniel *et al.* could not exclude the possibility that the reduced level of membrane-bound $\alpha 3$(M) reflected less efficient receptor assembly compared with the $\alpha 3$(I) subunit (Daniel *et al.* 2011). Notably, the reduced $\alpha 3$(M) surface level was shown to be independent of the subunit combination, as it was observed for $\alpha 3$ in combination with either $\beta 2$ or $\beta 3$ subunits. Amino acid substitution at the corresponding I/M site in the $\alpha 1$ subunit had a similar effect on cell surface presentation, indicating the importance of this change for receptor trafficking.

From my data thus far, I cannot directly assess the effects of editing on receptor trafficking. This is because the magnitude of the macroscopic currents ($\bar{I}$) reflects not only the number of receptors (N) but also their single-channel current (i) and their open probability (P_o), according to the relationship $\bar{I} = i * N * P_o(t)$. Nevertheless, the 4-fold difference in current density I observed with 1 mM GABA between cells expressing the edited $\alpha 3$(M) and the unedited $\alpha 3$(I) is at least consistent with the idea of reduced cell surface expression of the $\alpha 3$(M) subunit. This is supported by my

observations on *i* and P_o (described in Chapter 4). With lower GABA concentrations, other groups have also observed a ~2-fold differences in the current amplitude in cells expressing the edited $\alpha3(M)$ subunit compared with unedited $\alpha3(I)$ (Rula *et al.* 2008). Thus, my results, together with the findings from others, are suggestive of a role for $\alpha3$ editing in the trafficking of $\alpha3$-containing receptors that may facilitate the switch of subunit compositions during development.

3.8.1. Rectification

My initial experiments using bath application and voltage-jumps revealed no significant difference in rectification between the unedited $\alpha3(I)\beta2\gamma2L$ and edited $\alpha3(M)\beta2\gamma2L$ $GABA_A$ receptors. Nevertheless, the GABA-evoked currents from both of these isoforms were modulated by membrane voltage. With 3 μM GABA, the instantaneous and steady-state I/V relationships of both isoforms displayed a strong outward rectification. Interestingly, there was no significant difference in RI between instantaneous and steady-state current. This last observation differs from what has been reported for currents elicited by exogenous GABA in hippocampal neurons (Ransom *et al.* 2010), as well as at the late phase of IPSC (Pavlov *et al.* 2009)**,** where the outward rectification of the steady-state current was more pronounced than peak current. It is possible that this discrepancy reflects the relatively slow application of exogenous of GABA in my experiments or the prior holding voltage of the cells. I observed a similar lack of difference between instantaneous and steady-state I/Vs at high (1 mM) GABA, indicating that the voltage-dependent increase in conductance of GABA-evoked currents is primarily attributed to the intrinsic properties of the $GABA_A$ receptors rather than to the elevation of GABA concentrations.

It is also important to note that the experiments described in this chapter have determined the I/V relationship using either relatively slow ramp voltage changes (very different from many physiological processes, where voltage

change can occur on a millisecond timescale) or slow bath application of GABA.

A large body of evidence from cultured neurons and recombinant receptors suggests that GABA$_A$ receptors exhibit outward rectification in the presence of low GABA concentrations (Birnir *et al.* 1994, Pavlov *et al.* 2009, Pytel *et al.* 2006a, Pytel and Mozrzymas 2006b, Ransom *et al.* 2010, Rula *et al.* 2008, Weiss *et al.* 1988). Additionally, the amplitude and decay time of miniature GABA$_A$ receptor-mediated synaptic currents (mIPSCs) have been found to be significantly larger at positive voltages than negative voltages (Pytel and Mozrzymas 2006b). Although the mechanism underlying the voltage-dependent rectification of GABA$_A$ receptors has not been fully elucidated, it has been proposed that the membrane potential effects on GABA$_A$ receptor functioning are related to modulation of the receptor gating. Certainly, in many cases the rectification cannot simply be explained by Goldman rectification due to asymmetric Cl$^-$ concentrations across the membrane (see section 4.3) (but also see (Barker and Harrison 1988). Rectification has been attributed to voltage-dependent deactivation of GABA$_A$ receptors (Mellor and Randall 1998) though other mechanisms, such as voltage-dependence of desensitization, conductance and opening time may also play a role (Birnir *et al.* 1994, Yoon 1994). In particular, the outward rectification of GABA$_A$ receptors at low GABA concentrations has been suggested to be secondary to an increased GABA binding rate and a larger opening rate at depolarized potentials (Pytel *et al.* 2006a). At low GABA concentration, only a minority of GABA$_A$ receptors are bound and the outward rectification of the macroscopic current reflects a more effective recruitment into the open state due to both larger opening rate and binding rate (Pytel *et al.* 2006a). This is further supported in single-channel recordings from chick neurons where membrane voltage increased single-channel open probability (Weiss *et al.* 1988). In contrast, a more recent study has shown that rectification of GABA$_A$ receptor was inversely related to the degree of channel activation (O'Toole and Jenkins 2011). An increased outward rectification was observed when open probability was decreased. A similar phenomenon has been observed

for currents recorded from tonically active $GABA_A$ receptors (Pavlov *et al.* 2009, Ransom *et al.* 2010).

It is interesting to note that I observed concentration-dependent rectification in whole-cell ramp experiments, whereby the I/V relationships changed from outwardly rectifying to linear with increasing GABA concentration. The I/V relationship was close to linear with 1 mM GABA concentration as opposed to outwardly rectifying with 3 μM GABA (**Figure 3.7**). Other studies have also reported such a switch in rectification, although inward rectification rather than a linear response was seen with high GABA concentrations (Pytel *et al.* 2006a). It is possible that this variation is related to the fact that synaptic receptors and those in heterologous cells could differ in their subunit composition and therefore in kinetics and susceptibility to modulators (including voltage). Nevertheless, the altered rectification index at high GABA concentrations indicates a relative loss of rectification, due either to recruitment of less rectifying $GABA_A$ receptors or to a dependence of $GABA_A$ receptor rectification on GABA concentration (Pavlov *et al.* 2009).

Similar phenomena have been observed in other receptors. For example, the macroscopic I/V relationships for AMPA receptors were shown to be outwardly rectifying, with the degree of rectification greater in sub-saturating than in saturating glutamate concentrations (Prieto and Wollmuth 2010). In addition, it has been demonstrated that outward rectification of AMPA receptors involves both rectification of the single-channel conductance *and* voltage-dependent gating. A switch between different gating modes of AMPA receptors and its modulation by voltage were investigated further by Prieto and Wollmuth (Prieto and Wollmuth 2010). In a sub-saturating glutamate concentration, AMPA receptors were found to switch between a low open probability mode and a high open probability model in which the behaviour of the receptor was identical to that in saturating glutamate. These gating modes occurred at both negative and positive potentials, but the high open probability mode was more prominent at positive potentials. This voltage-dependent enhancement of the high open probability mode may be a

physiologically important mechanism, whereby local synaptic activity regulates the efficacy of glutamatergic synapses.

The rectification observed for the macroscopic GABAergic currents is unlikely to result from changes in single-channel conductance, as most studies show that, in symmetrical chloride concentrations, the single-channel I/V is linear (Bormann *et al.* 1987, Eghbali *et al.* 2003, Ma *et al.* 1994, Macdonald *et al.* 1989, Mistry and Hablitz 1990, Weiss *et al.* 1988). Indeed, it has been shown that membrane potential can modulate the time course of current responses elicited by exogenous GABA applications indicating that voltage might principally affect the GABA$_A$ receptor gating. Quantitative analysis based on model simulations has shown that the effect of membrane depolarization was to increase the rates of binding, desensitization and of opening as well as to slightly reduce the rate of exit from desensitization (Pytel *et al.* 2006a).

The physiological role of voltage-dependent GABA$_A$ receptor gating is not clear. It might be speculated that at depolarized membrane potentials, when the balance between inhibition and excitation is shifted towards the latter, increased binding and opening rates (leading to increased GABA-evoked conductances) might be of advantage for preventing excess excitation. The outward rectification of GABAergic macroscopic currents at low GABA concentration is likely to play an important role in enhancing the tonic inhibition mediated by ambient GABA in the absence of mIPSCs (Pytel *et al.* 2006a).

3.8.2. Differences with other studies

The residue in the $\alpha3$ subunit altered by A-to-I RNA editing is located near the extracellular end of TM3 and is believed to lie within the region of the helix that faces the TM2 domain. It is structurally adjacent to residues that can cross-link to TM2, and may influence conformational stability. Its location is therefore consistent with a possible role in channel gating. It has been shown that the functional effects of structural changes at the editing site are

not specific for the $\alpha3$ subunit, but are shared by the $\alpha1$ subunit, suggesting a common role for this region for the α subunits (Nimmich *et al.* 2009). Similarly, as the asparagine residue at this site in the $\alpha1$ subtype is replaced by a histidine residue, outward rectification is not observed, indicating that residues in the TM2-TM3 region can influence the regulation of GABA$_A$ receptor activity by voltage (Granja *et al.* 1998).

Although another group has addressed the effect of Gabra3 editing on rectification, the results they obtained differ from those I have presented here. Thus, Rula *et al.* (2008) reported that $\alpha3\beta3\gamma2L$ receptors containing the edited $\alpha3(M)$ subunit were less rectifying than those containing the unedited $\alpha3(I)$ subunit (Rula *et al.* 2008). The lack of effect of editing on rectification reported here (**Figure 3.7**) is therefore somewhat surprising.

An important consideration that could have affected the ability to resolve any difference in function caused by editing is the maintained identity of the transfected subunits. The apparent lack of effect of editing on rectification could be due to the possibility that the unedited $\alpha3(I)$ subunit was edited endogenously in tsA201 cells. However, this seemed unlikely. In the original study of Ohman *et al.*, it was shown that a Gabra3 mini-gene containing exon 9, including the I/M site, when expressed in HEK293 cells could be edited following transient transfection with ADAR1 or ADAR2 (Ohlson *et al.* 2007). However, transfection of the mini-gene together with an empty vector resulted in no editing, suggesting that HEK293 cells lack the enzymes necessary to produce 'endogenous editing' (Ohlson *et al.* 2007). As tsA201 cells are simply transformed HEK293 cells, altered only by the addition of a stably expressing SV40 temperature-sensitive T antigen, it is thus likely that they also lack the ability to endogenously edit the $\alpha3(I)$ subunit. This view was supported by the results of my experiments with non-editable Gabra3 cDNA (**Figure 3.12**).

The difference between my results and those of Rula *et al.* could, in theory, have been explained by the fact that a different experimental protocol was used. In particular, the ramp speed used by Rula was faster than the speed I

used. However, repeating my experiments at this faster ramp speed did not reveal any differences in the I/V relationships or RIs, suggesting that the slow ramp speed used in my original experiments was not obscuring a difference between edited and unedited subunits (**Figure 3.9**). Of note, at both ramp speeds, for a given subunit combination, RI values showed considerable variation. One possible explanation for the variability is that mixtures of receptors may exist in individual cells. For example, when expressing combinations of α, β and γ subunits, the cells may contain a mixture of receptors, with both tri-heteromeric (α, β and γ) and di-heteromeric (α and β) forms present (Boileau *et al.* 2002a). Boileau *et al.* showed the transfection ratio affects benzodiazepine potentiation of GABA$_A$ receptors; co-expression of equal ratios of α1, β2 and γ2 subunits in *Xenopus* oocytes produced a mixed population of α1β2 and α1β2γ2 receptors with variable benzodiazepine potentiation, whereas a higher ratio of γ2 subunit resulted in a purer population of α1β2γ2 receptors with a more consistent benzodiazepine potentiation (Boileau *et al.* 2002a). Indeed, this finding was borne out in a preliminary study carried out by my colleague Dr. M. Renzi, showing that a transfection ratio of 1:1:10 (as used in my recordings) favoured the inclusion of the γ2L subunit, to produce ternary α3β2γ2L GABA$_A$ receptors (see section 4.1). As the transfection ratio in Rula's study was 1:1:1, there is a possibility that patches expressing ternary α3β2γ2L GABA$_A$ receptors might also contain di-heteromeric α3β2 GABA$_A$ receptors. For this reason, I investigated the properties of receptors formed from α and β receptors only. Surprisingly, I found that α3(M)β3 receptors displayed *greater* outward rectification than α3(I)β3 receptors. Thus, at face value, an excess of $\alpha\beta$ receptors would not explain the results of Rula *et al.*, where α3(M)β3γ2L GABA$_A$ receptors displayed *less* outward rectification than α3(I)β3γ2L GABA$_A$ receptors.

My data has shown that varying the β subunit resulted in changes in rectification. Although β2 and β3 subunits have high structural homology and confer similar pharmacological properties (Smith *et al.* 2004), the β3 subunit is more highly expressed in the developing brain while the β2 subunit

predominates in the adult brain (Laurie *et al.* 1992a). Incorporation of the $\beta3$ subunit yielded receptors that exhibited less outward rectification than $\beta2$-containing receptors. The $\beta3$ subunit has a negatively charged glutamic acid located at the carboxyl-terminal end of the putative channel lining domain TM2, a region that is suggested to play an important role in determining the conductance and ionic selectivity of the channel pore (Smith and Olsen 1995). This change in charge could contribute to a lower chloride conductance for the channel. Mutagenesis of the positively charged residue introduced rectification of the whole-cell I/V relationship, suggesting that this region can influence conductive properties (Backus *et al.* 1993, Fisher 2002).

Overall, I conclude that RNA editing of Gabra3 significantly reduced current amplitude, possibly due to reduced cell surface expression. The reduction in current amplitude was independent of the specific subunit combination, as it was observed for $\alpha3$ in combination with either the $\beta2$ or $\beta3$ subunit. Although RNA editing of $\alpha3$ appears to have little or no effect on rectification, my result show that the β subunit isoform can influence the extent of rectification.

Chapter 4

Single-channel properties of α3-containing GABA_A receptors

The single-channel properties of GABA$_A$ receptor subtypes depend critically on their subunit composition. The gating characteristics of single channels influence the amplitude, shape and duration of the postsynaptic current. Single-channel kinetic analysis has been used extensively to study the biophysical and pharmacological properties of a numerous native and recombinant GABA$_A$ receptor subtypes. This has increased our knowledge concerning the gating of GABA$_A$ receptors and how drugs such as barbiturates and benzodiazepines (Mathers 1987, Porter *et al.* 1992, Rogers *et al.* 1994) influence this gating.

