GABA_A RECEPTORS OF THE RAT DENTATE GYRUS

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The errors have been underlined and the corrections are in bold where possible.

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| 1       | 2           | 3           | Sieghart | GABA receptors ...
| 1       | 2           | 11          | The GABA receptor ... | ...
| 1       | 2           | 23          | ... but unlike the GABA_A ... | ...
| 1       | 4           | 15          | ... examined, for example ... | ... examined in terms of their ...
| 1       | 6           | 18          | ... subpopulations confer different ... | subpopulations with different ...
| 1       | 8           | 7           | ... receptors conferring bicuculline ... | ...
| 1       | 8           | 27          | The nature of the ... | Investigations of the nature of the ...
| 1       | 10          | 3           | ... pentobarbitone and phenobarbitone). | ...
| 1       | 10          | 27          | ... conflicting _ _ whether the ... | ...
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| 1       | 13          | 6           | ... partiality towards ... | ...
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| 1       | 15          | 20          | 1605±199mM | 1605±199µM
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| 3       | Fig 3.1.B. | label       | ASCF | ACSF
| 3       | 20          | 16          | 10mM and 140mM ... | 5mM and 10mM ...
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| 3       | 38          | 27          | recover_ | recover.
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| 4       | 53          | 24          | ... so a straight _ was ... | ...
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... effects of 100µM bicuculline...
... decay of the currents was...
... different affinities to Zn²⁺.
... slowed down the rate of...

Algar

... were applied together.
... activated by a saturating...
... above, showed that the differences...
... observed, although...

... 7µM GABA and 10mM pentobarbital...
... possibility that these GABAₐ...
... application, this suggests...
... they had not adhered well...
... the presence of 0.5µM GABA, ...

Frequency
... states was obtained...
... conductance states...
... mean currents increase in ...
... were also modulated by ...


Seighart
I declare that the material presented in this thesis is my own work and contains no material previously published or written by another person. The research material in this thesis has not been submitted or accepted for the award of any other degree or diploma in any university.

Michelle S.F. Lim

[Signature]
This thesis is dedicated to my parents.

There is no doubt that I could not have achieved this without the both of you.
With all my love and gratitude, always.
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Last but by no means least, a humongous thank you to my Family both in Australia and around the world. You were always there with a great deal of support, encouragement and understanding. Love you all.
PUBLICATIONS


The GABA<sub>A</sub> receptor is a member of the ligand-gated ion channel family and approximately 30-40% of all central synapses use GABA as their neurotransmitter. The GABA<sub>A</sub> receptor is the target for many mood- and emotion- altering drugs such as general anaesthetics, benzodiazepines, steroids and alcohol. However, the mechanisms that underlie receptor activation by GABA and modulation by drugs are not well understood. The dentate gyrus is a region that develops postnatally and in situ hybridisation studies have indicated high expression of the α<sub>4</sub> and δ subunits in the mature GABA<sub>A</sub> receptor. Recombinant receptors expressing these subunits exhibit unique characteristics that will aid in the identification of these subunits in the dentate gyrus. By optimising neuronal dissociation techniques, isolated granule cells were obtained and whole-cell patch clamping performed. Several measurements were taken from whole-cell responses that include peak and plateau current amplitudes as well as exponential fits to the decay of the currents. These responses were compared to responses activated by GABA and depressed by either bicuculline or Zn<sup>2+</sup>. Dose-response curves were generated from peak and plateau current amplitudes and produced different EC<sub>50</sub> or IC<sub>50</sub> values. Compared to plateau currents, peak currents were less sensitive to GABA and Zn<sup>2+</sup> but more sensitive to bicuculline. Although bicuculline and Zn<sup>2+</sup> both depressed GABA-activated currents, bicuculline affected the faster exponential component while Zn<sup>2+</sup> affected the slower exponential component. Increasing concentrations of pentobarbital elicited a range of effects that include depression, enhancement and abolition of currents activated by GABA. In addition, 100µM pentobarbital did not directly activate the GABA<sub>A</sub> receptor. Single channel results obtained from the dentate gyrus region of hippocampal slices showed two characteristically different channels that could be modulated by pentobarbital and diazepam. Taken together, these results show at least two pharmacologically distinct responses that could be attributed to two different receptors. Subunit combinations were predicted and the two major GABA<sub>A</sub> receptor populations were proposed to be α<sub>4</sub>βγ<sub>2</sub> and α<sub>4</sub>βδ.
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<tr>
<td>A&lt;sub&gt;1&lt;/sub&gt;, A&lt;sub&gt;2&lt;/sub&gt;</td>
<td>amplitudes associated with the exponential time constants, pA</td>
</tr>
<tr>
<td>ACSF</td>
<td>artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine tri-phosphate, chloride salt</td>
</tr>
<tr>
<td>C</td>
<td>steady-state current amplitude, pA</td>
</tr>
<tr>
<td>c/a</td>
<td>cell-attached patch configuration</td>
</tr>
<tr>
<td>CA1-4</td>
<td>CA regions of the hippocampus, numbered 1-4</td>
</tr>
<tr>
<td>CaCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>calcium chloride</td>
</tr>
<tr>
<td>Cl&lt;sup&gt;-&lt;/sup&gt;</td>
<td>chloride ion</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DZ</td>
<td>diazepam</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>half-effective agonist concentration</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diaminetetra-acetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>half-effective antagonist concentration</td>
</tr>
<tr>
<td>I&lt;sub&gt;M&lt;/sub&gt;EAN</td>
<td>mean current</td>
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<tr>
<td>i/o</td>
<td>inside-out patch configuration</td>
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<td>IPSC&lt;sub&gt;s&lt;/sub&gt;</td>
<td>inhibitory postsynaptic currents</td>
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<td>potassium chloride</td>
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<td>sodium hydroxide</td>
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<td>PB</td>
<td>pentobarbital</td>
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<td>PEN</td>
<td>penicillin</td>
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<td>RT</td>
<td>room temperature</td>
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<tr>
<td>SEM</td>
<td>standard error of mean</td>
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<tr>
<td>TES</td>
<td>N-tris-(hydroxymethyl)-methyl-2-aminoethane-sulfonic acid</td>
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<td>τ&lt;sub&gt;1&lt;/sub&gt;, τ&lt;sub&gt;2&lt;/sub&gt;</td>
<td>exponential time constants, millisecond (ms)</td>
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<tr>
<td>V&lt;sub&gt;P&lt;/sub&gt;</td>
<td>applied pipette potential</td>
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CHAPTER 1

GENERAL INTRODUCTION

The GABA receptor is a chloride channel that is activated by GABA, GABA, and GABA agonists that are distinguished from each other by their unique pharmacology. The GABA-A receptor is an important therapeutic target in the central nervous system, and its activation by GABA-A agonists has been shown to produce a variety of effects, including sedation, anticonvulsant, and anesthetic actions (Enna, 1991; Steiner & Tatem, 1991; Steiner, 1992; Nakamura et al., 1995). The GABA-A receptor was first distinguished from the GABA-B receptor by Hill and Berlevy (1983) and has been shown to play a critical role in the regulation of various physiological processes, including sleep, wakefulness, and memory (Besson, 1993; Hugdahl, 1995; Steinman & Nafzgi, 1997; Ghayur & Drago, 1990). Since then, it has been recognized that the GABA-A receptor is a complex entity that is composed of several subunits, each with distinct pharmacological properties. It also forms a chloride channel, but unlike the GABA-B receptor, it is not modulated by benzodiazepines and barbiturates and is predominantly found in the vertebrate retina (Engelman et al., 1991; Qin & Dowling, 1990; Johnson, 1995). Recently, it has been recommended to include the GABA-A class as a member of the...
CHAPTER 1

GENERAL INTRODUCTION

1.1. THE GABA\textsubscript{A} RECEPTOR

\(\gamma\)-aminobutyric acid (GABA) is recognised as the major inhibitory neurotransmitter of the mammalian central nervous system (CNS). Approximately 30-40% of all central synapses use GABA as their transmitter (Sieghart, 1989; Zorumski & Isenberg, 1991). The earliest evidence of GABA as a neurotransmitter was reported by Bazemore et al. (1954) who reported that neuronal activity in crayfish stretch receptors was inhibited by mammalian brain extracts. This active inhibitory substance was identified soon after as GABA (Enna, 1983; Rabow et al., 1995).

The GABA receptor can be divided into three classes, GABA\textsubscript{A}, GABA\textsubscript{B} and GABA\textsubscript{C}, that are distinguished from each other by their unique pharmacology. The GABA\textsubscript{A} receptor is the major inhibitory receptor of the CNS and is the target site of many important therapeutic drugs that include sedatives, hypnotics, anxiolytics, anaesthetics and anticonvulsants (Enna, 1983; Sivilotti & Nistri, 1991; Seighart, 1992; Rabow et al., 1995). The GABA\textsubscript{B} receptor was first distinguished from the GABA\textsubscript{A} receptor when Hill and Bowery (1981) demonstrated that \(\beta\)-p-chlorophenyl-GABA (baclofen) bound selectively to a population of GABA recognition sites that was also insensitive to bicuculline (a GABA\textsubscript{A} receptor antagonist). GABA\textsubscript{B} receptors regulate potassium and calcium channels through G-protein and intracellular second-messenger pathways (Bormann, 1988; Nicoll, 1988; Sivilotti & Nistri, 1991; Kerr & Ong, 1995). Less common is the GABA\textsubscript{C} receptor that is defined by its insensitivity to both bicuculline and baclofen. It also forms a chloride channel but unlike the GABA\textsubscript{A} receptor, it is not modulated by barbiturates and benzodiazepines and is predominantly found in the vertebrate retina (Feigenspan et al., 1993; Qian & Dowling, 1993; Johnston, 1996). Recently it has been recommended to include the GABA\textsubscript{C} class as a subclass of the
GABA_A receptor since GABA_A and GABA_C receptor subunits have a high degree of amino acid sequence homology (Barnard et al., 1998).

The GABA_A receptor is a member of the ligand-gated ion channel family that also includes the nicotinic acetylcholine (nACh) and glycine receptors (Schofield et al., 1987; Hille, 1992; Barnard, 1992; Seighart, 1992; Macdonald & Olsen, 1994; Stroud et al., 1995; Rabow et al., 1995; Karlin & Akabas, 1995). The neuronal ACh receptor is so far the most widely studied of all ligand-gated ion channels (Conti-Tronconi & Raftery, 1982; Dani, 1989; Stroud et al., 1990; Cockcroft et al., 1990; Revah et al., 1991; Akabas et al., 1992; Devillers-Thiéry et al., 1993; Hille, 1992; Galzi et al., 1992; Akabas et al., 1994; Karlin & Akabas, 1995; Changeux, 1995; Vidal & Changeux, 1996; Wilson & Karlin, 1998). Using electron microscopy, Unwin (1993, 1995) showed that the ACh receptor is composed of five subunits with a central pore between them. The five common features of all ligand-gated ion channel family subunits are (Figure 1.1.; Schofield et al., 1987; Cockcroft et al., 1992; Seighart, 1992; Whiting et al., 1995):

1. A signal peptide is removed upon membrane translocation of the polypeptide chain;
2. An N-terminal extracellular agonist binding domain containing one or more sites for N-linked oligosaccharide attachment;
3. Four predicted transmembrane segments, termed M1, M2, M3 and M4;
4. A large variable intracellular region (between M3 and M4) that contains consensus sequences for regulatory sites of phosphorylation;
5. A short C-terminal region.

The GABA_A receptor is a protein complex of about 220-400kDa (Schwartz, 1988; Olsen & Tobin, 1990; Macdonald & Olsen, 1994). The binding of GABA to the extracellular binding site on the receptor opens up a chloride channel that drives the membrane potential towards the chloride equilibrium potential. Consequently, this decreases the likelihood of excitatory neurotransmitters generating an action potential (Rabow et al.,
The GABA<sub>A</sub> receptor is a protein complex of about 220-400kDa and is believed to be composed of five subunits with a central pore between them. Each subunit is composed of four transmembrane segments with extracellular and intracellular loops joining them together and it is believed that the second transmembrane segment, M2, lines the pore. Reproduced from Olsen & Tobin (1990).

**Figure 1.1.** The GABA<sub>A</sub> receptor is a protein complex of about 220-400kDa and is believed to be composed of five subunits with a central pore between them. Each subunit is composed of four transmembrane segments with extracellular and intracellular loops joining them together and it is believed that the second transmembrane segment, M2, lines the pore. Reproduced from Olsen & Tobin (1990).
The GABA<sub>A</sub> receptor is a locus for the action of many mood- and emotion-altering agents such as benzodiazepines, barbiturates, steroids and alcohol. Anxiety, sleep disorders and convulsive disorders have been effectively treated with therapeutic agents that enhance the action of GABA at the GABA<sub>A</sub> receptor (Figure 1.2).

1.2. SUBUNIT DIVERSITY & PHARMACOLOGY

Techniques such as in situ hybridisation (Shivers et al., 1989; Wisden et al., 1992; Laurie et al., 1992a,b; Serafini et al., 1998; Wegelius et al., 1998), immunohistochemistry with subunit-specific antisera (Houser et al., 1988; Möhler et al., 1992; Endo & Olsen, 1993; Fritschy et al., 1994), northern or western blot analysis (Quirk et al., 1994; Davies et al., 1997; Jechlinger et al., 1998) as well as reverse transcription - polymerase chain reaction (RT-PCR; Hadingham et al., 1993; Santi et al., 1994; Grigorenko & Yeh, 1994; Duncan et al., 1995; Ogurusu et al., 1995; Zeiter et al., 1996; Ruano et al., 1997; Tyndale et al., 1997; Berger et al., 1998; Brooks-Kayal et al., 1998) have revealed what GABA<sub>A</sub> receptor subunit isoforms are likely to be present in the various areas of the CNS. Based on the results of these studies, combinations of subunit isoforms have been expressed and functionally examined, for example whole-cell and single-channel characteristics, dose-response and current-voltage relationships and pharmacology. Examination of these recombinant receptors has unveiled particular characteristics unique to certain subunit isoforms and using this knowledge, we can predict the presence or absence of certain subunits in native neurons.

1.2.1. The Subunits

Currently 19 subunit isoforms have been identified and they are classed into seven subunit families: α<sub>1-6</sub>, β<sub>1-4</sub>, γ<sub>1-3</sub>, δ, ρ<sub>1-3</sub>, ε and π (Olsen & Tobin, 1990; Sivilotti & Nistri, 1991; Cockcroft et al., 1990; Macdonald & Olsen, 1994; Wang et al., 1994; Rabow et al., 1995; McKernan & Whiting, 1996; Davies et al., 1997; Hedblom & Kirkness, 1997; Boue-Grabot et al., 1998). Each subunit contains approximately 500 amino acids with about 70-80% amino acid sequence homology within a subunit family but only 30-40%
Figure 1.2. The GABA$_A$ receptor is modulated by various therapeutic drugs that act at different sites on the receptor. The subunit composition of the receptors determines the affinity for these drugs. Reproduced from McKernan & Whiting (1996).
homology between the subunit families (Olsen & Tobin, 1990; Burt & Kamatchi, 1991; Wieland et al., 1992; Seighart, 1992).

The α subunit is one of the more important subunits that is present in all GABA<sub>A</sub> receptors. The α<sub>1</sub> subunit appears to be the most abundant and widely distributed subunit isoform in the mammalian brain with high levels found in the cortex, hippocampus and cerebellum (Wisden et al., 1989, Laurie et al., 1992a,b; Wisden et al., 1992). In contrast, the least abundant of all α subunits are the α<sub>4</sub> and α<sub>6</sub> subunits. The α<sub>4</sub> subunit is located mostly in the thalamus and hippocampus with lower levels in the cortex, olfactory bulb and cerebellum while the α<sub>6</sub> subunit is found predominantly in the granule cell layer of the cerebellum (Wieland et al., 1992; Wisden et al., 1991, 1992; Laurie et al., 1992a,b; Rabow et al., 1995; Wafford et al., 1996; Benke et al., 1997). There is increasing evidence that shows that the α subunit determines the sensitivity of the GABA<sub>A</sub> receptor to GABA, barbiturates and benzodiazepines (Levitan et al., 1988a,b; Lüddens et al., 1990; Malherbe et al., 1990a; McKernan et al., 1991; Möhler et al., 1992; Macdonald & Olsen, 1994; Verdoorn, 1994; Gingrich et al., 995; Thompson et al., 1996; Huh et al., 1996; Thompson et al., 1996; Benke et al., 1997; Jechlinger et al., 1998). More recently the α subunit isoform has been shown to affect the rate of desensitisation (Im et al., 1995; Gingrich et al., 1995; Lavoie et al., 1997) as well as the direction of current rectification (Granja et al., 1998).

A β subunit is also found in all GABA<sub>A</sub> receptors and appears to be important for GABA sensitivity (Amin & Weiss, 1993; Hadingham et al., 1993; Zhang et al., 1995) as well as receptor desensitisation (Zhang et al., 1995; Lu & Huang, 1998). Of all the β subunit isoforms, the β<sub>2</sub> subunit is expressed most abundantly (Wisden et al., 1992; Laurie et al., 1992a,b) and is often associated with α<sub>1</sub> and γ<sub>2</sub> subunits (Benke et al., 1991b, 1994). The β subunit also appears to be important for producing functional GABA<sub>A</sub> receptors (Birnir et al., 1992) and there seems to be some regional specificity as to which β subunit isoform is expressed (Endo & Olsen, 1992; Hadingham et al., 1993). It has also been suggested that the β subunit determines the subcellular location of the GABA<sub>A</sub> receptor (Benke et al., 1994; Connolly et al., 1996).
Of all the γ subunits, the γ₂ subunit is the most abundant (Wisden et al., 1992; Laurie et al., 1992a,b) and has been associated with both the α₁ subunit as well as the β₂/β₃ subunit (Benke et al., 1991b). Western blot analysis has shown that the γ₁ subunit does not co-exist with another γ subunit but some receptors can contain both γ₂ and γ₃ subunits (Quirk et al., 1994). The γ subunit has been shown to affect receptor desensitisation (Dominguez-Perrot et al., 1996). In conjunction with the α subunit, the γ subunit confers benzodiazepine sensitivity (Horne et al., 1993; McKernan et al., 1995) and zinc insensitivity (Pritchett et al., 1989; Draguhn et al., 1990; Celentano et al., 1991). The best studied γ subunit is the γ₂L splice variant which when compared to the short version, γ₂S, has an extra eight amino acids in the intracellular loop between M3 and M4 (Whiting et al., 1990). These eight extra amino acids are thought to constitute an extra phosphorylation site which may be involved in ethanol sensitivity (Wafford et al., 1991).

The δ subunit is present in only a few regions of the CNS that include the cerebellum, thalamus and dentate gyrus of the hippocampus (Benke et al., 1991a; Wisden et al., 1992; Laurie et al., 1992a,b). In situ hybridisation studies showed that both δ and γ₂ mRNA were expressed in neurons but largely occurred in two distinct neuronal subpopulations confer different pharmacological characteristics (Shivers et al., 1989). Whiting et al. (1995) later showed that the δ and γ subunits do not co-exist together in combination with α and β subunits. This was supported by subunit-specific antibody studies that did not detect the δ subunit in combination with any γ subunit (Quirk et al., 1995; Araujo et al., 1998).

GABA receptors that contain the ρ subunits are classified as belonging to the GABAc receptor type and are found predominantly in the retina and lower levels in the thalamus, hippocampus and brain stem (Ogurusu et al., 1995; Wegelius et al., 1998). When ρ₁ subunits are expressed in Xenopus oocytes, they form functional homomeric GABA receptors that have properties different from those of the typical heteromeric GABAₐ receptor. These receptors are not blocked by bicuculline nor are they affected by baclofen. Furthermore, they are not modulated by barbiturates nor benzodiazepines but they are blocked by zinc (Qian & Dowling, 1993; Wang et al., 1994; Chang et al., 1995; Johnston, 1996).
Northern blot analysis showed that the ε subunit is relatively abundant in the amygdala and thalamus (Davies et al., 1997) and more recently, it has been found in the dentate gyrus of the human and rat (Hewson et al., 1998). When the ε subunit is co-expressed with either an α subunit (α1, α2 or α6) or a β subunit (β1 or β3), no GABA binding sites could be detected but combinations of the α-, β- and ε- subunits formed functional receptors. Unlike the γ subunit, the α/β/ε subunit combination is insensitive to benzodiazepines; this is similar to the δ subunit but the ε subunit can be distinguished from the δ subunit by its insensitivity to barbiturates. Furthermore, the ε subunit in combination with an α- and β- subunit produces a current-voltage relationship that is linear (Davies et al., 1997).

The π subunit is the most recent subunit isoform identified and displays 30-40% amino acid sequence homology with the β, δ and ρ subunits. The π subunit is expressed in relatively high amounts in several peripheral tissues (lung, thymus and prostate) and is especially abundant in the uterus but not detectable in the whole brain or pancreas. Binding studies have indicated that recombinant receptors containing the π subunit were insensitive to benzodiazepines and were pharmacologically more similar to the δ and ε subunits. It was concluded that the π subunit was most likely part of a α1/β1/γ2/π combination that lack Ro15-1788 (a benzodiazepine antagonist) binding sites and/or a mixed receptor population of α1/β1/γ2 and α1/β1/π subunits (Hedblom & Kirkness, 1997).

Typically 10 subunit isoforms can be found in any one region of the brain or in any one cell and may suggest the existence of multiple subunit combinations producing functional GABA_A receptors. Depending on the subunit combination, these receptors may exhibit different characteristics (such as rate of current decay or current rectification) and be affected to different extents by drugs such as bicuculline, picrotoxin, penicillin, barbiturates, benzodiazepines, alcohol and polyvalent cations (Peters et al., 1988; Macdonald et al., 1988; Lambert et al., 1995).
1.2.2. Bicuculline

Bicuculline is believed to be a competitive antagonist that selectively and reversibly blocks the inhibitory effect of GABA from the external side of the GABA<sub>A</sub> receptor (Curtis <i>et al.</i>, 1971; Nicoll & Wojtowicz, 1980; Simmonds, 1980; Akaike <i>et al.</i>, 1985a; Itabashi <i>et al.</i>, 1992; Macdonald & Olsen, 1994; Krishek <i>et al.</i>, 1996; Ueno <i>et al.</i>, 1997). Although no subunit isoform has been implicated with bicuculline sensitivity, Zhang <i>et al.</i> (1995) found β subunits of insect GABA<sub>A</sub> receptors conferring bicuculline sensitivity.

1.2.3. Picrotoxin

It is still not clear how picrotoxin acts on the GABA<sub>A</sub> receptor but it is generally accepted that picrotoxin binds to a site distinct from the GABA<sub>A</sub> binding site, that is, it does not compete with GABA (Takeuchi & Takeuchi, 1969; Curtis <i>et al.</i>, 1971; Nicoll & Wojtowicz, 1980; Simmonds, 1980; Olsen, 1981; Akaike <i>et al.</i>, 1987a; Yakushiji <i>et al.</i>, 1987; Itabashi <i>et al.</i>, 1992; Newland & Cull-Candy, 1992; Krishek <i>et al.</i>, 1996). It has been postulated that the site of picrotoxin block is exposed by the conformational change initiated by GABA binding to the receptor (Inoue & Akaike, 1988; Yoon <i>et al.</i>, 1993). Mutations made near the centre of the M2 region (T246F) of either α<sub>1</sub>, β<sub>2</sub> or γ<sub>2</sub> subunits caused the recombinant receptor to be picrotoxin-insensitive (Gurley <i>et al.</i> 1995; Akabas & Xu, 1995). It was proposed that picrotoxin blocks GABA-activated currents by acting at a site within the pore but does not itself physically block current flow.

1.2.4. Penicillin

Penicillin is generally accepted to be an open channel blocker of the GABA<sub>A</sub> receptor and its binding site is separate from the binding sites for bicuculline and picrotoxin (Curtis <i>et al.</i>, 1972; Hochner <i>et al.</i>, 1976; Pickles & Simmonds, 1980; Chow & Mathers, 1986; Akaike <i>et al.</i>, 1987a; Yakushiji <i>et al.</i>, 1987; Katayama <i>et al.</i>, 1992; Twyman <i>et al.</i>, 1992). The nature of the penicillin block has been inconclusive with reports of competitive block (Macdonald & Barker, 1977) and non-competitive block (Pickles &
Simmonds, 1980; Chow & Mathers, 1986) or even a mixture of both (Hochner et al., 1976). Penicillin has been shown to induce triphasic modulation of the GABA-activated current that consists of two modes of blockade (phasic and tonic) followed by a rebound current when penicillin plus GABA was removed (Katayama et al., 1992).

1.2.5. Benzodiazepines
The benzodiazepines are a group of drugs that have anxiolytic and anti-convulsant properties (Macdonald & Olsen, 1994; Jin et al., 1997). For GABA_A receptors to be sensitive to the benzodiazepines, they require a γ2 subunit in combination with an α subunit. Based on the benzodiazepine pharmacology, GABA_A receptors can be divided into a further three classes. The first receptor class has high affinity for the partial agonist, CL218872 and these receptors contain the α1 subunit isoform only. The second receptor class has a low affinity for CL218872 and these receptors contain the α2, α3 and α5 subunit isoforms. The third receptor class does not bind CL218872 and these receptors contain the α4 and α6 subunits (Squires et al., 1979; Faull & Villiger, 1988; Pritchett et al., 1989; Sieghart, 1989; Pritchett & Seeburg, 1990; Lüddens & Wisden, 1991; Wieland et al., 1992; Ruano et al., 1992; Yakushiji et al., 1993; Rabow et al., 1995; Mehta & Shank, 1995; Yeh & Grigorenko, 1995; Wafford et al., 1996; Knoflach et al., 1996; Nusser et al., 1996a; Jin et al., 1997; Benke et al., 1997).

1.2.6. Anaesthetics
General anaesthesia can be induced with a wide variety of chemical agents including inert rare gases, hydrocarbons, halocarbons, ethers, ketones, carbamates, barbiturates, amines, and steroids to name a few. Anaesthetics are hydrophobic and their potencies are highly correlated with their lipid solubility (Miller, 1985). A common feature of general anaesthetic agents is their ability to enhance neuronal inhibition through GABA_A receptors (Morrow et al., 1991; Lin et al., 1993; Davies et al., 1997). Pentobarbital and ethanol are two anaesthetics that will be examined here.
The barbiturates are a group of drugs that have anaesthetic, anti-convulsant and anxiolytic properties. The most well known barbiturate is pentobarbital (other derivatives include pentobarbitone and phenobarbitone). Pentobarbital has been demonstrated to have three major effects on currents activated by GABA. Low concentrations of pentobarbital enhance GABA-activated currents but millimolar concentrations depress currents activated by GABA. Pentobarbital has also been shown to directly activate the GABA_A receptor and responses are sensitive to bicuculline, picrotoxin and diazepam (Barker & Ransom, 1978; Nicoll & Wojtowicz, 1980; Schwartz et al., 1986; Mathers & Barker, 1980; Parker et al., 1986; Thompson et al., 1996; Akaike et al., 1987; Peters et al., 1988; Robertson, 1989; Yakushiji et al., 1989; Itabashi et al., 1992; Macdonald & Olsen, 1994; Rho et al., 1996). In addition, terminating the application of millimolar concentrations of pentobarbital (with or without GABA) produced a transient rebound current that could be blocked by bicuculline in a dose-dependent manner (Akaike et al., 1987b; Peters et al., 1988; Peters et al., 1988; Robertson, 1989; Yakushiji et al., 1989; Nakagawa et al., 1991; Itabashi et al., 1992; Rho et al., 1996). The modulatory effect of pentobarbital on currents activated by GABA do not appear to be dependent on specific combinations of GABA_A receptor subunits (DeLorey & Olsen, 1992; Lin et al., 1993). However, recent studies have shown that the β subunit may contribute to the potency of pentobarbital. Recombinant receptors expressing α1β2γ2, α1β3γ2 and α1γ2 had decreasing potencies for pentobarbital in that order (Harris et al., 1995). Furthermore, Thompson et al. (1996) showed that the direct effect of pentobarbital was influenced more by the type of β subunit present than the type of α subunit.

Ethanol has been reported to enhance currents activated by GABA but only with those receptors containing the γ2L splice variant. It was thought that the extra eight amino acids in the γ2L splice variant hold a phosphorylation site that is needed for enhancement of GABA-activated currents (Wafford et al., 1991). However, results appear to be conflicting whether the γ2L subunit isoform is required or not (Aguayo, 1990; Reynolds & Prasad, 1991; Cherubini et al., 1991; Reynolds et al., 1992; Criswell et al., 1993; Aguayo & Alarcón, 1993; Weiner, et al., 1994; Wafford et al., 1991, 1996; Tyndale et al., 1997).
1.2.7. Zinc

There are many polyvalent cations that can influence the GABA\textsubscript{A} receptor, including zinc, cadmium, nickel, cobalt, manganese and the lanthanides (Ma & Narahashi, 1993; Narahashi et al., 1994). Zinc is essential for the proper development of the nervous system and is concentrated in synaptic terminals in the cortex and hippocampus (Crawford & Connor, 1972; Frederickson et al., 1983; Assaf & Chung, 1984; Westbrook & Mayer, 1987; Xie & Smart, 1991; Smart et al., 1994; Gordey et al., 1995; Berger et al., 1998). Zinc acts as a reversible non-competitive blocker at an extracellular cation site on the GABA\textsubscript{A} receptor that is distinct from the benzodiazepine, barbiturate and picrotoxin recognition sites (Draguhn et al., 1990; Smart & Constanti, 1990; Legendre & Westbrook, 1991; Celentano et al., 1991; Chang et al., 1995; Berger et al., 1998). In contrast to the benzodiazepines, zinc-sensitivity requires the absence of the $\gamma$ subunit (Draguhn et al., 1990; Smart et al., 1991; Celentano et al., 1991; Schönrock & Bormann, 1993; Saxena & Macdonald, 1994; Chang et al., 1995). It also appears that the presence of certain $\alpha$ subunits may contribute to the sensitivity of the GABA\textsubscript{A} receptor to zinc (White & Gurley, 1995; Fisher & Macdonald, 1998).

1.3. THE HIPPOCAMPUS

The hippocampus is a very distinct cortical structure in the rat brain and is composed of several major regions that include the CA region (numbered 1 to 4) and the dentate gyrus. It is believed that GABA is one of the first functional neurotransmitter systems to develop in the hippocampus and GABA synapses may play a major role in neuronal maturation (Davies et al., 1998). The hippocampus is amenable to both electrophysiological and binding studies which makes it an ideal target for the studying the effects of various pharmacological agents on GABA\textsubscript{A} receptors (Kemp et al., 1987; Nicoll, 1988). The hippocampus is of considerable interest as it is believed to play an important role in the acquisition of memory and in other processes such as anxiety and epilepsy (Nicoll, 1988; Swerdlow, 1995; Vnek & Rothblat, 1996; Greenfield, 1997; Sperk et al., 1997).
Almost all subunit isoforms of the GABA<sub>A</sub> receptor are present in the hippocampus. Based on in situ hybridisation studies, the expression of GABA<sub>A</sub> receptor subunit isoforms vary throughout the development of the hippocampus. The α<sub>2</sub> subunit is very strongly expressed from the embryonic hippocampus into adulthood while the α<sub>6</sub> subunit is not expressed at all. The adult hippocampus predominantly expresses the α<sub>2</sub>, β<sub>3</sub> and γ<sub>2</sub> subunits with slightly less expression of the α<sub>1</sub>, α<sub>5</sub>, β<sub>1</sub> and δ subunits and even less of α<sub>3</sub>, α<sub>4</sub> and β<sub>2</sub> and none of the α<sub>6</sub>, γ<sub>1</sub> and γ<sub>3</sub> subunits (Figure 1.3; Montpied et al., 1988; Laurie et al., 1992a,b; Wisden et al., 1992; Yin & Lee, 1998). However, in the dentate gyrus, in situ hybridisation (Shivers et al., 1989; Wisden et al., 1992; Laurie et al., 1992a,b) and immunohistochemistry with subunit-specific antisera (Houser et al., 1988; Fritschy et al., 1994; Sperk et al., 1997) have demonstrated the strong presence of the α<sub>2</sub>, α<sub>4</sub>, β<sub>1</sub>, β<sub>3</sub> and γ<sub>2</sub>, with slightly less expression of the α<sub>1</sub>, β<sub>2</sub> and δ subunits. In total, there are about 10 subunit isoforms present in the adult dentate gyrus.

The dentate gyrus consists of just two cellular layers: an outer granule cell layer and a deeper polymorphic zone consisting mainly of interneurons. The principal cellular layer in the dentate gyrus is the “stratum granulosum” or “granule cell layer”. It was estimated that number of granule cells in Wistar rats at one month is about 700,000 and steadily increases to one million at four months. Granule cell numbers then decline to about 800,000 at one year (Cowan et al., 1980; Boss et al., 1985). In the rat, about 85% of granule cells are generated postnatally and the peak of cell proliferation occurs towards the end of the first postnatal week (Cowan et al., 1980; Baraban & Lothman, 1994). The dentate gyrus has been implicated in many functional processes that include synaptic plasticity in long term potentiation and temporal lobe epilepsy (Williamson et al., 1995; Tong et al., 1996).

There have been many studies investigating the electrical and morphological characteristics of the dentate gyrus (Cowan et al., 1980; Brown et al., 1981; McNaughton et al., 1981; Crunelli et al., 1983; Fricke & Prince, 1984; Fournier & Crepel, 1984; Crunelli et al., 1984; Boss et al., 1985; Lambert & Jones, 1990; Staley, 1994; Kneisler & Dingledine, 1995; Scharfman, 1995a,b; Kuhn et al., 1996; Jackson & Scharfman, 1996; Deller et al., 1996; Acsády et al., 1998). However, there are
The expression of GABA<sub>a</sub> receptor subunit isoforms varies throughout the development of the hippocampus. The α<sub>2</sub> subunit is very strongly expressed from the embryonic hippocampus into adulthood while the α<sub>6</sub> subunit is not expressed at all. The adult hippocampus is predominantly made up of the α<sub>2</sub>, β<sub>3</sub> and γ<sub>2</sub> subunits with slightly less of α<sub>1</sub>, α<sub>5</sub>, β<sub>1</sub> and δ subunits and even less of α<sub>3</sub>, α<sub>4</sub> and β<sub>2</sub> and none of the α<sub>6</sub>, γ<sub>1</sub> and γ<sub>3</sub> subunits. E14 is the rat embryo at day 14 after conception; PO is the day of the rat’s birth and P12 is postnatal day 12. (Reproduced from Laurie et al., 1992a)
comparatively far fewer studies investigating the pharmacology of the GABA\(_A\) receptor in this region. There could be several reasons for this. The dentate gyrus is a region that matures postnatally which would make standard neonatal neuronal cultures not feasible. Dentate gyrus neurons are also much smaller than the more resilient CAI neurons and I have observed that granule cells tend to be more sensitive to any disruptions, both physical and chemical, which could also explain the partiality towards CAI neurons.

