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receptor*

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An electrophysiological study of the interaction  
between Fenamate NSAIDs and the GABA<sub>A</sub>  
receptor.

Debra Patten  
Submitted for the Degree of Ph.D.

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19 JUL 2000



Dept. of Biological Sciences  
University of Durham  
March 1999

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### *Candidates Declaration*

The work presented in this thesis was composed by me and is an accurate record of the experimental work undertaken by me. This work, under the supervision of Dr. Robert Francis Halliwell, has been carried out at the Department of Biological Sciences, at the University of Durham, and has not been previously submitted for a higher degree.

"The copyright of this thesis rests with the author. No quotation from it should be published without their prior written consent and information derived from it should be acknowledged."

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**Debra Patten**

**An Electrophysiological Study of the Interaction between Fenamate NSAIDs and the GABA<sub>A</sub> receptor.**

**Submitted for the Degree of Ph.D. 1999**

**Abstract**

The effects of certain NSAIDs were determined on agonist-evoked responses recorded from rat neurones maintained *in vitro* using electrophysiological techniques.

Initially, the rat isolated vagus and optic nerves were employed. Alphaxalone, pentobarbitone, propofol and the NSAID, mefenamic acid (MFA), potentiated GABA-evoked responses of the vagus nerve. Propofol (1-100 $\mu$ M) selectively potentiated GABA and glycine-evoked responses of the rat vagus and optic nerves, but had little effect on nicotinic acetylcholine-,  $\alpha$ , $\beta$ -methylene-ATP or 5-hydroxytryptamine-mediated responses.

The interaction between MFA and ligand-gated receptors was investigated further using voltage-clamped rat hippocampal neurones maintained in culture. MFA (3-100 $\mu$ M) selectively, concentration-dependently and reversibly potentiated GABA-evoked responses, consistent with the observations made using the vagus nerve. MFA (3-100 $\mu$ M) however had little or no effect on glycine, AMPA, kainate or NMDA-receptor mediated responses.

A final series of experiments investigated the site and molecular mechanism of the interaction between MFA and the GABA-gated chloride ion channel. The potentiating effects of MFA (and other fenamates) were not the result of prostaglandin synthesis inhibition, since other NSAIDs did not modulate the GABA<sub>A</sub> receptor (GR). The actions of MFA were not mediated *via* the benzodiazepine site of the GR, nor were they due to inhibition of GABA-uptake or membrane perturbation. The modulatory effects of MFA were not use-dependent, but the potentiating effects of MFA were voltage-dependent, where the potentiation was 3-fold greater at -100mV than at +40mV, with no change in the equilibrium potential for GABA. MFA activated a current, in the absence of GABA. Hippocampal neurones varied in sensitivity to modulation by MFA and the anticonvulsant, loreclezole, which may indicate a degree of sub-unit selectivity.

These data are discussed in relation to the possible site and mechanism of action of fenamates at the GR, their similarities with other positive modulators of the GR and the neurophysiological implications of these findings.

## **CHAPTER ONE: GENERAL INTRODUCTION**

### **1.1. An Overview of the Hypothesis Addressed in this Thesis**

Non-steroidal anti-inflammatory drugs (NSAIDs) are prescribed for their analgesic, anti-inflammatory and anti-pyretic properties (Orme, 1990). Due to their efficacy and relative lack of toxicity, NSAIDs are the most widely consumed drugs world-wide with over 100 million prescriptions per year and an estimated seven-fold higher consumption of non-prescription drugs in the US alone (Roth, 1996). Perhaps, not surprisingly, these drugs are also a common source of self-poisoning and although most cases of NSAID overdose do not result in serious cases of toxicity, there have been some cases of NSAID overdose resulting in severe toxicity.

Mefenamic acid (Ponstan) is the fifth most commonly prescribed NSAID in the UK (McMurray *et al.*, 1987). It is a fenamate NSAID commonly prescribed for the relief of mild to moderate pain including that experienced during dysmenorrhoea and rheumatoid arthritis. The most common side effects observed with mefenamic acid treatment, like other NSAIDs, generally pertain to gastrointestinal disturbances. However, other adverse effects observed include headache, visual disturbances, dizziness, drowsiness and anxiety (Winder *et al.*, 1966). In cases of mefenamic acid overdose, the severity of these adverse drug effects is increased with symptoms of coma and seizures occurring in over one third of all overdose cases (Smolinske *et al.*, 1990). Interestingly, another NSAID, fenbufen, has been reported to dramatically increase the risk of convulsions in humans when co-prescribed with antimicrobial fluoroquinolones (Lietman, 1995).

Almost thirty years ago, Vane (1971) demonstrated that aspirin-like drugs, namely NSAIDs, inhibited prostaglandin synthesis, thus reducing inflammation and thereby symptoms of pain. Subsequently, over 40 NSAIDs used in clinical practice have been found to inhibit prostaglandin synthesis

(Cronstein and Weissman, 1995). In addition to inhibition of prostaglandin synthesis in the periphery, NSAIDs are known to penetrate the central nervous system (CNS; Bannwarth *et al.*, 1989) and have been shown to have direct effects on various neuronal (and non-neuronal) ion channels including non-selective cation channels (Lerma and Del Rio, 1991; Shaw *et al.*, 1995) and chloride channels (White and Alywin, 1990; McCarty *et al.*, 1993). Indeed the fenamate NSAIDs, for example, flufenamic acid and niflumic acid, are routinely used as pharmacological tools to block  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels when investigating  $\text{Ca}^{2+}$  permeable channels in *Xenopus* oocytes (Leonard and Kelso, 1990; White and Alywin, 1990; Vernino *et al.*, 1992 and Séguéla *et al.*, 1993). More recently, a number of the fenamate NSAIDs have also been shown to modulate rat brain  $\gamma$ -aminobutyric acid type-A ( $\text{GABA}_A$ ) receptors expressed in *Xenopus* oocytes (Woodward *et al.*, 1994). Moreover, a preliminary study by Halliwell *et al.*, (1994) demonstrated a marked positive modulation of GABA-mediated currents recorded in rat hippocampal neurones by mefenamic acid.

Modulation of the  $\text{GABA}_A$  receptor, which probably mediates the majority of inhibitory synaptic transmission in the mammalian brain, may have significant behavioural and psychological effects (for reviews see Matsumoto, 1989; Krosggaard-Larsen *et al.*, 1994). Indeed  $\text{GABA}_A$  receptors have been implicated in the regulation of appetite via  $\text{GABA}_A$  receptor modulation in hypothalamic satiety centres, in the mediation of analgesic effects present throughout the brain and spinal cord via pre- and post-synaptic mechanisms and in  $\text{GABA}_A$  receptor-mediated decreases in heart rate and blood pressure. GABA may also play a role in many other physiological and behavioural processes such as arousal, sexual behaviour, coma, stress, anxiety, depression, memory, thermal regulation, muscle relaxation and sleep. Many anaesthetic agents have been shown to produce sedation and anaesthesia probably by enhancing GABA-mediated synaptic transmission (Franks and Leib, 1994). GABA has also been linked with a number of neurological disorders such as schizophrenia, epilepsy, and Alzheimer's disease (for review see Krosggaard-Larsen *et al.*, 1994).

It is well established that antagonists of the GABA<sub>A</sub> receptor, such as bicuculline and picrotoxin are proconvulsant drugs (Curtis and Johnston 1974), and positive allosteric modulators of the GABA<sub>A</sub> receptor such as alphaxalone, propofol and diazepam enhance the inhibitory actions of  $\gamma$ -aminobutyric acid (GABA) and thereby reduce neuronal excitation. It is possible, therefore, that some of the actions of NSAIDs may be mediated through direct effects on the CNS. In particular, coma and/or convulsions, which result from fenamate overdose, suggest an interaction between these drugs and neuronal ligand-gated ion channels.

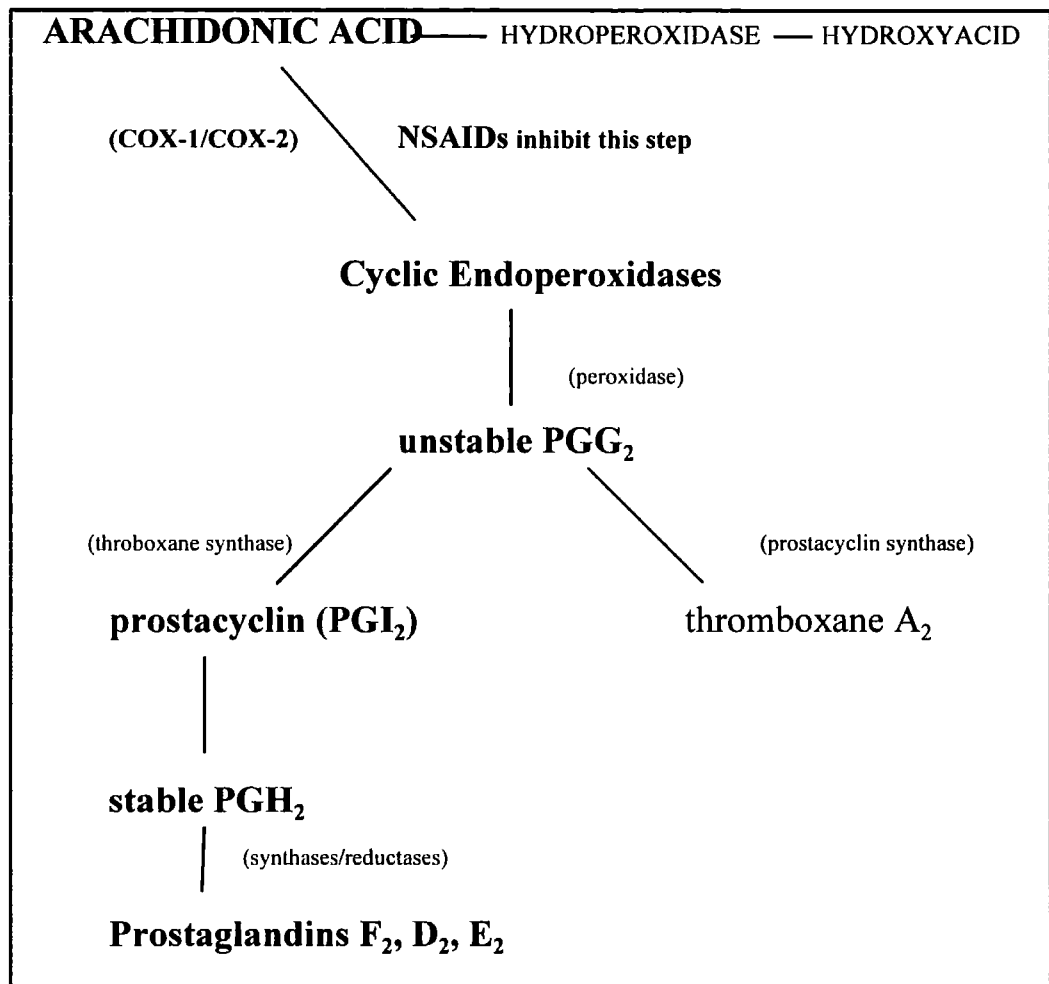
The experiments reported in this thesis examined the effects of selected NSAIDs on the major inhibitory and excitatory amino acid receptors in mammalian CNS neurones. The effects of these NSAIDs were compared to the effects of other experimental and clinically important modulators of these receptors, in an attempt to elucidate, in particular, the mechanism of action of fenamate NSAIDs within the CNS.

## **1.2. Non-Steroidal Anti-inflammatory Drugs (NSAIDs)**

### ***1.2.i. Mechanism of action of NSAIDs.***

In 1971, a number of investigators demonstrated that aspirin-like drugs (later known as NSAIDs) inhibited prostaglandin synthesis in a variety of cells and tissues from different species (Vane, 1971; Smith and Willis, 1971; Ferreira *et al.*, 1971). This important discovery led to the hypothesis that aspirin-like drugs mediated their therapeutic effects through the inhibition of prostaglandin synthesis (Flower and Vane, 1974).

Prostaglandin formation occurs via a two-step process. The first step involves the oxygenation of arachidonic acid by cyclo-oxygenase (COX) enzymes to form unstable prostaglandins known as PGG<sub>2</sub>. Subsequently, these unstable PGG<sub>2</sub> are converted into stable prostaglandins, PGH<sub>2</sub>, by peroxidases, which are then converted into prostaglandins of the D-, E- and F-series by individual synthases or reductases.



**Figure 1.1: Prostaglandin synthesis.** The flow diagram shows the metabolic pathway of arachidonic acid which leads to the production of prostaglandins F<sub>2</sub>, D<sub>2</sub> and E<sub>2</sub>. Inhibition of prostaglandin synthesis can occur by inhibiting the action of cyclo-oxygenase enzymes, which catalyse the conversion of arachidonic acid to cyclic endoperoxidases. NSAIDs inhibit prostaglandin synthesis by inhibiting cyclo-oxygenase enzymes, COX-1 and COX-2 (modified from Rang *et al.*, 1998).

Initially, COX was thought to be a single enzyme, producing inflammatory prostaglandins at the inflamed site and also homeostatic prostaglandins required for normal function within other tissues, such as the kidney and stomach (Hanes, 1990). Recently, a second COX enzyme has been isolated and subsequently, these enzymes have been termed COX-1 and COX-2. COX-1 has been described as having "constitutive activity" and is

expressed in most tissues throughout the body, including the gastrointestinal mucous (Kargman *et al.*, 1996; Ristimaki *et al.*, 1997). COX-2 has been described as having "inducible activity" (O'Bannion *et al.*, 1992); it is expressed in low levels throughout the body, including the gastrointestinal mucosa, but its expression can be up-regulated at sites of inflammation by cytokines and bacterial lipopolysaccharides. These enzymes are encoded by different genes and are regulated at transcriptional and post-transcriptional levels to different degrees, with the "inducible" COX-2 being regulated to a much higher degree than COX-1 (reviewed by Jouzeau *et al.*, 1997). It is commonly thought that the analgesic, anti-pyretic and anti-inflammatory properties of NSAIDs are related to their capacity to inhibit COX-2, whereas the anti-thrombotic and some adverse effects of certain NSAIDs, such as gastrointestinal toxicity, are related to their ability to inhibit COX-1 (Vane, 1994; Battistini *et al.*, 1994).

### **1.2.ii. Classification of NSAIDs**

Most clinically useful NSAIDs are weak organic acids which vary in their ability to inhibit prostaglandin synthesis. Most acidic NSAIDs bind extensively to plasma albumin, but differ in their binding capacity to other proteins and tissues (for review, see McCormack, 1994). Traditionally, NSAIDs were classified in accordance with their chemical grouping, namely: the carboxylic acids (which are sub-divided into salicylates, heteroarylacetic acids, propionic acids and fenamates), the pyrazoles and oxicams (Weissmann, 1991; Rang *et al.*, 1998, see Table 1.1).

Recently NSAIDs have been classified according to their relative inhibition of cyclo-oxygenase isoenzymes 1 and 2 (Jouzeau *et al.*, 1997; Frölich, 1997); this classification system may be more representative of the different therapeutic properties of NSAIDs. However, confusion arises from the diversity of biological systems used to assay COX selectivity such that their selectivity ratios are not directly comparable when obtained from different sources and under different experimental conditions (Vane *et al.*, 1997). For example, Young *et al.*, (1996) and others report that COX-1 and -2 sequence homology and mechanisms of inhibition (e.g. competitive, non-competitive,

reversible, irreversible) are strongly species-dependant, such that selectivity of rat enzymes did not correlate with human selectivity. Other studies, using assays of whole human blood have shown, for example, mefenamic acid to be COX-1 selective (Gierse *et al.*, 1995; Young *et al.*, 1996) whereas others indicate it is COX-2 selective (Cryer and Feldman, 1998). Indeed, Young *et al.*, (1996) using purified human recombinant cell-free COX enzymes, suggest mefenamic acid is COX-2 selective. In fact, from the available data, the primary mechanism of action of mefenamic acid in humans, for example, still remains unclear.

## NONSTEROIDAL ANTI-INFLAMMATORY DRUGS

### CARBOXYLIC ACIDS

#### SALICYLATES

Acetylsalicylic acid (aspirin)  
Salsalate  
Diflusal  
Fendosal

#### ACETIC ACIDS

Indomethacin  
Acemetacin  
Cinmetacin  
Sulindac  
Tolmetin  
Zomepirac  
Diclofenac  
Fenclofenac  
Isoxepac

#### PROPIONIC ACIDS

Ibuprofen  
Flurbiprofen  
Naproxen  
Ketoprofen  
Fenoprofen  
Benoxaprofen  
Indoprofen  
Pirprofen  
Carprofen

#### FENAMATES

Mefenamic acid  
Meclofenamic Acid  
Flufenamic Acid  
Niflumic Acid  
Tolfenamic

Phenylbutazone  
Feprazone  
Apazone  
Trimethazone  
Mofebutazone  
Kebutazone  
Suxibutazone

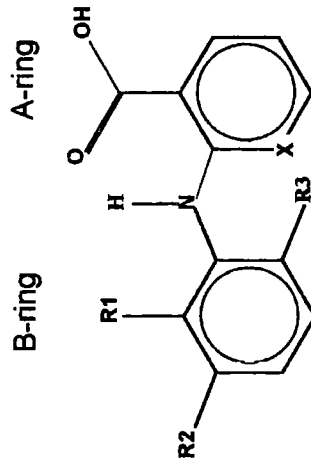
Piroxicam  
Isoxicam  
Tenoxicam

### PYRAZOLES

### OXICAMS

#### FENAMATES

#### Structure of Fenamates



Fenamate	R1	R2	R3	X
Mefenamic Acid	CH <sub>3</sub>	CH <sub>3</sub>	H	C
Meclofenamic acid	Cl	CH <sub>3</sub>	Cl	C
Flufenamic Acid	H	CF <sub>3</sub>	H	C
Tolfenamic Acid	Cl	CH <sub>3</sub>	H	C
Niflumic Acid	H	CH <sub>3</sub>	H	N

**Table 1.** showing (top) traditional NSAID classification system, based upon chemical structure and (bottom left) general structural representation of fenamates. Bottom right, shows table of group substitutions for a series of fenamates.



### **1.2.iii. Analgesic Effects of NSAIDs**

#### **1.2.iii. i. Peripheral antinociceptive effects of NSAIDs**

Prostaglandin production in the periphery causes vasodilation and increased vascular permeability resulting in inflammatory symptoms. In addition, prostaglandins serve to potentiate pain caused by other mediators of inflammation such as histamine and bradykinin (Lim *et al.*, 1964, Handwerker, 1976, Chahl and Iggo, 1977). Thus, NSAIDs inhibit the hyperalgesic state of peripheral afferent fibres caused by the concerted action of prostaglandins and other mediators of pain.

An early study using an animal model of pain elicited by bradykinin (Lim *et al.*, 1964) led to the development of a classification scheme for analgesic agents acting either centrally or peripherally. This study and others (for example Horton, 1964; Guzman, 1964; Juhlin and Michaelson, 1969; Crunkhorn and Willis, 1971 and Karim, 1971) indicated that, for analgesia, the inhibitory action of NSAIDs on prostaglandin synthesis is predominant in the periphery. However, in addition to their effects in the peripheral nervous system, it is clear that NSAIDs, depending on their lipophilicity, can readily cross the blood-brain-barrier and enter the CNS (Bannwarth *et al.*, 1989, 1990) where they can also inhibit prostaglandin synthesis and exert antinociceptive effects.

#### **1.2.iii. ii. Central antinociceptive effects of NSAIDs**

A number of studies have clearly shown that NSAIDs exert analgesic effects even when placed directly into the CNS, but the mechanisms behind this action are currently undefined. For example, early studies by Dubas and Parker (1971) demonstrated that sodium salicylate, injected subcutaneously, increased the nociceptive threshold to electrical stimulation of the lateral hypothalamus in rats and concluded that this structure is involved in a primary level of integration of behavioural responses to pain in the CNS and that aspirin exerts actions centrally. Later experiments demonstrated that acetylsalicylate inhibited electrically stimulated nociceptive responses in monkeys when micro-injected into the pre-optic region of the hypothalamus (Shyu *et al.*, 1984).

A number of investigators have demonstrated that NSAIDs administered *intrathecally* also exert antinociceptive effects. Using this method of administration in rats, acetylsalicylic acid, has been shown to attenuate acetic acid-induced writhing responses (Yaksh, 1982). Intrathecal administration of acetylsalicylic acid, ibuprofen and ketorolac trimethamine have also been shown to inhibit pain behaviour and thermal hyperalgesia evoked by intrathecal injection of NMDA (N-methyl-D-aspartate), AMPA ( $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and substance P (Malmberg and Yaksh, 1992). Jurna *et al.*, (1992) demonstrated that intrathecal injection of acetylsalicylic acid, salicylic acid and indomethacin depressed the activity of rat thalamic and spinal neurones observed following electrical stimulation of afferent C-fibres in the sural nerve. In human studies, high doses of acetylsalicylic, administered orally, reduced the amplitude of brain electrical potentials evoked by electrical stimulation of the tooth (Chen and Chapman, 1980) and intrathecal administration of lysine acetylsalicylic acid, an injectable acetylsalicylic acid derivative, produced pain relief in cancer patients (Devoghel, 1983).

In addition, increased prostaglandin synthesis in the brain has temperature-raising effects and actions of NSAIDs on brain prostaglandin levels may contribute to their antipyretic properties. Cooper *et al.*, (1982) have suggested that the antipyretic effects of NSAIDs are mediated through inhibition of prostaglandin synthesis in the hypothalamus, particularly in the preoptic region.

These studies suggest that spinally released prostaglandins are involved in the transmission of nociceptive stimuli and that NSAIDs may be effective in spinal antinociception. Importantly, these studies suggest that there may be a central component to the actions of NSAIDs.

### 1.2.iii. iii. *Alternative mechanisms for the analgesic effects of NSAIDs in the CNS*

A review in 1991 by McCormack and Brune concluded that there was little correlation between the analgesic effects of NSAIDs and their ability to inhibit prostaglandin synthesis *in vitro*, prompting the notion that other, or

mechanisms additional to prostaglandin synthesis inhibition, may contribute to the clinical effects of NSAIDs. Thus, NSAIDs have been shown to affect the synthesis and activity of other neuroactive substances involved in nociceptive responses in the dorsal horn. For example, an *in vivo* study by Durbourdieu and Dray (1989) reported that ketoprofen reduced substance P levels in the hypothalamus and spinal cord of rats. These actions may contribute to the effects of NSAIDs on spinal nociceptive processing (reviewed by McCormack, 1994). In addition, NSAIDs have also been shown to exert changes in membrane fluidity (Weissmann, 1991) and to enhance descending serotonergic inhibitory nociceptive pathways (McCormack 1994). Indeed, Shyu *et al.*, (1984), in an *in vivo* study, report a direct relationship between the analgesic effects of aspirin and increased central serotonergic activity in the control of dental pain in primates. Recently, NSAIDs have also been shown to modulate opioid peptide release (Herrero and Headley, 1996) and to potentiate the analgesic actions of  $\mu$ -opioid receptor agonists in the periaqueductal gray (PAG; Vaughan *et al.*, 1998). These additional pharmacological effects may contribute to the actions of NSAIDs in the CNS.

#### **1.2.iv. NSAIDs and neuroplasticity**

Arachidonic acid has been implicated in neuronal excitation and synaptic plasticity. It has been suggested that inhibition of COX enzymes by NSAIDs would lead to an accumulation of arachidonic acid, which may subsequently be diverted along lipoxygenase and epoxygenase metabolic pathways (Vaughan, 1998). Arachidonic acid has been shown to enhance synaptic transmission in the hippocampus (Williams *et al.*, 1989). It is released following glutamate receptor (particularly NMDA) activation (Dumuis *et al.*, 1988,1990; Lazarewicz *et al.*, 1988, 1990; Sanfeliu *et al.*, 1990), during long term potentiation (Lynch *et al.*, 1989) and has been proposed to rapidly inhibit glutamate uptake during long-term potentiation (Barbour *et al.*, 1989; Volterra *et al.*, 1992). Binding studies have also suggested that arachidonic acid and its metabolites may play a role in neuronal excitation, partly by inhibition of GABA-mediated neuronal

inhibition (Schwartz *et al.*, 1988; Schwartz and Yu, 1992; Koenig and Martin, 1992). Recently, Bezzi *et al.*, (1998) have shown that prostaglandins stimulate calcium-dependent glutamate release in astrocytes and that inhibition of prostaglandin synthesis by NSAIDs prevents this release of glutamate; these authors have suggested that NSAIDs may play a role in synaptic plasticity.

### **1.2.v. Novel Clinical Applications of NSAIDs**

#### **1.2.v. i. Ischaemia and Neuroprotection**

Post-ischaemic inflammation has been implicated in playing an important role in the delayed progression of damage to brain tissue (Kochanek and Hallenbeck, 1992; Feuerstein *et al.*, 1997). Following cerebral ischaemia, local expression of a cascade of inflammatory proteins is induced (for review see Feuerstein *et al.*, 1997) which includes COX-2, a mediator of the cytotoxic effects of inflammation (Seibert *et al.*, 1995; Smith and DeWitt, 1995). Nogawa *et al.*, (1997) have demonstrated in rats that cerebral ischaemia leads to the up-regulation of COX-2, (but not COX-1) expression, protein and reaction products (PGE<sub>2</sub>) within the injured site. The selective COX-2 inhibitor, NS-398, attenuated the ischaemic damage, suggesting that selective COX-2 inhibitors may be protective during the post-ischaemic period.

Chen *et al.*, (1998) have recently reported that fenamate NSAIDs (and the NMDA receptor antagonist MK-801) protect embryonic chick retinal neurones against glutamate-induced damage and ischaemia-induced injury. In particular, fenamates were protective against NMDA- and kainate-induced excitotoxicity. However, in the same study, voltage-clamp experiments using salamander retinal neurones, demonstrated that fenamates inhibited NMDA-evoked currents, but not kainic acid-evoked currents. The authors suggest that this neuroprotection by fenamates may be due to inhibition of chloride influx rather than AMPA/ kainate receptor blockade (Chen, *op. cit.*).

These data suggest that although inhibition of prostaglandin synthesis by NSAIDs undoubtedly plays a role in their neuroprotective effects during post-ischaemic inflammation, other mechanisms may contribute to these effects. The

neuroprotective properties of NSAIDs have also been implicated in other neurodegenerative disorders, such as Alzheimer's disease.

#### 1.2.v. ii. *Alzheimer's Disease*

Recent data also suggests that the neurodegeneration associated with Alzheimer's disease involves COX enzymes. Alzheimer's disease lesions are characterised not only by the presence of amyloid plaques and neurofibrillary tangles, but also by the accumulation of many inflammatory proteins, such as inflammatory cytokines, complement proteins and their regulators, which may promote neuronal death (McGeer *et al.*, 1994, 1995). These pro-inflammatory cytokines cause a marked induction of COX-2 enzyme levels (Hempel and Müller, 1995; Cochran and Vitek, 1996). These data have led to the hypothesis that patients taking NSAIDs to control other anti-inflammatory diseases, such as arthritis, may also have a reduced chance of developing Alzheimer's disease. Although there is no direct evidence to-date, a number of epidemiological studies have indicated that NSAIDs (and other anti-inflammatory treatments) may indeed delay the onset and slow the progression of neurodegenerative disorders such as Alzheimer's disease (McGeer *et al.*, 1996, Breitner, 1995, 1996). In addition, Breitner *et al.*, (1995) have reported a delayed onset of Alzheimer's Disease with NSAIDs and histamine H2 blocking drugs and suggest that the actions of these very different drugs may be linked to the actions of COX on the NMDA pathway to reduce NMDA-mediated glutamatergic excitotoxicity.

Together, the data above strongly indicate that NSAIDs exert analgesic and anti-inflammatory effects, not only in the periphery, but also within the CNS. The mechanisms underlying these actions may involve targets additional to COX enzyme inhibition.

A number of studies have demonstrated that NSAIDs modify the behavioural effects of chemically- and electrically-induced seizures in rodents. These data will now be reviewed.

### **1.2.vi. The effect of Prostaglandins on Seizure-models**

Prostaglandin presence in the CNS has been well-documented *in vivo* and *in vitro* (Eliasson, 1959; Samulesson, 1964; Wolfe, 1978; Galli *et al.*, 1980). Prostaglandin release appears to be stimulated by increased neuronal activity, for example, during chemically- or electrically-induced seizures (Bosisio *et al.*, 1976; Berchtold-Krantz *et al.*, 1981; Förstermann *et al.*, 1982; Baran *et al.*, 1987) and decreased by certain anti-convulsant drugs such as benzodiazepines and barbiturates (Wolfe, 1978; Steinhauer *et al.*, 1979). Interestingly, Förstermann *et al.*, (1984) found that spontaneously convulsing gerbils had unusually low prostaglandin levels and suggested that prostanoid deficiency, either due to reduced availability of arachidonic acid, impairment of cyclo-oxygenase activity or increased prostanoid metabolism, could be a contributing factor to seizure susceptibility.

Early studies demonstrated that administration of prostaglandins of the E series intracerebrally in unanaesthetized cats, intravenously in chicks (Horton, 1964) or intraperitoneally in rats (Haubrich *et al.*, 1973) produced sedative effects. Later, it was shown that intracerebral administration of these prostaglandins also inhibited pentylenetetrazole (PTZ)- (Bhattacharya and Sanyal, 1978; Rosenkranz and Killam, 1979) and also picrotoxin-, strychnine- and isoniazid-induced seizures in rats (Rosenkranz and Killam, 1979).

Several studies have shown that certain NSAIDs increase the susceptibility of experimental animals to seizures, possibly due to inhibition of prostaglandin synthesis. Indeed, clinical evidence shows that certain NSAIDs, such as mefenamic acid, administered in high doses in humans, can themselves be epileptogenic. However, some reports indicate an anti-convulsant effect of NSAIDs. These issues will now be discussed in detail below.

### **1.2.vii. NSAIDs used in seizure models**

Following a report that maternal indomethacin induced convulsions in a breast-fed infant (Eeg-Olofsson, 1978), Steinhauer *et al.*, (1979) and later Steinhauer and Hertting, (1981), investigated the effects of NSAIDs on chemically-induced seizures in mice. The authors demonstrated that ibuprofen, indomethacin, diclofenac and flurbiprofen lowered the convulsive threshold and LD<sub>50</sub> of PTZ-induced seizures and, also inhibited the increase of prostaglandins and thromboxane B<sub>2</sub> normally observed following chemically- and electrically-induced seizures in rodents. However aspirin did not affect either of these parameters. Moreover, the authors demonstrated that indomethacin also accelerated seizure onset.

Wallenstein (1985a) demonstrated that mefenamic acid, meclofenamic acid and ibuprofen (and paracetamol which is not an NSAID) delayed the onset of PTZ-induced seizures in rats and that, in addition, mefenamic acid and meclofenamic acid potentiated the excitatory effects of subconvulsive and convulsive doses of PTZ. In a study examining the effect of NSAIDs on the electrocorticogram recorded in rats, Wallenstein (1985b) found that mefenamic acid and meclofenamic acid produced dose-dependent increases in excitation (including seizure) whereas the non-fenamate NSAIDs, ibuprofen and indomethacin (and paracetamol) produced dose-dependent increases in sedation. Wallenstein (1987) later examined the effects of NSAIDs on penicillin-induced "primary generalised seizures" and "focal seizures" and demonstrated that ibuprofen (and paracetamol) inhibited the number and delayed the onset of penicillin-induced "primary generalised seizures" in rats, whereas mefenamic acid inhibited the incidence, but did not affect the onset of these seizures. In the penicillin-induced "focal seizure" model, mefenamic acid (and paracetamol) delayed the number and onset of seizures, whilst ibuprofen only delayed the onset and indomethacin only reduced the number of penicillin-induced seizures.

Ikonomidou-Turski *et al.*, (1988) demonstrated that a range of NSAIDs differentially modulated the threshold for pilocarpine-induced seizures in rats. Some NSAIDs, for example, sodium salicylate and phenylbutazone, were pro-convulsant, others (e.g. indomethacin and ibuprofen) had no effect, and one in

particular, mefenamic acid, was anti-convulsant. Interestingly, in this study, mefenamic acid not only prevented pilocarpine-induced seizures, but was also neuroprotective against the ensuing seizure-related brain damage. Wallenstein (1991) using behavioural and electrocorticogram studies, later demonstrated that mefenamic acid, but not ibuprofen, attenuated PTZ-induced seizures in kindled rats without effects on latency to onset, but that high doses of mefenamic acid also produced excitatory effects. More recently, Baran *et al.*, (1994) reported that indomethacin potentiated kainic-acid induced seizure activity in rats, whilst ibuprofen and the lipoxygenase inhibitor, ebselen, had no effect. However, the combined cyclo-oxygenase and lipoxygenase inhibitor, BW755C (3-amino-1-, *m*-(trifluoromethyl-phenyl)-2-pyrazoline) was neuroprotective and reduced the severity of PTZ-induced seizures. Most recently, Kaminski *et al.*, (1998) demonstrated, using an electrically-induced seizure model in mice, that a number of NSAIDs differentially increased the protective effects of two antiepileptic drugs (diphenylhydantoin and valproate magnesium), whilst having no effect on the threshold of electrically-induced seizures themselves: acetylsalicylic acid, ibuprofen, indomethacin, metamizole and piroxicam all potentiated the protective effects of valproate magnesium, whilst only ibuprofen and piroxicam increased the protective effects of diphenylhydantoin.

These data demonstrate that certain NSAIDs modify seizure activity. However, the evidence regarding their effects is conflicting, with some NSAIDs potentiating and others inhibiting seizure activity, depending on the seizure model employed. Intriguingly, mefenamic acid overdose in humans has been reported to result in convulsive seizures. In fact, Prescott *et al.*, (1981) reported that over one third of all overdose cases of mefenamic acid resulted in grand mal convulsions. Typical symptoms of such overdose cases include generalised seizures, clonic-tonic limb movements and lack of response to painful stimuli (Young *et al.*, 1979, Balali-Mood *et al.*, 1981, Shipton and Müller, 1985, Turnbull *et al.*, 1988). Interestingly, Shipton and Müller (1985) describe a case of mefenamic acid overdose resulting in a generalised convulsion where the patient was remarkably resistant to diazepam treatment, but did respond to



etomidate. However, other clinicians have reported successful treatment of mefenamic acid-induced generalised convulsions with diazepam (Gossinger *et al.*, 1982). In addition to seizures, other reported symptoms of mefenamic acid overdose include coma (Gossinger *et al.*, 1982, Hendrikse, 1988), which in one case was followed by a grand mal convulsion (Gossinger *et al.*, 1982).

In the main, it has been proposed that NSAIDs could produce their complex effects on seizure threshold and latency by their concurrent inhibition of cyclo-oxygenase activity. However, this hypothesis does not explain the range of effects exhibited by different NSAIDs, all of which have the ability, albeit to different degrees, to inhibit cyclo-oxygenase activity. For example, indomethacin and ibuprofen, which have no effect on pilocarpine-induced seizures, are more potent inhibitors of cyclo-oxygenase activity than sodium salicylate and phenylbutazone, both of which exacerbate pilocarpine-induced seizures (Ikonomidou-Turski *et al.*, 1988). In addition, this hypothesis does not explain the complex biphasic effects of mefenamic acid on chemically induced seizures. Recently, Wong (1993) demonstrated that relatively high doses of indomethacin (200-400mg/Kg), but not acetylsalicylic acid, afforded protection against chemically- and electrically induced seizures in mice. Wong suggested that this protection could be due to GABA-uptake inhibition rather than inhibition of cyclo-oxygenase activity and also suggested that the high dosages required are due to the "relative insensitivity of mice to CNS depression compared to humans."

In addition to inhibition of cyclo-oxygenase activity, a number of alternative explanations have been proposed. Bhattacharya and Sanyal (1978), for example, suggest a serotonergic, but not catecholamine-mediated component to the convulsive effects induced by PTZ. These authors demonstrated that the anti-convulsant effect of PGE-1 on PTZ-induced seizures was inhibited by p-chlorophenylalanine (PCPA; a selective serotonin synthesis inhibitor), methysergide (a mixed 5-HT<sub>1/2</sub> receptor antagonist) and 5,6-dihydroxytryptamine (DHT; an inductor of selective degeneration of serotonergic neurones), but not by the catecholamine synthesis inhibitor,  $\alpha$ -methyl-p-tyrosine (MPT), or the adrenoreceptor antagonists phenoxybenzamine (PBZ), propranolol or haloperidol.

Despite the clinical evidence, a paucity of studies investigating the mechanisms underlying the complex effects of NSAIDs on seizure activity has precluded a convincing explanation for these observations. Some electrophysiological studies, in a number of non-neuronal and a limited number of neuronal preparations, have revealed that NSAIDs may dramatically influence the behaviour of a variety of ion channels. These experiments, which may provide a valuable insight into the effects of NSAIDs on the CNS, will now be discussed in detail.

### **1.2.viii. Physiological effects of NSAIDs on Ion Channel function**

#### **1.2.viii. i. Non-neuronal Preparations**

A number of studies have demonstrated that NSAIDs modulate ion channel function in non-neuronal preparations.

Several studies have shown that certain fenamates induce intracellular  $\text{Ca}^{2+}$  release. McDougall, *et al.*, (1988) demonstrated that flufenamic acid and mefenamic acid uncoupled oxidative phosphorylation causing an inhibition of calcium uptake (with an  $\text{IC}_{50}$ s of  $7\mu\text{M}$  and  $68\mu\text{M}$ , respectively), and thereby increased cytosolic  $\text{Ca}^{2+}$  levels in mitochondria isolated from rat liver. Later, Northover *et al.*, (1990) showed that flufenamate ( $5\mu\text{M}$ ) induced intracellular  $\text{Ca}^{2+}$  release in isolated myocardial cells and Poronnik *et al.*, (1992), showed that flufenamic (with an  $\text{EC}_{50}$  of  $100\mu\text{M}$ ), mefenamic and niflumic acid (in descending order of potency) induced intracellular  $\text{Ca}^{2+}$  release in a mouse mandibular cell line. In addition, flufenamic acid (between  $37\text{-}500\mu\text{M}$ ) and mefenamic acid (with less potency) have been shown to directly activate potassium channels in human jejunum (Farrugia *et al.*, 1993a); similar results were obtained in canine jejunum (Farrugia *et al.*, 1993b). Niflumic acid (with a  $\text{K}_d$  of  $261\mu\text{M}$ ), flufenamic acid and mefenamic acid (although less potently than flufenamic acid also potentiated calcium-activated  $\text{K}^+$  channels in plasma membranes vesicles from pig coronary smooth muscle (reconstituted into lipid bilayers) by increasing open channel probability (Ottolia and Toro, 1994).

Fenamates have also been shown to inhibit non-selective cation channels in non-neuronal preparations. For example, flufenamic, mefenamic (both with  $IC_{50}$ s of  $10\mu\text{M}$ ), and niflumic acid (with an  $IC_{50}$  of  $50\mu\text{M}$ ) inhibited non-selective cation channels in rat exocrine pancreas; indomethacin, ibuprofen or aspirin did not modulate these channels (Gögelein *et al.*, 1990). Fenamates have also been shown to block non-selective cation channels in rat distal colon cells (Siemer and Gögelein, 1992), in murine L cells (Jung *et al.*, 1992), rat capillary endothelial cells (Popp and Gögelein, 1992) and in a mouse mandibular cell line (Poronnik *et al.*, 1992).

An early study by Cousin and Motais, (1979) demonstrated that niflumic and meclofenamic acid (with  $IC_{50}$ s of  $0.63\mu\text{M}$  and  $0.75\mu\text{M}$ , respectively), non-competitively inhibited anion transport in human erythrocytes. More recently, it has been shown that fenamates inhibit  $\text{Ca}^{2+}$ -activated chloride channels in certain epithelial cell types (Chao and Mochizuki, 1992). For example, niflumic and flufenamic acid inhibit chloride conductance in the basolateral membrane lining the ascending Loop of Henle in rabbit kidney (Wangemann, *et al.*, 1986). In addition, flufenamic and niflumic acid have been shown to inhibit  $\text{Ca}^{2+}$ -activated chloride conductance in *Xenopus* oocytes (White and Aylwin, 1990, Séguéla *et al.*, 1993; Woodward *et al.*, 1994). McCarty *et al.*, (1993) report that flufenamic acid ( $200\mu\text{M}$ ) inhibits the cystic fibrosis transmembrane conductance regulator chloride channel expressed in *Xenopus* oocytes by a voltage-dependent mechanism, suggesting open-channel blockade.