The biophysical characterization of GABA$_A$ receptors was initially carried out using noise analysis of currents from neurons in primary culture; these studies provided the first estimates of mean single-channel conductance and average channel open times (Jackson *et al.* 1982). The development of single-channel recording techniques provided further detail on the nature of channel events with the demonstration of multiple single-channel conductances (Bormann *et al.* 1987). GABA$_A$ receptors exhibit complex gating behaviours, indicating the presence of multiple open and closed states and of substate conductance levels. Both channel opening times and opening frequency are dependent on agonist concentration and the competitive antagonist, bicuculline, reduces the current by modulating both of these parameters (Macdonald *et al.* 1989, Twyman *et al.* 1990). Current flow through the activated GABA$_A$ receptors has been studied in both cell-attached and excised patches (Akk *et al.* 2004, Angelotti and Macdonald 1993, Fisher and Macdonald 1997, Haas and Macdonald 1999). The results indicate that the kinetic behaviour of this receptor is complex, even in the absence of modulating drugs. In addition, kinetic analysis of single-channel currents activated by high concentrations of agonist has provided additional and complementary information to that obtained at low agonist

concentrations. In the former case, discrete single-channel clusters separated by prolonged closed periods are presumed to reflect infrequent exit from desensitized states, allowing clusters of openings to be ascribed to the activity of single receptors (Brickley *et al.* 1999, Keramidas and Harrison 2008, Keramidas and Harrison 2010, Lema and Auerbach 2006, Newland *et al.* 1991). This approach has revealed heterogeneous behaviour of the GABA receptor channels in mammalian neurones that would not have been apparent at low agonist concentrations.

Editing of Gabra3 is developmentally regulated, and the expression of the unedited and edited $\alpha 3$ mRNAs in the mammalian brain is different, suggesting the possibility of distinct physiological roles for these receptors. While there have been recent reports describing the effects of editing on the kinetic properties examined at the whole-cell level (Nimmich *et al.* 2009, Rula *et al.* 2008), to date, there has been no study characterizing the effect of Gabra3 editing on single-channel currents. To investigate the influence of Gabra3 editing on conductance and kinetics of $GABA_A$ receptors I examined the single-channel properties of receptors in tsA201 cells transiently transfected with $\beta 2$ and $\gamma 2L$ subunit together with either the unedited or the edited $\alpha 3$ subunit cDNAs.

4.1 Cell-attached single-channel openings

I made cell-attached recordings from tsA201 cells expressing $\alpha 3$ subunit-containing $GABA_A$ receptors. As co-expression of α, β and γ subunits could give rise to di-heteromeric $\alpha 3$: $\beta 2$ instead of tri-heteromeric $\alpha 3$: $\beta 2$: $\gamma 2$ $GABA_A$ receptors (Boileau *et al.* 2002a), I used a cDNA ratio of 1:1:10 to favour the formation of tri-heteromeric receptors. As discussed earlier, the choice of this ratio was influenced by preliminary studies from my colleague Dr Massimiliano Renzi. When $\alpha 3$, $\beta 2$ and $\gamma 2L$ subunits were transfected at a 1:1:2 ratio there was a heterogeneity of single-channel conductance that was greatly reduced when a ratio of 1:1:10 was employed. Consistent with this, expression of $\alpha 3$ and $\beta 2$ subunits alone (without $\gamma 2L$) gave raise to low

conductance openings that were absent from the 1:1:10 recordings. Heterogeneity of single-channel conductance has also been observed in $\alpha 1$ subunit-containing GABA$_A$ receptors. Recombinant receptors composed of $\alpha 1$ and $\beta 1$ subunits display a markedly lower single-channel conductance and altered gating properties in comparison with receptors composed of α and β subunits in combination with the $\gamma 2$ subunit (Angelotti and Macdonald 1993).

With 100 μM GABA included in the patch pipette, single-channel openings were seen in approximately 60% of patches. Both unedited (I) and edited (M) $\alpha 3\beta 2\gamma 2L$ GABA$_A$ receptors exhibited broadly similar single-channel activity. Active periods occurred as discrete clusters of openings and shuttings, separated by quiescent periods generally lasting anything from ~15 ms to several minutes (**Figure 4.1**).

In the presence of GABA I observed long clusters of channel activity that could be distinguished by apparent differences in intra-cluster open probability (P_o). A similar kinetic phenomenon was reported for $\alpha 1\beta 1\gamma 2s$ GABA$_A$ receptors recorded in the cell-attached configuration (Lema and Auerbach 2006). In this earlier study, clusters were extracted for division into bursts by applying a critical shut time (t_{crit}) that marked the end of a burst (Colquhoun and Hawkes 1995). Three modes were observed, with P_o values of ~ 0.8 (H-Mode), ~0.6 (M-Mode) and ~0.2 (L-Mode). The M-Mode was the most prevalent and the lowest P_o mode (L-Mode) was the least prevalent. In fact, a preliminary study performed by my colleague Dr Massimiliano Renzi also showed distinct bursting patterns, or 'modes', in cell-attached patches containing $\alpha 3\beta 2\gamma 2L$ GABA$_A$ receptors. His data suggested that the openings also displayed three distinct modes (**Figure 4.2**).
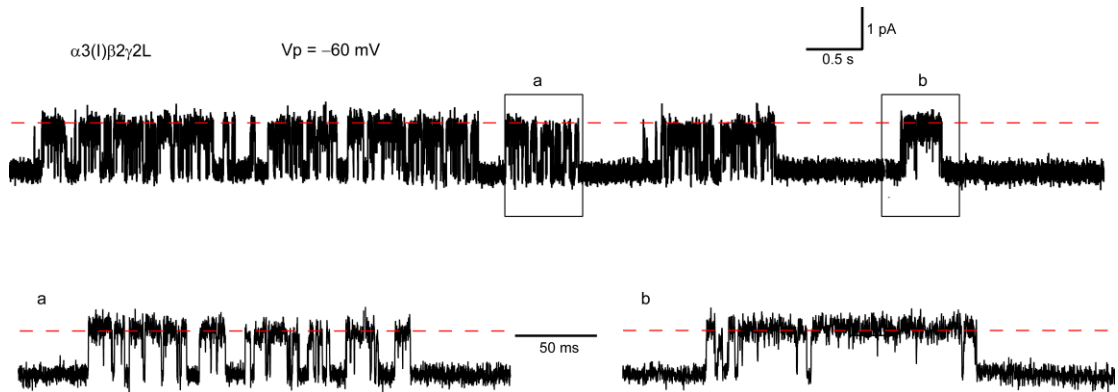
Figure 4.1 Single-channel openings from α3β2γ2L GABA$_A$ receptors.
A representative trace of recording from a cell-attached patch on a tsA201 cell expressing rat α3(I), β2, and γ2L GABA$_A$ receptor subunits with 100 μM GABA in the pipette. Clusters of single-channel openings (upward deflection of the trace indicates outward current corresponding to Cl$^-$ inward flow) are separated by closed intervals. Two clusters (boxed) were selected by eye, and represent apparently different gating modes, with medium (**a**) and high (**b**) open probability.
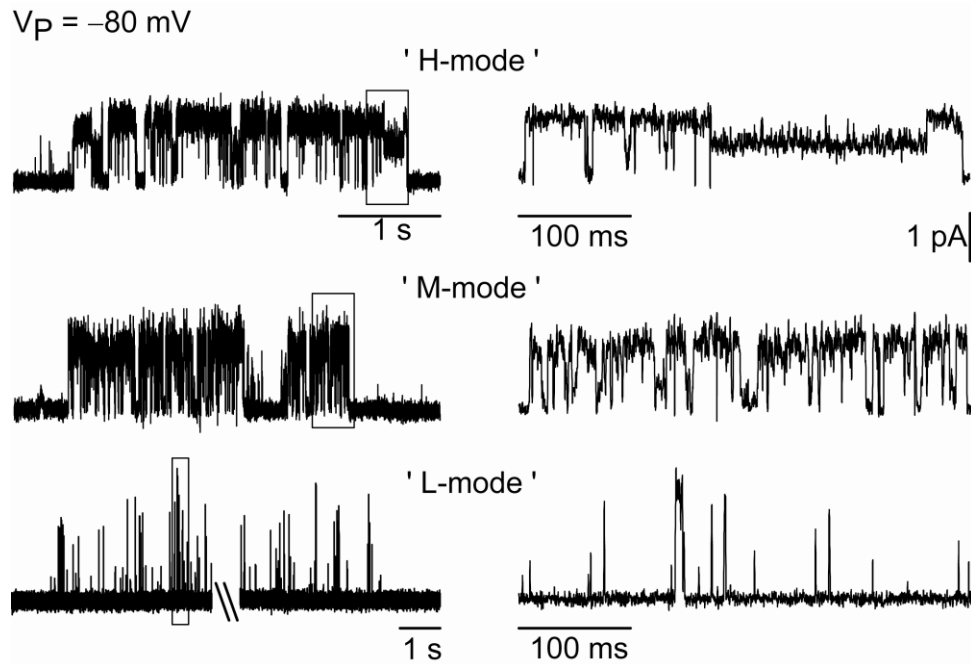
Figure 4.2 Single-channel recordings obtained from tsA201 cells expressing α3-subunit containing GABA$_A$ receptors were kinetically heterogeneous.

Three distinct gating modes were observed based on open probability (P_o), as described by (Lema and Auerbach 2006)). Typical clusters with high-, medium- and low-P_o ('H-mode', 'M-mode' and 'L-mode', respectively) are shown for the unedited α3(I)β2γ2L GABA$_A$ receptors in response to 1 mM GABA. The V_p was −80 mV. Note that the cluster used to illustrate the L-mode (containing a 5 s break) is longer than the ones showing M- or H-modes. In most patches, one gating mode was predominant within each patch, with the H-mode the most and the L-mode the least frequently as observed. This property was common to both the edited α3(M)β2γ2L and unedited α3(I)β2γ2L GABA$_A$ receptors. Transitions from one gating mode to another were observed not only between but also within clusters.

4.2 Single-channel conductance

Figure 4.3 illustrates selected recordings obtained from tsA201 cells expressing $\alpha3\beta2\gamma2L$ GABA$_A$ receptors in the presence of 100 μM GABA. Single-channel current amplitudes were determined by generating amplitude histograms for selected segments of record and fitting the histograms with Gaussian curves (**Figure 4.3**). The area under each Gaussian peak represents the relative frequency of occurrence of events at the corresponding amplitude level. For both isoforms, the lower amplitude occurred either as brief transitions from the larger amplitude openings, as shown in **Figure 4.3b** for a record from cell expressing $\alpha3(M)\beta2\gamma2L$ GABA$_A$ receptors, or as occasionally as separate active periods, as illustrated by the cluster of activity in **Figure 4.3c** from a cell expressing $\alpha3(I)\beta2\gamma2L$ receptors. In 118 patches containing $\alpha3(I)$ receptors the main cluster current ranged from 0.67-1.15 pA ($V_p = +60$ mV). This corresponds to chord conductances of 11.2-19.2 pS (assuming a reversal of 0 mV; but see section 4.3). For 22 patches containing $\alpha3(M)$-containing receptors, the corresponding values were 0.48-1.02 pA and 8.0-17.0 pS for main cluster current and chord conductances, respectively.

Sub-conductance openings were evident in some (~1 %) patches. A wide range of conductance levels has been reported for chloride channels activated by GABA and most of the studies have also reported the presence of multiple conductance levels (Bormann *et al.* 1987, Brickley *et al.* 1999, Newland *et al.* 1991). Although the incidence of sub-conductance openings in most of the studies was not quantified, it seems that the incidence of sub-conductance openings observed in my experiments is lower than that observed by others. The overall variation in conductance levels is likely due to the presence of different populations of receptor subtypes, as GABA$_A$ receptors from different preparations show considerable diversity in their gating characteristics (Bormann *et al.* 1987, Brickley *et al.* 1999, Newland *et al.* 1991). The smallest conductance value measured depends on the neuronal type. For example, GABA$_A$ receptors in cultured mouse spinal cord cells exhibit two conductance levels of about 30 pS and 19 pS, when

measured with 145 mM Cl⁻ present on each membrane face (Hamill *et al.* 1983). At the same time, GABA$_A$ receptors studied in hippocampal neurons have been reported to exhibit only a single conductance level of about 20 pS at 0 mV (Bormann 1988, Gray and Johnston 1985). In addition, it is not surprising that the recording mode influences conductance measurements. Thus, Bormann *et al.* demonstrated that in cell-attached patches, the single-channel slope conductances close to 0 mV were 28, 17 and 10 pS; while in outside-out patches with equal extracellular and intracellular concentrations of Cl⁻ (145 mM), the conductance values were 44, 30, 19 and 12 pS (Bormann *et al.* 1987). In the former case, both the unknown membrane voltage and the likely unequal concentrations of permeating ions on either side of the membrane necessitate slope conductance measurements and complicate interpretation. Nevertheless, it is clear that the discrete distribution of conductance levels is a common property of many GABA$_A$ receptors. This may be correlated with subunit composition but could also involve differences in the presence of intracellular receptor-associated proteins and/or post-translational mechanisms (e.g. phosphorylation). Another concern when making comparisons between studies is the possibility of differing resolutions due to filtering or different signal-to-noise ratios. Thus, the low occurrence of resolved subconductance states in my recording could reflect genuine channel differences or a relatively high background noise level. For example, the range of baseline SD values for α3(I) and α3(M) were 0.13-0.15 pA (n = 24) and 0.09-0.11 pA (n = 15) respectively (V_p = −60 mV) at 100 μM GABA. Due to the paucity of clear examples of low conductance data, only the high (main) conductance currents were analysed further.