Hippocampal slices obtained from adult rats, mice or guinea pigs are the most commonly used preparation to avoid this age-related problem of the dentate gyrus. Using intracellular recording techniques, Biscoe & Duchen (1980) electrically stimulated the mossy fibre area of the dentate gyrus and antidromically evoked depolarising postsynaptic potentials that were bicuculline-sensitive. Edwards _et al._ (1990) recorded both miniature and stimulus-evoked inhibitory postsynaptic currents (IPSCs) from the granule cell layer of the dentate gyrus and were able to block these stimulus-evoked IPSCs completely with 2µM bicuculline. They also observed that desensitisation of the stimulus-evoked IPSCs was biphasic and two time constants could be fitted with the slow time constant contributing about half of the total current. Spontaneous IPSCs (sIPSCs) which were generated by the spontaneous release of endogenous GABA were characterised in the dentate gyrus by Soltesz & Mody (1994). They showed that hilar neurons had slower rise and decay time constants than granule cells thereby revealing powerful GABAergic inhibitory characteristics in the hilar neurons.

Studies of single channels activated by GABA in the dentate gyrus have been somewhat sparse but one study by Birnir _et al._ (1994), characterised the GABA\(_A\) receptor in this region and obtained many interesting results. Single-channel currents were activated in rat hippocampal slices by low concentrations of GABA (0.5µM to 5µM) in cell-attached and inside-out patches. It was observed that these GABA-activated channels had multiple conductance states and could be divided, based on their maximum conductance states, into high (greater than 40pS) and low (less than 40pS) conductance channels. Both high and low conductance channels exhibited rectification that was either outward or more linear. They speculated the existence of multiple GABA\(_A\) receptor types to explain the different single-channel characteristics and concluded that these single channels may contribute to a tonic inhibitory mechanism.
More recently, there has been increasing interest in the dentate gyrus as it provides the opportunity to look at the maturing GABA\textsubscript{A} receptor from the neonate to adult. Liu \textit{et al.} (1996) investigated stimulus-evoked IPSCs in developing granule cells of hippocampal slices and found that biphasic desensitisation was more prominent in immature neurons than in adult neurons. They also showed that this biphasic desensitisation appeared to be due to two GABAergic inputs into the granule cells. The faster and shorter phase was produced by polysynaptic inputs (via interneurons and glutamate receptors) while the later and longer phase was produced by monosynaptic inputs (via interneurons) with the proportions of each input changing during development. In all neurons, this longer and later phase was almost completely eliminated by 10\textmu M bicuculline. Later they (Liu \textit{et al.}, 1998) found a third component that was relatively bicuculline-insensitive. In immature neurons, about 30\% of the current was insensitive to 10\textmu M bicuculline while only 10\% of this current was left in mature neurons. However, 100\textmu M bicuculline did eliminate these currents. They then speculated that this bicuculline-insensitive current could be mediated by a GABA\textsubscript{A} receptor that was unique in subunit composition and was functionally and anatomically separate. Hollrigel & Soltesz (1997) also compared immature and adult GABA\textsubscript{A} receptor-mediated postsynaptic currents of dentate gyrus granule cells and observed that both rise and decay times were significantly longer in immature neurons than in adult rats. In addition, there was different sensitivity to the subunit-specific benzodiazepine modulator zolpidem and the divalent ion zinc. Zolpidem increased the decay time constant of immature IPSCs with a reduced potency compared with adults and, while zinc decreased the decay time constant in immature neurons, no effect was seen in adult neurons.

While hippocampal slices have proven to be a useful preparation for studying the dentate gyrus, focal application of GABA to mimic the fast time course of presynaptic release of GABA cannot be achieved in this preparation. If drugs are not applied to the receptor quickly, synchronous activation of GABA\textsubscript{A} receptors cannot occur so that currents are smaller and slower. An ideal way to avoid this is to apply GABA and associated modulators directly onto an isolated neuron. One of the first studies to isolate granule cells of the dentate gyrus was performed by Mody \textit{et al.} (1989) who investigated various properties of the dentate gyrus to determine the viability of dissociated neurons. They
were able to activate NMDA receptors by applying L-aspartate and specifically block
these receptors with Mg$^{2+}$. In addition, voltage-dependent Ca$^{2+}$ channels were also
observed in the presence of extracellular TTX and intracellular Cs$^+$. Taken together, this
revealed receptors that behaved normally and that were not affected by the dissociation
process. A more comprehensive study on dissociated granule cells was described by
Kapur & Macdonald (1996) who investigated the pharmacological properties of several
benzodiazepines and Zn$^{2+}$. Using a U-tube multi-puffer system they were able to apply
drugs with an average 10-90% rise time of 54±5ms. They obtained a GABA EC$_{50}$ of
46µM with a Hill coefficient of 1.2 and in the presence of 10µM GABA, the EC$_{50}$ for
diazepam was 158nM. In the presence of 30µM GABA, the IC$_{50}$ for Zn$^{2+}$ was 29µM. As
half of their responses were sensitive to loreclezole, an anti-epileptic drug which has
more effect on receptors containing the β$_1$ subunit than on receptors containing other β
subunit isoforms, they proposed the idea of multiple GABAA receptors. More recently
they (Kapur & Macdonald, 1999) compared the effects of GABA and associated
modulators in the dentate gyri of young (7-14 days old) and adult (45-52 days old) rats.
They observed several differences. Compared to adult rats, younger rats had smaller
maximal GABA-activated currents (476±65pA vs. 893±160pA), lower affinity for
GABA (EC$_{50}$ of 40±8 vs. 31±10µM) and diazepam (EC$_{50}$ of 648nM vs. 24±7nM) but
higher affinity for zinc (IC$_{50}$ of 40±5 vs. 103±19µM) and furosemide (IC$_{50}$ of 492±79µM
vs. 1605±199mM). In addition, there were fewer granule cells that were sensitive to
loreclezole (30% vs. 70%) in younger rats than in adult rats. They proposed the
possibility of these young rats expressing a pharmacologically distinct GABAA receptor
and concluded that the maturation of GABAA receptors lasts for at least 45 days.

1.4. AIMS

There has been growing interest in the dentate gyrus region of the hippocampus,
particularly so because it develops postnatally. While most studies have concentrated on
the morphological and electrical properties of the dentate gyrus, comparatively there are
far fewer studies investigating the pharmacology of GABAA receptors in this region.
My aim in this thesis is to functionally characterise the GABA_A receptor of the dentate gyrus using electrophysiological techniques. In order to achieve this aim, the project was threefold:

1. To simplify and optimise dissociation techniques so that isolated granule cells of the dentate gyrus can be studied.

2. To functionally characterise the GABA_A receptor of the dentate gyrus using whole-cell patch clamp techniques and to obtain a pharmacological profile of these receptors.

3. To study single-channel characteristics of GABA_A receptors present in the dentate gyrus and to obtain more information on the effects of various drugs.

As reviewed above, there is a strong correlation between the effect of GABA_A receptor modulators and the presence of certain subunits in these receptors. Using the information gathered from the following pharmacological experiments, we can also speculate on the presence of certain subunits.
CHAPTER 2

METHODS AND MATERIALS

2.1 ACUTE DISSOCIATION

The optimization procedure of acutely dissociating samples will be described in depth in the present chapter but the final method is not short nor trivial.

Table 1. The sample was incubated at 37°C with the dissociating enzyme for 30 minutes. After the complete digestion of the tissue, the solution was cooled immediately immersed in ice-cold artificial cerebral spinal fluid (ACSF) containing NaCl, 245 mM; CaCl₂, 2.46 mM; KCl, 29.76 mM; MgSO₄, 25.8 mM; NaHCO₃, 26.12 mM; and glucose, 10.5 mM. This solution was gassed with 95% O₂ and 5% CO₂ and maintained at 37°C.

7.4. The cytocentrifuged slices were incubated in half the mitral body tissue in a 50 mm diameter glass chamber containing the above-mentioned ACSF. After slicing was complete, slicing about 10-15 minutes, they were then placed in a water bath at a temperature of 37°C for 10 minutes in pre-cooled water. At the same time and under the same conditions, the enzyme solution (which consisted of 25 mM HEPES, 10 mM NaCl, 0.5 mM CaCl₂, 0.5 mM EDTA, and 12.4 mM sucrose) was also allowed to activate. After 10 minutes, the enzyme solution was then added to the slices and incubated at 37°C for a further 15 minutes. After this incubation, the slices were removed and the enzyme solution replaced with fresh carbogated ACSF. This was then incubated for a further 30 minutes at 37°C. Slices were then removed from the water bath and left for about 30 minutes to allow the slices to cool down to room temperature.
Outlined below is the methodology used in this thesis. Details of materials and equipment can be found at the end in Table 2.1.

2.1. ACUTE DISSOCIATION

The optimisation process of acutely dissociating neurons will be discussed in-depth in the next chapter but the final method is outlined here.

Wistar rats aged 16 to 21 days old were swiftly killed by decapitation and within 30 seconds of the decapitation, the brain was quickly removed and immediately immersed in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 26 NaHCO₃, 20 glucose, 3 KCl, 2.6 CaCl₂, 2.5 NaH₂PO₄ and 1.3 MgSO₄. The pH of this solution when equilibrated with carbogen gas (95% O₂ and 5% CO₂) was approximately 7.4. The cerebellum was removed and the brain cut in half along the mid-sagittal plane. After blotting on filter paper, the two hemispheres were super-glued to the cutting stage and immediately submerged in ice-cold ACSF which was bubbled with carbogen. Slices 500µm thick were cut using a vibrating microslicer using the highest vibration speed and the slowest advance. Slices were then put into a 50ml beaker containing carbogenated ACSF. After slicing was complete, yielding about 10-12 slices, they were then put into a water bath at a temperature of 35°C for 30 minutes to pre-incubate. At the same time and under the same conditions, the enzyme solution which consisted of 20Units/ml lyophilised papain, 1.1mM cysteine, 0.22mM EDTA and 13.4µM mercaptoethanol was allowed to activate. After 30 minutes, the enzyme solution was then added to the slices and incubated at 35°C for a further 30 minutes. After this incubation session, slices were removed and the enzyme solution replaced with fresh carbogenated ACSF. This was then incubated for a further 30 minutes at 35°C. Slices were then removed from the water bath and left for about 30 minutes to allow the slices to cool down to room temperature.
When ready to patch, about four to five dentate gyri were carefully dissected free from the hippocampal slice and gently triturated in about 2ml of extracellular bath solution using a polished Pasteur pipette. Neurons were then placed in the patch clamp recording bath and allowed to adhere to the glass bottom of the recording bath (usually about an hour). This prevented isolated neurons from being washed away when a high rate of bath solution exchange was required with the fast-perfusion method described below.

2.2. HIPPOCAMPAL SLICES

A similar method to the above was used to produce brain slices for single channel recording. After decapitating the rat and adhering the brain to the vibrating microslicer stage, 400µm slices were cut. On ice and in carbogenated ACSF, the hippocampus was dissected free from the rest of the brain slice and placed in a 50ml beaker containing carbogenated ACSF. This beaker was placed in a 35°C water bath and incubated for one hour. After this incubation session, the beaker was removed from the water and kept at room temperature until all the hippocampal slices were used. An adapted recording bath was used which had a ring of mesh placed approximately at half the depth of the bath. Just before patching, three to four hippocampi were placed on this ring and a U-shaped platinum wire (with nylon threads glued across) was placed on top to sandwich the hippocampi and prevent them from moving and floating away. Using a dissecting microscope with a light source from below, slices were easily visualised and regions readily identified.

2.3. SOLUTIONS

2.3.1. Whole-Cell Solutions

Extracellular bath solution consisted of (in mM) 140 NaCl, 5.4 KCl, 1.8 CaCl₂, 1.0 MgCl₂ and 10 TES. The pipette solution consisted of (in mM) 140 NaCl, 2 MgCl₂, 1 CaCl₂, 5 EGTA, 10 TES and 4mM ATP (which was added on the day of recording).
Extracellular and pipette solutions were adjusted to a pH of 7.4 with 10mM NaOH. The extracellular solution was filtered with a vacuum pump using a 0.45µm filter and the pipette solution was filtered through a disposable 0.22µm filter before use.

GABA and drugs: bicuculline (BIC), picrotoxin (PIC), penicillin (PEN), pentobarbital (PB), diazepam (DZ), zinc (Zn²⁺) and ethanol (EtOH) were easily dissolved in extracellular bath solution with the exception of picrotoxin and diazepam. Picrotoxin was sonicated for more than five minutes in bath solution. Diazepam was first dissolved in (dimethylsulfoxide) DMSO and then in extracellular bath solution to give a 200µM diazepam stock solution in 27mM DMSO.

2.3.2. Single-Channel Solutions

Bath solution consisted of (in mM) 140 NaCl, 5 KCl, 1.8 CaCl₂, 1 MgCl₂ and 10 TES and the pipette solution consisted of (in mM) 140 choline chloride, 1 MgCl₂, 1.8 CaCl₂, and 10 TES. On a few occasions choline chloride was replaced with 145 NaCl for cell-attached experiments. Using the Nernst Equation, equilibrium potentials were calculated for Cl⁻ and Na⁺; K⁺ was absent in all pipette solutions. In cell-attached (c/a) patches, intracellular Cl⁻ and Na⁺ concentration was assumed to be 10mM and 140mM, respectively. For a cell-attached patch, the driving force potential is equal to \( V_m - E_i - V_p \); where \( V_m \) is the membrane potential (assumed to be about -70mV), \( E_i \) is the equilibrium potential of the ion and \( V_p \) is the applied pipette potential. If the driving force potential is 0mV, then \( -V_P = E_i - V_m \). For inside-out patches, \( -V_P = E_i \).

<table>
<thead>
<tr>
<th>Ions (pip soln)</th>
<th>Pipette Potential (-V_P (c/a patches))</th>
<th>Pipette Potential (-V_P (i/o patches))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl⁻ (CholCl)</td>
<td>2mV</td>
<td>-1mV</td>
</tr>
<tr>
<td>Cl⁻ (NaCl)</td>
<td>1mV</td>
<td>0mV</td>
</tr>
<tr>
<td>Na⁺ (NaCl)</td>
<td>71mV</td>
<td>-1mV</td>
</tr>
</tbody>
</table>

Nernst Equation:

\[ E_i = 58.2 \log_{10} \left( \frac{[I]_o}{[I]_i} \right) \]

Pipette Potential:

\[ -V_P = E_i - V_m \]

\( V_m = -70mV \)
Both bath and pipette solutions were adjusted to pH of 7.4 with 1mM NaOH. For cell-attached patches and inside-out patches, GABA was dissolved in the pipette solution and filtered through a 0.22µm filter before use. Pentobarbital and diazepam were either dissolved in bath solution and applied through the bath or they were dissolved with GABA in the pipette solution and filtered. Like whole-cell solutions, the bath solution was filtered through a vacuum pump using a 0.45µm filter.

2.4. ELECTROPHYSIOLOGY

2.4.1. Whole-Cell Electrophysiology

Whole-cell currents were obtained using standard whole-cell, tight-seal recording techniques (Hamill et al., 1981). Patch pipettes were made from borosilicate glass and had resistances between 5MΩ and 12MΩ after fire-polishing. After establishing a whole-cell recording, series resistance was compensated to 80% or above. All drugs were applied to the single neuron using a fast perfusion system (Figure 2.1.). This system is a set of stainless steel delivery tubes with internal diameters between 200µm and 300µm. This is situated directly in the recording bath and positioned so that under optimum conditions, the rate of solution exchange across the cell is less than a millisecond (Birnir et al., 1995). This solution exchange is also dependent on the flow of bath solution (approximately 10ml/min) through the recording bath. Whole-cell currents were obtained at a pipette potential of -40mV and experiments were carried out at room temperature (22 to 25°C). Currents were recorded with an Axopatch 1a amplifier, low-pass filtered at 500Hz at a gain of 1 or 2mV/pA with a sample interval of 1ms. Currents were also simultaneously stored on computer using the program CAPTURE and on video tape after being digitised at 44kHz using a pulse code modulator.

2.4.2. Single-Channel Electrophysiology

Single-channel experiments performed on dissociated neurons were achieved by obtaining either cell-attached or inside-out patch configurations. In the absence of GABA in the pipette solution, GABA was applied through an internal perfusion system.
Figure 2.1. Schematic diagram of the fast perfusion system used in whole-cell experiments. A. A series of seven stainless steel tubes sits strategically in the bath such that when B. a granule cell is placed in front of one tube, the flow of the bath solution carries the drug released from the perfusion tube to the cell. This attempts to ensure synchronous activation of GABA\(_A\) receptors.
A bolus of GABA preceded by air to prevent leakage or diffusion of GABA from the tube was injected through a fine polyethylene tube running to within 0.5-1 mm of the patch pipette tip. Single channel experiments performed on hippocampal slices were accomplished by visualising either the granule cell layer of the dentate gyrus region or the pyramidal cell layer of the CA1 region in the hippocampal slice. These layers are composed primarily of the soma of these neurons and were "blindly patched" with positive pressure inside the pipette (Blanton et al., 1989). Both cell-attached and inside-out patch configurations were obtained with pipette resistances ranging between 18 MΩ and 25 MΩ after fire-polishing and backfilling with pipette solution. Quiet patches were held for greater than five minutes before any drugs were applied. With GABA (0.5 µM and 20 µM) present in the pipette solution, either pentobarbital (100 µM) or diazepam (1 µM) was applied through the bath or were dissolved in the pipette solution with GABA; the reason for using pentobarbital and diazepam is their lipid solubility (Miller, 1985) which was ideal for cell-attached and inside-out patch experiments. If there was channel activity, currents were recorded at various potentials to determine if the nature of this current was Cl⁻, that is, the current reversed closed to 0 mV. Single channels were recorded with an Axopatch 1B amplifier. Currents were recorded at a gain of 100, 200 or 500 mV/pA, low-pass filtered at 5 kHz and then digitised at 44 kHz via a pulse code modulator and stored on video tape. For analysis, currents were played back from the video tape, digitised at 10 kHz and stored on computer using the CAPTURE program.

2.5. DATA ANALYSIS

2.5.1. Whole-Cell Data Analysis

All whole-cell currents were analysed using the CHANNEL2 computer program. Current rise times were obtained by measuring the time it took for the current to rise from 10% to 90% of the peak current amplitude. The half-decay time was calculated by measuring the time it took for the current to decay to half of the peak current amplitude. The protocol for testing drugs was to apply the control solution (either 100 µM or 7 µM GABA), then the test solution (either drug or another concentration of GABA) and then after a minimum of one minute washout with bath solution, the control solution was
reapplied and the response compared to the initial control solution response. To
determine the level of recovery from the test solution, the ratio of the two control
responses was calculated and expressed as a percentage. The minimum acceptable level
of recovery was 50%. Comparisons were made between pools of data with recoveries of
80-100%, 50-80% and 50-100% and no significant differences could be found. The
average of the two control responses was then calculated and the degree of current
enhancement or depression caused by the test solution was determined against this
averaged response.

Three sets of data were measured from the whole-cell response: peak current, mean
current calculated over a one second period and plateau current. Dose-response curves
were constructed from these three currents with GABA concentrations ranging from
1µM to 1mM. Dose-response curves were also constructed with increasing drug
(bicuculline, Zn$^{2+}$ or pentobarbital) concentrations in the presence of either 100µM or
7µM GABA depending on the effect of the drug. With currents normalised to the
maximum current response, these dose-response curves were constructed by best fitting
a Hill type equation (see Equations 2.1. and 2.2.; from Aguayo et al., 1994) to the
average of the data points and weighted with one standard error of mean (±1SEM). The
computer program SlideWrite Plus was used to generate these curves as well as to
calculate the half-effective agonist (EC$_{50}$) or antagonist (IC$_{50}$) concentrations and the Hill
coefficient (h). Standard error of mean (±1SEM) vertical bars are shown on all graphs
unless obscured by the symbol.

\[
I = I_{\text{max}} \left( \frac{[\text{GABA}]^h}{(\text{EC}_50)^h + [\text{GABA}]^h} \right) \quad \text{Equation 2.1.}
\]

\[
I = I_{\text{max}} \left( \frac{[\text{DRUG}]^h}{(\text{IC}_50)^h + [\text{DRUG}]^h} \right) \quad \text{Equation 2.2.}
\]
The program PeakFit was used to best fit one or two exponentials (Equation 2.3.) to the inverted whole-cell current response (Figure 2.2.). Comparisons of the time constants and associated amplitudes were made between GABA and GABA plus drug (bicuculline, Zn$^{2+}$ or pentobarbital) to determine the effects of these drugs on the current decay. Coefficients of determination ($r^2$ values) were acceptable if they were greater than 0.98 for decay currents produced by 100µM GABA in the absence or presence of other drugs; this standard was relaxed with fits produced by 7µM GABA as decay currents could not be easily fitted. Both component amplitudes ($A_1$ and $A_2$) and steady-state current amplitude ($C$) were measured in picoAmperes (pA), with time ($t$) and the time constants ($\tau_1$ and $\tau_2$) measured in milliseconds (ms). Time constants and their associated amplitudes were grouped into two sets with $\tau_1$ defined as times that fell between 0ms to 599ms and $\tau_2$ defined as having times greater and including 600ms.

$$I = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) + C$$  

Equation 2.3.

Voltage ramps from -100mV to +100mV were generated by the computer within two seconds during the plateau phase of the whole-cell response. A voltage ramp applied before GABA application (Ο) was subtracted from a voltage ramp applied in the presence of 100µM GABA (Ω) in order to get the “true” GABA current-voltage relationship (Figure 2.3.). In addition, the voltage ramp applied after GABA washout (Ω) was subtracted from the voltage ramp taken before GABA application (Ο) so that any deterioration in the seal could be assessed. Only I-V traces showing no deterioration were used.

When comparisons were made between two sets of data, any significant differences were determined by using a two-tailed Student’s $t$-test ($p<0.05$). Prior to this, the F-test was used to determine if there were any significant differences between the variances of the two samples and if not, the variances were then pooled together. If there was a
Figure 2.2. Exponential fitting to decaying currents. A. A whole-cell response, B. was inverted and only the decaying current was fitted with, C. one or two exponentials using the program PeakFit. For the decaying current shown, two exponentials were fitted and the resultant exponential fit is:

\[ I = A_1 \cdot \exp(-t/\tau_1) + A_2 \cdot \exp(-t/\tau_2) + C \]

where \( t \) = time.
Figure 2.3. Schematic diagram of where computer generated -100/+100mV voltage ramps (thick lines) were applied. The “true” GABA I-V relationship is calculated from subtracting the current at ① from ②. To check that these ramp protocols did not affect the health and stability of the whole-cell seal, a ramp at ③ was subtracted from ①; a horizontal line at 0pA would result if there was no deterioration. Only I-V traces showing no deterioration were used. The thin bar above the current trace represents the duration of 100μM GABA application.
significant difference in the variances, they were assumed to be unequal and treated accordingly.

2.5.2. Single-Channel Data Analysis

Single channel currents were analysed using the computer program CHANNEL2. No further filtering was used while measuring conductance states, mean currents and the generation of the all-points amplitude and frequency histograms. On many occasions conductance states could not be determined so mean currents (I\text{MEAN}) were measured instead. Mean currents were calculated using the maximum duration possible which ranged from as low as 1.7 seconds up to 50 seconds. However, for comparing pooled results, current traces that were 10 seconds long were used to take advantage of as many current traces as possible. For the all-points amplitude and frequency histograms, a 20 second current trace was used unless stated otherwise; both the open and closed threshold levels were set as close to baseline as possible but above the baseline noise level so that any subconductance states could be detected by CHANNEL2. Bursts were defined as openings separated by closures of longer than 1 ms. For open, closed and burst time frequency histograms, bin widths ranged from 0.2-0.5 ms. Single-channel traces shown are 400 ms in length and computer (Gaussian) filtered at 2 kHz with every two points dropped. As convention dictates for cell-attached and inside-out patches (Sakmann & Neher, 1995), all traces have been inverted and potentials are shown as the negative of the applied pipette potential (-V\text{P}). Therefore, channel events opening upwards are outward currents (Cl\textsuperscript- moving inward) and downward events are inward currents (Cl\textsuperscript- moving outward) with respect to the interior of the cell.
Table 2.1. Source of materials and equipment used in experiments.

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<th>Materials</th>
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<td>agar</td>
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<td>ATP-Na₂</td>
<td>Sigma</td>
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<tr>
<td>bicuculline methiodide</td>
<td>Sigma</td>
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<td>borosilicate glass capillaries</td>
<td>Clark Electromedical Instruments (GC150F-15)</td>
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<td>CaCl₂</td>
<td>Ajax Chemicals</td>
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<td>CAPTURE</td>
<td>Written by Michael Smith &amp; Phil Berg, JCSMR</td>
</tr>
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<td>cathode ray oscilloscope CHANNEL2</td>
<td>Tektronix (5223 Digitizing Oscilloscope)</td>
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<tr>
<td>choline chloride</td>
<td>Written by Michael Smith, JCSMR</td>
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<tr>
<td>computer</td>
<td>Sigma</td>
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<td>cysteine</td>
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<td>Sigma</td>
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<td>EtOH, absolute</td>
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<td>- 0.45µm filter</td>
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Table 2.1. continued...

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<td>picrotoxin</td>
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<td>List-Medical (L/M-3P-A)</td>
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<td>Narashige</td>
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<td>pulse code modulator</td>
<td>Sony (PCM-501Es)</td>
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<td>Advanced Graphics Software Inc.</td>
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<td>Skin Graft Knife Blades</td>
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<td>AWA (AV-64 HQ)</td>
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<td>water bath</td>
<td>Julabo SW-20C</td>
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<td>ZnCl₂</td>
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CHAPTER 3

ACUTE DISSOCIATION OF DENTATE GYRUS NEURONS
ACUTE DISSOCIATION OF DENTATE GYRUS NEURONS

3.1. INTRODUCTION

Expression systems, cultured neurons or hippocampal slices are the most common preparations used when characterising the GABA$_A$ receptor. Each of these preparations has its advantages and disadvantages but contribute to the study of the GABA$_A$ receptor in enormous ways.

Expression systems (eg. Sf9 cells, Xenopus laevis oocytes, CHO or HEK cell lines) have allowed researchers to manipulate the expression of various combinations of subunit isoforms. In addition, site-directed mutagenesis has also allowed structure-function studies giving us insights into how the GABA$_A$ receptor may work. However, as we still do not know what and how many subunits make up the native GABA$_A$ receptor, the use of native tissues will never be superseded.

Cultured neurons are a very versatile preparation and are relatively easy to prepare and once cultured, the neurons generated can last several days. Cultured neurons are ideal for single channel studies as excised patches are easy to obtain. While it is possible to perform whole-cell studies on cultured neurons, this preparation does pose some practical problems. For example, it is difficult to perform millisecond drug perfusion experiments (that try and mimic presynaptic release of neurotransmitter) and even more difficult to target a single neuron. Furthermore, it is conceivable that neurons grown in culture conditions do not follow the same pattern of development as neurons grown in vivo. Brooks-Kayal et al. (1998) compared acutely dissociated hippocampal neurons from postnatal day five rat pups with embryonic hippocampal neurons on days 1-21 in culture. They showed that cultured neurons aged between days 14-21 exhibited similar subunit expression levels found in the 5-day old acutely dissociated neurons. They concluded that GABA$_A$ subunit expression develops similarly but later than in cultured neurons. However, the dentate gyrus region is unique and differs from most other parts of the central nervous system. Most cultures are generated from foetal or newborn...
neurons and, because the dentate gyrus is a region that develops postnatally, culturing neurons from this region is not feasible. Furthermore, there may be many aspects of the in vivo environment that foster the development of the GABA<sub>A</sub> receptor in the dentate gyrus that the culture environment cannot provide.

Hippocampal slices are the most natural of all the preparations described so far. However, due to the neuronal denseness of this preparation, not only is individual cell identification unresolvable (although infra-red technology has allowed identification of individual neurons in slices up to 500µm thick: Sakmann & Neher, 1995) but as with cultured neurons, it does have its limitations. For example, millisecond perfusion of drugs onto single neurons is not possible. Without infra-red technology, the ability to resolve individual neurons in slices is impossible but it is possible to “blind patch” (Blanton et al., 1989) into regions where there are high densities of cell bodies, such as the pyramidal cell layer of the CA1-4 regions and the granule cell layer of the dentate gyrus region. Using a dissecting microscope with a light source from below, these cell body layers are more light permeable than the rest of the hippocampus.

More recently, acutely dissociating cells by using a combination of enzymes and mechanical trituration has been performed on a wide variety of cell types. This preparation not only allows millisecond perfusion of drugs but is a particularly useful preparation for studying the dentate gyrus. As the dentate gyrus develops postnatally, dissociation of dentate gyrus neurons on any postnatal day can be advantageous. There are many different dissociation methods that are currently being used and are quite involved and complicated (refer to Appendix I). Many change solutions several times or use several enzymes together that are likely to affect the welfare of the cell. This fact is important for neurons as they are particularly sensitive to changes in pH, oxygen availability, temperature and ionic environment. It is also vital that neurons are manipulated as little as possible and handled gently.

My aim in this chapter was to optimise and simplify the dissociation techniques that are currently being used. The goal was to reduce the concentrations of all enzymes and associated activators, the enzyme incubation time, the amount of tissue manipulation and solution changes. By reducing the degree of neuronal damage, healthier isolated neurons
are produced that are easier to patch clamp. Numann & Wong (1984) describes a fairly simple dissociation method for hippocampal neurons. It meets most of the goals outlined above and is the basis of the final method used in this study.

### 3.2. METHODS & MATERIALS

To optimise conditions for producing viable and healthy cells, various conditions and solutions were tested (range of conditions are listed in Table 3.1.). To reduce the possibility of animal, temperature and time variability, the various tests were conducted at the same time on the same sample split into two or more portions.

#### 3.2.1. Brain Preparations

Two brain preparations were tested to determine the effectiveness of the enzyme treatment without compromising the health of dentate gyrus neurons. Where possible, all dissections were performed in carbogenated and icy artificial cerebrospinal fluid (ACSF) as well as on ice to minimise neuronal deterioration.

##### 3.2.1.1. Whole Dentate Gyrus.

The first preparation tested was the whole dentate gyrus. It was thought that by keeping the bulk of glial cells and other supporting structures of the dentate gyrus, they would protect the granule cells from irreparable harm. After decapitation, the whole brain was removed and cut in half along the mid-sagittal plane. In a petrie dish wedged into a larger ice-filled petrie dish, one hemisphere of the brain was pinned down around the perimeter with the cut side facing up as shown in Figure 3.1.A. In carbogenated ACSF, the whole hippocampus was dissected out and in turn was pinned down around the perimeter. The dentate gyrus was then unrolled downwards, (starting from a blood vessel that runs perpendicular to the dentate sulcus) from the rest of the hippocampus by gently teasing and coaxing the folded dentate gyrus region away from the dentate sulcus, much like opening a closed fist. The dentate gyrus was then cut into about 3 pieces and placed
Table 3.1. Listed below are the variables that were manipulated. Combinations of each of these conditions were trialed within the ranges quoted. Details of each set of conditions tested can be seen in Appendix II.

<table>
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<tr>
<th>VARIABLES</th>
<th>DETAILS</th>
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<td>Whole Dentate Gyrus</td>
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<td>Hippocampal Slices</td>
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<td></td>
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Figure 3.1. Preparing the dentate gyrus. **A.** Each hemisphere is pinned down and the hippocampus removed (left). Half the hippocampus (right) is then pinned down, the dentate gyrus unrolled (small arrow) from the blood vessel (dotted line), dissected free from the rest of the hippocampus and then cut into small pieces. This is repeated for the other half of the hippocampus. **B.** These small pieces of dentate gyrus or hippocampal slices are placed onto a mesh within a beaker initially containing bubbled ACSF.
within a meshed ring situated inside a 50ml beaker containing 8ml bubbled ACSF (Figure 3.1.B.). A nylon mesh was glued to this ring that served as a platform for the dentate gyrus pieces to lie on. This allowed sufficient aeration of all sides of the dentate gyrus pieces. This was repeated for the other hemisphere of the brain.

3.2.1.2. Hippocampal Slices

This preparation took a little more time but allowed greater exposure of the dentate gyrus to the enzyme solution while still having the support of the surrounding tissue. After decapitating and removing the brain from the skull, the brain was cut along the mid-sagittal plane. The cut sides of the two hemispheres were quickly blotted on filter paper to dry these surfaces. Both these cut sides were then glued to the vibrating microslicer stage and placed into the microslicer chamber containing carbogenated and icy ACSF. Using the highest vibration and the slowest advance, 500µm thick slices were cut. The hippocampus was then dissected free from the surrounding brain tissue and then placed into a 50ml beaker containing 8ml of bubbled ACSF as shown in Figure 3.1.B. Occasionally the dentate gyrus was removed from the rest of the hippocampus and then placed into the beaker containing 8ml of carbogenated ACSF.

3.2.2. Dissociation Solutions

There are two major components that make up the dissociation solution. The first is the artificial cerebrospinal fluid (ACSF). This makes up the bulk of the solution and attempts to mimic the physiological milieu that the rat brain is used to. The second component is the enzyme solution which is composed of the enzyme papain as well as the associated activators that are needed to make the enzyme work.

3.2.2.1. Artificial Cerebrospinal Fluid

A volume of 250ml of ACSF was made daily. Prepared previously, frozen stocks of (in M): 1 KCl, 1 CaCl₂, 1 MgSO₄ and 1 NaH₂PO₄ were made using MilliQ water, filtered through a 0.22µM disposable filter and aliquoted into 5ml portions; a portion of each stock was thawed and used each week. Powdered NaCl, glucose and Na₂CO₃ were
weighed out each day and dissolved in approximately 100ml of 4°C-chilled doubled-distilled water. The appropriate volume of the stock solutions was added and the volume brought up to 250ml. The final concentration of this solution was (in mM): 126 NaCl, 26 NaHCO₃, 20 glucose, 3 KCl, 2.6 CaCl₂, 2.5 NaH₂PO₄ and 1.3 MgSO₄. After thoroughly mixing together, the solution was placed in the freezer for about one hour and allowed to become icy but not frozen solid. When this solution was bubbled with carbogen (a mixture of 95% oxygen and 5% carbon dioxide) the pH was about 7.4.