#### 1.2.viii. ii. Neuronal Preparations.

There have been few studies investigating the actions of NSAIDs on ion channel function in neuronal preparations. However, early studies demonstrated that salicylic acid, albeit at millimolar (1-30mM) concentrations, inhibited chloride ion permeability and increased potassium ion permeability in buccal ganglion neurones of the marine mollusc, *Navanax inermis* (Barker and Levitan, 1971). Neto (1980) later demonstrated that salicylic acid (2-5mM) reduced the spike amplitude, and at higher concentrations (10-20mM), blocked

conduction of the compound action potentials recorded extracellularly in rabbit vagus and frog sciatic nerves.

Interestingly, Shaw *et al.*, (1995) demonstrate non-selective cation channel block with flufenamic acid (300-500 $\mu$ M), but not mefenamic acid in molluscan neurones, and also showed that at these concentrations flufenamic, but not mefenamic acid, also induced intracellular  $Ca^{2+}$  release in these molluscan neurones. Lerma and Martin del Rio, (1991) reported that niflumic and flufenamic inhibited NMDA-gated cation channel in mouse spinal cord neurones, with  $IC_{50}$  values of  $\approx 350\mu$ M. More recently, Chen *et al.*, (1998) report an inhibition of NMDA, but not kainate-mediated responses, by mefenamic acid, meclofenamic acid and flufenamic acid (all at 1mM), recorded in salamander retinal ganglion neurones.

The studies reviewed above clearly reveal that NSAIDs influence the behaviour of a variety of ion channels. However of particular interest are three studies which demonstrate that fenamates modulate neuronal  $GABA_A$  receptors. A radio-ligand binding study by Evonuk and Skolnick (1988) demonstrated that niflumic acid inhibited  $Cl^-$ -modulated [ $^{35}S$ ]-t-butylbicyclophosphorothionate (TBPS) binding to rat neuronal  $GABA_A$  receptors and suggested that niflumic acid acts at or near a binding site within the  $GABA$ -gated  $Cl^-$  channel. More recently, Woodward *et al.*, (1994) demonstrated that fenamates modulated rat cortical  $GABA_A$  receptors expressed in *Xenopus* oocytes. Furthermore, a preliminary study by Halliwell *et al.*, (1994) demonstrated that mefenamic acid was a potent modulator of native  $GABA_A$  receptors in rat hippocampal neurones; these studies will be discussed in detail below.

In light of the observations reviewed above, the effects of NSAIDs upon neuronal ligand-gated ion channels have been investigated, with particular focus on the  $GABA_A$  receptor. For this reason, the general properties of ligand-gated ion channels and, in particular, the  $GABA_A$  receptor, will now be discussed.

### **1.3. Ligand-Gated Ion Channels-general characteristics**

Ligand-gated ion channels probably mediate the majority of fast inhibitory and excitatory synaptic transmission in the mammalian CNS. These channels comprise a multi-subunit assembly forming a central ion selective channel and associated ligand binding sites. Neuronal excitation is usually associated with the opening of cation channels and subsequent membrane depolarisation. Neuronal inhibition, by contrast, is often associated with an increase in anion/chloride ion permeability and subsequent membrane hyperpolarisation. The GABA<sub>A</sub> receptor, like the nicotinic acetylcholine (nACh), strychnine-sensitive glycine, 5-hydroxytryptamine<sub>3</sub> (5-HT<sub>3</sub>), ionotropic glutamate and recently, the purinoceptor P<sub>2X</sub> receptor, is part of the ligand-gated ion channel super-family (Unwin *et al.*, 1993; Schofield *et al.*, 1987). The structure of the nACh receptor is the best characterised of this receptor super-family. However, since the GABA<sub>A</sub> receptor is a major focus of this thesis, the properties of GABA<sub>A</sub> receptors will be reviewed in detail below.

### **1.4. The $\gamma$ -Aminobutyric Acid Type A (GABA<sub>A</sub>) Receptor**

#### ***1.4.i. $\gamma$ -Aminobutyric Acid (GABA)***

GABA has been known to act as a neurotransmitter in mammals for over thirty years. Early studies demonstrated that GABA had marked depressant effects on mammalian cerebral cortical neurones (Hayashi, 1956), and that GABA could inhibit chemically-induced seizure activity in the motor cortex of the dog (Hayashi, 1959). Later, seminal electrophysiological studies performed by Krnjevic and Schwartz, (1966, 1967) compared the properties of inhibitory postsynaptic potentials (IPSPs) and GABA-induced membrane hyperpolarization in cat neocortical neurones and found that in both cases these events were chloride-dependent and had similar reversal potentials. GABA is now thought to be the major inhibitory amino acid neurotransmitter in the vertebrate CNS with between 20 to 50% of all central synapses using

GABA as their neurotransmitter, depending on brain region (Bloom and Iverson, 1971; Bormann, 1988; Young & Chu, 1990). GABA is found predominantly within the CNS with only small amounts being located in the periphery. It is a neutral amino acid which is synthesised in GABAergic nerve terminals by the action of the decarboxylating enzyme, glutamic acid decarboxylase (GAD) on L-glutamic acid (Roberts And Frankel, 1950). It is released from nerve terminals in response to a stimulus in a calcium-dependent manner (Bradford, 1970) and following release; it is removed from the synaptic cleft by high affinity sodium-dependent uptake systems (Iverson and Neal, 1968). GABA is the endogenous ligand for a diversity of GABA receptors.

#### **1.4.ii. GABA Receptor Classification**

Three different receptor classes for GABA have been defined in terms of physiology and pharmacology namely, GABA<sub>A</sub>, GABA<sub>B</sub> and GABA<sub>C</sub> receptors. Of these the GABA<sub>A</sub> receptor has been the most extensively characterised.

The first GABA receptor subtype to be described was later defined as the GABA<sub>A</sub> receptor. GABA<sub>A</sub> receptors have been shown to be directly associated with a Cl<sup>-</sup> channel (Bormann, 1988; Silvilotti and Nistri, 1991) and are located pre- and post-synaptically throughout the CNS. GABA influences neuronal excitability and affects glial cells at GABA<sub>A</sub> receptors by increasing permeability to chloride ions (Curtis *et al.*, 1968; Krnjevic, 1974; Olsen, 1982) usually causing membrane hyperpolarization in neurones and depolarisation in glial cells. GABA<sub>A</sub> receptors are activated by GABA, muscimol and isoguvacine, inhibited competitively by bicuculline and, non-competitively, by picrotoxin, and are subject to allosteric modulation by a number of chemically diverse allosteric modulators which will be discussed in detail below.

GABA<sub>B</sub> receptors have been shown to be coupled to Ca<sup>2+</sup> or K<sup>+</sup> ionophores via G-proteins (Bormann 1988; Bowery 1993); they are located pre- and post-synaptically throughout the CNS and are composed of heterogeneous subtypes (Bonnanno *et al.*, 1993). Recently, Kaupmann *et al.*, (1997) have

cloned a GABA<sub>B</sub> receptor, which is a 7-transmembrane domain protein, bearing similarities to metabotropic glutamate receptors. GABA<sub>B</sub> receptors are bicuculline-insensitive, stimulated by GABA and baclofen (Bowery *et al.*, 1980; Hill and Bowery, 1981) and inhibited by phaclofen (Kerr *et al.*, 1987) and CGP 54626 ([S-(R\*,R\*)]-3-[[1-(3,4-dichlorophenyl) ethyl] amino-2-hydroxypropyl] (cyclohexylmethyl) phosphinic acid; Froestl *et al.*, 1996).

GABA<sub>C</sub> receptors (Drew *et al.*, 1984; Cutting *et al.*, 1991; Polenzani *et al.*, 1991) may represent a simpler homo-oligomeric type of ligand-gated ion channel, having simpler pharmacology than GABA<sub>A</sub> receptors. They are stimulated by GABA, muscimol, cis-4-aminocrotonic acid (CACA; a conformationally restricted analogue of GABA) and its trans-isomer (trans-4-aminocrotonic acid; TACA) (Sivilotti and Nistri, 1989, 1991; Feigenspan *et al.*, 1993; Lukasiewicz *et al.*, 1994; Dong *et al.*, 1994), but are insensitive to baclofen and bicuculline (Quian and Dowling, 1993; Feigenspan *et al.*, 1993; Dong *et al.*, 1994). Unlike GABA<sub>A</sub> receptors, GABA<sub>C</sub> receptors are not modulated by benzodiazepines, barbiturates (Sivilotti and Nistri, 1991; Bormann and Feigenspan, 1995) or neurosteroids (Feigenspan *et al.*, 1993). GABA<sub>C</sub> receptors have recently been localised to sub-populations of vertebrate retinal neurones (Cutting *et al.*, 1991, Feigenspan *et al.*, 1993; Quian and Dowling, 1993; Lukasiewicz *et al.*, 1994; Dong *et al.*, 1994) and are thought to contain the recently discovered  $\rho$ -subunit (Cutting *et al.*, 1991, 1992).

Thus, a diversity of receptor classes exists for which GABA is the endogenous ligand. This diversity is further increased by the existence of a number of subtypes of the GABA receptor which differ in terms of physiology and pharmacology.

### **1.4.iii. Structure of the GABA<sub>A</sub> receptor**

Determination of the molecular structure of the GABA receptor was aided by the discovery that the receptor protein could be photo-affinity labelled by [<sup>3</sup>H]-flunitrazepam (Möhler *et al.*, 1980). A single polypeptide was labelled in crude brain homogenates with a molecular weight of 51kD. Subsequently, the existence of additional benzodiazepine binding polypeptides was demonstrated (Sieghart and Drexler, 1983). On the basis of these and similar biochemical studies (e.g. Sigel *et al.*, 1983), the GABA<sub>A</sub> receptor was proposed to form a hetero-oligomeric complex of about 200-300kD composed of 2 or more different polypeptides ( $\alpha$  and  $\beta$ ). Later, five different GABA<sub>A</sub> receptor subunit subtypes (each between 48-56kDa.) were identified on the basis of sequence analysis (Schofield *et al.*, 1987; Olsen and Tobin, 1990; Levitan *et al.*, 1988; Pritchett *et al.*, 1989; Burt & Kamatchi 1991). Electron microscope and image analysis studies have revealed that the GABA<sub>A</sub> receptor probably comprises a pentameric subunit organisation surrounding a central pore (Nayeem *et al.*, 1994). Hydropathy analysis of the sequences of GABA<sub>A</sub> receptor subunits predicts an extracellular N-terminal of about 200 amino acids containing three glycosylation sites, and four putative  $\alpha$ -helical hydrophobic transmembrane (TM) spanning regions (TM1-4; Schofield *et al.*, 1987; Olsen and Tobin, 1990; Burt and Kamatchi, 1991; Wisden and Seeburg, 1992). A large intracellular loop exists between TM3 and TM4, which contains phosphorylation sites thought to be responsible for receptor assembly and regulation (for review see Moss and Smart, 1996). The N-terminus is thought to be involved in ligand recognition. As in the nicotinic acetylcholine (nACh) receptor (Leonard *et al.*, 1988; Sakmann, 1992; Lester, 1992; Akabas, 1992, 1994; Unwin, 1993), the TM2 region of the GABA<sub>A</sub> receptor is thought to be involved in the formation of the channel wall (Xu and Akabas, 1993,1996).



**KEY:**

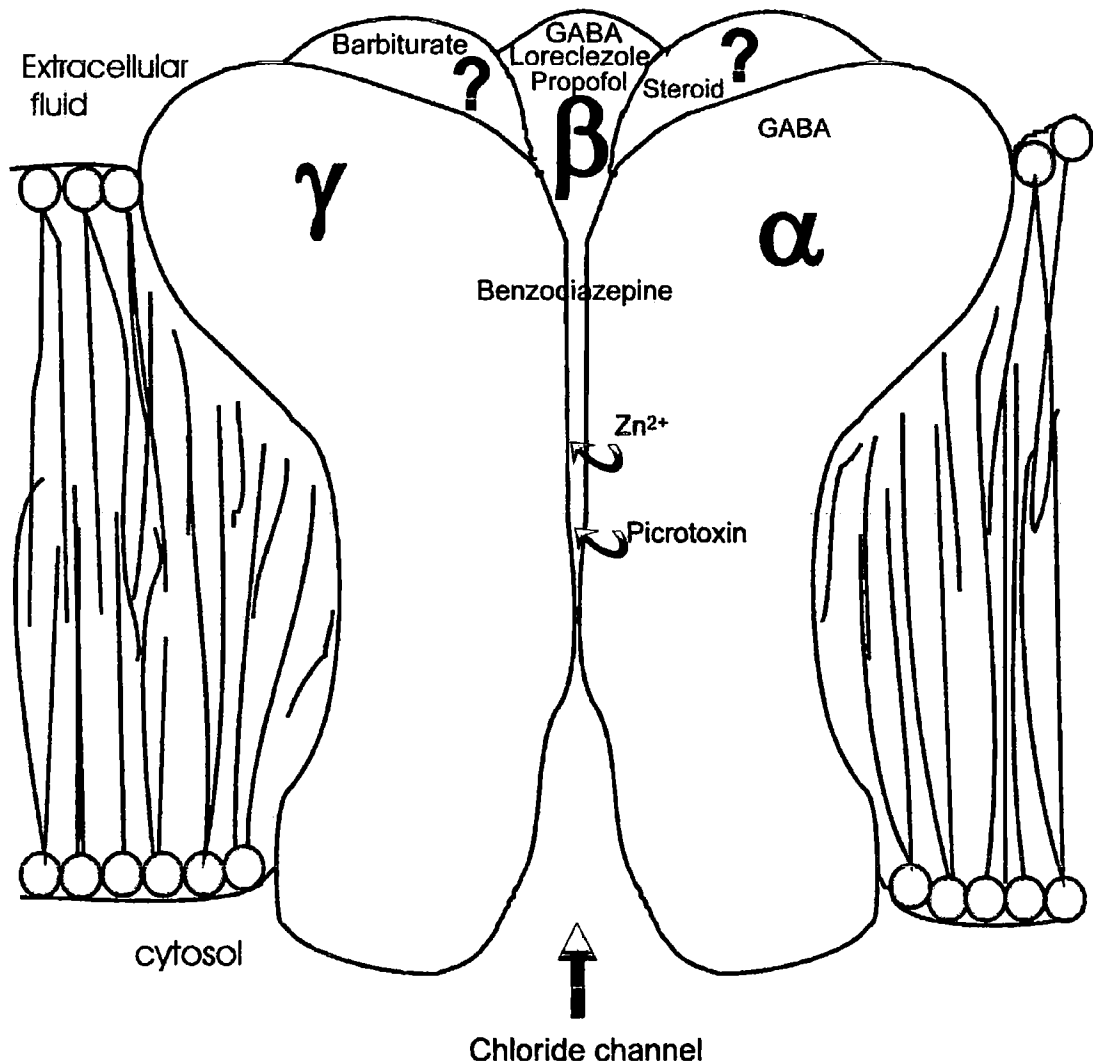
$\alpha$ -GABA, BDZ<sub>1</sub> & BDZ<sub>2</sub>

$\beta$ -GABA, Loreclezole & propofol

$\gamma$ -Ethanol, BDZ<sub>1</sub> & BDZ<sub>2</sub>

?- unknown site for steroids/barbiturates

Channel - picrotoxin/ Zn<sup>2+</sup>



**Figure 1.2: Schematic representation of the GABA<sub>A</sub> receptor:** showing a pentameric subunit organisation surrounding a central pore. Known binding sites on specific subunits for GABA and some positive allosteric modulators of the GABA<sub>A</sub> receptor are shown (see key).  $\alpha$ - and  $\gamma$ - subunits are shown adjacent to each other comprising the benzodiazepine binding site. The  $\beta$ -subunit, carrying binding sites for loreclezole (etomidate) and propofol is shown between two other unknown subunits, which may also be involved in ligand binding. Binding sites for barbiturates and steroids may be present on all subunits. (Schematic modified from McKernan and Whiting, 1996 ).

#### **1.4.iv. Biophysical Properties of the GABA<sub>A</sub> receptor**

The GABA<sub>A</sub> receptor is an anion-selective channel. Electrophysiological studies by Bormann *et al.*, (1987) using cultured mouse spinal neurones revealed the following permeability sequence (relative to chloride ions) for the GABA<sub>A</sub> receptor: SCN<sup>-</sup> > I<sup>-</sup> > NO<sub>3</sub><sup>-</sup> > Br<sup>-</sup> > NO<sub>2</sub><sup>-</sup> > Cl<sup>-</sup> > HCO<sub>3</sub><sup>-</sup> and an ionic conductance sequence which was reversed in order. These authors estimated an effective pore diameter of 5.6nm for the GABA<sub>A</sub> receptor. In mammalian neurones a gradient of chloride ions exists across the membrane. Activation of GABA<sub>A</sub> receptors results in movement of chloride ions in or out of cells, depending on the electrochemical driving force present.

Development of the single-channel recording technique (Hamill *et al.*, 1981) has enabled the characterisation of GABA<sub>A</sub> receptors at the single-channel level. Using this technique, Bormann *et al.*, (1987) described multiple conductance levels of the GABA<sub>A</sub> receptor in outside-out patches of cultured mouse spinal neurones and found a predominant conducting state of 30pS with three less frequently occurring states of 44, 19 and 12 pS. Similar experiments conducted by Macdonald *et al.*, (1989a), again in cultured mouse spinal neurones, also revealed main conductance states of 27-30pS and two less frequently occurring states of 17-19pS and 11-12pS for GABA<sub>A</sub> receptors.

The single-channel gating properties of the main conductance level of GABA<sub>A</sub> receptors in cultured mouse spinal neurones has been well characterised (Sakmann *et al.*, 1983; Macdonald *et al.*, 1989a; Twyman *et al.*, 1990; Twyman and Macdonald, 1992). These investigators have shown that the GABA<sub>A</sub> receptor has three different open states. For example, low concentrations (0.5μM) of GABA open channels with a mean open duration of 0.5ms whereas higher concentrations (up to 5μM) open channels with mean durations of 2.6 and 7.6ms (Macdonald *et al.*, 1989a; Macdonald and Olsen, 1994). Multiple closed states, with different mean closed durations, also exist for the GABA<sub>A</sub> receptor, namely two brief closed states with mean durations of 0.2 and 1.4ms states with time constants which are concentration independent, and at least three longer closed states with time constants which decrease with increasing agonist concentration. Perhaps, not surprisingly, several investigators have shown that the gating properties of recombinant GABA<sub>A</sub>

receptor single channels vary according to subunit subtype (reviewed by Macdonald & Olsen, 1994).

#### **1.4.v. Subunit Heterogeneity & Stoichiometry of GABA<sub>A</sub> Receptors**

The GABA<sub>A</sub> receptor is formed by the assembly of five subunits to produce a ligand-gated ion channel complex (Macdonald & Olsen, 1994; Nayeem *et al.*, 1994). To date, molecular cloning and expression studies have revealed the presence of at least nineteen different, but structurally similar, gene products encoding for GABA<sub>A</sub> receptor subunit isoforms. Subsequently, six distinct glycoprotein classes have been identified on the basis of predicted amino acid sequences. These contain the following isoforms:  $\alpha$ 1- $\alpha$ 6,  $\beta$ 1- $\beta$ 4,  $\gamma$ 1- $\gamma$ 3,  $\delta$ ,  $\epsilon$  (Davies *et al.*, 1997a; Whiting *et al.*, 1997) and  $\pi$  (Heblom and Kirkness, 1997). These polypeptides are all approximately 50 kD in size, with four putative transmembrane spanning regions. In addition, some of the GABA<sub>A</sub> receptor genes undergo alternative splicing and splice variants exist for the genes encoding  $\alpha$ 6,  $\beta$ 2,  $\beta$ 4 and for  $\gamma$ 2- subunits. For example, the rat cerebellar  $\alpha$ 6 splice variant has a short sequence of amino acids missing from the N-terminus which renders the receptor non-functional when in combination with other subunits (Korpi *et al.*, 1994). In the chick, two splice variants have been identified for  $\beta$ 2 and  $\beta$ 4 subunits (Bateson *et al.*, 1991; Harvey *et al.*, 1994). In addition, two splice variants have been identified for  $\gamma$ 2- subunits,  $\gamma$ 2S and  $\gamma$ 2L, in human, rat and bovine tissue, with the  $\gamma$ 2L splice variants containing an 8-amino acid insert with a consensus substrate sequence for phosphorylation (Whiting *et al.*, 1990; Kofuji *et al.*, 1991). In the CNS, GABA<sub>A</sub> receptors comprise combinations of  $\alpha$ - and  $\beta$ - subunits, together with one or more of the  $\gamma$ -,  $\delta$ - or  $\epsilon$ - subunits (McKernan and Whiting, 1996; Davies *et al.*, 1997a; Barnard *et al.*, 1998). To date, in humans, the  $\pi$ -subunit has been located mostly in peripheral tissues, particularly in the uterus, but it has also been identified in small amounts in the hippocampus and temporal cortex (Heblom and Kirkness, 1997). In addition,  $\rho$ -subunits have been found to make up GABA<sub>A</sub> receptors only in the retina (Cutting *et al.*, 1991, 1992 and Ogurusu and Shingai, 1996).

The amino acid sequence homology between subunit classes is about 30-40%, but within a subunit class, it is 60-80%. A separate gene encodes for each subunit and these genes have been shown to cluster together on different chromosomes. For example, in the human genome, genes encoding  $\alpha 1$ ,  $\alpha 6$ ,  $\beta 2$  and  $\gamma 2$  subunits are clustered onto chromosome 5 (Wilcox *et al.*, 1992; Hicks *et al.*, 1994; Buckle *et al.*, 1989), genes encoding  $\alpha 2$ ,  $\beta 1$  and  $\gamma 1$  are clustered onto chromosome 4 (Wilcox *et al.*, 1992; Buckle *et al.*, 1989) and genes encoding  $\alpha 5$ ,  $\beta 3$  and  $\gamma 3$  are clustered onto chromosome 15 (Wagstaff *et al.*, 1991; Knoll *et al.*, 1993; Greger *et al.*, 1995).

Immunoprecipitation and *in situ* hybridization studies have revealed the existence of a large diversity of GABA<sub>A</sub> receptor isoforms in native cells which differ in terms of their subunit combination. These different receptor isoforms are known to predominate in different brain regions (Wisden *et al.*, 1992; Rabow *et al.*, 1995). In fact, *in situ* hybridisation studies have indicated that an overlapping regional distribution of the various subunits exists throughout the brain. In a comprehensive *in situ* hybridization study, Wisden *et al.*, (1992) mapped the mRNA distribution of 13 GABA<sub>A</sub> receptor subunit-encoding genes in adult rat brain and demonstrated that the mRNAs of the rat GABA<sub>A</sub> receptor subunits were present in a complex, overlapping pattern. For example the mRNA distribution of the  $\alpha 1$  subunit was ubiquitous throughout the brain, whereas the mRNA of the  $\alpha 6$  subunit was confined to cerebellar granule cells (Wisden *et al.*, 1992, Laurie *et al.*, 1992). A number of studies have shown that most GABA<sub>A</sub> receptor subunits are expressed in the hippocampus (reviewed by McKernan and Whiting, 1996). Wisden *et al.*, (1992) demonstrated that in the hippocampus,  $\alpha 1-5$  mRNA were expressed throughout, with the  $\alpha 2$ -subunit mRNA being the most prevalent, particularly in dentate gyrus cell layers and in the CA1 and CA3 regions.  $\alpha 3$  mRNA expression was highest in the dentate granule cells, but was also present, to a lesser degree, in pyramidal cells.  $\alpha 5$  mRNA expression levels, virtually absent from all other areas of the brain in this study, was highest in CA1 and CA3 regions, but was also present in the dentate gyrus (see also Thompson *et al.*, 1992).  $\beta 1$ ,  $\beta 2$  and  $\beta 3$  mRNAs were

found throughout the hippocampus with  $\beta 1$  and  $\beta 3$  being predominant and  $\beta 1$  being rarely expressed elsewhere in rat brain. All  $\gamma$ -subunit mRNAs were expressed in the hippocampus, with the  $\gamma 2$ -subunit being the most common and  $\gamma 3$  being relatively uncommon. Finally, the  $\delta$ -subunit mRNA distribution was found to be confined to dentate granule cells.

Although the number of possible combinations of these native isoforms is vast, only twelve to twenty-four combinations are thought to exist in the native form (e.g. Smith and Olsen, 1995). Studies involving the expression of GABA<sub>A</sub> receptor subunits in *Xenopus* oocytes and mammalian cells suggest the occurrence of an ordered assembly of GABA<sub>A</sub> receptor subunits into a preferred complex of functional GABA<sub>A</sub> receptors, rather than a random assembly of subunits (Macdonald & Olsen, 1994).

A number of possible subunit stoichiometries has been shown to exist in mammalian brain. For example, immunoprecipitation studies (Khan *et al.*, 1994, 1996; Pollard *et al.*, 1995) have indicated that two different  $\alpha$ -subunits,  $\alpha 1$  and  $\alpha 6$ , can co-occur in cerebellar receptors. In similar studies, other investigators have demonstrated the co-occurrence of different  $\beta$ -subunits and that this co-occurrence varies with brain region. For example, Li and De Blas, (1997) have indicated that 33% of rat cerebral cortex GABA<sub>A</sub> receptors contain both  $\beta 1$  and  $\beta 2$  subunits and that 19% contain both  $\beta 1$  and  $\beta 3$  subunits; also  $\beta$  subunit co-occurrence was seen predominantly in the hippocampus with the least occurring in the cerebellum. Additionally, using immunoprecipitation techniques in whole rat brain, Benke *et al.*, (1996) demonstrated that  $\gamma 2$  and  $\gamma 3$  subunits co-assembled in native GABA<sub>A</sub> receptors. Immunoprecipitation studies in  $\alpha 6$ -containing cerebellar granule cells indicated that 10%, 51% or 21% of these cells contained homogeneous  $\beta 1$ ,  $\beta 2$  or  $\beta 3$  subunits, respectively and that two different  $\beta$ -subunits were present in 18% of these cells (Jechlinger *et al.*, 1998).

Kirsch *et al.*, (1995), for example, have demonstrated that any ternary combination of  $\alpha$ ,  $\beta$  and  $\gamma$  subunits expressed recombinantly in oocytes or cultured cells can form functional membrane-bound receptors. In fact, the triple combination of  $\alpha 1\beta 2\gamma 2$  has been suggested to constitute the majority of GABA<sub>A</sub>

receptor mRNAs in many neurone populations in the brain (McKernan *et al.*, 1991; McKernan and Whiting, 1996). In the case of the native receptor, however, it has been suggested that the combination of specific subunit subtypes may be genetically predetermined for given cell populations (McKernan and Whiting, 1996).

It is now increasingly recognised that the precise subunit composition of the receptor confers distinct pharmacological and physiological properties to GABA<sub>A</sub> receptors (Mihic *et al.*, 1995; McKernan and Whiting, 1996). These properties will be discussed in detail where appropriate.

Additionally, GABA<sub>A</sub> receptor function may be modified by agents that increase protein phosphorylation. Phosphorylation is a process whereby receptor structure is covalently modified and it is catalysed by enzymes known as protein kinases, for example, cyclic adenosine monophosphate-dependent protein kinase (PKA), protein kinase C (PKC) and protein kinase G (PKG) calmodulin dependent protein-kinase II and tyrosine kinases. *In vitro* recombinant studies (reviewed by Moss and Smart, 1996) suggest that the  $\beta$  and  $\gamma 2$  subunits are the primary sites of GABA<sub>A</sub> receptor phosphorylation.

Phosphorylation of GABA<sub>A</sub> receptors in different neuronal preparations may cause enhancement or inhibition of GABA<sub>A</sub> receptor function. For example, in mouse spinal neurones, phosphorylation by PKA decreases GABA<sub>A</sub> receptor activation (Porter *et al.*, 1990), whereas in rat retinal cells PKA phosphorylation may increase receptor function (Veruki and Yeh, 1992, 1994) and may also be involved in receptor assembly (Angelotti *et al.*, 1993). PKC-mediated phosphorylation usually results in inhibition of GABA<sub>A</sub> receptor function. For example, early studies using rat or chick brain mRNA injected into *Xenopus* oocytes, demonstrated, for the first time, that activation of PKC by phorbol esters caused inhibition of GABA-evoked whole-cell currents (Sigel and Baur, 1988; Moran and Dascal, 1989). Several other investigators have also demonstrated inhibition of GABA<sub>A</sub> receptor function by PKC-mediated phosphorylation (for review see Moss and Smart, 1996). In contrast, tyrosine phosphorylation has been reported to enhance GABA<sub>A</sub> receptor function in

superior cervical ganglion neurones by increasing mean open time and open channel probability (Moss *et al.*, 1995).

## **1.5. GABA<sub>A</sub> Receptor Pharmacology: Agonists and Antagonists**

The GABA<sub>A</sub> receptor possesses binding sites for many chemically diverse compounds. Included amongst these are sites for agonists, partial agonists, competitive-antagonists and positive and negative allosteric modulators. A description of some of the important agonist and antagonists which have contributed to the characterisation of the GABA<sub>A</sub> receptor is presented below, followed by a description of the allosteric modulators of the GABA<sub>A</sub> receptor which are central to this thesis.

### **1.5.i. Agonists**

GABA is the primary endogenous ligand for the GABA<sub>A</sub> receptor. It is a flexible molecule which can adopt a number of low energy conformations allowing it to interact with different GABA receptors, enzymes and transporters (Johnston *et al.*, 1978). Another GABA<sub>A</sub> agonist, which is more potent than GABA itself is muscimol, the naturally occurring isoxazole analogue obtained from the hallucinogenic mushroom *Amanita muscaria*. Curtis *et al.*, (1971) demonstrated that GABA (0.5M) and imidazole acetic (0.5M) acid were approximately equipotent as depressants of cat spinal interneurons when applied iontophoretically, whereas muscimol (0.5M) was much more potent. The potency ratio was reflected by the currents required to produce equal diminution of neuronal firing (20nA for GABA compared to 1nA for muscimol in one cell; firing (2nA for GABA compared to 3nA for imidazole acetic in a second cell). Similar experiments by Krosggaard-Larsen *et al.*, (1977) have shown that THIP (4,5,6,7-tetrahydroisoxazolo-[4,5-c]pyridin-3-ol), a conformationally restricted, bicyclic synthetic analogue of muscimol, is more selective for GABA<sub>A</sub> receptors than muscimol or GABA, but is equipotent with GABA and less potent than muscimol in terms of its ability to inhibit neuronal activity in the cat spinal cord, *in vivo* (Krosggaard-Larsen *et al.*, 1977). Isoguvacine (1,2,3,6-

tetrahydropyridine-4-carboxylic acid), a compound where the isoxazole of THIP has been substituted by a carboxyl group, however, is equipotent with muscimol in its ability to inhibit neuronal activity in the cat spinal cord, *in vivo* (Krogsgaard-Larsen *et al.*, 1977) and demonstrates some selectivity for  $\beta$  subunits (Bureau and Olsen, 1990). Studies in rat cerebral cortex slices demonstrate that neither THIP nor isoguvacine (0.5-1mM) affect GABA transaminase activity or GABA uptake (Krogsgaard-Larsen *et al.*, 1977). ZAPA (Z-3-[(aminoiminomethyl)thio]prop-2-enoic acid) is a conformationally restricted isothiuronium analogue of GABA and it is selective for low affinity GABA<sub>A</sub> receptors, which are modulated by benzodiazepines. ZAPA facilitates the binding of diazepam (EC<sub>50</sub> 0.19 $\mu$ M for ZAPA and 0.46 $\mu$ M for GABA; Allan *et al.*, 1986) and displaces the low affinity binding of GABA to rat brain membranes (IC<sub>50</sub> in washed synaptosomal membranes for inhibition of [3H] GABA binding – GABA 70 $\mu$ M and ZAPA 46 $\mu$ M; Allan *et al.*, 1991). (+)-TACP ((+)-trans-(1S,3S)-3-aminocyclopentane-1-carboxylic acid) is a stereoisomer of a cyclopentane analogue of GABA (Allan *et al.*, 1979). (+)-TACP is a potent GABA<sub>A</sub> agonist, which does not interact with GABA enzymes or transport systems.

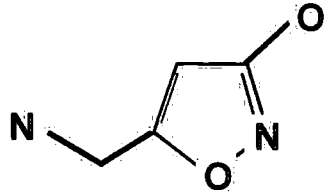
### **1.5.ii. Partial Agonists**

A number of partial agonists also exist, for example, 4-PIOL (5-(4-piperidyl) isoxazol-3-ol), thio-THIP, (Krogsgaard-Larsen *et al.*, 1994), piperidine-4-sulphonic acid and other related compounds (Falch *et al.*, 1985). 4-PIOL is a “non-fused” THIP analogue which is approximately 200 times less potent than isoguvacine as an agonist, with an EC<sub>50</sub> of 91 $\mu$ M in whole-cell voltage-clamped hippocampal neurones, and 30 times less potent than bicuculline methochloride as an antagonist (Kristiansen *et al.*, 1991). Thio-THIP appears to be a low-efficacy partial agonist in human brain recombinant receptors expressed in oocytes (Krogsgaard-Larsen *et al.*, 1994), but a full agonist in cat spinal dorsal horn interneurons where it has half the potency of THIP or GABA when these agonists are applied electrophoretically at concentrations of 0.2M (Krogsgaard-Larsen *et al.*, 1983).

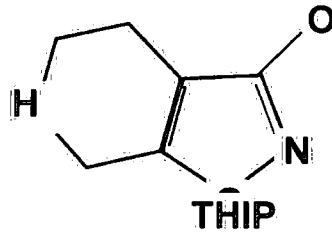




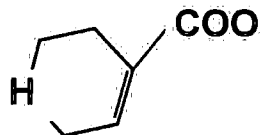
**$\gamma$ -Aminobutyric Acid**



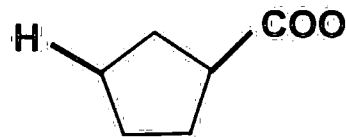
**Muscimol**



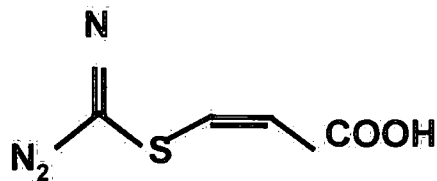
**THIP**



**Isoguvacine**



**(+)-TACP**



**ZAPA**

**Figure 1.3:** Shows the GABA<sub>A</sub> receptor agonists, GABA, muscimol, THIP, isoguvacine, (+)-TACP and ZAPA.

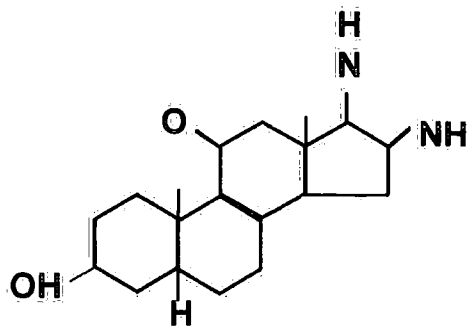
### 1.5.iii. Competitive Antagonists

Competitive antagonists of the GABA<sub>A</sub> receptor are thought to act at GABA recognition sites. In 1970, bicuculline, a convulsant compound from the plant *Dicentra cucullaria*, was found to antagonise the inhibitory actions of GABA in cat spinal Renshaw cells, whereby bicuculline (10mM) was found to considerably reduce the depressant action of electrophoretically-applied GABA on neuronal excitability (Curtis et al., 1970). Bicuculline is a phthalide isoquinoline alkaloid and is structurally similar to the GABA<sub>A</sub> receptor agonist, muscimol (Andrews and Johnston, 1979). In addition, other convulsant isoquinoline alkaloids, such as (+) hydrastine and corlumine have been associated with GABA antagonism (Curtis and Johnston, 1974). Securinine, from the plant *Securinega suffruticosa*, is a convulsant indolizidine alkaloid which is a selective GABA<sub>A</sub> receptor antagonist. Securinine induced tonic seizures in mice with a CD<sub>50</sub> dose four times less potent than that of bicuculline (28±3mg/kg versus 8±4mg/kg), but was approximately 7 times less potent than bicuculline in inhibiting [<sup>3</sup>H]GABA binding to rat brain membranes (Beutler et al., 1985). In the same study, electrophysiological experiments conducted in cat spinal cord neurones revealed that securinine blocked the inhibitory action of GABA, but not glycine. Electrophysiological studies conducted in the cat spinal cord in vivo revealed that SR95531 (5mM), ("gabazine" or 2-(3-carboxypropyl)-3-amino-6-p-methoxyphenylpyridazinium bromide), was a selective GABA<sub>A</sub> receptor antagonist (Gynther and Curtis, 1986). In addition, Michaud et al., (1986) demonstrated in a microiontophoretic in vivo study that SR95531 (5mM) and bicuculline methochloride (5mM), with equal potency, inhibited GABA-elicited responses recorded extracellularly in the rat cuneate nucleus in a dose-dependent, competitive and reversible manner for ejection currents up to 100nA.

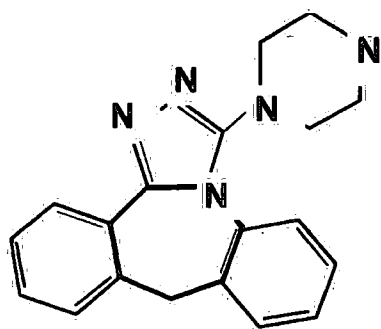
RU5135 (3- $\alpha$ -hydroxy-16-imino-5 $\beta$ -17-aza-androstan-11-one) is an aminidine steroidal compound which is the most potent GABA<sub>A</sub> receptor antagonist known, being about 214 times more potent than bicuculline in the rat cuneate nucleus slice with a pA<sub>2</sub> value of 8.31 (Simmonds and Turner, 1985). RU5135, however, also acts as a glycine antagonist in the rat optic nerve (pA<sub>2</sub>

= 7.67; Simmonds and Turner, 1985) and in the cat spinal cord *in vivo* (Curtis and Malik, 1985).

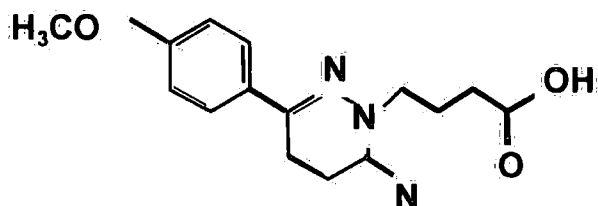
Pitrazepin (3-(piperazinyl-1)-9H-dibenz(c,f)triazolo(4,5-a)azepine) (and a number of other N-aryl piperazines) is known to be a potent, but not selective, GABA<sub>A</sub> receptor antagonist. In binding studies, pitrazepin reversed the inhibitory effect of 1 $\mu$ M GABA on 35S-TBPS binding with an EC<sub>50</sub> OF 0.36 $\mu$ M and was found to be 4 to 5 times more potent than bicuculline (Squires and Saederup, 1987). In electrophysiological studies in the cat spinal cord *in vivo*, pitrazepin hydrochloride (5mM) blocked GABA- and glycine- mediated neuronal inhibition (Curtis and Gynther, 1986). Other competitive GABA<sub>A</sub> receptor antagonists include (+)-tubocurarine (5mM), which apart from being an acetylcholine nicotinic antagonist, also weakly antagonised cortical GABA<sub>A</sub> and glycine receptors (Hill *et al.*, 1972) and cuneate nucleus glycine receptors (Hill *et al.*, 1973).