Interestingly, a concentration-dependent effect on the main conductance was observed. For α3(I)β2γ2L GABA$_A$ receptors at high GABA concentrations (1 and 10 mM), the chord conductance (16.8 $\pm$ 0.48 pS and 20.7 $\pm$ 0.83 pS, n = 80 and 45, respectively) was significantly increased compared to that observed at a non-saturating (100 μM) GABA concentration (13.3 $\pm$ 0.11 pS, n = 128; both P < 0.0001). A similar increase in chord conductance (P < 0.0001) was also observed for α3(M)β2γ2L receptors (at 1 and 10 mM 18.2 $\pm$

0.47 pS and 18.0 $\pm$ 0.72 pS, n = 64 and 13, respectively) compared to 10.6 $\pm$ 0.53 pS (n = 20) with 100 μM GABA.
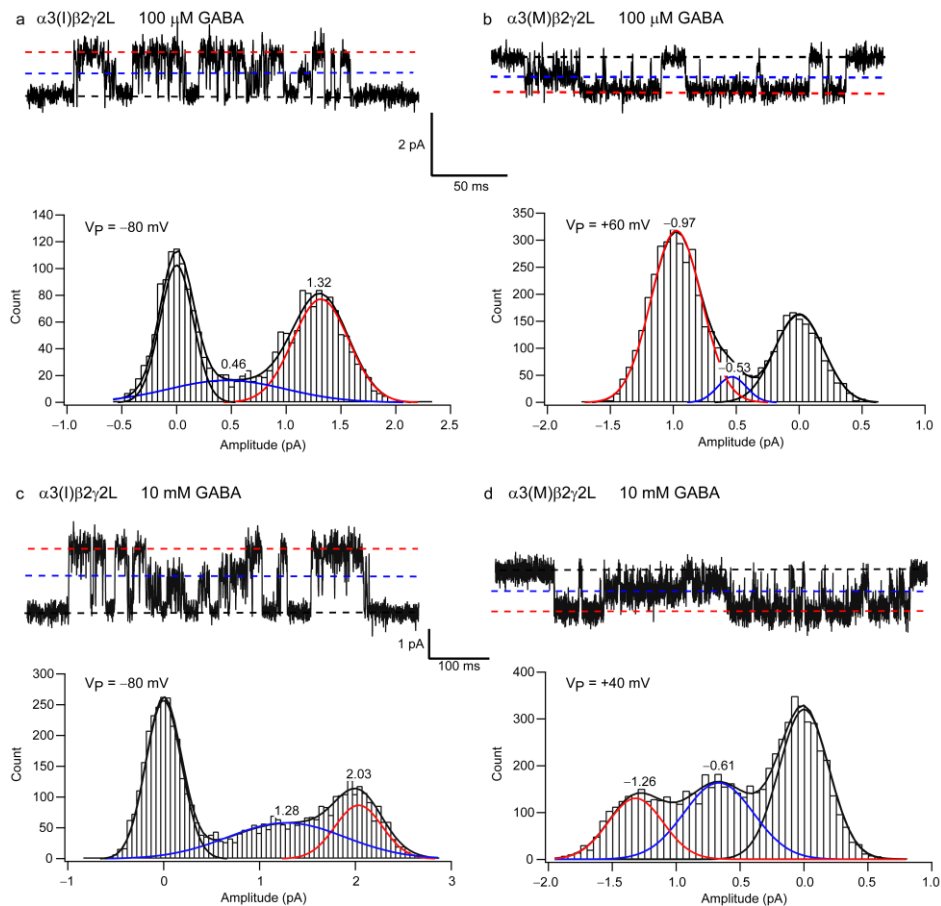
Figure 4.3 Multiple conductance states are present in α3β2γ2L GABA$_A$ receptors.

A single cluster selected to illustrate the presence of sub-conductance levels in patches expressing the unedited α3(I)β2γ2L (**a**) and the edited α3(M)β2γ2L (**b**) receptors. The dotted lines indicate the shut level and the different conductance levels. **a**) Current levels in channels activated by 100 μM GABA in a cell-attached patch expressing the α3(I)β2γ2L receptors at pipette potential (V_p) of −80 mV. **b**) Current levels in channels activated by 100 μM GABA in a cell-attached patch expressing the α3(M)β2γ2L receptors (V_p = +60 mV). All-point amplitude histograms were constructed from short recordings from the same patch as shown. Amplitude distribution was fitted by the sum of Gaussian curves and the values indicated at the peak of each Gaussian curve represent the mean amplitude (pA). Multiple openings with various current amplitudes were also obtained in the presence of 10 mM GABA. **c** and **d**) Current levels in GABA-activated channels in a cell-attached patch expressing the α3(I)β2γ2L and α3(M)β2γ2L receptors at (V_p = −80 and +60 mV respectively).

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4.3 Current-voltage relationships

To estimate the single-channel slope conductance and investigate the possibility of single-channel rectification, currents were recorded over a range of voltages (-100 to $+60$ mV) in each patch. To obtain estimates of single-channel amplitudes at each pipette potential, amplitude histograms were plotted and fitted with Gaussian curves. Examples of these plots are shown in **Figure 4.4** for the accompanying recordings. I/V relationships were constructed from the main amplitude only, as the amplitudes of the minor peaks were less reliably resolved. The average main current amplitudes were plotted as a function of pipette potential (V_p) for both isoforms (**Figure 4.5**).

An I/V relationship for unedited $\alpha3(I)\beta2\gamma2L$ GABA$_A$ receptors is illustrated in **Figure 4.5a**. The positive and negative limbs of the I/V plots seemed to show some slight outward rectification: the slope conductance at depolarized potentials (i.e. when V_p was negative) was 16.2 ± 2.0 pS ($n = 3$). Although this appeared greater than that seen at hyperpolarized potentials (8.9 ± 2.5 pS, $n = 3$) this difference was not statistically significant ($P = 0.25$ Wilcoxon matched pairs test). The RI, calculated as the ratio of slope conductance at negative and positive V_p, was 1.87 ± 0.39. For the edited $\alpha3(M)\beta2\gamma2L$ GABA$_A$ receptors (example in **Figure 4.5b**), mean slope conductances at both negative and positive V_p were obtained only for two patches, but again, these were similar (mean of 10.4 pS at negative V_p and 8.9 pS at positive V_p). The corresponding mean RI was 1.16.

Figure 4.4 Single-channel currents at different voltages.
a) Representative traces of GABA-evoked single-channel currents recorded in a cell-attached patch at the pipette potentials (V_p) indicated. tsA201 cells were transfected with rat unedited $\alpha 3$, $\beta 2$ and $\gamma 2L$ $GABA_A$ receptor subunits in 1:1:10 ratio. Inclusion of 100 μM GABA induced single-channel openings at all voltages tested. **b**) All-points amplitude histograms for sections of data containing predominantly single channel openings were fitted with the sum of two Gaussians, representing closed (black) and open (red) state current distributions. The peak current amplitudes in the examples shown were 1.20, -1.35 and -1.73 pA at V_p -80, $+60$ and $+80$ mV, respectively.

Figure 4.5 c and **d** show pooled I/V plots, fit using the Goldman-Hodgkin-Katz current equation (Goldman 1943, Hodgkin and Katz 1949), as described by Bormann *et al.* (Bormann *et al.* 1987). The current, *i*, of monovalent anions, A, flowing through an electrically homogeneous membrane channel can be described by:

$$i = P_\mathrm{A} F \psi \frac{[\mathrm{A}]_o - [\mathrm{A}]_i \mathrm{e}^\psi}{1 - \mathrm{e}^\psi}$$

where

$$\psi = -\frac{VF}{RT}$$

and P_A is the single-channel permeability of the anion A, *V* is membrane potential, *F* is Faraday's constant, *R* is the universal gas constant, and *T* is the temperature. With cell-attached recordings, the experimenter does not determine the intracellular ion concentrations. The constant field equation predicts that rectification will occur when the chemical driving force on chloride ion is greater in one direction than the other. Thus, outward rectification is expected at negative reversal potentials, linear I/Vs with symmetrical [Cl⁻], and inward rectification at positive reversal potentials.

Channels were recorded at only four or five voltages, making it difficult to produce compelling fits. Moreover, the predicted [Cl⁻]$_i$ (>100 mM in both cases) was higher than might have been expected (Darman *et al.* 2001, Monette and Forbush 2012). Nevertheless, the I/Vs did not display rectification that differed substantially from that which could be attributed to GHK rectification. This echoes the lack of obvious rectification seen in the comparisons of slope conductance.
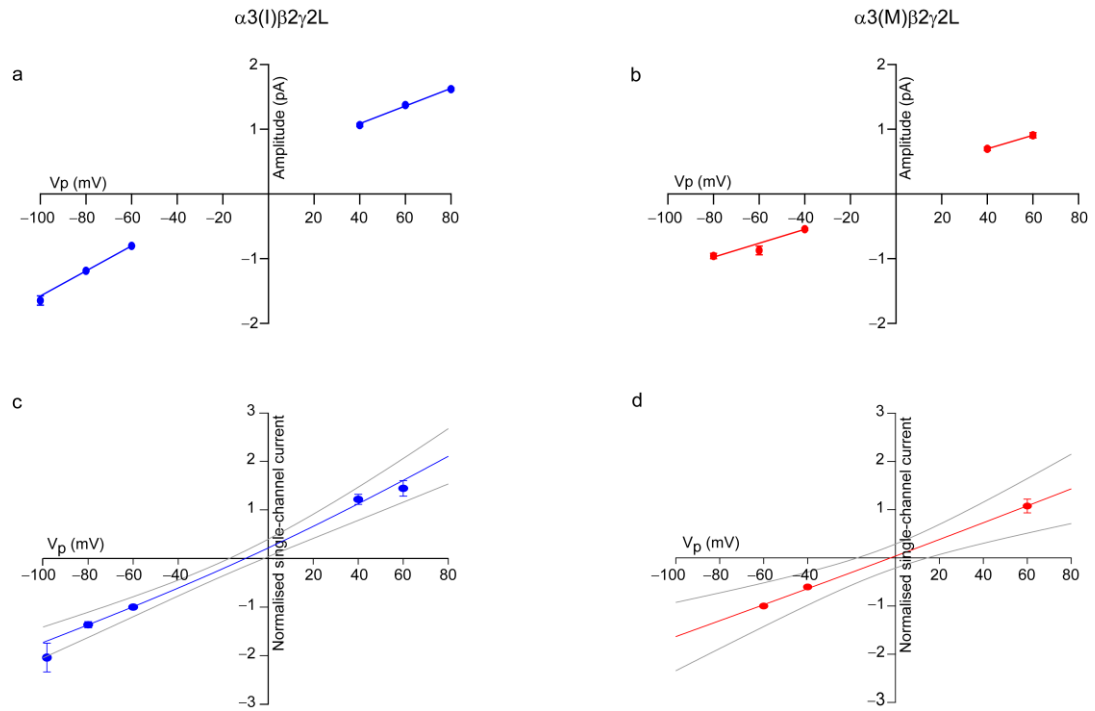
Figure 4.5 I/V relationships of $\alpha3\beta2\gamma2L$ GABA$_A$ receptors.
a and **b**) Individual I/V plots showing the single-channel activity recorded from patches expressing unedited $\alpha3(I)\beta2\gamma2L$ or edited $\alpha3(M)\beta2\gamma2L$ GABA$_A$ receptors in the presence of 100 μM GABA. Only the main conductance openings were plotted as a function of pipette potential (V_p). Data are shown as mean $\pm$ SEM. **c** and **d**) Pooled normalised data ($n = 3$ patches each) fit with the Goldman-Hodgkin-Katz current equation. Data were normalised to the current at $V_p = -60$ mV. Grey lines denote 95% confidence bands. For the $\alpha3(I)$ cells, the fitted parameters were permeability coefficient p = $4.57e^{-10}$ cm^3 s^{-1} and [Cl$^-$]$_i$ = 101 mM. For the $\alpha3(M)$ cells the fitted parameters were permeability coefficient p = $3.15e^{-10}$ cm^3 s^{-1} and [C$^-$l]$_i$ = 138 mM. For details, see text.

4.4 Analysis of open probability and its concentration dependence

In a separate set of experiments, single-channel currents were recorded with different GABA concentrations in the patch electrode. Initially, I determined the intra-cluster open probability (P_o) from Gaussian fits to all-point amplitude histograms of clusters selected by eye (Clampfit; pCLAMP) according to the following equation:

$$P_o = \frac{A_o}{(A_o + A_c)}$$

where A_o is the area under the curve during openings and A_c is area under the curve reflecting the closed state.

The probability being open as a function of GABA concentration (0.1-10 mM) was examined across multiple patches; for each patch 13-127 clusters were analysed. At each concentration these displayed a wide range of P_o values. For examples, at 10 mM GABA, the P_o values ranged 0.39-0.94 and 0.39-0.85 for α3(I)- and α3(M)-subunit containing GABA$_A$ receptors, respectively. For the edited α3(M)β2γ2L GABA$_A$ receptors, the mean intra-cluster P_o values were 0.74 $\pm$ 0.03 (10 mM, n = 3 patches), 0.81 $\pm$ 0.01 (1 mM, n = 3) and 0.75 $\pm$ 0.03 (100 μM, n = 3). Slightly (but consistently) lower intra-cluster P_o was obtained for the unedited α3(I)β2γ2L GABA$_A$ receptors (0.69 $\pm$ 0.02, 0.65 $\pm$ 0.03 and 0.72 $\pm$ 0.01 with 10 mM, 1 mM and 100 μM GABA, n = 3, 4 and 3, respectively) (**Table 4.1; Figure 4.8a**).