3.2.2.2. Enzyme Solution

Two enzyme solutions were tested. The first consisted of papain in suspension and cysteine. This was added directly to 10ml of ACSF (containing the brain preparation). The second solution was lyophilised (powdered) papain that was combined with cysteine and/or EDTA and mercaptoethanol in an initial volume of 2ml. This was later added to 8ml of ACSF (containing the brain preparation). Various concentrations of each of these components were tested and details are given below. The starting concentrations of each of these components were based on what has been commonly used in the past (see Appendix I) and the recommendations given by the manufacturer (Worthington Biochemical Corp, 1993).

3.2.3. Variable Physical Conditions

There were five other aspects of the dissociation procedure that were manipulated: hippocampal slice thickness, temperature, incubation time, solution mixing and mechanical trituration. Three thicknesses were trialed: 400µm, 500µm and 600µm. Slices could not be too thin otherwise exposure to enzyme would be too much while slices could not be too thick otherwise exposure to enzyme would not be enough. Most incubations were performed in a water bath using temperatures ranging from room temperature (about 23°C) up to 37°C. The duration of the incubation time ranged from 15 minutes up to 2½ hours. In addition, various durations (ranging from 0 to 60 minutes) of pre-enzyme and post-enzyme incubation sessions were also tested to see if none, one or both incubations were required. Occasionally a magnetic stirrer was used to keep the solution well mixed and aerated. It was later found that adjusting the bubbling strength of
the carbogen gas also accomplished the same goal. The enzyme-treated dentate gyrus preparations were mechanically triturated using a fire-polished Pasteur pipette to liberate single neurons; the degree of this fire-polishing was varied. Details of all these conditions will be described further in the appropriate sections below.

3.3. RESULTS

3.3.1. Brain Preparations

Details of each set of conditions tested on the brain preparations are summarised in Appendix II. One to four sets of conditions were imposed on the same brain preparation obtained from the same rat and assessed. The criteria for this assessment were based on the appearance of the granule cell and success of patch clamping. A score between 0 and 3 was given: 0 for none and 3 for healthy cells liberated. Each set of conditions is numbered (#1-73) so that identification of the conditions discussed below can be related to Appendix II.

3.3.1.1. Whole Dentate Gyrus

While this preparation initially looked promising, isolated neurons were not consistently produced. Damaged neurons would appear speckled or grainy and sometimes misshapen. Besides the small number of neurons, they were also difficult to patch clamp and whole-cell seals often unattainable. It is possible that these results coincided with the use of papain in suspension (reasons described below). Alternatively, it is also possible that these dentate gyrus pieces were not exposed long enough to the enzyme and this would have a two-fold effect. Firstly, the supporting connective tissues holding the granule cells and other neurons together would not have been sufficiently broken down. Secondly, because of inadequate enzyme treatment, mechanically triturating the preparation would tear and shred the neurons apart. This would yield unhealthy and reduced numbers of viable neurons.
3.3.1.2. Hippocampal Slices

Slice thicknesses of 400µm, 500µm and 600µm were tested. The thickness of the slice would determine the degree of exposure to enzyme as well as the degree of support given to dentate gyrus neurons. 500µm thick slices proved to be superior to the other thicknesses tested for two reasons. Firstly, the slice was thin enough to distinguish the various parts of the hippocampus under a low resolution dissecting microscope. Secondly, it was thick enough for the dentate gyrus neurons to have the support and protection of surrounding glial cells and other supporting connective tissues.

Hippocampal slices were used in two ways. Firstly the whole hippocampal slice was treated with enzyme and only prior to patching was the dentate gyrus removed from the rest of the hippocampus. Secondly, just after slicing the dentate gyrus was dissected free from the rest of the hippocampus and only the dentate gyrus was treated with enzyme. It was found that exposing the whole hippocampal slice to the enzyme yielded healthier and more numerous neurons. A likely reason for this is that the dentate gyrus is surrounded by the rest of the hippocampus that cushions the dentate gyrus from the physical handling as well as to some extent, the enzyme treatment.

3.3.2. Enzyme Solution

Most studies tend to use papain or trypsin for dissociating hippocampal neurons (see Appendix I) and according to Worthington Biochemical Corp. (1993), papain and trypsin have strong digestive capabilities. Trypsin often requires inactivation by a trypsin inhibitor and as one of the primary aims of this optimisation process was to reduce the number of solution changes or exposure to other chemicals, using trypsin would defeat this purpose. Papain is a sulphydryl protease that exhibits wide specificity and degrades most protein substrates (Worthington Biochemical Corp., 1993). Furthermore, papain is also easily inactivated by exposing it to air (Arnon, 1970). Two preparations of papain were used and treated differently, the first was a suspension and the second was a lyophilised powder. The papain suspension was combined with cysteine and added directly to the brain preparation after the slices were pre-incubated. Papain in suspension was used initially and while at the beginning some neurons were successfully liberated,
with time the effect of papain (obtained from the same bottle) diminished. It would appear that the strength of the enzyme decreased from the time the bottle was opened, probably the result of air exposure. This was evident from the reduced number of neurons and whole-cell seals were difficult to obtain indicating damaged cell membranes. Powdered papain was tested and proved to be a more stable preparation by consistently producing high numbers of isolated neurons. The powdered papain was combined with cysteine and/or EDTA and mercaptoethanol which was then activated for 20 to 45 minutes at a temperature ranging from approximately 25°C up to 37°C. Most studies (see Appendix I) use about 20Units/ml (20U/ml) of papain although there were a few that have used 25U/ml. Here, papain concentrations of 5, 10, 15, 20, 24 and 25U/ml (#19-23, 54-55, 68-69) were tested but it became evident that 20U/ml was the best concentration to use.

Papain requires a free sulfhydryl group for its catalytic activity and activation is achieved by mild reducing agents such as cysteine. Most studies found the added cysteine sufficient for papain activation but Worthington Biochemical Corp. (1993) suggests activating papain in a solution containing 5.5mM cysteine, 1.1mM EDTA and 0.067mM mercaptoethanol at a temperature of 25°C for 30 minutes. While the recommended cysteine concentration was 5.5mM (Worthington Biochemical Corp., 1993), most studies have used concentrations of about 1mM or below in about 20U/ml of papain (Huettner & Baughman, 1986; Shen et al., 1988; Tse et al., 1992; Suszkiw et al., 1992; Sayer et al., 1992; Alzheimer et al., 1993; Fraser et al., 1995). Here, cysteine concentrations of 1, 2 and 5mM were tested. It was generally found that the lower concentrations of cysteine generated healthier neurons. It is possible that combinations or lack of EDTA, mercaptoethanol and temperature (#19-32) or incubation times (#66-67) could have enhanced or diminished the effectiveness cysteine on papain; both over- and under- digestion of the dentate gyrus would not produce viable granule cells. Most studies rarely used EDTA to aid in papain activation. EDTA chelates heavy metal ions such as Cd²⁺, Zn²⁺, Fe²⁺, Cu²⁺, Hg²⁺ and Pb²⁺ that are inhibitory for papain. However, there were two studies that used EDTA and despite using the same papain and cysteine concentrations (20U/ml and 1mM, respectively), Huettner & Baughman (1986) used an EDTA concentration of 5mM while Suszkiw et al. (1992) used 0.5mM. Here, EDTA concentrations of zero to 0.5mM were tested (#19-22, 45-53). It became evident that
EDTA was required in the dissociation process but 0.5mM was too high. EDTA concentration was reduced down to 0.22mM and was sufficient to produce viable neurons. Even less used in dissociation procedures is the reducing agent mercaptoethanol which is used for breaking disulfide bonds. While this reagent was not thoroughly investigated, a concentration of either zero or 13.4μM was tested (#52-53). Overall, it was found that both EDTA and mercaptoethanol were required for the dissociation process.

The presence of Ca$^{2+}$ in the dissociation solutions was important to ensure healthy neurons, particularly when slicing the brain (Aitken et al., 1995; Lipton et al., 1995) and during the dissociation process (Alzheimer et al., 1993; Itier et al., 1996). However, Kaneda et al. (1988) reported that changes in the Ca$^{2+}$ concentration between 1µM and 2mM with a calcium-EGTA buffered solution had no effect on the apparent survival rate of isolated hippocampal neurons. Calcium concentrations were varied from 0mM to 3mM during the enzyme treatment. Without Ca$^{2+}$ (#59) or in the presence of 0.15mM Ca$^{2+}$ (#19-22), no healthy neurons were liberated, supporting earlier reports that Ca$^{2+}$ is important for neurons. Similarly, at a higher Ca$^{2+}$ concentration of 3mM (#52-55), this was also harmful to neurons and could be attributed to insufficient activation of papain; described earlier, divalent ions hinder the activation of papain. It is also possible that the physical moving of slices to beakers containing different concentrations of Ca$^{2+}$ could have affected the health of the neurons. Therefore, the best conditions would be to leave the neurons in the same Ca$^{2+}$ concentration throughout the whole dissociation procedure. ACSF contains a Ca$^{2+}$ concentration of 2.6mM and it was decided that manipulating the concentration of EDTA (that would chelate Ca$^{2+}$) would be a less harmful way of reducing the Ca$^{2+}$ concentration.

3.3.3. Variable Physical Conditions

As outlined previously, there were five physical aspects of the whole dissociation process that were manipulated. The thickness of the hippocampal slice has already been addressed leaving temperature, incubation times, solution mixing and mechanical trituration to be discussed.
Throughout the whole dissociation procedure, either dentate gyrus pieces or hippocampal slices were placed in a 50ml beaker. This was then placed into a larger container with a volume of water that was level to at least 10mls in a 50ml beaker. This container was always located in a water bath that was pre-heated for about an hour to the desired temperature. To try and maintain a constant temperature within the beaker, it was covered with Parafilm except for a small space allowing a thin tube carrying carbogen to the dissociation solution. This also discouraged any evaporation of the dissociation solution. The temperatures tested ranged from room temperature (about 23°C) up to 37°C. Temperatures of 30°C and below (#36-39, 54-58) did not appear to be beneficial while 37°C (#42-44) appeared to generate healthy neurons. However, 37°C was thought to be too hot so the temperature was reduced to 35°C but appeared to have little difference.

The time between decapitation and patch clamping should be as short as possible. Throughout the whole dissociation procedure, the most time consuming step was the incubation sessions. Both pre- and post- enzyme incubations were also tested to see if they were necessary. The enzyme incubation times ranged from 15 minutes up to 2½ hours while pre- and post- enzyme incubation sessions ranged from 0 to 60 minutes. Neurons appeared healthier when both pre- and post- enzyme incubation sessions were included (#63-65 vs. 14-15, 38-41). Furthermore, unequal duration of the pre- and post-enzyme incubation sessions was observed to be damaging as well (#47-51, #54-58). The duration of the enzyme incubation did not appear to be too important with times ranging from 30 minutes (#25-28, 34-35) up to 2 hours (#8-9, 12-13). As the goal was to reduce the time between decapitation and patch clamping, 30 minutes of each session were tested and proved to be the best conditions for liberating healthy neurons. It was thought that the pre-enzyme incubation session was required to soften and relax the tissue that would make the preparation more amenable to enzyme digestion while the post-enzyme incubation step at 35°C would allow the tissue to recover.

Most studies used a magnetic stirrer to keep the solutions well mixed and aerated (Numann & Wong, 1986; Kay & Wong, 1986; Huettner & Baughman, 1986; Sah et al., 1988; Shen et al., 1988; Drake et al., 1991; Newland & Cull-Candy, 1992; Fraser et al.,
1995). Stirring the dissociation solution with a magnetic bar was tested with pieces of dentate gyrus (#42-43) and proved to be reasonably successful. However, stirring with this method can only be done at room temperature. Therefore, the only way to thoroughly mix the dissociation solution and maintain it at the optimal temperature of 35°C was to bubble the solution with carbogen. Although the degree of bubbling strength cannot be quantified, bubbling should be reasonably strong but not to the extent where hippocampal slices are moving.

Mechanical trituration is an important process to liberate isolated neurons from dentate gyrus pieces or slices. It was at this stage where dentate gyrus pieces were at a disadvantage. It is possible that these pieces were not sufficiently treated with enzyme and therefore trituration shredded these dentate gyrus pieces. However, dentate gyrus slices (that were dissected free from enzyme-treated hippocampal slices) often survived this process very well. Pasteur pipettes were prepared by fire polishing the tips to various sizes. Fire polishing ensured a well-rounded tip that would not tear the dentate gyrus slices as they were being triturated. Internal diameters of approximately one millimetre were used; this size was approximately the same width as the dentate gyrus slice so that slices were easily squeezed through but were not squashed. Typically, five to six dentate gyrus slices in about 2ml of extracellular bath solution were triturated two to three times and the success of this entire dissociation procedure could be gauged by how well the dentate gyrus was triturated. Often the extracellular bath solution would go slightly cloudy indicating isolated neurons. If the solution turned very cloudy, this would often indicate over-digestion of the dentate gyrus while if the solution remained clear, the preparation was most likely under-digested. After allowing the triturated pieces to settle, the supernatant was removed and placed into the patch clamp recording bath containing about 1ml of extracellular bath solution. Granule cells were allowed to settle for about an hour before fast-perfusion experiments were performed.

3.3.4. Morphology of Dissociated Neurons

Using the dissociation method based on the optimum conditions found in this study (summarised in Chapter 2), 20 to 50 neurons were routinely dissociated but it was fairly common for neuron numbers to get up to 70. Granule cells of the dentate gyrus were
distinguished from other neuronal types (mainly interneurons) by their small size (about 10µm in diameter), characteristic pear-shaped soma and a single large principal dendrite with possibly some intact processes as seen in Figure 3.2. Using an inverted microscope with a light source from above, granule cells appeared uniformly shiny. If granule cells looked speckled or grainy and misshapen, this was a good indication that these neurons were over- or under-digested and/or mechanical trituration was too harsh.

Although the aim of this optimisation process was to produce healthy isolated dentate gyrus granule cells, comparisons were made with CA1 neurons. From the same animal and undergoing the same sets of conditions as the dentate gyrus, isolated CA1 neurons were produced more consistently than dentate gyrus neurons. It would appear that CA1 neurons were more amenable to the conditions imposed on it compared to dentate gyrus neurons.

In summary, several aspects of the dissociation method were manipulated to optimise the conditions to produce healthy and numerous granule cells. Papain was chosen over other enzymes as inactivation did not require additional chemicals to be added to the dissociation solution. Of all the brain preparations tested, the most successful preparation was 500µm hippocampal slices. Only prior to patch clamping was the dentate gyrus dissected free from the rest of the slice and gently triturated. In contrast to many other studies, the duration of each incubation session, the number of solution changes and tissue handling were all reduced. Furthermore, solution mixing by bubbling with carbogen was shown to be superior to stirring with a magnetic bar.

3.4. DISCUSSION

This study describes a method that consistently yielded relatively high numbers of isolated dentate gyrus granule cells. Past dissociation methods (see Appendix I) outline many different steps that require different solutions and one or more enzyme incubation sessions.
Figure 3.2. Two typical examples of dentate gyrus granule cells that are seen under the inverted microscope. The cell bodies (approximately 10µm in diameter) are shiny and have one principal dendrite. On occasions secondary processes can be seen but most are removed through the enzymatic and trituration procedures.
The first step in any dissociation procedure is to prepare the tissue for enzyme incubation. Here, several brain preparations were tested using the whole dentate gyrus cut into about six pieces or hippocampal slices that were treated with enzyme either before or after the dentate gyrus was dissected free. The best preparation found was hippocampal slices at a thickness of 500µm. This thickness was proven to be better than the other thicknesses tested as it was thick enough to protect granule cells but thin enough to identify regions of the hippocampus with a low resolution dissecting microscope. It was also found that healthier granule cells were produced when the dentate gyrus was removed from the rest of the hippocampus after enzyme treatment. It is possible that the surrounding hippocampal tissue protected the dentate gyrus from too much physical damage and/or too much exposure to enzyme.

Vorobjev (1991) proposed a method of vibrodissociation of hippocampal CA1 neurons in which a local application of vibration was used to liberate isolated neurons from the whole slice. It is interesting that in a subsequent paper (Vorobjev et al., 1993), papain was added before the vibrodissociation step. They did not give any explanation for using papain but it is likely that not enough neurons were released from the slice just by vibrodissociation itself. Thus, it appears that enzyme treatment is necessary to breakdown the connective tissue surrounding the neurons together in the slice. Although enzyme treatment could affect the physiological conditions of the cells, it was believed to be unlikely (Gray & Johnston, 1985; Huguenard & Algar, 1986; Mody et al., 1989). Preliminary studies done by Huettner and Baughman (1986) found that enzymes such as trypsin, collagenase, dispase, hyaluronidase and neuroaminidase proved inferior to papain for the dissociation of postnatal tissue. They did not discuss any details but it is possible that they could not completely inactivate trypsin and therefore over-digested the tissue. According to Worthington Biochemical Corp. (1993), the other enzymes are weaker and therefore under-digestion of the tissue could have occurred. Typically with these weaker enzymes, a combination of one or two are used together or sequentially. It is reasonable to suggest that healthier neurons would be produced if exposed to fewer enzymes and of course one that can be easily inactivated. After the hippocampal slices have been treated with enzyme, the enzyme solution is replaced with fresh ACSF. As papain is inactivated by exposure to air (Arnon, 1970), together this would ensure that most of the papain would have been removed and/or inactivated.
It was found that higher incubation temperatures were more conducive to the health of the neurons. Aitken et al. (1995) and Lipton et al. (1995) also observed that physiological temperatures produced healthier hippocampal slices. They believe that damaged processes would seal up better at physiological temperatures rather than at temperatures below 30°C. At a temperature of 35°C, pre- and post-enzyme incubation sessions were also found to be necessary. Pre-enzyme incubation was thought to soften the tissue to make it more amenable to the digestive effects of papain while the post-enzyme incubation session allowed the tissue to recover. Most studies tend to expose the tissue for more than an hour to the enzyme. Performing both pre- and post-enzyme incubation steps allowed the enzyme treatment to be shortened. It was interesting to observe that unequal durations of pre- and post-enzyme incubations were harmful to the health of the neurons. However, it is possible that other conditions influenced this outcome.

Keeping the dissociation solution well mixed and the hippocampal slices well aerated throughout the dissociation procedure were vital factors. Too much bubbling would cause the slices to be tossed around and damaged while not enough bubbling would not produce sufficient aeration or movement of the dissociation solution around the hippocampal slice. Most studies use mechanical trituration as a way of liberating isolated neurons from the digested tissue. It is important that the tip of the Pasteur pipette is well fire polished to a diameter about the size of dentate gyrus pieces or slices.

Another important observation was made throughout this study. There were other environmental conditions imposed on the rat before the dissociation procedure that affected the success of obtaining isolated neurons. During winter, the success of producing healthy neurons was much higher than during summer. This may suggest that live rats preferred cooler temperatures to warmer ones or there are seasonal changes in the neurons themselves. This preference for cooler temperatures was supported during a time when the air conditioner in the facility that houses the rats broke down. For about six months during the summer, temperatures were suspected to be consistently above 30°C and both dissociated neurons and hippocampal slices (for single channel work) were very poor and very few results could be obtained during this time.
The basis for this optimisation process was on a dissociation method described by Numann & Wong (1984) and differs in many respects to the final method presented here. The thickness of the hippocampal slice was reduced from 700µm to 500µm. They added 15mg of papain to the dissociation solution while 20U/ml papain was activated first in a solution containing cysteine, EDTA and mercaptoethanol before adding to the slices. Instead of stirring and aerating the dissociation solution with a small magnetic bar and carbogen blown across the surface of the solution, the solution was mixed and aerated with direct bubbling of the dissociation solution. Incubation with papain was reduced from two hours down to 30 minutes but two incubation sessions of 30 minutes were performed before and after the enzyme incubation.

In conclusion, various aspects of the dissociation procedure were tested and many interesting observations were made. This dissociation method (summarised in Chapter 2) now allows reasonable numbers of healthy neurons to be consistently produced and whole-cell seals that are easily obtained.
CHAPTER 4

WHOLE-CELL CHARACTERISTICS

4.1. INTRODUCTION

While most studies have predominated on the morphological and electrical properties of the dentate gyrus, there are far fewer studies investigating the pharmacology of GABA_A receptors in this region. However, there has been growing interest in the dentate gyrus because it develops postnatally and is allowed examination of the developing GABA_A receptor. Young and adult granule cells exhibited different sensitivities to various pharmacological agents and these differences were attributed to changingnoradrenergic combinations in the GABA_A receptor subunits; these studies were reviewed in Chapter 1.

A more general approach was taken by relating the GABA_A receptors to the entire dentate gyrus. For this in this chapter, we measure the GABA_A receptor using electrophysiological

4.1. METHODS & MATERIALS

The method of studying dentate gyrus neurons was described in Chapter 2 and discussed further in Chapter 2. Whole-cell solutions, electrophysiology, and data analysis were also described in Chapter 2.
WHOLE-CELL CHARACTERISTICS

4.1. INTRODUCTION

While most studies have concentrated on the morphological and electrical properties of the dentate gyrus, there are far fewer studies investigating the pharmacology of GABA_A receptors in this region. However, there has been growing interest in the dentate gyrus because it develops postnatally and has allowed examination of the developing GABA_A receptor. Young and adult granule cells exhibited different sensitivities to various pharmacological agents and these differences were attributed to changing subunits combinations as the GABA_A receptor matures; these studies were reviewed in Chapter 1.

A more general approach was used here to study the GABA_A receptors of the mature dentate gyrus. My aim in this chapter was to characterise the GABA_A receptor using electrophysiological methods.

4.2. METHODS & MATERIALS

The method of acutely dissociating dentate gyrus neurons was described in Chapter 2 and discussed further in Chapter 3. Whole-cell solutions, electrophysiology and data analysis were also described in Chapter 2.
4.3. RESULTS

4.3.1. GABA-Activated Currents

4.3.1.1. Analysis Of Currents Activated By GABA

Application of 100µM GABA to isolated granule cells caused a rapid increase in current that desensitised to a steady-state plateau current amplitude (at the holding potential of -40mV). When the application of GABA was terminated, the current returned to the original baseline current level. Obtaining a complete portrait of the GABA-activated response was achieved by making five (or six) measurements from each whole-cell response. This included: ① maximum peak current measured from the baseline prior to GABA application to the maximum response produced by GABA; ② mean (or average) current calculated over a one second period starting from the time of GABA application; ③ steady-state plateau current measured from the baseline prior to GABA application to the steady-state current level obtained from prolonged exposure to GABA; ④ rise time defined as the time it took for the current to rise from 10% to 90% of the peak current; ⑤ half-decay time defined as the time it took for the current to decay to half the amplitude of the peak current, and where required, ⑥ one or two exponentials were fitted to the decay of the current of the whole-cell response. This information was then used to compare responses produced by GABA with responses produced by GABA in combination with modulators of the GABA_A receptor. Illustrated in Figure 4.1. is a typical response to 100µM GABA with each point on the trace denoting one millisecond. Values measured from this GABA-activated response are also shown. As previously reported, the current decay is commonly best fitted with two exponentials (Puia et al., 1994; Celentano & Wong, 1994; Zemková et al., 1995; Jones & Westbrook, 1995; Rabow et al., 1995; Martina et al., 1996; Galarreta & Hestrin, 1997; Hollrigel & Soltesz, 1997) and this was observed here as well; these results will be shown later.

Responses to GABA and associated modulators in the dentate gyrus have recently been reported to depend on the maturity of the rat. For example, 10µM GABA was applied to granule cells isolated from 45-52 day old rats and were observed to have higher maximal current amplitudes than granule cells taken from younger rats aged 7-14 days (Kapur & Macdonald, 1999). In 261 neurons activated by a saturating concentration of GABA (100µM), the peak, mean and plateau current amplitudes varied considerably. Peak
Figure 4.1. A typical response to 100µM GABA applied for the duration of the bar above the trace; each point on the trace represents one millisecond. Five measurements were obtained from each response and where required, exponentials were fitted to the decay phase of the whole-cell response (see Equation 2.3.). This provides a method to quantify and compare GABA-activated responses to those modulated by GABA<sub>A</sub> receptor drugs. * In this particular response, the plateau current could not be accurately measured as the decaying current did not reach a steady-state level before GABA was removed.
current amplitudes ranged from 155pA to 2459pA with an average of 1009±34pA. Similarly, mean currents ranged from 60pA to 1894pA with an average of 623±22pA and plateau currents ranged from 3pA to 664pA with an average of 135±7pA (Figure 4.2.A.). Also shown are three examples of currents activated by 100µM GABA that were selected to represent the range of current amplitudes seen in these granule cells (Figure 4.2.B.). In addition, current rise times ranged from 4ms to 150ms with an average rise time of 25±1ms. Also the current half-decay time ranged from 115ms to 4568ms with an overall average half-decay time of 890±43ms. In 261 neurons activated by 100µM GABA, two exponentials (see Equation 2.3.) were fitted to the decay of the currents. The average time constants were 200±8ms for $\tau_1$ and 1938±94ms for $\tau_2$. The average amplitudes associated with these time constants were 262±18pA for $A_1$ and 535±27pA for $A_2$ with an average steady-state amplitude (C) of 85±10pA. Occasionally, extremes in current decay were observed and examples are shown in Figure 4.3. All other responses fell between these two extremes with the majority of responses (72%) having characteristics more similar to Figure 4.3.D. The measurements for peak, mean and plateau current amplitudes as well as rise and half-decay times are shown for these examples. In both cases, two exponentials were fitted to the current decay and differed the most in the slow time constant ($\tau_2$) and steady-state amplitude (C).

### 4.3.1.2. GABA Dose-Response Curves

In isolated granule cells, Kapur & Macdonald (1996, 1999) found no significant correlations between granule cell maturity and GABA sensitivity. They reported an EC$_{50}$ value of 31±10µM for adult rats while younger rats had an EC$_{50}$ of 40±8µM. In this study, GABA concentrations ranging between 1µM and 1mM were directly applied to isolated granule cells and typical current responses are shown in Figure 4.4. Taken from a single neuron, the current traces show consecutive applications of 100µM, 1mM, 1µM and 10µM GABA. As the full range of GABA concentration could not be applied to a single neuron, typical current responses of these “test concentrations” were compiled from five different neurons. The respective 100µM GABA control responses (of these “test concentrations”) were normalised to the peak amplitude of the 100µM GABA shown in Figure 4.5. The factor by which each of these control responses were normalised to, the corresponding “test concentration” was also scaled by the same factor.
Figure 4.2. Varying magnitudes of current amplitudes were seen. A. 100μM GABA elicited a range of peak, mean and plateau current amplitudes (n=261). The closed symbols represent individual experiments and the open symbol represents the average. The average peak, mean and plateau currents were 1009±34pA, 623±22pA and 135±7pA, respectively. B. Examples of the responses generated by 100μM GABA; GABA was applied for the duration of the bar above the current traces; the peak current amplitude responses were 2346pA, 1382pA and 156pA, respectively.
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<tr>
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<th>Response 1</th>
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<tr>
<td>Peak Current</td>
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<td>531pA</td>
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<td>275pA</td>
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<tr>
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**Figure 4.3.** Extreme time courses of currents activated by 100µM GABA. Responses tended to be more like response 1. The most notable difference is in the rate of current decay. In this example, the half-decay time was 18 times longer in 2 than in 1 and is reflected in the longer slow time constant, $\tau_2$. The steady-state amplitude (C) also differed between the two responses. * In response 2, the plateau current could not be accurately measured as the decaying current did not reach a steady-state level before GABA was removed.
Figure 4.4. Consecutive paired responses generated by applying 100µM, 1mM, 1µM and 10µM GABA to a single neuron.
Figure 4.5. Current responses to increasing concentrations of GABA. As the full range of GABA concentrations could not be tested on a single neuron, these responses were taken from five neurons and their respective control peak current amplitudes were normalised to the peak current amplitudes of the 100µM GABA response shown in this figure (see text for explanation).
Thus, each of the “test concentrations” have also been normalised to the 100µM GABA response shown in Figure 4.5.

Dose-response curves have usually been generated from only GABA-activated peak currents (White, 1992; Schönrock & Bormann, 1993; Takahashi et al., 1994; Fraser et al., 1995; Rho et al., 1996; Itier et al., 1996, Kapur & Macdonald, 1996, 1999) with little attention to mean and plateau currents. It was thought that these latter currents, particularly the plateau currents, were just as important as the peak currents activated by GABA. Those receptors active during the plateau phase of the whole-cell current are most likely to be the receptors seen during single-channel recording. A range of peak, mean and plateau current amplitudes were observed when plotted against GABA concentration (Figures 4.6.A., 4.7.A. & 4.8.A.). The scatter was reduced when these values were normalised (I') to 100µM GABA and expressed as a percentage (Figures 4.6.B., 4.7.B. & 4.8.B.). Clearly, current amplitude increased as GABA concentration increased. These values were then averaged and normalised (I") to the maximum amplitude response and expressed as a percentage. A Hill type equation was fitted (see Equation 2.1.) giving an EC50 of 14µM, 7µM and 4µM for peak, mean and plateau currents, respectively. The associated Hill coefficients were 1.3, 2.1 and 2.9 (Figures 4.6.C., 4.7.C. & 4.8.C. and Table 4.1.A.). Compared to the dose-response curve for the plateau current, the dose-response curve for the peak current was shifted to the right. That is, peak currents are less sensitive to GABA than the plateau currents. The higher Hill coefficient for plateau currents, although not well determined, may indicate greater binding site co-operativity for those receptors active at this time. As peak and plateau currents measure the two extreme ends of the GABA-activated response, it was expected that measurements taken from mean currents would fall between these two extreme ends. As predicted, the EC50 for mean currents was 7µM GABA which did fall between the EC50 values for peak and plateau currents. These differences in EC50 for peak and plateau currents could be a result of several possibilities. It may represent two different GABA_A receptor populations that activate or desensitise at different rates in response to GABA and having their own unique sensitivity to GABA. Alternatively, perhaps desensitised GABA_A receptors have properties different from those receptors that have not been previously activated.
Figure 4.6. Peak currents activated by increasing concentrations of GABA. A. Peak current amplitudes obtained for each concentration of GABA. Filled symbols represent 6-65 results for each concentration of GABA. Open circles denote the average current amplitude with the vertical bars showing ±1SEM. B. The peak current amplitudes were normalised ($I'$) to the peak current response produced by 100µM GABA and expressed as a percentage. C. The averaged peak current amplitudes were normalised to the maximum current response ($I''$) and expressed as a percentage. The solid line shows the fit to a Hill type equation (Equation 2.1.).
Figure 4.7. Mean currents activated by increasing concentrations of GABA. **A.** Mean current amplitudes obtained for each concentration of GABA. Filled circles represent 6-65 results for each concentration of GABA. Open circles denote the average current amplitude with the vertical bars showing ±1SEM. **B.** The mean current amplitudes were normalised (I') to the mean current response produced by 100µM GABA and expressed as a percentage. **C.** The averaged mean current amplitudes were normalised to the maximum current response (I'') and expressed as a percentage. The solid line shows the fit to a Hill type equation (Equation 2.1.).
Figure 4.8. Plateau currents activated by increasing concentrations of GABA. A. Plateau current amplitudes obtained for each concentration of GABA. Filled circles represent 6-65 results for each concentration of GABA. Open circles denote the average current amplitude with the vertical bars showing ±1SEM. B. The plateau current amplitudes were normalised (I') to the plateau current response produced by 100µM GABA and expressed as a percentage. C. The averaged plateau current amplitudes were normalised to the maximum current response (I") and expressed as a percentage. The solid line shows the fit to a Hill type equation (Equation 2.1.).
Table 4.1. The EC\textsubscript{50} and IC\textsubscript{50} for peak, mean and plateau currents for A. GABA, B. bicuculline and, C. Zn\textsuperscript{2+}. All data was best fitted with a Hill type equation (see Equations 2.1. & 2.3.) except for the mean current data for Zn\textsuperscript{2+} which could only be statistically fitted with a straight line. The degree of best fit ($r^2$) are also shown.

A.

<table>
<thead>
<tr>
<th>GABA</th>
<th>EC\textsubscript{50} (µM)</th>
<th>Hill Coefficient</th>
<th>$r^2$</th>
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<tr>
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<tr>
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<td>0.983</td>
</tr>
<tr>
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B.

<table>
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<th>$r^2$</th>
</tr>
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<tbody>
<tr>
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<td>0.990</td>
</tr>
<tr>
<td>Mean</td>
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<td>1.6</td>
<td>0.997</td>
</tr>
<tr>
<td>Plateau</td>
<td>36</td>
<td>2.2</td>
<td>0.858</td>
</tr>
</tbody>
</table>

C.

<table>
<thead>
<tr>
<th>Zn\textsuperscript{2+}</th>
<th>IC\textsubscript{50} (µM)</th>
<th>Hill Coefficient</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak</td>
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<td>4.5</td>
<td>0.71</td>
</tr>
<tr>
<td>Mean</td>
<td>55</td>
<td>-</td>
<td>0.941</td>
</tr>
<tr>
<td>Plateau</td>
<td>2</td>
<td>4.99</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>2.7</td>
<td></td>
</tr>
</tbody>
</table>
4.3.1.3. Effects Of Increasing GABA Concentration On Current Time Course

It has been reported that with higher concentrations of GABA, the extent and rate of current decay is faster (Frosch et al., 1992; Oh & Dichter, 1992; Zemková et al., 1995; Gingrich et al., 1995). While this has never been quantified in the dentate gyrus, it is obvious from the current traces that the half-decay times are faster with higher concentrations of GABA (Kapur & Macdonald, 1996, 1999). Here, the effect of increasing GABA concentration on the time course was assessed.

With increasing GABA concentration, the time it took for the current to rise to 10-90% of the peak current amplitude decreased, showing a concentration-dependence of the current rise times (Figure 4.9.A.). It should be noted that at low concentrations of GABA (1µM and 3µM), a detectable current was not always evoked hence the absence or the low number of results for these concentrations. Despite this, currents activated by 3µM GABA produced the slowest rise time with an average of 260±140ms. The fastest average rise time of 19±2ms was recorded for currents activated by 100µM GABA; this rise time was not significantly faster than the rise time of 23±4ms that was generated for currents activated by 1mM GABA.