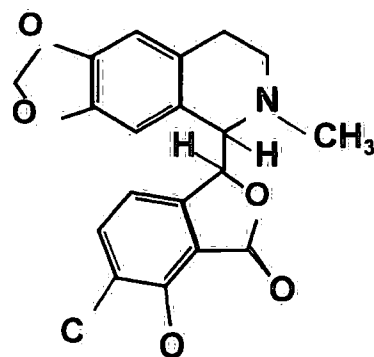
**RU5135**



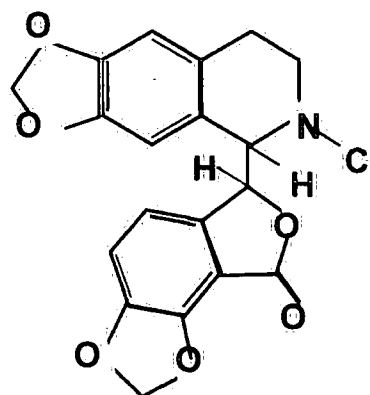
**Pitrazepine**



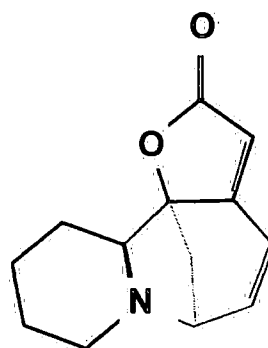
**SR95531**



**(+) Hydrastine**



**Bicuculline**



**Securinine**

**Figure 1.4:** Showing chemical structures of competitive antagonists of the GABA<sub>A</sub> receptor in order of potency : RU5135> pitrazepine> SR95531 ≈ (+) hydrastine > bicuculline > securinine.

### **1.5.iv. Non-competitive antagonists**

Picrotoxin, obtained from the poisonous plant *Anamirta cocculus*, is an equimolar mixture of a potent convulsant, picrotoxinin, and a less potent convulsant, picrotin (Curtis and Johnston, 1974). Picrotoxin is a non-competitive antagonist of the GABA<sub>A</sub> receptor, which is thought to directly block the chloride channel, rather than binding to the GABA recognition site. In contrast to bicuculline (10 $\mu$ M), picrotoxin (10 $\mu$ M) can act as an antagonist whether applied intra- or extracellularly to voltage-clamped bullfrog dorsal root ganglion cells (Akaike *et al.*, 1985). Also, in the rat cuneate nucleus, synergistic antagonism of GABA-evoked currents by a combination of picrotoxin and bicuculline suggested different sites of action for these compounds (Simmonds, 1980). Indeed, picrotoxinin binding sites, identified with [<sup>35</sup>S]-TBPS, are known to be closely associated with the chloride channel of the GABA<sub>A</sub> receptor. Apart from antagonising GABA-mediated responses, at higher concentrations, picrotoxin has also been reported to antagonise the neuronal effects of glycine in neurones (Davidoff and Aprison, 1969) and in spinal neurones (Curtis *et al.*, 1969). In addition, iontophoretic application of picrotoxin, albeit at higher concentrations (a saturated solution) than those required for GABA antagonism, antagonized the inhibitory effect of 5-HT on visual cortex neurones of the rat *in vivo* (Mayer and Straughan, 1981).

Furosemide is a chloride transport blocker which has been shown to antagonise the action of GABA in a number of preparations, for example in rat cuneate nucleus slices (Simmonds, 1982), guinea pig ileum (Taniyama *et al.*, 1988) and frog sensory neurones (Inomata *et al.*, 1988). Simmonds, (1982) demonstrated that furosemide (0.1-1mM) antagonised muscimol-evoked responses in rat cuneate nucleus slices in a non-competitive manner. In addition, furosemide antagonises recombinant GABA<sub>A</sub> receptors expressed in oocytes in a subunit-selective manner. Electrophysiological experiments by Korpi *et al.*, (1995) have shown that furosemide potently antagonizes  $\alpha 6\beta 2\gamma 2s$ - (IC<sub>50</sub>  $\approx$  10 $\mu$ M), but not  $\alpha 1\beta 2\gamma 2s$ -containing receptors (IC<sub>50</sub> > 3mM). Binding studies by the same authors also indicated that furosemide was selective for

$\beta 2/3\gamma 2$ -containing receptors and was ineffective at  $\alpha 1/6\beta 1\gamma 2s$ -containing receptors.

$Zn^{2+}$  (50-300 $\mu$ M) has also been shown to inhibit GABA-evoked responses in rat neurones (Smart and Constanti, 1990; Smart, 1992). Patch-clamp studies of embryonic and adult sympathetic neurones performed by Smart, (1992) revealed that antagonism of GABA-evoked currents by  $Zn^{2+}$  was subject to a developmental influence, whereby embryonic neurones were much more sensitive to inhibition than adult neurones. This study demonstrated that  $Zn^{2+}$  did not affect the main single-channel conductance and mean open and shut times, but rather reduced the opening frequency of the GABA-gated Cl<sup>-</sup> channel. Moreover, recombinant studies have shown that only hetero-oligomeric recombinant GABA<sub>A</sub> receptors, devoid of a  $\gamma$ -subunit are sensitive to  $Zn^{2+}$  inhibition (Draguhn *et al.*, 1990; Smart *et al.*, 1991).

Penicillin is approximately 100 times less potent than bicuculline (Curtis and Johnston, 1974). Penicillin (100-5000 $\mu$ M) has been shown to concentration-dependently antagonise GABA<sub>A</sub> receptor-mediated events recorded in voltage-clamped mouse spinal cord neurones by shortening the duration of channel openings and producing simple open channel blockade of the GABA<sub>A</sub> receptor (Twyman *et al.*, 1992).

Other non-competitive GABA<sub>A</sub> receptor antagonists or more correctly, negative allosteric modulators (for review see Johnston, 1996), include convulsant  $\beta$ -carbolines and quinolone antibiotics together with NSAIDs, these will be discussed below.

### **1.6. Positive Allosteric Modulators of the GABA<sub>A</sub> Receptor**

A variety of compounds do not interact directly with the GABA binding site but, instead, bind to additional allosteric modulatory sites present on GABA<sub>A</sub> receptors. This allosteric binding elicits a conformational change in the GABA<sub>A</sub> receptor which subsequently influences the properties of other binding sites present on the receptor, thus modulating GABA-induced chloride ion flux.

Johnston (1996) has reviewed over 100 compounds which act at the GABA<sub>A</sub> receptor. An overview of the clinical effects, electrophysiology and pharmacology of the major classes of positive allosteric modulators of the GABA<sub>A</sub> receptor, together with a review of the recent evidence of novel modulators including fenamates will be presented below. These data form the background to the experimental work described later in this thesis.

### **1.6.i. Benzodiazepine Modulation of the GABA<sub>A</sub> Receptor**

The first benzodiazepines were introduced into the clinic in the early 1960s, but it was not until some years later that it was recognised that benzodiazepines exerted their therapeutic effects by facilitating GABA-mediated neurotransmission. For example, experiments by Costa *et al.*, (1975) revealed the extraordinary potency ( $\leq 1\mu\text{M}$ ) with which diazepam antagonised convulsions elicited by blockade of GABA synthesis in mice. Subsequently, benzodiazepines have become amongst the most widely used drugs, employed for their anticonvulsant, sedative-hypnotic, anxiolytic and muscle relaxant properties.

On the basis upon early radio-ligand binding studies using rat brain membranes, it was thought there was a single class of benzodiazepine receptors. These studies first revealed the existence of benzodiazepine-specific high-affinity binding sites, in close association with GABA<sub>A</sub> receptors (Braestrup and Squires, 1977; Möhler and Okada, 1977). Initially, benzodiazepine receptors were classified according to their regional distribution, i.e. "central" and "peripheral" type benzodiazepine receptors, which appeared to be pharmacologically distinct (Braestrup and Squires, 1977). Peripheral benzodiazepine receptors have since been shown to be largely associated with the outer mitochondrial membrane (Verma and Synder, 1989) and to be involved in controlling steroidogenesis. However, these receptor types have also been identified within the CNS and thus benzodiazepine receptor subtypes may be re-defined as a "neuronal and non-neuronal benzodiazepine receptors" (Gardner *et al.*, 1992).

"Central" or "neuronal" benzodiazepine receptors associated with GABA<sub>A</sub> receptors have subsequently been classified into subtypes according to their pharmacology. Benzodiazepines, such as diazepam or flunitrazepam, increase the potency of GABAergic ligands at GABA<sub>A</sub> receptors (Study and Barker, 1981), whereas, other benzodiazepines, for example, methyl 6,7-dimethoxy-4-ethyl- $\beta$ -carboline-3-carboxylate (DMCM), decrease the potency of GABAergic ligands. Both of these effects are blocked by benzodiazepine antagonists, such as flumazenil (Nutt *et al.*, 1982; Polc *et al.*, 1982). The availability of compounds such as the triazolopyridazine, CL 218872, the imiadazopyridine, zolpidem and certain  $\beta$ -carbolines, such as DMCM have indicated the existence of other classes of benzodiazepine receptors (Braestrup *et al.*, 1982; Braestrup *et al.*, 1984). These compounds displaced [<sup>3</sup>H]-benzodiazepine binding in a biphasic manner and had different affinities for benzodiazepine receptors in the cerebellum, compared to other brain regions (e.g. Seighart, 1989; Arbilla and Langer, 1986).

Subsequently, these benzodiazepine subtypes have been termed BDZ<sub>1</sub> types, BDZ<sub>2</sub> types and BDZ<sub>3</sub> types. BDZ<sub>1</sub> type receptors have greater affinity for the C1 218,872, zolpidem and certain  $\beta$ -carbolines than BDZ<sub>2</sub> type receptors. BDZ<sub>2</sub> type receptors, in contrast, have low affinity for these compounds, but high affinity for flunitrazepam. BDZ<sub>3</sub> type receptors are selective for the negative allosteric modulator, Ro15-4513, and insensitive to diazepam.

Recombinant studies have shown that benzodiazepine modulation has been shown to be absolutely dependent upon the presence of the  $\gamma$ -subunit in recombinant GABA<sub>A</sub> receptors, with  $\alpha$ -subunits modulating benzodiazepine sensitivity. Electrophysiological studies by Pritchett *et al.*, (1989) demonstrated that co-expression of the human  $\gamma$ 2 subunit, together with  $\alpha$ 1 and  $\beta$ 1 in HEK 293 cells, mediated GABA<sub>A</sub> receptor responses which were potentiated by flunitrazepam and that this potentiation was abolished by the benzodiazepine antagonist, Ro15-1788. To date, recombinant BDZ<sub>1</sub>, BDZ<sub>2</sub> and BDZ<sub>3</sub> type receptors may be distinguished not only by their pharmacology but also by the presence of different  $\alpha$ -subunits. The pharmacological and electrophysiological properties of BDZ<sub>1</sub> type receptors appear to depend on the presence of the  $\alpha$ 1



together with another  $\beta$  and the  $\gamma 2$  subunit. BDZ<sub>1</sub> type receptors constitute the predominant GABA<sub>A</sub> receptor subtype throughout the CNS. Autoradiographic studies of human brain by Faull *et al.*, (1987) and Faull and Villinger, (1988) demonstrated that BDZ<sub>1</sub> receptors were concentrated in the cerebellum but were scarce in the hippocampus, whereas BDZ<sub>2</sub> type receptors were concentrated in the hippocampus, striatum and spinal cord. BDZ<sub>2</sub> type receptor pharmacological and electrophysiological properties appear to depend on the presence of the  $\alpha 2, 3$  or  $5$  together with another  $\beta$  and the  $\gamma 2$  subunit. The BDZ<sub>3</sub> type receptor is the least common subtype, being restricted to cerebellar granule cells (Lüddens *et al.*, 1990) which contain the  $\alpha 6$  subunit together with  $\beta 2$  and  $\gamma$  subunits.

Electrophysiological studies in a variety of neuronal preparations have indicated that benzodiazepines enhance the actions of GABA at the GABA<sub>A</sub> receptor but are unable to directly activate the GABA<sub>A</sub> receptor in the absence of GABA (e.g. Study and Barker, 1981). Fluctuation analysis studies on mouse spinal neurones demonstrated that diazepam increased GABA<sub>A</sub> receptor open channel frequency, without effects on channel conductance or open time (Study and Barker, 1981). Subsequently, single channel studies have shown that this increase in opening frequency is due to increased occurrence of bursting activity, rather than increases in burst duration or single-channel events (Macdonald and Twyman, 1992; Macdonald and Olsen, 1994).

### **1.6.ii. Steroidal Modulation of the GABA<sub>A</sub> Receptor**

Almost 60 years ago, Hans Selye (1941) described the potent sedative-anaesthetic properties of the ring A-reduced metabolites of progesterone and deoxycorticosterone. Subsequently, these "neuroactive steroids" have been shown to rapidly alter CNS excitability and also give rise to rapid behavioural changes *via* non-genomic mechanisms. Binding studies using synaptosomal membrane preparations from rat forebrain demonstrated that certain synthetic and endogenous steroids at physiologically relevant concentrations, were

potent allosteric modulators of the GABA<sub>A</sub> receptor (Harrison and Simmonds, 1984, Majewska *et al.*, 1986).

To date, the 3 $\alpha$ -hydroxy ring A-reduced pregnane steroids are the most widely studied "neuroactive steroids." Allopregnanolone (5 $\alpha$ -pregnan-3 $\alpha$ -ol-one; 3 $\alpha$ -OH-DHP) and allotetrahydrodeoxycorticosterone (5 $\alpha$ -pregnan-3 $\alpha$ ,21-diol-20-one; 5 $\alpha$ THDOC), at physiologically relevant concentrations, have been shown to be amongst the most potent of the known ligands for the GABA<sub>A</sub> receptor (Callachan *et al.*, 1987; Peters *et al.*, 1988). It has been suggested that these steroids may be endogenous GABA<sub>A</sub> receptor ligands, producing sedative and/or anxiolytic effects. Early electrophysiological studies conducted in voltage-clamped bovine chromaffin cells and rat hippocampal neurones demonstrated that alphaxalone (Cottrell *et al.*, 1987) and 5 $\beta$ -pregnan-3 $\alpha$ -ol-20-one and 5 $\beta$ -pregnan-3,20-dione (Callachan *et al.*, 1987; Harrison *et al.*, 1987) reversibly and dose-dependently potentiated GABA-evoked chloride currents in these cells. Conversely, steroids such as RU5135, pregnenolone sulphate and dehydroepiandrosterone sulphate (DHEAS; for review see Lambert *et al.*, 1995) have been shown to antagonise GABA<sub>A</sub> mediated chloride currents.

Like a number of other positive allosteric modulators of the GABA<sub>A</sub> receptor, steroids have been shown, at concentrations higher than those required for potentiation of the GABA response, to directly activate the GABA<sub>A</sub> receptor in the absence of GABA. For example, using voltage-clamped bovine chromaffin cells, Cottrell *et al.*, (1987) demonstrated that alphaxalone (>1 $\mu$ M) and Callachan *et al.*, (1987) demonstrated that 5 $\beta$ -pregnan-3 $\alpha$ -ol-20-one and 5 $\beta$ -pregnan-3,20-dione, directly elicited a membrane current in the absence of GABA, which was potentiated by diazepam and phenobarbitone and inhibited by bicuculline and also had a reversal potential similar to currents elicited by GABA.

Although a number of studies have shown that GABA<sub>A</sub> receptor subunit composition may exert some influence the action of steroids at this receptor

site, generally steroidal effects at the GABA<sub>A</sub> receptor are considered to be relatively subunit-independent (Lambert *et al.*, 1995). For example, two-point voltage-clamp studies conducted by Belelli *et al.*, (1996), using human ( $\alpha_1$ - $\beta_1\gamma_2L$ ) recombinant GABA<sub>A</sub> receptors expressed in *Xenopus* oocytes, demonstrated that the maximal potentiation of GABA-evoked responses by pregnanediols was not influenced by  $\alpha$ -subtype. However, this study also demonstrated that in  $\alpha_2$ -containing receptors such pregnanediols were less potent than in  $\alpha_1$ - or  $\alpha_3$ -containing receptors.

Due to the relative potency and selectivity of action of steroids it is thought that they interact at a specific binding site on the GABA<sub>A</sub> receptor. The notion that these steroids, which are highly lipophilic, could exert their short-term effects by perturbation of cell membranes has been questioned by the fact that intracellularly applied steroids are inert on GABA<sub>A</sub> receptors (Lambert *et al.*, 1990). Single-channel studies conducted in voltage-clamped mouse spinal neurones and bovine chromaffin cells have revealed that steroids, like barbiturates, increase average channel open time duration by increasing the probability of the channel being in long duration open states and, like benzodiazepines, increase the frequency of single channel openings (Callachan *et al.*, 1987; Lambert *et al.*, 1987; Barker *et al.*, 1987; Twyman and Macdonald, 1992).

### **1.6.iii. Barbiturate Modulation of the GABA<sub>A</sub> Receptor**

Barbiturates have been long been used since as sedative-hypnotics, anticonvulsants and anaesthetics and have been shown to potentiate GABA<sub>A</sub>-mediated inhibition in a manner distinct to benzodiazepines.

The potentiating effects of barbiturates do not appear to be subunit dependent; recombinant studies have shown that GABA- induced chloride flux in homomeric channels consisting of either  $\alpha$ -,  $\beta$ -,  $\gamma_2$  or  $\delta$  subunits could be stimulated by barbiturates, indicating that a barbiturate binding site could be

present on each of these subunits, in either homo- or hetero-oligomeric formations (Seighart, 1995). However, Thompson *et al.*, (1996) have demonstrated in *Xenopus* oocytes expressing human recombinant GABA<sub>A</sub> receptors that the degree of maximum potentiation evoked by barbiturates on submaximal GABA-evoked responses is dependent on the presence of the  $\alpha$ -subunit,  $\alpha 6$  conferring the greatest and  $\alpha 1$  conferring the lowest maximal potentiation.

In contrast, high concentrations of barbiturates have been shown to directly activate GABA<sub>A</sub> receptors in the absence of GABA (Macdonald and Barker, 1979; Bormann, 1988). Recombinant studies indicate that different domains on the  $\beta$  subunits may be required for channel activation by barbiturates (Amin and Weiss, 1993), in particular the  $\beta 1$  subunit (Pritchett *et al.*, 1989; Mohler *et al.*, 1990; Sanna *et al.*, 1994) and also the  $\beta 3$  subunit (Cestari *et al.*, 1994) have been implicated. Murine  $\beta 1$  homomeric channels expressed in *Xenopus* oocytes are activated by pentobarbitone, but not by GABA, muscimol or isoguvacine (Krishek *et al.*, 1995) thus, reinforcing the notion that the dual action of barbiturates indicates at least two distinct sites of interaction for barbiturates on the GABA<sub>A</sub> receptor: one for direct activation and another for potentiation. However, it has been reported in *Xenopus* oocytes expressing recombinant GABA<sub>A</sub> receptors that the degree of direct activation of the GABA<sub>A</sub> receptor by pentobarbitone was also influenced by the presence of the  $\alpha$ -subunit.  $\alpha 6$ -containing receptors expressed in *Xenopus* oocytes mediated a maximal response to pentobarbitone which was larger than that obtainable with a maximal concentration of GABA (Thompson *et al.*, 1996). These authors also report that in  $\alpha 6$ -containing receptors, the type of  $\beta$ -subunit present did not markedly influence the direct action of pentobarbitone, whereas in  $\alpha 1$ -containing receptors, pentobarbitone was more potent and efficacious in  $\beta 3$ -containing receptors, compared to  $\beta 2$ - and  $\beta 1$ -containing receptors. Wafford *et al.*, (1996) report that pentobarbitone potentiated, but did not directly activate  $\alpha 4\beta 1\gamma 2$  subunits expressed in *Xenopus* oocytes.

In addition, binding studies conducted in rat cerebral cortex membrane preparations revealed that barbiturates increased the rate of GABA<sub>A</sub> receptor desensitisation (Cash and Subbarao, 1988). Thus, additional barbiturate binding sites on the GABA<sub>A</sub> receptor may contribute to the modulation of GABA<sub>A</sub> receptor desensitisation.

A number of single channel studies in voltage-clamped mouse spinal neurones have revealed that barbiturates enhance GABA<sub>A</sub>-receptor mediated events by increasing mean channel open duration time, but have no effect on opening frequency or receptor conductance (Study and Barker, 1981, Macdonald and Twyman, 1992; Macdonald and Olsen, 1994).

#### ***1.6.iv. Modulation of the GABA<sub>A</sub> Receptor & Other Neuronal Ligand-gated Ion Channels by propofol.***

Propofol (2,6 di-isopropylphenol) is a chemically novel, intravenous general anaesthetic agent (James & Glen, 1980, Smith *et al.*, 1994) which has also been reported to have mood-altering properties (Zacny *et al.*, 1992), anti-emetic effects (Borgeat *et al.*, 1992, Borgeat *et al.*, 1994a, Scher *et al.*, 1992) and neuroprotective effects during neurological insults (Kochs *et al.*, 1992; Weir *et al.*, 1989; Hans *et al.*, 1994). Propofol has also been used successfully in the treatment of status epilepticus (Wood *et al.*, 1988, MacKenzie *et al.*, 1990, Borgeat *et al.*, 1994b). Although there have been no reports of propofol causing epileptiform activity in non-epileptic patients to date (Mahla *et al.*, 1991), the effects of propofol on the electroencephalogram (EEG) activity of epileptic patients appears to be variable, with some patients experiencing increased and others reduced neuronal excitation (Samra *et al.*, 1993).

Pharmacological evidence suggests that the sedative, hypnotic and anaesthetic actions of propofol might be, in part, explained by their action at the GABA<sub>A</sub> receptor-gated chloride channel. Initial electrophysiological studies reported that propofol enhanced synaptic inhibition in the cat spinal cord (Lodge

and Anis, 1984). Whole-cell voltage-clamp studies later demonstrated that propofol concentration-dependently potentiated GABA-evoked membrane currents, which desensitised with high concentrations of propofol, and directly activated GABA<sub>A</sub> receptors in bovine adrenomedullary chromaffin cells, native rodent cortical neurones and in murine hypothalamic GT1-7 neurones (Hales and Lambert, 1991; Hara *et al.*, 1994; Orser *et al.*, 1994; Adodra and Hales, 1995). These experiments revealed that propofol caused an increase in the probability of the GABA<sub>A</sub>-gated chloride channel being in the conducting state, without a significant effect on the single channel conductance (Hales and Lambert, 1991; Orser *et al.*, 1994) or on the reversal potential of GABA (Hales and Lambert, 1991; Hara *et al.*, 1993). In addition, propofol increased the frequency, but not duration of GABA-activated single channel events (Orser *et al.*, 1994). Electrophysiological studies (Hales and Lambert, 1991; Hara *et al.*, 1993) and radio-ligand binding studies (Concas *et al.*, 1991 and 1994) have indicated the existence of separate binding sites for propofol, steroids and barbiturates.

At clinically relevant concentrations (up to 22 $\mu$ M in plasma; Vyuck *et al.*, 1992), in the absence of GABA, propofol has also been shown to directly activate the GABA<sub>A</sub> receptor, in a bicuculline-sensitive manner (Hales and Lambert, 1991; Hara *et al.*, 1993). Recently, studies using different human brain GABA<sub>A</sub> receptor subunit isoforms and mRNAs from mouse brain expressed in *Xenopus* oocytes have demonstrated that this direct activation requires the presence of the  $\beta$  subunit (e.g. Sanna *et al.*, 1995ab; Hill-Venning *et al.*, 1997). In *Xenopus* oocytes expressing human recombinant GABA<sub>A</sub> receptors, the presence of the  $\beta_2$ -subunit conferred a slightly greater sensitivity for direct activation than  $\beta_1$ -subunit (Hill-Venning *et al.*, 1997).

Potentiation of GABA-evoked responses was thought to be subunit-independent (Sanna *et al.*, 1995b; Davies *et al.*, 1997b). However, more recent studies demonstrate that expression of  $\alpha$  and  $\gamma_{2L}$ -subunits influences the direct and modulatory effects of propofol on the GABA<sub>A</sub> receptor. Lam and Reynolds, (1998) have shown using human recombinant GABA<sub>A</sub> receptors in *Xenopus*

oocytes, that the efficacy of propofol potentiation was greater in  $\alpha_1\beta_2$  than in  $\alpha_1\beta_2\gamma_{2L}$  receptor isoforms and that potentiation of the  $\alpha_2\beta_2\gamma_{2L}$  receptor isoform by propofol occurred with higher affinity and lower efficacy than in the  $\alpha_1\beta_2\gamma_{2L}$  receptor isoform. These authors also demonstrated that the presence of the  $\gamma_{2L}$  subunit decreased the sensitivity of the  $\alpha_1\beta_2$  receptor isoform to the direct effects of propofol and that replacement of the  $\alpha_1$ - with the  $\alpha_2$ -subunit subtype increased the receptor sensitivity to the direct effects of propofol. Additionally, human recombinant GABA<sub>A</sub> receptors in *Xenopus* oocytes containing  $\alpha_4$ -subunit subtypes were subject to allosteric modulation, but not direct activation by propofol (Wafford *et al.*, 1996). Kradowski *et al.*, (1998) have reported that a point-mutation in the TM3 region of the  $\beta_1$ -subunit (M286W) of human recombinant GABA<sub>A</sub> receptors expressed in HEK cells, abolished the potentiation of GABA by propofol without affecting the ability of propofol to directly activate the GABA<sub>A</sub> receptor. Conversely, a mutation in the TM2 region of the  $\beta_1$  subunit  $\alpha_2\beta_1$ (S265I) did not greatly affect potentiation, but reduced direct activation by propofol. Kradowski *et al.*, (1997) have also demonstrated that the efficacy of propofol for modulation, but not its potency, was influenced by the presence of the  $\alpha$ -subunit: presence of  $\alpha_1$  enabled a greater maximal potentiation than  $\alpha_6$ ; in contrast, the direct activation evoked by propofol was greater in  $\alpha_6$ - than in  $\alpha_1$ -containing receptors. In addition, Jones *et al.*, (1995) and Kradowski *et al.*, (1997) have reported that modulation by propofol is independent of the  $\gamma$ -subunit.

A number of behavioural studies have attributed the CNS excitation observed following propofol-mediated general anaesthesia in mice to glycine antagonism (Al Muhandis *et al.*, 1991; Dolin *et al.*, 1992; Bansinath *et al.*, 1995). However, voltage-clamp studies have shown that propofol potentiated native strychnine-sensitive glycine receptor-mediated currents in murine spinal neurones (Hales and Lambert 1991), but neither potentiated nor directly activated these receptors in hippocampal neurones (Hara *et al.*, 1994).

Barann *et al.*, (1993) initially reported that propofol non-competitively inhibited the 5-HT-induced influx of  $^{14}\text{C}$ -guanidinium through 5-HT<sub>3</sub> receptors of N1E-115 neuroblastoma cells and concluded that the 5-HT<sub>3</sub> receptor could be a target for general anaesthetic agents like propofol. However, a later binding study by Appadu and Lambert, (1996) using the same preparation, concluded that propofol exerts little direct effects on 5-HT<sub>3</sub> receptors at clinically relevant concentrations (<22 $\mu\text{M}$  in plasma, Vyuck *et al.*, 1992). Voltage-clamp studies performed in homomeric 5-HT<sub>3</sub> receptors expressed in *Xenopus* oocytes by Machu and Harris (1994) examined the effects of propofol on 5-HT<sub>3</sub> receptors expressed in *Xenopus laevis* oocytes and found that over the therapeutic concentration range (1-22 $\mu\text{M}$ , Vuyk *et al.*, 1992), propofol did not enhance or inhibit 5-HT<sub>3</sub> receptor-mediated responses.

A number of electrophysiological investigations have examined the effects of propofol on nicotinic acetylcholine receptors (nAChR). Voltage-clamp studies on clonal BC3H-1 cell (an embryonic muscle type nAChR with a subunit stoichiometry of  $\alpha 2\beta\gamma\delta$ ) by Watchtel and Wegrzynowicz (1992) and Dilger *et al.*, (1994) have demonstrated, that propofol (81 $\mu\text{M}$ ; Watchtel and Wegrzynowicz, 1992 and 25-250 $\mu\text{M}$ ; Dilger *et al.*, 1994) decreased channel open time. Dilger *et al.*, (1994) observed that propofol decreased the channel open time, but did not cause flickering or decreased amplitude of the channel. The authors suggest that the anaesthetic effects of propofol on nAChRs may be in part the result of propofol binding to the nAChR, though not necessarily in the channel pore, and an ensuing "interruption of the flow of ions through the pore of the channel" (Dilger *et al.*, 1994, 1995).

Early studies revealed that propofol inhibits NMDA-receptor mediated polysynaptic reflexes in cat spinal cord (Lodge & Amis, 1984). Yamakura *et al.*, (1995) in a voltage-clamp study, demonstrated that propofol (50-1000 $\mu\text{M}$ ) only slightly inhibited current responses to AMPA-, kainate- and NMDA-selective glutamate receptor channels expressed in *Xenopus* oocytes and concluded that NMDA receptor activity is only slightly suppressed throughout propofol-



maintained anaesthesia. Voltage-clamp studies in cultured mouse hippocampal neurones have also demonstrated that propofol, at concentrations outside the therapeutic range, inhibited the NMDA receptor subtype of the glutamate receptor family with an  $IC_{50}$  of  $160\mu\text{M}$  (Orser *et al.*, (1995). These authors suggest that this inhibition occurs through an allosteric modulation of the channel rather than a blocking action on the ion channel.

#### **1.6.v. Loreclezole & its Action at the GABA<sub>A</sub> Receptor**

The triazole derivative loreclezole (Z)-1-[2-chloro-2-(2,4-dichlorophenyl)ethenyl]-1,2,4-triazole or R 72 063) is a broad-spectrum antiepileptic agent which reduces seizure activity and increases seizure threshold in animal models based on chemical or electrical stimulation (Waqueier *et al.*, 1990; Ashton *et al.*, 1992, Green *et al.*, 1996) but, unlike many other anticonvulsants, does not induce sedative-hypnotic effects (Waqueier *et al.*, 1990, Ashton *et al.*, 1992). These effects are thought to be, at least in part, due to positive allosteric modulation of the GABA<sub>A</sub> receptor.

A number of electrophysiological studies have demonstrated the potentiating effects of loreclezole at human recombinant GABA<sub>A</sub> receptors expressed in *Xenopus* oocytes. Convincing data from recombinant studies has demonstrated that loreclezole acts at a novel allosteric site located on  $\beta$ 2- or  $\beta$ 3 GABA<sub>A</sub> receptor subtypes (Wafford *et al.*, 1994). Point-mutation analysis has identified a single amino acid in the TM2 region of  $\beta$ 2 (asparagine 289) and  $\beta$ 3 (asparagine 290) subunits which confers sensitivity to loreclezole-induced channel modulation; this sensitivity was some 300-fold higher in  $\beta$ 2- or  $\beta$ 3-containing receptors than  $\beta$ 1-containing receptors where the amino acid at this corresponding position is serine (Wafford *et al.*, 1994; Wingrove *et al.*, 1994; Donnelly and Macdonald, 1996).

Recently, Kapur and Macdonald (1996) have demonstrated that only 50% of whole-cell voltage-clamped dentate granule cells were sensitive to modulation by loreclezole and suggested that these were not  $\beta$ 1-containing

receptors. Additionally, Xue *et al.*, (1996) in a binding study, have demonstrated a greater potency of loreclezole and a higher GABA sensitivity in the cerebellum and thalamus where there is a relatively higher expression of  $\beta 2$  and  $\beta 3$  subunits compared to other areas of the CNS. Stevenson *et al.*, (1995) have shown that potentiation of GABA-evoked currents by methyl-6,7-dimethoxy-4-ethyl- $\beta$ -carboline (DMCM) is dependent on the presence of the same amino acid residue which confers loreclezole sensitivity in  $\beta 2$  or  $\beta 3$  subunits, providing evidence that the low affinity site for  $\beta$ -carboline potentiation is indeed the loreclezole site. Further evidence supporting the importance of the  $\beta$ -subunit in loreclezole-induced modulation of the GABA<sub>A</sub> receptor was given by Whitemore *et al.*, (1996) who report that loreclezole induced a strong modulation of  $\beta 2\gamma 2L$  receptors expressed in *Xenopus* oocytes, but that there was little difference between modulation of  $\alpha 1\beta 2\gamma 2L$  and  $\alpha 4\beta 2\gamma 2L$  receptors, suggesting that the modulation induced by loreclezole is not greatly influenced by the presence of the  $\alpha$ -subunit. Interestingly, Donnelly and Macdonald (1996) have described a second, subunit independent, voltage-independent and non-competitive inhibitory action of loreclezole on GABA<sub>A</sub> receptors. These investigators have shown that loreclezole enhances the degree and rate of desensitisation of GABA-mediated currents recorded from recombinant GABA<sub>A</sub> receptor containing  $\alpha 1$ ,  $\alpha 5$ , or  $\alpha 6$  together with  $\gamma 2L$  and  $\beta 1$ , 2 or 3 subunits, expressed in fibroblasts, and also in native GABA<sub>A</sub> receptors from mouse cortex.

Several binding studies (Vaught and Waquier, 1991, Van Rijn and Willems van Bree, 1993, Xue *et al.*, 1996; Sanna *et al.*, 1995; Ghiani *et al.*, 1996; Green *et al.*, 1996) have demonstrated that the actions of loreclezole at the GABA<sub>A</sub> receptor do not appear to be mediated through the benzodiazepine, barbiturate, steroid or propofol site. However, Green *et al.*, (1996) also report, using rat cerebral cortex membrane preparation, that although loreclezole interacts specifically with an allosteric site on the  $\beta$ -subunit of the GABA<sub>A</sub> receptor, it also alters the binding characteristics of other modulatory sites for chlormethiazole and pentobarbitone.

In addition, several binding studies (Sanna *et al.*, 1995c; Ghiani *et al.*, 1996; Sanna *et al.*, 1996) and electrophysiological studies (Sanna *et al.*, 1996) have indicated that loreclezole (at concentrations higher than those required for potentiation), like propofol, can directly activate the GABA<sub>A</sub> receptor in the absence of GABA. To date, however no study has determined the effects of loreclezole at the single channel level.

#### **1.6.vi. Negative Allosteric Modulators of the GABA<sub>A</sub> Receptor**

A number of negative allosteric modulators also exist for the GABA<sub>A</sub> receptor (reviewed by Johnston, 1996) including the  $\beta$ -carboline, DMCM, diazepam-binding inhibitor (DBI) and the  $\beta$ -substituted  $\gamma$ -butyrolactone,  $\beta$ -ethyl- $\beta$ -ethyl- $\gamma$ -butyrolactone ( $\beta$ -EMGBL). A novel negative allosteric modulation occurring at the GABA<sub>A</sub> receptor involves an interaction between certain quinolone antimicrobial agents and NSAIDs.

#### **1.6.vii. NSAIDs, Quinolones & the GABA<sub>A</sub> Receptor**

Quinolones are widely prescribed as antimicrobial agents with a relatively low incidence of side effects, such as headaches, insomnia and convulsive seizures. However, certain quinolones when taken in combination with the NSAID, fenbufen, cause a marked increase in the incidence and/or severity of these adverse side effects which can lead to convulsive seizures in humans (Simpson and Brodie, 1985; Anastasio *et al.*, 1988) and in animals (Murayama *et al.*, 1987; Akahane *et al.*, 1989; Hirai *et al.*, 1989).

Subsequently, it has been shown that certain quinolones act as antagonists at the GABA<sub>A</sub> receptor (Akaike *et al.*, 1991; Halliwell *et al.*, 1991; Shirasaki *et al.*, 1991) and that this antagonism is greatly increased in the

presence of biphenyl acetic acid (BPAA), the active metabolite of the NSAID, fenbufen (Akaike *et al.*, 1991; Halliwell *et al.*, 1991; 1995; Shirasaki *et al.*, 1991; Green and Halliwell 1997). In agreement with these electrophysiological studies, several binding experiments have demonstrated the inhibition of [<sup>3</sup>H]muscimol and [<sup>3</sup>H]GABA binding and modulation of [<sup>35</sup>S]-TBPS binding by certain quinolones and BPAA at neuronal GABA<sub>A</sub> receptors (Hori *et al.*, 1987; Yamamoto *et al.*, 1988; Akahane *et al.*, 1989; Squires and Saederup, 1993; Domagala, 1994). Moreover, Akahane *et al.*, (1989) have demonstrated a close correlation between the epileptogenic activity of quinolones and BPAA and their inhibitory potencies for [<sup>3</sup>H] muscimol binding to GABA<sub>A</sub> receptors. Recently, it has been shown that a hybrid molecule of norfloxacin and BPAA is also a potent antagonist at native GABA<sub>A</sub> receptors (Imanish *et al.*, 1996; Ito *et al.*, 1996).

The antagonism induced by quinolones and BPAA has been shown to be selective for the GABA<sub>A</sub> receptor since this combination of drugs have little or no effect at neuronal 5-HT<sub>3</sub>, P<sub>2x</sub> and nACh receptors of the vagus nerve (Green and Halliwell, 1997), ionotropic glutamate receptors in hippocampal neurones (Akaike *et al.*, 1991; Halliwell *et al.*, 1995) or recombinant glutamate and 5-HT<sub>3</sub> receptors expressed in *Xenopus* oocytes (Kawakami *et al.*, 1997). In addition, Green and Halliwell, (1997) have shown a marked decrease in the synergy of antagonism evoked by the action of certain quinolones and BPAA on GABA<sub>A</sub> receptors of the optic nerve suggesting that regional differences exist across the CNS to quinolones and NSAIDs, perhaps reflecting differences in GABA<sub>A</sub> receptor subunit composition.

### **1.7. Fenamate Modulation of GABA<sub>A</sub>-gated-chloride channels**

Woodward *et al.*, (1994) were the first to demonstrate that fenamate NSAIDs both potentiated and inhibited GABA-activated currents recorded from rat brain GABA<sub>A</sub> receptors, expressed in *Xenopus* oocytes. In descending order of potency, flufenamic, meclofenamic, mefenamic and niflumic acid, (10μM)

reversibly potentiated currents evoked by low concentrations of GABA. In contrast, currents evoked by high concentrations of GABA were non-competitively inhibited by these fenamates, with a reverse order of potency. Woodward *et al.*, (1994) also reported that MFA had the highest efficacy in terms of its facilitatory effects, but was the weakest inhibitor, and that the opposite was the case for niflumic acid. The agonist concentration was shown to be the critical determinant between facilitatory and inhibitory effects of fenamates.

In rat cortex GABA<sub>A</sub> receptors expressed in *Xenopus* oocytes, the only other (non-fenamate) NSAIDs which produced facilitatory effects at similar concentrations were sulindac (an acetic acid with effects similar to niflumic acid) and diflusal (salicylic acid with effects similar to flufenamic and meclofenamic acid). At ten-fold higher concentrations, fenoprofen, indomethacin, ibuprofen, naproxen and piroxicam evoked modest potentiations (<120%), whereas flurbiprofen and phenylbutazone caused modest inhibitions in GABA-evoked currents (Woodward *et al.*, 1994). As a positive modulator of GABA-evoked responses, MFA was shown to be 10 000 times more potent than ethanol, equipotent with  $\alpha$ - and  $\delta$ -hexachlorohexane, twice as potent as pentobarbitone and 100 times *less* potent than allopregnanolone. In this study it was also shown that the modulatory effects of MFA, together with pentobarbitone or  $\delta$ -hexachlorohexane, were additive (Woodward *et al.*, 1994).

The potentiating effects of mefenamic acid on rat brain GABA<sub>A</sub> receptors expressed in *Xenopus* oocytes were insensitive to the benzodiazepine antagonist, flumazenil. In the presence of the weak steroid antagonist, 5 $\beta$ -pregnan-3 $\beta$ ol-20-one (3 $\beta$ -OH-DHP) the MFA-potentiated GABA response amplitude was not appreciably decreased, but instead, a synergistic increase on response decay rate was observed (Woodward *et al.*, 1994). In addition, Woodward *et al.*, (1994) have demonstrated that only extracellular application of mefenamic acid resulted in appreciable modulation of GABA-mediated responses, arguing strongly for a membrane-bound recognition site for fenamates, rather than a non-specific membrane perturbation effect.

A preliminary study by Halliwell *et al.*, (1994) demonstrated that mefenamic acid concentration-dependently potentiated submaximal GABA-evoked currents in voltage-clamped rat hippocampal neurones in terms of amplitude and duration. In contrast, Shirasaki *et al.*, (1991) demonstrated a suppression of GABA-evoked ( $EC_{60-70}$ ) responses by mefenamic acid recorded in dissociated rat hippocampal neurones.

The data reviewed above suggest that fenamates may modulate neuronal GABA<sub>A</sub> receptor function. However, no study to date has investigated this interaction on neuronal (or recombinant) GABA<sub>A</sub> receptors .