In an attempt to more accurately characterize the kinetic differences between α3(I)- and α3(M)-containing receptors, the duration and P_o of identified bursts of openings were measured using QuB software (http://www.qub.buffalo.edu). The method of analysis was adapted from a previous single-channel analysis of α3β3γ2S GABA$_A$ receptors (Keramidas and Harrison 2010). Briefly, periods of continuous activity were initially selected by eye and extracted to separate files within QuB for further analysis. These selected segments were discrete and separated from each

other by quiescent periods lasting at least 100 ms (**Figure 4.6**). Isolated brief openings that sometimes occurred between clusters and multiple openings within active periods were excluded during cluster selection and not included in the analysis. The segmented *k*-means algorithm was used to idealize the data, and the first and last shutting events of discrete active periods were dropped from the idealized data. The segments were then divided into clusters of activity by applying a critical shut time (t_{crit}) that marked the end of a burst (Colquhoun and Hawkes 1995). The values for t_{crit} were determined for each patch by generating shut-time dwell histograms of the idealized selected segments of data. The shut-time dwell histograms for both isoforms were best fitted with the sum of three exponential distributions, while the open-time dwell histograms were best fitted with the sum of two exponential distributions (**Figure 4.7**). To examine the burst properties of these isoforms, the t_{crit} representing the termination of bursts was defined between the brief closed components. Some selected segments produced several t_{crit} values, and the longest one (between the two longest components) was chosen and used to divide the segments. Using these techniques, the selected t_{crit} was similar between the two isoforms; the values at 1 mM GABA ranged between 7 and 18 ms for the unedited $\alpha3$(I)- and from 3 to 42 ms for the edited $\alpha3$(M)-containing receptors. The system dead time was 50-150 μs. The resulting clusters were analysed for intra-burst P_{o} and cluster duration.
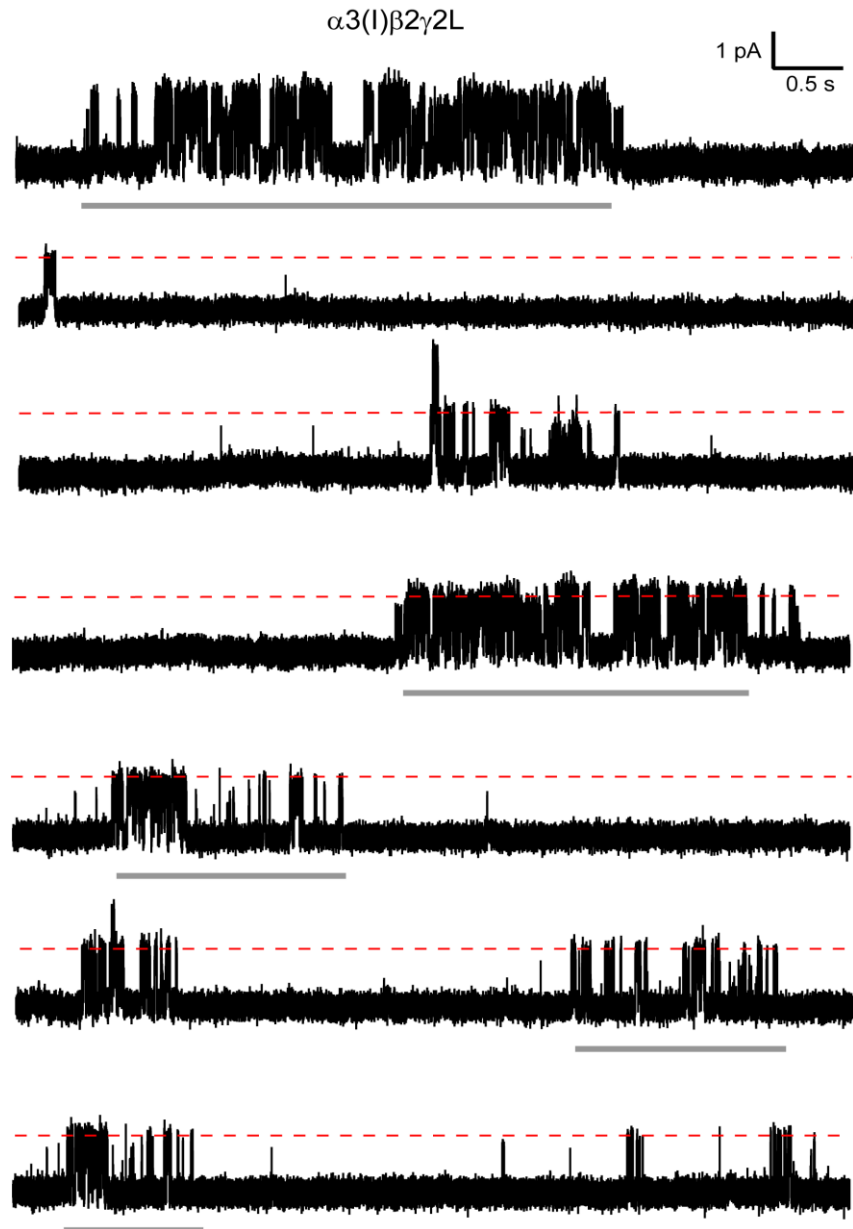
Figure 4.6 Selecting segments of the record for kinetic analysis.
A recording from a patch expressing the unedited $\alpha3(I)\beta2\gamma2L$ GABA$_A$ receptors in response to 10 mM GABA ($V_P = -60$ mV). Stretches of single-channel activity were selected by eye and subsequently divided into discrete clusters by applying a critical shut time (t_{crit}) (Keramidas and Harrison 2010). Segments with a high conductance and intermediate to high intra-cluster P_o were included in the analysis (underlined with grey bars). Segments were excluded if they exhibited extremely low P_o, contained overlapping activity from multiple channels, occurred as isolated openings, or were mainly of the low single-channel conductance.
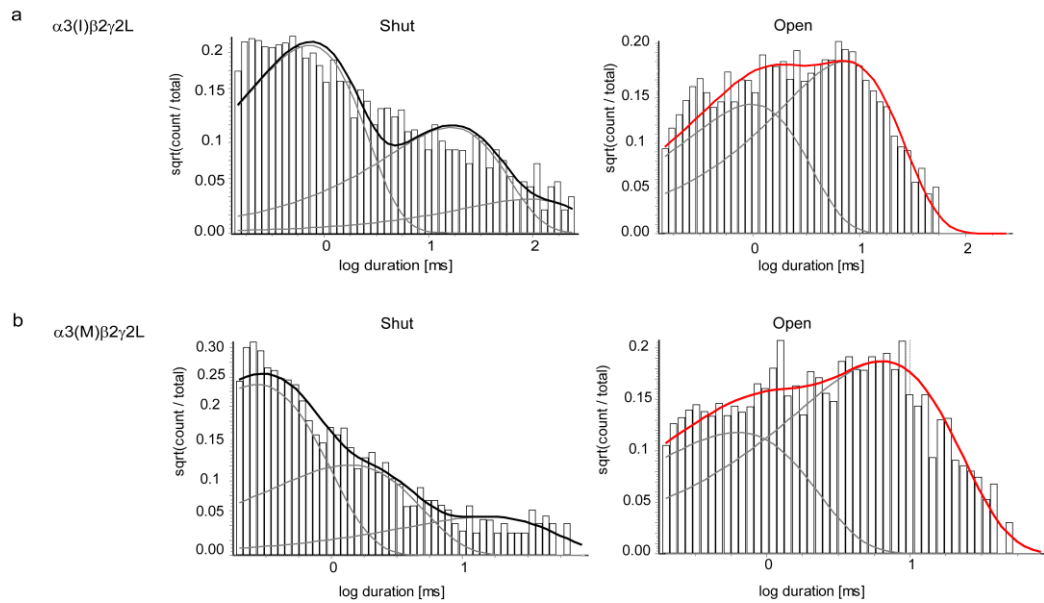
Figure 4.7 Dwell-time distributions for α3 subunit-containing GABA_A receptors.

Examples of the dwell-time distributions for apparent shut and open times of the unedited α3(I)β2γ2L (**a**) and the edited α3(M)β2γ2L (**b**) GABA_A receptors in response to 10 mM GABA. Dwell time distribution histograms for the shut and open time were fitted with the sum of two to three exponential functions. The solid lines represent the mixture of probability density functions together with the individual components.

Currents recorded in additional GABA concentrations (0.01, 0.1 and 10 mM) were analysed in the same way as the data obtained at 1 mM GABA. With 0.1 and 10 mM GABA, clusters of channel activity were observed for both receptor isoforms. Similar t_{crit} ranges were obtained for the two isoforms at 100 μM GABA; 6-37 ms for the unedited α3(I)β2γ2L GABA$_A$ receptors and 3-64 ms for the edited α3(M)β2γ2L GABA$_A$ receptors. However, at 10 mM the records had very few clusters of openings, possibly due to desensitization at this high GABA concentration. Only one patch for each isoform yielded sufficient activity at 10 mM to allow kinetic analysis. In this case, the t_{crit} values were 7.49 ms for the unedited α3(I)β2γ2L GABA$_A$ receptors and 3.90 ms for the edited α3(M)β2γ2L GABA$_A$ receptors. Conversely, data recorded at 10 μM GABA was not included in the analysis because this concentration induced mostly very short, scattered activity with very few complex bursts that meant it was generally difficult to isolate segments of activity that appeared to arise from a single-channel.

Interestingly, burst length was not obviously dependent on GABA concentration. This is inconsistent with the concentration-dependent effect on burst length reported by Keramidas and Harrison (Keramidas and Harrison 2010). However, although the burst lengths were quite variable, those from edited α3(M)β2γ2L GABA$_A$ receptors were consistently longer on average than those of the unedited α3(I)β2γ2L GABA$_A$ receptors at the same concentration (**Table 4.1; Figure 4.8c**). Exposing the unedited α3(I)β2γ2L GABA$_A$ receptors to 100 μM elicited bursts that were 177 $\pm$ 71 ms long ($n =$ 127 bursts from 3 patches). For the edited α3(M)β2γ2L GABA$_A$ receptors, the corresponding value was 319 $\pm$ 200 ms ($n =$ 20 bursts from 3 patches). The respective mean cluster lengths in response to 1 mM GABA were 160 $\pm$ 61 ms ($n =$ 80 bursts from 4 patches) and 339 $\pm$ 206 ms ($n =$ 63 bursts from 3 patches).

Similarly, the intra-burst P_o appeared slightly greater for the edited α3(M)β2γ2L GABA$_A$ receptors (**Table 4.1; Figure 4.8b**). For the unedited α3(I)β2γ2L GABA$_A$ receptors, the intra-burst P_o values were: 0.77 $\pm$ 0.04 ($n =$

3; 100 μM GABA), 0.73 $\pm$ 0.01 (n = 4; 1 mM GABA) and 0.74 (n = 1; 10 mM GABA). For the edited α3(M)β2γ2L GABA$_A$ receptors, the intra-burst P_o values were: 0.76 $\pm$ 0.06 (n = 3; 100 μM GABA), 0.82 $\pm$ 0.03 (n = 3; 1 mM GABA) and 0.79 (n = 1; 10 mM GABA). As noted, for intra-burst P_o and burst length the expected concentration-dependence was not obvious. Comparison with data from Keramidas and Harrison (Keramidas and Harrison 2010) (**Figure 4.8**) makes it clear that this likely reflects the fact that the GABA concentrations I used were at the plateau region of the concentration-response curve. Nevertheless, the values of both intra-burst P_o and burst length were broadly similar to the previously published measurements for α3β3γ2S (edited) GABA$_A$ receptors (Keramidas and Harrison 2010). My results, together with those of Keramidas and Harrison (2010), indicate that the α3 subunit-containing GABA$_A$ receptors have a higher intra-burst P_o and longer burst length than GABA$_A$ receptors containing the α1 subunit (**Figure 4.8**).

Table 4.1 The mean intracluster P_o, intraburst P_o and burst length ($\pm$ s.e.m.) of the $\alpha3(I)\beta2\gamma2L$ and $\alpha3(M)\beta2\gamma2L$ GABA$_A$ receptors over a range of GABA concentrations.

GABA concentration (mM)	Mean intracluster P_o (n)	
	$\alpha3(I)\beta2\gamma2L$	$\alpha3(M)\beta2\gamma2L$
0.1	0.72 ± 0.01 (3)	0.74 ± 0.03 (3)
1	0.65 ± 0.03 (4)	0.81 ± 0.01 (3)
10	0.69 ± 0.02 (3)	0.75 ± 0.03 (3)

GABA concentration (mM)	Mean intraburst P_o	
	$\alpha3(I)\beta2\gamma2L$	$\alpha3(M)\beta2\gamma2L$
0.1	0.77 ± 0.04 (3)	0.76 ± 0.06 (3)
1	0.73 ± 0.01 (4)	0.82 ± 0.03 (3)
10	0.74 (1)	0.79 (1)

GABA concentration (mM)	Mean burst length (ms) (n)	
	$\alpha3(I)\beta2\gamma2L$	$\alpha3(M)\beta2\gamma2L$
0.1	177 ± 71 (3)	319 ± 200 (3)
1	160 ± 61 (3)	339 ± 206 (3)
10	122 (1)	137 (1)
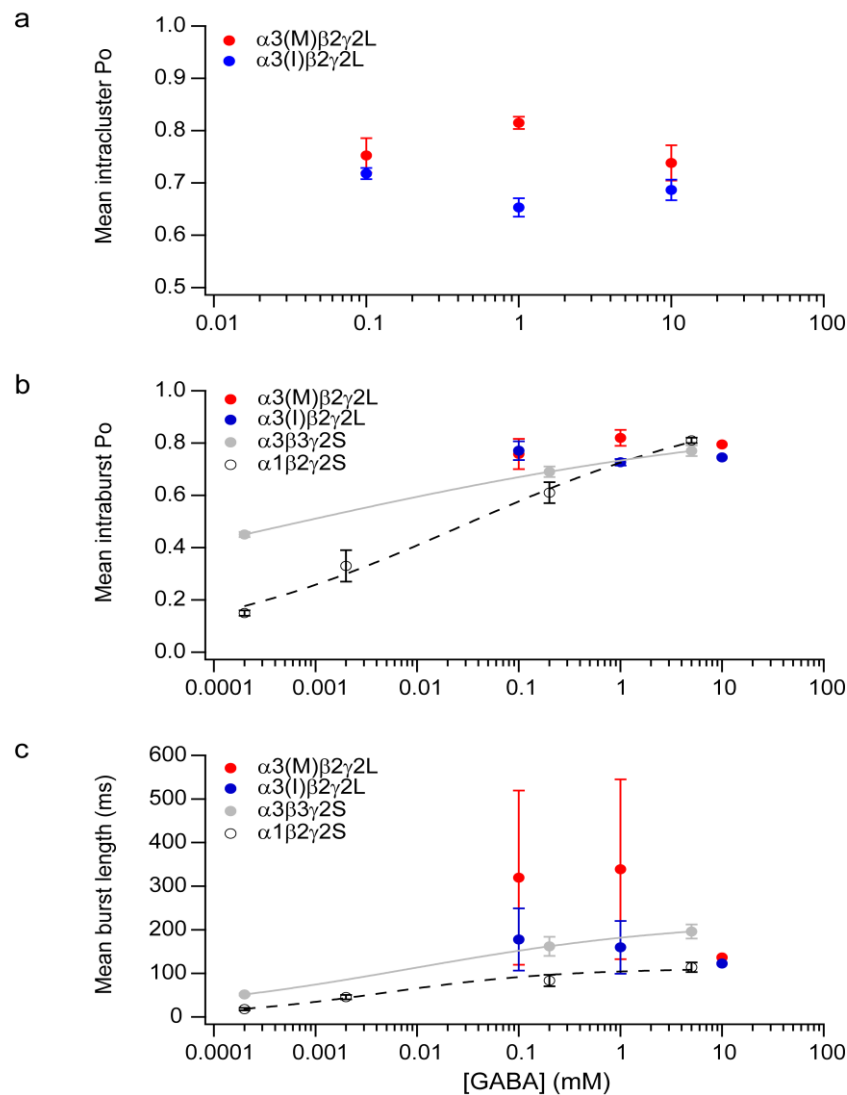
Figure 4.8 Intracluster P_o, intraburst P_o and burst length of α3 subunit-containing GABA$_A$ receptors.
a) Plot of mean intracluster P_o as a function of GABA concentration for α3β2γ2L GABA$_A$ receptors (pClamp analysis). **b** and **c**) Plots of mean intraburst P_o and burst durations as a function of GABA concentration for α3β2γ2L GABA$_A$ receptors (QUB analysis). The values were compared with α3β3γ2S (grey lines) and α1β2γ2S (dashed black lines) GABA$_A$ receptors from (Keramidas and Harrison 2010). The data for the α3β3γ2S and α1β2γ2S GABA$_A$ receptors were fitted to Hill equations. For intraburst P_o, the maximum, EC_{50} and n_H values of α1β2γ2S were 0.97, 29 μM and 0.30. The corresponding values for α3β3γ2S were 0.92, 0.3 μM and 0.16. For mean cluster length, the maximum, EC_{50} and n_H values with α1β2γ2S were 111.4 ms, 5 μM and 0.50; whereas the corresponding values for α3β3γ2S were 222.4 ms, 9 μM and 0.32.