Also showing a concentration-dependence, the time it took for the current to decay to half the maximum peak current amplitude decreased as the concentration of GABA was increased (Figure 4.9.B.). Again, the absence or the low number of results was due to low GABA concentrations not evoking any detectable current. Nevertheless, the slowest average half-decay time of 2768±245ms was recorded for currents activated by 5µM GABA; this half-decay time was not significantly slower than 2666±1673ms which was recorded for currents activated by 3µM GABA. Currents activated by 1mM GABA produced the fastest average half-decay time of 380±90ms.

In summary, a method of describing various aspects of GABA-activated currents has been outlined that provides a way of quantifying and comparing GABA-activated responses with those in the presence of GABA_A receptor modulators. It was observed that currents activated by 100µM GABA generated a range of peak, mean and plateau current amplitudes as well as half-decay times. Dose-response curves generated for GABA-activated peak, mean and plateau currents showed different sensitivities to
Figure 4.9. Effect of GABA concentration ([GABA]) on the time course of currents. As the concentration of GABA was increased, both A. rise times (for the current to rise from 10 to 90% of the peak current) and B. half-decay times (for the current to decay to half of the peak current) decreased. Closed symbols represent individual results and the open symbol represents the average with the vertical bars showing ±1SEM.
GABA. The EC₅₀ value for peak currents was 3½ times higher than for plateau currents with the EC₅₀ for mean currents falling between the two. Both rise and half-decay times were dependent on the GABA concentration and the time course of currents speeded up with increasing concentrations of GABA.

4.3.2. Effects Of Bicuculline Currents Activated By GABA

4.3.2.1. Bicuculline Dose-Response Curves

Bicuculline, a competitive antagonist (Simmonds, 1980; Akaike et al., 1985a, 1987a; Yakushiji et al., 1987; Nakagawa et al., 1991), has been reported to eliminate synaptic currents activated by GABA at concentrations below 10µM in the dentate gyrus (Edwards et al., 1990; Liu et al., 1998). In addition, Nakagawa et al. (1991) obtained a bicuculline IC₅₀ of 0.75µM for peak currents in acutely dissociated nucleus solitarii neurons of the rat. The sensitivity of currents activated by GABA to low concentrations of bicuculline was explored here.

Bicuculline concentrations ranging from 0.5µM to 300µM was applied with 100µM GABA. Each bicuculline concentration was tested on 4-8 neurons. Illustrated in Figure 4.10.A., are consecutive applications of bicuculline with 100µM GABA onto a single neuron. As the full range of bicuculline concentration could not be tested on a single neuron, typical current responses showing the effect of increasing bicuculline concentration on currents activated by 100µM GABA were selected from five different neurons. The 100µM GABA control responses from each of these typical bicuculline responses were normalised to the peak current amplitude of the 100µM GABA response shown in Figure 4.10.B. The factor by which each of these control responses were normalised to, the same factor was applied to the corresponding bicuculline response. Thus, each of the bicuculline responses have also been normalised to the 100µM GABA response shown in Figure 4.10.B. From these current traces, it is clear that as the concentration of bicuculline increases, the amplitudes of peak, mean and plateau currents diminishes. As the peak and plateau currents appear to differ the most, these will be presented in detail here but all currents were examined (Table 4.1.B.).
Figure 4.10. Comparison of the effects of increasing bicuculline (BIC) concentration on currents activated by 100µM GABA. A. 30µM and 300µM bicuculline (with 100µM GABA) was applied to a single neuron. B. As the full range of bicuculline concentrations could not be tested on a single neuron, these responses were compiled from five neurons and their respective control peak amplitudes were normalised to the peak amplitude of the 100µM GABA response shown above (see text for explanation).
The peak and plateau current amplitudes generated by 100µM GABA with increasing concentrations of bicuculline, show a spread of current amplitudes although not to the same extent as the currents activated by GABA alone (Figures 4.11.A & 4.12.A.). The scatter was reduced when these current amplitudes were normalised (I') to 100µM GABA and expressed as a percentage (Figures 4.11.B. & 4.12.B.). These values were then averaged and normalised (I'') to the maximum amplitude response and expressed as a percentage. A Hill type equation was fitted (see Equation 2.2.) giving an IC₅₀ of 7µM for peak currents and a Hill coefficient of 1.6 (Figure 4.11.C.). Plateau currents had an IC₅₀ of 36µM and a Hill coefficient of 2.2 (Figure 4.12.C. and Table 4.1.B.). Therefore, GABA-activated peak currents were about five times more sensitive to bicuculline than plateau currents.

4.3.2.2. Effects Of Increasing Bicuculline Concentration On Current Time Course

The current traces also show that with increasing concentrations of bicuculline, the rate of current decay appears to decrease (Akaike et al., 1987a; Frosch et al., 1992). This was explored further by examining the effects of increasing bicuculline concentration on the rise and half-decay times of currents activated by GABA. A range of current rise times was obtained for currents activated by 100µM GABA in the presence of increasing bicuculline concentration. However, on average there appear to be no significant effect of bicuculline on the GABA-activated current rise times (Figure 4.13.A.).

Similarly, a range of half-decay times was recorded for currents activated by 100µM GABA in the presence of increasing concentrations of bicuculline. In contrast to the rise times, there appears to be a general increase in the half-decay times which then declines as high concentrations of bicuculline (>100 µM) began to abolish the GABA-activated current (Figure 4.13.B.). The fastest half-decay time of 461±76ms was recorded for currents modulated by 0.5µM bicuculline (with 100µM GABA). The slowest half-decay time of 1521±408ms was recorded for currents modulated by 10µM bicuculline (with 100µM GABA). Above this concentration, the half-decay time decreased to 0ms when currents could no longer be detected with 300µM bicuculline.
Figure 4.11. Inhibitory effects of bicuculline on peak currents generated by 100µM GABA. A. Peak current amplitudes obtained for each concentration of bicuculline. Filled symbols represent 4-36 results for each concentration of bicuculline. Open circles denote the average current amplitude with the vertical bars showing ±1SEM. B. The peak current amplitudes were normalised (I') to the peak current response produced by 100µM GABA and expressed as a percentage. C. The averaged peak current amplitudes were normalised to the maximum current response (I'') and expressed as a percentage. The solid line shows the fit to a Hill type equation (Equation 2.2.).
Figure 4.12. Inhibitory effects of bicuculline on plateau currents generated by 100µM GABA. A. Peak current amplitudes obtained for each concentration of bicuculline. Filled symbols represent 4-36 results for each concentration of bicuculline. Open circles denote the average current amplitude with the vertical bars that show ±1SEM. B. The plateau current amplitudes were normalised (I') to the plateau current response produced by 100µM GABA and expressed as a percentage. C. The averaged plateau current amplitudes were normalised to the maximum current response (I") and expressed as a percentage. The solid line shows the fit to a Hill type equation (Equation 2.2.).
Figure 4.13. Effects of bicuculline on time course of current activated by 100µM GABA. A. Rise times and B. half-decay times were plotted against increasing bicuculline concentrations. Each symbol represents 2-36 results for each concentration of bicuculline. The open symbol denote the averages with the vertical bars showing ±1SEM.
In summary, the peak, mean and plateau currents activated by 100µM GABA showed differential sensitivity to bicuculline. The IC$_{50}$ for the peak current was about five times smaller than the IC$_{50}$ value for plateau currents. The GABA-activated rise times does not appear to be significantly affected by increasing bicuculline concentration but there does appear to be some influence on the half-decay times. With increasing concentrations of bicuculline, the half-decay times increased until the GABA-activated current was abolished. In contrast to synaptic currents that could be abolished by concentrations below 10µM bicuculline, here peak currents could not be consistently abolished even with 100µM bicuculline. A possible explanation could come from peak currents generated by receptors that contain a subunit combination different from that found for receptors generating the plateau current. However, desensitised receptors may confer different pharmacology and cannot be disregarded.

4.3.3. Effects of Zn$^{2+}$ On Currents Activated By GABA

4.3.3.1. Zn$^{2+}$ Dose-Response Curves

Kapur & Macdonald (1996, 1999) speculated the possibility of two GABA$_A$ receptor populations based on the incomplete inhibition of GABA-activated currents by Zn$^{2+}$. It has been previously reported that zinc sensitivity is associated to the absence of a $\gamma$ subunit (Draguhn et al., 1990; Smart et al., 1991; Celentano et al., 1991; Schönrock & Bormann, 1993; Chang et al., 1995). However, a small degree of diazepam sensitivity was found (in this study and by Kapur & Macdonald, 1996) in the dentate gyrus granule cells which would imply the presence of the $\gamma$ subunit. Since peak, mean and plateau currents showed differential sensitivity to both GABA and bicuculline, this was also examined for Zn$^{2+}$.

Zn$^{2+}$ concentrations ranging from 1µM to 3mM was applied with 100µM GABA. Each Zn$^{2+}$ concentration was tested on 4-10 neurons. Illustrated in Figure 4.14.A. are consecutive applications of Zn$^{2+}$ with 100µM GABA onto a single neuron. In this case, the response produced by 1mM Zn$^{2+}$ does not reflect the overall average response seen at this concentration. Again, the full range of Zn$^{2+}$ concentration could not tested on a single neuron so typical current responses showing the effect of increasing Zn$^{2+}$
Figure 4.14. Comparison of the effects of increasing Zn$^{2+}$ concentrations on currents activated by 100µM GABA. **A.** 1µM and 1mM Zn$^{2+}$ (with 100µM GABA) was applied to a single neuron. The response shown for 1mM Zn$^{2+}$ from this neuron does not reflect the average response. **B.** As the full range of Zn$^{2+}$ concentrations could not be tested on a single neuron, these responses were taken from five neurons and their respective control peak amplitudes were normalised to the peak amplitude of the 100µM GABA response shown above (see text for explanation).
concentration on currents activated by 100µM were selected from five different neurons. The 100µM GABA control responses from each of these typical Zn\(^{2+}\) responses were normalised to the peak current amplitude of the 100µM GABA response shown in Figure 4.14.B. The factor by which each of these control responses were normalised to, the same factor was applied to the corresponding Zn\(^{2+}\) response. Thus, each of the Zn\(^{2+}\) responses have also been normalised to the 100µM GABA response shown in Figure 4.14.B. In addition to the depressed peak current amplitude, the plateau current was almost totally abolished with Zn\(^{2+}\) concentrations above 300µM. The abolition of plateau currents with high concentrations of Zn\(^{2+}\) has been reported in the hippocampus (Legendre & Westbrook, 1991; Kapur & Macdonald, 1996, 1997, 1999; Tietz et al., 1999) but was not examined in detail.

The peak and plateau current amplitudes generated by 100µM GABA with increasing concentrations of Zn\(^{2+}\) show a range of current amplitudes (Figures 4.15.A & 4.16.A.). The scatter was reduced when these current amplitudes were normalised (I') to 100µM GABA and expressed as a percentage (Figures 4.15.B. & 4.16.B.). These values were then averaged and normalised (I'') to the maximum amplitude response and expressed as a percentage. A Hill type equation was fitted (see Equation 2.2.) giving an IC\(_{50}\) of 92µM and a Hill coefficient of 4.5 for peak currents (Figure 4.15.C.). Two Hill type equations could be fitted to the plateau currents giving IC\(_{50}\)'s of 2µM and 300µM with a Hill coefficient of 4.99 and 2.7, respectively. However, the fits of the two Hill type equations were not well fitted to the data points due to a lack of data around the IC\(_{50}\) value. This may explain the relatively high Hill coefficient values. While only the peak and plateau current results are shown here, mean currents were also examined. A Hill type equation could not be fitted to the mean current data so a straight was statistically best fitted, the half-way point of this straight line calculated and then related to the Zn\(^{2+}\) concentration. This gave an “IC\(_{50}\)” of about 55µM (Table 4.1.C.).

Kapur & Macdonald (1999) showed that GABA-activated peak currents were not completely inhibited by 1mM Zn\(^{2+}\) and this was explored further. In the presence of 3mM Zn\(^{2+}\), currents activated by 100µM GABA had peak current amplitudes that were 0.48±0.05 of control, while the plateau currents were completely abolished (n=4). Although the current deteriorated and no recovery could be obtained, at the maximum
Figure 4.15. Inhibitory effects of Zn$^{2+}$ on peak currents generated by 100µM GABA. A. Peak current amplitudes obtained for each concentration of Zn$^{2+}$. Filled symbols represent 3-40 results for each concentration of Zn$^{2+}$. Open circles denote the average current amplitude with the vertical bars showing ±1SEM. B. The peak current amplitudes were normalised (I') to the peak current response produced by 100µM GABA and expressed as a percentage. C. The averaged peak current amplitudes were normalised to the maximum current response (I'') and expressed as a percentage. The solid line shows the fit to a Hill type equation (Equation 2.2.).
A. Plateau Current Amplitudes

B. Normalised Plateau Currents

C. Dose-Response Curve

Figure 4.16. Inhibitory effects of Zn\(^{2+}\) on plateau currents generated by 100µM GABA. A. Plateau current amplitudes obtained for each concentration of Zn\(^{2+}\). Filled symbols represent 3-40 results for each concentration of Zn\(^{2+}\). Open circles denote the average current amplitude with the vertical bars showing ±1SEM. B. The plateau current amplitudes were normalised (I') to the plateau current response produced by 100µM GABA and expressed as a percentage. C. The averaged plateau current amplitudes were normalised to the maximum current response (I'') and expressed as a percentage. The symbol at 300µM Zn\(^{2+}\) represents only one data point. The solid line shows the fit to two Hill type equations (Equation 2.2.).
4.3.3.2. Effects Of Increasing \( \text{Zn}^{2+} \) Concentration On Current Time Course

The current traces in Figure 4.14.B. show a faster time course of GABA-activated currents with increasing concentrations of \( \text{Zn}^{2+} \). This was quantified by examining both rise and half-decay times. A range of current rise times was obtained with increasing \( \text{Zn}^{2+} \) concentration on currents activated by 100µM GABA. However, on average there appear to be no significant effect of \( \text{Zn}^{2+} \) on the GABA-activated current rise times (Figure 4.17.A.).

In contrast to the rise times, as the concentration of \( \text{Zn}^{2+} \) increased, the half-decay times of GABA-activated currents decreased (Figure 4.17.B.). The slowest half-decay time of 840±88ms was recorded for currents activated by 1µM \( \text{Zn}^{2+} \) (with 100µM GABA). The fastest half-decay time of 126±11ms was recorded for 300µM \( \text{Zn}^{2+} \) (with 100µM GABA) which was not significantly different from that recorded for 3mM \( \text{Zn}^{2+} \) (139±47ms).

In summary, the peak, mean and plateau currents activated by 100µM GABA showed differential sensitivity to \( \text{Zn}^{2+} \). The single IC50 for peak current fell between the two IC50 values for the plateau current. This may suggest the presence of two or three receptor populations which will be discussed later. Increasing \( \text{Zn}^{2+} \) concentration did not appear to affect the 100µM GABA-activated current rise times but the half-decay times was speeded up. While both \( \text{Zn}^{2+} \) and bicuculline specifically depress currents activated by GABA, it appears that they have opposite effects on the rate of current decay. This could be due to the mechanism of block used by these drugs or the effect of these drugs on different populations of receptors.

4.3.4. General Pharmacology Of GABA\(_{\text{A}}\) Receptors

In addition to the GABA\(_{\text{A}}\) receptor modulators already investigated, the effects of other drugs on the GABA-activated peak, mean and plateau currents were examined. The
Figure 4.17. Effects of Zn$^{2+}$ on the time course of currents activated by 100µM GABA. A. Rise times and B. half-decay times were plotted against increasing Zn$^{2+}$ concentrations. Each closed symbol represents 4-41 responses for each concentration of Zn$^{2+}$. Open symbols denote the average with vertical bars showing ±1SEM.
effect of these drugs on the GABA-activated current rise and half-decay times were also evaluated.

4.3.4.1. Drugs That Depress Currents Activated By GABA

Both picrotoxin (Takeuchi & Takeuchi, 1969; Curtis et al., 1971; Nicoll & Wojtowicz, 1980; Simmonds, 1980; Akaike et al., 1987a; Yakushiji et al., 1987; Itabashi et al., 1992; Newland & Cull-Candy, 1992; Krishek et al., 1996) and penicillin (Curtis et al., 1972; Hochner et al., 1976; Pickles & Simmonds, 1980; Chow & Mathers, 1986; Akaike et al., 1987a; Yakushiji et al., 1987; Katayama et al., 1992; Twyman et al., 1992) depress GABA-activated currents and are believed to act at different sites on the receptor. 100µM picrotoxin was applied together with 100µM GABA to nine neurons (Figure 4.18.A.). This produced an average peak, mean and plateau current that was 0.6±0.04, 0.2±0.02 and 0.5±0.05 times the control, respectively (Figure 4.21.A. & Table 4.2.). The average current rise time was 2.4±0.4 of control and the average half-decay time was 0.1±0.02 of control (Figure 4.22.A. & Table 4.2.). Similarly, 10mM penicillin was applied with 100µM GABA to seven neurons (Figure 4.18.B.). This produced an average peak, mean and plateau current that was 0.1±0.03, 0.06±0.01 and 0.2±0.04 times the control, respectively (Figures 4.21.A. & Table 4.2.). The average current rise time was 5.5±3.1 of control and the average half-decay time was 0.05±0.01 times the control (Figure 4.22.A. & Table 4.2.).

Terminating the application of 100µM GABA with either 100µM picrotoxin or 10mM penicillin, caused a rebound current to occur (Figure 4.18.). This rebound current was quantified by measuring the peak amplitude of the rebound current from the baseline prior to drug application. When picrotoxin (with GABA) was removed, the rebound current amplitude (80±16pA) was about 0.2 times the peak current amplitude generated by both picrotoxin and GABA (432±54pA). The opposite was true for penicillin with a rebound current amplitude (310±51pA) that was 6.3 times larger than the current amplitude generated by penicillin and GABA (49±8pA). A likely reason for these different current amplitudes could be attributed to the blocking mechanism used by these two drugs. To examine this, the peak current amplitude for each response activated by GABA was compared with the corresponding rebound current amplitude generated by
Figure 4.18. Effects of GABA<sub>A</sub> receptor antagonists on currents activated by 100µM GABA. A. 100µM picrotoxin decreased GABA-activated peak, mean and plateau currents as well as the rise and half-decay times (n=9). B. 10mM penicillin also reduced GABA-activated peak, mean and plateau currents as well as the rise and half-decay times (n=7). See Table 4.2. for values.
picrotoxin or penicillin with GABA (Figure 4.19.). It would be expected, for example, that in the case of a simple open channel block the amount of current activated by GABA would be about the same when the block is removed provided that dissociation of GABA is much slower than removal of drug block. If this is true, then the rebound current amplitude should be approximately the same as the GABA-activated peak current amplitude. For the rebound currents involving picrotoxin, despite the range of peak current amplitudes activated by GABA alone, when picrotoxin plus GABA was removed the rebound peak current amplitudes were all about the same reduced magnitude (Figure 4.19.A.). These results would indicate that an open channel blocking mechanism may be unlikely. In contrast, for the rebound currents involving penicillin, there appears to be some correlation between the peak current amplitude activated by GABA and the amplitude of the rebound current. It was observed that the larger the GABA-activated peak current amplitude, the larger the rebound peak current amplitude (Figure 4.19.B.). Although there was a relatively small range of drug application times, there also appears to be some relationship between the duration of penicillin (with GABA) and the amplitude of the rebound current. The ratio of the rebound current amplitude to the GABA-activated peak current amplitude was calculated and the general trend was the longer the drug application, the larger the rebound peak current amplitude (Figure 4.19.C.). This would have to be tested further by comparing the amplitude of the rebound current to much longer and shorter drug application times. Nevertheless, these results support the well regarded notion that penicillin is an open channel blocker. Using the peak current amplitudes as a gauge for the numbers of channels open, it was found that the larger the GABA-activated peak current amplitude (the more channels that are open and blocked by penicillin), the larger the rebound current (the more channels that are then unblocked and free to carry current). It could also be speculated that desensitised receptors can be blocked (and then unblocked) as it was found that the longer the drug application, the larger the rebound current amplitude. These ideas will be explored further in the Discussion below.

Shown in Figure 4.21.A. is a comparison of the peak, mean and plateau currents activated by GABA with the current responses in the presence of bicuculline, picrotoxin, penicillin and Zn$^{2+}$. Compared to plateau currents activated by 100µM GABA, peak currents were more sensitive to the depressant effects 100µM bicuculline and 10mM
Figure 4.19. Comparison of 100μM GABA-activated peak current amplitudes to the rebound peak current amplitudes. Terminating the application of 100μM GABA with A. 100μM picrotoxin (n=9) or B. 10mM penicillin (n=7) produced a rebound current response that was always smaller than the GABA-activated current. Individual experiments are denoted by the symbols with the dotted line leading to the corresponding rebound current. C. Table showing the ratio of the penicillin rebound peak current amplitude to the GABA-activated peak current amplitude. These calculations indicate a correlation between the magnitude of the rebound current amplitude to the duration of the penicillin (with GABA) application.
penicillin. In contrast, compared to the peak currents, plateau currents activated by GABA were more sensitive to the depressant effects of 100µM picrotoxin and 100µM Zn²⁺. The effect of these drugs on the magnitude of the GABA-activated mean currents were generally similar to the effects seen for the peak currents although picrotoxin depressed the GABA-activated mean current to a greater extent than the peak and plateau currents. Apart from 100µM bicuculline, these drugs slowed down the rise times and speeded up the half-decay times of currents activated by 100µM GABA; 100µM bicuculline speeded up both the rise and half-decay times of the GABA-activated current (Figure 4.22.A.).

4.3.4.2. Drugs That Enhance Currents Activated By GABA

Pentobarbital (Barker & Ransom, 1978; Nicoll & Wojtowicz, 1980; Mathers & Barker, 1980; Akaike et al. 1985b; Schwartz et al. 1986; Parker et al., 1986; Akaike et al., 1987; Peters et al., 1988, Robertson, 1989; Yakushiji et al., 1989; Itabashi et al., 1992; Macdonald & Olsen, 1994; Rho et al., 1996) and diazepam (Squires et al., 1979; Sigel & Baur, 1988; Pritchett et al., 1989; Möhler et al., 1990; Lüddens & Wisden, 1991; Itabashi et al., 1992; Horne et al., 1993; Schönrock & Bormann, 1993; Yakushiji et al., 1993; Aguayo et al., 1994; Zemkova et al., 1995; McKernan et al., 1995; Rabow et al., 1995; Benke et al., 1997; Eghbali et al., 1997; Kapur & Macdonald, 1996, 1999; Tietz et al., 1999) are specific GABAA receptor modulators. Depending on their concentration, both have enhancing and depressant effects on currents activated by GABA. Ethanol has also been reported to enhance GABA-activated currents but in contrast to the multiple effects observed with pentobarbital and diazepam, it was revealed that ethanol only enhanced the currents generated by GABAA receptors containing the γ2₉ splice variant. However, there appear to be conflicting opinions on this (Aguayo, 1990; Reynolds & Prasad, 1991; Cherubini et al., 1991; Reynolds et al., 1992; Criswell et al., 1993; Aguayo & Alarcón, 1993; Weiner, et al., 1994; Wafford et al., 1996; Tyndale et al., 1997). Enhancement of 7µM GABA-activated currents by these three modulators was examined.

100µM pentobarbital gave peak, mean and plateau currents activated by 7µM GABA that were 2.1±0.2, 2±0.3 and 1.3±0.1 times the control, respectively (n=9; Figures
In the presence of pentobarbital, the average current rise time was 1.3±0.3 and the average half-decay time was 1.3±0.2 times the control (Figure 4.22.B. & Table 4.2.). In addition, when the application of pentobarbital (with GABA) was terminated, a small rebound current occurred in three out of the nine neurons. These rebound currents as well as the other effects reported for pentobarbital were examined further and will be presented in the next chapter. 5µM diazepam enhanced peak, mean and plateau currents activated by 7µM GABA, that were on average 1.3±0.1, 1.3±0.1 and 1.1±0.1 times the control, respectively (n=13; Figures 4.20.B., 4.21.B. & Table 4.2.). In the presence of diazepam, the average rise time was 1.4±0.1 and the average half-decay time was 1.2±0.2 times the control (Figure 4.22.B. & Table 4.2.). The relatively small GABA-activated current enhancement by diazepam was not a result of current run-down. Current run-down is believed to be caused by phosphorylation (Moss et al., 1992; Gyenes, et al., 1994; Krishek et al., 1994) and as ATP was present in all recording patch pipettes, current run-down was minimised. Furthermore, the degree of current enhancement by diazepam was also fairly consistent and a good level of current recovery was obtained in all 13 responses. Similarly, 10mM ethanol enhanced the peak and mean currents activated by 7µM GABA to 1.3±0.1 and 1.1±0.1 times the controls. However, GABA-activated plateau currents were depressed by ethanol and were 0.7±0.1 times the control (n=11; Figures 4.20.C., 4.21.B. & Table 4.2.). In contrast to the other two drugs, ethanol speeded up the current time course with the average rise and half-decay times that were 0.9±0.1 and 0.8±0.1 times the control (Figure 4.22.B. & Table 4.2.).

Comparison of the peak and plateau currents activated by 7µM GABA show that peak currents were more sensitive to the enhancing effects of all three modulators. While 100µM pentobarbital and 5µM diazepam enhanced the GABA-activated plateau current, 10mM ethanol depressed the plateau current. The effect of these modulators on the magnitude of the GABA-activated mean currents were very similar to the effects seen for the peak currents (Figure 4.21.B.). Both 100µM pentobarbital and 5µM diazepam slowed down the rise and half-decay times of currents activated by 7µM GABA but 10mM ethanol speeded up the time course (Figure 4.22.B.).
Figure 4.20. Effects of GABA<sub>A</sub> receptor agonists on currents activated by 7µM GABA, A. 100µM pentobarbital increased GABA-activated peak, mean and plateau currents as well as the rise and half-decay times (n=9). B. 5µM diazepam increased GABA-activated peak, mean and plateau currents as well as the rise and half-decay times (n=13). C. 10mM ethanol also increased the GABA-activated peak and mean currents but decreased the plateau currents, rise and half-decay times (n=11). See Table 4.2. for values.
Figure 4.21. The effect of GABA<sub>A</sub> receptor modulators on peak, mean and plateau current amplitudes activated by A. 100µM GABA or B. 7µM GABA. For comparison, GABA is included in the graph with all other drugs (including 100µM bicuculline and 100µM Zn<sup>2+</sup>) normalised and expressed as a percentage of the GABA response (dashed 100% line). Each bar shows the average effect for that modulator and the vertical bars show ±1SEM.
Figure 4.22. The effect of GABA_A receptor modulators on rise and half-decay times of currents activated by either A. 100µM GABA or B. 7µM GABA. For comparison, GABA is included in the graph with all other drugs (including 100µM bicuculline and 100µM Zn^{2+}) normalised and expressed as a percentage of the GABA response (dashed 100% line). Each bar shows the average effect for that modulator and the vertical bars show ±1SEM.
Table 4.2. Summary of the general pharmacology of GABA<sub>A</sub> receptors in the dentate gyrus. The values are expressed as a percentage of either 100µM GABA for the antagonists or 7µM GABA for the agonists.

<table>
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<tr>
<th>Drug</th>
<th>n</th>
<th>Peak</th>
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<th>Plateau</th>
<th>Rise Times</th>
<th>Half-Decay Times</th>
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<td>100</td>
<td>100</td>
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<tr>
<td>+ 100µM picrotoxin</td>
<td>9</td>
<td>60±4</td>
<td>18±2</td>
<td>45±5</td>
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<td>7</td>
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<td>6±1</td>
<td>20±4</td>
<td>552±312</td>
<td>5±1</td>
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<tr>
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<td>2±1</td>
<td>8±5</td>
<td>42±16</td>
<td>45±40</td>
</tr>
<tr>
<td>+ 100µM Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>10</td>
<td>81±4</td>
<td>66±4</td>
<td>51±9</td>
<td>135±14</td>
<td>76±6</td>
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<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<td>9</td>
<td>205±21</td>
<td>200±26</td>
<td>134±10</td>
<td>130±34</td>
<td>131±15</td>
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<tr>
<td>+ 5µM diazepam</td>
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In summary, peak and plateau currents as well as the time course of currents activated by GABA were modulated differently by these drugs. When the application of picrotoxin, penicillin or pentobarbital (with GABA) was terminated, a rebound current occurred. Comparison of the GABA-activated current with the rebound current for picrotoxin and penicillin, suggests different mechanisms for current depression. Diazepam slightly enhanced both peak and plateau currents activated by 7µM GABA. However, ethanol enhanced only the GABA-activated peak current but depressed the GABA-activated plateau current. These results may be attributed to the presence of certain subunits and will be discussed in Chapter 7.

4.3.5. Current-Voltage Relationship

Birnir et al. (1994) showed differing degrees of single-channel current outward rectification in the dentate gyrus. They speculated that these differences could be due to different GABA_A receptor populations. As described in Chapter 2, voltage ramps from -100mV to +100mV were generated by the computer within two seconds during the plateau phase of the whole-cell response. A voltage ramp applied before GABA application was subtracted from a voltage ramp applied in the presence of 100µM GABA in order to get the “true” GABA current-voltage relationship (Figure 2.3.). Out of nine neurons, eight exhibited inwardly rectifying currents (Figure 4.23.A.) while only one exhibited outwardly rectifying currents (Figure 4.23.B.). In all neurons, the rectifying current reversed at 0mV which was the calculated Cl^- reversal potential. The quality of these ramps were determined by comparing the currents generated by voltage ramps before and after GABA application and they showed no indication of deterioration. Therefore, it is unlikely that these rectifying currents are a result of unstable membrane potentials or deteriorating seals.

4.3.6. Indications Of At Least Two GABA_A Receptor Populations

All neurons generally had very similar GABA-activated current characteristics. However, as the numbers of neurons that were tested increased, it became evident that a wide range of half-decay times for currents activated by GABA could be obtained (Figure...
Figure 4.23. Mixed current rectification was seen in nine isolated granule cells. A. Eight neurons exhibited current rectification that was inward. B. A single neuron exhibited outwardly rectifying currents. These results could indicate the presence of two different receptor populations.
Furthermore, on very rare occasions (eight out of 261 responses that were activated by 100µM GABA) a hump was observed early in the decay of the current (Figure 4.24.). There also appeared to be a correlation between the size of the hump and the half-decay time of the GABA-activated current measured in the same neuron. Those GABA-activated currents that had a faster half-decay time, had a more pronounced hump while currents that had a slower half-decay time had a much smaller hump. It is possible that these faster and slower decay of the currents could represent two different GABA_A receptor populations having different activation and inactivation properties. If the numbers of these two receptor populations differ enormously in any one neuron, that could explain the magnitude of this hump. For example, if the numbers of receptors yielding faster activation and inactivation properties ("fast receptors") was larger than those receptors yielding slower activation and inactivation properties ("slow receptors"), this could produce the large hump seen in Figure 4.24.A. That is, as a greater number of "fast receptors" are inactivating together, the "slow receptors" would still be activating causing a pronounced hump. Conversely, if there is a larger population of "slow receptors" than "fast receptors", then the current hump in Figure 4.24.B. could occur. That is, the smaller population of inactivating "fast receptors" is obscured by the more prominent inactivation of the "slow receptors" and consequently, a much smaller hump.

In addition, the mixed current rectification seen here could be explained by the existence of two receptor populations (Figure 4.23.). So far, only those receptors containing the α_4 or α_6 subunits have been associated with inwardly rectifying currents (Granja et al., 1998). Therefore, it can be inferred that the one outwardly rectifying current shown here contained another isoform of the α subunit family. However, more neurons would have to be tested to confirm this as well as to get a more accurate representation of the relative numbers of these two receptor populations.

If these receptor populations could be separated by their different activation and inactivation rates, then perhaps they could also have different pharmacological profiles. As earlier results showed, increasing concentrations of bicuculline and Zn^{2+} had different effects on the half-decay times of currents activated by 100µM GABA. As the concentration of bicuculline increased, the half-decay time of the GABA-activated current slowed down (Figure 4.13.B.). In contrast, increasing concentrations of Zn^{2+} had
Figure 4.24. In 8 out of 261 responses generated by 100µM GABA, a current hump (arrow) was observed during the decay phase of the whole-cell current response. It was observed that: A. a faster half-decay time produced a larger current hump, and B. a slower half-decay time produced a smaller current hump. These results may suggest the presence of two unequal receptor populations with different kinetics.
the opposite effect of speeding up the half-decay time of the GABA-activated current (Figure 4.17.B.). This was examined more closely by fitting exponentials (see Equation 2.3.) to the decay of the currents.

Since 100µM bicuculline depressed nearly all of the current activated by 100µM GABA, a much lower concentration was used. Using 3µM bicuculline ensured that a significant proportion of the GABA-activated current remained which allowed easier exponential fitting. Illustrated in the Figure 4.25., one or two exponentials and steady-state current amplitudes (C) were fitted to the decay of currents generated in one neuron by 100µM GABA (Figure 4.25.A.) and 3µM bicuculline plus 100µM GABA (Figure 4.25.B.). Displayed on the left are the individual exponential fits and shown on the right are the sum of the exponentials superimposed over the decay of the current trace. A table of these exponentials fits is shown in Figure 4.25.C. The decay of the currents were scaled to the same peak amplitude and show the effect of the absent $A_1/\tau_1$ exponential on the time course of currents activated by GABA and bicuculline (Figure 4.25.D.). Average results for fits to decay of currents generated by 100µM GABA gave two exponentials with amplitudes and time constants of $180\pm27\text{pA} & 279\pm79\text{ms}$ ($A_1$ & $\tau_1$) and $318\pm95\text{pA} & 2610\pm459\text{ms}$ ($A_1$ & $\tau_2$). Then, in the presence of 3µM bicuculline, only one exponential of $283\pm65\text{pA} & 1944\pm509\text{ms}$ ($A_2$ & $\tau_2$) could be fitted to the decay of the current. The amplitude and time constant for $A_2$ and $\tau_2$ recorded for GABA only and in the presence of bicuculline were not significantly different ($n=5$; Table 4.3.). Therefore, it would appear that 3µM bicuculline eliminates the faster exponential ($A_1/\tau_1$) without significantly affecting the slower exponential ($A_2/\tau_2$). These results agree with the earlier results that showed that in addition to the depressed current amplitude, increasing concentrations of bicuculline slowed down the half-decay times of currents activated by 100µM GABA. This can now be attributed to the gradual disappearance of the $A_1/\tau_1$ exponential with increasing bicuculline concentration and the apparent insensitivity of the $A_2/\tau_2$ exponential to bicuculline.