### **1.8. Aims of This Study**

The main focus of this study was to investigate the actions of fenamate NSAIDs on a series of neuronal ligand-gated ion channels. The initial studies examined the action of a range of allosteric modulators, and the NSAID, mefenamic acid, on GABA<sub>A</sub> receptor-mediated responses recorded from the rat vagus nerve employing a grease-gap recording technique (Marsh, 1989). The grease-gap recording technique is a relatively simple, but robust recording system which allows long-term and stable recordings to be made from a variety of neuronal preparations, including the rat vagus and optic nerves. The rat vagus nerves express a number of extra-synaptic receptors, such as the GABA<sub>A</sub>, 5-HT<sub>3</sub>-, nicotinic acetylcholine, P<sub>2x</sub> receptors and the optic nerve expresses strychnine-sensitive glycine receptors and GABA<sub>A</sub> receptors, which allow the collection of quantitative pharmacological data on a number of drug-receptor interactions to be obtained (e.g. Marsh, 1989; Ireland and Tyres, 1987; Trezise *et al.*, 1993). Thus, the effects of mefenamic acid were compared with other allosteric modulators of the GABA<sub>A</sub> receptor, and in particular, propofol. Additional experiments investigated the action of propofol on 5-HT<sub>3</sub>-, nicotinic acetylcholine- and P<sub>2x</sub> receptor-mediated responses of the rat vagus nerve and strychnine-sensitive glycine receptor-mediated responses of the rat optic nerve.

To further elucidate the mechanism of action and selectivity of fenamates on neuronal ligand-gated ion channels, investigations were carried out using the patch-clamp technique (Hamill *et al.*, 1981) to make recordings from cultured single hippocampal neurones. This technique allows the experimenter to control the internal and external ionic environments of the membrane in order to make high fidelity recordings of agonist-gated channel activity. Primary cultures of hippocampal neurones possess morphological and neurophysiological characteristics similar to those found *in vivo* and are easily accessible under patch-clamp recording conditions. These cells also express a number of ligand-gated ion channels, again with characteristics similar to those seen *in vivo*. Further experiments also investigated the actions of other fenamates at the GABA<sub>A</sub> receptor in order to gain some insight into the structure activity relationship of fenamates.

## **CHAPTER TWO: GENERAL METHODS**

### **2.1. Extracellular Recording Methods**

Electrophysiological recordings were made from freshly excised rat vagus and optic nerves. Agonist-evoked changes in membrane potential were recorded across a high resistance grease-gap using extracellular electrodes. The method employed is an adaptation of that described by Marsh (1989). A description of this method together with the dissection procedures employed is described below.

#### ***2.1.i. Vagus Nerve Dissection***

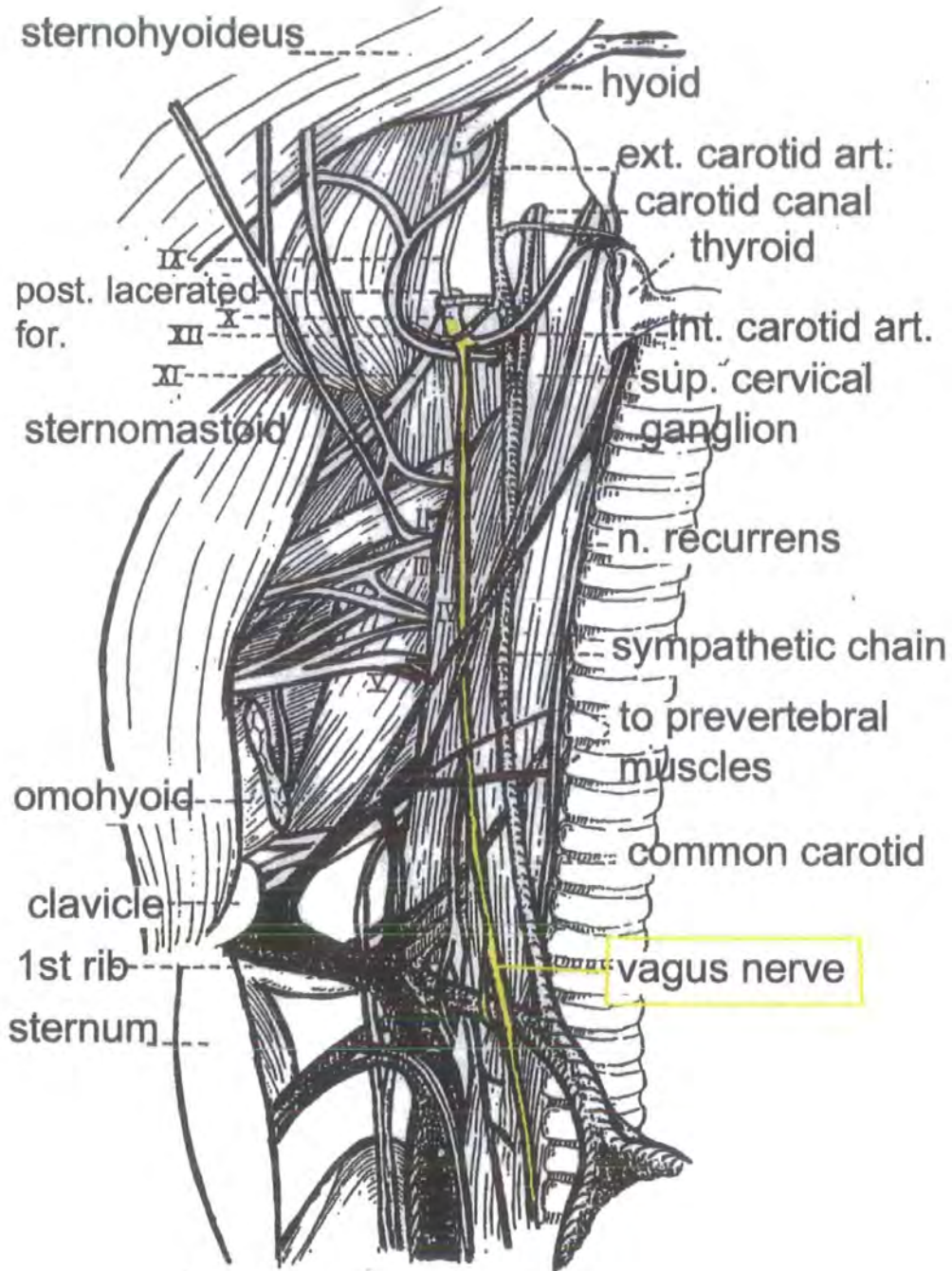
Male Sprague-Dawley rats (100-250g) were killed by a rising concentration of CO<sub>2</sub> (a Schedule 1 method of euthanasia). The skin overlying the throat area was removed to expose the thyroid gland, the sternomastoideus, posterior digastricus and sternohyoideous muscles; these were carefully removed (see figure 2.1). Using a binocular dissecting microscope (Nikon SMZ-2B) the vagus nerves could then be seen adjacent to the common carotid arteries (which run bilateral to the trachea). The vagus nerves were carefully separated from the carotid arteries and cut away at the point at which they entered the thorax. The freed vagus nerves (15-25mm) were then transferred to a 35mm dish of cold, oxygenated, physiologically balanced salt solution (PBS) and the connective tissue sheaths surrounding the nerves were removed using watchmakers forceps. The removal of the sheath was essential to facilitate drug penetration.

#### ***2.1.ii. Optic Nerve Dissection***

Male Sprague-Dawley rats (100-250g) were killed by a rising concentration of CO<sub>2</sub> (a Schedule 1 method of euthanasia) and were then decapitated. Skin overlying the back of the neck to the snout area was removed to expose the skull. The dorsal surface of the skull was bisected using bone scissors and the bone was gently broken away using artery forceps to reveal the frontal cortex. The bone of the orbits above the zygomatic arch was also broken away. The oculomotor muscles were teased away using watchmaker's forceps



to expose the optic nerves. The brain was gently lifted from the cavity of the skull using a spatula and the optic nerves were severed at the optic chiasma. The optic nerves were removed from the back of the eyeballs using small dissecting scissors. The freed optic nerves were then transferred to a 35mm dish of cold, oxygenated PBS.



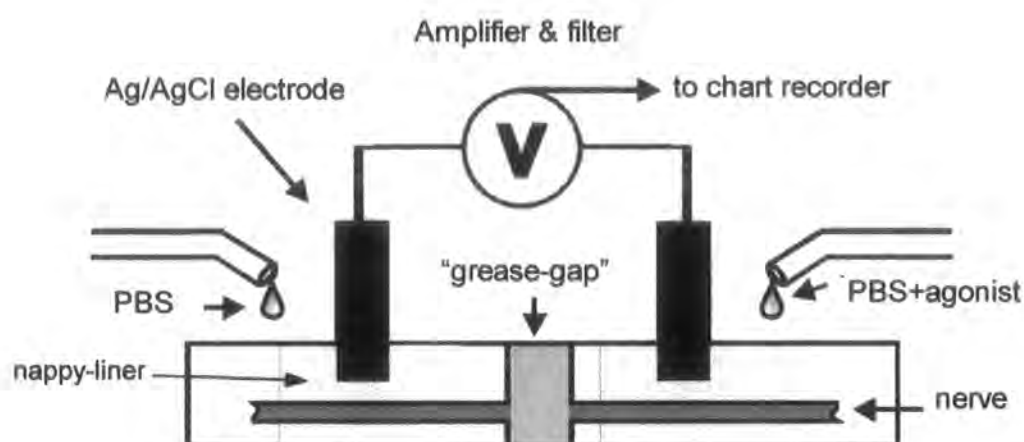
**Figure 2.1** : shows a schematic of the rat thorax showing the position of the vagus nerve (highlighted in yellow) in relation to major blood vessels and muscles. This schematic was modified from Marsh (1989).

## **2.2. Extracellular Recording From The Excised Rat Vagus and Optic Nerves**

A thin seam of grease, (approximately 2mm deep x 2mm wide) (BDH High Vacuum Silicone Grease) was placed midway across the width of a microscope slide. A piece of nappy liner (Boots one-way nappy liner) was placed on each side of the grease seam, as shown in figure 2.2. The nappy liner served to facilitate superfusion of recording PBS and drugs onto and off the nerve. The vagus or optic nerve was placed on top of the microscope slide and onto the nappy liner, so that half of it lay on either side of the grease barrier. A second layer of grease was then placed on top of the first layer, thus, forming a high resistance seal around the nerve trunk. The grease seam also served to hold the nerve in position on the slide. The slide was secured onto a Perspex frame and housed inside a Faraday cage.

Silver-silver chloride electrodes (RC1 electrodes, Clark Electromedical) were positioned on either side of the grease seam, adjacent to the free ends of the nerves; these electrodes were held in position by brackets clamped onto the Perspex frame.

Agonist-evoked changes in DC-potential across the silver-silver chloride electrodes were relayed through miniature coaxial cable to a Neurolog Amplifier (Digitimer, NL106) and a Neurolog Filter (Digitimer, NL125). Signals were low-pass filtered at DC-50Hz. and the recordings were displayed on a Kipp-Zonen flatbed pen chart recorder. Agonist-evoked changes in DC-potential were recorded between 0.03mV and 5mV, under these conditions. Baseline noise levels were approximately  $\leq 0.02\text{mV}$  in amplitude and were mainly attributable to perfusion drip; improvement in these baseline noise levels could often be achieved by re-positioning the electrodes and/or the perfusion pipes.



**Figure 2.2: A simple schematic of the grease-gap technique.** A vagus or optic nerve was placed across a grease-gap barrier onto a microscope slide, which was mounted onto a perspex holder and housed inside a Faraday cage (not shown). The nerve was perfused with a physiologically balanced solution (PBS). Agonist-evoked responses were recorded, extracellularly, amplified, filtered and displayed on a flatbed pen chart recorder. Drugs were dissolved in PBS and delivered to the nerve *via* the perfusion system.

### 2.2.i. Drugs and Solutions

PBS was made up in ultra pure water (Milli-QPLUS) with the following salts (all B.D.H. Ltd., Poole, U.K. except where stated otherwise; concentrations in mM): NaCl (118.0),  $\text{KH}_2\text{PO}_4$  (1.18), KCl (4.7),  $\text{MgSO}_4$  (1.18),  $\text{CaCl}_2$  (2.5), Glucose (11.0), HEPES (Sigma;10.0). The PBS was then titrated to pH 7.2 using concentrated (2.5M) hydrochloric acid.

$\gamma$ -amino butyric acid (GABA) and glycine (both Sigma, Poole, U.K.) were dissolved in PBS as 10mM stock solutions and serially diluted in PBS as required.  $\alpha,\beta$  methylene adenosine tri-phosphate ( $\alpha,\beta$ -MeATP), 5-hydroxytryptamine (5-HT) and dimethyl-phenylpiperazinium (DMPP; all from Sigma) were dissolved in PBS as 1mM stock solutions and serially diluted as required in PBS. Sodium pentobarbitone (Sigma) and alphaxalone (a gift from Dr. David Gemmel, Akzo Nobel, Organon Laboratories, Newhouse, Scotland) were also made up as 1mM stock solutions in PBS. Propofol (also a gift from Dr. David Gemmel) was dissolved in absolute ethanol as a 10mM stock solution

and then serially diluted in PBS. The maximal concentration of ethanol used was 20 $\mu$ M. In selectivity studies, the final concentration of ethanol was 2 $\mu$ M. Picrotoxin (Sigma), suramin (R.B.I., Minnesota, U.S.A.), hexamethonium and strychnine (both Sigma) were all made up as 1mM stocks in PBS and diluted in PBS as required. MDL72222 (1 $\alpha$ H,3 $\alpha$ ,5 $\alpha$ H-tropan-3-yl-3,5-di-chlorobenzoate; R.B.I.) was dissolved in 50% ethanol, as a 1mM stock, and diluted as required in PBS. All solutions and drugs were made up as fresh stocks at the beginning of each experiment.

### **2.2.ii. Experimental Protocol**

All experiments were conducted at room temperature. PBS and drugs were dripped onto the free ends of the nerves at a rate of 2ml/min using a variable speed peristaltic pump (Gilson Minipuls3), via 21G hypodermic needles connected to flexible polythene tubing (internal diameter=0.76mm, Portex, U.K.). Concentration-response curves to each agonist were determined by perfusing different concentrations of agonist, in a quasi random order, onto one end of the preparation only. Pilot experiments indicated that an agonist contact time of approximately 1.5-2 minutes was sufficient to evoke a response in which an equilibrium peak was clearly discernible. Thus, in all experiments, agonists were perfused onto the nerves for 1.5-2 minutes. Application of agonist to the preparation once every 10 $\pm$ 1 minutes for GABA and once every 15 $\pm$ 1 minutes for 5-HT,  $\alpha$ , $\beta$ MeATP and DMPP was sufficient to minimise any sensitisation/desensitisation of the preparation to the agonist.

In experiments investigating drug effects, three control sub-maximal agonist-evoked responses were obtained, followed by three agonist-evoked responses in the presence of each modulator or antagonist concentration. Pilot experiments demonstrated that all compounds produced their maximal effect after 15 minutes pre-incubation; thus, in most cases (except where specified), a 15-20 minute drug pre-incubation period was employed. At the end of such a

protocol the preparation was washed with PBS and control agonist-evoked responses re-established to determine the reversibility of the drug action.

### **2.2.iii. Data analysis**

Agonist-evoked responses were measured at their peak amplitude and are expressed as the arithmetic mean ( $\pm$ s.e.mean of n experiments) of the responses before addition of any drugs. Agonist-evoked responses in the presence of drugs are expressed as the percentage of the response in their absence (control). Data were pooled and mean log [agonist]-response plots and log[modulator] response plots were fitted with a sigmoidal function using a four-parameter logistic equation (sigmoidal concentration-response curve with a variable slope; Graphpad Prism™ v2.0) to determine EC<sub>20</sub> and EC<sub>50</sub> ( $\pm$ 95% confidence intervals; C.I.) values. Control concentration-response curves to agonists were normalised by expressing all responses relative to the concentration that evoked a maximum response for each agonist. The equation used to fit the concentration-response relationship was:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC}_{50} - X) \text{HillSlope})})$$

where X is the logarithm of concentration of drug. Y is the response, which starts at Bottom and goes to Top with a sigmoid shape. "Pseudo" Hill slopes were calculated from the curve. Care must be taken in interpreting these data since the final concentration of agonist which reaches the receptor may differ from the concentration exogenously applied. For example, the degree of agonist uptake/degradation within the nerve is unknown and may reduce agonist concentrations at receptor sites.

## **2.3. WHOLE-CELL VOLTAGE-CLAMP RECORDING METHODS**

### **Hippocampal Cell Culture**

In agreement with Bottenstein (1986), neuronal survival in serum-supplemented media has been found to be variable and varied with different batches of serum. Thus, a variety of experimental procedures and culture media

conditions were employed to improve neuronal viability. All cell culture solutions and media types were filter sterile (0.22 $\mu$ m GS filters, Millipore).

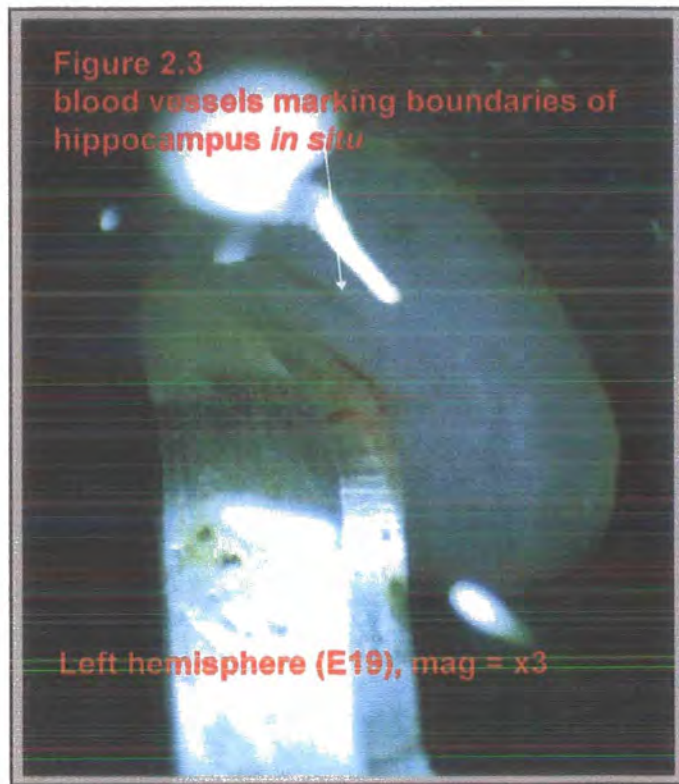
### **2.3.i. Hippocampal Dissection**

Embryonic (E17-19 days gestation) rat hippocampal neurones were isolated and cultured using the method described by Halliwell *et al.*, (1989). Time-mated Wistar rats were killed by cervical dislocation and the embryos (usually around 10-12) were removed and placed into a beaker containing chilled Hanks' balanced salt solution (HBSS; GIBCO). Embryonic brains were removed rapidly and aseptically, using small dissection scissors and a scapula, and placed into petri dish of chilled HBSS. Hippocampi were dissected out under a dissecting microscope (Nikon SMZ-2B) into a 35mm dish containing HBSS as follows: following excision of the embryonic brain, the cerebellum was removed using a small scalpel and discarded. The cerebral hemispheres were separated along the midline fissure, using the blunt edge of the scalpel, and the underlying midbrain was removed. Hippocampi were dissected free from each cerebral hemisphere along the length of the blood vessel as indicated in figure 2.3 and subsequently then chopped into small fragments (2mm<sup>3</sup>).

The tissue was incubated at 37°C, for 60 minutes, in 10mls of filtered enzyme solution (0.22 $\mu$ m GS filters, Millipore) containing the following (all BDH, except where indicated; in mM): NaCl (116.0), KCl (5.4), NaHCO<sub>3</sub> (26.0), NaH<sub>2</sub>PO<sub>4</sub> (1.0), CaCl<sub>2</sub> (1.5), MgSO<sub>4</sub> (1.0), EDTA (Sigma; 0.5), glucose (25.0), DL cysteine (Sigma;1.0) and papain (Sigma; 20units/ml). Dissociation of the tissue was facilitated by gently shaking the flask periodically. The tissue fragments were then washed in 5mls of filtered HBSS containing bovine serum albumin (BSA) and ovomucoid (both Sigma, at 1mg/ml each) and were transferred to another sterile test tube containing 3-4mls of the same solution. The tissue was dissociated mechanically by gentle trituration using a fire polished Pasteur pipette. The upper layer of dissociated cells was layered onto 5mls of HBSS containing BSA and ovomucoid (at 10mg/ml each); this process was repeated until all of the tissue was dissociated. The cell suspension was centrifuged at 100g for 10 minutes. After discarding the supernatant, the cells were re-suspended in 4mls of media 1 or media 2 (described later). Cells were

plated out at a density of approximately  $1-2 \times 10^5$ /35mm Primaria culture dishes (Falcon, Becton Dickinson) and incubated in 1.5mls of culture media 1 or 2, at 37°C, 95% air/ 5% CO<sub>2</sub> and 100% relative humidity. Subsequently, every 5-7 days, approximately two-thirds of the media was replaced with fresh media.



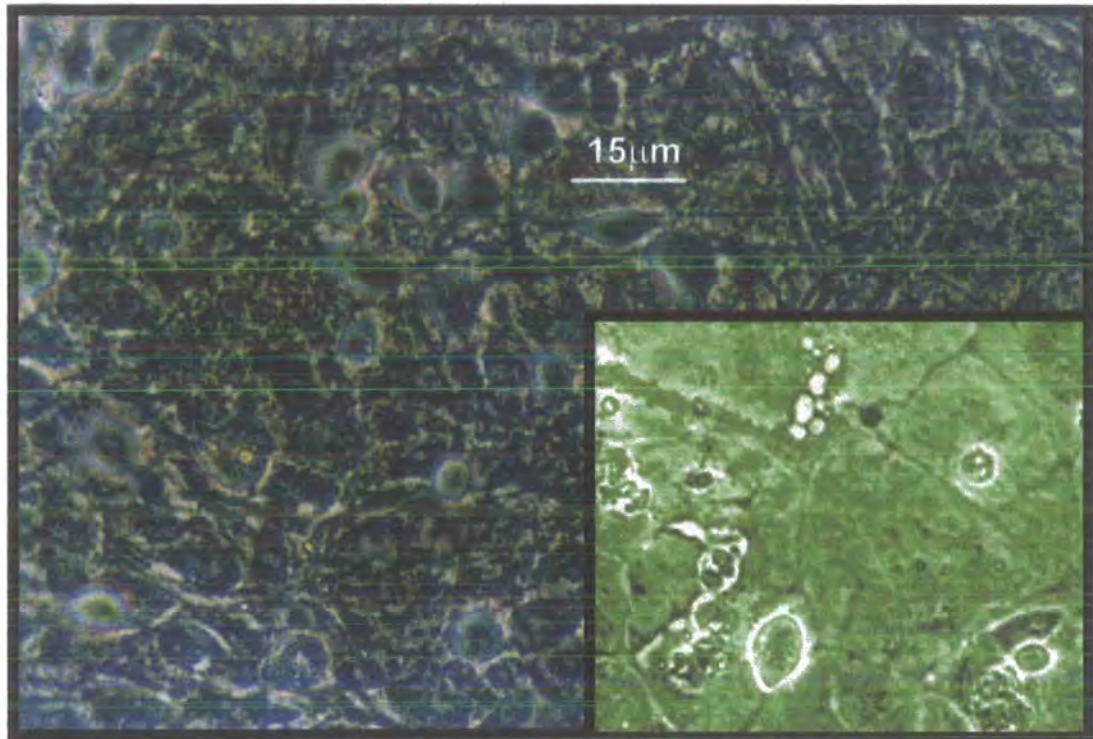


**Figure 2.3:** photograph (x3 magnification) shows the left hemisphere of an embryonic rat brain (19 days gestation) with a scalpel blade underlying the hippocampus. The arrows indicate marker blood vessels around boundaries of hippocampus.

### 2.3.ii. Methods for cell culture maintenance

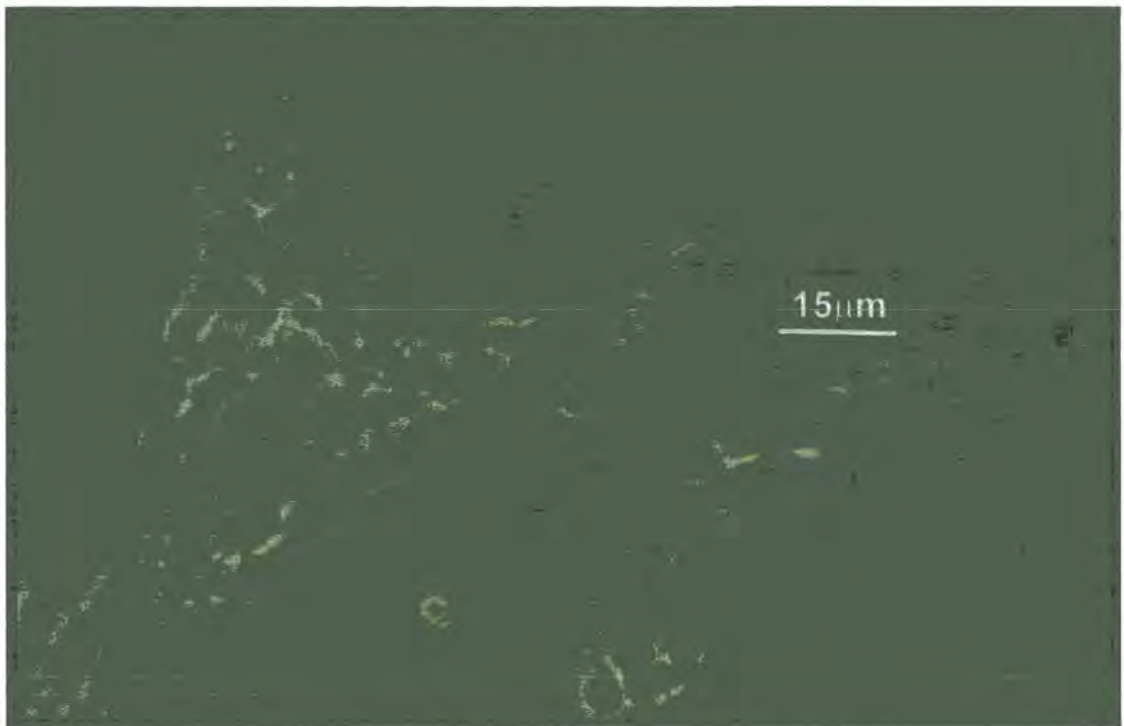
Four different methods were employed for the maintenance of rat hippocampal neuronal primary cultures. These are now described below.

**Method 1:** Hippocampal neurones were plated into culture *media 1*, containing 88% (v/v) minimal essential medium, 5% (v/v) heat-inactivated fetal calf serum, 5% (v/v) heat-inactivated horse serum, penicillin/streptomycin (5000i.u./ml-5000ug/ml), glutamine (2mM) (all supplied by GIBCO) and glucose (20mM). When background glial cells reached confluence (usually within 5-7 days), the media was replaced with fresh media containing the mitotic inhibitor cytosine furano-arabioside ( $10\mu\text{M}$ ; Sigma) for 48 hours. This method was employed for a period of two years from the commencement of this study until Summer 1996, when the serum batch was identified as a critical factor for neuronal survival. Until this time neuronal cell cultures were reliably maintained for up to 40 days (figure 2.4-main image). After Summer 1996, neuronal viability deteriorated after a period of 5 days in culture, critically depending on serum batch, (figure 2.4-insert).



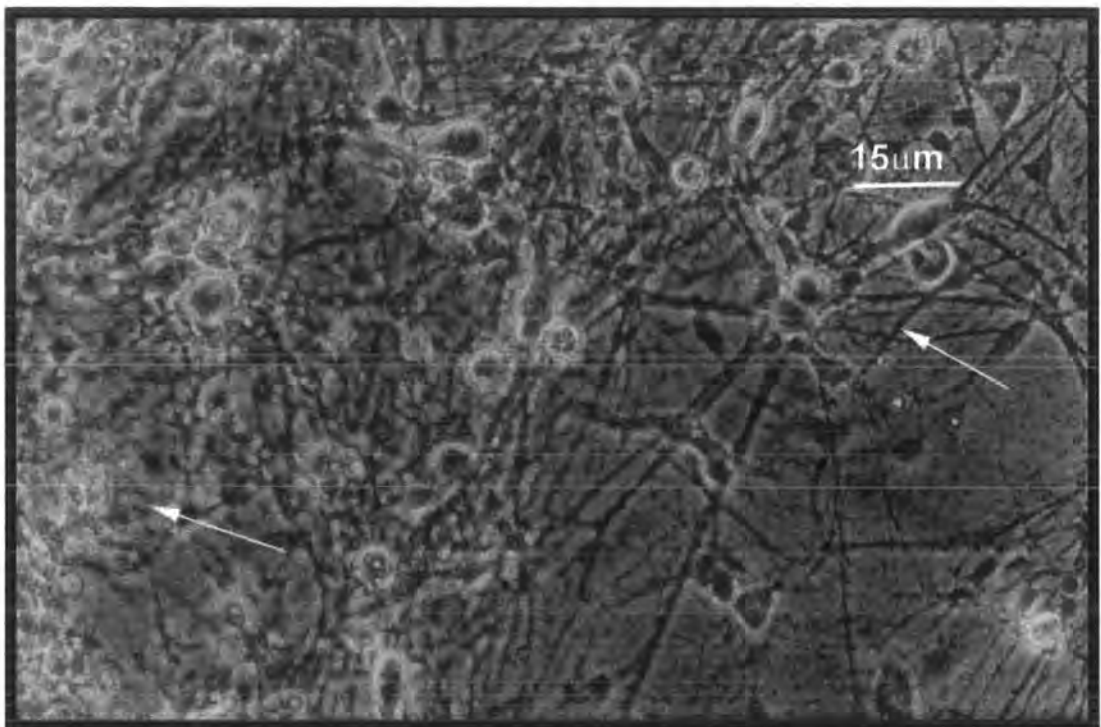
**Figure 2.4:** **Main image:** shows a phase-contrast photomicrograph (x500 magnification) of a primary culture of embryonic rat hippocampal neurones 20 days in culture maintained using method 1, **bottom right : insert** shows a phase-contrast photomicrograph (using a green filter) of a primary culture of embryonic rat hippocampal neurones 20 days in culture (x 500 magnification) using a different batch of serum. Both primary cultures were derived from the same embryos.

**Method 2:** cells were grown in a serum-free media (*media 2*) containing 97.5% (v/v) "Neurobasal" media supplemented with 1% B-27 supplement (v/v), 1% penicillin-streptomycin (v/v) and 0.5% glutamax (v/v) (all GIBCO). Neurobasal media does not support rapid glial development, subsequently neurones survived in clusters or clumps and were unevenly distributed in the culture dishes, (see figure 2.5). This method maintained phase-bright, pyramidal-shaped hippocampal neurones which survived in culture for up to 35 days. Many of these neurones grew larger ( $\approx 25\mu\text{m}$  in diameter) when compared to those maintained in serum-based media ( $15\mu\text{m}$  in diameter). Neuronal survival was best in areas of the culture dish where cell density was low, thus plating density was lowered using this method. Following difficulty experienced in whole-cell patch-clamp recording from cells grown in serum-free *media 2* and the observation that hippocampal neurones appeared unusually large in size compared to those grown in serum-based media 1, the osmolarity of *media 2* was measured and found to be hypo-osmotic at  $199\pm 1$  mosmol ( $n=3$ ) relative to the recording solution ( $264\pm 2$ ,  $n=3$ ). Thus, to optimise recording conditions, the osmolarity of *media 2* was adjusted from 199 mosmol to 265 mosmol by the addition of glucose. This markedly improved recording success with some cells allowing stable recordings which lasted up to 2.5 hours.



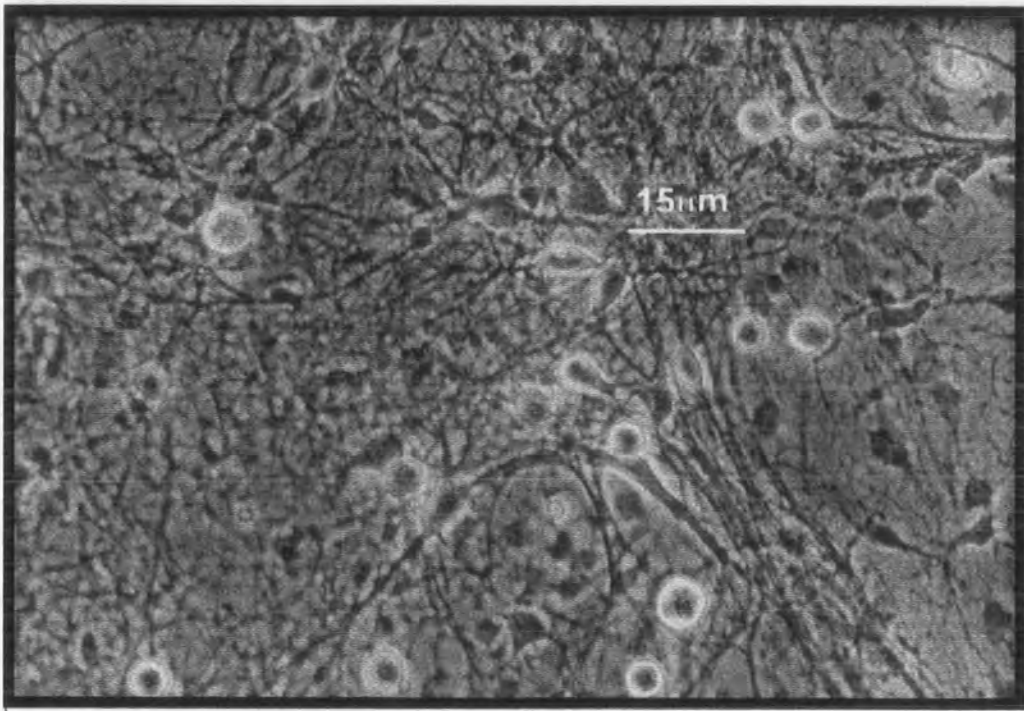
**Figure 2.5:** shows a phase-contrast photomicrograph (x500 magnification) of a primary culture of embryonic rat hippocampal neurones 7 days in culture, maintained using method 2. Using this method, large neurones (c.f. method 1), which had a "clumped" distribution, were frequently observed and glial development was reduced.

**Method 3:** cells were grown in the serum-supplemented *media 1* for between 3-4 days and then transferred into glucose-supplemented *media 2*, as previously described. Undefined media supplements, such as serum, contribute variable amounts of hormones, growth factors, vitamins, substratum modifying proteins, and glycoproteins (for example, fibronectin) which may promote rapid glial and neuronal development (Bottenstein, 1986). Initial plating in serum-supplemented media facilitated the development of a confluent glial layer for neuronal attachment. This method sustained phase-bright, pyramidal-shaped hippocampal neurones in culture for up to 30 days. Although cells were dispersed evenly without obvious clumping using this method, cell death was considerably higher in these cultures, see figure 2.6 (top left). Those surviving cells, however, did enable stable recordings, which lasted over 2 hours in duration.



**Figure 2.6:** shows a phase-contrast photomicrograph (x500 magnification) of a primary culture of embryonic rat hippocampal neurones 21 days in culture (x 500 magnification) maintained using method 3. Figure shows evenly distributed neurones on bed of glial cells. **Bottom left arrow** indicates granulated cells which are undergoing cell death, **top right hand arrow** indicates phase-bright cells.

**METHOD 4:** cells were over-layered onto a previous culture (at least 7 days old in serum-based media) and grown in media 2 or in the serum-supplemented media 1 for 3-4 days and then transferred into media 2. Both of these methods sustained phase-bright, pyramidal-shaped hippocampal neurones in culture for up to 30 days, (see figure 2.7). Again, these cells enabled stable recordings, some of which lasted over 2 hours in duration.



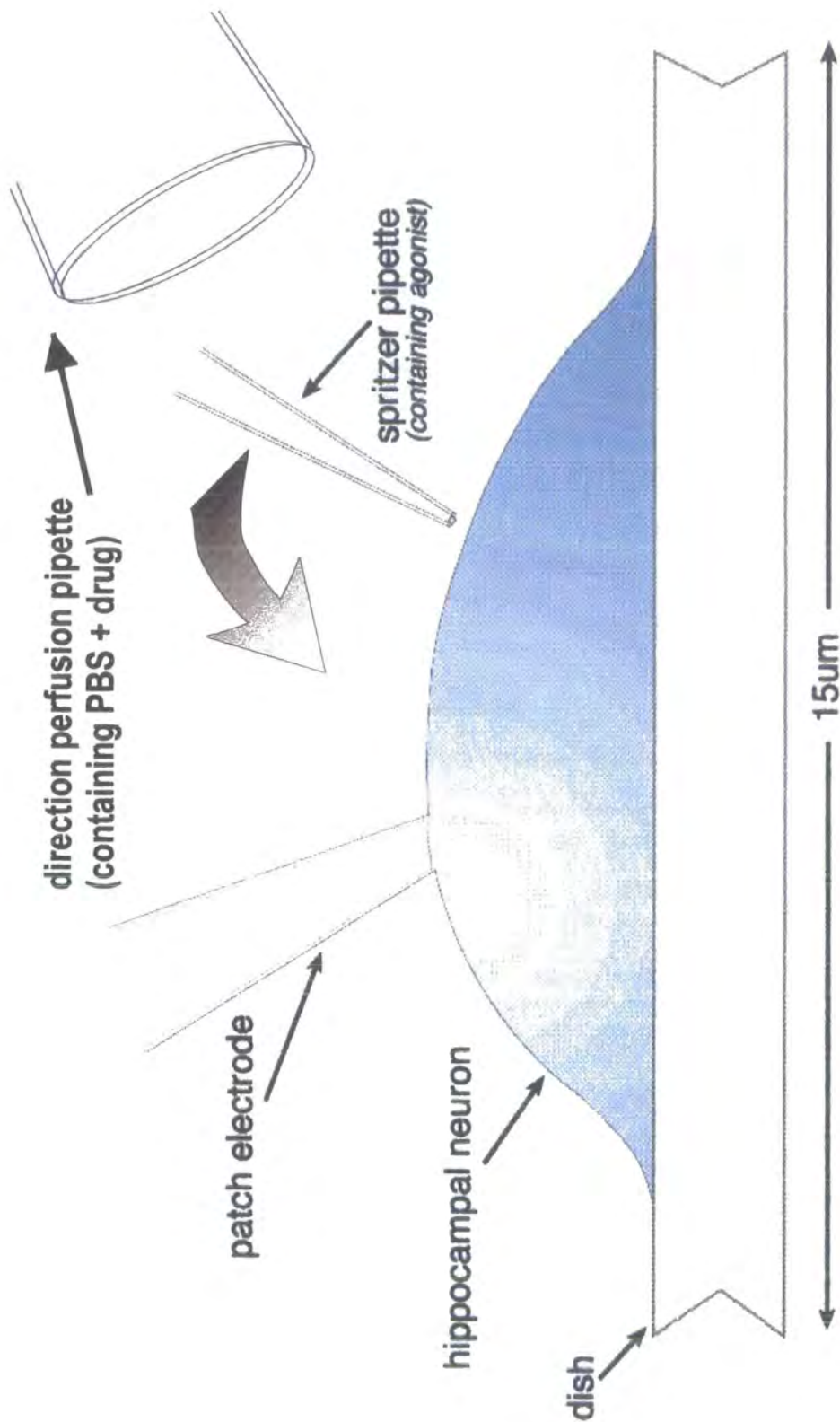
**Figure 2.7:** shows a phase-contrast photomicrograph (x500 magnification) of a primary culture of embryonic rat hippocampal neurones 21 days in culture maintained in culture using method 4. Phase-bright cells are evenly distributed on glial feeder layer.

#### **2.4. Whole-Cell Voltage Clamp Recording**

Whole-cell currents were recorded from voltage-clamped hippocampal neurones using the patch clamp technique (Hamill *et al.*, 1981). Agonists were pressure applied at 1.4kPa and between 0.02-0.033Hz. Cells were superfused with a physiologically balanced solution (PBS) containing 0.3 $\mu$ M tetrodotoxin (TTX, except where stated) at a rate of approximately 2ml/min. All drugs were dissolved in PBS containing 0.3 $\mu$ M TTX. All experiments were carried out at ambient room temperature (20-23°C). All responses were measured at their peak amplitude. Responses in the presence of drugs are expressed as the mean  $\pm$  s.e.m of control.