4.5 Discussion

While some studies have examined the effects of $\alpha 3$ editing at the whole-cell level, the single-channel characteristics of receptors containing the unedited $\alpha 3$ subtype have not been described. Channel gating properties influence the amplitude and duration of the postsynaptic response to GABA. Therefore, any differences in these properties that result from Gabra3 editing could alter the effectiveness of GABAergic neurotransmission. Of note, the isoforms of the GABA$_A$ $\alpha 3$ subunit show distinct expression patterns through development, suggesting that if these isoforms confer different channel/receptor properties, these may be of developmental relevance.

Channel openings from both isoforms were of relatively long duration, and exhibited multi-opening bursts. In general, the single-channel kinetic properties of the unedited $\alpha 3(I)\beta 2\gamma 2L$ GABA$_A$ receptors, were consistent with the single-channel traces published for recombinant (edited) $\alpha 3\beta 3\gamma 2S$ GABA$_A$ receptors (Keramidas and Harrison 2010) and native $\alpha 3$-containing receptors in dopaminergic neurons of the rat substantia nigra pars compacta (Guyon *et al.* 1999).

Both isoforms exhibited a similar slope conductance at hyperpolarized potentials ($+V_p$), but the unedited $\alpha 3(I)\beta 2\gamma 2L$ GABA$_A$ receptors displayed a greater tendency toward a higher slope conductance at depolarizing potential ($-V_p$) compared to the edited $\alpha 3(M)\beta 2\gamma 2L$ GABA$_A$ receptors. However, this change was not statistically significant and fitting of the GHK current equation did not reveal any trend beyond that expected for GHK rectification. An outwardly rectifying GABA$_A$ receptor with a high conductance at depolarized potentials has been described previously (Birnir *et al.* 2000, Gage and Chung 1994). But the lack of obvious rectification of single GABA-activated channels in my recordings is similar to the findings of many previous investigations (Allen and Albuquerque 1987, Bormann *et al.* 1987, Curmi *et al.* 1993, Fatima-Shad and Barry 1992, Gray and Johnston 1985, Hamill *et al.* 1983, Valeyev *et al.* 1999, Weiss *et al.* 1988). The relative

linearity of the single-channel I/Vs suggests that the outward rectification observed in whole-cell currents reflects voltage-dependent gating and not rectification of individual channels. Interestingly, a recent study has suggested that the nonlinearity of the I/V relationship for whole-cell $GABA_A$ responses is inversely related to channel open probability (O'Toole and Jenkins 2011). The authors found that currents were outwardly rectifying at low P_o and linear at high P_o – the magnitude of outward currents was greater than the magnitude of inward currents in low P_o conditions but at high P_o the desensitization of outward currents was enhanced, resulting in a linearization of the I/V relationship. The magnitude of current was increased by increasing GABA concentrations but the degree of current rectification was reduced (i.e. responses became more "ohmic"). However, this did not occur when current amplitude was increased by changing the electrical driving force on the permeant ion, suggesting that rectification was linked to the direction of chloride flux and not caused by a direct action of membrane potential on ion channel (as occurs with voltage-gated ion channels). The link between the degree of channel activation and the amount of rectification was explained by the channel gating behaviour in relation to GABA concentrations. At low P_o, the more frequent presence of gating elements within the pore was suggested to hinder ion permeation directionally, generating an asymmetry similar to that observed in low intracellular chloride condition. At high P_o, the ion channel behaved as a simple ohmic pore, with a liner relationship between the magnitude of current and the electrical driving force contributed by the membrane potential. With prolonged GABA application, the open channel was assumed to transition to a non-conducting desensitized state. It was suggested that the asymmetry established by primary channel gating was masked by the enhanced desensitization of outward currents, which served to linearize the I/V relationship (O'Toole and Jenkins 2011).

Interestingly, as mentioned earlier, my results showed that the P_o (both intra-cluster and intra-burst) of the $\alpha3(I)\beta2\gamma2L$ $GABA_A$ receptors (which displayed a tendency toward outwardly rectification) were generally lower than the edited $\alpha3(M)\beta2\gamma2L$ $GABA_A$ receptors.

Sub-conductance levels were also observed in both isoforms. The presence of multiple conductance levels has been described for both native and recombinant GABA$_A$ receptors containing different subunit combinations (Angelotti and Macdonald 1993, Birnir *et al.* 2000, Fisher and Macdonald 1997, Gage and Chung 1994). The existence of multiple levels has not yet received a definitive interpretation but can be explained in several ways. There may be variable conformational states of a single channel, each dependent on the binding of an increasing number of agonist molecules and associated with a different conductance. However, Guyon *et al.* found a high number of conductance levels (more than five) even in patches where the P_o was extremely low (Guyon *et al.* 1999). An alternative hypothesis postulated that several channels could open synchronously (Gage and Chung 1994). The smallest conductance value would correspond to the open state of one single elementary conducting pathway (possibly one GABA$_A$ receptor channel) and the other levels would be due to the synchronized co-operative opening of a variable number of elementary conducting pathways. The potential dependence of the number of sub-conductance levels would be due to potential dependence of the number of conducting conformations (Gage and Chung 1994). Although the basis for the multiple conductance states remains unknown, the broad range of GABA$_A$ receptor channel conductance levels reported in these studies may reflect the configuration or combination of different receptor subunits or the distribution of charges within the ion channel. Moreover, rectification of the GABA$_A$ receptors may be related to an effect of potential on coupling of elementary pores (Gage and Chung 1994).

Because the intracellular chloride concentration and membrane voltage are unknown when using cell-attached patches, I reported slope conductance rather than chord conductance in the present study. As most studies measure the chord conductance, I could not compare my data directly with values in the literature. Nevertheless, the conductances for the $\alpha3\beta2\gamma2L$ GABA$_A$ receptors I reported here were similar to previously published values for $\alpha3\beta3\gamma2S$ GABA$_A$ receptor (Keramidas and Harrison 2010). However, variable conductance levels have been reported and the predominant main conductance state of the GABA$_A$ receptors recorded from the outside-out

patches is about 27-31 pS (Macdonald and Olsen 1994, Twyman 1991). The heterogeneity of observed single-channel conductances of GABA$_A$ receptors could be due to different recording configuration used as well as subunit composition in the preparation. In particular, as noted earlier, the single-channel properties of GABA$_A$ receptor subtypes depend critically on their subunit composition (Angelotti and Macdonald 1993, Lorez *et al.* 2000, Verdoorn *et al.* 1990). I found that recombinant receptors composed of α3 and β2 subunits alone display a markedly lower single-channel conductance (~ 6 pS) in comparison with receptors composed of α3 and β2 subunits in combination with the γ2 subunit (~ 18 pS). Similarly, in human embryonic kidney cells, α1β2 receptors have a main conductance level of 11 pS; whereas α1β2γ2 receptors had a main conductance level of 30 pS (Angelotti and Macdonald 1993). Thus my data is consistent with the prevailing view that the γ2 subunit greatly enhances the efficacy of GABA by determining open conformations of high conductance and long lifetime, and by prolonging the time the receptors remain in the active bursting state (Boileau *et al.* 2002a, Lorez *et al.* 2000).

At least two (and up to three) bursting patterns, or 'modes', have been described for α3β2γ2L GABA$_A$ receptors in the presence of a given GABA concentration. From my data, the most prevalent modes were M- and H-mode but L-mode was occasionally seen in several patches. The origin of modal bursts is unknown, but this type of behaviour has been reported in NMDA receptors (Popescu and Auerbach 2004), GABA$_A$ receptors (Lema and Auerbach 2006), glycine receptors (Plested *et al.* 2007), and nicotinic acetylcholine receptors (Auerbach and Lingle 1986). For all of the above, single channel recordings were done in the cell-attached configuration which might seem to implicate intracellular modulators of channel activity in modal gating. However, modes have also been seen in excised patches including modal switching within the same cluster of activity (Keramidas and Harrison 2010).

In **Figure 4.8**, the kinetic features of α3β2γ2L GABA$_A$ receptors were compared with those reported for the α1β2γ2S GABA$_A$ receptor in the

literature. $\alpha1\beta2\gamma2$ receptors have been extensively studied, and are believed to be the most abundantly expressed among all GABA$_A$ receptors in the CNS (Whiting 2003). The marked differences in the intra-cluster P_o and the cluster length between the two receptor subtypes could account for the differences in activation, deactivation and desensitization kinetics seen for currents mediated by $\alpha1$ and $\alpha3$ subunit-containing receptors (Gingrich *et al.* 1995, Verdoorn 1994). Receptors containing the $\alpha3$ subunit are characterized by slow deactivation, slow desensitization onset and low affinity. Such a particular kinetic pattern has been shown to play an important role in the regulation of network temporal resolution in early development (Ortinski *et al.* 2004). Since Gabra3 editing is also developmentally regulated and gives rise to different expression patterns of the edited isoform in different brain regions, it is important to understand whether the unedited and edited isoform confer different kinetic properties. I found that the cluster lengths of the unedited $\alpha3(I)\beta2\gamma2L$ GABA$_A$ receptors were consistently shorter than the corresponding cluster lengths for the edited $\alpha3(M)\beta2\gamma2L$ GABA$_A$ receptors at a given GABA concentration. This was also true for the intra-cluster and intra-burst P_o. These properties could, to some extent, affect the deactivation phase of IPSCs. It has also been suggested that the relatively high P_o and channel conductance may contribute to the slow decay phase of the IPSCs and increase the total charge transferred through the membrane (Guyon *et al.* 1999, Keramidas and Harrison 2010).

As it is difficult to relate these steady-state data to what may occur at the level of the synapse, in the next chapter I report a series of experiments using rapid agonist application to study the macroscopic kinetics of receptors containing different $\alpha3$ isoforms.

Chapter 5

Effects of α3 RNA editing on properties of macroscopic currents evoked by rapid GABA application

Fast GABAergic synaptic inhibition in the brain reflects the response to a brief but very rapidly rising GABA transient (for review see (Farrant and Kaila 2007). The processes of channel activation, deactivation and desensitization govern the characteristics and duration of the post-synaptic currents under such non-equilibrium conditions. The effects of any differences in the rates of activation and deactivation on the kinetics of the synaptic currents are clear. Desensitization, the decline of responsiveness in the continued presence of GABA (Celentano and Wong 1994, Dominguez-Perrot *et al.* 1996, Haas and Macdonald 1999, Jones and Westbrook 1995), although generally considered as a phenomenon that reduces the peak of IPSCs, can, in fact, enhance GABAergic transmission by prolonging IPSCs (Jones and Westbrook 1995). The high-affinity, long-lived desensitized states delay unbinding of GABA, allowing additional late openings to occur before unbinding and slowing deactivation.

The sum total of $GABA_A$ receptors available for activation at the time of synaptic release is an important factor in controlling the inhibitory contribution of any synapse. This parameter is defined by the actual number of $GABA_A$ receptors that become occupied with agonist following release, and by the fraction of non-desensitized receptors present within the total population (Jones and Westbrook 1996). It seems likely that, when released, the contents of a single GABA-containing vesicle can transiently desensitize a proportion of postsynaptic $GABA_A$ receptors. Desensitization produced in this way may contribute to short-term plasticity of GABAergic transmission (Jones and Westbrook 1996).

Not all GABA$_A$ receptors exhibit the same rates of desensitization and deactivation. It is known that α subunits are critical determinants of ligand binding and of activation, deactivation and desensitization kinetics of GABA-induced responses of native and recombinant GABA$_A$ receptors (Gingrich *et al.* 1995, Lavoie *et al.* 1997, McClellan and Twyman 1999, Verdoorn 1994). Such α subunit dependence of GABA$_A$ receptor function is significant given the differential localization of these subunits in the CNS (Laurie *et al.* 1992a, Pirker *et al.* 2000, Wisden *et al.* 1992). Receptors containing the $\alpha3$ subunit are characterized by slower rates of deactivation and onset of desensitization than most other α subunit subtypes, including the $\alpha1$ (Gingrich *et al.* 1995, Picton and Fisher 2007). Such a peculiar kinetic pattern has been shown to play an important role in the regulation of the network temporal characteristics in early development (Ortinski *et al.* 2004). Although the physiological relevance of the spatial and temporal differential expression of $\alpha3$-containing receptors in the brain is incompletely understood, it is likely that the $\alpha3$ subunit could efficiently serve to provide a prolonged and sustained synaptic GABAergic signal. Of note, the prevalence of the $\alpha3$ subunit can be influenced by pathological changes in the brain. Thus, an increase in $\alpha3$ mRNA is observed during epileptogenesis (Brooks-Kayal *et al.* 1998) while a reduction is often observed following seizure onset (Poulter *et al.* 1999). Additionally, animals lacking the $\alpha3$ subunit exhibit abnormalities in sensorimotor processing similar to those observed in schizophrenic patients (Yee *et al.* 2005).