Similarly Zn$^{2+}$ had differential effects on the two exponential components. This is illustrated in Figure 4.26. where exponentials were fitted to the decay of the currents generated in one neuron by 100µM GABA (Figure 4.26.A.) and 100µM Zn$^{2+}$ plus 100µM GABA (Figure 4.26.B.). Displayed on the left are the individual exponential fits
Figure 4.25. One or two exponentials were fitted to the decay currents generated by A. 100µM GABA and, B. 3µM bicuculline plus 100µM GABA (see Equation 2.3.). The exponentials are shown individually on the left with the sum of these exponentials superimposed over the actual decay current trace on the right. C. Table shows time constants, amplitudes and degree of fit ($r^2$). D. Decay currents were scaled to the same peak amplitude and show the absence of the fast exponential with 3µM bicuculline.
Figure 4.26. Two exponentials were fitted to the decay currents generated by A. 100μM GABA and, B. 100μM Zn\(^{2+}\) plus 100μM GABA (see Equation 2.3.). The exponentials are shown individually on the left with the sum of these exponentials superimposed over the actual decay current trace on the right. C. Table shows time constants, amplitudes and degree of fit (\(r^2\)). D. Decay currents were scaled to the same peak amplitude and show the effect of the reduced slow exponential with 100μM Zn\(^{2+}\).
Table 4.3. Time constants and associated amplitudes for A. 100µM GABA ± 3µM bicuculline and B. 100µM GABA ± 100µM Zn$^{2+}$.

A.

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<th>$\tau_2$ (ms)</th>
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<th>$\tau_1$ (ms)</th>
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and shown on the right are the sum of the exponentials superimposed over the decay of the current trace. A table of these exponentials are shown in Figure 4.26.C. The decay of the currents were scaled to the same peak amplitude and show the effect of the reduced $A_2/\tau_2$ exponential on the time course of currents activated by both GABA and Zn$^{2+}$ (Figure 4.26.D.). Average results for fits to decay of currents generated by 100µM GABA gave two exponentials with amplitudes and time constants of 233±49pA & 198±24ms ($A_1$ & $\tau_1$) and 456±134pA & 1465±117ms ($A_2$ & $\tau_2$). In the presence of 100µM Zn$^{2+}$, two exponentials of 285±94pA & 215±19ms ($A_1$ & $\tau_1$) and 260±69pA & 1039±142ms ($A_2$ & $\tau_2$) were fitted to the decay of current ($n=6$; Table 4.3.). The only significant difference between the exponential fits for GABA alone and in the presence of Zn$^{2+}$ was that the $A_3/\tau_2$ exponential was significantly smaller and faster for current decays in the presence of Zn$^{2+}$. Therefore, it would appear that 100µM Zn$^{2+}$ reduces both the amplitude and time constant of the slower exponential ($A_3/\tau_2$) without significantly affecting the faster exponential ($A_1/\tau_1$). These results agree with earlier results that showed that in addition to the depressed current amplitude, Zn$^{2+}$ speeded up the half-decay times of GABA-activated currents. This can now be attributed to the gradual reduction in the $A_2/\tau_2$ exponential with increasing Zn$^{2+}$ concentration and the apparent insensitivity of the $A_1/\tau_1$ exponential to Zn$^{2+}$.

If GABA-activated peak and plateau currents were dominated by two different exponential components, this could conceptually simplify and consolidate the results presented so far. That is, those receptors active during the generation of peak currents, are assigned the fast ($A_1/\tau_1$) exponential while those receptors active during the generation of plateau currents, are assigned the slow ($A_2/\tau_2$) time exponential. These receptors could also be defined as the “fast receptors” and “slow receptors” described earlier (that is, {peak currents = $A_1/\tau_1$ exponential = “fast receptors”} and {plateau currents = $A_2/\tau_2$ exponential = “slow receptors”}). If this is true, then earlier results which showed that GABA-activated peak and plateau currents had different sensitivities to both bicuculline and Zn$^{2+}$, would agree with these latest results. GABA-activated peak currents were about five times more sensitive to bicuculline than the GABA-activated plateau currents (Table 4.1.B.). Therefore, at a bicuculline concentration of 3µM (the concentration used when fitting exponentials to the decay of the currents), it would be expected that GABA-activated peak currents would be depressed to a greater extent
than the plateau currents. This correlates well with the exponential fits to the decay of the currents where the fast \((A_1/\tau_1)\) exponential (ie. peak currents) was abolished while the slow \((A_2/\tau_2)\) exponential (ie. plateau currents) was not significantly affected. Furthermore, 10µM bicuculline has been shown to abolish synaptic GABA-activated currents which could be mediated by the “fast receptors” here. Therefore, these “slow receptors” could be characterised by their apparent insensitivity to bicuculline.

Furthermore, the \(Zn^{2+}\) dose-response curves generated for peak and plateau currents activated by 100µM GABA could also suggest the possibility of two or three receptor populations. According to the degree of maximum current depression by 3mM \(Zn^{2+}\), it could be inferred that approximately half of the receptors generating the peak currents were sensitive to \(Zn^{2+}\) while the other half was insensitive (Figure 4.15.C.). For plateau currents, it would appear that approximately half of the receptors generating the plateau current have a higher affinity for \(Zn^{2+}\), while the other half has a lower affinity for \(Zn^{2+}\) (Figure 4.16.C.). Recalling earlier results, the \(Zn^{2+}\) IC\(_{50}\) for GABA-activated peak currents was 92µM while the two IC\(_{50}\) values for GABA-activated plateau currents were 2µM and 300µM (Table 4.1.C.). This could be simply attributed to the existence of three populations of receptors having different affinities \(Zn^{2+}\). Alternatively, there could be two receptor populations, with one having two affinity states; this could fit in with the two receptor populations assigned an exponential each. At the \(Zn^{2+}\) concentration of 100µM, it would be expected that at least half of the GABA-activated peak current would be abolished. In addition, approximately half of the GABA-activated plateau current was also abolished by 100µM \(Zn^{2+}\) with the other half untouched. Therefore, one receptor population could have intermediate and low \(Zn^{2+}\) affinity states (IC\(_{50}\)’s of 92µM and 300µM \(Zn^{2+}\)), while the other receptor population has a high \(Zn^{2+}\) affinity state (IC\(_{50}\) of 2µM \(Zn^{2+}\)). That is, “fast receptors” have two lower affinity states and are less sensitive to \(Zn^{2+}\) than the “slow receptors” that only have a single higher affinity state. If this is true, this would correlate well with exponential fits to the decay of the currents where the fast \((A_1/\tau_1)\) exponential (ie. peak currents or “fast receptors”) was not significantly affected while the slow \((A_2/\tau_2)\) exponential (ie. plateau currents or “slow receptors”) was totally abolished.
Exponentials were also fitted to the decay of the currents modulated by pentobarbital and diazepam (with 7µM GABA). The results for pentobarbital will be shown in the next chapter. 5µM diazepam had little effect on the peak, mean and plateau currents activated by 7µM GABA. This apparent insensitivity to diazepam was reflected in the exponential fits to the decay of the currents (results not shown). A single exponential was fitted to the GABA-activated current decay and produced an average amplitude of 327±77pA with a time constant of 2275±221ms. Similarly, a single exponential was fitted to the decay of the currents modulated by 5µM diazepam with an average amplitude of 426±83pA and a time constant of 2683±304ms; this exponential was not significantly different from the exponential fitted for GABA alone. Several conclusions could be drawn from this. It is possible that this concentration of diazepam (5µM) may be too high to enhance GABA-activated currents; Kapur & Macdonald (1996, 1999) reported nanomolar EC50 values for diazepam in dentate gyrus granule cells. Alternatively, these GABA-activated currents may be simply insensitive to diazepam. At this low concentration of GABA (7µM), the majority of receptors activated would be those generating the plateau currents. It is possible that “slow receptors” are resistant to the modulatory effects of diazepam.

In summary, the current hump observed in a few GABA-activated responses as well as the existence of mixed current rectification characteristics, suggest the presence of at least two GABA_A receptor populations. This was further supported by the effect of both bicuculline and Zn^{2+} on the exponential fits to the decay of the currents generated by 100µM GABA. These differing effects on the fast and slow time constants could be correlated with the sensitivity of the GABA-activated peak and plateau currents to these two drugs.

4.4. DISCUSSION

My aim in this chapter was to characterise and better understand the GABA_A receptor pharmacology of dentate gyrus neurons. Several aspects of GABA-activated whole-cell currents were measured and this proved to be a useful protocol for quantifying and
comparing responses. 100µM GABA elicited a wide range of current amplitudes with peak, mean and plateau currents exhibiting different sensitivities to GABA, bicuculline and Zn\(^{2+}\). As was predicted, the mean currents activated by GABA had values that fell between the GABA-activated peak and plateau current values. GABA-activated mean currents had values that were more similar to the GABA-activated peak current values; this was most likely due to the relatively short one-second time constraint imposed on mean current calculations. As drug applications varied for each experiment, this one second duration was common to all responses as drug application times were never shorter than one second. For a more accurate representation of mean currents, they would have to be calculated over a much longer period of time. While the other drugs (picrotoxin, penicillin, diazepam and ethanol) were not examined as thoroughly as bicuculline and Zn\(^{2+}\), they were shown to affect the GABA-activated peak, mean and plateau currents differently. In addition, while in the most part the current time course generated by 100µM GABA was fairly consistent, extremes in the half-decay time could be measured. It was also shown that bicuculline and Zn\(^{2+}\) had opposite effects on the GABA-activated time course.

The characteristics of currents activated by GABA reported here are similar to those observed by Kapur & Macdonald (1996, 1999) in adult rats. Despite the obvious difference in the age of the rats, there were also a few other notable distinctions. For currents activated by 10µM GABA, younger rats had maximal peak current amplitudes of 476±65pA which was almost half the size of that found in adult rats (893±65pA). In this study, 100µM GABA produced an average maximum current of 1009±34pA and it was not unusual for peak currents to be larger than 2nA. It is likely that this saturating concentration of GABA ensured that a large proportion, if not all, receptors were activated. Furthermore, it is possible that 100µM GABA also allowed the range of current amplitudes to be observed. Kapur & Macdonald (1996, 1999) obtained an EC\(_{50}\) of about 40µM GABA for peak currents for both young and adult rats. Here, an EC\(_{50}\) of 14µM GABA for peak currents was measured. As the age of the rats cannot account for these differences, a possible explanation could come from the different rates of drug application. They reported an average 10-90% current rise time of about 54±5ms while the average 10-90% current rise time obtained here was 25±1ms but times of as short as 4ms were achievable (n=261). It is likely that a faster application of GABA and other
drugs allowed synchronous activation of receptors that would produce larger peak current amplitudes as well as the higher apparent affinity for GABA.

Nevertheless, this still does not explain the range of GABA-activated peak current amplitudes observed in this study. A possible explanation comes from Liu et al. (1996) who studied evoked postsynaptic currents in the granule cells of hippocampal slices. They reported a wide range of membrane properties at any postnatal age from 10 to 28 days which suggested that there were different degrees of physiological maturity. The maturity of the granule cells was assessed and divided according to their input resistances. Compared to mature granule cells, immature neurons had higher input resistances, lower resting membrane potentials, action potentials that had a lower amplitude and slower time course. That is, on any given day between 10 to 28 days old, a gamut of membrane properties was observed. It is possible that the range of amplitudes observed here could be due to the physiological age of the individual granule cell rather than the actual age of the rat. However, Hollrigel & Soltesz (1997) examined GABA receptor-mediated postsynaptic currents in the dentate gyrus and reported that while the rise and decay time constants were significantly longer in immature (postnatal day 0-14) granule cells than in adult neurons (>postnatal day 21), the amplitude of the current did not change during development.

The half-decay times obtained for response activated by 100µM GABA was fairly consistent. Occasionally extremes in the half-decay time were seen that ranged from as short as a few hundred milliseconds to as long as a few seconds. This was sometimes seen as a stray point as in Figure 4.9.B. with 100µM GABA. Generally, currents activated by GABA that had faster half-decay times also had smaller steady-state current amplitudes. The opposite was true for GABA-activated currents that had slower half-decay times. This characteristic trait was explored further but as the majority of GABA-activated currents were fairly uniform, to detect any changes in the decay of the currents generated by GABA and/or drug, a more sensitive method was needed. Thus, the sum of one or two exponentials with a steady-state current amplitude (see Equation 2.3.) were fitted to the decay of the currents generated by GABA and the effects of drugs on these exponentials were examined. In a few studies, two exponentials have been fitted to the decay phase of whole-cell currents. The time constants obtained from adult
hippocampal CA1 pyramidal neurons were 650±240ms and 3750±2000ms (Martina et al., 1996), much longer than the average time constants reported here of 200±8ms and 1938±94ms. In neonatal anterior pituitary neurons, the time constants found were 1400±500ms and 5300±1400ms (Zemkova et al., 1995), again much longer than the time constants measured here. Both claim solution exchanges around the cell in less than 30ms which is slightly longer than the average rise time of 25±1ms measured here. The obvious differences in neuronal type and age of the rat, and therefore the subunit composition of these GABAA receptors, could account for these much longer time constants. In recombinant studies, Gingrich et al. (1995) found that substituting the α3 subunit for the α1 subunit slowed down rate of desensitisation by half. Therefore, it is possible that the presence of certain subunits or subunit isoforms can explain the faster time constants measured here.

The pharmacological profile of these granule cells was similar to that reported for GABAA receptors in the hippocampus (Kemp et al., 1987; Zhang et al., 1993; Aguayo et al., 1994; Kapur & Macdonald, 1996, 1997, 1999; Tietz et al., 1999) and in other regions of the central nervous system (Akaike et al., 1987a; Yakushiji et al., 1987; Inoue & Akaike, 1988; Nakagawa et al., 1991; Katayama et al., 1992; Newland & Cull-Candy, 1992; Zemkova et al., 1995). Bicuculline, Zn2+, picrotoxin and penicillin all depressed currents activated by 100µM GABA. Bicuculline has been reported to slow down desensitisation (Akaike et al., 1987a; Frosch et al., 1992) which was measured here as longer half-decay times of the currents generated by bicuculline (with GABA). Exponentials fitted to the decay of the currents generated by 100µM GABA and depressed by 3µM bicuculline showed the relative insensitivity of the slow time constant to bicuculline while the fast time constant was completely abolished. In contrast, increasing Zn2+ concentration speeded up the half-decay times of currents activated by GABA. This was also seen by Berger et al. (1998) but others have observed Zn2+ to not affect the rate of current desensitisation (Akaike et al., 1987a; Legendre & Westbrook, 1991). It is possible that these opposing views can be explained by the different types of neurons used and hence different subunit composition. This decrease in the half-decay time in the presence of Zn2+ was also exhibited as a reduction in the slow exponential (A2/τ2) while the fast exponential (A1/τ1) was unchanged. In adult rats, Kapur & Macdonald (1999) calculated a Zn2+ IC50 of 103±19µM for peak currents activated by
30µM GABA. This was not significantly different from the Zn\textsuperscript{2+} IC\textsubscript{50} of 92µM for peak currents activated by 100µM GABA measured here. All three Zn\textsuperscript{2+} IC\textsubscript{50} values obtained for the peak and plateau currents activated by GABA, fall within the reported range of Zn\textsuperscript{2+} IC\textsubscript{50} values (Nakagawa et al., 1991; Hales et al., 1992; Smart et al., 1994). In addition, it has been commonly observed that millimolar concentrations of Zn\textsuperscript{2+} completely abolished the plateau currents activated by GABA (Legendre & Westbrook, 1991; Celentano \textit{et al}, 1991; Kapur & Macdonald, 1996, 1999; Tietz et al., 1999). Zn\textsuperscript{2+} sensitivity has been associated with the absence of the γ subunit (Draguhn \textit{et al}., 1990; Smart \textit{et al}., 1991; Celentano \textit{et al}., 1991; Schönrock & Bormann, 1993; Chang \textit{et al}., 1995). Thus, if two receptor populations exist in these neurons, then the γ subunit would be present in at least half of the receptors generating plateau currents. This would also apply for the receptors generating peak currents. This will be further explored in Chapter 7.

Terminating the application of either picrotoxin or penicillin (with GABA) produced a rebound current. Such rebound currents have been reported for penicillin but not for picrotoxin in native tissues. Although not discussed, Yakushiji \textit{et al}. (1987) showed in their current traces obtained from frog dorsal root ganglion neurons, a rebound current for picrotoxin at depolarised potentials. It is generally believed that picrotoxin is a non-competitive inhibitor (Takeuchi & Takeuchi, 1969; Simmonds, 1980; Akaike \textit{et al}., 1985a, 1987a; Yakushiji \textit{et al}., 1987). Inoue & Akaike (1988) proposed from their whole-cell studies in frog sensory neurons that picrotoxin binds to a site inside the Cl\textsuperscript{−} channel either through open or closed channels. That is, picrotoxin could act as an open channel blocker. This was later supported by a cysteine mutagenesis study by Akabas & Xing (1995) who also suggested that picrotoxin binds to a site within the channel lumen. However, there is increasing evidence that points to a much more complicated mechanism of block. Using dissociated rat sympathetic neurons, Newland & Cull-Candy (1992) examined the effect of picrotoxin in outside-out patches activated by GABA. While their results support the earlier conclusion that picrotoxin is not a competitive antagonist, they also show that picrotoxin is unlikely to be an open channel blocker. Instead, they propose a complex channel blocking mechanism where picrotoxin stabilises a closed form of the receptor channel and/or causes allosteric block of the channel. Yoon \textit{et al}. (1993) examined the effect of picrotoxin on GABA-activated currents in cultured...
hippocampal neurons and observed that picrotoxin block was use-dependent, suggesting that the site of picrotoxin block is exposed by the conformational change initiated by GABA binding to the receptor. They also concluded that picrotoxin was not an open channel blocker. This study supports the notion that picrotoxin may not be an open channel blocker. If it was, it might be expected that there would be a relationship between the amplitudes of the GABA-activated peak current and the rebound peak current. Despite the amplitude of the GABA-activated peak current, all rebound currents were of a similarly reduced amplitude. In contrast, penicillin has been well established to be an open channel blocker (Curtis et al., 1972; Hochner et al., 1976; Pickles & Simmonds, 1980; Chow & Mathers, 1986; Akaike et al., 1987a; Yakushiji et al., 1987; Katayama et al., 1992; Twyman et al., 1992). Yakushiji et al. (1987) observed in frog dorsal root ganglion neurons a rebound current for penicillin that occurred more consistently at higher concentrations of penicillin (≥1mM). Katayama et al. (1992) also observed triphasic modulation by penicillin of GABA-activated currents in frog sensory neurons. This triphasic modulation consisted of a phasic (peak current) and tonic (plateau current) blockade followed by the rebound current. They observed a correlation between the degree of phasic blockade and the rebound current which increased when penicillin concentration was increased. They also suggested that the generation of these rebound currents was based on the removal of GABA receptor-desensitisation. Taken together, this could explain the relationship between the larger rebound current amplitudes and the larger GABA-activated peak currents. That is, in neurons where a large GABA-activated peak current was induced, it is likely that a greater number of receptors could go into a desensitised state. When desensitisation was removed, a large rebound current would occur. It was speculated earlier that perhaps desensitised receptors could be blocked by penicillin. A weak correlation was made here where larger rebound currents occurred with a longer application of penicillin (with GABA) but this would have to be confirmed by varying the application times of penicillin (with GABA). This idea supports the earlier proposition by Katayama et al. (1992) who postulated that penicillin may create a new state of blockade in desensitised receptors.

As pentobarbital has many effects on the GABA-activated currents, these effects will be further explored and discussed in the next chapter. In this study, diazepam appeared to have little effect on either the GABA-activated peak or plateau currents and this could be
due to several possibilities. It has been shown that micromolar concentrations of diazepam can inhibit GABA-activated responses (Itabashi et al., 1992; Eghbali et al., 1997) but there are other studies that have indicated otherwise (Zemková et al., 1995).

However, Kapur & Macdonald (1999) observed an EC\textsubscript{50} of nanomolar concentrations in the dentate gyrus so it could be possible that at the micromolar diazepam concentration tested here, the enhancing effects of diazepam on GABA-activated currents were non-existent. Alternatively, the apparent lack of enhancement of GABA-activated currents by diazepam could be due to the subunit composition of these receptors. In situ hybridisation has detected a strong presence of the α\textsubscript{4} subunit in the dentate gyrus (Laurie et al., 1992a; Wisden et al., 1992) and those receptors that contain the α\textsubscript{4} or α\textsubscript{6} subunits are known to be diazepam-insensitive (Huh et al., 1996; Knoflach et al., 1996; Benke et al., 1997). The final modulator investigated here was ethanol. Enhancement of GABA-activated currents by ethanol has been proposed to be related to the presence of the long splice variant of the γ\textsubscript{2} subunit (Wafford et al., 1991). As there was some enhancement of the GABA-activated current with diazepam, these ethanol results would support the presence of the γ subunit in at least some receptors despite the apparent inhibition of GABA-activated currents with Zn\textsuperscript{2+}. This conflict will be discussed further in Chapter 7.

Current rectification has always been a controversial topic with many reports that claim rectification does not occur in either whole-cell or single-channel preparations (Biscoe & Duchen, 1985; Huguenard & Algar, 1986; Allen & Albuquerque, 1987; Bormann, 1988; Bormann et al., 1988; Macdonald et al., 1989a). There are reports of rectification in whole-cell configurations but not in single-channels in patches and it has been postulated that the whole-cell current rectification was actually a result of a voltage-dependence of channel open probability (Segal & Barker, 1984a; Bormann et al., 1987; Weiss et al., 1988; Robertson, 1989). Nevertheless, there are many reports of outward (Gray & Johnston, 1985; Parker et al., 1986; Itabashi et al., 1992; Newland & Cull-Candy, 1992; Fatima-Shad & Barry, 1992, 1993; Curmi et al., 1993; Birnir et al., 1994; Gage & Chung, 1994; Kaneda et al., 1995; Eghbali et al., 1997; Liu et al., 1998) and inward current rectification (Thomson, 1988; Staley, 1994; Fraser et al., 1995) in many different native tissues in both whole cells and patches. It is possible that these conflicting results could be due to a number of variables such as temperature (Huguenard & Algar, 1986;
Kettenmann et al., 1987) and the maturity of the GABA<sub>A</sub> receptor (Allen & Albuquerque, 1987; Gray & Johnston, 1985; Liu et al., 1998) affecting the nature and degree of rectification. The presence of certain subunits have also been shown to have a large influence on the magnitude (Levitan et al., 1988; Blair et al., 1988; Verdoorn et al., 1990) as well as the direction (Granja et al., 1998) of the current rectification. Thus, the mixture of both inward and outward current rectification observed here could be attributed to the subunit composition of these GABA<sub>A</sub> receptors. These results could also support the existence of multiple receptor types and will be discussed further in Chapter 7.

In conclusion, the major findings of this chapter are that peak and plateau currents activated by GABA exhibit different pharmacological profiles. Compared to GABA-activated plateau currents, GABA-activated peak currents were less sensitive to GABA and Zn<sup>2+</sup> but more sensitive to bicuculline. The Zn<sup>2+</sup> IC<sub>50</sub> for GABA-activated peak currents fell between the two Zn<sup>2+</sup> IC<sub>50</sub> values calculated for the GABA-activated plateau current. Increasing concentrations of GABA and Zn<sup>2+</sup> speeded up but bicuculline slowed down the half-decay time generated for GABA-activated currents. This was reflected in the exponential fits. There were also indications of the presence of at least two different receptor populations and together with the effect of Zn<sup>2+</sup>, diazepam, ethanol and current rectification characteristics, the subunit composition of these receptors can be proposed. This will be revealed in Chapter 7.
CHAPTER 5
PENTOBARBITAL

Of the drugs that act on GABA, pentobarbital investigated in the present study more closely mimicked pentobarbital produced a pronounced potentiation. This finding and the other characteristic effects of pentobarbital were identical in both the presence and absence of the dentate gyrus.

8.2. METHODS & MATERIALS

The method of activity discriminating dentate gyrus output was described in Chapter 8 and discussed further in Chapter 1. Whole-cell recordings, electrophysiological and statistical analyses were also described in Chapter 2.
5.1. INTRODUCTION

Pentobarbital has been shown to affect GABA-activated currents in several ways and, as described in Chapter 1, there appear to be three major effects. GABA-activated currents are potentiated by pentobarbital concentrations below 500µM while at millimolar concentrations, these currents are blocked. Also at millimolar concentrations, pentobarbital alone can directly activate a current that is sensitive to bicuculline, picrotoxin and diazepam (Barker & Ransom, 1978; Nicoll & Wojtowicz, 1980; Mathers & Barker, 1980; Akaike et al. 1985b; Schwartz et al. 1986; Parker et al., 1986; Akaike et al., 1987b; Peters et al., 1988, Robertson, 1989; Yakushiji et al., 1989; Nakagawa et al., 1991; Itabashi et al., 1992; Macdonald & Olsen, 1994; Rho et al., 1996). Furthermore, upon the removal of millimolar pentobarbital alone or in combination with GABA, a transient rebound current was produced that could be inhibited by bicuculline in a dose-dependent manner (Akaike et al., 1987b; Peters et al., 1988; Robertson, 1989; Yakushiji et al., 1989; Nakagawa et al., 1991; Itabashi et al., 1992; Rho et al., 1996).

Of the drugs that act on GABAA receptors investigated in the previous chapter, only pentobarbital produced a pronounced potentiation. This finding and the other characteristic effects of pentobarbital were examined further in the granule cells of the dentate gyrus.

5.2. METHODS & MATERIALS

The method of acutely dissociating dentate gyrus neurons was described in Chapter 2 and discussed further in Chapter 3. Whole-cell solutions, electrophysiology and data analysis were also described in Chapter 2.
5.3. RESULTS

5.3.1. Pentobarbital Modulation Of GABA-Activated Currents

The enhancing effects of pentobarbital have commonly been demonstrated with low concentrations of GABA. Here, the magnitude of this enhancement was examined and compared with both low (7µM) and high (100µM) GABA concentrations. These concentrations were specifically selected as 7µM GABA was the lowest GABA concentration that gave reproducible responses and 100µM GABA was chosen as currents were maximally activated by this concentration.

5.3.1.1. Effects Of Increasing Pentobarbital Concentration On 7µM GABA-Activated Currents

In the presence of 7µM GABA, pentobarbital concentrations ranging between 0.1µM and 10mM were applied together. Each concentration of pentobarbital was applied to 3-9 neurons. Figure 5.1. shows paired responses to 7µM GABA alone (left column) and then in the presence of 10µM, 100µM and 10mM pentobarbital (right column). 10µM pentobarbital depressed the current generated by 7µM GABA whereas 100µM pentobarbital enhanced the current. However, 10mM pentobarbital blocked the peak current and upon the termination of drug application, a large rebound current occurred. As it was not possible to apply the full range of pentobarbital concentrations onto a single neuron, typical responses were selected and compiled to show the range of pentobarbital effects on GABA-activated currents (Figure 5.2.). For each of the current responses, the 7µM GABA control response was normalised to the 7µM GABA response shown in the figure. The factor by which each of these control responses were normalised to, was also applied to the corresponding pentobarbital-modulated response. Pentobarbital concentrations between 0.1µM and 10µM depressed the GABA-activated current with 10µM pentobarbital giving a peak current that was 0.6±0.04 times the control peak current. Concentrations of 50µM, 100µM and 1mM pentobarbital gave GABA-activated peak currents that were 1.7±0.1, 2.1±0.2 and 2±0.2 times their control peak currents, respectively. However, 10mM pentobarbital gave a GABA-activated peak current that was 0.1±0.03 times the control. Removal of millimolar pentobarbital (with 7µM GABA) produced a rebound current response that appeared to be concentration-
Figure 5.1. Increasing pentobarbital concentrations had four effects on 7µM GABA-activated currents. A. 10µM pentobarbital depressed the GABA-activated current. B. 100µM pentobarbital enhanced the GABA-activated current. C. 10mM pentobarbital blocked the GABA-activated peak current and when the application of drug was terminated, a large rebound current occurred.
Figure 5.2. Comparison of the effects of increasing pentobarbital concentration on currents activated by 7µM GABA. As the full range of pentobarbital concentrations could not be tested on a single neuron, these responses were taken from eight neurons and their respective control peak amplitudes were normalised to the peak amplitude of the 7µM GABA response shown above (see text for explanation). Concentrations below 10µM pentobarbital depressed GABA-activated currents while 50µM, 100µM and 1mM pentobarbital enhanced the GABA-activated current. 10mM pentobarbital markedly depressed the GABA-activated peak current. Removal of pentobarbital concentrations above 100µM (with GABA), a rebound current was elicited that appeared to be pentobarbital concentration-dependent.
dependent (see 1mM and 10mM pentobarbital in Figure 5.2.). These rebound currents will be further examined below.

Apart from 10mM pentobarbital, a range of peak current amplitudes were produced by increasing concentration of pentobarbital on currents activated by 7µM GABA (Figure 5.3.A.). This scatter was reduced when these values were normalised (I') to 7µM GABA and expressed as a percentage (Figure 5.3.B.). Clearly, GABA-activated peak current amplitude was depressed or enhanced by low or high concentrations of pentobarbital. These values were then averaged and the data points joined as it was impossible to fit a Hill type equation (see Equation 2.1.). The shape of the dose-response curves for peak and plateau currents were similar. However, plateau currents appear to be more sensitive to the depressant effects of pentobarbital at concentrations between 0.1µM and 10µM but less sensitive to the enhancing effects of 50µM and 100µM pentobarbital (Figure 5.3.C.). Of the low pentobarbital concentrations that depressed the GABA-activated current, maximum current depression occurred with 10µM pentobarbital with peak currents that were 0.6±0.04 of the control and plateau currents that were 0.3±0.0.05 of the control (n=3). Of the three pentobarbital concentrations that enhanced the GABA-activated currents, 100µM pentobarbital enhanced GABA-activated peak currents that were 2.1±0.2 times the control and the plateau currents that were 1.3±0.1 times the control plateau current (n=9). 1mM pentobarbital enhanced GABA-activated peak currents that were 2±0.2 times the control, much the same enhancement as obtained with 100µM pentobarbital. Maximum enhancement of plateau currents activated by GABA occurred with 1mM pentobarbital that was 1.8±0.2 times the control (n=3); this was not significantly lower than the value obtained for the GABA-activated peak current with this pentobarbital concentration. 10mM pentobarbital, the highest concentration tested, produced GABA-activated peak and plateau currents that were 0.1±0.03 and 0.2±0.04 of the control (n=8).

5.3.1.2. Effects Of Increasing Pentobarbital Concentration On 100µM GABA-Activated Currents

Considering the unique effects of pentobarbital with low GABA concentrations, these effects were examined on currents activated by saturating concentration of GABA
Figure 5.3. Modulating effects of pentobarbital on peak currents generated by 7µM GABA. A. Amplitudes of the peak currents are plotted against pentobarbital concentrations and show variation in current amplitudes. B. Expressed as a percentage, peak currents were normalised (I') to the 7µM GABA peak current response (dashed line at 100%). C. The dose-response curves for peak (▲) and plateau (●) currents show different sensitivities for pentobarbital. The dashed line denotes the 100% response to the 7µM GABA.
(100µM). Three pentobarbital concentrations were selected: 10µM which produced the largest depression, 100µM which produced a large enhancement and 10mM which consistently blocked the GABA-activated current. Neither depression nor enhancement of 100µM GABA-activated peak or plateau currents were observed with 10µM pentobarbital (n=5). 100µM pentobarbital did not significantly enhance the 100µM GABA-activated peak currents that were 1.1±0.02 times the control peak current amplitude but the plateau current was 0.8±0.1 of the control plateau current amplitude (n=3). However, 10mM pentobarbital depressed the peak and plateau GABA-activated currents which were 0.1±0.02 and 0.3±0.3 times their respective controls, much the same depression as obtained with 7µM GABA (n=3; Figures 5.4. & 5.5.A.).

These results together with the GABA dose-response curves (Figure 4.6.C. - 4.8.C.), indicate that 100µM GABA maximally activates the GABA<sub>A</sub> receptor and that no further current enhancement could be obtained with 100µM pentobarbital. Why 10µM pentobarbital could depress 7µM GABA-activated currents but not 100µM GABA-activated currents cannot be explained and requires further investigation. Peak and plateau currents activated by 7µM or 100µM GABA were blocked by 10mM pentobarbital to a similar degree which would suggest that the block was not dependent on the GABA concentration.

5.3.1.3. Direct Activation By Pentobarbital

Direct activation of GABA<sub>A</sub> receptors has been reported in many tissues which could also be inhibited by bicuculline and picrotoxin and potentiated with diazepam (Barker & Ransom, 1978; Nicoll & Wojtowicz, 1980; Mathers & Barker, 1980; Akaike et al. 1985b; Schwartz et al. 1986; Parker et al., 1986; Akaike et al., 1987b; Peters et al., 1988, Robertson, 1989; Yakushiji et al., 1989; Itabashi et al., 1992; Macdonald & Olsen, 1994; Rho et al., 1996). In order to determine whether pentobarbital alone could evoke a current, 100µM pentobarbital was directly applied to neurons. This concentration of pentobarbital was chosen as it maximally enhanced currents activated by 7µM GABA. In 12 neurons where GABA-activated currents could be produced, 100µM pentobarbital alone did not elicit any detectable response (Figure 5.5.B.). That is, at this concentration
Figure 5.4. The effect of pentobarbital on 100µM GABA-activated currents. Both 10µM and 100µM pentobarbital appear to have no effect on the currents generated by 100µM GABA. However, 10mM pentobarbital depressed the GABA-activated peak current and when both drugs were removed, a rebound current was elicited.
Figure 5.5. A. Comparison of the effects of 10µM, 100µM and 10mM pentobarbital concentrations on peak, mean and plateau currents activated by 7µM or 100µM GABA. Pentobarbital alone did not directly activate GABA_A receptors (n=12).
of pentobarbital, direct activation of GABA$_A$ receptors does not occur and therefore probably does not contribute to the enhancement of currents activated by 7µM GABA.