Whole-cell voltage-clamp recordings were made from hippocampal neurones maintained in culture between 10 to 35 days. The culture media for hippocampal neurones was replaced by the external recording solution (bath solution, see below) containing tetrodotoxin (TTX; 0.3 $\mu$ M) and cells were viewed under a phase-contrast inverted microscope (Nikon TMS) at a magnification of x200. The microscope and micromanipulators used to position the microelectrodes were mounted on a vibration-free air table (Wentworth, U.K.) and housed in a copper Faraday cage (built in house).

Thin glass pipettes (Kimble, soda-lime non-heparinized microhaematocrit tubes) of approximate tip diameter 1-5 $\mu$ m and tip resistance of between 1-3M $\Omega$  were manufactured using a Narishige PB-7 (Tokyo, Japan) two-stage electrode puller. These pipettes were coated at the tip with Sylgard (Dow Corning, Belgium) and fire-polished just prior to use using a fire-polisher built in-house. The pipettes were filled with a conducting salt solution (internal solution; detailed below) containing 2mM Mg-ATP which served to maintain intracellular ATP stores and thus reduce rundown of GABA currents as previously described by Stelzer *et al.*, (1988). A silver chloride coated silver wire, together with the electrolyte-containing pipette formed the recording electrode which was connected to a low-noise, high gain current amplifier (Axopatch 200A, California, U.S.A.). Another silver/silver-chloride RC1-type bath electrode (Clark Electromedical, U.K.) was connected to the virtual ground of the amplifier's headstage.



**Figure 2.8 : A schematic of the patch-clamp recording technique.** Schematic shows a hippocampal neuron adhered to the base of a culture dish. Recordings were made using the patch electrode, (shown on the far left) which is continuous with cell membrane. A spritzer pipette delivering agonist to the cell is shown on the far right. Drugs and PBS were delivered to the cell using a directional perfusion system (not shown to scale); the gray arrow indicates the direction of flow solutions over the cell.

Once a suitable cell was selected on the basis of cell morphology (phase-bright, pyramidal shaped neurons), the pipette electrode was advanced towards the cell using a piezo-driven micromanipulator (Burleigh Patch Clamp Driver, PCS-250, New York, U.S.A.) and, simultaneously, a low voltage square wave pulse (0.2-0.6mV, 4.5ms duration, 5Hz, Grass S48 Stimulator, Warwick, U.S.A.) was delivered to the pipette electrode tip to enable the monitoring of seal resistance using the Axon 200A amplifier. As the pipette electrode tip touched the cell, the current recorded was reduced, thus indicating an increase in seal resistance. Application of negative pressure (*via* a 5ml syringe attached to the amplifier headstage with Nalgene tubing) then allowed the formation of a "gigaseal"; this was indicated by a sharp increase in seal resistance with almost zero current recorded. Once a "gigaseal" was obtained ( $>20\text{G}\Omega$ ), capacitance transients due to the patch pipette and cell membrane were electronically cancelled and the cell was voltage clamped at -60mV unless otherwise stated. Further application of negative pressure caused the patch of membrane below the pipette tip to rupture, allowing the internal milieu of the cell to be accessed and subsequently dialysed with internal solution. At this point the whole cell membrane capacitance and series resistance transient currents were electronically and iteratively cancelled using the amplifier's whole-cell capacitance and series resistance circuitry. Currents were recorded and low pass filtered at 2kHz with an eight-pole Bessel filter before digitisation, storage and display.

Agonists were applied to cells under voltage-clamp by pressure ejection (0.02-0.03Hz, 1.4kPa, 10-100ms duration) from the tip of a "spritzer" microelectrode (tip diameter  $\leq 20\mu\text{m}$ ) using a picospritzer device (General Valve Corporation, New Jersey, U.S.A.). The spritzer pipette was positioned in close proximity to the neurone (80-100 $\mu\text{m}$  laterally from cell and  $\approx 50\mu\text{m}$  above cell) using a Narishige Joystick micromanipulator (Narishige, Tokyo, Japan). Drugs were perfused onto cells from the tip of a plastic cannula (tip diameter, 2mm) positioned in close proximity to the cell ( $\sim 300\mu\text{m}$  laterally from cell and  $\sim 200\mu\text{m}$  above cell) using a Prior micromanipulator. This directional, gravity-feed perfusion system was manufactured in-house by the experimenter.



Where drugs were applied to the cell interior, the time constant for the diffusional exchange of the drug between the recording pipette and the cell interior was calculated according to methods described by Pusch and Neher (1988). The diffusional exchange rate between the patch pipette solution and the cell depends on a number of factors: the molecular weight of the drug, the cell-pipette access resistance and the cell volume (which may be estimated from the cell capacitance, assuming a specific membrane capacitance of  $1\mu\text{F}/\text{cm}^2$ ; Pusch and Neher, 1988). Under the present recording conditions, (using the equation  $\tau = (0.6 \pm 0.17) \cdot R_A \cdot M^{1/3}$  and a correction factor for hippocampal cells; Pusch and Neher, 1988) the time constant ( $\tau$ ) for diffusional exchange of mefenamic acid between the pipette recording solution and the cell interior was calculated to be approximately 71 secs. Drugs were allowed to dialyse the cell interior for a duration four times in excess of the calculated time constant. Whole-cell currents were monitored on a storage oscilloscope (Tectronix 2212, Holland) and recorded onto a two-channel chart recorder (Lectromed, Jersey, U.K.) and on a Digital Audio Tape Recorder (DAT; Biologic DTR-1202, France). The DAT recorder signal was sampled at a frequency of 48KHz and was low-pass filtered using "Digital Comb and FIR filters."

#### **2.4.i. Drugs and Solutions**

Bath solution was made up in ultra pure water (Milli-QPLUS) with the following salts (all BDH, except where stated; concentrations in mM): NaCl (140.0), KCl (2.8),  $\text{MgCl}_2$  (2.0),  $\text{CaCl}_2$  (1.0), HEPES (Sigma; 10.0). Tetrodotoxin (Sigma,  $0.3\mu\text{M}$ ) was also added to the bath solution to block spontaneous voltage-dependent sodium channel activity. NMDA-evoked currents were recorded in the nominal absence of  $\text{Mg}^{2+}$  and in the presence of glycine ( $1\mu\text{M}$ ). Internal solution was made up in ultra pure water (Milli-QPLUS) with the following salts (all BDH; concentrations in mM): KCl (140.0) or  $\text{CaCl}_2$  (140.0),  $\text{MgCl}_2$  (2.0),  $\text{CaCl}_2$  (0.1), EGTA (Sigma; 1.1), HEPES (Sigma; 10.0) MgATP (Sigma; 2). These solutions were then titrated to pH 7.2 using concentrated hydrochloric acid and were filtered before use ( $0.22\mu\text{m}$  GV filters, Millipore, U.K.).

GABA, glycine, sodium pentobarbitone and strychnine (Sigma) were dissolved in bath solution as 10mM and 1mM stock solutions, respectively. AMPA, kainate and NMDA (Sigma) were dissolved in 0.1NaOH as 1mM stocks. D(-)-2-amino-5-phosphonopentanoic acid (D-AP5), and 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX; both Tocris-Cookson, Bristol, U.K.) were dissolved in water as 1mM stocks. Propofol, alphaxalone (both gifts from Dr. David Gemmel, Akzo Nobel, Organon Laboratories, Newhouse, Scotland), diazepam and flumazenil (SIGMA) were dissolved in absolute ethanol as a 1mM stock solutions (the final concentration of ethanol used was always less than 0.1%). Bicuculline was dissolved in 0.1ml of hydrochloric acid (1M) and further diluted with water to form to a 10mM stock. The fenamate NSAIDs: mefenamic acid, flufenamic acid, meclofenamic acid, tolfenamic acid and niflumic acid, were made up as 10mM stock solutions in 0.1M NaOH. The NSAIDs, indomethacin, ibuprofen, diflusal and biphenylacetic acid (BPAA; all Sigma) were made up as 10mM stock solutions in ethanol. Loreclezole (Janssen Research Foundation) was made up as a 1mM stock in DMSO. All drugs were serially diluted in bath solution as required.

#### **2.4.ii. Experimental Protocol.**

GABA (10 $\mu$ M) and other agonists were delivered to the cell via pressure ejection as previously described until a series of submaximal control agonist-evoked responses, consistent in amplitude and duration, were obtained prior to drug application using the directional perfusion system. Drugs were washed off once a clear asymptotic drug effect was observed. Control responses were re-established before further drug applications.

#### **2.4.iii. Data Analysis**

Submaximal agonist-evoked responses were measured at their peak amplitude, and in some cases duration of charge, and are expressed as the

arithmetic mean ( $\pm$ s.e.mean of n experiments) of the response before addition of any drugs. Such experiments from different neurones were pooled and log[modulator]-response plots were constructed. Log[modulator]-response plots were fitted to a sigmoidal function as described above (page 56) to determine  $EC_{50}$  ( $\pm$ 95% C.I.) values. The equation used to fit the concentration-response relationship was:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{Log}EC_{50} - X) \text{HillSlope})})$$

where X is the logarithm of concentration, Y is the response which starts at Bottom and goes to Top with a sigmoid shape. Log[inhibitor]-response plots were fitted point to point and  $IC_{50}$  ( $\pm$ 95% C.I.) values were interpolated. "Pseudo" Hill slopes (see page 58) are calculated from the curve.

## **CHAPTER THREE: RESULTS**

### **Section 3.1: Modulation of agonist-evoked responses of the rat vagus by mefenamic acid**

#### **1. Introduction**

Woodward *et al.*, (1994) have reported that fenamate NSAIDs modulated rat brain GABA<sub>A</sub> receptors expressed in *Xenopus* oocytes and a preliminary study by Halliwell *et al.*, (1994) reported modulation of GABA-evoked currents in voltage-clamped rat hippocampal neurones by mefenamic acid. It was of interest, therefore, to further examine the effects of mefenamic acid on neuronal GABA<sub>A</sub> receptors and other ligand-gated ion channels.

The rat isolated vagus and optic nerves have proved useful preparations to examine the mechanism, site and selectivity of compounds acting on neuronal receptors, since a number of ligand-gated ion channels are present on these nerves (e.g. Marsh, 1989). In the first instance, experiments were undertaken to establish the action of known modulators of the GABA<sub>A</sub> receptor and other ligand-gated ion channels present on the vagus and optic nerves, in order to ascertain the integrity of these preparations.

#### **2. Procedures**

Superfusion of submaximal concentrations of agonist onto either side of the vagus or optic nerves evoked a change in membrane polarisation in most preparations. Agonist contact time was standardised at  $2 \pm 0.5$  min, a time which produced a clearly discernible peak agonist-evoked response. Since response amplitude varied between different sides of the nerve, most experiments were carried out on the side of the nerve which produced the largest agonist-evoked response. Agonists were applied to the nerve at regular intervals. To avoid receptor desensitisation, the intervals between agonist application were  $10 \pm 0.5$  min,  $15 \pm 0.5$  min,  $15 \pm 0.5$  min and  $15 \pm 0.5$  min for GABA-, 5-HT-,  $\alpha, \beta$ -MeATP- and DMPP- evoked responses, respectively. Control agonist-evoked responses were found to remain stable for up to 14 hours. Whenever possible,

vagus nerves were obtained from small rats (~150g) since such nerves produced larger responses to known concentrations of GABA than those from larger rats. However, optic nerves (12-15mm in length) were generally obtained from larger rats (~250g) since in smaller rats, dissection only produced very short lengths of optic nerve (~10mm) and these were more difficult to set up for extracellular recording.

The experimental protocol and data analysis were carried out as described in Methods.

### **3. Pharmacological characterisation of agonist-evoked responses in the vagus and optic nerves**

The experiments reported here have investigated the pharmacology of GABA-, 5-HT-,  $\alpha,\beta$ -MeATP- and DMPP- evoked responses of the vagus nerve and GABA- and glycine-evoked responses of the optic nerve. Further studies examined the action of certain known allosteric modulators of the GABA<sub>A</sub> receptor. Sodium pentobarbitone, alphaxalone and the intravenous anaesthetic agent, propofol, were the compounds investigated since their modulation of the GABA<sub>A</sub> receptor in other neuronal preparations has been well characterised (for review see: Seighart, 1995; Johnston, 1996).

## **4. Results**

### **4.i. Pharmacological characterisation of agonist-evoked responses in the rat isolated vagus nerve.**

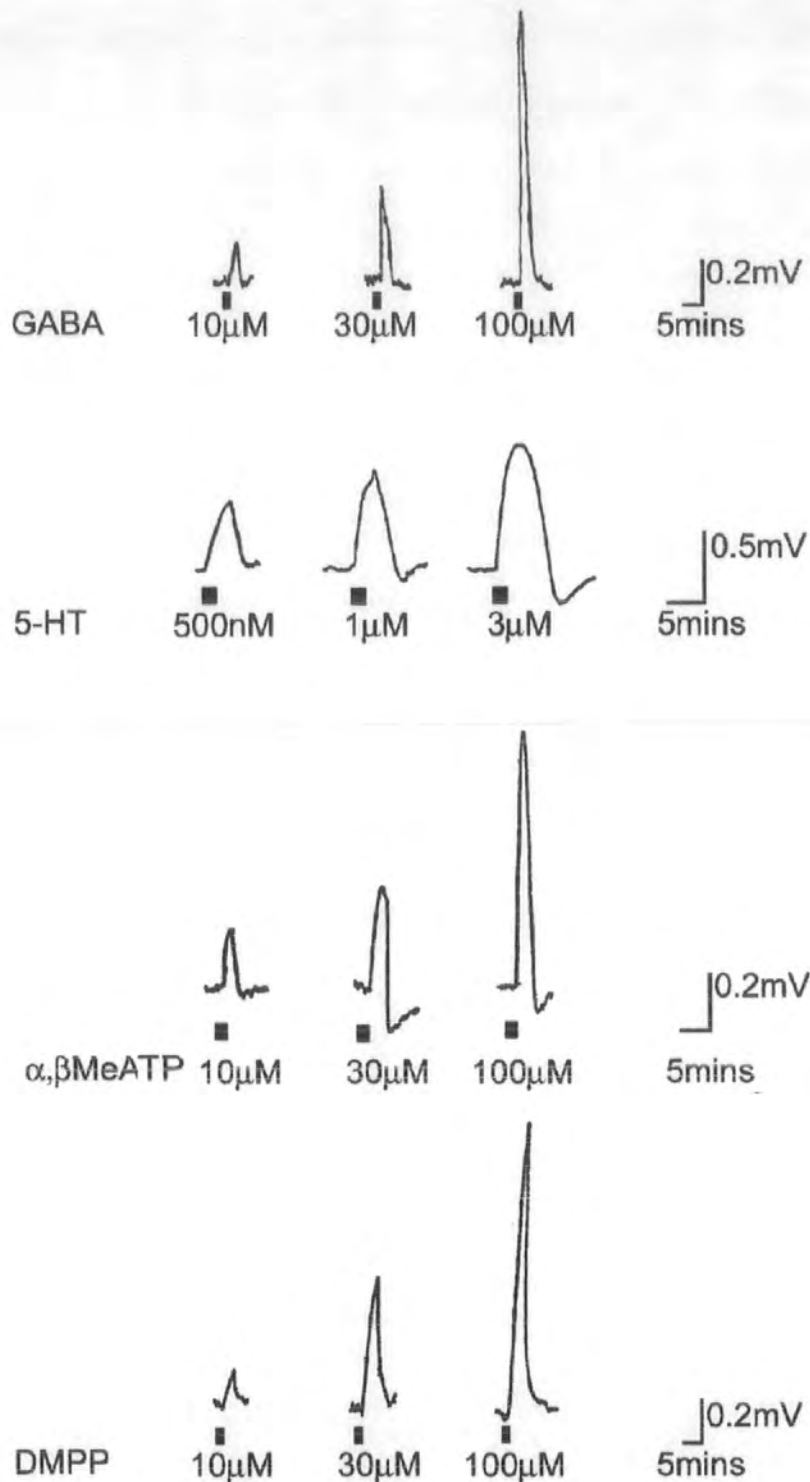
Using the extracellular recording technique, concentration response curves to GABA (3-3000 $\mu$ M), 5-HT (100nM-10 $\mu$ M),  $\alpha,\beta$ -MeATP (3-500 $\mu$ M) and DMPP (3-1000 $\mu$ M) were determined in the isolated vagus nerve. The EC<sub>50</sub> values (geometric mean and 95% confidence intervals) for GABA, 5-HT,  $\alpha,\beta$ -MeATP and DMPP-evoked responses were found to be 34 $\mu$ M (25-43 $\mu$ M, n=5), 0.8 $\mu$ M (0.6-1.0 $\mu$ M, n=4), 48 $\mu$ M (34-62 $\mu$ M, n=5) and 33 $\mu$ M (6-61 $\mu$ M, n=12), respectively. From these data, concentrations approximating these EC<sub>50</sub>

values of 50 $\mu$ M, 0.5 $\mu$ M, 30 $\mu$ M and 30 $\mu$ M for GABA, 5-HT,  $\alpha,\beta$ -MeATP and DMPP, respectively, were employed in further experiments. The EC<sub>20</sub> value for GABA-evoked responses was found to be 13 $\mu$ M, however, for convenience a value of 10 $\mu$ M was employed in further experiments. The mean amplitude of responses evoked by 10 $\mu$ M GABA was found to be 0.1 $\pm$ 0.01mV (n=101). The mean amplitude of responses evoked by 5-HT (0.5 $\mu$ M),  $\alpha,\beta$ -MeATP (30 $\mu$ M) and DMPP (30 $\mu$ M) were found to be 0.36 $\pm$ 0.03mV (n=41), 0.62 $\pm$ 0.06mV (n=41) and 1.98 $\pm$ 0.55mV (n=16) respectively. In experiments where the modulatory effects of drugs were investigated against control agonist-evoked responses, the drug-induced effect on the agonist-evoked response is represented as a percentage change of the control agonist-evoked response. The results of these experiments are summarised in figures 3.1 and 3.2.

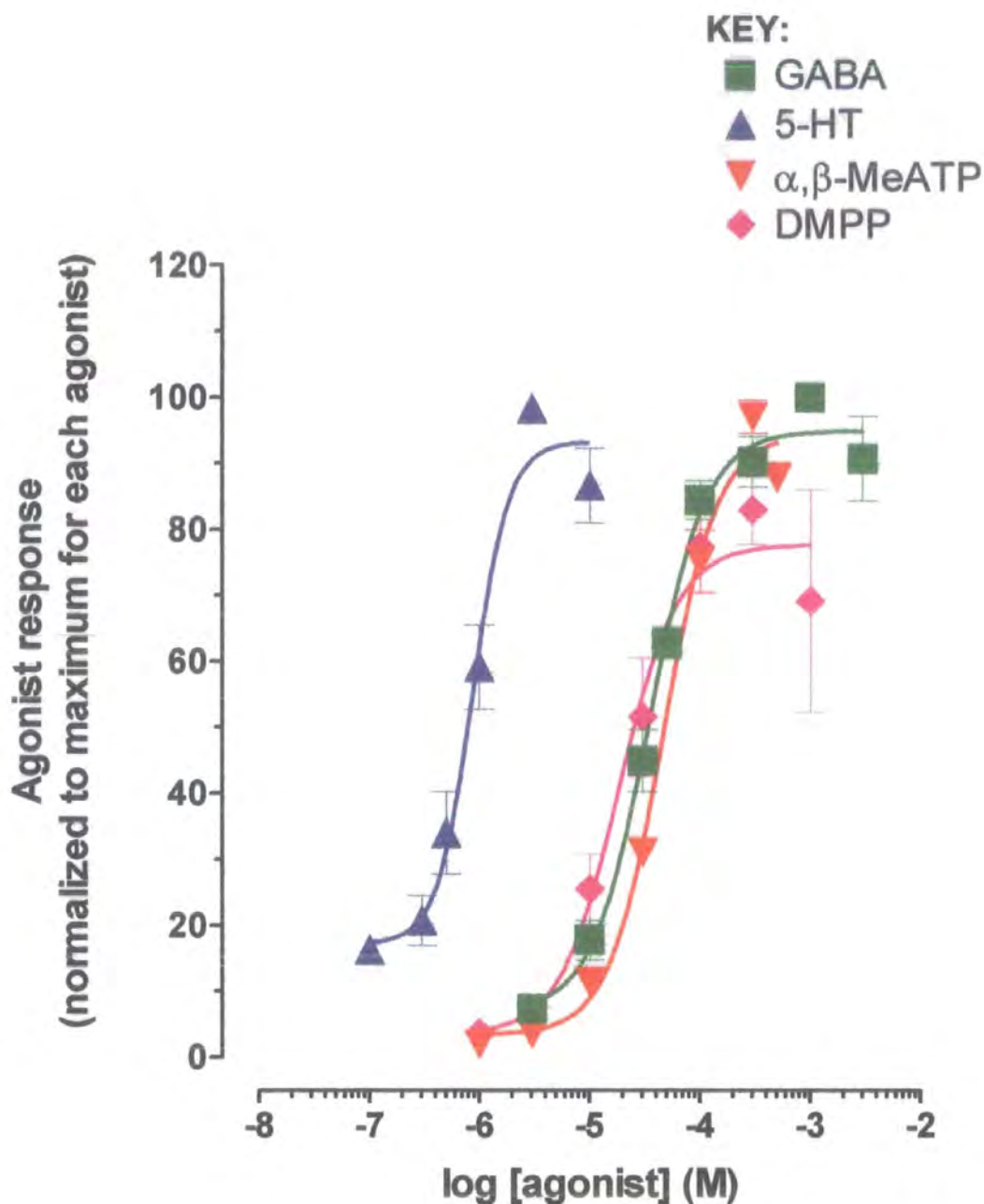
In the isolated rat vagus nerve, concentrations approximating EC<sub>50</sub> concentrations of agonist-evoked responses, were recorded in the presence of their respective antagonists. Submaximal (50 $\mu$ M) GABA-evoked responses were inhibited by 1 $\mu$ M picrotoxin to 55 $\pm$ 8% (n=5, p<0.02) of control. Submaximal (0.5 $\mu$ M) 5-HT-evoked responses were inhibited by 0.1 $\mu$ M MDL-72222 (3-tropanyl-3,5-dichlorobenzoate) to 17 $\pm$ 5% (n=7, p<0.0001) of control. Submaximal (30 $\mu$ M)  $\alpha,\beta$ -MeATP-evoked responses were inhibited by 30 $\mu$ M suramin to 37 $\pm$ 4% (n=5, p<0.0003) of control and submaximal (30 $\mu$ M) DMPP-evoked responses were inhibited by hexamethonium (300 $\mu$ M) to 29 $\pm$ 13 (n=6, p<0.003) of control. These data are consistent with the activation of neuronal GABA<sub>A</sub>, 5-HT<sub>3</sub>, P<sub>2x</sub> and nicotinic ACh receptors, respectively. All drug effects were reversible upon wash. The results of these experiments are summarised in a histogram (figure 3.4); chart recorder traces of these results are shown in figure 3.3.

#### **4.ii. Pharmacological characterisation of agonist-evoked responses in the rat isolated optic nerve.**

Using the extracellular recording technique the concentration response relationships for GABA and glycine (both 0.1-10mM) were determined in the rat

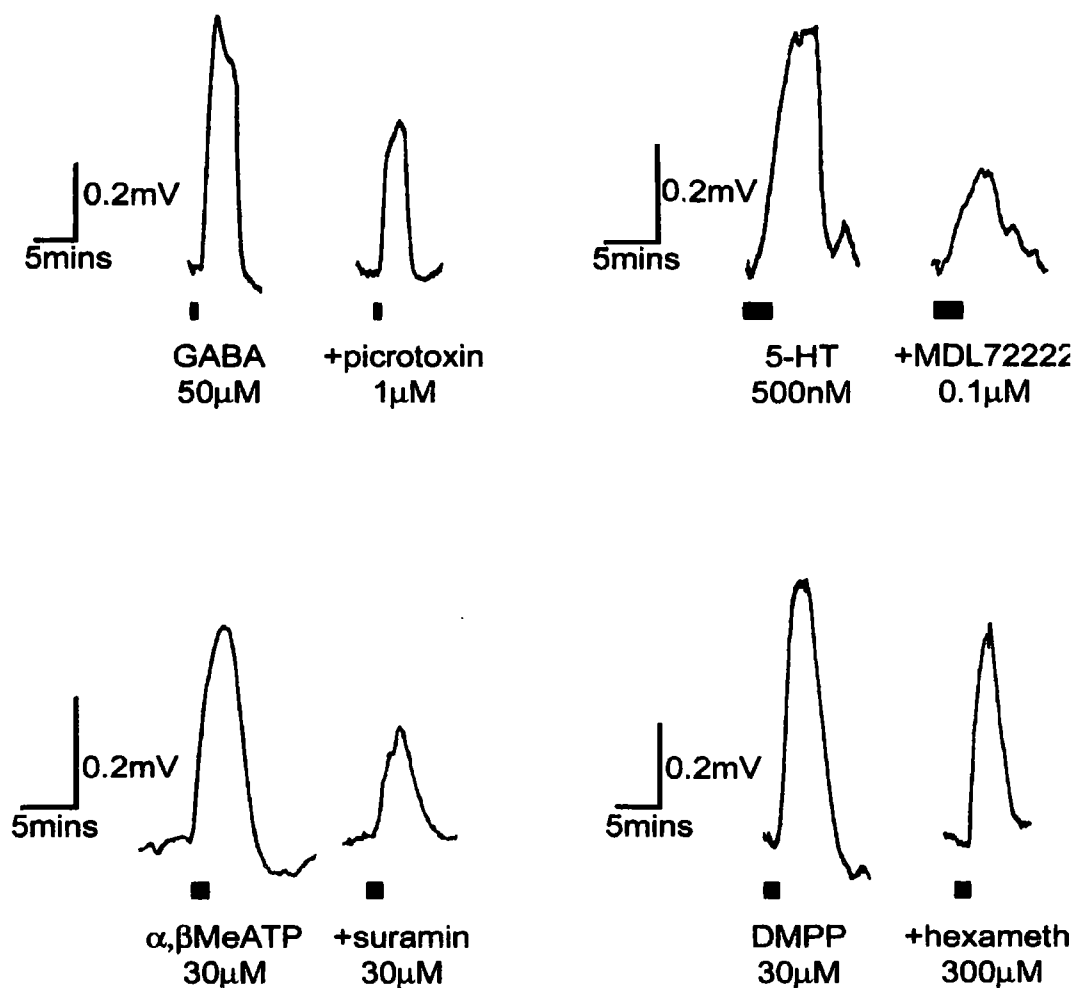


**Figure 3.1: GABA, 5-HT,  $\alpha,\beta\text{-MeATP}$  and DMPP evoked concentration-dependent responses in the rat isolated vagus nerve.** The figure shows chart recorder traces of GABA-, 5-HT-,  $\alpha,\beta\text{-MeATP}$ - and DMPP- evoked responses. The agonist applied is indicated to the left of the appropriate traces. The concentration of agonist applied to the nerve is shown below the corresponding response and the agonist contact time is represented by the solid bar beneath each trace.

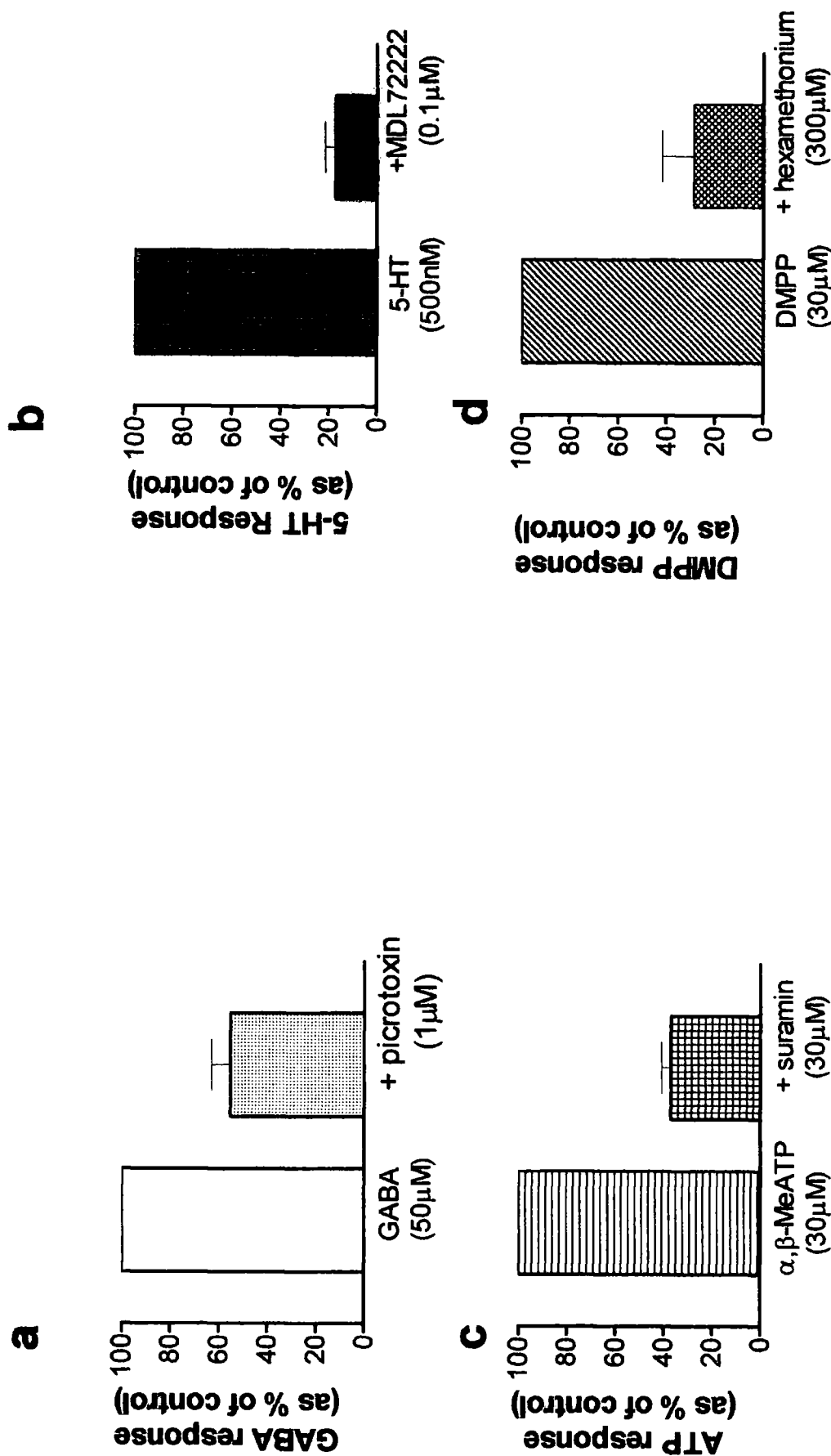


**Figure 3.2: GABA, 5-HT,  $\alpha, \beta$ -MeATP and DMPP evoke concentration-dependent agonist-evoked responses in the rat isolated vagus nerve.** The figure shows mean log concentration-response curves for GABA-, 5-HT-,  $\alpha, \beta$ -MeATP- and DMPP- evoked responses. The  $\log_{10}$  of the agonist concentration is shown on the abscissa and the response amplitude is shown on the ordinate. Each agonist-evoked response has been normalized to its respective maximum response. Each data point is the mean  $\pm$  s.e.m (represented by vertical lines) of 5, 6, 2-5 and 3-12 experiments for GABA, 5-HT,  $\alpha, \beta$ -MeATP and DMPP, respectively. The "pseudo Hill slopes" calculated for GABA, 5-HT,  $\alpha, \beta$ -MeATP and DMPP, were 0.3, 1.3, 0.4 and 0.6, respectively.





**Figure 3.3: GABA-, 5-HT-,  $\alpha,\beta$ MeATP- and DMPP-evoked responses of the rat isolated vagus nerve are inhibited by drugs acting at the GABA<sub>A</sub> receptor, 5-HT<sub>3</sub>, P<sub>2x</sub> and nACh receptors, respectively.** The figure shows actual chart recorder traces of submaximal ( $EC_{50}$ ): GABA-evoked responses in the absence and presence of picrotoxin (1μM); 5-HT-evoked responses in the absence and presence of MDL-72222 (0.1μM);  $\alpha,\beta$ MeATP-evoked responses in the absence and presence of suramin (30μM) and DMPP-evoked responses in the absence and presence of hexamethonium (300μM). The agonist concentration applied to the nerve is indicated below its corresponding response and agonist contact time is represented by the solid bar beneath each response.



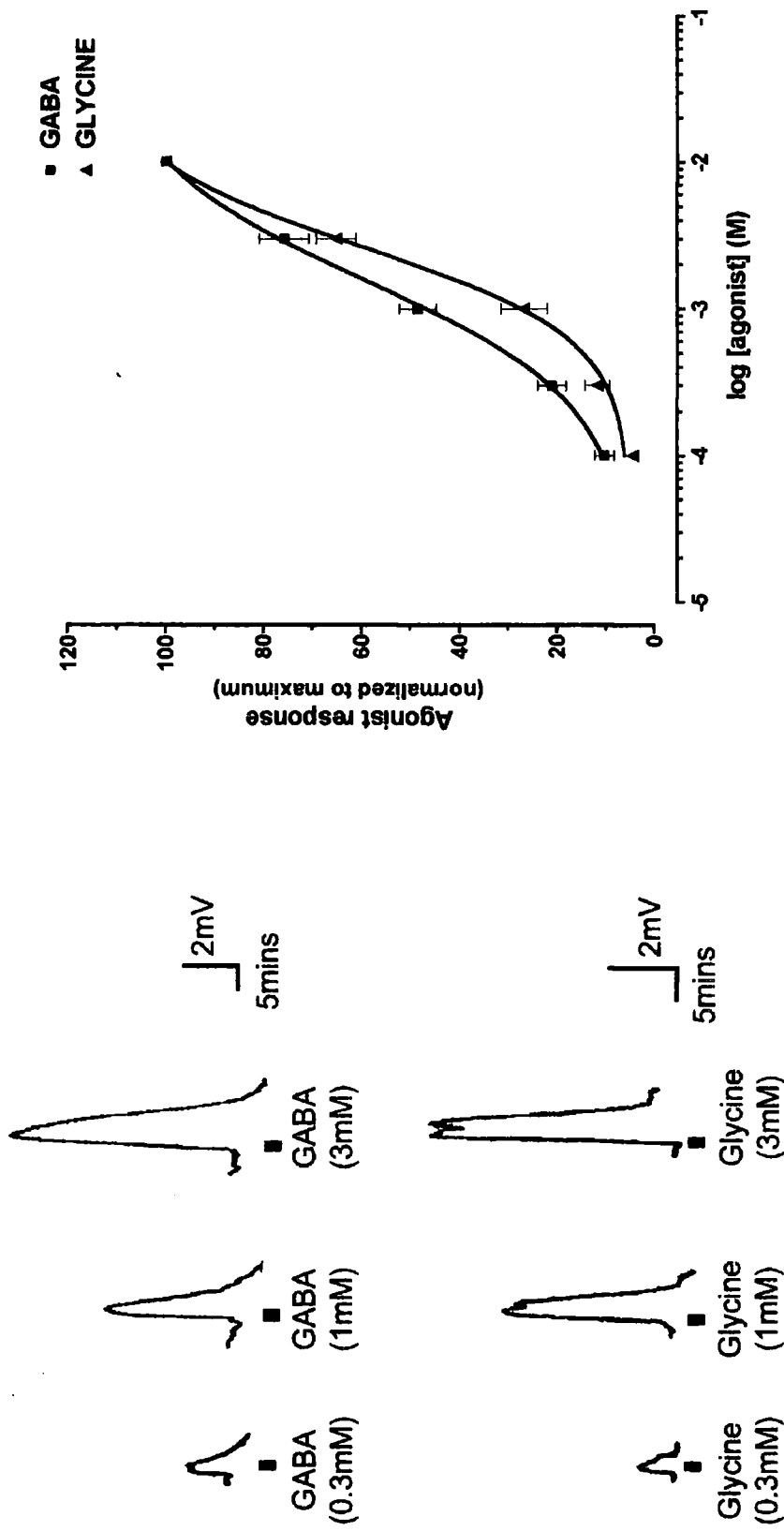
**Figure 3.4:** GABA-, 5-HT-,  $\alpha, \beta$  MeATP- and DMPP-evoked responses of the rat vagus nerve are inhibited by drugs acting at the GABA<sub>A</sub>, 5-HT<sub>1</sub>, P<sub>2x</sub> and nACh receptors, respectively. Histograms summarize the inhibitory effects of (a): picrotoxin (1µM) on submaximal (50µM) GABA-evoked responses, (b): MDL-72222 (0.1µM) on submaximal (0.5µM) 5-HT-evoked responses, (c): suramin (30µM) on submaximal (30µM)  $\alpha, \beta$ MeATP-evoked responses and (d): hexamethonium (300µM) on submaximal (30µM) DMPP-evoked responses. Each data bar is the mean  $\pm$  s.e.m (represented by the vertical lines) of 5, 7, 5 and 6 experiments for GABA-, 5-HT-,  $\alpha, \beta$ MeATP- and DMPP-evoked responses, respectively.

isolated optic nerve. The  $EC_{50}$  values (geometric mean and 95% confidence intervals) for GABA and glycine-evoked responses were found to be 1.6mM (0.9-2.5mM, n=9) and 2.6mM (2.0-3.5mM, n=8), respectively. From these data concentrations approximating the  $EC_{20}$  values of 0.3mM and 1mM for GABA and glycine, respectively, were interpolated and used in further experiments. The mean amplitude of responses evoked by 300 $\mu$ M GABA and 1mM glycine were found to be  $0.22\pm 0.02$ mV (n=15) and  $0.27\pm 0.03$ mV (n=20), respectively. In experiments where the modulatory effects of drugs were investigated against control agonist-evoked responses, the drug-induced effect on the agonist-evoked response is represented as a percentage change of the control agonist-evoked response. These data are summarised in figure 3.5.

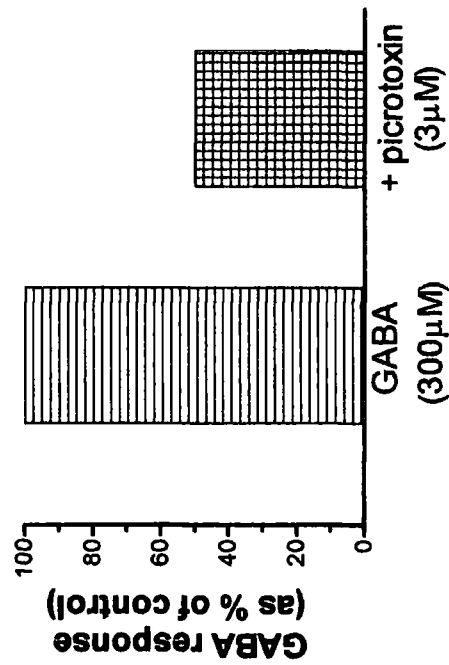
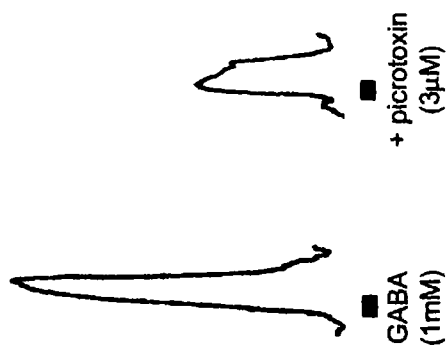
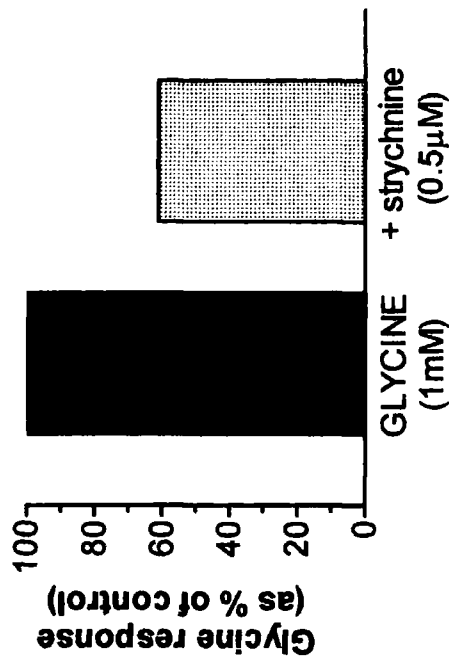
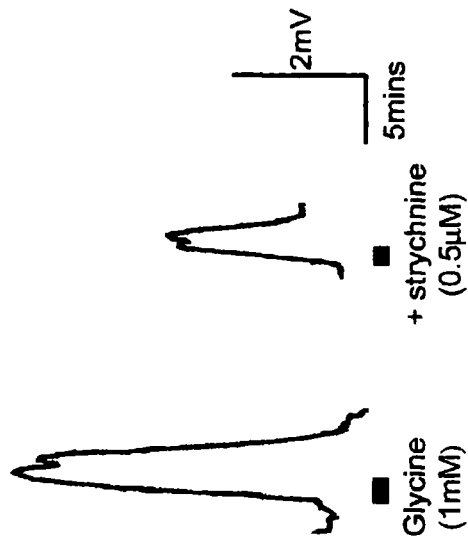
In the rat isolated optic nerve, submaximal GABA-evoked responses (0.3mM) were inhibited by 3 $\mu$ M picrotoxin to  $50\pm 9$  % (n=3,  $p<0.02$ ) of control. Submaximal glycine-evoked responses (1mM) were abolished by 1 $\mu$ M strychnine and inhibited by 0.5 $\mu$ M strychnine to  $41\pm 12\%$  (n=4,  $p<0.02$ ) of control. These data are consistent with activation of neuronal GABA and strychnine-sensitive glycine receptors, respectively. These data are summarised in a histogram (figure 3.6).