Importantly, given that the single channel behaviour described in the previous chapter is consistent with the idea that rectification of macroscopic currents most likely reflects voltage-dependent effects on gating (rather than permeation), it is important to investigate the possible effects of $\alpha3$ editing on channel kinetics and rectification under non-equilibrium conditions that mimic those found at the synapse. To do this I used an ultrafast drug delivery system allowing rapid changes of extracellular solution (see **Methods** section 2.5)

5.1 GABA$_A$ receptor rectification in excised patches

To assess the effect of Gabra3 editing on rectification under non-equilibrium conditions, GABA (10 mM) was applied for 100 ms at various holding potentials and the current amplitude was measured both at the peak (the first 5 ms of the current – 'instantaneous') and at the end of the application (the last 20 ms of the current – 'steady-state') (**Figure 5.1**). As can be seen in **Figure 5.2**, both α3(I)β2γ2L and α3(M)β2γ2L receptors exhibited inward rectification (RI < 1). With 10 mM GABA, the RIs of instantaneous I/V relationships of unedited α3(I)- and edited α3(M)-containing receptors were 0.28 ± 0.13 ($n = 6$) and 0.57 ± 0.14 ($n = 4$), respectively ($P = 0.16$, unpaired t-test). The corresponding RIs of the steady-state I/V relationships were 0.42 ± 0.20 ($n = 6$) and 0.34 ± 0.04 ($n = 4$) ($P = 0.77$, unpaired t-test). With a lower concentration of GABA (1 mM) the I/Vs were more linear, with instantaneous RIs for unedited α3(I)- and edited α3(M)-containing receptors (both $n = 5$) of 0.71 ± 0.15 and 0.79 ± 0.09. The corresponding steady-state RIs were 0.80 ± 0.16 and 1.01 ± 0.17 (**Figure 5.3** and **Table 5.1**).

Importantly, there was no difference in rectification between receptors containing the α3(I) and α3(M) isoforms (**Figure 5.2 and 5.3**). This was also true of β3-containing receptors, as described for whole-cell ramps with α3β3γ2L receptors (see section 3.5). Although β3-containing receptors displayed significantly greater inward rectification that those containing the β2 subunit, α3(I)β3γ2L receptors did not differ from α3(M)β3γ2L receptors (**Table 5.1**). Interestingly, a difference in the rectification of β3- and β2-containing receptors was also seen when comparing α1βxγ2L receptors (**Figure 5.4**). Thus, the instantaneous I/V relationships of the α1β2γ2L receptors were less inwardly rectifying (RI 0.87 ± 0.04, $n =3$) than those of the α1β3γ2L GABA$_A$ receptors (RI 0.61 ± 0.09, $n =7$; $P = 0.028$, unpaired t-test). For the steady-state I/Vs, the same trend was apparent but this did not reach statistical significance (0.75 ± 0.11 and 0.59 ± 0.09, $P = 0.33$).

For $\beta2$-containing receptors, the rectification observed in outside-out patches was different from that seen in whole-cell recordings (Chapter 3). Comparing currents recorded in response to 1 mM GABA: (a) whole-cell responses to bath applied GABA assessed using voltage steps showed outward rectification; (b) whole-cell responses to locally applied GABA assessed using voltage ramps showed linear I/Vs; (c) patch responses rapidly applied GABA at different voltages ramps exhibited mild inward rectification. Factors that differed between these recordings were the speed and duration of GABA exposure and the physical environment of the receptors. Thus, one concern with comparisons of rectification between the whole cell and excised patch configurations is that channel behaviour might be altered if patch excision disrupts interactions with cytoplasmic factors or with the cytoskeleton. For example, phosphorylation and interactions with clustering proteins are known to affect GABA$_A$ receptor function (Chen *et al.* 2000, Jones and Westbrook 1997). However, it is also possible that the differences are related to the technical limitations in exchanging solutions around whole cells on a time scale sufficiently rapid to resolve fast processes causing a failure to reach the 'true' peak with slower, localized drug applications.
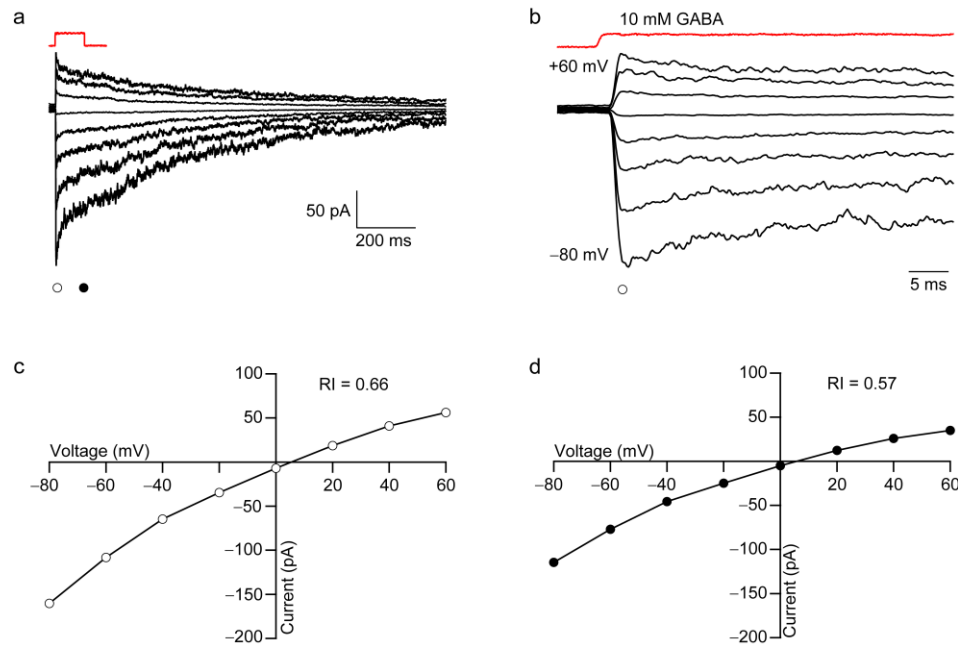
Figure 5.1 Experimental protocol for measurement of I/V properties of macroscopic GABA-evoked currents.
a) Currents were evoked at several command voltages by ultrafast application of 10 mM GABA (100 ms duration) onto patches containing edited α3(M)β2γ2L GABA$_A$ receptors. The GABA application is indicated by the red trace. **b**) An expanded section of the first part ($\sim$ 30 ms) of **a**. Voltage steps ranging from $-$80 mV to $+$60 mV in $+$20 mV increments were applied. **c** and **d**) Corresponding I/V relationships for the instantaneous (o) and the steady-state ($\bullet$) currents respectively. Instantaneous currents were measured at the first 2 ms of the response, whereas steady-state currents were measured during the last 20 ms of the response.
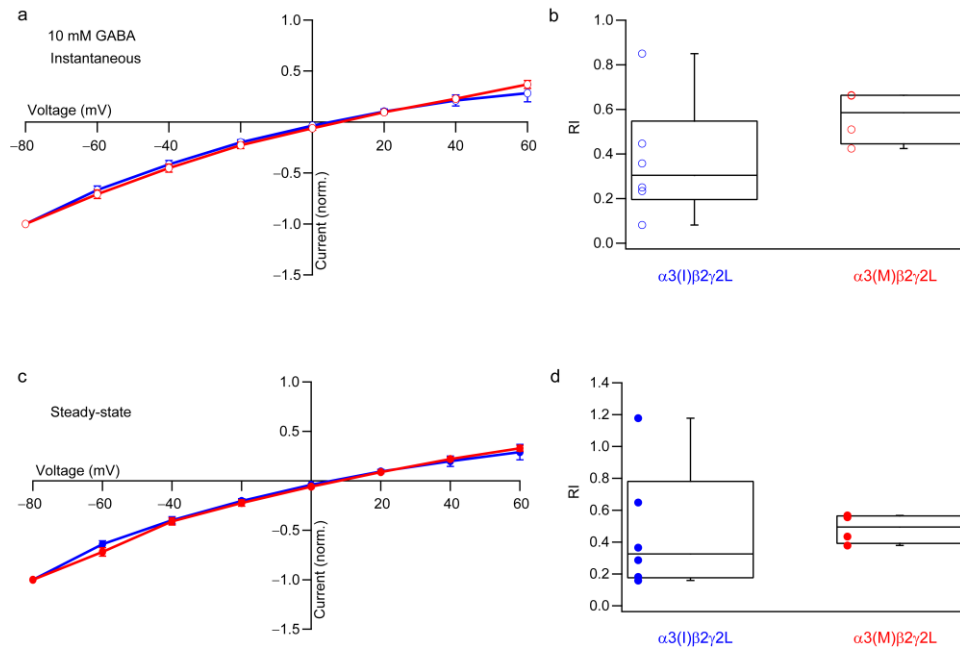
Figure 5.2 Inwardly rectifying I/V relationships of $\alpha3\beta2\gamma2L$ GABA$_A$ receptors with 10 mM GABA.
Normalised I/V relationships of unedited (I) and edited (M) $\alpha3\beta2\gamma2L$ GABA$_A$ receptors obtained with jumps into 10 mM GABA. The instantaneous (**a**) and steady-state (**c**) I/V relationships of both isoforms display inward rectification. Data are presented as mean and SEM (n = 6 and 4). **b** and **d**) Boxplots of instantaneous and steady-state RIs for unedited (I) and edited (M) $\alpha3\beta2\gamma2L$ GABA$_A$ receptors.
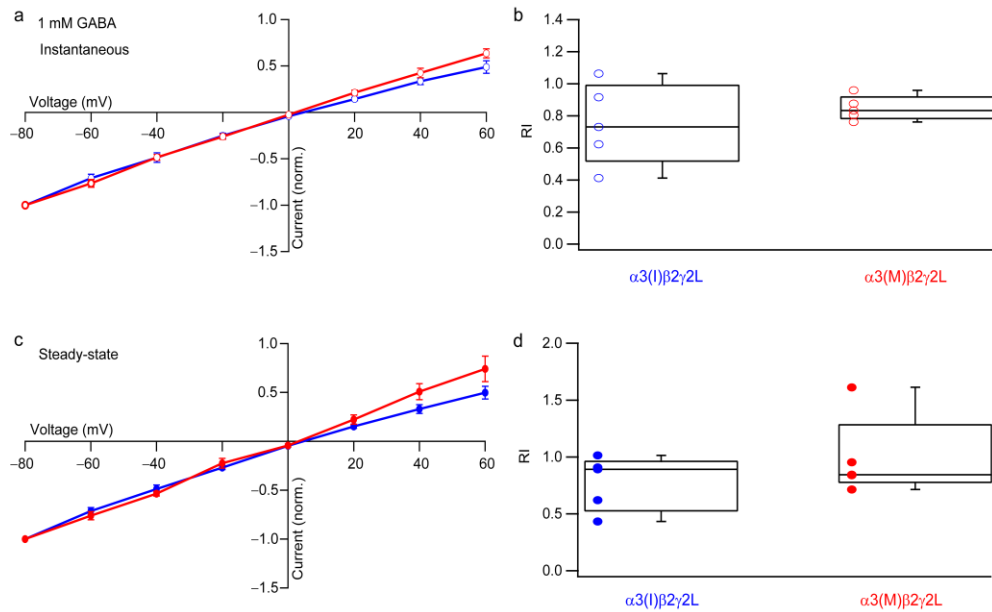
Figure 5.3 I/V relationships of $\alpha 3\beta 2\gamma 2L$ GABA$_A$ receptors with 1 mM GABA.
Normalised instantaneous (**a**) and steady-state (**c**) I/V relationships of unedited (I) and edited (M) $\alpha 3\beta 2\gamma 2L$ GABA$_A$ receptors obtained with jumps into 1 mM GABA (both $n = 5$). The instantaneous and steady-state I/V relationships of both isoforms are essentially linear. (**b**) and (**d**) Boxplots of instantaneous and steady-state RIs.