5.3.1.4. Effects Of Increasing Pentobarbital Concentration On Current Time Course

It has been reported that pentobarbital increases the rate of current decay (Parker et al., 1986) and this was examined. The time for the current to rise from 10 to 90% of the peak amplitude and the time for the current to decay to 50% of the peak amplitude were measured. As pentobarbital alone did not elicit any response, any changes in the rate of current rise and half-decay could only be the result pentobarbital affecting the GABA-activated current. Increasing concentrations of pentobarbital did not have any significant effect on the rise or half-decay times of currents activated by 7µM GABA (Figure 5.6.A.). 10mM pentobarbital which blocked GABA-activated currents, had the fastest half-decay time with an average of 100±76ms (n=8). Similarly, no significant differences were found between 10µM and 100µM pentobarbital on the rise and half-decay times of currents activated by 100µM GABA (Figure 5.6.B.). 10mM pentobarbital again produced the fastest half-decay time with an average of 10±2ms (n=3).

The effects of 10µM, 100µM and 10mM pentobarbital on the time course of currents activated by either 7µM or 100µM GABA were compared. In the presence of these pentobarbital concentrations, the rise times of currents activated by 7µM GABA were approximately ten times slower than the currents activated by 100µM GABA. In addition, the effect of these pentobarbital concentrations on the half-decay times of currents activated by 7µM GABA were about three times slower than the currents activated by 100µM GABA. This together with the results above, the differences in the time course could only be attributed to the different concentrations of GABA. This is supported by earlier results which showed that currents activated by 7µM GABA had rise times that were three to ten times slower and half-decay times that were two to five times slower than the currents activated by 100µM GABA (Figure 4.9.). Therefore, pentobarbital does not appear to have any significant effect on the rise and half-decay times of currents generated by either 7µM or 100µM GABA.
A. 7µM GABA

Figure 5.6. The rise and half-decay times of increasing pentobarbital concentrations on currents activated by either 7µM or 100µM GABA. Each solid symbol represents a single experiment (3-21 experiments for each pentobarbital concentration) with the average±1SEM superimposed. A. In the presence of 7µM GABA, increasing concentrations of pentobarbital had little effect on the overall rise and half-decay times. B. Similarly, apart from 10mM pentobarbital which markedly depressed the GABA-activated current, the rise and half-decay times were not significantly different.
5.3.1.5. Decay Time Constants & Amplitudes

Measuring half-decay times can obscure changes in kinetics. Therefore, the decay of the currents were fitted with the sum of one or two exponentials (see Equation 2.3.). Illustrated in Figure 5.7., one exponential and a steady-state current amplitude (C) was fitted to the decay of the currents produced by 7µM GABA (Figure 5.7.A.) and 100µM pentobarbital plus 7µM GABA (Figure 5.7.B.). Displayed on the left are the individual components of the fit and shown on the right are the sum of the components superimposed over the decay of the current trace. The table shows the time constants and associated amplitudes (Figure 5.7.C.). The decay of the currents were scaled to the same peak amplitude and show similar decays (Figure 5.7.D.). The differences towards the end of the decay of the currents were probably due to the relatively large plateau current of the 7µM GABA-activated response. Average results for fits to decay of currents generated by 7µM GABA gave an amplitude and time constant of 270±64pA and 2844±462ms. Then in the presence of 100µM pentobarbital, a single exponential component was fitted to the decay of the current giving an amplitude and time constant of 563±163pA and 2964±890ms. The amplitude was doubled in the presence of pentobarbital but the time constants were not significantly different (n=4; Table 5.1.A.). These results agree with earlier findings that showed that the half-decay times were not significantly affected by pentobarbital. Therefore, it appears that pentobarbital enhances the amplitude of the GABA-activated current without affecting its time course.

Likewise, illustrated in Figure 5.8., two exponentials were fitted to the decay of the currents generated by 100µM GABA (Figure 5.8.A.) and 100µM pentobarbital plus 100µM GABA (Figure 5.8.B.). Displayed on the left are the individual exponentials and steady-state currents and shown on the right are the sum of these exponentials components superimposed over the decay of the current trace. The table shows the time constants and associated amplitudes (Figure 5.8.C.). The decay of the currents were scaled to the same peak amplitude and they show nearly identical time courses. Average results for fits to decay of currents generated by 100µM GABA gave amplitudes and time constants of 440±119pA & 213±51ms (A₁ & τ₁) and 576±119pA & 1357±271ms (A₂ & τ₂). Then in the presence of 100µM pentobarbital, two exponential components were fitted to the decay of the currents giving amplitudes and time constants of 488±112pA & 253±14ms (A₁ & τ₁) and 636±163pA & 1387±277ms (A₂ & τ₂). The
Figure 5.7. A single exponential and a steady-state amplitude current (C), was fitted to the decay currents generated by A. 7µM GABA and, B. 7µM GABA plus 100µM pentobarbital (see Equation 2.3.). The two components are shown individually on the left with the sum of these components superimposed over the actual decay current trace on the right. C. Table shows time constants, amplitudes and degree of fit ($r^2$). D. The decay currents were scaled to the same peak amplitude and show small differences.
Figure 5.8. Two exponentials and a steady-state current amplitude (C), was fitted to the decay currents generated by A. 100µM GABA and, B. 100µM GABA plus 100µM pentobarbital (see Equation 2.3.). These three components are shown individually on the left with the sum of these components superimposed over the actual decay current trace on the right. C. Table shows time constants, amplitudes and degree of fit ($r^2$). D. The decay currents were scaled to the same peak amplitude and do not show any differences.
Table 5.1. Time constants, associated amplitudes and degree of fit for currents activated by: **A.** 7μM GABA ± 100μM pentobarbital, **B.** 100μM GABA ± 100μM pentobarbital and, **C.** 100μM pentobarbital + 7μM GABA or 1mM GABA.

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### B. 100μM GABA + 100μM Pentobarbital

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### C. 100μM Pentobarbital

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amplitudes and time constants were not significantly different in the presence of 100µM pentobarbital compared to 100µM GABA alone (n=3; Table 5.1.B.). As previously shown by the current traces, 100µM pentobarbital caused little enhancement of currents activated by 100µM GABA.

For comparison, the decay of the currents produced by 7µM and 1mM GABA in the presence of 100µM pentobarbital were examined (details of these experiments are described later). As the currents produced by 1mM GABA were not significantly different from the currents produced by 100µM GABA, the comparison between these experiments and those above were considered acceptable. Illustrated in Figure 5.9., one or two exponentials and a steady-state current was fitted to the decay of the currents generated by 7µM GABA plus 100µM pentobarbital (Figure 5.9.A.) and 1mM GABA plus 100µM pentobarbital (Figure 5.9.B.). Displayed on the left are the individual components fitted to the decay of the currents and shown on the right are the sum of these components superimposed over the decay of the currents. The table shows the time constants and associated amplitudes (Figure 5.9.C.). The decay of the currents were scaled to the same peak amplitude and they show the influence of the additional exponential with 1mM GABA. Average results for fits to the decay of the currents generated by 7µM GABA plus 100µM pentobarbital gave an amplitude and time constant of 526±116pA & 2505±172ms (A₂ & τ₂). When the concentration of GABA was increased to 1mM, two exponential components were fitted to the decay of the currents giving average amplitudes and time constants of 226±140pA & 137±68ms (A₁ & τ₁) and 492±57pA & 2502±700ms (A₂ & τ₂). Whether one or two exponentials were fitted to the decay of the currents, the slower time constant and steady-state current amplitude were very similar (n=3; Table 5.1.C.).

In summary, increasing pentobarbital concentrations appear to have three different effects on currents activated by 7µM GABA. Between 0.1µM and 10µM pentobarbital, the GABA-activated current was depressed with 10µM pentobarbital causing the greatest depression. Concentrations between 50µM and 1mM pentobarbital enhanced the GABA-activated current with 100µM pentobarbital causing the largest enhancement. However, 10mM pentobarbital abolished almost all of the GABA-activated current. Generally, there were no differences in the effects of pentobarbital on GABA-activated
Figure 5.9. One or two exponentials plus a steady-state current amplitude (C) was fitted to the decay currents generated by A. 7µM GABA plus 100µM pentobarbital and, B. 1mM GABA plus 100µM pentobarbital (see Equation 2.3.). These components are shown individually on the left with the sum of these components superimposed over the actual decay current trace on the right. C. Table shows time constants, amplitudes and degree of fit ($r^2$). D. The decay currents were scaled to the same peak amplitude and show the influence of the fast time constant with 1mM GABA.
peak and plateau currents but plateau currents did appear to be more sensitive to the depressant and less sensitive to the enhancing effects of pentobarbital than the peak currents. In contrast to the currents activated by 7µM GABA, the depressant effect of 10µM pentobarbital and the enhancing effect of 100µM pentobarbital on currents activated by 100µM GABA were no longer observed. Although, a similar degree of current block by 10mM pentobarbital was found in currents activated by both 7µM and 100µM GABA. 100µM pentobarbital alone did not directly elicit a response so any effect on the time course of the GABA-activated current could only be due to the modulatory effects of pentobarbital on the GABA_A receptor. It was found that pentobarbital had no effect on the time course of currents activated by either 7µM or 100µM GABA. Thus, pentobarbital appears to only affect the amplitude without affecting the time course of GABA-activated currents.

5.3.2. GABA-Activated Currents In The Presence Of 100µM Pentobarbital

5.3.2.1. Effects Of 100µM Pentobarbital On GABA Dose-Response Curves

It has been reported that pentobarbital shifts the GABA dose-response curve to the left without affecting the maximum peak current and this was explained as an increase in the apparent affinity of GABA for the receptor (Barker & Ransom, 1978; Willow & Johnston, 1980; Nicoll & Wojtowicz, 1980; Alger & Nicoll, 1982; Akaike et al., 1985b; Parker et al., 1986; Nakagawa et al., 1991; Itabashi et al., 1992). To see if a similar shift could be obtained in these dentate gyrus neurons, 100µM pentobarbital with increasing concentrations of GABA (1µM to 1mM) were applied together and the peak and plateau currents examined (Figure 5.10.A.). Results were obtained from 3-6 neurons for each concentration of GABA tested. These values were averaged and normalised to the maximum response (I") and expressed as a percentage. A Hill type equation (see Equation 2.1.) was fitted to the peak current values and the apparent affinity for GABA was increased by the presence of 100µM pentobarbital. The GABA EC_{50} for peak currents decreased from 14µM to 3µM in the presence of 100µM pentobarbital. As expected, the maximum peak current was not affected (Figure 5.10.B.). In contrast, a Hill type equation could not be fitted to the plateau currents. Compared to the control
A. **Peak Currents**

![Graph showing peak currents](image)

**Plateau Currents**

![Graph showing plateau currents](image)

B.

<table>
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**Figure 5.10.** The effect of 100µM pentobarbital on the GABA dose-response curves. A. In the presence of 100µM pentobarbital, dose-response curves were constructed from peak and plateau currents generated by increasing concentrations of GABA (solid lines). These responses were compared to the peak and plateau current dose-response curves generated by GABA alone (dotted lines). All values have been normalised (I") to the maximum current and expressed as a percentage. B. A Hill type equation (see Equation 2.1.) was fitted to the data points and the table shows the EC₅₀, Hill coefficient and maximum current (I_MAX).
(7µM GABA plus 100µM pentobarbital) when GABA was increased to 19µM, the plateau current was 0.5±0.06 times the control (n=3). This depression in the plateau current was similar to the earlier result which showed that 100µM pentobarbital depressed the plateau current generated by 100µM GABA to 0.8±0.07 of the control (Figure 5.5.A.). Nevertheless, the apparent affinity for GABA-activated plateau currents appear to have also increased (see 1µM GABA in Figure 5.10.A.) but this could not be determined with any confidence from the data.

5.3.2.2. Effects Of 100µM Pentobarbital On GABA-Activated Current Time Course

It has been reported that increasing GABA concentration increases the rate of current decay that was further accelerated by the presence of 100µM pentobarbital (Parker et al., 1986). Firstly, rise times were measured from responses activated by increasing concentrations of GABA in the presence of 100µM pentobarbital. The average rise time for 1µM GABA was 900±153ms and as the concentration of GABA was increased, the rise times decreased to 20±10ms for 1mM GABA (Figure 5.11.A.). In the presence of 100µM pentobarbital, the half-decay times for currents activated by 1µM GABA could not be determined as drug application was terminated before the current could decay to half the peak amplitude. However, from the data obtained, there was an initial decrease in the average half-decay times from 1567±146ms for currents activated by 7µM GABA to 698±174ms for currents activated by 19µM GABA (Figure 5.1.B.). The half-decay times then levelled off to 862±300ms for currents activated by 1mM GABA which was not significantly different from the half-decay time obtained for currents activated by 19µM GABA. This follows a similar trend shown for the current time course for currents activated by GABA alone (Figure 4.9.)

Described earlier, one or two exponentials and steady-state current amplitudes were fitted to the decay of the currents produced by 7µM and 1mM GABA in the presence of 100µM pentobarbital (Figure 5.9.). It was found that they had similar time constants of 2505±172ms and 2502±700ms, respectively. However, decay of the currents generated by 1mM GABA (with pentobarbital) required another exponential component. This additional exponential had an average amplitude and time constant of 226±140pA and 137±68ms (Table 5.1.C.).
A. The rise time gets faster with increasing GABA concentrations.

B. The half-decay time also gets faster with increasing GABA concentrations. Half-decay times could not be determined for 1µM GABA (with pentobarbital) as drug application was removed before the current could decay to half.

Each open circle represents one result with 3-21 results for each GABA concentration; the filled symbol denotes the average with the vertical bars showing ±1SEM.

**Figure 5.11.** The effect of 100µM pentobarbital on the time course of currents activated by increasing concentrations of GABA. A. The rise time gets faster with increasing GABA concentrations. B. The half-decay time also gets faster with increasing GABA concentrations. Half-decay times could not be determined for 1µM GABA (with pentobarbital) as drug application was removed before the current could decay to half. Each open circle represents one result with 3-21 results for each GABA concentration; the filled symbol denotes the average with the vertical bars showing ±1SEM.
In summary, 100µM pentobarbital caused the GABA-activated peak and plateau current dose-response curves to shift to the left. In the presence of 100µM pentobarbital, the time course for currents became faster as the concentration for GABA was increased. This followed a similar trend observed for the time course of currents activated by only GABA. A similar exponential was fitted to the decay of the currents activated by 7µM and 1mM GABA in the presence of 100µM pentobarbital. However, an additional exponential was needed for the decay of the current generated by 1mM GABA.

5.3.3. Rebound Current Responses

Terminating the application of pentobarbital alone or in combination with GABA causes a rebound current that is sensitive to bicuculline. (Akaike et al, 1987b; Peters et al., 1988; Robertson, 1989; Yakushiji et al., 1989; Nakagawa et al., 1991; Itabashi et al., 1992; Rho et al., 1996). They postulated that pentobarbital blocks the GABAA channel and by removing the agonist(s), the channel rapidly unblocks and is then available to carry current again. Earlier results showed that removing 7µM GABA and pentobarbital concentrations above 100µM, produced a rebound current response that appeared to be dependent on the concentration of pentobarbital (Figure 5.2.). That is, the higher the concentration of pentobarbital, the larger the rebound current. All rebound peak current amplitudes were measured from the baseline prior to drug application and this is illustrated in Figure 5.12.A1. These current amplitudes were averaged and normalised to the control (7µM GABA) first and then to the maximum amplitude (Ip). This was expressed as a percentage and is illustrated in Figure 5.12.A2. An approximation of the concentration that would elicit half the maximum amplitude rebound current response was measured to be about 1mM pentobarbital (with 7µM GABA).

Terminating the application of either 7µM or 100µM GABA with 10mM pentobarbital, produced similar rebound current responses that were not significantly different (Figures 5.12.B. & 5.12.C.). On average, these rebound peak current amplitudes were 1025±143pA for 7µM GABA (n=8) and 1135±476pA for 100µM GABA (n=3). These results would suggest that the magnitude of the rebound current was not dependent on
Figure 5.12. The effect of pentobarbital concentration on the rebound current. A1. Rebound peak current amplitudes are plotted against increasing concentrations of pentobarbital in the presence of 7µM GABA. Each point represents 3-8 neurons. A2. Current amplitudes were averaged and then normalised (I') to the maximum response generated by 7µM GABA and expressed as a percentage. Vertical bars (unless obscured by the symbol) show ±1SEM. B. Current responses to 7µM GABA in the absence (left) and presence (right) of 10mM pentobarbital. C. Current responses to 100µM GABA in the absence (left) and presence (right) of 10mM pentobarbital.
the concentration of GABA. This then raises the question of whether pentobarbital can block closed GABA_{A} channels so that when pentobarbital is unblocked, a greater number of channels are open. In an attempt to answer this question, peak currents activated by 7µM and 100µM GABA were compared to their corresponding rebound peak current amplitudes generated by the removal of GABA and 10mM pentobarbital. In all eight rebound peak current amplitudes produced by removing 7µM GABA and 10mM pentobarbital, they were always 1.5 to 2.7 times greater than the peak current amplitudes produced by 7µM GABA alone (Figure 5.13.A.). If the peak current amplitude could be correlated with a relative number of activated GABA_{A} receptors, it could then be speculated that more channels had to be open to carry the larger rebound current than compared to the number of channels activated by 7µM GABA alone. Clearly this could not be an open channel block mechanism as observed with penicillin in the previous chapter. One possible explanation for this larger rebound current could be that pentobarbital can block closed or desensitised channels. Removing 100µM GABA and 10mM pentobarbital caused a rebound current that had similar amplitudes to the peak currents activated by 100µM GABA alone (Figure 5.13.B.). Thus, it would appear that the magnitude of the rebound current was already at its maximum amplitude when 7µM GABA and 10mM pentobarbital was removed. If pentobarbital can indeed block closed or desensitised channels, then the concentration of GABA would not necessarily matter. If this is true, then the similarities in the amplitudes of the rebound current generated by removing either 7µM or 100µM GABA (with pentobarbital) are justified. Other possible explanations for these rebound currents will be discussed later.

In summary, removing 7µM GABA with pentobarbital concentrations above 100µM, caused a rebound current that was dependent on the concentration of pentobarbital. Although not accurately determined, a half-maximal rebound current response could be produced by removing 1mM pentobarbital plus 7µM GABA. Furthermore, the amplitude of the rebound peak current does not appear to be dependent on the concentration of GABA. Removing either 7µM or 100µM GABA with 100µM pentobarbital produced nearly identical rebound peak current amplitudes. There could be several possibilities for this similarity and this will be discussed below.
Figure 5.13. Comparison of the effect of GABA concentration on the rebound peak current amplitude. Each symbol represents one experiment with the dotted line leading to the corresponding rebound current response elicited by the removal of 10 mM pentobarbital plus GABA. A. Peak currents activated by 7 µM GABA were compared to the corresponding rebound peak current amplitudes. In all cases, the rebound current amplitudes were larger than the GABA-activated peak current amplitudes (n=8). B. Peak currents activated by 100 µM GABA were compared to the corresponding rebound peak current amplitudes. In all cases, the rebound current was slightly smaller (n=3).
5.4. DISCUSSION

Pentobarbital had five major effects on currents activated by 7µM GABA. Pentobarbital concentrations between 0.1µM and 10µM depressed the current with 10µM pentobarbital causing the greatest reduction in both peak and plateau currents. Concentrations between 50µM and 1mM pentobarbital enhanced the GABA-activated currents. Both 100µM and 1mM pentobarbital produced similar enhancements of the currents activated by 7µM GABA. However, 10mM pentobarbital abolished nearly all of the GABA-activated current. Removing pentobarbital concentrations of 100µM and above produced rebound current responses that were dependent on the concentration of pentobarbital but not on the concentration of GABA. Finally, 100µM pentobarbital alone did not directly activate the GABA<sub>A</sub> receptor.

In a similar study by Kapur & Macdonald (1997), 10µM GABA was co-applied with increasing concentrations of pentobarbital (1-300µM) to isolated granule cells of the dentate gyrus. The pentobarbital dose-response curve had an EC<sub>50</sub> of 42±15µM and they did not observe any GABA-activated current depression with low concentrations of pentobarbital. This could perhaps be explained by their use of older rats (28-35 days) and also the possibility that GABA<sub>A</sub> receptors may have been different. Due to the depressed GABA-activated currents caused by low pentobarbital concentrations in this study, a Hill type equation could not be fitted. Conceivably, the shape of the dose-response curve could be indicative of more than one binding site for pentobarbital. It is possible that there could be two binding sites, one for depression and the other for enhancement. If the binding site for enhancement has a lower affinity for pentobarbital than the binding site for depression, then low concentrations of pentobarbital could depress currents activated by GABA, as observed here. As the concentration of pentobarbital increases, then enhancement of the GABA-activated current would occur. However, this would not explain the consistent exaggerated reduction of GABA-activated currents observed with 10µM pentobarbital. Alternatively, the shape of the dose-response curve could represent more than one receptor. In frog sensory neurons, Akaike <i>et al.</i> (1987b) found that both the activation and inactivation of pentobarbital-induced Cl<sup>-</sup> currents were composed of a fast and a slow component. In addition to the different kinetics, they also had different
affinities for bicuculline and picrotoxin. They proposed that these two components could represent two pentobarbital receptor-ionophore complexes.

It is commonly believed that the current block caused by millimolar concentrations of pentobarbital could be a result of a direct block of the GABA$_A$ receptor pore (Peters et al., 1988; Yakushiji et al., 1989; Itabashi et al., 1992). This was supported by studies that examined the rebound current responses caused by removing GABA and millimolar pentobarbital. They explained this rebound current as the activation of undesensitised receptors when the channel becomes unblocked (Akaike et al., 1987b, Robertson, 1989). Alternatively, Itabashi et al. (1992) suggested that pentobarbital directly affects the affinity of GABA binding to its receptor rather than the Cl$^-$ channel itself. While this rebound current has been shown to be sensitive to inhibition by bicuculline in a dose-dependent manner (Akaike et al., 1987b), no one has yet investigated the effects of the open channel blocker, penicillin on these rebound currents. This could perhaps confirm that pentobarbital does indeed block the channel pore, if the channel is open. A study by ffrench-Mullen et al. (1993) found that in acutely dissociated hippocampal CA1 neurons, there was no use-dependence in the pentobarbital block which indicated that pentobarbital can bind to and block closed channels (ffrench-Mullen et al. 1993). The results found in this study would support that conclusion as the rebound current amplitudes were nearly identical despite the concentration of GABA. This could be tested further by increasing the concentration of pentobarbital to above 10mM to see if the amplitude of the rebound current could be increased. Alternatively, when the application of GABA and pentobarbital was terminated, the subsequent declining concentrations of both these agonists could perhaps reach a combination (for example, <7µM GABA and ~100µM pentobarbital) that would activate and enhance any receptors that have just been unblocked or reverted from a desensitised state. In support of this idea, the rebound currents (generated by removing 7µM GABA and 10mM pentobarbital) was about twice the amplitude of that measured for the currents activated by 7µM GABA alone (Figure 5.13.A.). This was similar to the two-fold current amplitude increase with 100µM pentobarbital by currents activated by 7µM GABA (Figure 5.3.C.). This could also be happening with the rebound currents generated by removing 100µM GABA and 10mM pentobarbital. As 100µM pentobarbital did not significantly affect currents activated by 100µM GABA (Figures 5.4. & 5.5.A.) it would
be expected that the rebound current amplitude would be similar to the peak current amplitudes produced by 100µM GABA, as was observed here. It is clear that pentobarbital has a complex modulatory effect on the GABA_A receptor which cannot be simply explained by a simple block of the channel. There could be a combined effect of both a simple direct block of the channel pore coupled with a conformational change in the receptor as a result of pentobarbital binding.

Peters et al. (1988) observed that directly applying only 5mM pentobarbital to isolated bovine adrenomedullary chromaffin cells, caused a rebound current that was of a greater amplitude than the initial peak current response. This was also observed by Rho et al. (1996) using 3mM pentobarbital in cultured rat hippocampal neurons. They both attributed this characteristic to the blockade of channels by pentobarbital. In the presence of GABA, Rho et al. (1996) derived an estimate of the blocking potency of pentobarbital which was 3mM. In this study, an approximate value for the concentration that could produce a half-maximal amplitude rebound current response was 1mM pentobarbital (with 7µM GABA). Both Akaike et al. (1987b) and Itabashi et al. (1992) also reported a pentobarbital concentration-dependence on the amplitude of the rebound currents but did not give an EC_{50} value as a Hill type equation could not be fitted properly.

In contrast to many other reports (Barker & Ransom, 1978; Nicoll & Wojtowicz, 1980; Mathers & Barker, 1980; Akaike et al. 1985b; Schwartz et al. 1986; Parker et al., 1986; Akaike et al., 1987b; Peters et al., 1988, Robertson, 1989; Yakushiji et al., 1989; Itabashi et al., 1992; Macdonald & Olsen, 1994; Rho et al., 1996), GABA_A receptors of the dentate gyrus could not be directly activated by 100µM pentobarbital. The most likely reason for this is the subunit composition of these receptors. As discussed earlier, GABA_A receptors of the dentate gyrus region show positive antibody staining for the α_A subunit. Wafford et al. (1996) showed that α_A-containing receptors could not be directly activated by the two anaesthetics, pentobarbital and propofol.

As expected, the effect of 100µM pentobarbital on the GABA dose-response curve was to shift the curve to the left without affecting the maximum peak current. This effect of pentobarbital has been explained as an increase in the apparent affinity of GABA for the receptor (Barker & Ransom, 1978; Willow & Johnston, 1980; Nicoll & Wojtowicz,
Here, the GABA-activated peak current EC50 value decreased from 14µM to 3µM in the presence of 100µM pentobarbital with no significant change in the Hill coefficient. The apparent affinity for plateau currents activated by increasing GABA concentration in the presence of 100µM pentobarbital appear to have also increased. Despite the inability to fit a Hill type equation to these values, 1µM GABA produced a larger plateau current amplitude in the presence of 100µM pentobarbital than alone (Figure 5.10.A).

There have been many studies that have documented a prolongation of the GABA-activated current with pentobarbital. In cultured mammalian central neurons, Barker & Ransom (1978) observed that in addition to an increase in conductance caused by 100µM pentobarbital, there was also a variable slowing down of the kinetics. Similarly, in cultured rat hippocampal neurons, Segal & Barker (1984b) saw that in the presence of 100µM pentobarbital, the decay time constant produced by GABA-activated currents were on average 232±30% longer. Again, in isolated hippocampal neurons the rate of the GABA-activated current decay was 1.8 times slower than the GABA-activated current in the presence of 100µM pentobarbital (Huguenard & Alger, 1986). However, this study showed that 100µM pentobarbital had no effect on the half-decay times of currents activated by either 7µM or 100µM GABA, except for 10mM pentobarbital which abolished the GABA-activated current (Figure 5.6.). Apart from the increase in current amplitude, 100µM pentobarbital appeared to have no effect on the time constants fitted to the decay of the currents generated by GABA. In fact, any changes seen in the exponential fits could be attributed to the concentration of GABA. The half-decay times produced by currents activated by 7µM or 100µM GABA in the presence of 100µM pentobarbital (Figure 5.6.) were very similar to the half-decay times produced by currents activated by 7µM or 100µM GABA alone (Figure 4.9.).

In conclusion, the effect of pentobarbital appears to be quite different in the dentate gyrus. Currents activated by 7µM GABA are not only enhanced, but they can be depressed (≤10µM) or abolished (10mM) with pentobarbital. Where GABA-activated currents were enhanced by pentobarbital, only the current amplitude was increased but no effect on the current decay could be attributed to pentobarbital. Pentobarbital did not
directly activate GABA_A receptors which could be explained by the presence of the α_4 subunit. Also discussed were several explanations for the rebound currents.
CHAPTER 6

SINGLE-CHANNEL CHARACTERISTICS

6.2. METHODS & MATERIALS

The method of projecting dissociated neurones and brain slices to record single-channel activity, electrophysiological and data analysis were described in Chapter 5.

6.3. RESULTS

6.3.1. Single Channels Activated By GABA

In other regions of the central nervous system, there have been many single-channel studies that have characterized the GABA\textsubscript{A} receptor, with most of these studies...
SINGLE-CHANNEL CHARACTERISTICS

6.1. INTRODUCTION

Studies of single channels activated by GABA in the dentate gyrus have been somewhat sparse. Birnir et al. (1994) characterised the GABA_A receptor in this region and obtained many interesting results; reviewed in Chapter 1. They speculated the existence of multiple GABA_A receptor types to explain the different single-channel characteristics and concluded that these single channels may contribute to a tonic inhibitory mechanism.

My aim in this chapter was to further characterise single channels activated by GABA in the dentate gyrus region and to observe the modulatory effects of pentobarbital and diazepam on GABA-activated and spontaneous channels. These two drugs were selected based on their lipid solubility (Miller, 1985) which is advantageous when using cell-attached and inside-out patch configurations. Because of their lipid solubility, these drugs are able to cross the lipid bilayer and pass into the recording patch electrode to modulate the GABA_A receptor.

6.2. METHODS & MATERIALS

The method of preparing dissociated neurons and brain slices as well as single-channel solutions, electrophysiology and data analysis were described in Chapter 2.

6.3. RESULTS

6.3.1. Single Channels Activated By GABA

In other regions of the central nervous system, there have been many single channel studies that have characterised the GABA_A receptor, with most of these studies
performed on cultured neurons (Allen & Albuquerque, 1987; Bormann et al., 1987; Huck & Lux, 1987; Bormann & Kettenmann, 1988; Weiss, 1989; Macdonald et al., 1989a; Smith et al., 1989; Fatima-Shad & Barry, 1992; Twyman et al., 1992; Curmi et al., 1993; Fatima-Shad & Barry, 1993; Eghbali et al., 1997). Hippocampal slice preparations have also been used (Gray & Johnston, 1985; Kaneda et al., 1995; Birnir et al., 1994) and have been particularly useful when studying the dentate gyrus. Cultured neurons are often obtained from foetal or newborn animals which would not be feasible for the dentate gyrus that develops postnatally. Furthermore, it is not known to what extent the culture environment influences the development of these neurons. In this study, both hippocampal slices and dissociated neurons were used although dissociated neurons were the preferred preparation as it would have been easier to relate the whole-cell results of the previous chapters to the following single-channel results. However, it was quickly found that the success rate of obtaining results from dissociated neurons was low (details will be explained later) and therefore, hippocampal slices had to be used. Results from both preparations will be presented.

6.3.1.1. Acutely Dissociated Neurons

Using the same acute dissociation method described in Chapter 2, isolated granule cells were obtained and cell-attached patches were attempted. Out of 338 isolated cells patched, only 57 (17%) were successful. The definition of this success was determined by meeting all the following criteria: the cell-attached patch had to last for more than 30 seconds, had very little leak and currents activated by GABA reversed at the Cl\(^{-}\) equilibrium potential of 0mV. Out of those 57 neurons, 47 patch pipettes contained only pipette solution while the other 10 patch pipettes contained 7µM GABA with the pipette solution. Out of those 47 patches, 39 patches were quiet and showed no channel activity. The remaining eight patches exhibited spontaneous currents and will be examined later. In 14 cell-attached patches that were silent for about five minutes, low concentrations of GABA (0.5µM, 1µM or 10µM) were internally perfused resulting in six patches that exhibited channel activity, six patches that remained quiet despite numerous injections of GABA into the patch pipette and two patches that deteriorated before any detectable current could be obtained.
In the six cell-attached patches that were activated by GABA, channel activity consisted of fast channel openings and closings to several conductance states that could not be accurately determined. These channels were labelled as "spiky channels" for the purpose of distinguishing this type of channel activity from the more traditional square-like channels. As convention dictates for cell-attached and inside-out patches (Sakmann & Neher, 1995), all traces have been inverted and potentials are shown as the negative of the applied pipette potential (-Vp). Therefore, channel events opening upwards are outward currents (inward Cl⁻ movement) and downward events are inward currents (outward Cl⁻ movement) with respect to the interior of the cell. Illustrated in Figure 6.1. is an example of spiky channels activated by GABA in a dissociated neuron. Prior to the injection of 0.5µM GABA into the patch pipette, no channel activity was observed for about six minutes at -Vp's of +40, 0 or -40mV. Mean currents were calculated for a 20 second period and these values reflected the absence of any channel activity (Figure 6.1.A1.). Approximately five minutes after injecting a bolus of 0.5µM GABA into the patch pipette, channel activity began to occur; the delay in the appearance of channel activity could be attributed to the time it took for GABA to diffuse down the patch pipette to the surface of the membrane. Illustrated in Figure 6.1.A2., currents activated by 0.5µM GABA reversed at a -Vp of 0mV with a mean current of 0.05pA measured from a 20 second current trace. At -Vp of +40mV and -40mV, the mean currents measured from a 20 second current trace were 1.03pA and -0.42pA, respectively; these values indicate outwardly rectifying currents. As the only major ion reversing at 0mV is Cl⁻, and the fact that these patches were silent before GABA application, suggests that the channel activity observed was caused by GABA.

In 10 cell-attached patches where 7µM GABA was present inside the pipette prior to patching, six patches exhibited channel activity that occurred within a couple of seconds after making the cell-attached seal. The other four cell-attached patches presented no detectable channel activity. Illustrated in Figure 6.1.B., currents activated by 7µM GABA were also characteristically spiky and were outwardly rectifying. At -Vp's of +40mV and -40mV, the mean currents measured from a 20s current trace were 0.95pA and -0.09pA. With two of these spiky channels, 100µM pentobarbital was perfused through the bath and these results will be examined later.
Figure 6.1. Isolated granule cells activated by GABA. A1. In a cell-attached patch at $-V_p$'s of +40, 0 and -40mV, no channel activity was observed for over six minutes. A2. A bolus of 0.5µM GABA was injected into the patch pipette which resulted in spiky channel activity ($n=6$). Each trace is 400ms long but mean currents ($I_{MEAN}$) were measured in a 20s period. B. Currents were also activated by 7µM GABA in a cell-attached patch and reversed at 0mV.
In an attempt to reduce the spikiness of these channels, 200µM EDTA was included in the pipette solution of six cell-attached patches (results not shown). It was thought that EDTA could chelate any cations that might be contributing to the short open time of these channels. With only 200µM EDTA in pipette solution present in the patch pipette, six cell-attached patches showed no channel activity for over five minutes. Injecting a bolus of either 0.5µM (n=1), 1µM (n=4) or 10µM (n=1) GABA into the patch pipette, three patches remained silent while the other three patches exhibited spiky channel activity. Thus, it appears that EDTA does not help to increase the open time of these spiky channels activated by GABA. It is possible that the spiky channel activity seen in cell-attached patches could be caused by intracellular mechanisms so inside-out patches were attempted. However, all attempts proved unsuccessful as the isolated neuron would lift off the bottom of the recording bath. Although these dissociated neurons were not washed away by the high rate of bath solution flow, they were not adhered well enough for a patch to be ripped off. While dissociated neurons have proven to be a very useful preparation for whole-cell experiments, obtaining single-channel results from this preparation was more arduous. It was concluded that for single-channel experiments, dissociated neurons took too long to prepare and together with the relatively limited number of neurons available to patch and the low data yield, it was not economical to continue.