#### **4.iii. Allosteric modulation of the GABA-mediated response in the rat isolated vagus nerve.**

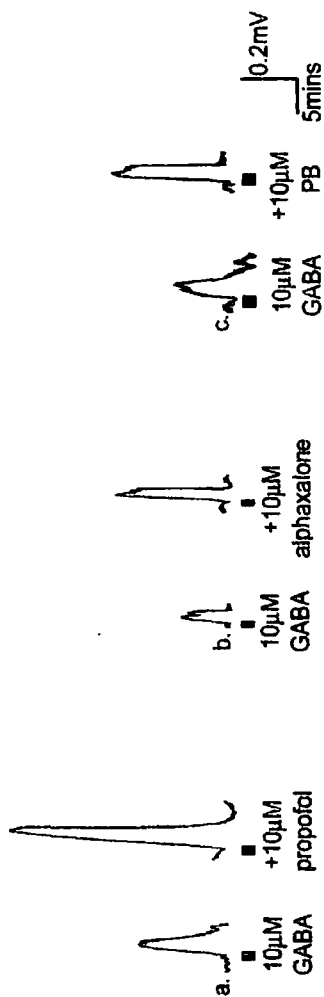
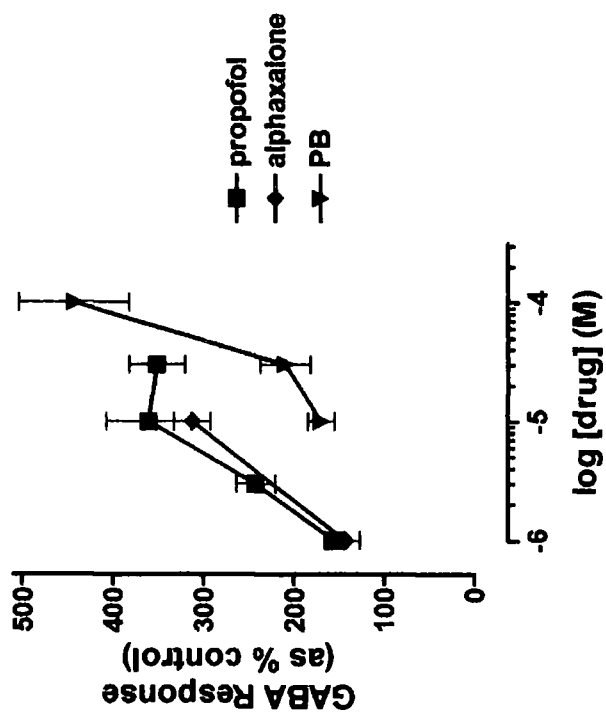
Submaximal GABA-evoked responses of the vagus nerve were concentration-dependently and reversibly potentiated by three chemically diverse drugs acting at the GABA<sub>A</sub> receptor: submaximal (10 $\mu$ M) GABA-evoked responses were potentiated by sodium pentobarbitone (10-100 $\mu$ M), alphaxalone (1-10 $\mu$ M) and propofol (1-30 $\mu$ M). The maximum potentiation evoked by sodium pentobarbitone (10 $\mu$ M), alphaxalone (10 $\mu$ M) and propofol (10 $\mu$ M) was found to be to 170% (n=2),  $312\pm 18\%$  (n=4,  $p<0.002$ ) and  $361\pm 47\%$  (n=15,  $p<0.0001$ ) and of control, respectively. The results of these experiments are summarised in figure 3.7.



**Figure 3.5: GABA and glycine evoke concentration-dependent responses of the rat isolated optic nerve. Left: chart recorder traces of GABA- and glycine-evoked responses. The agonist and concentration applied to the nerve is given below the response and the agonist contact time is represented by the solid line beneath the trace. Right: shows mean log concentration-response curves for GABA and glycine. The  $\log_{10}$  of the agonist concentration applied to the nerves is shown on the abscissa and the peak response amplitude is shown on the ordinate. For each nerve, agonist-evoked responses have been normalized to their respective maximum response. Each data point is the mean  $\pm$  s.e.m (represented by the vertical lines) of 6-9 and 1-9 experiments for GABA and glycine, respectively.**



**Figure 3.6: GABA- and glycine-evoked responses are antagonised by picrotoxin and strychnine, respectively in the rat isolated optic nerve.** Top: shows chart recorder traces of GABA- and glycine-evoked responses in the absence and presence of their respective antagonists. The drug concentration applied to the nerve is indicated below the GABA (0.3mM)- or glycine (0.3mM)-evoked response and the agonist contact time is represented by the solid bar beneath each response. The calibration bars (shown on the right) apply to both GABA- and glycine-evoked responses. GABA- and glycine-evoked responses are taken from the same optic nerve. Bottom: shows histograms summarizing the inhibitory effects of (left) picrotoxin (3µM) on the GABA-evoked response and the inhibitory effects of (right) strychnine (0.5µM) on glycine-evoked responses. Each data point is the mean  $\pm$  s.e.m (represented by the vertical bars) of 3 and 4 experiments for GABA and glycine, respectively.



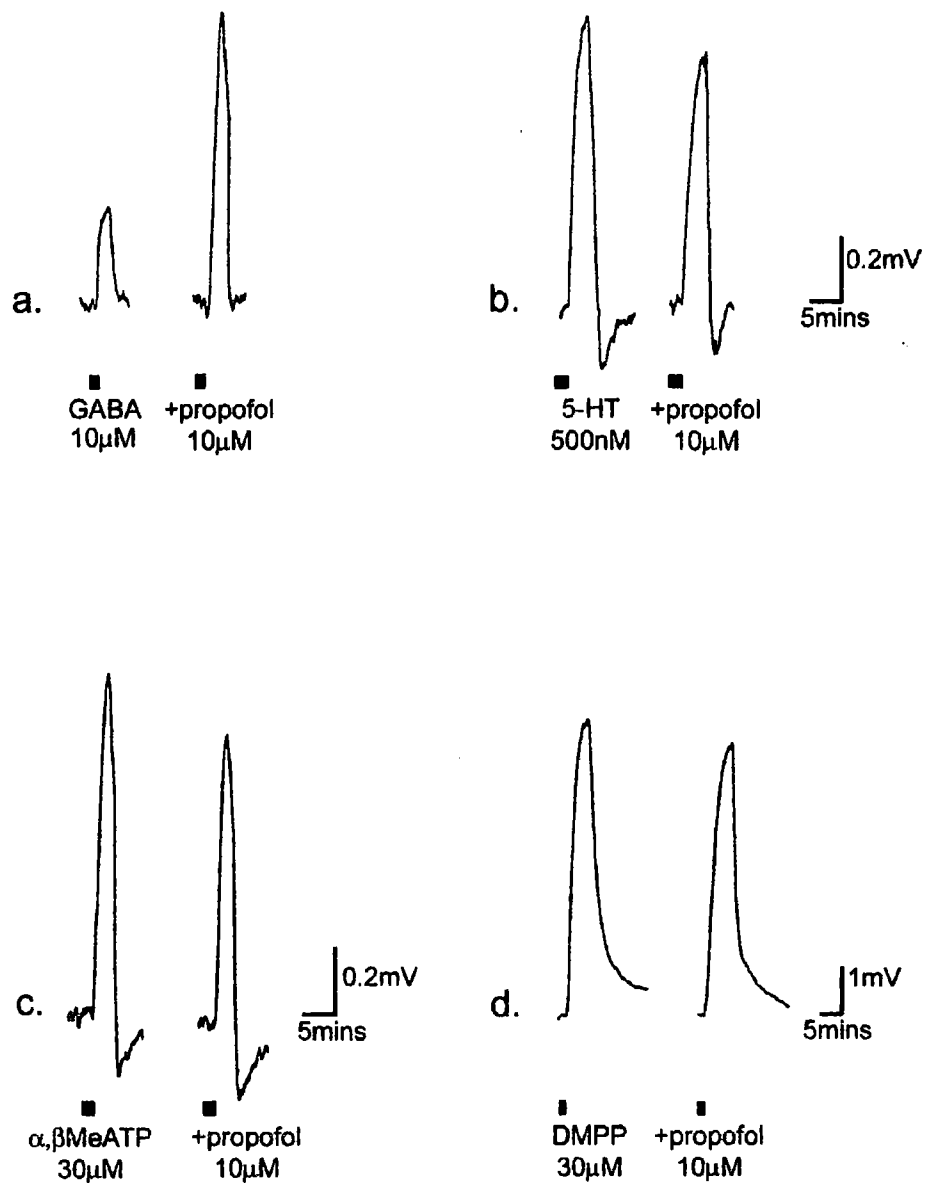
**Figure 3.7: GABA -evoked responses of the rat isolated vagus nerve are potentiated by propofol, sodium pentobarbitone and alphaxalone. Left: Chart recorder traces of submaximal ( $EC_{20}$ ) GABA-evoked responses potentiated by a. propofol, b. alphaxalone and c. sodium pentobarbitone (all at  $10\mu M$ ). The drug concentration applied to the nerve is indicated below its corresponding response and agonist contact time is represented by the solid bar beneath each response. Traces are taken from different vagus nerves. Right: mean log concentration-effect curves for potentiation of submaximal GABA ( $10\mu M$ )-evoked responses by propofol ( $1-30\mu M$ ), alphaxalone ( $1-10\mu M$ ) and sodium pentobarbitone ( $10-100\mu M$ ). The  $\log_{10}$  concentration of modulator is plotted on the abscissa and the response, as a percentage of the control GABA response is plotted on the ordinate. Each data point represents the mean of 7-15, 2-4 and 2-13 experiments for propofol, alphaxalone and sodium pentobarbitone, respectively.**

These modulatory effects of propofol, alphaxalone and pentobarbitone are consistent with those of other investigators (e.g. electrophysiological studies by Peters *et al.*, 1988 and Hales and Lambert, 1991) and taken together are indicative of activation of a neuronal GABA<sub>A</sub> receptor. Collectively, the above data validated the use of the vagus and optic nerves as suitable preparations to examine modulation of neuronal GABA<sub>A</sub>, 5-HT<sub>3</sub>, nACh, P<sub>2X</sub> receptors and the strychnine-sensitive glycine receptors.

However, also noteworthy in these studies, was the marked and consistent modulation of the GABA-evoked response in the rat vagus nerve by propofol. Since the selectivity of this agent was unknown, the vagus and optic nerves were employed to determine the action of propofol at the 5-HT<sub>3</sub>, nicotinic acetylcholinergic (nACh) and P<sub>2X</sub> receptors of the rat isolated vagus nerve and at the GABA<sub>A</sub> and the strychnine-sensitive glycine receptor of the rat isolated optic nerve.

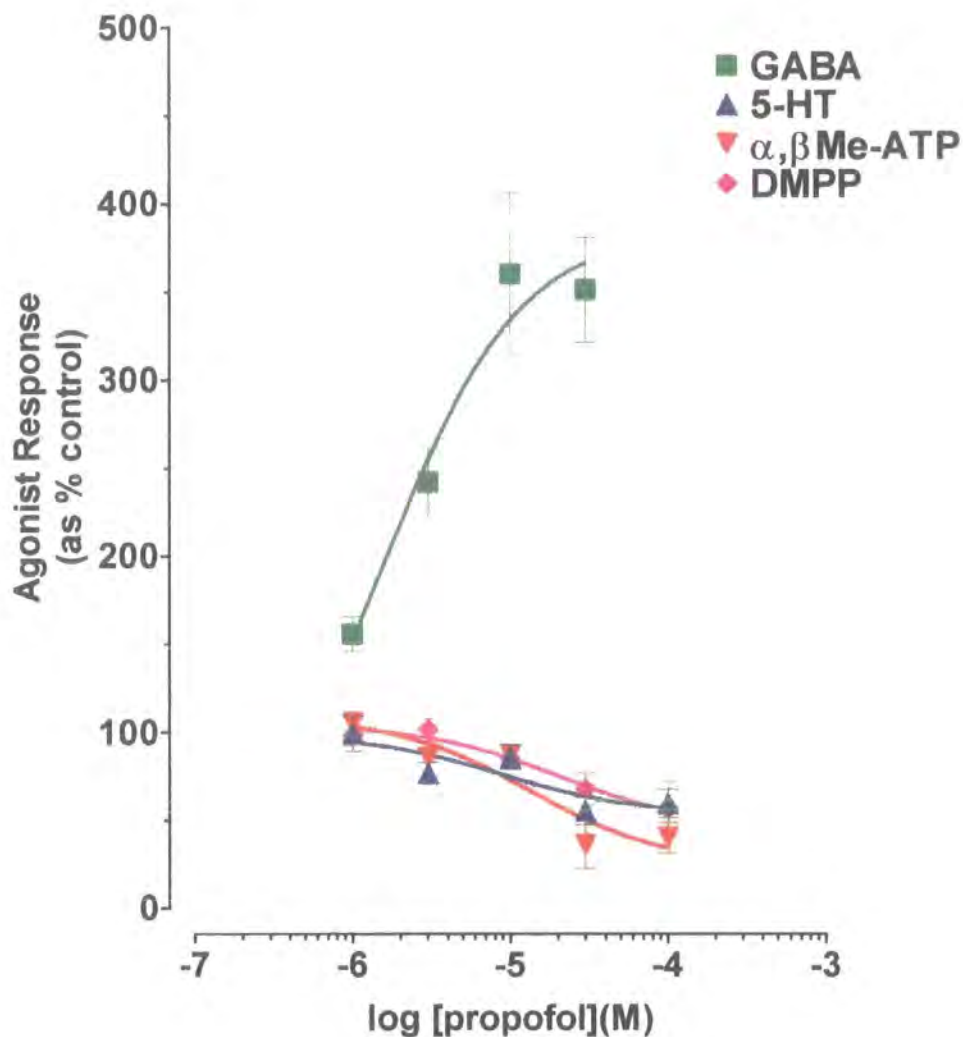
#### **4.iv. Selectivity of action of propofol on vagus and optic nerves.**

The effects of propofol (1-100 $\mu$ M) were examined on neuronal GABA<sub>A</sub>, 5-HT<sub>3</sub>, P<sub>2X</sub> and nACh receptors on the rat isolated vagus nerve. Propofol concentration-dependently potentiated submaximal (10 $\mu$ M) GABA-evoked responses to a maximum of 360 $\pm$ 45% (n=15; p<0.0001) of control in the presence of 10 $\mu$ M propofol. In contrast, 5-HT (0.5 $\mu$ M),  $\alpha$ , $\beta$ -MeATP (30 $\mu$ M) and DMPP (30 $\mu$ M)-evoked responses were little or unaffected by propofol (1-10 $\mu$ M). In the presence of 10 $\mu$ M propofol, the 5-HT,  $\alpha$ , $\beta$ -MeATP and DMPP-evoked responses were reduced to 86 $\pm$ 7% (n=6, p<0.1), 88 $\pm$ 5% (n=5, p<0.07) and 85 $\pm$ 7% (n=5, p<0.1) of control, respectively. In the presence of 100 $\mu$ M propofol the 5-HT,  $\alpha$ , $\beta$ -MeATP and DMPP-evoked responses were reduced to 60 $\pm$ 8% (n=6), 40 $\pm$ 9% (n=5) and 57 $\pm$ 16% (n=5) of control, respectively. 100 $\mu$ M propofol only transiently potentiated the GABA response, therefore, its effects are presented at t=15 mins. The results of these experiments are summarised in figures 3.8 and 3.9.



**Figure 3.8: Propofol potentiates submaximal GABA-evoked responses, but has little or no effect on 5-HT-,  $\alpha,\beta$ MeATP- and DMPP-evoked responses in the rat isolated vagus nerve.** Chart recorder traces of agonist-evoked responses (all approximating their  $EC_{50}$ , except for GABA which  $\approx EC_{20}$ ) in the absence and presence of propofol (10 μM) are shown. Top calibration bars apply to traces a and b. The agonist concentration applied to the nerve is indicated below the response and the agonist contact time is represented by the solid bar beneath each response. Agonist-evoked responses are taken from different nerves.





**Figure 3.9: Propofol potentiates submaximal GABA-evoked responses, but has little or no effect on 5-HT-,  $\alpha, \beta$  MeATP- or DMPP-evoked responses in the rat isolated vagus nerve.** Mean log concentration-effect curves for agonist-evoked responses (all EC<sub>50</sub>, except GABA which  $\approx$  EC<sub>20</sub>) in the presence of propofol (1-100  $\mu$ M). The log<sub>10</sub> propofol concentration is shown on the abscissa and the response, as a percentage of the control agonist response, is shown on the ordinate. Each data point represents the mean  $\pm$  s.e.m (represented by the vertical bars) of 5-15 experiments.

The effect of propofol (10 $\mu$ M) on submaximal GABA- and glycine - evoked responses of the optic nerve was examined. Propofol (10 $\mu$ M) potentiated submaximal (EC<sub>20</sub>) GABA (0.3mM) and glycine (1mM)-evoked responses to 307 $\pm$ 12% (n=3, p<0.009) and 124 $\pm$ 6 % (n=7, p<0.03) of control, respectively. These data are summarised in figure 3.10.

Thus, having validated the vagus nerve as a simple neuropharmacological assay, the effects of mefenamic acid on GABA-evoked responses of the vagus nerve were determined.

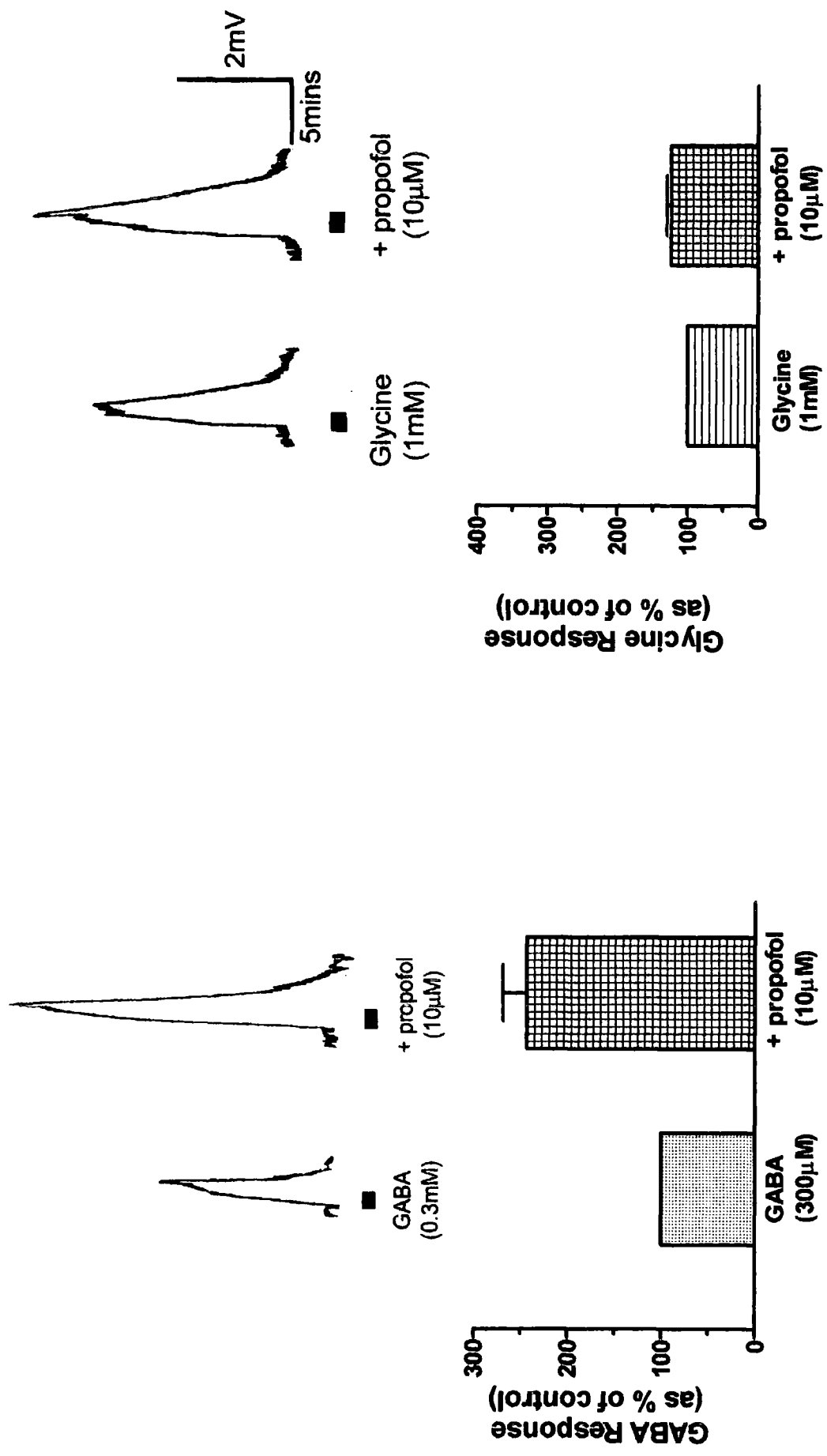
#### ***4.v. Mefenamic acid potentiates the GABA-mediated response in the rat isolated vagus nerve.***

Submaximal (10 $\mu$ M) GABA-evoked responses of the vagus nerve were concentration-dependently and reversibly potentiated by mefenamic acid (10-100 $\mu$ M). The maximum potentiation evoked mefenamic acid (10 $\mu$ M) was found to be to 164  $\pm$  14% (n=3, p<0.02) of control. The results of these experiments are summarised in figure 3.11.

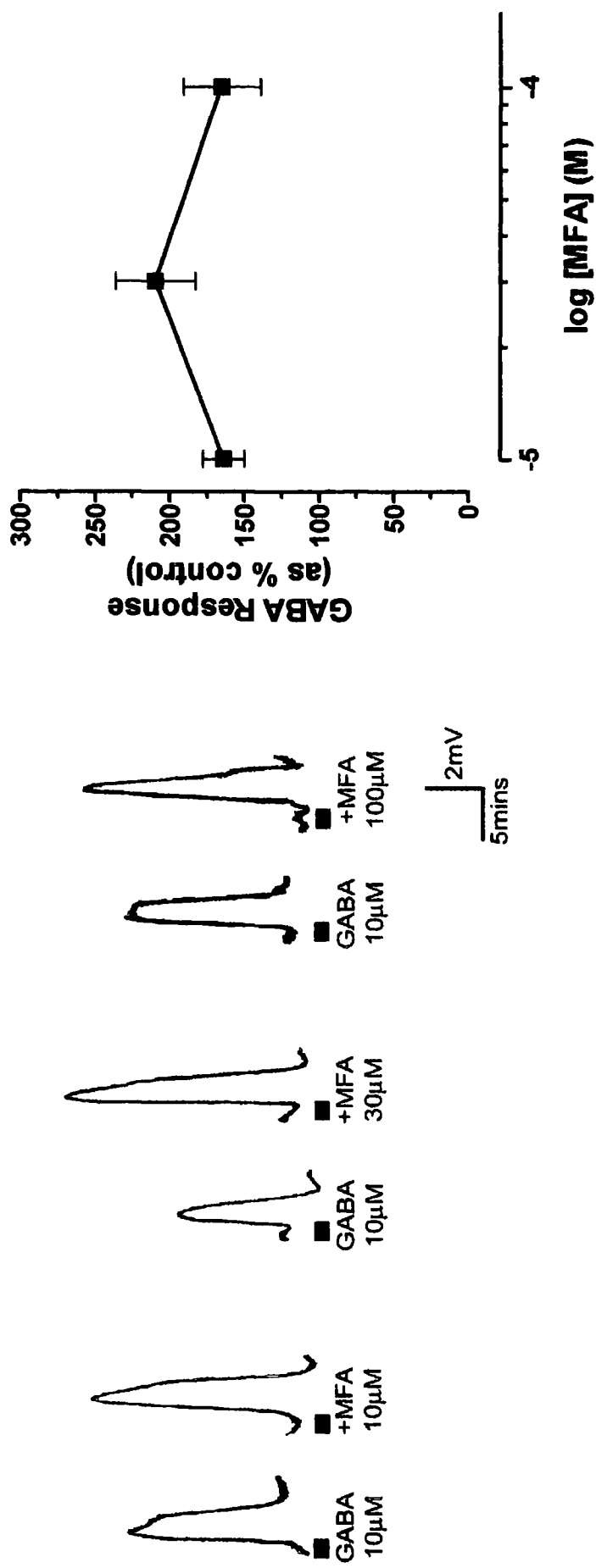
## **5. Discussion**

### ***5.i. Characterisation of agonist-evoked responses in the vagus and optic nerves***

GABA, 5-HT,  $\alpha$ , $\beta$ -MeATP and DMPP evoked concentration-dependent depolarisation's of the rat isolated vagus nerve. The EC<sub>50</sub> calculated for GABA-evoked responses was found to be 34 $\mu$ M; this is consistent with the EC<sub>50</sub> values of 28 $\mu$ M and 69 $\mu$ M for GABA-evoked depolarisation's of the rat vagus nerve reported by Ireland and Tyers (1987) and Green and Halliwell (1997), respectively. The EC<sub>50</sub> calculated for the 5-HT-evoked responses was found to be 0.8 $\mu$ M; this is in agreement with the work of Trezise (1993) who found that 5-HT evoked concentration-dependent depolarisation's of the vagus nerve with an EC<sub>50</sub> of 0.48 $\mu$ M and Green and Halliwell (1997), who report a value of 0.8 $\mu$ M. Ireland and Tyres (1987) and Green and Halliwell (1997) also report



**Figure 3.10: Propofol potentiates submaximal GABA- and glycine-evoked responses in the rat isolated optic nerve. Top: shows chart recorder traces of submaximal GABA- and glycine-evoked responses in the absence and presence of propofol (10 μM); calibration bars shown apply to both sets of traces. The agonist concentration applied to the nerve is shown below the response and the agonist contact time is represented by the solid bar beneath each trace. Traces are taken from different nerves. Bottom: histograms summarizing the potentiating effect of propofol (10 μM) on submaximal GABA (0.3 mM; on the left)- and glycine (1 mM; on the right)-evoked responses. Each data bar is the mean ± s.e.m (represented by the vertical lines) of 3 and 7 experiments for GABA and glycine, respectively.**



**Figure 3.11: Mefenamic acid concentration-dependently potentiates submaximal GABA-evoked responses in the rat isolated vagus nerve. Left:** shows chart recorder traces of submaximal GABA-evoked responses in the absence and presence of 10µM mefenamic acid (left), 30µM mefenamic acid (middle) and 100µM mefenamic acid (right); calibration bars apply to each set of traces. The agonist contact time is represented by the solid bar beneath each trace. Responses were taken from different vagus nerves. **Right:** Mean log concentration-effect plot for GABA-evoked responses in the presence of mefenamic acid (10-100µM). The log<sub>10</sub> mefenamic acid concentration is shown on the abscissa and the GABA response, as a percentage on control, is shown on the ordinate. Each data point represents the mean of 3 experiments.

DMPP-evoked depolarisations of the vagus nerve with  $EC_{50}$  values of  $35\mu\text{M}$  and  $13\mu\text{M}$ , respectively; an  $EC_{50}$  value of  $33\mu\text{M}$  was determined in this study. Trezise *et al.*, (1993) and Green and Halliwell (1997) have reported that  $\alpha,\beta$ -MeATP concentration-dependently depolarises the rat vagus nerve with  $EC_{50}$  values of  $25\mu\text{M}$  and  $26\mu\text{M}$ , respectively; these data are in accordance with the  $EC_{50}$  value of  $48\mu\text{M}$  reported in this study.

GABA and glycine evoked concentration-dependent depolarisation's of the rat optic nerve with  $EC_{50}$  values of  $1.6\text{mM}$  and  $2.6\text{mM}$ , for GABA and glycine, respectively. These data are in agreement with those of Green and Halliwell, (1997) who report  $EC_{50}$  values of  $1.1\text{mM}$  and  $1.7\text{mM}$ , for GABA and glycine, respectively. Also, Simmonds (1983) reports concentration-dependent depolarizations of the isolated optic nerve by the GABA analogue, muscimol, and glycine with interpolated  $EC_{50}$  values of  $\approx 10\mu\text{M}$  and  $\approx 1.4\text{mM}$ , respectively.

Agonist-evoked responses had characteristic profiles: 5-HT-evoked responses were observed to develop relatively slowly when compared to GABA-evoked responses and, occasionally, a small "after-hyperpolarising" response was observed on washout of higher concentrations of 5-HT; similar observations have been reported by Azami and colleagues, (1985).

Responses evoked by  $\alpha,\beta$ -MeATP typically reached peak more rapidly than GABA-evoked responses and an after-hyperpolarising response was frequently observed, especially on washout of higher concentrations ( $>100\mu\text{M}$ ) of  $\alpha,\beta$ -MeATP. In addition,  $\alpha,\beta$ -MeATP-evoked responses were observed to fade rapidly, particularly with higher concentrations and in the continued presence of the drug; this phenomena has been previously reported to occur in other preparations such as guinea-pig bladder (Kasakov and Burnstock, 1983). These observations are also in good agreement with those outlined by Trezise *et al.*, (1993).

DMPP-evoked responses were also slower in onset than GABA-evoked responses and no after-hyperpolarising responses were observed. In addition, higher concentrations of DMPP evoked the largest depolarization's of the vagus nerve, (up to  $5\text{mV}$  in some cases). The concentration of DMPP which evoked a maximal response appeared to be variable ( $10\text{-}1000\mu\text{M}$ ); for this reason,

DMPP-evoked responses were normalised to the response evoked by 300 $\mu$ M DMPP. A possible explanation for this phenomenon could be that at concentrations of DMPP greater than 300 $\mu$ M other receptor types may be recruited (e.g. DMPP may promote the pre-synaptic release of acetylcholine from post-ganglionic nerve terminals, Wingard *et al.*, 1991). Other investigators have also limited their DMPP concentration range to 3-300 $\mu$ M for nicotinic receptor activation of the vagus nerve (e.g. Green and Halliwell, 1997).

GABA-, 5-HT-,  $\alpha,\beta$ -MeATP- and DMPP-evoked responses were inhibited by picrotoxin, MDL 72222, suramin and hexamethonium, respectively. It is well established that picrotoxin non-competitively inhibits GABA<sub>A</sub>-mediated responses (Akaike, 1985). In this study, GABA-evoked responses were inhibited to 55% of control by picrotoxin (1 $\mu$ M); Green and Halliwell (1997) report similar inhibition by picrotoxin in the vagus nerve with an IC<sub>50</sub> value of 3.6 $\mu$ M. Bowery and Brown (1974) demonstrated antagonism of extracellularly recorded GABA-evoked responses recorded in the rat superior cervical ganglion by somewhat higher concentrations of picrotoxin; in this study the IC<sub>50</sub> for picrotoxin was 37 $\mu$ M.

MDL 72222 has been shown to act as a potent and selective antagonist of 5-HT-mediated depolarisation's of nodose and superior cervical ganglion cells (Azami *et al.*, 1985 ) and has also been shown to lack affinity for 5-HT<sub>1</sub> and 5-HT<sub>2</sub> recognition sites in rat brain membranes (Fozard, 1984). In this study MDL 72222 (0.1 $\mu$ M) virtually abolished 5-HT<sub>3</sub>-mediated responses of the vagus nerve. Similar observations were made in the vagus nerve by Green and Halliwell (1997) and Azami *et al.*, (1985) in rabbit nodose ganglia and superior cervical ganglia.

Suramin has been reported to antagonise ATP-activated channels (Nakazawa *et al.*, 1990; Evans *et al.*, 1992). However, it lacks selectivity for P<sub>2x</sub> subtypes (Evans, 1992). Consistent with data published by Trezise *et al.*, (1993), suramin inhibited  $\alpha,\beta$ -MeATP-evoked depolarizations of the rat vagus nerve supporting the proposal that these responses are predominantly mediated through P<sub>2x</sub> receptors.

In this study, DMPP-evoked responses of the rat vagus nerve were inhibited to 71% of control by hexamethonium (300 $\mu$ M). Ireland and Tyres (1987) and Green and Halliwell (1997) also report similar levels of inhibition of DMPP-evoked responses of the rat vagus by hexamethonium. These pharmacological data are in accordance with nACh receptor activation.

In the optic nerve, GABA and glycine-evoked responses were inhibited by picrotoxin and strychnine, respectively. These data are consistent with those previously reported by Green and Halliwell (1997) who report antagonism of glycine-evoked responses in the optic nerve by strychnine (3 $\mu$ M) and Simmonds (1983) who reported antagonism by strychnine between 0.1 and 10 $\mu$ M in the optic nerve.

#### **5.ii. Allosteric modulation of the GABA-mediated response in the rat isolated vagus nerve.**

The positive allosteric modulation of the GABA<sub>A</sub> receptor by several CNS-depressant drugs, such as sodium pentobarbitone, alphaxalone and propofol, is well documented (for review see Seighart, 1995). In the vagus nerve, propofol was the most potent of the modulators examined and the relative order of potency, in descending order, was as follows propofol > alphaxalone > sodium pentobarbitone. These effects of propofol, alphaxalone and pentobarbitone are consistent with those of other investigators using single cell preparations (e.g. Peter *et al.*, 1988 and Hales and Lambert, 1991) and taken together, are consistent with activation of a neuronal GABA<sub>A</sub> receptor. In binding studies (e.g. Concas *et al.*, 1990), alphaxalone is usually reported to be a more potent modulator of the GABA<sub>A</sub> receptor than propofol. However in the vagus nerve, propofol is almost equipotent with alphaxalone.

The pharmacology of the GABA, 5-HT,  $\alpha$ , $\beta$ -MeATP and DMPP-evoked responses in the rat isolated vagus nerve is consistent with the activation of GABA<sub>A</sub>, 5-HT<sub>3</sub>, P<sub>2x</sub> and nACh receptors, respectively. Similarly, the pharmacology of the GABA and glycine-evoked responses in the rat isolated optic nerve is consistent with the activation of GABA<sub>A</sub> and strychnine-sensitive

glycine receptors, respectively. These data, therefore, support the use of the vagus and optic nerves as appropriate preparations with which to examine modulation of neuronal ligand-gated ion channel function.

### **5.iii. Selectivity of action of propofol on vagus and optic nerves**

The vehicle for experiments investigating the action of propofol was ethanol. A number of studies have demonstrated that millimolar concentrations of ethanol interact with ligand-gated ion channels such as the GABA<sub>A</sub>, 5-HT<sub>3</sub>, nACh, P<sub>2x</sub> and strychnine-sensitive glycine receptors.

For example, Aguayo and Pancetti (1994) have demonstrated that ethanol (0.5-850mM) potentiated GABA-evoked responses in ethanol-sensitive voltage-clamped mouse hippocampal neurones between 110 and 430% of control, respectively (EC<sub>50</sub>=502mM). These authors have also demonstrated that ethanol (1-425mM) concentration-dependently potentiated ethanol-sensitive voltage-clamped mouse hippocampal neurones; a potentiation of 150% of control being achieved with 100mM ethanol. In voltage-clamped mouse spinal neurones, ethanol (1-400mM) potentiated glycine-evoked currents between approximately 102 and 190% of control, respectively. Additionally, Machu and Harris (1994) have demonstrated using 5-HT<sub>3</sub> receptors expressed in voltage-clamped *Xenopus* oocytes that ethanol (25-200mM) potentiated 5-HT evoked currents (EC<sub>50</sub>=100mM) where the maximal potentiation obtainable was 50% of control.

More recently, Li, *et al.*, (1998) have demonstrated using ATP-evoked currents recorded from voltage-clamped bull frog dorsal root ganglion cells that ethanol (50-100mM) inhibited neuronal P<sub>2x</sub> purinoceptors by shifting the ATP-evoked dose-response curve to the right, possibly via an allosteric mechanism rather than competitive inhibition. Cardoso *et al.*, (1999) have also shown using human nicotinic acetylcholine receptor subunits expressed in *Xenopus* oocytes that ethanol (75mM) potentiated acetylcholine-induced currents in  $\alpha_2\beta_4$ ,  $\alpha_4\beta_4$ ,  $\alpha_2\beta_2$  and  $\alpha_4\beta_2$  receptor constructs, where the potentiation was due to an increase in E<sub>max</sub>, rather than a change in EC<sub>50</sub> or the Hill coefficient. The same



study also demonstrated that  $\alpha_3\beta_2$  and  $\alpha_3\beta_4$  receptor constructs were insensitive to modulation by ethanol, whereas homomeric  $\alpha_7$  receptors were significantly inhibited by lower concentrations (25-50mM) of ethanol. Another recent patch-clamp study (Aistrup *et al.*, 1999) using cultured rodent cortical neurones has demonstrated that ethanol (3-300mM) reversibly enhanced high affinity neuronal acetylcholine-induced currents which were insensitive to  $\alpha$ -bungarotoxin (the potentiation ranging from  $7.7\pm 5\%$  to  $192\pm 52\%$ ), whereas ethanol (10-300mM) weakly inhibited low affinity neuronal acetylcholine-induced currents which were sensitive to  $\alpha$ -bungarotoxin, (the inhibition ranging from  $5\pm 5\pm 4\%$  to  $29\pm 6\%$ ).

In selectivity studies the final concentration of ethanol was  $2\mu\text{M}$  and therefore less than the concentrations of ethanol required to produce modulation of neuronal GABA<sub>A</sub>, 5-HT<sub>3</sub>, nACh, P<sub>2x</sub> or strychnine-sensitive glycine receptors.

Propofol concentration-dependently and reversibly potentiated GABA<sub>A</sub> receptor-mediated responses in the rat isolated vagus nerve. The mean maximal potentiation of GABA-evoked responses was 360% and was achieved with  $10\mu\text{M}$  propofol and the EC<sub>50</sub> for this potentiating effect was  $1.5\mu\text{M}$ . These data are in good agreement those of Hara *et al.*, (1994) who report a concentration-dependent potentiation of submaximal GABA-evoked currents in rat hippocampal neurones by propofol with an EC<sub>50</sub> of  $1\mu\text{M}$  (interpolated) and a 3-fold potentiation of the GABA-evoked current by  $10\mu\text{M}$  propofol.