	RI			
	mean ± s.e.m. (*n*)			
	α3β2γ2L		α3β3γ2L	
Subtype	Instantaneous	Steady-state	Instantaneous	Steady-state
α3(I)	0.71 ± 0.15 (*5*)	0.80 ± 0.16 (*5*)	0.38 ± 0.05 (*8*) ∗	0.33 ± 0.03 (*8*) ∗∗
α3(M)	0.79 ± 0.09 (*5*)	1.01 ± 0.17 (*5*)	0.46 ± 0.05 (*9*) ∗∗	0.49 ± 0.07 (*9*) ∗∗

Table 5.1 Rectification indices of α3βxγ2L subunit isoforms in the presence of 1 mM GABA.
β subunit content has a significant effect on rectification. ∗ indicates $P < 0.05$ and ∗∗ indicates $P < 0.01$ when compared with α3β2γ2L GABA$_A$ receptors (unpaired *t*-test).
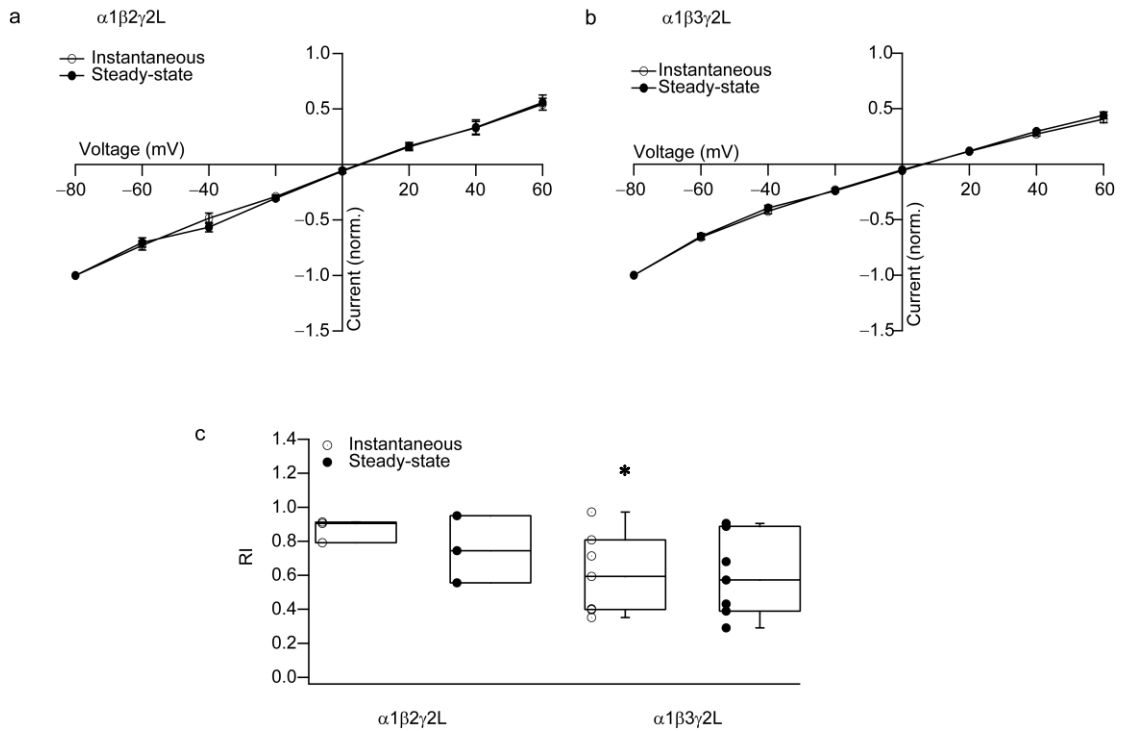
Figure 5.4 I/V relationships of α1 subunit-containing GABA_A receptors with 1 mM GABA.
Normalised instantaneous and steady-state I/V relationships of $\alpha1\beta2\gamma2L$ (**a**) and $\alpha1\beta3\gamma2L$ (**b**) GABA_A receptors obtained with jumps into 1 mM GABA ($n = 3$ and 7). Corresponding boxplots in (**c**). ∗ denotes $P < 0.05$ compared to instantaneous RI of $\alpha1\beta2\gamma2L$ receptors (unpaired t-test).

5.2 Effects of RNA editing on the macroscopic kinetic properties of α3 subunit-containing GABA$_A$ receptors

I next examined more closely the kinetic behaviour of receptors containing the different α3 isoforms. For this I used α3β3γ2L receptors, as the β3 subunit afforded more consistent expression. Application of 1 mM GABA (100 ms) produced rapidly activating, slowly bi-exponentially decaying currents from outside-out patches. A representative response from a patch containing α3(M)β3γ2L receptors is shown in **Figure 5.5a**. On average, currents from unedited α3(I)β3γ2L receptors activated more rapidly than those from the edited α3(M)β3γ2L receptors (**Figure 5.5b**): the 10-90% rise times for currents were 4.4 ± 0.7 ms (n = 7) and 11.9 ± 1.7 ms (n = 9), respectively (P = 0.003, unpaired t-test).

The desensitization and deactivation phases of the currents were each fit with the sum of two exponential components. The unedited α3(I) receptors exhibited a small fast component of desensitization that was essentially absent from the responses of the edited α3(M) receptors (**Table 5.2**). Thus, the fast time constant of desensitization was approximately 9-fold faster in the α3(I)- compared with the α3(M)-containing receptors. However, the relative contribution of this component was small (<20%) and the weighted-mean time constants of desensitization were similar (**Figure 5.5c**; **Table 5.2**). The mean time constants were 96.3 ± 14.4 ms (n = 7) and 107.2 ± 14.3 ms (n = 9) for the unedited (I) and edited (M) receptors, respectively (P = 0.60, unpaired t-test). Unfortunately, longer duration pulses would be required to determine whether additional, slower phases of desensitization exist. Entry into long-lived desensitized states would be expected to influence the duration of the synaptic current, the spread of the synaptic signal and the response to repetitive stimulation (Jones and Westbrook 1995, Overstreet *et al.* 2000).

As shown in **Figure 5.5d** and **Table 5.2**, following the jump out of GABA the unedited α3(I)β3γ2L receptors deactivated ~20% slower than the edited α3(M)β3γ2L receptors. Of note, α3(I)β3γ2L receptors have previously been

reported to exhibit a slower deactivation than edited $\alpha3(M)\beta3\gamma2L$ receptors, with an apparent decrease in the relative contribution from the fast component, rather than a change in the time constants (Nimmich *et al.* 2009). However, from my data, neither the weighted time constants nor the fractional contributions of the fast component differed significantly between the two isoforms (P = 0.30 and 0.12, respectively; unpaired *t*-tests) (**Table 5.2**).
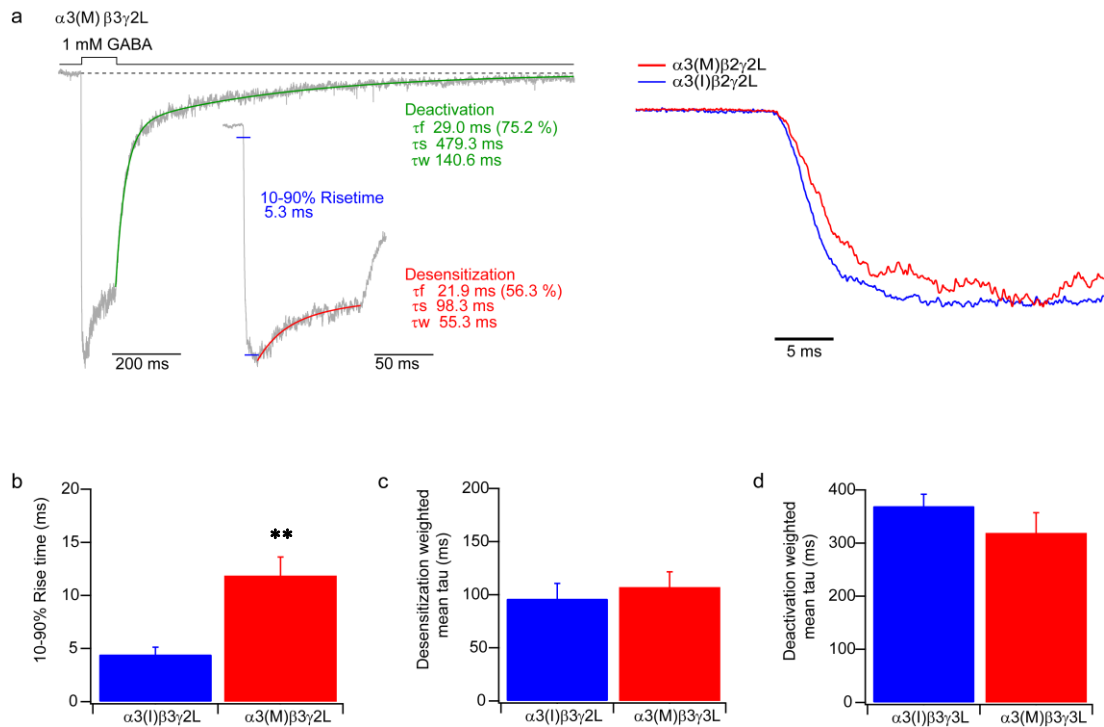
Figure 5.5 RNA editing does not alter desensitization or deactivation of α3β3γ2L GABA$_A$ receptor currents evoked by 1 mM GABA.
a) A representative current evoked by ultrafast application of 1 mM GABA to an excised patch from a tsA201 cell expressing edited α3(M)β3γ2L GABA$_A$ receptors. The GABA pulse of 100 ms duration is shown by the step above the trace. Desensitization and deactivation were each fitted with the sum of two exponential components. Current rise time (as indicated by the time elapsed between 10 and 90% of the peak current) was also measured. The inset shows an expanded section of the initial phase of the currents; the α3(I)β3γ2L receptors can be seen to have a faster activation than the α3(M)β3γ2L receptors. Quantification of the macroscopic current properties is shown as bar plots for **b**) current rise time, **c**) weighted mean time constants of desensitization, and **d**) weighted mean time constants of deactivation α3(I)β3γ2L ($n = 7$) and α3(M)β3γ2L ($n = 9$) GABA$_A$ receptors. ∗∗ indicates $P < 0.01$ (unpaired t-test). Individual time constants are given in **Table 5.2**.

	α3(I)β3γ2L (*n* = 7)	α3(M)β3γ2L (*n* = 9)
I_{GABA} (pA)	131.38 ± 16.7	44.22 ± 11.4 ***
10-90% Rise time (ms)	4.44 ± 0.7	11.88 ± 1.7 **
Desensitization		
τ1 (ms)	10.32 ± 2.3	91.83 ± 17.9 ***
τ2 (ms)	113.77 ± 13.9	120.50 ± 13.3
% τ1	19.40 ± 6.8	52.73 ± 6.4 ***
Weighted mean τ (ms)	96.25 ± 14.4	107.20 ± 14.3
Deactivation		
τ1 (ms)	123.31 ± 26.1	156.40 ± 49.0
τ2 (ms)	545.82 ± 53.8	521.43 ± 34.6
% τ1	40.03 ± 7.1	55.23 ± 6.0
Weighted mean τ (ms)	369.69 ± 22.3	319.26 ± 37.7

Table 5.2 Editing effects on macroscopic kinetics of α3β3γ2L GABA$_A$ receptors. Data are shown as mean ± SEM. ∗∗ indicates $P < 0.01$ and ∗∗∗ indicates $P < 0.001$ when compared with the unedited α3(I)β3γ2L GABA$_A$ receptors (unpaired *t*-test). Visual inspection, and examination of the standard deviation of the residuals, showed that for both receptor isoforms, fits of current deactivation required two components with >4-fold difference in their time constants. For desensitization, α3(I)β3γ2L clearly required two components with >10-fold difference in their time constants. α3(M)β3γ2L desensitization was less clearly fit by a double exponential (although an F-test was not used to compare goodness of fit). Nevertheless, for consistency across the isoforms α3(M)β3γ2L deactivation was also fit with two components (although in this case the time constants were not greatly different).

5.3 Discussion

My recordings of macroscopic currents in response to rapid GABA application showed that exchanging the isoleucine residue encoded by the DNA sequence for the methionine residue produced by RNA editing led to only modest functional consequences for $\alpha3\beta2\gamma2L$ receptors. Activation (10-90% rise time) became faster but I found no significant change in desensitization or deactivation.

Editing of the $\alpha3$ subunit has previously been found to produce a speeding of deactivation, attributable to an increase in the relative contribution of the fast component of the decay (Nimmich *et al.* 2009, Rula *et al.* 2008). This led to the suggestion that IPSCs in $\alpha3$-expressing neurons would decay more slowly early in development, when the I/M site is unedited. GABAergic neurotransmission onto neurons expressing the unedited $\alpha3$ subunit might therefore be expected to be more effective, due to a longer-lasting conductance change, compared to neurons expressing the edited subunit. As this early expression of the unedited $\alpha3$ subunit would occur at a developmental stage when GABA may be depolarizing, it has been proposed that a prolonged action may serve to enhance 'excitatory' actions of GABA and that editing of $\alpha3$ may contribute to the alteration of the $GABA_A$ receptor properties in a manner that is temporally linked to the shift from depolarizing to hyperpolarizing actions of GABA (Nimmich *et al.* 2009, Rula *et al.* 2008).

Although I observed a trend towards a faster deactivation of the $\alpha3$(M) receptors, the 20% decrease in mean τ_w was less than the 50-70% change reported by others (Nimmich *et al.* 2009, Rula *et al.* 2008). Whether my failure to detect a speeding in deactivation reflected mundane experimental differences (inadequate cell numbers or inappropriate protocol of GABA exposure) is unclear. Certainly, while the slow time constants of deactivation I observed (both ~550 ms) were comparable to those reported by (Nimmich *et al.* 2009), the fast time constants for both $\alpha3$(I) and $\alpha3$(M) were very much slower than previously reported (~5-fold). This may reflect the fact that I

attempted to determine both desensitization and deactivation with a single 100 ms-long jump into GABA, rather than separate protocols (of 400 and 5 ms) as used by Nimmich *et al.* It is interesting to note that (as described in Chapter 4) cluster lengths of the unedited $\alpha3(I)\beta2\gamma2L$ GABA$_A$ receptors were consistently shorter than the corresponding cluster lengths for the edited $\alpha3(M)\beta2\gamma2L$ GABA$_A$ receptors at a given GABA concentration. This would at least be consistent with a potential effect of editing on current deactivation. However, of note, in both of the other studies the cells were transfected with $\alpha/\beta/\gamma$ subunit plasmids at a ratio of 1:1:1, whereas I used an excess of $\gamma2L$ (1:1:10) to avoid the formation of $\alpha\beta$-only receptors (see Chapters 3 and 4). As the deactivation of $\alpha\beta$ is faster than that of $\alpha\beta\gamma$ receptors (Haas and Macdonald 1999, Tia *et al.* 1996), an editing-induced alteration in the efficiency of assembly or enhanced degradation (see Chapter 3), ultimately affecting the ratio of surface $\alpha\beta$ and $\alpha\beta\gamma$ receptors, could conceivably contribute to the greater speeding of deactivation observed in the other studies.

Chapter 6

General discussion

RNA editing provides a post-transcriptional mechanism to increase heterogeneity of gene products. RNA editing by ADARs of the $\alpha 3$ subunit of GABA$_A$ receptors (Gabra3 transcript) results in an isoleucine codon being altered to a methionine codon. This I/M site resides at position 342 in the third transmembrane domain of the $\alpha 3$ subunit. The experiments described in this thesis were aimed at characterizing the effects of these changes on the functional properties of $\alpha 3$-containing GABA$_A$ receptors. The impetus for the studies came from the novelty of the newly discovered Gabra3 RNA editing site and the recognition that editing can have dramatic effects on the function of other ion channels (as exemplified by the GluA2 subunit of the AMPA-type glutamate receptor) and the observation that changes in $\alpha 3$ expression and editing were temporally correlated with the developmental shift from depolarising to hyperpolarising GABA$_A$ receptor-mediated signalling (Ohlson *et al.* 2007).