6.3.1.2. Hippocampal Slices.

Hippocampal slices have been successfully used to study the dentate gyrus region before (Birnir et al., 1994). Together with the ease of preparing slices and the greater number of patches possible, it was expected that results would be more forthcoming. Out of 1163 attempted patches on hippocampal slices, 588 (51%) patches were successful; again this success was determined by the quality of the seal and currents activated by GABA reversed at the pipette potential of 0mV. Of those 588 patches, GABA and/or drug was either present in the patch pipettes or bath perfused onto 463 (79%) patches while the other 125 (21%) patches were not exposed to any drug. Of those 463 patches exposed to GABA and/or drug, 253 (55%) patches exhibited channel activity that reversed at the pipette potential of 0mV and the other 210 (45%) patches were quiet. In 125 patches where GABA was absent from the pipette solution, 61 (49%) patches exhibited
spontaneous currents and the other 64 (51%) patches were devoid of any detectable channel activity; these results will be presented later. Both cell-attached and inside-out data have been combined as there were no observable differences between the two configurations.

In the 253 patches exhibiting channel activity, the pipette solution in 132 patch pipettes contained 0.5µM GABA while 74 patch pipettes contained 20µM GABA only. In another five pipettes, either 5µM or 100µM GABA was present but these concentrations were not investigated further (results not shown). Due to the high incidence of silent patches in the presence 0.5µM GABA, 20µM GABA was chosen to ensure a higher rate of channel activation. In addition, 17 other pipettes contained 100µM pentobarbital and 25 pipettes contained 1µM diazepam together with 20µM GABA in their pipette solutions; these results will be discussed later. Of these 253 patches, 132 patches (52%) exhibited spiky channels that were observed previously with the dissociated neurons. It was difficult to analyse these spiky channels as no conductance levels could be accurately determined so they were omitted from the initial analysis of GABA-activated channels. Nevertheless, those channels in which conductance levels could be determined, some displayed several (or multiple) conductance states. The most common conductance state was determined by the frequency of channel openings to that level; the maximum conductance state is not necessarily the same as the most common conductance state. These conductance states were pooled into 10pS bins and are shown in Figure 6.2. Single-channel currents activated by 0.5µM GABA (n=49) had a most common conductance level of around 51-60pS while for 20µM GABA (n=41), the most common conductance level was around 21-30pS. Why a higher common conductance level was seen with 0.5µM GABA than with 20µM GABA was puzzling. Furthermore, in the presence of either 0.5µM or 20µM GABA, single-channel conductances above 100pS could occasionally be observed. These would often occur in conjunction with other conductance levels and were not very common. Illustrated in Figure 6.3. are two cell-attached patches activated by 0.5µM GABA at a -V_P of +40mV and exhibited multiple conductance states. The first cell-attached patch exhibited three prominent conductance states of 37pS, 54pS and 77pS (Figure 6.3.A.) and the second cell-attached patch had conductance states of 14pS, 39pS and 51pS (Figure 6.3.B.). Although not shown, these
Figure 6.2. The range of single-channel conductance states observed with channels activated by either A. 0.5µM GABA (n=49) or B. 20µM GABA (n=41). Conductance levels have been pooled into 10pS bins.
Figure 6.3. Single channels exhibiting multiple conductance states. Taken from two different cell-attached patches, these currents were activated by 0.5µM GABA and exhibited multiple conductance states at a $-V_p$ of +40mV.
currents reversed at the pipette potential of 0mV and were subsequently modulated by either pentobarbital or diazepam, indicating that these currents were caused by GABA.

On several occasions multiple channels were also observed, typically two or sometimes three channels. Often these channels would also exhibit multiple conductance states. Shown in Figure 6.4.A., in the presence of 0.5µM GABA two 71pS channels were recorded in a cell-attached patch at a -Vp of +40mV. Illustrated in Figure 6.4.B., in the presence of 20µM GABA, a 48pS and a 46pS channel were recorded in a cell-attached patch at -Vp of +40mV. These channels also had subconductance states of 30pS and 21pS, respectively. Again not shown, these currents reversed at the pipette potential of 0mV and with only Cl− being the major ion reversing at this -Vp, it is likely that these channels were mediated by GABA.

Mentioned previously, spiky channels contribute about 52% of all the single channels activated by GABA in this study. Both spiky channels and the square-like channels were often present in the same patch which is illustrated in Figure 6.5. These spiky channels were determined to be activated by GABA for several reasons. The baseline level was steady and therefore spiky channels would not be an indication of seal deterioration; often associated with deteriorating seals is a fluctuating baseline. Spiky channels could not be the result of other ion movements as shown in Chapter 2. The pipette (which contained choline chloride more often than NaCl) and bath solutions were designed such that the only major ion that reversed at the pipette potential of 0mV was Cl−. Finally, these spiky channels could be modulated by GABA receptor modulators and these results will be presented next.

In summary, both dissociated neurons and hippocampal slice preparations were used to study currents activated by GABA in the dentate gyrus. While dissociated neurons proved to be a very useful preparation for whole-cell studies, it was not so for single channel studies. Currents activated by GABA obtained from hippocampal slices showed a range of conductance states. On several occasions more than one channel was also activated. In the presence of 0.5µM GABA, at a -Vp of +40mV the most common conductance state was about 50pS. In addition, spiky channels were also prominent and were thought to be mediated by GABA.
Figure 6.4. Multiple channels activated by GABA in two cell-attached patches. A. In the presence of 0.5µM GABA, two 71pS channels were observed at a -Vp of +40mV. B. In the presence of 20µM GABA, two channels of 48pS and 46pS with subconductance states of 30pS and 21pS were seen. The solid lines indicate the closed state for the two channels (C1 and C2) with subconductance states indicated by the dotted lines.
Figure 6.5. Mixed single-channel characteristics. In the presence of 0.5μM GABA, a 51pS channel as well as spiky channel activity were recorded from a cell-attached patch at $-V_p$ of +40mV. Shown above are continuous traces with each trace 400ms in duration. From the end of the second trace, the 50pS channel returned and is illustrated in figure 6.3.B.
6.3.2. Single-Channel Currents Modulated By Pentobarbital

Pentobarbital has been shown to modulate currents activated by GABA by decreasing the frequency of channel openings and increasing the average mean open time (Mathers & Barker, 1980; Study & Barker, 1981; Jackson et al., 1982; Mathers, 1985; Mathers, 1987; Macdonald et al., 1988; Twyman et al., 1989). In addition, pentobarbital has also been reported to promote burst-like openings (Mathers, 1985; Macdonald et al., 1988) but has no effect on single-channel conductance (Study & Barker, 1981; Twyman et al., 1989; Macdonald et al., 1989b). In this study, because multiple conductance states were observed in any one patch, in order to determine the effect of pentobarbital on the GABA_A receptor, the maximum conductance states were compared to avoid any bias with the degree of potentiation. However, as no clear conductance states could be determined for spiky channel activity, mean currents (I_{MEAN}) were measured for the maximum duration possible which ranged from 1.7 to 50 seconds. A concentration of 100µM pentobarbital was chosen based on the results obtained from the previous whole-cell results; 100µM pentobarbital maximally enhanced the whole-cell currents while concentrations lower or higher than this concentration depressed currents activated by GABA.

In the presence of 0.5µM GABA, channels were modulated by 100µM pentobarbital in three out of four cell-attached patches (Table 6.1.A.). At a -V_p of +40mV, the maximum conductance states for these three channels activated by GABA were 64pS, 71pS and 51pS. When 100µM pentobarbital was perfused through the bath, only in two of these patches was the maximum conductance state increased to 71pS (from 64pS) and 62pS (from 51pS); a 50pS and a 45pS channel was seen in the other patch. However, in all three patches the most common conductance state increased (from 64pS to 71pS, 30pS to 50pS and 39pS to 51pS). Illustrated in Figure 6.6. is an example of the increase in maximum conductance state produced by the presence of 100µM pentobarbital. Recorded from a cell-attached patch at a -V_p of +40mV, a maximum conductance state of 51pS was measured in the presence of 0.5µM GABA. With the addition of 100µM pentobarbital, the maximum conductance state increased to 62pS. This increase was also reflected in the all-points amplitude histograms. Compared to the histogram measured for GABA alone, the amplitude peak was shifted to the right as well as showing an
Figure 6.6. Pentobarbital increased the maximum conductance state. Recorded from a cell-attached patch at \(-V_p\) of +40mV, the maximum conductance state of 51pS for currents activated by 0.5μM GABA increased to 62pS in the presence of 100μM pentobarbital. This increase in conductance was reflected in the amplitude histograms generated from a 20s current trace and showed a shift to the right of the amplitude peak together with an increase in the probability of the channel opening to that higher amplitude.
increase in the probability of the channel opening to that higher amplitude in the presence of 100µM pentobarbital.

Pentobarbital has been reported to shift the frequency histograms for both open times and burst times to the right but the closed time histograms were unchanged (Mathers & Barker, 1980; Study & Barker, 1981; Jackson et al., 1982; Mathers, 1985; Mathers, 1987; Macdonald et al., 1988; Twyman et al., 1989; Macdonald et al., 1989a,b). These trends were also observed here and are illustrated in Figure 6.7. Histograms on the left are generated from currents activated by 0.5µM GABA while histograms on the right are generated from currents activated by 0.5µM GABA plus 100µM pentobarbital; all frequency histograms were generated from 20s current traces recorded at a -Vp of +40mV. To quantify and compare, one or two exponential components \[A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2)\], where \(A_{1/2}\) are the probability amplitudes and \(\tau_{1/2}\) are the time constants] were fitted to the frequency histograms. For open time histograms (bin widths of 0.3ms), in the presence of 0.5µM GABA two exponential components were best fitted that produced two time constants of 0.1ms and 7.3ms; with the 0.1ms time constant smaller than the bin width (of 0.3ms), this time constant is unlikely to be significant. The probability amplitudes associated with these time constants were 0.08pA and 0.04pA. In the presence of 0.5µM GABA plus 100µM pentobarbital, time constants of 7.2ms and 11ms were fitted to the open time histogram. The probability amplitudes associated with these time constants were 0.02pA and 0.02pA which were much smaller than those fitted for GABA alone. Clearly in the presence of pentobarbital, the open time of channels activated by GABA were much longer. In contrast, pentobarbital did not appear to affect the closed time histograms (bin widths of 0.2ms). For 0.5µM GABA alone, only one exponential component with a time constant of 0.5ms could be fitted to the closed time histogram. In the presence of 0.5µM GABA plus 100µM pentobarbital, a time constant of 0.6ms was fitted to the closed time histogram. The probability amplitudes of the time constants (0.19pA and 0.18pA) were nearly identical for both GABA alone and in the presence of pentobarbital. Similar to the open time histograms, two exponential components were fitted to the burst time frequency histograms; bursts were defined as openings separated by closings greater than 1ms and pooled into bin widths of 0.5ms. In the presence of 0.5µM GABA alone, time constants of 2.5ms and 25.8ms were fitted with probability amplitudes of 0.01pA and 0.02pA. In the presence of 0.5µM GABA
Figure 6.7. Single-channel kinetics of GABA-activated currents modulated by pentobarbital. Compared to the currents activated by 0.5µM GABA, the frequency histograms of A. open time and C. burst time were shifted to the right, but the B. closed time histograms were relatively unchanged in the presence of 100µM pentobarbital. Histograms were generated from a 20s current trace recorded at a -Vp of +40mV.
plus 100µM pentobarbital, time constants of 4.2ms and 32ms were fitted and the associated probability amplitudes were 0.01pA and 0.01pA, respectively. Therefore, in agreement with previous reports, the effect of 100µM pentobarbital on currents activated by 0.5µM GABA were to lengthen the open and burst times without affecting the closed times.

As expected, the mean currents activated by GABA were increased in the presence of pentobarbital. Currents activated by 0.5µM GABA had mean current values of 0.9pA, 1.6pA and 1.4pA that were increased to 1.5pA, 1.8pA and 1.6pA, respectively, in the presence of 100µM pentobarbital (Table 6.1.A.); in Patch 2 of this Table, the mean current was measured in a region where only one channel was active. This increase in mean current was also observed for spiky channels. Shown in Figure 6.8.A. is a result obtained from a cell-attached patch on a dissociated neuron. In the presence of 7µM GABA, the mean current at a -V_P of +80mV was 0.1pA. Similarly, at a -V_P of -80mV a mean current of -0.2pA was measured. Perfusing 100µM pentobarbital through the bath caused the mean currents to increase to 0.4pA at a -V_P of +80mV and to -0.5pA at -V_P of -80mV. In a second cell-attached patch on an isolated neuron, the mean current at a -V_P of -80mV, increased from -0.6pA for 7µM GABA alone to -0.96pA when 100µM pentobarbital was perfused through the bath. In the hippocampal slice preparation where 0.5µM GABA was present in the pipette, when 100µM pentobarbital was perfused through the bath, a detectable effect was observed in two out of seven patches. The other five patches displayed initial channel activity but became silent; despite numerous bath applications of pentobarbital, the channel remained quiet. The present study did not determine why these channels went quiet and this requires further investigation. Nevertheless, mean currents recorded in two inside-out patches at a -V_P of +40mV had initial mean currents of 0.2pA and 0.1pA. When 100µM pentobarbital was perfused through the bath, the mean currents increased to 3.3pA and 0.6pA, respectively (Table 6.1.B.). An example of these spiky channels modulated by pentobarbital is shown in Figure 6.8.B. Recorded from an inside-out patch at a -V_P of +40mV, in the presence of 0.5µM GABA a mean current of 0.1pA was measured from a 50s current trace; the 400ms trace shown in the figure shows a more active region of the current trace. When 100µM pentobarbital was perfused through the bath, the mean current increased to 0.6pA.
Figure 6.8. Pentobarbital modulated spiky channels activated by GABA. A. Recorded in a cell-attached patch on an isolated neuron, currents activated by 7µM GABA generated similar mean currents at Vp’s of +80mV and -80mV. Perfusing 100µM pentobarbital through the bath caused the mean currents to increase. B. Recorded in an inside-out patch from a slice at a Vp of +40mV, the mean current activated by 0.5µM GABA increased with the addition of 100µM pentobarbital. Current traces show (400ms) the more active regions while mean currents were measured for 20s and 50s, respectively.
Due to the low number of results obtained so far, these next experiments were performed in hippocampal slices to obtain more information. Despite the difficulty in determining the actual effect of pentobarbital, these results followed the same trends as above. Currents activated by the presence of 20µM GABA in the pipette solution, were measured at a $-V_p$ of +40mV. These currents were compared to other currents that were activated by the presence of 20µM GABA plus 100µM pentobarbital at the same $-V_p$; although not shown, these currents reversed at a $-V_p$ of 0mV. For these pooled results, a range of maximum conductance states were obtained and where conductance states could not be determined, mean currents were measured for 10s; this duration was chosen so that a maximum number of patches could be analysed. In current traces where conductance states could be determined, the average maximum conductance was 41±2pS (ranged from 18pS to 59pS) for currents activated by 20µM GABA at a $-V_p$ of +40mV (n=41). In the presence of 20µM GABA plus 100µM pentobarbital, the average maximum conductance was 46±5pS (ranged from 30pS to 66pS) at a $-V_p$ of +40mV (n=8). That is, an overall increase in the maximum conductance state was observed (Table 6.1.C). For those currents where only mean currents could be calculated, currents activated by 20µM GABA produced an averaged mean current of 0.12±0.04pA at a $-V_p$ of +40mV (n=7). In the presence of 20µM GABA plus 100µM pentobarbital, the averaged mean current was increased to 0.66±0.2pA at a $-V_p$ of +40mV (n=6; Table 6.1.D.). In agreement with previous results, mean currents activated by 20µM GABA were increased in the presence of 100µM pentobarbital.

In summary, pentobarbital increased the maximum conductance state as well as the mean currents of channels activated by GABA. In the presence of 100µM pentobarbital, the all-points amplitude histograms for channels activated by 0.5µM GABA were shifted to the right together with an increase in the probability of channels opening to a higher amplitude. Both open and burst time frequency histograms were shifted to longer times while the closed time histograms were unaffected by the presence of 100µM pentobarbital. Generally pentobarbital had a greater effect on the time constants with only small changes in the probability amplitudes associated with these time constants.
Table 6.1. The effect of 100µM pentobarbital on currents activated by GABA at a $-V_p$ of +40mV in the dentate gyrus. A. & C. Multiple conductance states were measured; in bold are the most common conductance states for each patch. B. & D. Mean currents ($I_{\text{MEAN}}$) were calculated for spiky channels for the duration stated for that patch.

<table>
<thead>
<tr>
<th>Patch</th>
<th>0.5µM GABA (pS)</th>
<th>+100µM PB (pS)</th>
<th>Ratio</th>
</tr>
</thead>
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<td>9</td>
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<td></td>
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<td>71</td>
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</tr>
<tr>
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<td>$I_{\text{MEAN}}$ (20s)</td>
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<td>+100µM PB ($I_{\text{MEAN}}$ pA)</td>
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<td>------</td>
<td>-----------------</td>
<td>-----------------</td>
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<td>6</td>
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<td>20µM GABA + 100µM Pentobarbital (maximum conductance state, n=7)</td>
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<tr>
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<td>46±5pS (range: 30-66pS)</td>
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<td>D.</td>
<td>20µM GABA (ave$I_{\text{MEAN}}$ ± 1SEM, n=7)</td>
<td>20µM GABA + 100µM PB (ave$I_{\text{MEAN}}$ ± 1SEM, n=6)</td>
<td>Ratio</td>
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<tr>
<td></td>
<td>0.12±0.04pA</td>
<td>0.66±0.2pA</td>
<td>5.5</td>
</tr>
</tbody>
</table>
6.3.3. Single-Channel Currents Modulated By Diazepam

Diazepam has been shown to increase the frequency of single-channel openings as well as increase the mean open time without altering the conductance (Study & Barker, 1981; Mathers, 1987; Bormann, 1988). However, Eghbali et al. (1997) and Guyon et al. (1999) recently reported a benzodiazepine-concentration dependence of single-channel conductances. In two out of three patches where conductance states could be measured, 1µM diazepam perfused through the bath caused an increase in the maximum conductance state (Table 6.2.A.). In the third patch, despite an initial 77pS channel at a -VP of +40mV which became silent, subsequent applications of diazepam (or pentobarbital) had no effect and the channel remained quiet. The cause of the channels becoming silent cannot be explained and because these channels went silent before diazepam or pentobarbital application, it cannot be determined whether these drugs depressed channel activity. While these channels did reverse at the -VP of 0mV, it is possible that these channels were not mediated by GABA or alternatively, these channels could represent another GABA\text{A} receptor population. Illustrated in Figure 6.9. is an example of the increase in maximum conductance state produced by the presence of 1µM diazepam. Recorded from an inside-out patch at a -VP of +40mV, a maximum conductance of 77pS was measured in the presence of 0.5µM GABA. With the addition of 1µM diazepam, the maximum conductance state increased to 91pS. This increase was also reflected in the all-points amplitude histograms. Compared to the histogram measured for GABA alone, the amplitude peak was shifted to the right as well as showing a decrease in the probability of the channel opening to lower conductance states in the presence of 1µM diazepam. Also evident in the amplitude histograms were the 37pS and 54pS subconductance states. When 1µM diazepam was added, the amplitude histogram clearly shows an increase in the maximum conductance state as well as the probability of the channel preferring the higher 91pS conductance state. The amplitude histogram for 0.5µM GABA plus 1µM diazepam would indicate that the probability of opening is reduced but these histograms could only be generated from a 2s current trace, and therefore may not be an accurate representation of the effect of diazepam on channel open probability. Furthermore, open, closed and burst time frequency histograms could not be generated for this patch for the same reason. The second patch which also showed an increase in the maximum conductance state could
Figure 6.9. Diazepam increased the maximum conductance state. Recorded from a cell-attached patch at a $-V_p$ of +40mV, the maximum conductance state of 77pS for currents activated by 0.5µM GABA increased to 91pS in the presence of 1µM diazepam. This increase in conductance was reflected in the all-points amplitude histograms which showed a shift to the right of the amplitude peak together with decrease in the probability of the channel opening to a lower amplitude. These histograms were generated from a 2s current trace.
not be used to generate histograms as spiky channel activity was dispersed throughout which would have produced inaccurate frequency histograms.

The mean currents calculated for these two patches were 1.6pA and 0.4pA at a \(-V_p\) of +40mV. In the presence of 1µM diazepam, the mean currents increased to 2.2pA and 0.6pA, respectively (Table 6.2A). This increase in mean current was also observed for spiky channels. In three out of nine patches exhibiting spiky channel activity, the addition of 1µM diazepam through the bath caused an increase in the mean current activated by 0.5µM GABA. The remaining six patches remained silent despite initial channel activity and subsequent application of 1µM diazepam. Nevertheless, in one cell-attached patch and in two inside-out patches at a \(-V_p\) of +40mV, the mean currents were 0.9pA, 0.7pA and 0.6pA. Perfusing 1µM diazepam through the bath caused the mean currents to increase to 1.8pA, 2.6pA and 0.8pA respectively (Table 6.2B). An example of these spiky channels modulated by diazepam is shown in Figure 6.10. Recorded from a cell-attached patch at a \(-V_p\) of +40mV, in the presence of 0.5µM GABA a mean current of 0.9pA was measured from a 6.7s current trace. When 1µM diazepam was perfused through the bath, the mean current increased to 1.8pA.

Again to obtain more information, currents activated by 20µM GABA were compared to currents activated by 20µM GABA plus 1µM diazepam at a \(-V_p\) of +40mV in hippocampal slices. For these pooled results, a range of maximum conductance states were obtained and where conductance states could not be determined, mean currents were measured for 10s. In the presence of 20µM GABA, the average maximum conductance state was 41±2pS (ranged from 18pS to 59pS) at a \(-V_p\) of +40mV (n=41). In the presence of 20µM GABA plus 1µM diazepam, the average maximum conductance was 53±5pS (ranged from 21pS to 80pS) at a \(-V_p\) of +40mV (n=20). That is, an overall increase in the maximum conductance state was observed (Table 6.2C). For those currents where only mean currents could be calculated, currents activated 20µM GABA produced an averaged mean current of 0.12±0.04pA at a \(-V_p\) of +40mV (n=7). In the presence of 20µM GABA plus 1µM diazepam, the averaged mean current was increased to 0.66±0.12pA (n=6; Table 6.2D). In agreement with previous results, mean currents activated by 20µM GABA were increased in the presence of 1µM diazepam.
Figure 6.10. Diazepam modulated spiky channels activated by 0.5µM GABA. Recorded in a cell-attached patch at a -Vp of +40mV, the mean current activated by 0.5µM GABA doubled in amplitude with the addition of 1µM diazepam.
Table 6.2. The effect of 1µM diazepam on currents activated by GABA at a \(-V_p\) of +40mV in the dentate gyrus. A. & C. Multiple conductance states were measured; in **bold** are the most common conductance state for that patch. B. & D. Mean currents (\(I_{\text{MEAN}}\)) calculated for spiky channels for the duration stated for that patch.

### A.**

<table>
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<tr>
<th>File</th>
<th>0.5µM GABA (pS)</th>
<th>+1µM Diazepam (pS)</th>
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</tr>
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<td>1.4</td>
</tr>
<tr>
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<tr>
<td>(I_{\text{MEAN}})</td>
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<td>0.6pA</td>
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### B.**

<table>
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<th>0.5µM GABA ((I_{\text{MEAN}},) pA)</th>
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<td>Patch 3 (25s; i/o)</td>
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### C.**

<table>
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<th>20µM GABA + 1µM DZ (max. conductance state, n=20)</th>
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<td>41±2pS (range: 18-59pS)</td>
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### D.**

<table>
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<th>20µM GABA (ave(I_{\text{MEAN}}) ± 1SEM, n=7)</th>
<th>20µM GABA + 1µM DZ (ave(I_{\text{MEAN}}) ± 1SEM, n=6)</th>
<th>Ratio</th>
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<tbody>
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<td>0.12±0.04pA</td>
<td>0.66±0.12</td>
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</table>
In summary, diazepam increased the maximum conductance state as well as the mean currents of channels activated by GABA. Open, closed and burst time frequency histograms could not be generated due to the short duration of the current traces.

6.3.4. Spontaneous Channel Activity

Spontaneous currents have been observed in outside-out patches from neurons but rarely in cell-attached or inside-out patches (Hamill et al., 1983; Huck & Lux, 1987; Weiss et al., 1988; Macdonald et al., 1989a). In this study, channel activity that occurred in the absence of GABA and reversed at a pipette potential (-V<sub>P</sub>) of 0mV as expected for Cl<sup>-</sup> currents if the Cl<sup>-</sup> equilibrium potential is close to the membrane potential, were defined as spontaneous currents. These spontaneous currents were examined in the dentate gyrus region (from isolated neurons and hippocampal slices) as well as the CA1 region (from hippocampal slices) for comparison using either cell-attached or inside-out patches.

6.3.4.1. Dentate Gyrus Region

In eight cell-attached patches on isolated neurons, spontaneous currents were seen at -V<sub>P</sub>'s of +40mV and -40mV. These spontaneous currents reversed at a -V<sub>P</sub> of 0mV. An example of these spontaneous currents is illustrated in Figure 6.11. All mean currents were calculated from 20s current traces. In one cell-attached patch, mean currents of 0.6pA and -0.7pA were recorded at -V<sub>P</sub> of +40mV and -40mV. Similarly, in another cell-attached patch, mean currents of 1.1pA and -1.2pA were recorded at -V<sub>P</sub> of +40mV and -40mV.

Of 125 cell-attached and inside-out patches in hippocampal slices, spontaneous channel activity occurred in 61 (49%) patches while the other 64 (51%) patches exhibited no spontaneous channel activity. Of these 61 patches, multiple conductance states could be measured in 25 patches while the other 36 patches exhibited spiky channel activity. Of those 25 patches, conductance states ranged from 13pS to 112pS and the most common conductance state could not be determined (ranged from 13pS to 100pS). Illustrated in Figure 6.12.A, is a spontaneous channel exhibiting multiple conductance states.
Figure 6.11. Spontaneous currents obtained from isolated granule cells. Recorded from two cell-attached patches, mean currents were measured from 20s current traces. The spontaneous currents also reversed at the Cl⁻ reversal potential of 0mV.
Figure 6.12. Spontaneous currents seen in the dentate gyrus region. A. Recorded in a cell-attached patch at a \(-V_p\) of +40mV, multiple conductance states of 39pS, 64pS and 100pS were measured. B. Recorded from another cell-attached patch at a \(-V_p\) of +40mV, two channels of 24pS and 27pS were measured. Solid lines denote the closed levels for each channel and the dotted lines denote conductance states. C. In an inside-out patch at a \(-V_p\) of +40mV, the mean current measured in a 20s current trace was 0.2pA. When 1\(\mu\)M diazepam was perfused through the bath, the mean current increased to 0.5pA.
Recorded from a cell-attached patch at a $-V_p$ of +40mV, the most common conductance state was 100pS with two other subconductance states of 64pS and 39pS. Occasionally two channels could be measured in the same patch. Shown in Figure 6.12.B. are two channels of 24pS and 27pS recorded in a cell-attached patch at a $-V_p$ of +40mV.

In six of seven patches exhibiting spontaneous currents, 100µM pentobarbital (n=3) or 1µM diazepam (n=4) perfused through the bath and had no effect on these currents. In the other patch, spontaneous currents recorded (inside-out patch) at a $-V_p$ of +40mV, the mean current measured in a 20s current trace was 0.2pA. When 1µM diazepam was perfused through the bath, the mean current increased to 0.5pA (Figure 6.12.C. & Table 6.3.A.). The other six patches initially showed spontaneous channel activity but similar to previous trends, these patches became silent and when either pentobarbital or diazepam was perfused through the bath, the patch remained quiet.

In 64 patches that did not exhibit spontaneous channel activity, when 100µM pentobarbital (n=6) or 1µM diazepam (n=5) was perfused through the bath, no channel activity could be seen. This would agree with the whole-cell results which showed that in the absence of GABA, the application of pentobarbital alone elicited no response. Currently there are no reports of diazepam directly activating the GABA$_A$ receptor and these results would support this. Alternatively, it is possible that no GABA$_A$ receptors were present in these patches.

In summary, spontaneous channel activity in the dentate gyrus were present in about half of the attempted cell-attached or inside-out patches. These spontaneous currents exhibited multiple conductance states which ranged from 13pS up to 112pS and they also appeared to be insensitive to both pentobarbital and diazepam. Despite spontaneous channel activity reversing at a $-V_p$ of 0mV, it is possible that these channels are not mediated by GABA. Only in one case did the mean currents increased in the presence of 1µM diazepam.
6.3.4.2. CA1 Region

For comparison, spontaneous currents from the CA1 region of the hippocampus were also examined. In 94 patches, 62 (66%) displayed spontaneous currents and the other 32 (34%) exhibited no detectable channel activity. Of the 62 patches exhibiting spontaneous currents, multiple conductance states were measured in 24 patches while the other 38 patches exhibited spiky channel activity. Of those 24 patches, conductance states ranged from 14pS to 70pS and the most common main conductance state was about 21pS. Illustrated in Figure 6.13.A. are four continuous current traces (each 400ms in duration) taken from a cell-attached patch at a -V_p of +40mV. These spontaneous currents exhibited multiple conductance states with the most common conductance state of 70pS.

The purpose of examining the CA1 region was to compare the spontaneous currents of this region to the dentate gyrus region. As only one result was obtained in the dentate gyrus with diazepam, only the effects of diazepam were investigated in the CA1 region. In 20 patches exhibiting spontaneous currents, when 1µM diazepam was perfused through the bath, 11 patches showed an increase in the mean currents calculated from 10s current traces. The averaged mean current at a -V_p of +40mV was 0.4±0.2pA. When 1µM diazepam was perfused through the bath, the mean current increased to 1.9±0.3pA (Table 6.3.B.). This increase in the mean currents caused by diazepam is illustrated in Figure 6.13.B. For spontaneous currents recorded from an inside-out patch at a -V_p of +40mV, the mean current measured in a 10s current trace was 0.1pA. With the addition of 1µM diazepam, the mean current increased to 1.7pA. In the other nine patches, diazepam appeared to have no effect on patches that initially showed spontaneous channel activity but went quiet and remained quiet after diazepam application.

In summary, spontaneous currents recorded from the CA1 region of the hippocampus occurred at a higher incidence than spontaneous currents recorded from the dentate gyrus region. The conductance states in the CA1 region were generally smaller than the conductance states measured in the dentate gyrus. In addition, there was a far greater number of spontaneous currents that were modulated by diazepam in the CA1 region than in the dentate gyrus. Furthermore, these preliminary results suggest that the degree...
Figure 6.13. Spontaneous currents seen in the CA1 region of the hippocampus. A. Recorded in a cell-attached patch at a -V_p of +40mV, multiple conductance states could be seen; the most common conductance state was 70pS. B. Recorded from an inside-out patch at -V_p of +40mV, the spontaneous mean current measured in a 10s current trace was 0.1pA. When 1µM diazepam was perfused through the bath, the mean current increased to 1.7pA.
Table 6.3. The effect of 1 µM diazepam on spontaneous currents recorded from the A. dentate gyrus and B. CA1 regions of the hippocampus. Mean currents were measured in both cell-attached (c/a) and inside-out (i/o) patches in either 20s or 10s current traces at a $-V_p$ of +40mV.

### A. DENTATE GYRUS

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### B. CA1

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</tr>
<tr>
<td><strong>average ±1SEM</strong></td>
<td><strong>0.4 ± 0.2</strong></td>
<td><strong>1.9 ± 0.3</strong></td>
<td><strong>4.8</strong></td>
</tr>
</tbody>
</table>
of mean current enhancement also appear to be greater in the CA1 region than in the dentate gyrus.

6.4. DISCUSSION

Dissociated neurons proved to be a very easy and useful preparation for whole-cell experiments but presents many practical problems for single-channel experiments. In contrast to the whole-cell experiments where neurons could be easily lifted off the bottom of the recording bath, this virtue posed problems when excised patches were required. The major obstacle using dissociated neurons, even when using cell-attached configurations, was obtaining a stable patch. As the statistics show, only 17% of attempted patches were successful. This could be due to a number of reasons. Firstly, it is possible that the dissociation method affects the cell membranes of the granule cells. It cannot be determined whether it is the enzymatic treatment or the mechanical trituration that is causing this but it is believed that enzyme treatment does not affect the physiological conditions of the cells (Gray & Johnston, 1985; Huguenard & Algar, 1986; Mody et al., 1989). Secondly, the relatively low number of isolated neurons tested compared to the number of attempted patches on the hippocampal slices could also account for the low statistical success. Thirdly, as stated previously in Chapter 3, seasonal and temperature variations appear to affect the health of the neurons and may have contributed to the low success rate. As a result, an alternative preparation had to be used. Hippocampal slices have been successfully used before to obtain single-channel data from the dentate gyrus region (Birnir et al., 1994) and have many advantages over dissociated neurons. Apart from less physical handling of the preparation, hippocampal slices were much easier and less time consuming to prepare. Slices also presented far many more opportunities to patch which therefore increased the likelihood of obtaining results.