Hales and Lambert (1991) have reported a concentration-dependent, reversible potentiation of GABA-evoked currents by propofol ( $1.7$ - $16.6\mu\text{M}$ ) with a maximal potentiation of  $>850\%$  of control with  $8.4\mu\text{M}$  propofol in both voltage-clamped bovine chromaffin cells and rat cortical neurones. Additionally, propofol potentiated GABA-evoked currents in clonal murine hypothalamic GT1-7 neurones with an EC<sub>50</sub> of  $5\mu\text{M}$  and levels of potentiation with  $10\mu\text{M}$  propofol were  $\approx 700\%$  of control (Adodra and Hales, 1995). In contrast, Orser *et al.*, (1994) report potentiation of submaximal GABA-evoked currents in voltage-clamped murine hippocampal neurones with higher concentrations of propofol ( $2$ - $100\mu\text{M}$ ) and, for example, a potentiation of  $269\%$  of control with  $50\mu\text{M}$

propofol. The variation in potency and efficacy of propofol may be due to the use of different preparations and/or different vehicles and also due to different concentrations of GABA employed. For example, Hara *et al.*, (1994) demonstrated that propofol (1 $\mu$ M) produced a 3-fold potentiation of currents elicited by 1 $\mu$ M GABA, but only a 1.1 fold potentiation of currents elicited by 100 $\mu$ M GABA.

The concentration-dependent potentiation of GABA-evoked responses in the vagus nerve resulted in a bell-shaped curve where potentiations evoked with higher concentrations of propofol (> 10 $\mu$ M) were reduced in magnitude. These data are in agreement with Hara *et al.*, (1994) and Hales and Lambert (1991) who also report that higher concentrations of propofol ( $\approx$ 10 $\mu$ M) caused a reduction in the level of potentiation. Similar observations were made by Orser *et al.*, (1994) with higher propofol concentrations (50 $\mu$ M). This reduced level of potentiation may be due to desensitization, since propofol, at these high concentrations can also directly activate the GABA<sub>A</sub> receptor (Hara *et al.*, 1993, Orser *et al.*, 1994; Adodra and Hales, 1995).

Low concentrations of propofol (1-10 $\mu$ M) had little effect on 5-HT-evoked responses of the vagus nerve, but higher concentrations ( $\geq$ 30 $\mu$ M) inhibited these responses. These data are in good agreement with the work of Machu and Harris (1994) who report that propofol (1.1-22 $\mu$ M) did not modulate the function of homomeric murine 5-HT<sub>3</sub> receptors expressed in *Xenopus* oocytes. Barann *et al.*, (1993) have reported a non-competitive inhibition of maximal 5-HT-induced <sup>14</sup>C-guanidinium flux in N1E-115 mouse neuroblastoma cells by propofol (1-30 $\mu$ M). In a more recent voltage-clamp study using N1E-115 cells, Barann *et al.*, (1998) have reported that propofol inhibited maximally-evoked 5-HT currents with an IC<sub>50</sub> of 56 $\mu$ M; these data are in agreement with the inhibitory effect observed on 5-HT-evoked responses of the vagus nerve with higher concentrations of propofol.

Propofol (1-10 $\mu$ M) had little effect on  $\alpha$ , $\beta$ -meATP-evoked responses of the vagus nerve, but higher ( $\geq$ 30 $\mu$ M) concentrations of propofol inhibited these

responses. In contrast, a recent voltage-clamp study by Furuya *et al.*, (1999) has demonstrated that propofol (30-100 $\mu$ M) did not affect ATP-evoked responses on neuronal P<sub>2x</sub> purinoceptors in a rat pheochromocytoma cell line. No other study has investigated the action of propofol on P<sub>2x</sub> receptors in native neurones.

Propofol (1-10 $\mu$ M) had little effect on DMPP-evoked responses of the vagus nerve, but higher ( $\geq$ 30 $\mu$ M) concentrations of propofol inhibited these responses. Watchtel and Wegrzynowicz (1992) and Dilger *et al.*, (1994) have demonstrated, using voltage-clamped muscle-like clonal BC3H-1 cells expressing nACh receptors, that propofol (81 $\mu$ M and 25-250 $\mu$ M, respectively) inhibited these receptors by decreasing channel open time. In agreement, a recent voltage-clamp study by Furuya *et al.*, (1999) also demonstrated that higher concentrations of propofol (30-100 $\mu$ M) inhibited neuronal nACh-receptor mediated responses recorded in a rat pheochromocytoma cell line (PC12). The data presented in this study, therefore, are in agreement with the data of Watchtel and Wegrzynowicz (1992), Dilger *et al.*, (1994) and Furuya *et al.*, (1999) who also report inhibition neuronal nACh-receptor mediated responses by propofol (>30 $\mu$ M).

However, two recent voltage-clamp studies of  $\alpha$ 4 $\beta$ 2-containing neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes revealed a concentration-dependent inhibition of acetylcholine-evoked currents by propofol with IC<sub>50</sub> values of 4.5 $\mu$ M (Violet *et al.*, 1997) and 19 $\mu$ M, (Flood *et al.*, 1997). Moreover, Flood *et al.*, (1997) also report that this propofol-induced inhibition did not occur in homomeric  $\alpha$ 7-containing nicotinic acetylcholine receptors, suggesting that the inhibitory effect of propofol is subunit-dependent. The higher potency of propofol reported by Violet *et al.*, (1997) and Flood *et al.*, (1997) may be attributed to the use of recombinant neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes compared to native neuronal receptors, as in this study, and cell line preparations containing muscle type nicotinic acetylcholine receptors (Watchtel and Wegrzynowicz, 1992; Dilger *et al.*, 1994) or neuronal nicotinic acetylcholine receptors (Furuya

*et al.*, 1999). Additionally, the higher potency of propofol (Violet *et al.*, 1997 and Flood *et al.*, 1997) and may also be due to the reported subunit dependency of propofol (Flood *et al.*, 1997).

Concentrations of propofol (10 $\mu$ M) which caused a maximal potentiation of GABA-evoked responses of the vagus nerve, evoked a marked potentiation of GABA-mediated responses (to 307% of control) and a modest potentiation (to 124% of control) of glycine-mediated responses of the optic nerve. Hales and Lambert (1991) also report a modest potentiation of glycine-evoked responses in cultured murine spinal neurones by propofol (8.4-16.8 $\mu$ M). However, in contrast Hara *et al.*, (1994) report that propofol (1-5 $\mu$ M) had no effect on glycine-mediated responses of voltage-clamped, cultured hippocampal neurones. A possible explanation for this discrepancy may be attributed to the lower concentration of propofol employed by Hara *et al.*, (1994) and/or the different preparations employed.

#### **5.iv. Mefenamic acid potentiates the GABA-mediated response in the rat isolated vagus nerve.**

The NSAID, mefenamic acid, also concentration-dependently potentiated submaximal GABA-evoked responses of the vagus nerve. These data are in agreement with Woodward *et al.*, (1994) and Halliwell *et al.*, (1994) who demonstrated that mefenamic acid concentration-dependently potentiated submaximal GABA-evoked currents in *Xenopus* oocytes and expressing rat brain mRNA and in voltage-clamped rat hippocampal neurones, respectively. However, both Woodward *et al.*, (1994) and Halliwell *et al.*, (1994) have reported higher levels of potentiation by mefenamic acid than those observed in the vagus nerve. Such differences in the potency and efficacy of mefenamic acid may be attributed to the use of different preparations. To further investigate the actions of mefenamic acid on native GABA<sub>A</sub> receptors its effects were determined on rat hippocampal neurones, using the whole-cell patch-clamp technique to record from single neurones maintained in culture.

## **CHAPTER THREE:RESULTS**

### **Section 3.2**

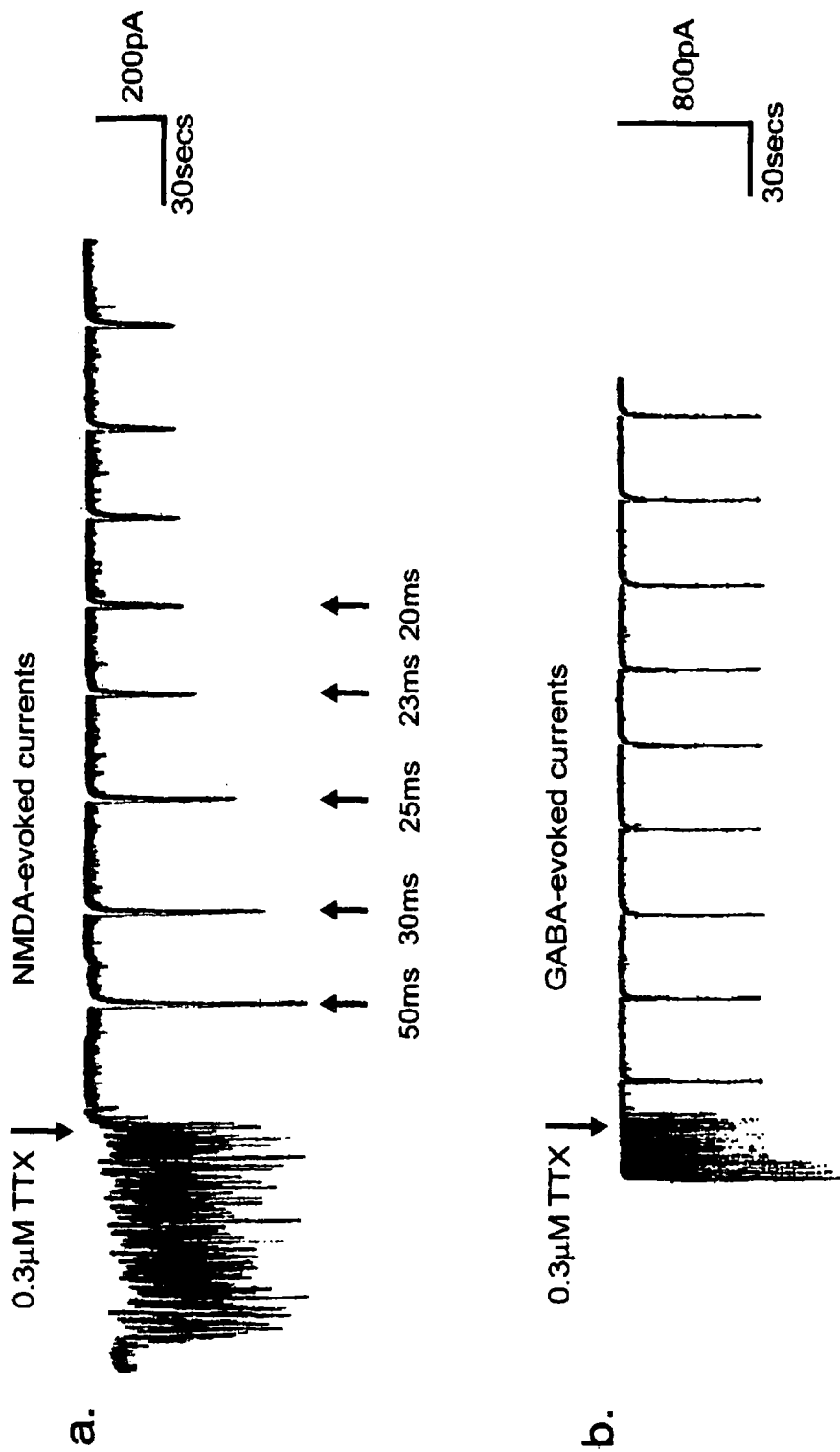
#### **Electrophysiological and neuropharmacological properties of cultured rat embryonic rat hippocampal neurones**

##### **1. Introduction**

The vagus and optic nerves have proved to be useful preparations for determining drug action at certain neuronal ligand-gated ion channels. However, in order to determine the precise mechanism/s of drug action at these receptor sites, the patch-clamp technique (Hamill *et al.*, 1981) was employed. This technique allows the experimenter to make high fidelity recordings of agonist-evoked ion channel activity, whilst maintaining precise control over the neuronal membrane potential and the internal and external ionic environments of the cell. A directional perfusion system, used in conjunction with this technique, enables the experimenter to rapidly perfuse known concentrations of drugs in close proximity to the cell under investigation. Thus, further experiments investigating the action of fenamate NSAIDs on neuronal ligand-gated ion channels were performed utilising cultured rat hippocampal neurones, maintained under voltage-clamp conditions. This chapter details the physiological and pharmacological properties of whole-cell voltage-clamped hippocampal neurones maintained in culture (see methods).

##### **2. Electrophysiological Characteristics of Hippocampal Neurones**

Hippocampal neurones, maintained in culture and initially identified by their morphological characteristics, exhibited spontaneous synaptic activity when held under voltage- or current-clamp in normal bathing solution. This spontaneous activity was more pronounced when cells were bathed in  $Mg^{2+}$ -free bathing solution (see figure 4.1a). In addition, cells grown in Neurobasal media alone often exhibited marked spontaneous activity compared to cells



**Figure 4.1 : Hippocampal neurones exhibit spontaneous activity which is inhibited by tetrodotoxin. The figure shows chart recorder traces of agonist-evoked currents in the presence and absence of TTX (0.3 μM). Trace a: shows NMDA-evoked currents recorded in Mg<sup>2+</sup>-free Bath solution. Note the high level of spontaneous activity at the beginning of the trace which is inhibited by TTX-containing bath solution (downward arrow); also note the reduction in response amplitude with decreasing NMDA exposure (agonist duration is indicated below arrows). Trace b: shows GABA-evoked currents recorded in Mg<sup>2+</sup>-containing Bath solution. Note the spontaneous activity at the beginning of the trace which is inhibited by the addition TTX-containing bath solution (downward arrows indicate the onset of perfusion of TTX-containing bath solution).**

maintained in serum based media, perhaps due to the clumped distribution of neurones observed when using Neurobasal media (see Methods: figure 2.5). Routine addition of tetrodotoxin (TTX; 0.3 $\mu$ M) to the bathing solution either diminished or abolished this activity (figure 4.1a,b). Thus all recordings, unless otherwise stated, were made in the presence of TTX. From a random sample of hippocampal neurones investigated, the average resting membrane potential ( $E_m$ ) was found to be  $-59.5 \pm 4$  mV ( $n=25$ ). The average hippocampal cell membrane capacitance, series resistance, electrode resistance and membrane resistances, under the recording conditions described in Methods, were calculated from a random sample of 24 hippocampal neurones voltage-clamped at  $-60$  mV and are presented in the table below (table 4.1). An example of mean action current amplitude and duration from one cell is also given in this table whereby the amplitude and duration of 20 fast events were measured and averaged.

Mean cell capacitance (pF)	26.7 $\pm$ 5.6
Mean access resistance (M $\Omega$ )	10.5 $\pm$ 0.6
Mean electrode resistance (M $\Omega$ )	2.1 $\pm$ 0.06
Mean membrane resistance (M $\Omega$ )	8.4 $\pm$ 0.54
Mean action current amplitude (pA)	3185
Mean action current rise time (ms)	0.024

**Table 4.1:** table shows mean cell membrane capacitance, series resistance, electrode resistance and membrane resistances calculated from a random sample of 24 cells. Mean action current amplitude and rise time from 20 fast events recorded from one cell is also given.

### 3. Procedures

Hippocampal neurones held under voltage-clamp at  $-60$  mV, unless otherwise stated, displayed rapid inward currents in response to pressure-

applied (10-100 millisecond pulses) GABA, glycine, NMDA (N-methyl-D-aspartate), AMPA ( $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionate) and kainic acid. Agonist-evoked responses increased in amplitude with longer duration pulses of agonist exposure. At the beginning of each experiment, an agonist pulse duration was selected which was at most 10% of the maximum response obtainable. All drug effects were determined against submaximal agonist-evoked control responses and were perfused onto the cell until a maximal inhibitory or potentiating effect was observed. Upon removal of the drug, a series of control responses were obtained before further drug applications. The experimental protocol and data analysis were carried out as previously described (see Methods).

#### **4. Results - Ligand-gated Anion Channels**

##### ***4.i. Physiological and pharmacological properties of GABA-evoked currents***

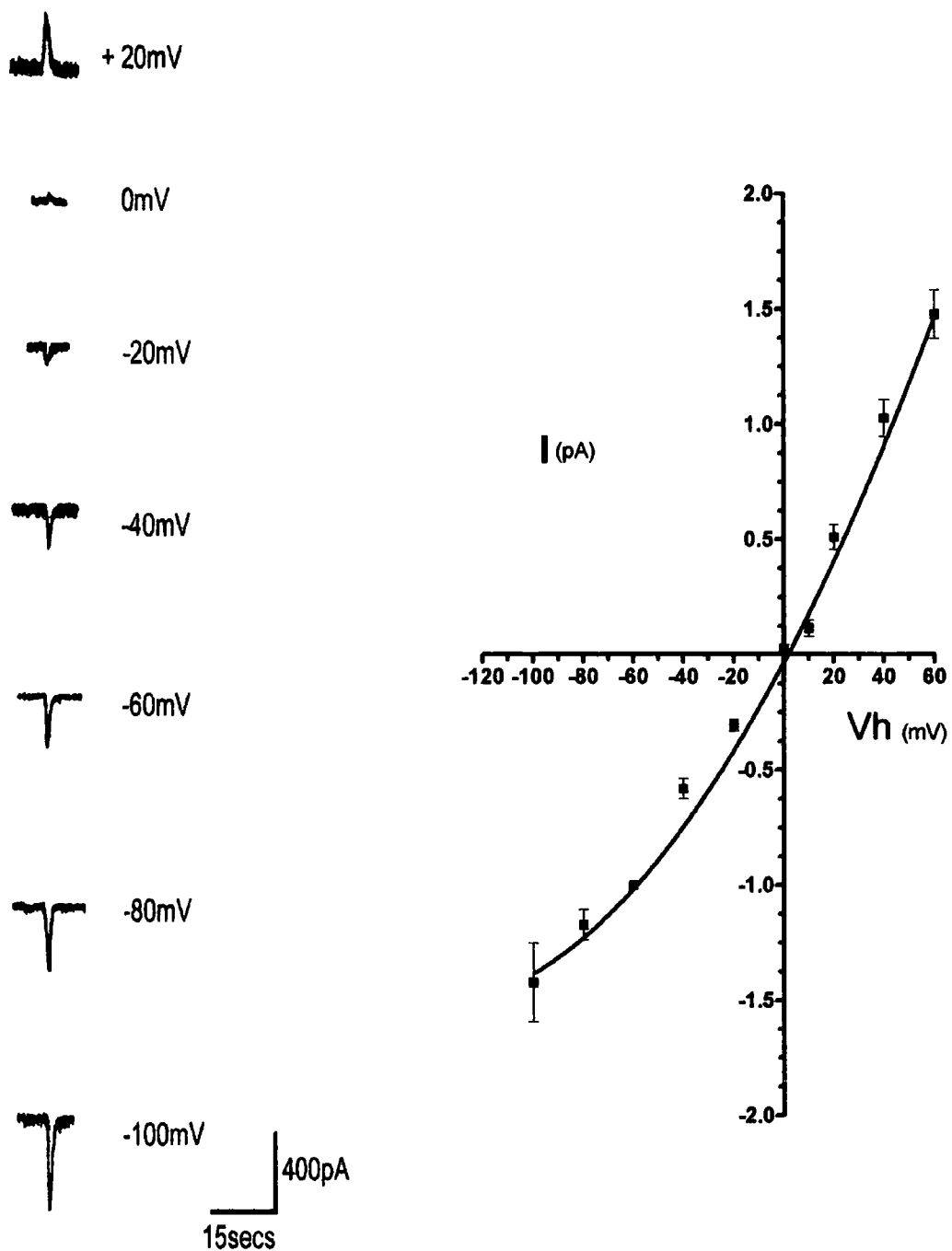
###### *(a) I-V Relationship*

GABA (10 $\mu$ M) pressure-applied (1.4kPa, at a rate of 0.02-0.03Hz and between 20-100ms in duration) to voltage-clamped hippocampal neurones ( $V_h$ =-60mV) evoked rapidly activating currents of 150-2400pA in amplitude in all cells tested (n=302 neurones; e.g. cell 2.16). The GABA whole-cell current-voltage relationship (I-V) for hippocampal neurones voltage-clamped between -100mV and +60mV was determined (using a CsCl-based internal and a normal external bathing solution as described in Methods). The GABA I-V (all responses were normalised to a holding potential of -60mV) showed outward rectification and reversed at 1.5 mV (n=4-20) (interpolated from the intercept of the curve on the x-axis using Graph Pad Prism Version 2.0a<sup>TM</sup>); this value is consistent with the  $E_{(Cl)}$  predicted from the Nernst equation of 0mV with equal chloride across the membrane (figure 4.2).

###### *(b) Pharmacology of the GABA<sub>A</sub> receptor*

Bicuculline (0.1-3 $\mu$ M), a competitive GABA<sub>A</sub> receptor antagonist (e.g. Simmonds, 1982) concentration-dependently and reversibly inhibited





**Figure 4.2: The GABA-evoked whole-cell current-voltage relationship for voltage-clamped hippocampal neurones.** Left: shows actual chart recorder traces of single GABA ( $10 \mu\text{M}$ )-evoked currents recorded over a range of holding potentials given to the right of the currents (cell 3.75). Right: the graph shows the GABA-evoked current amplitude (normalized to a holding potential of  $-60\text{mV}$ ; I, on the ordinate, in pA) plotted against the holding potential ( $V_h$ , on the abscissa, in mV). The GABA current reversed direction at  $1.5 \text{ mV}$  and displayed outward rectification. Each data point represents the mean  $\pm$  s.e.m (represented by the vertical bars) of 4-20 experiments.

submaximal GABA (10 $\mu$ M)-evoked whole-cell currents. From a series of concentration-effect studies, an IC<sub>50</sub> of 0.5 $\mu$ M was determined for bicuculline. (figure 4.3).

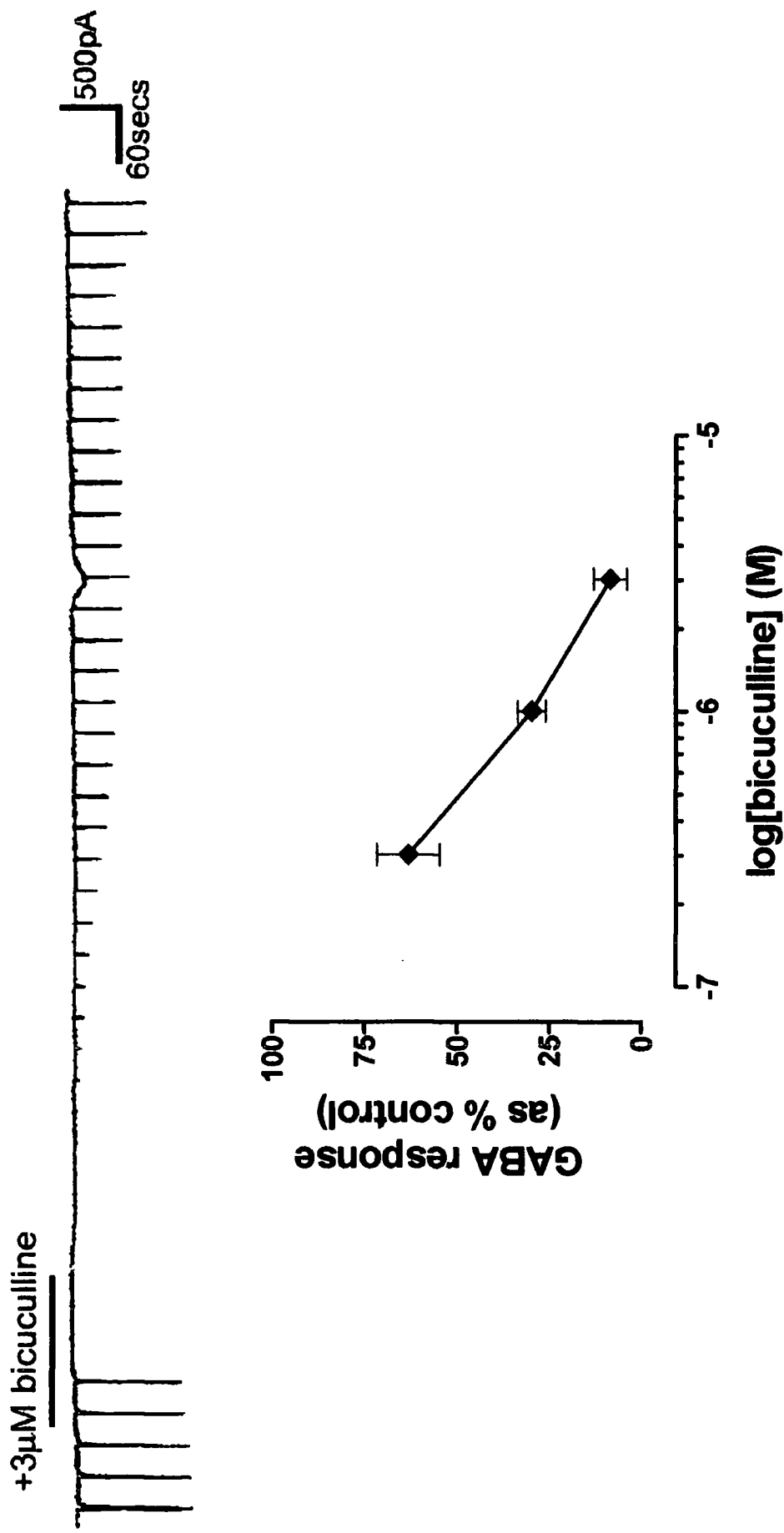
Alphaxalone (0.1-3 $\mu$ M), propofol (1-10 $\mu$ M), loreclezole (3-30 $\mu$ M), diazepam (0.1-1 $\mu$ M), and sodium pentobarbitone (10-300 $\mu$ M) concentration-dependently potentiated submaximal GABA (10 $\mu$ M)-evoked currents with EC<sub>50</sub> values of 4 $\mu$ M (2 $\mu$ M-7 $\mu$ M, n=2-4), 6 $\mu$ M (3 $\mu$ M-12 $\mu$ M, n=6-10), 4 $\mu$ M (1 $\mu$ M-16 $\mu$ M, n=4-11), 0.6 $\mu$ M (0.3 $\mu$ M-1.2 $\mu$ M, n=2-7) and 64 $\mu$ M (33 $\mu$ M-121 $\mu$ M, n=3-14), respectively (see figure 4.5).

The potency sequence for these modulators, in descending order is as follows: diazepam > alphaxalone = loreclezole > propofol > sodium pentobarbitone. Mean maximal potentiations  $\pm$  s.e.m evoked by alphaxalone (10 $\mu$ M), propofol (10 $\mu$ M), loreclezole (10 $\mu$ M), diazepam (1 $\mu$ M), and sodium pentobarbitone (300 $\mu$ M) were 975% (n=2), 421 $\pm$ 35% (n=10), 204 $\pm$ 11% (n=6), 231 $\pm$ 25% (n=11) and 393 $\pm$ 30% (n=3), respectively. Additionally, alphaxalone ( $\geq$ 1 $\mu$ M), propofol ( $\geq$ 1 $\mu$ M), loreclezole ( $\geq$ 10 $\mu$ M) and sodium pentobarbitone ( $\geq$ 10 $\mu$ M), but not diazepam, activated an inward transmembrane current (see figure 4.4).

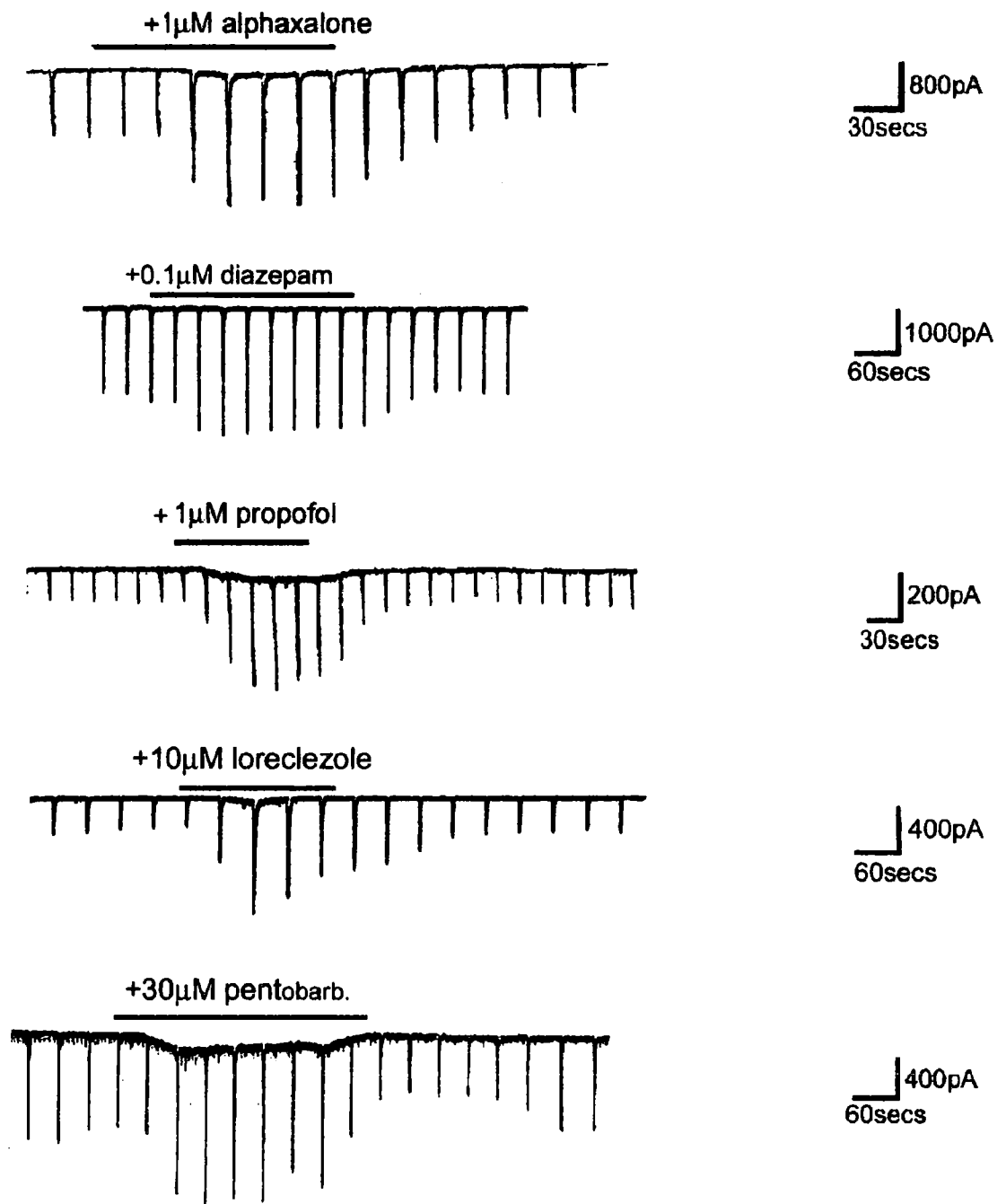
These data are in agreement with those of other investigators, although alphaxalone is usually reported to be more potent than determined here (e.g. Peters *et al.*, 1988). Collectively these data are consistent with activation of a neuronal GABA<sub>A</sub> receptor gated chloride ion channel.

#### **4.ii. Physiological and pharmacological properties of glycine-evoked currents**

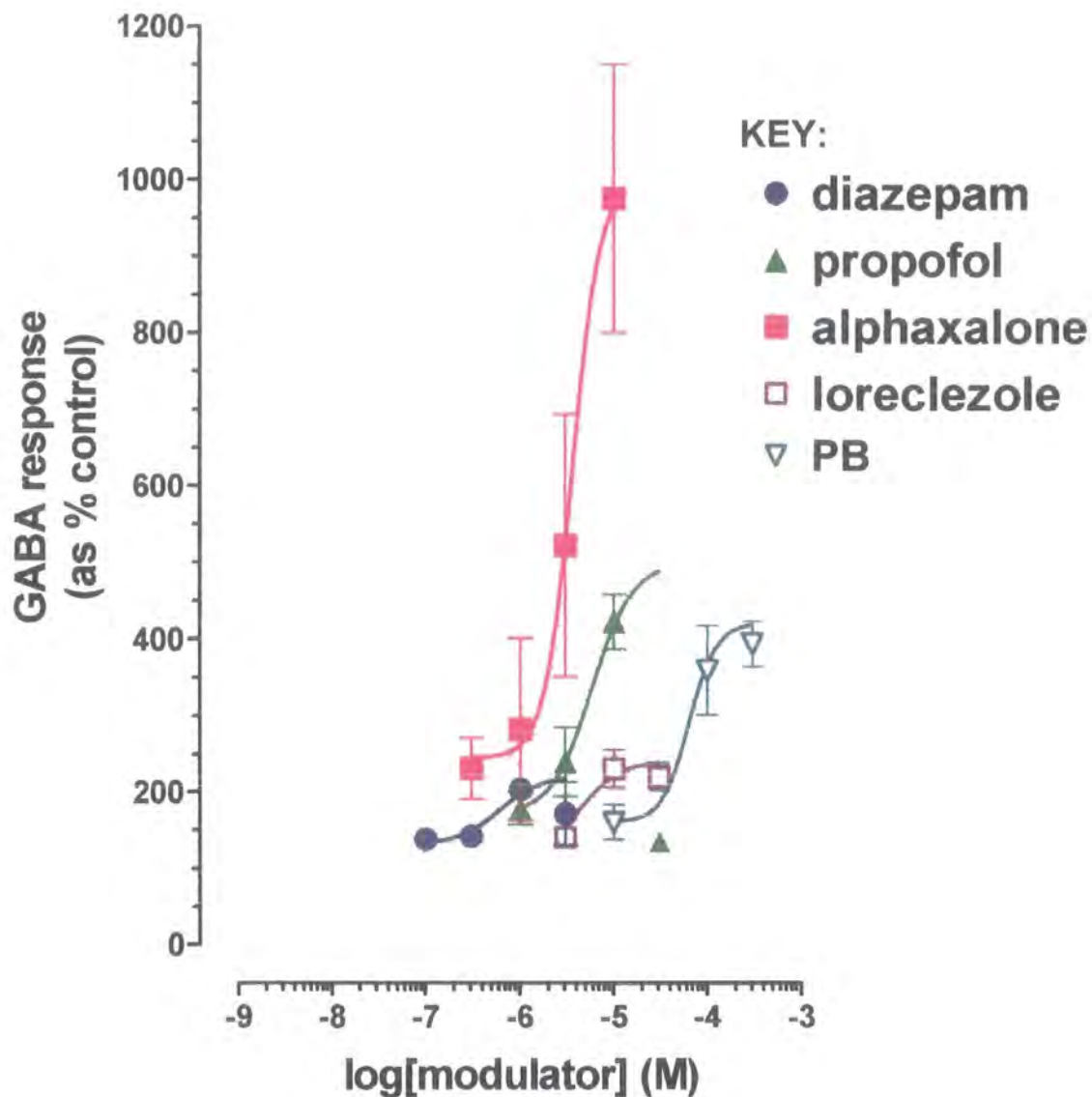
Glycine (100 $\mu$ M) pressure-applied (1.4kPa, at a rate of 0.02-0.03Hz and between 20-130ms in duration) to voltage-clamped hippocampal neurones (V<sub>h</sub>=-60mV) evoked rapidly activating currents of 100-1800pA in amplitude in all cells tested (n=14 neurones; e.g. cell 2.92).



**Figure 4.3: Bicuculline antagonises GABA-evoked responses in whole-cell voltage-clamped hippocampal neurones. Top: Chart recorder trace of GABA (10µM)-evoked whole-cell currents recorded before, in the presence of and following removal of bicuculline (3µM). Drug contact time is indicated by the solid line above the trace. Bottom: Graph shows the log<sub>10</sub> concentration of bicuculline (on the abscissa) plotted against GABA-evoked current, as percent of control on the ordinate. Each data point represents the mean ± s.e.m of 3-6 experiments (represented by the vertical lines).**



**Figure 4.4: Positive allosteric modulation of GABA-evoked whole-cell currents in voltage-clamped rat hippocampal neurones.** Chart recorder traces of submaximal GABA-evoked responses ( $V_h = -60\text{mV}$ ) potentiated by alphaxalone ( $1\mu\text{M}$ ), diazepam ( $0.1\mu\text{M}$ ), propofol ( $1\mu\text{M}$ ), loreclezole ( $10\mu\text{M}$ ) and sodium pentobarbitone ( $30\mu\text{M}$ ). All of the modulators shown, except diazepam, evoke a small transmembrane current. Drug contact time is represented by the solid line above each trace. Each chart recorder trace is recorded from a different neuron.



**Figure 4.5: Positive allosteric modulation of GABA-evoked responses in whole-cell voltage-clamped hippocampal neurones.** Graph shows the  $\log_{10}$  concentration of modulator (on the abscissa) plotted against GABA-evoked current, as percent of control on the ordinate. Each data point represents the mean  $\pm$  s.e.m (represented by the vertical lines) of 2-14 experiments.

### *(a) Pharmacology of the glycine receptor*

Strychnine (0.1 and 0.3 $\mu$ M), a potent glycine receptor antagonist (e.g. Simmonds, 1983) concentration-dependently and reversibly inhibited submaximal glycine-evoked (100 $\mu$ M) whole-cell currents. Strychnine (0.1 $\mu$ M) reversibly inhibited submaximal glycine-evoked (100 $\mu$ M) whole-cell currents to 17 $\pm$ 5% (n=4). Strychnine (0.3 $\mu$ M) reversibly inhibited submaximal glycine-evoked (100 $\mu$ M) whole-cell currents to 5 $\pm$ 4% (n=3) of control. (figure 4.6).

## **5. Results- Ligand-gated Cation Channels**

### **Ionotropic Glutamate Receptors**

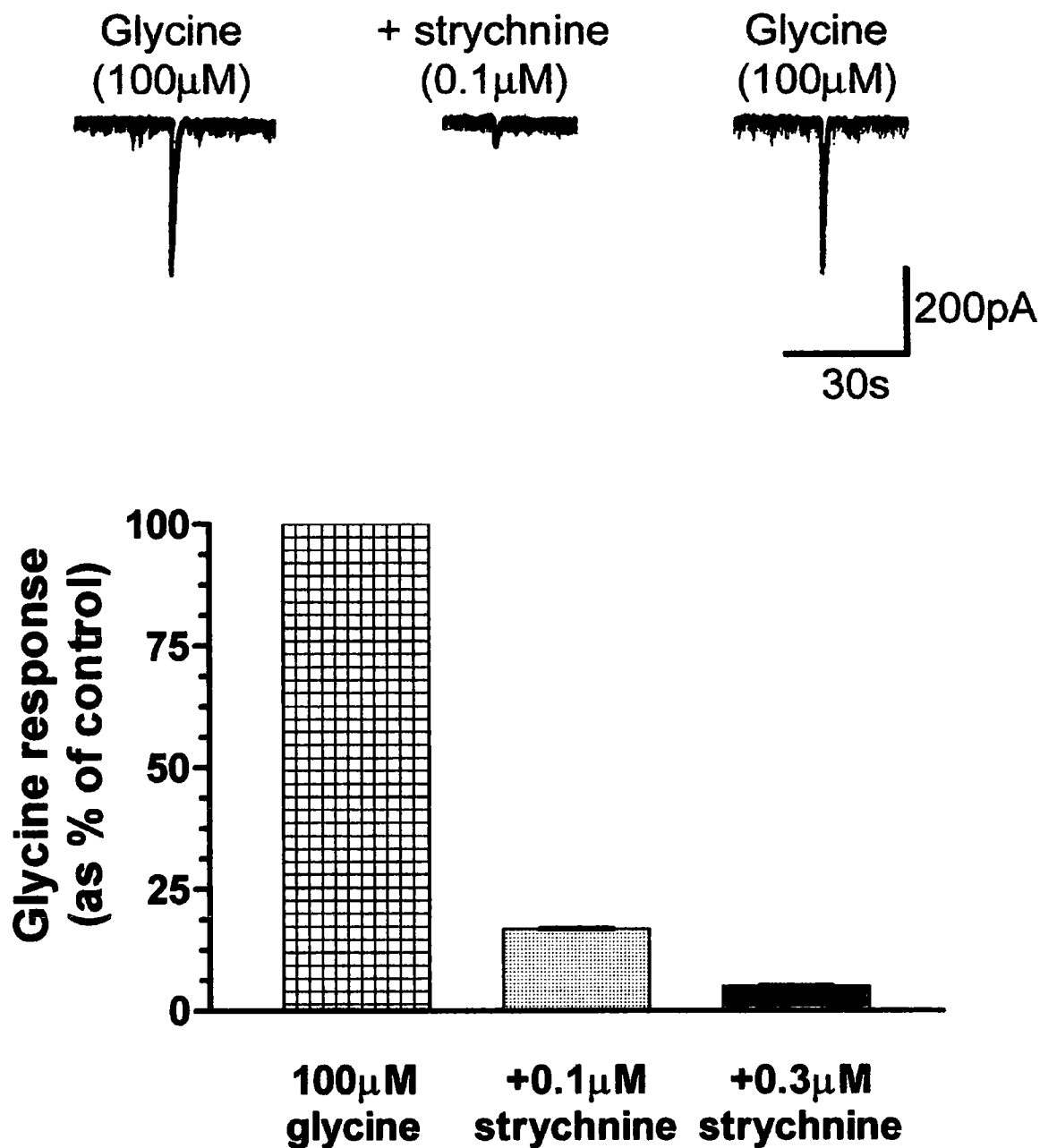
#### **5.i. Physiological and pharmacological properties of NMDA-evoked currents**

##### *(a) I-V Relationship*

NMDA (100 $\mu$ M) pressure-applied (1.4kPa, at a rate of 0.02-0.03Hz and between 15-120ms in duration) to voltage-clamped hippocampal neurones ( $V_h$ =-60mV) evoked rapidly activating currents of 180-1280pA in amplitude in all cells tested (n=21 neurones; e.g. cell 9.2). The whole-cell NMDA current-voltage relationship for hippocampal neurones voltage-clamped between -100mV and +40mV was determined in the absence (n=6) and presence (n=4) of Mg<sup>2+</sup> (2mM). In the absence of Mg<sup>2+</sup>, NMDA-evoked currents varied in an approximately linear fashion over a range of holding potentials. Mg<sup>2+</sup> blocked the NMDA-gated channel in a voltage-dependent manner with current flow being reduced at negative holding potentials, and a region of negative slope conductance occurring between -40 and -80mV (figure 4.7).