I found that editing of Gabra3, causing an I/M alteration in the third transmembrane domain, resulted in various changes in the functional properties of $\alpha 3$-containing GABA$_A$ receptors. These changes were modest, but included lower current densities with the edited $\alpha 3$(M) subunit, slower channel activation, a trend toward longer burst lengths and slightly greater intra-burst P_o, and slowed fast component of desensitization. I found that, like other GABA$_A$ receptor subtypes, responses from $\alpha 3$ subunit-containing GABA$_A$ receptors were modulated by voltage, the modulatory effect being observed as a non-linearity or rectification of the macroscopic I/V relationship. Although the extent of rectification was seen to vary with protocol and GABA concentration, I saw no effect of $\alpha 3$ editing on the voltage-dependence. Thus, the I/V relationships of both unedited and edited isoforms were not different

when they were determined in the whole-cell recording configuration from ramp data or from data obtained by stepping the membrane potential in the continued presence of GABA.

6.1 RNA editing and receptor trafficking

It has become clear that the stability and activity of $GABA_A$ receptors at synapses can be dynamically modulated by receptor trafficking and phosphorylation (Brandon *et al.* 2002, Kittler and Moss 2003). My results from whole-cell experiments, where there was a reduced current density with $\alpha3(M)\beta2\gamma2L$ receptors, would tend to support the idea (Daniel *et al.* 2011) that RNA editing may facilitate the switch of subunit compositions during development. Of particular additional interest may be the interplay between $\alpha3$ and $\alpha1$ subunits.

As discussed in Chapter 3, editing of the $\alpha3$ subunit appears to have a negative impact on surface expression of the receptors. My own findings (and those of Rula *et al.*) of reduced current density with $\alpha3(M)$-containing receptors, support data showing decreased surface labelling in HEK293 cells (immunofluorescence or biotinylation) of $\alpha3(M)\beta2/3\gamma2L$ compared to $\alpha3(I)\beta2/3\gamma2L$ receptors (Daniel *et al.* 2011). These authors showed that a corresponding mutation in $\alpha1$ (I315M) also reduced receptor surface expression, and noted the similarity of this effect to that of a nearby mutation $\alpha1(A322D)$. This mutation was previously identified as a cause of an autosomal dominant form of juvenile myoclonic epilepsy (ADJME) (Cossette *et al.* 2002), which causes the $\alpha1(A322D)$ subunit to fold incorrectly, leading to its degradation via the proteasome and lysosome (Bradley *et al.* 2008, Gallagher *et al.* 2007). Of interest was the observation that the residual $\alpha1(A322D)$ subunit expression exerted a 'dominant negative' effect that reduced $GABA_A$ receptor expression in HEK293T cells to a greater extent than would be expected from reduced copies of the gene alone, and that this was associated with markedly reduced expression of $\alpha3\beta2\gamma2$ receptors (Ding

et al. 2010). It was suggested that α1(A322D) subunits may preferentially associate with α3 rather than α1 subunits thus trapping them as nonfunctional α1/α3 oligomers in the ER, potentially resulting in an epilepsy phenotype that differs from loss of α1 alone. As these experiments were performed with the edited form of α3, it would be interesting to determine whether the unedited α3(I) form behaved similarly, particularly as the α1(A322D) mutation is associate with a juvenile onset epilepsy.

6.2 RNA editing and rectification

The apparent lack of effect of editing contrasts with the result of Rula *et al.* (2008). As discussed in Chapter 3, one possibility I considered was that differences in the results could reflect differences in the proportion of receptors with tri-heteromeric (α, β and γ) and di-heteromeric (α and β) forms. I used a transfection ratio of 1:1:10 (α:β:γ) that, as suggested by the pharmacological and single-channel studies of Boileau *et al.* in *Xenopus* oocytes (Boileau *et al.* 2002a) and mammalian cell lines (Boileau *et al.* 2005), and by our single-channel recordings, favoured the inclusion of the γ2L subunit, to produce ternary α3β2γ2L GABA$_A$ receptors. By contrast, Rula *et al.* (2008) used a transfection ratio of 1:1:1, raising the possibility that the cells may have expressed both α3β2γ2L and α3β2 GABA$_A$ receptors. Interestingly, another study of α3 editing (Nimmich *et al.* 2009) also used a transfection ratio of 1:1:1, but commented that they did not observe a 'high affinity component' in their GABA concentration-response relationships, as might have been expected if high affinity $\alpha$$\beta$ heteromers were present (EC_{50} of ~60 μM for α3(M)β3γ2L *versus* ~3 μM for α3(M)β3). These authors also observed pharmacological properties of the receptors that were consistent with the presence of the γ2 subunit (see below). It is not clear why these studies come to different conclusions about the receptor assemblies for given transfection ratios. The sensitivity of the different assays is likely to be one factor, but the presence or absence of low-conductance openings (Chapter 4) is surely a straightforward indicator. Setting aside these

arguments, in the end the issue of $\alpha\beta\gamma$ *versus* $\alpha\beta$ receptors appeared not to account for the discrepancy in editing effects on rectification, as I found that $\alpha3(M)\beta3$ receptors displayed *greater* outward rectification than $\alpha3(I)\beta3$ receptors. This would not explain the results of Rula *et al.*, where $\alpha3(M)\beta3\gamma2L$ GABA$_A$ receptors showed *less* outward rectification than $\alpha3(I)\beta3\gamma2L$ receptors.

Many studies have identified rectification of GABA$_A$ receptor-mediated currents that is independent of Goldman rectification (i.e. that does not arise simply from asymmetrical chloride concentrations). In a recent study, O'Toole and Jenkins (2012) found that outward rectification (with recombinant $\alpha1\beta2\gamma2S$ receptors) was inversely related to the degree of channel activation – rectification was greatest at low channel open probability. Thus, they observed (as did I; **Fig. 3.8**) that at higher GABA concentrations ramp-generated I/V relationships became more linear. A link between the degree of channel activation and the amount of rectification was suggested after introducing a deleterious gating mutation – $\alpha1(L277A)$ – to hinder channel gating, and thus decrease P_o. It was shown that the decrease in channel P_o was associated with increased outward rectification. Ordinarily, as demonstrated by Bormann *et al.* (Bormann *et al.* 1987), increasing chloride concentration equally on both sides of the membrane increases channel conductance by increasing the number of charge carriers available. O'Toole and Jenkins suggested that at low P_o, the more frequent presence of gating elements within the pore may hinder ion access to parts of the pore, which would result in *charge asymmetry* and thus rectification. The linearization of the I/Vs at high P_o was suggested to result from an enhanced desensitization of outward currents (O'Toole and Jenkins 2011). These authors also put forward an alternative view, namely that outward rectification, seen as enhanced magnitude of outward currents, rather than reflecting charge asymmetry, would occur if the inward permeability of chloride (P_{Cl}) was greater than the outward permeability. Their calculations indeed suggested that, at low P_o, depolarization increased P_{Cl}, and that there was an *asymmetry of chloride permeation*. How these suggested

mechanisms of rectification relate to the possibility of voltage-dependence changes in gating and to studies that have shown apparently ohmic behaviour of single-channel conductance (see Chapter 4) remain to be resolved. Nevertheless, this increase in macroscopic conductance with depolarization could serve as a protective feedback mechanism. Synaptically released (or ambient) GABA may be more effective, when the membrane is depolarized. This would facilitate a return to the resting membrane potential, and protect the neuron from a large prolonged depolarization.

6.3 RNA editing and receptor kinetics

Effects of Gabra3 editing on macroscopic receptor kinetics (activation, deactivation and desensitization) were examined in Chapter 5. The $\alpha3(I)$-containing receptors gave currents that activated more rapidly, with a faster rise time and had a faster desensitization onset than the receptors containing edited $\alpha3(M)$ subunits. On average the weighted-mean time constant of deactivation was slowed slightly, but this was not statistically significant. The modest differences in desensitization between the isoforms were largely in the relative contributions of the components and, in part, consistent with the findings of others (Nimmich *et al.* 2009, Rula *et al.* 2008). The kinetic properties of native $GABA_A$ receptors vary with the type of neuron and its stage of development. Typically, the IPSC decay is found to be slower in neurons early in development, changing to more rapid decay with adulthood (Takahashi 2005). Very different IPSC decay rates are seen in different kinds of neurons expressing different subunit types (see (Eyre *et al.* 2012). Since RNA editing of the $\alpha3$ subunit increases throughout development, others have postulated that GABAergic neurotransmission mediated by $\alpha3$-containing receptors may be more effective early in development, with greater GABA sensitivity and slower decay rates conferred by the unedited $\alpha3$ isoform (Nimmich *et al.* 2009, Rula *et al.* 2008).

6.4 Influence of subunit heterogeneity

In addition to RNA editing, subunit heterogeneity among $\alpha 3$ subunit-containing GABA$_A$ receptors could have an impact on receptor properties (Sieghart and Sperk 2002). Initially, I used the $\alpha 3\beta 2\gamma 2L$ combination to examine the RNA editing effects. Since the $\beta 3$ is more highly expressed in the developing brain (Laurie *et al.* 1992b), it is likely that the GABA$_A$ receptors containing the $\alpha 3$ subunit may express different β subunit in immature and adult brains. Accordingly, I also made recordings with the $\beta 3$ subunit. I found that incorporation of $\beta 3$ subunit significantly increased current amplitudes and reduced rectification in all studies. The $\beta 3$ subunit has a negatively charged glutamic acid in the second transmembrane domain, a region that is suggested to play an important role in determining the conductance and ionic selectivity of the channel pore (Smith and Olsen 1995). This change in charge could influence chloride conductance for the channel. Indeed, mutagenesis of the positively charged residue introduced rectification of the whole-cell I/V relationship, suggesting that this region can influence conductive properties (Backus *et al.* 1993, Fisher 2002). Taken together, the likely changes in the subunit composition of $\alpha 3$-subunit containing GABA$_A$ receptors suggest the possible existence of molecularly and functionally distinct immature and adult forms. Although I saw differences between $\alpha 3\beta 2\gamma 2L$ and $\alpha 3\beta 3\gamma 2L$ receptors, with $\beta 3$ subunit-containing receptors having less outward rectification than $\beta 2$-containing receptors, I observed no effect of $\alpha 3$-editing on rectification.

6.5 Future directions

In my experiments I used transient expression of recombinant GABA$_A$ receptors in a non-neuronal cell line. Accordingly, there is the potential for differences in channel characteristics compared to native receptors because of neuron-specific processes that regulate receptor function. Neuronal receptors may be differentially subject to modulation by post-translational modifications such as phosphorylation (Brandon *et al.* 2002), interactions

with cytoskeletal proteins and differences in assembly and membrane targeting (Chen and Olsen 2007, Fritschy *et al.* 1998, Fritschy *et al.* 2012, Tretter *et al.* 2011, Wu *et al.* 2012).

Expression of selected subunit combinations in cell lines that do not normally express functional GABA$_A$ receptors allows one to regulate the subunit composition of the receptors and thus describe the characteristics of a homogeneous population of receptors. However, it is also likely that neurons will produce a heterogeneous population of α3 editing forms, especially early in development. This raises the possibility that mixed receptors may be formed, carrying one edited and one unedited α3 subunit, or receptor populations containing both α3 and other α isoforms (such as α1) (Benke *et al.* 2004, Duggan *et al.* 1991). Accordingly, any further studies in neurons would be required to verify any predicted functional impact of RNA editing of the α3 subunit. For example, with the possibility of heterogeneous populations of α3 editing forms, this would raise a question of whether the kinetic properties of these receptors are intermediate, or might they be dominated by the characteristics of one of the isoforms?

The I/M editing site in α3 is adjacent to the extracellular TM2-TM3 linker region, suggested to be important in channel gating (Ernst *et al.* 2005). This linker region is thought to contribute to a water-filled cavity, which forms a binding pocket for volatile anaesthetics and ethanol (Campagna-Slater and Weaver 2007, Jung and Harris 2006, Krasowski and Harrison 2000, Wang *et al.* 2010). It has been reported that several residues within TM3 are accessible to the aqueous solution, and that changes in its secondary structure occur with GABA binding (Williams and Akabas 1999). Mutations of residues within the TM2-TM3 region affect GABA$_A$ receptor sensitivity to modulation by neurosteroids (Williams 2011), ethanol (Ueno *et al.* 1999), and protons (Wilkins *et al.* 2005). While this could suggest that α3 editing in TM3 might potentially influence GABA$_A$ receptor pharmacology, the group of Fisher have shown that the effects of the benzodiazepine-site positive modulators diazepam and zolpidem, the neurosteroid alphaxalone, the

intravenous anaesthetics pentobarbital and etomidate, and the $\alpha3$-selective modulators SB-205384 and stiripentol, albeit at single concentrations, were not influenced by the editing state (Fisher 2009, Nimmich *et al.* 2009).

Although further studies of the effects of $\alpha3$ editing of drug action might appear unlikely to reveal differences between isoforms, the effects of $\alpha3$ editing on trafficking would seem a worthwhile topic of further study, as this has been only partially addressed. $GABA_A$ receptors are crucial in the control of cell and network activity, therefore modulating their cell surface stability can have major consequences for neuronal excitation.

Finally, the balanced development of excitatory and inhibitory inputs has been characterised as an activity-dependent process (Eichler and Meier 2008, Fritschy 2008). The developmental shift from depolarization to hyperpolarization in $GABA_A$ receptor-mediated transmission is paralleled by changes in $GABA_A$ receptor subunit expression. Thus a marked switch from $\alpha3$- or $\alpha2$- to $\alpha1$ subunit-containing receptors is linked with faster IPSC decay in the adult compared to the neonate. Of interest, a recent study in primary cultures of cerebellar stellate/basket neurons has demonstrated that the shift in the polarity of chloride gradient during development could modulate the sequential expression of specific $GABA_A$ receptor subtypes (Succol *et al.* 2012). Intracellular chloride concentration was found to regulate the shift of $\alpha3/\alpha1$ and δ subunit expression and consequently the decay kinetics of GABAergic IPSCs and the extent of tonic inhibition. How intracellular chloride might act as a second messenger to influence transcription, translation, trafficking, targeting or post-transcriptional modifications is unclear, but a wider examination of the activity-dependent regulation of $\alpha3$ subunit and their editing in other neurons would be of interest.

Chapter 7

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