In this study, it was found that single channels activated by GABA had multiple conductance states that ranged from as low as 8pS to beyond 100pS. Multiple conductance states have been detected in many native tissues (Hamill et al., 1983; Segal
& Barker, 1984; Gray & Johnston, 1985; Bormann & Clapham, 1985; Allen & Alburquerque, 1987; Bormann et al., 1987; Bormann, 1988; Bormann & Kettenmann, 1988; Weiss et al., 1988; Macdonald et al., 1989a; Smith et al., 1989; Moss et al., 1990; Mathers, 1991; Yeh et al., 1991; Guyon et al., 1999) as well as recombinant GABA<sub>A</sub> receptors (Blair et al., 1988; Verdoom et al., 1990; Angelotti & Macdonald, 1993; Saxena et al., 1994; Fisher & Macdonald, 1997). On average, currents activated by 0.5µM GABA appeared to have three or four conductance states with a most common conductance state of about 50pS. High conductance states are not unusual as Smith et al. (1989) observed a GABA-activated conductance state of 71pS in cultured mouse spinal cord neurons. As indicated by the different conductance states exhibited in recombinant GABA<sub>A</sub> receptors (Blair et al., 1988; Verdoorn et al., 1990; Angelotti & Macdonald, 1993; Saxena et al., 1994; Kaneda et al., 1995; Fisher & Macdonald, 1997), the range and magnitude of conductances states found could be attributed to the subunit composition of the GABA<sub>A</sub> receptor that is unique to both tissue and animal.

While no detailed current-voltage studies were performed, comparing the conductances of currents activated by GABA at both depolarised and hyperpolarised potentials indicated inward or outward current rectification or no current rectification at all. These results support the earlier findings by Birnir et al. (1994) where both outwardly rectifying currents and non-rectifying currents were observed. In addition, these mixed rectifying single-channel currents also support the inwardly and outwardly rectifying currents observed in the whole-cell experiments. Currently only the α subunit has been implicated in the nature of rectification (Granja et al., 1998). As with the whole-cell results, those channels exhibiting inwardly rectifying currents are likely to contain the α<sub>4</sub> subunit (Granja et al., 1998) while the other channels exhibiting outwardly rectifying currents, and possibly the non-rectifying currents, probably contain other α subunit isoforms.

In the presence of GABA, pentobarbital shifted the open time and burst time frequency histograms to longer times while the closed time frequency histograms were unchanged. Two exponential components were fitted to the open and burst time frequency histograms while only one exponential component was fitted to the closed time histograms. Macdonald et al. (1989b) found that pentobarbital did not increase the mean
open time by changing the time constants but instead by increasing the relative frequency of occurrence of the slowest time constant. In contrast, the opposite was observed here where the faster time constant was replaced by a much longer one and the probability amplitudes associated with these time constants were much smaller than with GABA alone. In agreement with their results, pentobarbital had no effect on the time constant and probability amplitude for the closed time histograms. In the presence of pentobarbital, the time constants fitted to the burst time histograms were longer and the probability amplitudes unchanged compared to GABA alone. They found pentobarbital had little effect on the first two time constants but the third time constant was significantly longer. In addition, the probability amplitudes were also significantly altered with an increase in the probability amplitude of the third component but decreases in the probability amplitudes of the two other components. They concluded that pentobarbital increases the relative frequency of the third burst component and alters the rate constants which regulate the open states. The preliminary results here show that pentobarbital promoted longer bursts as well as an increase in the frequency of these bursts.

In contrast to previous observations (Study & Barker, 1981; Mathers, 1987; Bormann, 1988; Twyman et al., 1989; Macdonald et al., 1989b), both pentobarbital and diazepam increased the maximum conductance state of the channel activated by GABA. Two recent studies (Eghbali et al., 1997; Guyon et al., 1999) showed diazepam increasing the maximum conductance state. Eghbali et al. (1997) proposed many possible causes for this conductance increase but favoured the idea of multiple diazepam binding sites that when activated, promote synchronous openings and closings of clustered pores which is seen as an increase in conductance. Guyon et al. (1999) observed that there was an increase in the occupancy of the higher conductance states while there was a decrease in the lower conductance states. This study supports that observation (see Figure 6.9.). It is possible that the conductance increase seen with pentobarbital could be mediated by a similar mechanism. As there was some diazepam sensitivity observed at the single-channel level in these neurons, this could account for the small increase in the peak current amplitude observed in the whole-cell experiments.

It is evident from the high percentage of spiky currents that these channels may be just as important as the more commonly reported square-like channels. However, it cannot be
determined whether they are from the same or different channels. It is possible that the 
subunit composition of these spiky channels may be the cause of such characteristic 
kinetics. It has been generally found that receptors lacking a γ subunit, confer single-
channel currents that have shorter open lifetimes. In human embryonic kidney cells, 
Verdoorn et al. (1990) expressed the α1β2 and α1γ2 subunit combinations and found that 
the α1β2 channels had a lower most common conductance state as well as shorter mean 
open times than the α1γ2 channels. Similar results were also obtained by Angelotti & 
Macdonald (1993). They transfected mouse L929 cells with a α1β1 and α1β1γ2s subunit 
combination and observed that on average, the α1β1γ2s channels had a higher most 
common conductance state and were opened almost three times as long as the α1β1 
channels. Furthermore, Fisher & Macdonald (1997) found that by expressing the δ and 
γ2L subunits with α1 and β3 in mouse L292 cells, cells expressing the α1β3δ combination 
tended to exhibit channels that had shorter open times than α1β3γ2L channels. When 
exponentials were fitted to the open time distributions, they found that the α1β3δ 
receptors lacked the longest open state. As both the δ and γ subunits are expressed in the 
dentate gyrus, they could confer the different single-channel kinetics observed here; 
several studies have indicated that the δ and γ subunits do not co-localise together 
(Shivers et al., 1989; Whiting et al., 1995; Quirk et al., 1995). This will be explored 
further in Chapter 7. Spiky channels were also be modulated by pentobarbital and 
diazepam. Because no clear conductance states could be measured, mean currents were 
calculated over the longest duration possible. Compared to currents activated by GABA 
alone, both pentobarbital and diazepam increased the mean currents.

It was also observed that not all patches could be activated by GABA and/or modulated 
by pentobarbital and diazepam. It is possible that the cell-attached or inside-out patch did 
not contain any GABA<sub>A</sub> receptors or if there were GABA<sub>A</sub> receptors present, perhaps 
the receptor was not activated. Results from Chapter 4 showed that receptors active 
during peak currents had a lower affinity for GABA than receptors active during plateau 
currents. As will be discussed in Chapter 7, if these peak and plateau currents were 
generated by two different receptor populations that had different affinities for GABA, 
perhaps one population of receptors were not activated by 0.5µM GABA. In addition, 
there were other patches that initially showed channel activity but went quiet and 
remained quiet after numerous applications of either diazepam or pentobarbital. It was
not possible to determine if pentobarbital and diazepam depressed channel activity as channel activity ceased before application of these drugs were possible. Although these channels were Cl\(^-\) selective as currents reversed at a \(-V_P\) of 0mV, it is possible that these channels were not mediated by GABA. Alternatively, these channels could represent another \(\text{GABA}_A\) receptor population.

Spontaneous channel activity has been documented in various tissues but no detailed studies have yet been performed until recently (Hamill \textit{et al.}, 1983; Huck & Lux, 1987; Weiss \textit{et al.}, 1988; Macdonald \textit{et al.}, 1989a; Otis & Mody, 1992a; Häusser & Clark, 1997; Hollrigel \textit{et al.}, 1998). In this study, spontaneous currents occurred less frequently in the dentate gyrus (49\%) than in CA1 neurons (66\%). Furthermore, spontaneous currents in the dentate gyrus were generally insensitive to diazepam while CA1 neurons were more sensitive to diazepam. It is possible that these spontaneous currents recorded in the dentate gyrus are not mediated by \(\text{GABA}_A\) receptors. However, the whole-cell results have established the presence of the \(\alpha_4\) subunit and receptors containing this subunit are insensitive to diazepam (Huh \textit{et al.}, 1996; Benke \textit{et al.}, 1997). In addition to the greater number of CA1 spontaneous currents that were modulated by diazepam, there were also indications that the magnitude of this current enhancement was greater in the CA1 than in dentate gyrus neurons. Hamill \textit{et al.} (1983) speculated that such spontaneous currents were activated by GABA released from nerve terminals still attached to the patch. Although not thoroughly studied, Huck & Lux (1987) supported this idea as spontaneous currents observed in an outside-out patch could be inhibited by bicuculline. Similarly, studies investigating spontaneous inhibitory postsynaptic currents have also been explained by the overspill of synaptically released GABA (Alger & Nicoll, 1982; Otis & Mody, 1992a,b; Brickley \textit{et al.}, 1996). The release of endogenous amino acids such as GABA, glutamate, aspartate and taurine have been observed in hippocampal slices under cell-damaging conditions (Saransaari & Oja, 1998), such as slicing and hypoxia during patching. If GABA is released and subsequently trapped in the patch pipette when a cell-attached or inside-out patch is made, GABA could possibly activate \(\text{GABA}_A\) receptors in the slices. However, this is unlikely as positive pressure was applied when making a cell-attached or inside-out patch in hippocampal slices. In addition, this could not explain spontaneous currents observed in isolated neurons. Therefore, it would appear that these currents are produced by receptors that
spontaneously open in the absence of GABA and may play an important role in providing background tonic inhibition (Otis & Mody, 1992b; Soltesz et al., 1995; Brickley et al., 1996; Häusser & Clark, 1997). Especially with CA1 neurons, diazepam enhanced spontaneous channel activity and it is possible that these spontaneously opening receptors may be a target for benzodiazepines as well as other drugs such as general anaesthetics.

In conclusion, single-channel currents activated by GABA were modulated by pentobarbital and diazepam. The most striking result was that these modulators increased the maximum conductance state to a higher level. Spontaneous channel activity was more abundant in CA1 neurons and were modulated by diazepam to a greater extent than in dentate gyrus neurons.
CHAPTER 7

GENERAL DISCUSSION
7.1. SUMMARY

To recapitulate, the major findings from this study are:

- A new dissociation method was described which produced relatively high numbers of healthy neurons. Whole-cell seals were easily obtained and rapid perfusion of drugs onto isolated granule cells permitted fast activation of GABA\_A receptors.

- Currents activated by 100µM GABA displayed a range of characteristics including peak current amplitudes, current half-decay times and current rectification.

- Receptors producing peak currents were pharmacologically different from receptors producing plateau currents. Compared to those receptors producing plateau currents, receptors producing peak currents had a lower affinity for GABA and Zn\_2\+ but a higher affinity for bicuculline.

- Two exponential components were fitted to currents activated by GABA. Bicuculline and Zn\_2\+ differentially affected these exponential components and supported the results obtained from the dose-response curves. In contrast, pentobarbital and diazepam had no effect on any of the exponential components.

- Pentobarbital had several different effects on currents activated by GABA. Low micromolar concentrations depressed, high micromolar concentrations enhanced and high millimolar concentrations of pentobarbital markedly depressed GABA-activated currents. The GABA\_A receptor could not be directly activated by 100µM pentobarbital alone.

- GABA activated two characteristically different types of single channel activity in the dentate gyrus. The maximum conductance state or the mean current was
increased by the presence of pentobarbital or diazepam. Spontaneous channel activity was more abundant in the CA1 region than in the dentate gyrus. CA1 spontaneous currents were also modulated to a greater extent than in the dentate gyrus.

Reviewed in Chapter 1, there have been many studies that have demonstrated the strong presence of the α2, α4, β1, β3 and γ2 subunits and slightly less of the α1, β2 and δ subunits in the dentate gyrus (Houser et al., 1988; Shivers et al., 1989; Wisden et al., 1992; Laurie et al., 1992a,b; Benke et al., 1994; Fritschy et al., 1994; Sperk et al., 1997). In total, there are at least ten subunit isoforms present in the adult dentate gyrus and it is possible that several subunit combinations exist to produce several functional GABA_A receptors. The results obtained in this study have indicated the presence of at least two GABA_A receptor populations.

Recent studies have reported that the γ and δ subunits do not co-exist together (Shivers et al., 1989; Quirk et al., 1995; Araujo et al., 1998). Therefore, associated with either the γ2 or δ subunits, there could be the α4, α2 or α1 subunits with any one of the three β subunits. In situ hybridisation studies have detected co-localisation of the α1, β1 and γ2 subunit mRNAs in granule cells of the dentate gyrus (Möller et al., 1990; Malherbe et al., 1990b) while immunoprecipitation studies have shown a strong association of the β3 subunit to the α2 but not the α1 subunit (Benke et al., 1994). Nusser et al. (1995) demonstrated that there was a compartmentalised distribution of the GABA_A receptor on the surface of the dentate gyrus granule cell and showed strong immunoreactivity of the α1 and β2/β3 subunits on the soma, axon initial segment, proximal and distal dendrites. Benke et al. (1997) also showed that immunopurified receptors containing the α4 subunit were immunoreactive with the α1, α2, α3, β23 and γ2 subunits but not to the α5, γ1 and γ3 subunits. These results together with results obtained from pharmacological studies of recombinant GABA_A receptors, the presence of certain subunit isoforms can be predicted for the receptors examined here.

Considering the pharmacological differences between the peak and plateau currents, each of these currents can be described by a GABA_A receptor containing different subunit
isoforms as illustrated in Figure 7.1. For ease of discussion, the terms defined in Section 4.3.6. will be used here. The “fast receptors” are defined as the receptors producing peak currents and the “slow receptors” are defined as those receptors producing plateau currents.

There are strong indications that the \( \alpha_4 \) subunit is present in both “fast” and “slow” receptors. Firstly, diazepam only slightly enhanced peak and plateau GABA-activated currents (Figure 4.20.B.), 100\( \mu \)M pentobarbital did not directly activate the GABA\( \text{A} \) receptor (Figure 5.5.B.) and plateau currents were inwardly rectifying (Figure 4.23.A.). When the \( \alpha_4 \) subunit is in combination with a \( \beta \) or a \( \gamma_2 \) subunit, these receptors are insensitive to diazepam (Wisden et al., 1991; Benke et al., 1997). It has also been reported that receptors containing the \( \alpha_4 \) subunit cannot be directly activated by pentobarbital while receptors containing other \( \alpha \) subunits can be directly activated by pentobarbital (Wafford et al., 1996). The \( \alpha_4 \) subunit has also been shown to produce inwardly rectifying currents (Granja et al., 1998). However, one outwardly rectifying current was observed and this would indicate the presence of another \( \alpha \) subunit (Mohler et al., 1990; Malherbe et al., 1990b; Nusser et al., 1995; Escalpeze et al., 1996).

The “fast receptors” are predicted to contain the \( \gamma \) subunit based on some insensitivity of GABA-activated peak currents to \( \text{Zn}^{2+} \) (Figure 4.14.). Receptors containing the \( \gamma \) subunit have been reported to be insensitive to the depressant effects of \( \text{Zn}^{2+} \) (Draguhn et al., 1990; Smart et al., 1991; Celentano et al., 1991; Schonrock & Bormann, 1993; Saxena & Macdonald, 1994; Chang et al., 1995). However, at maximal \( \text{Zn}^{2+} \) concentration, approximately half of the peak current could be depressed by \( \text{Zn}^{2+} \) which would suggest the absence of the \( \gamma \) subunit. In addition, those receptors containing the \( \gamma_2 \) subunit are likely to be the \( \gamma_{2L} \) splice variant as ethanol enhanced currents activated by GABA (Figure 4.20.C.; Wafford et al., 1991). It is also possible that “fast receptors” could contain the \( \delta \) subunit as GABA-activated peak currents were fairly insensitive to diazepam (Figure 4.20.B.; Saxena & Macdonald, 1994). In contrast, the “slow receptors” are likely to contain the \( \delta \) subunit because GABA-activated plateau currents were abolished by \( \text{Zn}^{2+} \) (Figure 4.14.; Saxena & Macdonald, 1994).
Figure 7.1. Based on the functional and pharmacological characteristics obtained, subunit combinations were predicted for the receptors producing peak currents and the receptors producing plateau currents.
Furthermore, Nusser et al. (1998) examined the segregation of different GABA<sub>A</sub> receptors on membranes of cerebellar granule cells. They showed that δ-subunit containing receptors were localised exclusively to extrasynaptic membranes while there was a high occurrence of γ-subunit containing receptors in synaptic membranes. They proposed that phasic inhibition was mediated by these γ-subunit containing receptors while the δ-subunit containing receptors mediated tonic inhibition. This arrangement fits in well with the subunit combinations proposed here for the “fast” and “slow” receptors.

However, it may not be that simple. There were also indications that “slow receptors” could contain a γ subunit since single channels activated by GABA were enhanced by diazepam (Figures 6.9 & 6.10.). It is unlikely that this current enhancement by diazepam was generated by “fast receptors” considering the time frame of these single channel experiments. Diazepam was applied well after five minutes of making a cell-attached or inside-out patch and these “fast receptors” would have already gone into a desensitised state. As the γ and δ subunits are thought to not co-exist with each other in a single receptor, it is possible that there are two “slow receptors” each containing either a γ or δ subunit. Single channel studies have showed that receptors containing the γ subunit have longer mean open times compared with receptors that contain the δ subunit or lack the γ subunit (Verdoorn et al., 1990; Angelotti & Macdonald, 1993; Fisher & Macdonald, 1997). Both long (square-like) and short (spiky) mean open time single channels were observed in this study and they could also be modulated by diazepam (Figures 6.9 & 6.10. and Table 6.2.). However, this would suggest that receptors containing the δ can also be modulated by diazepam which contradict Saxena & Macdonald’s (1994) results.

It would be expected that single channels activated by 0.5µM GABA were generated by the “slow receptors”; according to the whole-cell results, “slow receptors” had a higher affinity for GABA than “fast receptors” (Table 4.1.). However, there is evidence that suggest that the presence of certain α subunits can influence GABA sensitivity (Levitan et al., 1988a,b; McKernan et al., 1991; Möhler et al., 1992; Verdoorn, 1994; Gingrich et al., 1995; Knoflach et al., 1996; Benke et al., 1997). Thus, it is possible that a minor population of “fast receptors” exhibiting high affinity for GABA could be present. If this is true then these “fast receptors” that also contain the γ subunit could explain the GABA-activated single channel current enhancement by diazepam. More than one α subunit has also been reported to exist in a single receptor and it is possible that
depending on the subunit arrangement of these receptors, the affinities for GABA and other drugs may vary (Duggan et al., 1991; Pollard et al., 1993; Verdoorn, 1994; Ebert et al., 1994; Nusser et al., 1996b; Serafini et al., 1998).

While only one possibility was discussed to explain the pharmacological results presented here, there is another possibility that should not be disregarded. It is possible that desensitised receptors could exhibit different pharmacological and kinetic characteristics. Both Celentano & Wong (1994) and Berger et al. (1998) observed that fast and slow components fitted to currents activated by GABA yielded different affinities for GABA. However, when pre-desensitisation experiments were performed where low concentrations of GABA were pre-applied, both the fast and slow components were not significantly different suggesting the presence of a single receptor population. However, neither investigated the effects of drugs on these components although Berger et al. (1998) did examine the effect of Zn$^{2+}$. They showed that the Zn$^{2+}$ IC$_{50}$ values obtained for the two components were not significantly different which supported their earlier conclusion of only a single receptor population. This differs from the results presented here where GABA-activated peak and plateau currents were affected differently by Zn$^{2+}$ (Table 4.1.C.) and therefore suggests the existence of at least two GABA$_A$ receptor populations.

Many studies have indicated the presence of at least 10 subunit isoforms in any one region or in any one cell type. Therefore, the existence of several functional GABA$_A$ receptors consisting of different subunit combinations is feasible. Although RT-PCR would appear to be the next step in identifying what subunit isoforms exist in a single neuron, it would not tell us whether the mRNA is actually translated into a functional receptor nor would it tell us what subunit isoforms combine to produce one or more functional receptors. Thus, pharmacological studies such as the one presented here is really the only way to identify subunit isoforms. In the future, perhaps drugs will be found or synthesised to target specific subunit isoforms which will make identification easier and clearer.
7.2. CONCLUSIONS

Many interesting results were obtained in this study that include the possibility of multiple functional receptor types. Using both whole-cell patch clamp techniques and single-channel recordings, these receptors had different affinities for GABA and drugs that act on the GABA<sub>A</sub> receptor such as bicuculline, Zn<sup>2+</sup>, pentobarbital and diazepam. The obvious advantage of having multiple GABA<sub>A</sub> receptor populations is diversity that would contribute to different mechanisms of inhibitory control.

As more functional and pharmacological information is obtained, we can better understand the GABA<sub>A</sub> receptors of the dentate gyrus and certainly the whole central nervous system. The hippocampus has been implicated in many physiological and pathological processes and together with the wide variety of clinical drugs that act on the GABA<sub>A</sub> receptor, it is important to unveil the secrets of this receptor. Perhaps in the future we will be able to relate a GABA<sub>A</sub> receptor subunit combination to a pathological disease and maybe subunit-specific drugs can be fabricated to treat these diseases.
CHAPTER 8
APPENDICES
APPENDIX I

Survey of dissociation techniques used in the CNS of various animal species. Refer to list at the end for abbreviations.

<table>
<thead>
<tr>
<th>Author</th>
<th>Animal Age Tissue</th>
<th>Technique</th>
<th>Solutions</th>
<th>Enzyme (Units or mg or µg per mL)</th>
<th>Pre-Incubation Conditions</th>
<th>Enzymatic Treatment Conditions</th>
<th>Post-Enzyme</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Numann &amp; Wong</td>
<td>Guinea pig adult</td>
<td>700µm slices cut in pieces</td>
<td>PSS</td>
<td>Papain: 15mg</td>
<td>-</td>
<td>2 hours RT carbogen stirred</td>
<td>triturated</td>
<td>intracellular recordings</td>
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<tr>
<td>(1984)</td>
<td>CA1 pyramidal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gray &amp; Johnston</td>
<td>Guinea pig adult</td>
<td>300µm slices</td>
<td>PSS</td>
<td>Pronase: 0.5-1mg</td>
<td>30-45mins 32°C oxygenated</td>
<td>30mins 32°C oxygenated</td>
<td>triturated</td>
<td>single-channel (i/o)</td>
</tr>
<tr>
<td>(1985)</td>
<td>Hippo pyramidal</td>
<td></td>
<td>pH 7.3</td>
<td>Thermolysin: 0.3mg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Engelhardt et al.</td>
<td>Pig adult</td>
<td>anterior horns dissected out</td>
<td>pH 7.4</td>
<td>no enzyme</td>
<td>-</td>
<td>-</td>
<td>triturated</td>
<td>morphology</td>
</tr>
<tr>
<td>(1985)</td>
<td>Spinal motoneurones</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>filtered</td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Species</td>
<td>Tissue/Cell Type</td>
<td>Preparation Method</td>
<td>Buffer/Condition</td>
<td>Enzyme</td>
<td>Time (Min./Hours)</td>
<td>Stimulus</td>
<td>Technique</td>
</tr>
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<td>------------------------</td>
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<tr>
<td>Kay &amp; Wong (1986)</td>
<td>Guinea pig</td>
<td>650µm slices</td>
<td>PIPES, pH 7</td>
<td>Trypsin: 0.6-0.8mg in PIPES</td>
<td>PIPES</td>
<td>1.5 hours, 32°C, 100% O₂, stirred</td>
<td>microscopy in HEPES</td>
<td></td>
</tr>
<tr>
<td>Huguenard &amp; Alger (1986)</td>
<td>Guinea pig</td>
<td>700µm slices</td>
<td>PSS</td>
<td>Papain: 1.5mg OR Trypsin: 1mg</td>
<td>-</td>
<td>1-3 hours 31°C oxygenated</td>
<td>trituated</td>
<td>whole-cell</td>
</tr>
<tr>
<td>Huettner &amp; Baughman (1986)</td>
<td>Rat</td>
<td>500µm slices</td>
<td>EBSS</td>
<td>Papain: 20U Cysteine: 1mM EDTA: 5mM</td>
<td>-</td>
<td>1.5 hours 37°C carbogen gently stirred</td>
<td>trituated</td>
<td>IHC Intracellular recordings Microscopy</td>
</tr>
<tr>
<td>Allen &amp; Albuquerque (1987)</td>
<td>Rat embryo</td>
<td>minced</td>
<td>HEPES/PBS pH 7.3</td>
<td>Trypsin: 0.25%</td>
<td>-</td>
<td>15mins 35.5°C</td>
<td>trituated</td>
<td>single-channel (i/o)</td>
</tr>
<tr>
<td>Sah et al. (1988)</td>
<td>Guinea pig</td>
<td>600µm slices</td>
<td>KREBS</td>
<td>Papain: 24U Cysteine: 5mM</td>
<td>-</td>
<td>~90mins RT rapidly stirred</td>
<td>trituated</td>
<td>whole-cell</td>
</tr>
<tr>
<td>Shen et al. (1988)</td>
<td>Turtle</td>
<td>100-400µm slices</td>
<td>HEPES</td>
<td>Papain: 1.6mg Cysteine: 1mM</td>
<td>-</td>
<td>5-15mins RT gently stirred</td>
<td>trituated</td>
<td>whole-cell</td>
</tr>
<tr>
<td>Authors</td>
<td>Species</td>
<td>Age</td>
<td>Slice Thickness</td>
<td>Medium</td>
<td>Enzyme(s)</td>
<td>pH</td>
<td>Temp</td>
<td>Ox Gen</td>
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<tr>
<td>Alho et al.</td>
<td>Rat</td>
<td>1 day old</td>
<td>400µm slices</td>
<td>not stated</td>
<td>Trypsin: 0.025%</td>
<td>-</td>
<td>not stated</td>
<td>triturated in 0.01% DNase + 0.05% Tissue Culture</td>
</tr>
<tr>
<td>Kaneda et al.</td>
<td>Rat</td>
<td>3 days - adult</td>
<td>500µm slices</td>
<td>HEPES pH 7.4</td>
<td>Collagenase: 0.1% Pronase E: 0.2%</td>
<td>60mins 37°C 100% O₂</td>
<td>90-150mins 37°C 100% O₂</td>
<td>15-60mins 37°C 100% O₂ dissect region and triturate</td>
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<td>Rat &amp; cat</td>
<td>Adult</td>
<td>dorsal laminectomy</td>
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<td>Trypsin: 0.5% Collagenase: 0.5%</td>
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<td>Mody et al.</td>
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<td>Adult and aged</td>
<td>400-450µm slices</td>
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<td>Pronase: 1.5mg</td>
<td>1 hour 32°C carbogen</td>
<td>one slice 30mins 32°C carbogen</td>
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<td>600µm slices cut in pieces</td>
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<td>~90mins RT carbogen</td>
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<td>650µm slices isolate region</td>
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<td>Trypsin inhibitor then triturated</td>
<td>whole-cell labelling</td>
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<td>HEPES</td>
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<td>1-7 hours RT → 30°C carbogen</td>
<td>1-5 seconds; RT microvibrate slice directly</td>
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<td>Rat 12-18 days Nucleus tractus solitarius</td>
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<td>KREBS</td>
<td>Dispase: 0.2mg</td>
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<td>1 hour 37°C</td>
<td>micro-punch region then triturated</td>
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<td>ganglia cleaned and cut into pieces</td>
<td>MEM buffered with HEPES</td>
<td>Collagenase: 266U Trypsin: 11000U DNase: 3µg</td>
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<td>Collagenase 15mins at 37°C gently agitated Centrifuged → Trypsin + DNase 30mins at 37°C</td>
<td>triturated</td>
<td>whole-cell single-channel</td>
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<td>Tse et al. (1992)</td>
<td>Rat 25-30 days CA1 astrocytes</td>
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<td>morphology whole-cell</td>
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<td>Rat 4-7 days Basal forebrain, hippo (CA1-3)</td>
<td>400µm slices cut in pieces</td>
<td>GBSS HBBS Trypsin: 0.25% in HBBS</td>
<td>regions dissected out in GBBS</td>
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<td>I’cytochem whole-cell</td>
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<td>Papain: 19U Cysteine: 0.01mM</td>
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<td>90mins 28°C HEPES oxygenated</td>
<td>chopped in HEPES at RT for 6-8 hours, triturated</td>
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<td>Rat 1-3 months Globus pallidus</td>
<td>450-550µm slices, region dissected out</td>
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<td>ACSF (pH 7.4) HEPES</td>
<td>Pronase: 1-1.5mg</td>
<td>1 hour RT oxygenated</td>
<td>30-45mins 35°C oxygenated</td>
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<td>Trypsin: 0.4mg Collagenase: 0.3mg DNase: 0.1mg</td>
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<td>vigorous shaking then + 0.4mg Ti</td>
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<td>Rat and Cat 10-21d and 2-5m Sensorimotor cortex pyramidal</td>
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<td>Papain: 19U Cysteine: 0.01mM</td>
<td>1 hour 30-32°C carbogen</td>
<td>90 mins 28°C HBSS</td>
<td>cut into pieces, 21-24°C, HBSS carbogen</td>
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<td>Age/Slice Size</td>
<td>Tissue/Region</td>
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<td>Enzymes 2</td>
<td>Enzymes 3</td>
<td>Duration</td>
<td>Temperature</td>
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<td>Kawahara et al. (1993)</td>
<td>Rat 21 days</td>
<td>500µm slices</td>
<td>SCN neurons</td>
<td>Pronase: 0.02% Thermolysin: 0.02%</td>
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<td>30 mins at 37°C with each enzyme</td>
<td>SCN punctured whole-cell</td>
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<td>Hara et al. (1993)</td>
<td>Rat 1-2 weeks</td>
<td>400µm slices</td>
<td>PSS</td>
<td>Pronase: 0.01% Protease: 0.01%</td>
<td>50 mins RT</td>
<td>20 mins at 31°C with each enzyme</td>
<td>CAI isolated at RT then triturated whole-cell</td>
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<td>Rat 2 weeks</td>
<td>200-500µm slices at RT</td>
<td>ACSF, pH 7.4 HEPES, pH 7.3</td>
<td>Papain: 0.1mg</td>
<td>1 hour 30°C ACSF carbogen</td>
<td>1-7 hours 30°C ACSF carbogen</td>
<td>Local vibro-dissociation to liberate neurons whole-cell</td>
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<td>French-Mullen et al. (1993)</td>
<td>Guinea Pig Mature</td>
<td>cut in pieces</td>
<td>PIPES/PSS: pH 7 HEPES/DMEM</td>
<td>Trypsin: 0.5mg</td>
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<td>40-50 mins 37°C O₂</td>
<td>&lt;2 hours RT then triturated in HEPES/DMEM whole-cell</td>
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<td>Baraban &amp; Lothman (1994)</td>
<td>Rats 5-30 days</td>
<td>400-450µm slices</td>
<td>ACSF</td>
<td>Protease: 30mg</td>
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<td>33 mins PIPES carbogen</td>
<td>30-45 mins PIPES carbogen triturated whole-cell</td>
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<td>Takahashi et al. (1994)</td>
<td>Rat 7-12 days Visual cortex</td>
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<td>HEPES pH 7.2-7.4</td>
<td>Pronase: 0.14mg Thermolysin: 0.14mg</td>
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<td>30mins at 31°C with each enzyme</td>
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<td>Aguayo et al. (1994)</td>
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<td>HEPES, pH 7.4</td>
<td>Trypsin: 0.25%</td>
<td>-</td>
<td>20-25mins 36.5°C</td>
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<td>Fraser et al. (1995)</td>
<td>Rat 2-6 weeks CA1 astrocytes</td>
<td>500µm slices</td>
<td>ACSF, pH 7.35</td>
<td>Papain: 24-28U Cysteine: 1mg/100U Papain Kynurenic Acid: 1mM</td>
<td>-</td>
<td>1 hour carbogen stirred</td>
<td>region dissected then triturated at RT when needed</td>
<td>Fluorometry IHC whole-cell</td>
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### List of Abbreviations for Appendix I

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ACSF</td>
<td>artificial cerebrospinal fluid</td>
<td>Hippo</td>
<td>hippocampus</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
<td>ICC</td>
<td>immunocytochemistry</td>
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<td>c/a</td>
<td>cell-attached patch</td>
<td>IHC</td>
<td>immunohistochemistry</td>
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<tr>
<td>d</td>
<td>days</td>
<td>i/o</td>
<td>inside-out patch</td>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
<td>m</td>
<td>months</td>
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<td>DG</td>
<td>dentate gyrus</td>
<td>MEM</td>
<td>Minimum Essential Medium</td>
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<td>DRG</td>
<td>dorsal root ganglion</td>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>EBSS</td>
<td>Earle’s Balanced Salt Solution</td>
<td>PIPES</td>
<td>piperazine-N,N’-bis[2-ethanesulfonic acid]</td>
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<td>FCS</td>
<td>Fetal Calf Serum</td>
<td>PSS</td>
<td>Physiological Salt Solution (saline)</td>
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<td>GBSS</td>
<td>Gey’s Balanced Salt Solution</td>
<td>RT</td>
<td>room temperature</td>
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<td>Hank’s Balanced Salt Solution</td>
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<td>N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid</td>
<td>TI</td>
<td>Trypsin Inhibitor</td>
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APPENDIX II

The dissociation conditions tested on brain preparations and assessed based on the appearance of the granule cell and success in patch clamping. A score between 0 and 3 are given; 0 denotes no neurons and 3 denotes many healthy neurons liberated. One line across is one set of conditions tested; often the same brain preparation is split into two and another set of conditions imposed on this second set.

- Slices (listed in µm) or whole dentate gyrus cut into several pieces (listed as “whole DG”) were used.
- Occasionally the dentate gyrus (→ DG) was removed from the slices prior to enzyme treatment.
- Two types of papain were used, suspension (S) or powdered (P).
- M-ETOH = mercaptoethanol.
- Three incubation times were used (pre-enzyme, enzyme and post-enzyme) - where incubation sessions were not done, these are shown as “-”.
- Occasionally enzyme incubations were performed at room temperature (RT) and/or with stirring via a magnetic stirrer.

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<th>#</th>
<th>Preparation (slice / whole)</th>
<th>Age (days)</th>
<th>Papain (Units/mL)</th>
<th>Cysteine (mM)</th>
<th>EDTA (mM)</th>
<th>M-ETOH (µM)</th>
<th>CaCl₂ (mM)</th>
<th>Temp (°C)</th>
<th>Time (mins)</th>
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<td>30, 30, -</td>
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* Protease inhibitors were added to the 30 minute post-incubation session. Concentrations of either 174µg/mL PMSF, 50µg/mL Antipain or 0.5µg/mL Leupeptin were tested.
REFERENCES


REFERENCES


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