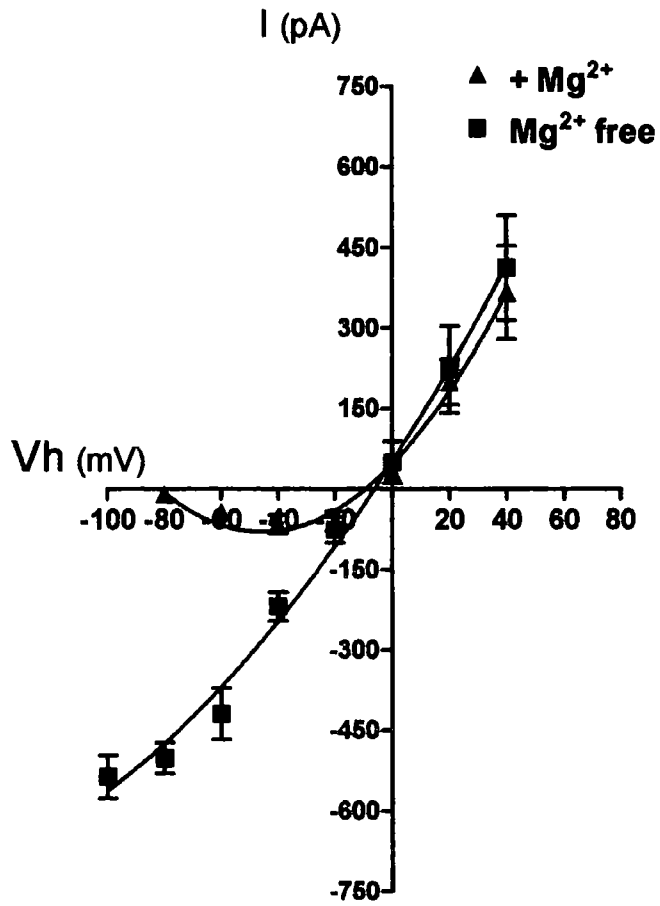
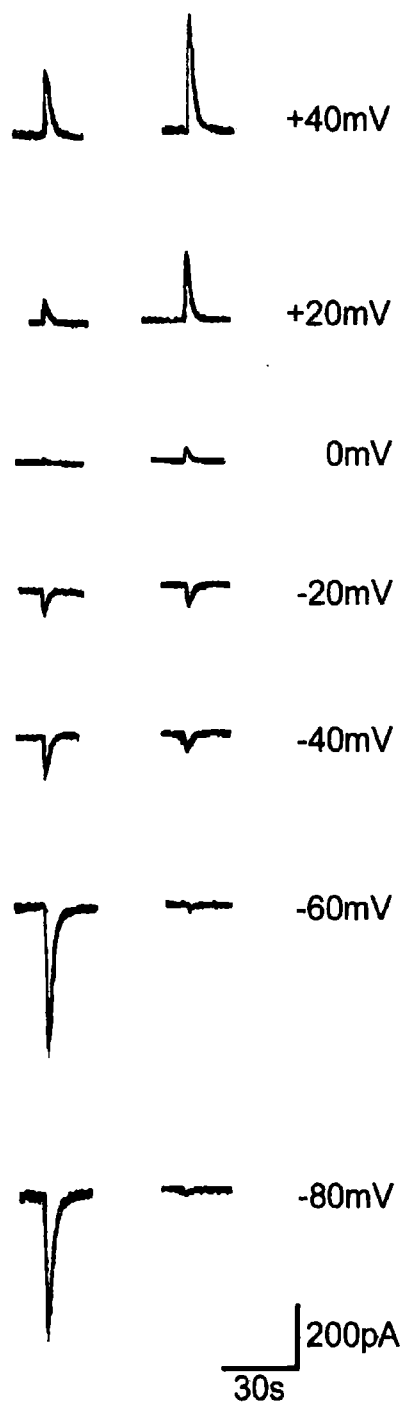
##### *(b) Pharmacology of the NMDA-gated receptor*

In agreement with Johnston and Ascher (1987), NMDA-evoked currents were potentiated by glycine (1 $\mu$ M) to 228 $\pm$ 20% of control (n=8). Glycine is thought to act as a co-agonist for the NMDA receptor, thus glycine (1 $\mu$ M) was routinely added to the external bathing solution when recording NMDA-evoked currents (see Methods). The competitive NMDA receptor antagonist D-APV



**Figure 4.6: Strychnine inhibits glycine-evoked responses in voltage-clamped hippocampal neurones.** Top: shows chart recorder traces of glycine (100µM)-evoked whole-cell currents ( $V_h = -60\text{mV}$ ) before (left), in the presence of (middle) and following removal of (right) strychnine (0.1µM). Bottom: histogram summarising the inhibitory effect of strychnine (0.1, 0.3µM) on submaximal (100µM) glycine-evoked responses. Each data point represents the mean  $\pm$  s.e.m of 3-4 experiments (represented by the vertical lines).

Mg<sup>2+</sup> free    Mg<sup>2+</sup> containing



**Figure 4.7: The NMDA-evoked whole-cell current-voltage relationship for voltage-clamped hippocampal neurones. Left:** shows actual chart recorder traces of single NMDA (100µM)-evoked currents recorded over a range of holding potentials (given to the right of the currents) in the absence and presence of Mg<sup>2+</sup> (2mM). **Right:** the graph shows the NMDA-evoked current amplitude (I; on the ordinate, in pA), in the absence (■) and presence (▲) of Mg<sup>2+</sup>(2mM), plotted against the holding potential (Vh; on the abscissa, in mV). Each data point represents the mean ± s.e.m (represented by the vertical bars) of 4-6 experiments.



(0.1-10 $\mu$ M), (Evans *et al.*, 1982) concentration-dependently and reversibly inhibited submaximal NMDA -evoked (100 $\mu$ M) whole-cell currents.

From a series of concentration-effect studies, D-APV (0.1-10 $\mu$ M) reversibly inhibited submaximal NMDA-evoked (100 $\mu$ M) currents with an IC<sub>50</sub> of 0.15 $\mu$ M (figure 4.8).

## **5.ii Physiological and pharmacological properties of AMPA-evoked currents**

### *(a) I-V Relationship*

AMPA (100 $\mu$ M), pressure-applied (1.4kPa, at a rate of 0.02-0.03Hz and between 15-85ms in duration) to voltage-clamped hippocampal neurones (V<sub>h</sub>=-60mV) evoked rapidly activating currents of 220-860pA in amplitude all cells tested (n=4 neurones; e.g. cell 3.31). The AMPA current-voltage relationship for hippocampal neurones whole-cell voltage-clamped between -100mV and +100mV revealed an approximately linear relationship (figure 4.9).

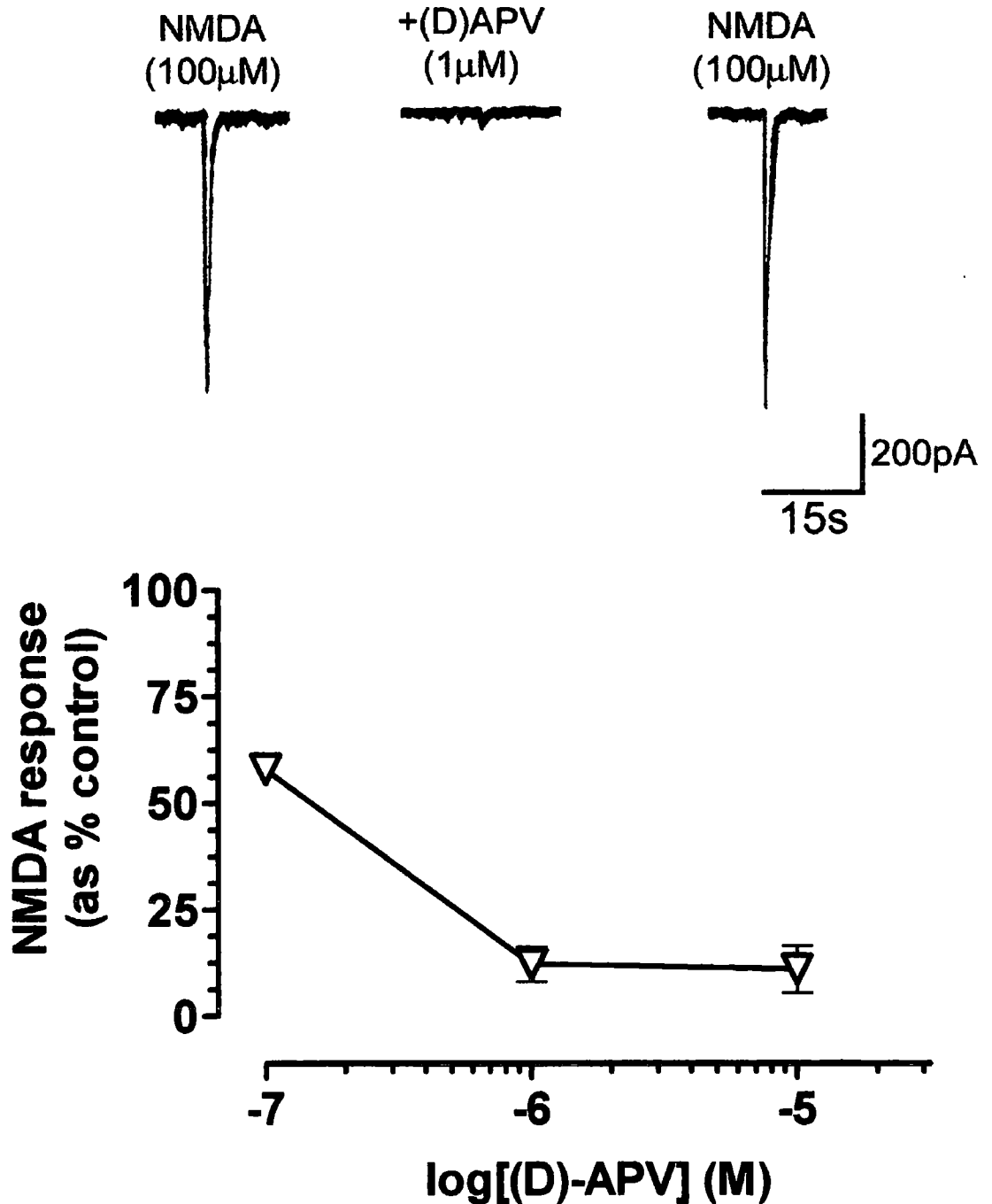
### *(b) Pharmacology of the AMPA -gated receptor*

The competitive AMPA/kainate receptor antagonist CNQX (3 and 10 $\mu$ M), (Stein *et al.*, 1992) concentration-dependently and reversibly inhibited submaximal AMPA -evoked (100 $\mu$ M) whole-cell currents to 31 $\pm$ 3% (n=5) and 6 $\pm$ 3% (n=5) of control, respectively (figure 4.10).

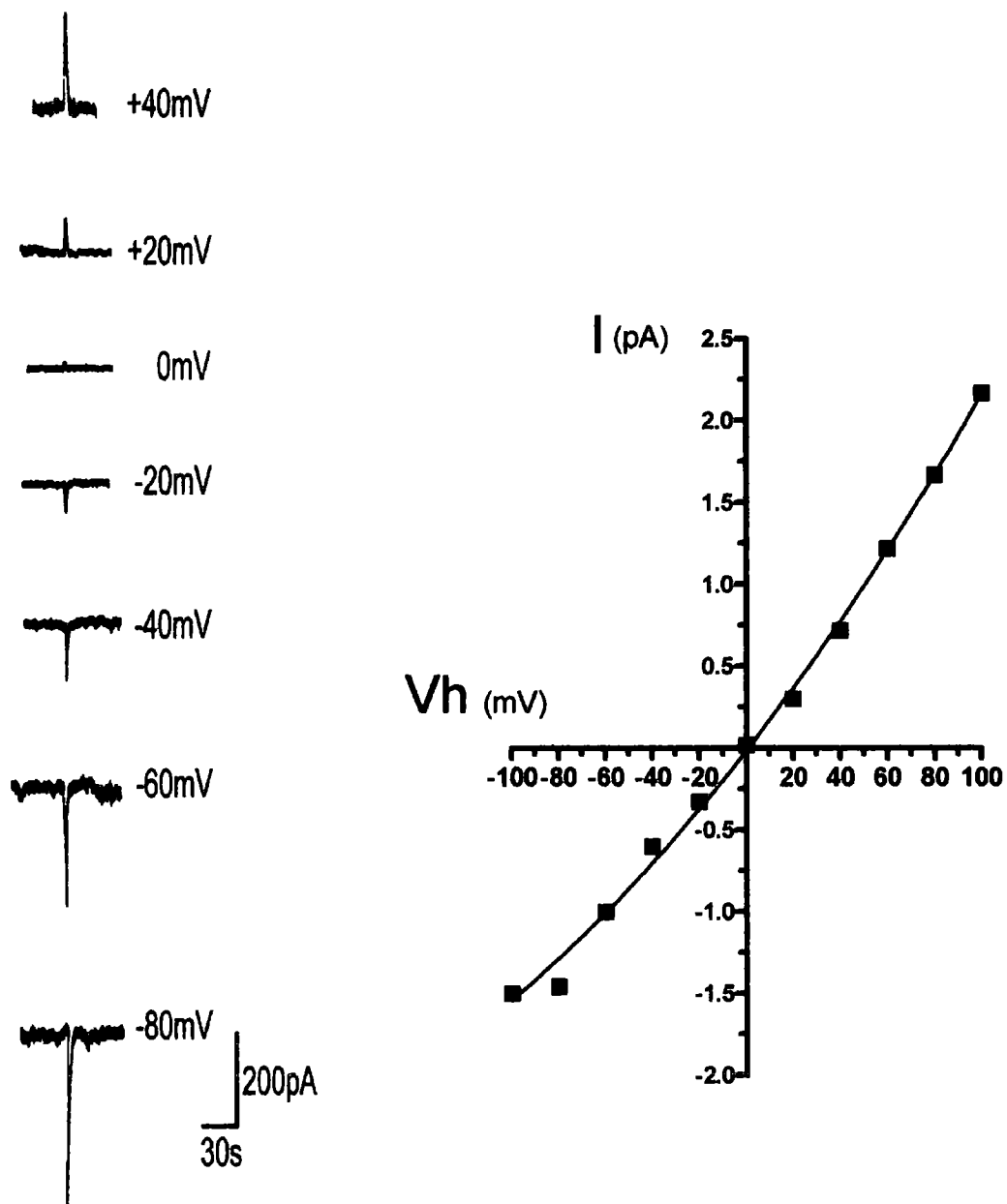
## **5.iii. Physiological and pharmacological properties of kainic acid-evoked currents**

### *(a) I-V Relationship*

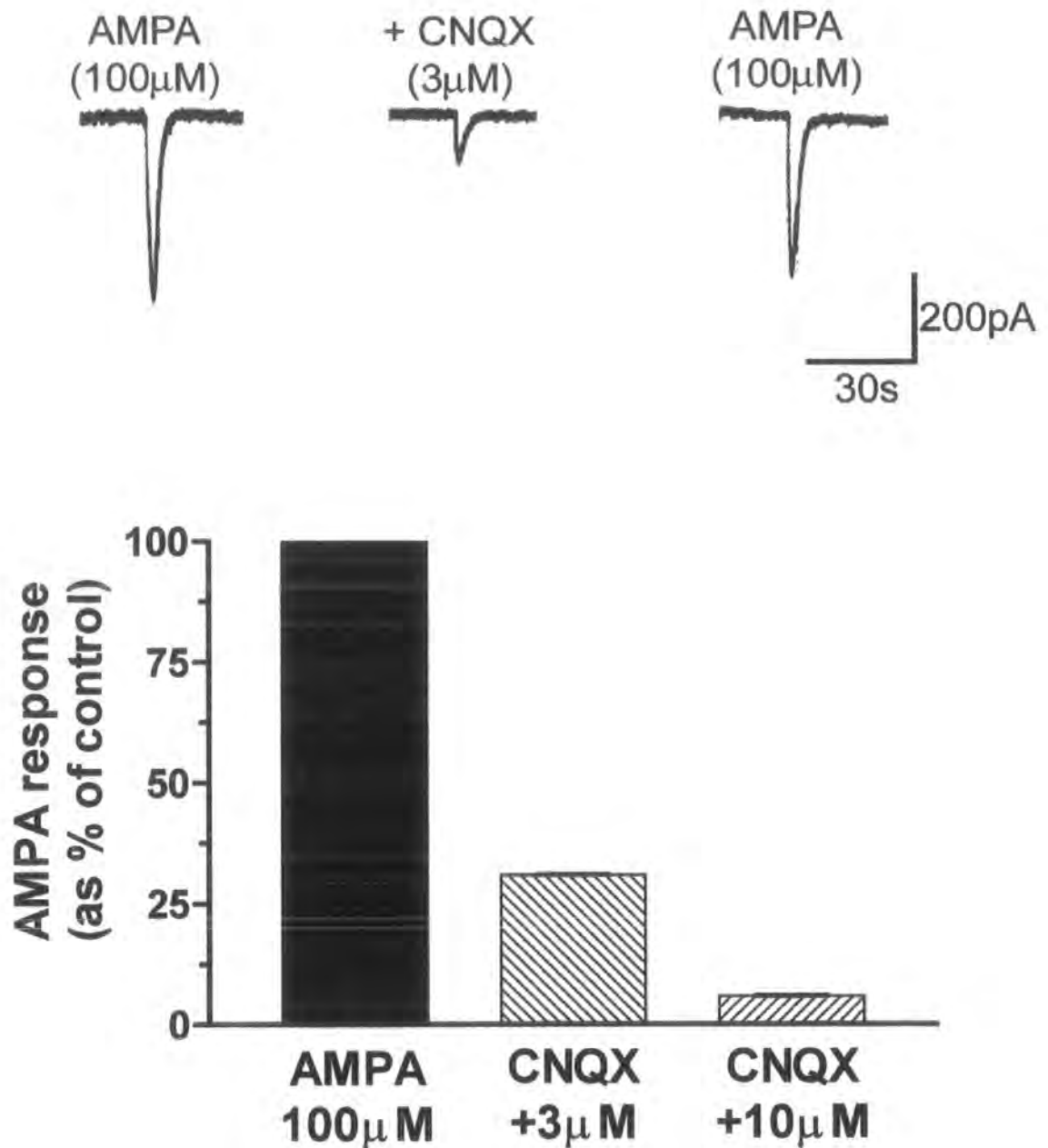
Kainate (100 $\mu$ M), pressure-applied (1.4kPa, at a rate of 0.02-0.03Hz and between 20-150ms in duration) to voltage-clamped hippocampal neurones (V<sub>h</sub>=-60mV) evoked rapidly activating currents of 180-960pA in amplitude all cells tested (n=9 neurones; e.g. cell 2.130). The kainate current-voltage



**Figure 4.8: (D)-APV antagonises NMDA-evoked responses in voltage-clamped hippocampal neurones.** Top: shows actual chart recorder traces of single NMDA (100  $\mu$ M)-evoked whole-cell currents ( $V_h = -60$ mV) before (left), in the presence of (middle) and following removal of (right) (D)-APV (1  $\mu$ M). Bottom: the graph shows the  $\log_{10}$  concentration of (D)-APV (on the abscissa) plotted against the NMDA-evoked current, as percent of control on the ordinate. Each data point represents the mean  $\pm$  s.e.m of 3-4 experiments (represented by the vertical lines).



**Figure 4.9: The AMPA-evoked whole-cell current-voltage relationship for voltage-clamped hippocampal neurones.** Left: shows actual chart recorder traces of AMPA ( $100\mu\text{M}$ )-evoked currents recorded over a range of holding potentials (given to the right of the currents). Right: Graph shows the AMPA-evoked current amplitude (normalized to a holding potential of  $-60\text{mV}$ ;  $I$ , on the ordinate, in pA) plotted against holding potential ( $V_h$ , on the abscissa, in mV). The AMPA current reversed direction at  $0.75\text{mV}$  and was approximately linear between  $-100\text{mV}$  and  $+100\text{mV}$ . Each data point represents the mean of 2 experiments.



**Figure 4.10: CNQX inhibits AMPA-evoked responses in whole-cell voltage-clamped hippocampal neurones.** Top: shows actual chart recorder traces of AMPA (100 μM)-evoked whole-cell currents ( $V_h = -60\text{mV}$ ) before (left), in the presence of (middle) and following removal of (right) CNQX (3 μM). Bottom: histogram summarising the inhibitory effect of CNQX (3, 10 μM) on submaximal (100 μM) AMPA-evoked responses. Each data point represents the mean  $\pm$  s.e.m of 4-5 experiments (represented by the vertical lines).

relationship for hippocampal neurones whole-cell voltage-clamped between -100mV and +60mV revealed an approximately linear relationship (figure 4.11).

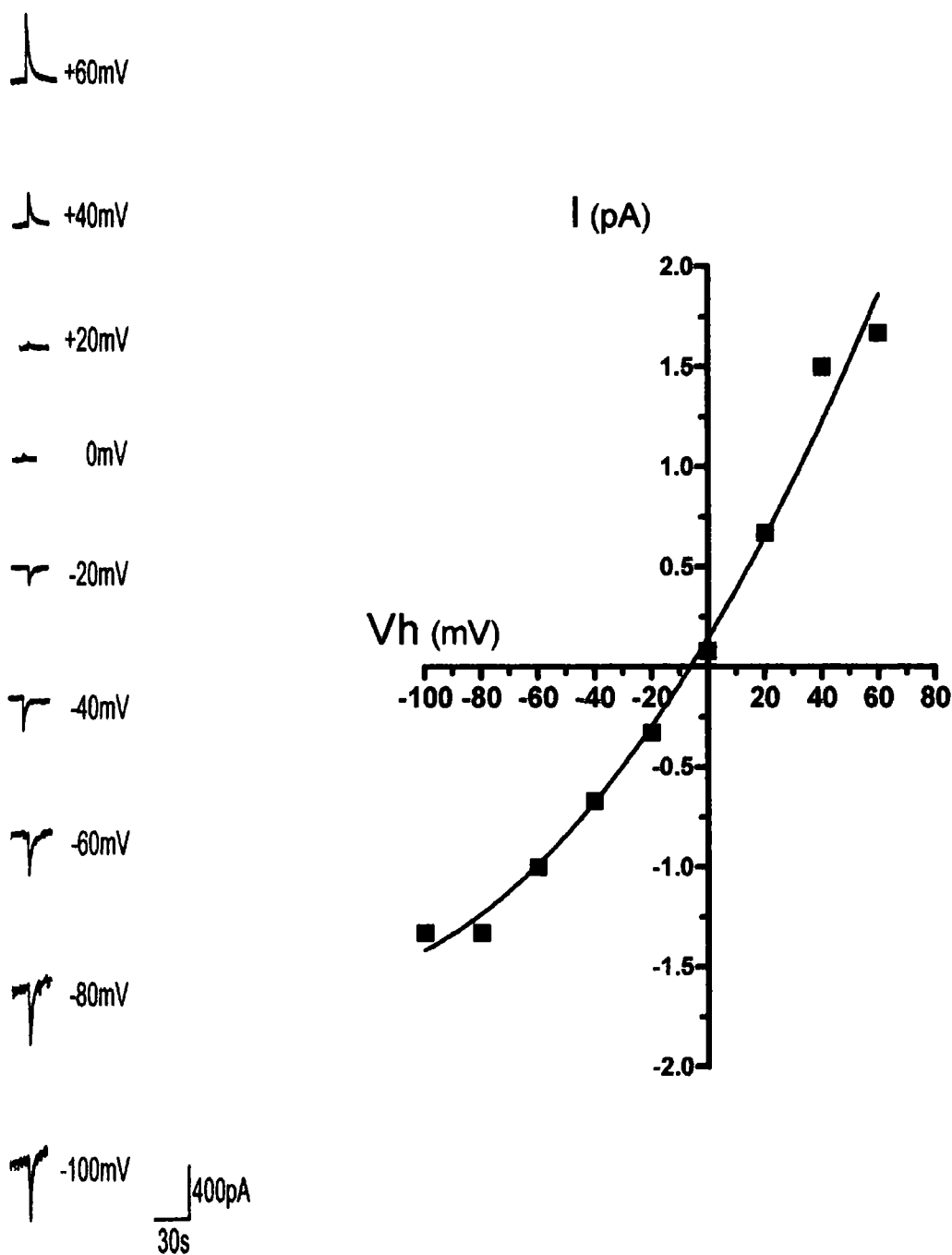
*(b) Pharmacology of the kainate-gated receptor*

The competitive AMPA/kainate receptor antagonist CNQX (1-10 $\mu$ M), (Stein *et al.*, 1992) concentration-dependently and reversibly inhibited submaximal kainic acid-evoked (100 $\mu$ M) whole-cell currents. From a series of concentration-effect studies CNQX (1-10 $\mu$ M) reversibly inhibited submaximal kainic acid-evoked (100 $\mu$ M) whole-cell currents. CNQX (1 $\mu$ M) inhibited kainic acid-evoked currents to 47% (n=1). CNQX (3 $\mu$ M) inhibited kainic acid-evoked currents to 33% (n=2) and CNQX (10 $\mu$ M) inhibited kainic acid-evoked currents to 9% (n=2) of control, respectively (figure 4.12).

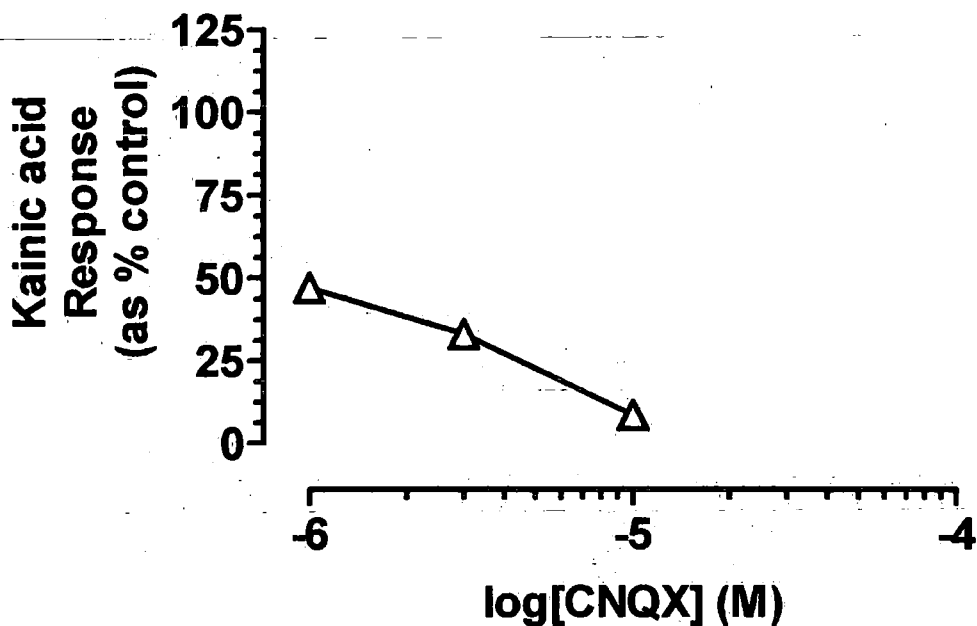
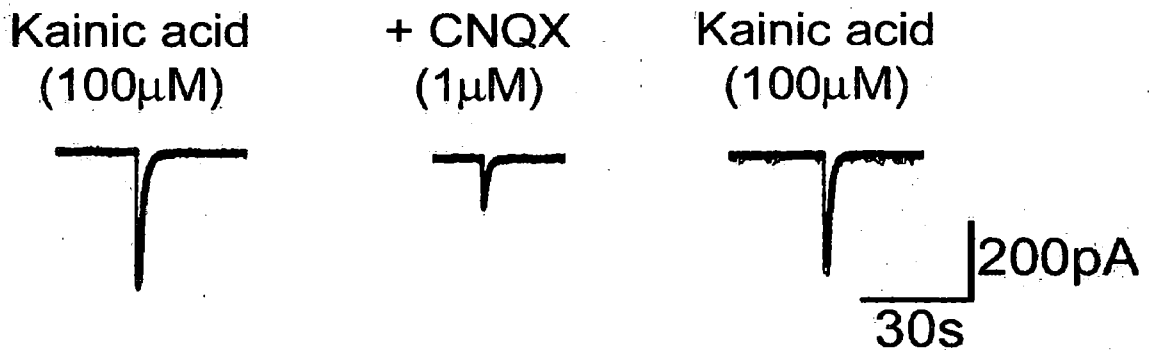
## **6. Discussion**

Those rat hippocampal neurones which were successfully maintained in culture were viewed under phase-contrast microscopy and visualized as phase-bright cells with pyramidal shaped cell-bodies. These cells were spontaneously active, in the absence of any external chemical or electrical stimuli, and responded to extracellularly applied agonists. These morphological and physiological characteristics are similar to those described by other investigators (Segal, 1983; Segal and Barker, 1984; Ozawa and Yuzaki, 1984; Valeyev *et al.*, 1993, 1995).

GABA-evoked whole-cell responses from voltage-clamped rat hippocampal neurones were pharmacologically and physiologically consistent with the activation of a neuronal GABA<sub>A</sub> receptor. For example, the GABA-evoked current was antagonized by bicuculline and potentiated by known positive allosteric modulators of the GABA<sub>A</sub> receptor, such as pentobarbitone, alphaxalone, propofol and loreclezole. Similar observations have been made by other investigators recording GABA-evoked currents in voltage-clamped rat hippocampal neurones (e.g. Segal and Barker, 1984; Hara *et al.*, 1994; Valeyev *et al.*, 1995) and in other whole-cell voltage-clamp studies using neuronal



**Figure 4.11: The kainic acid-evoked whole-cell current-voltage relationship for voltage-clamped hippocampal neurones. Left:** shows actual chart recorder traces of single kainic acid ( $100\mu\text{M}$ )-evoked currents recorded over a range of holding potentials, shown to the right of each current. **Right:** the graph shows the kainic acid-evoked current amplitude (normalized to a holding potential of  $-60\text{mV}$ ;  $I$ , on the ordinate, in pA) plotted against holding potential ( $V_h$ , on the abscissa, in mV). The kainic acid current reversed direction at  $-6.2\text{ mV}$  and was approximately linear between  $-100\text{mV}$  and  $+100\text{mV}$ . Each data point represents 1 experiment.



**Figure 4.12: CNQX antagonises kainic acid-evoked responses in whole-cell voltage-clamped hippocampal neurones.** Top: shows actual chart recorder traces of kainic acid (100 $\mu$ M)-evoked whole-cell currents ( $V_h = -60$ mV) before (left), in the presence of (middle) and following removal of (right) CNQX (1 $\mu$ M). Bottom: the graph shows the  $\log_{10}$  concentration of CNQX (on the abscissa) plotted against the kainic acid-evoked current, as percent of control, on the ordinate. Each data point represents the mean of 1-2 experiments.

preparations (e.g. Peters *et al.*, 1988; Wafford *et al.*, 1994). The potency sequence in descending order of potency for these modulators was: diazepam > alphaxalone > loreclezole  $\approx$  propofol > pentobarbitone. These data are in good agreement with the observations made in the rat vagus nerve.

Additionally, the GABA-evoked current reversed direction at 2.3mV ; this value is consistent with the reversal potential of 0mV for the chloride ion, when chloride is equally distributed across the cell membrane. These data are in agreement with those of Segal and Barker, 1984; Ozawa and Yuzaki, 1984 and Valeyev *et al.*, 1993.

Glycine-evoked whole-cell responses from voltage-clamped rat hippocampal neurones were pharmacologically and physiologically consistent with activation of a neuronal strychnine-sensitive glycine-gated chloride ion channel. For example, the glycine-evoked current was antagonized by strychnine. Similar observations have been made by other investigators recording glycine-evoked currents in voltage-clamped rat hippocampal neurones (e.g. Segal and Barker, 1984; Ito and Cherubini, 1991, Hara *et al.*, 1994).

NMDA-evoked whole-cell responses from voltage-clamped rat hippocampal neurones were pharmacologically and physiologically consistent with activation of a neuronal ionotropic glutamate receptor. The NMDA-evoked current was antagonized by (D)-APV and potentiated by glycine in agreement with Evans *et al.*, (1982) and Johnston and Ascher, (1987), respectively. The NMDA-IV plot was essentially linear between the holding potentials of -100mV and +40mV, but in the presence of  $Mg^{2+}$ , a voltage-dependent inhibition of the NMDA-evoked current was observed at holding potentials more negative than -20 mV . These data are consistent with those of Nowak *et al.*, (1984) and Mayer and Westbrook, (1985).

AMPA-evoked whole-cell responses from voltage-clamped rat hippocampal neurones were pharmacologically and physiologically consistent with activation of a neuronal ionotropic glutamate receptor. The AMPA-evoked current was antagonized by CNQX and produced an approximately linear I-V plot. These data are commensurate with the work of Stein *et al.*, (1992) and Jonas *et al.*, (1992).



Kainic acid-evoked whole-cell responses from voltage-clamped rat hippocampal neurones were pharmacologically and physiologically consistent with activation of a neuronal ionotropic glutamate receptor. In agreement with Stein *et al.*, (1992) and Jonas *et al.*, (1992), respectively, the kainic acid-evoked current was antagonized by CNQX and produced an approximately linear I-V plot.

These data support the use of hippocampal neurones and the whole-cell voltage-clamp recording technique for examining the mechanism and selectivity of action of drugs on neuronal ligand-gated ion channels.

## **CHAPTER THREE: RESULTS**

### **Section 3.3: Determination on the effect, site and molecular mechanism of action of fenamates at neuronal GABA<sub>A</sub> receptors**

#### **1. Introduction**

Woodward *et al.*, (1994) have reported a bi-directional modulation of GABA-evoked responses recorded from *Xenopus* oocytes injected with rat brain mRNA. Moreover, preliminary studies in native neurones, outlined in the previous chapter, revealed that mefenamic acid evoked a modest potentiation of GABA-evoked responses recorded from rat vagus nerves. In order to further investigate the site and mechanism underlying the effect of mefenamic acid on neuronal GABA<sub>A</sub> receptors, experiments were conducted using whole-cell voltage-clamped hippocampal neurones.

#### **2. Procedures**

All hippocampal neurones in these experiments were voltage-clamped at -60mV, unless otherwise stated. Brief pressure application (20-100ms) of GABA to cells resulted in rapidly activating inward currents in all hippocampal neurones, with the amplitude increasing with longer pulses of GABA. All drug effects were determined against submaximal GABA-evoked responses and all responses were measured at their peak. All recordings were made in the presence of TTX (see Methods). Drugs were directionally perfused onto the cell until a maximal effect was observed. Upon removal of the drug, a further series of control responses were obtained before additional drug applications. The experimental protocol and data analysis were carried out as previously described (see Methods).

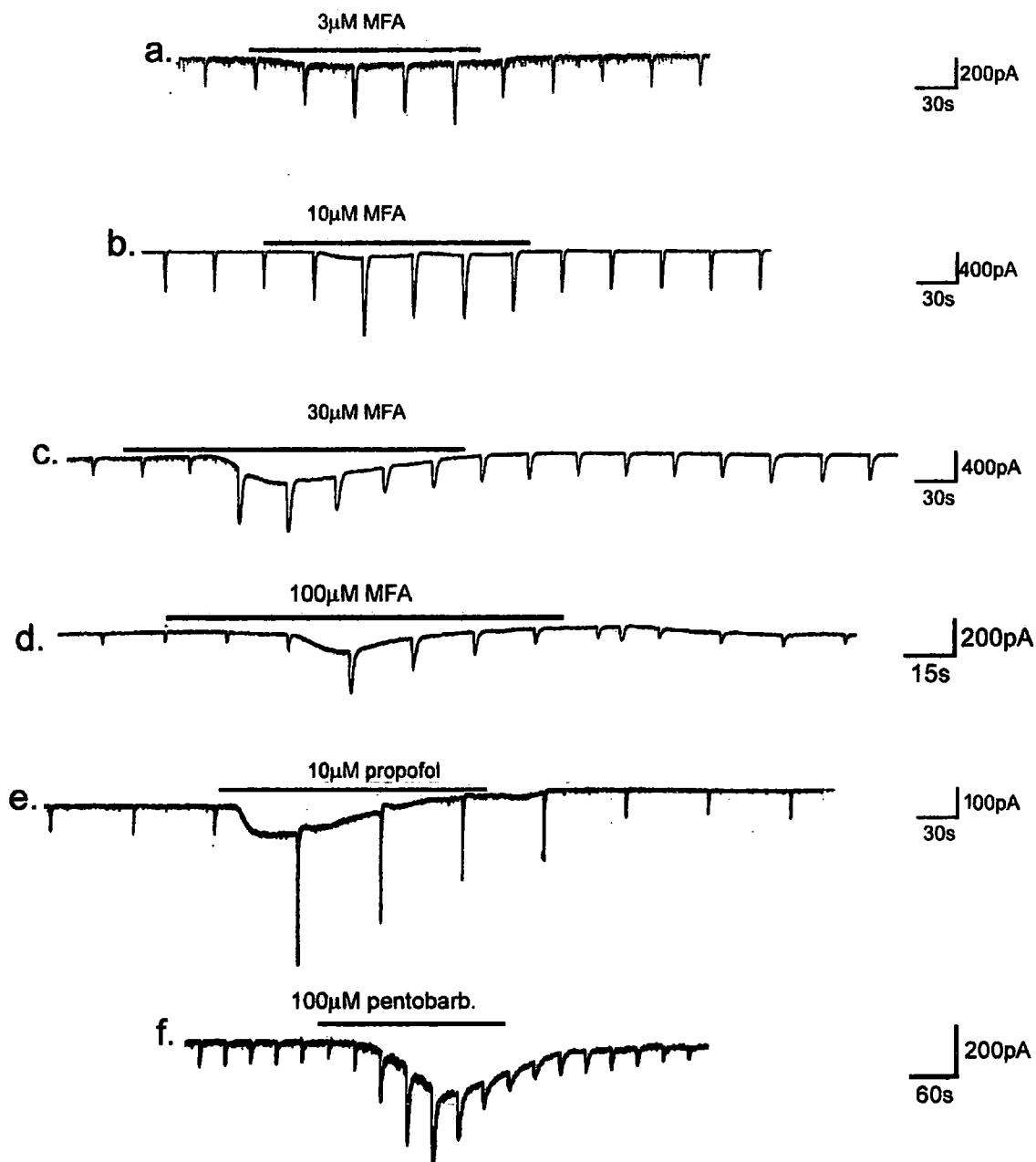
### 3. Results

#### 3.i. Mefenamic acid potentiates GABA-evoked whole cell currents recorded in voltage-clamped hippocampal neurones

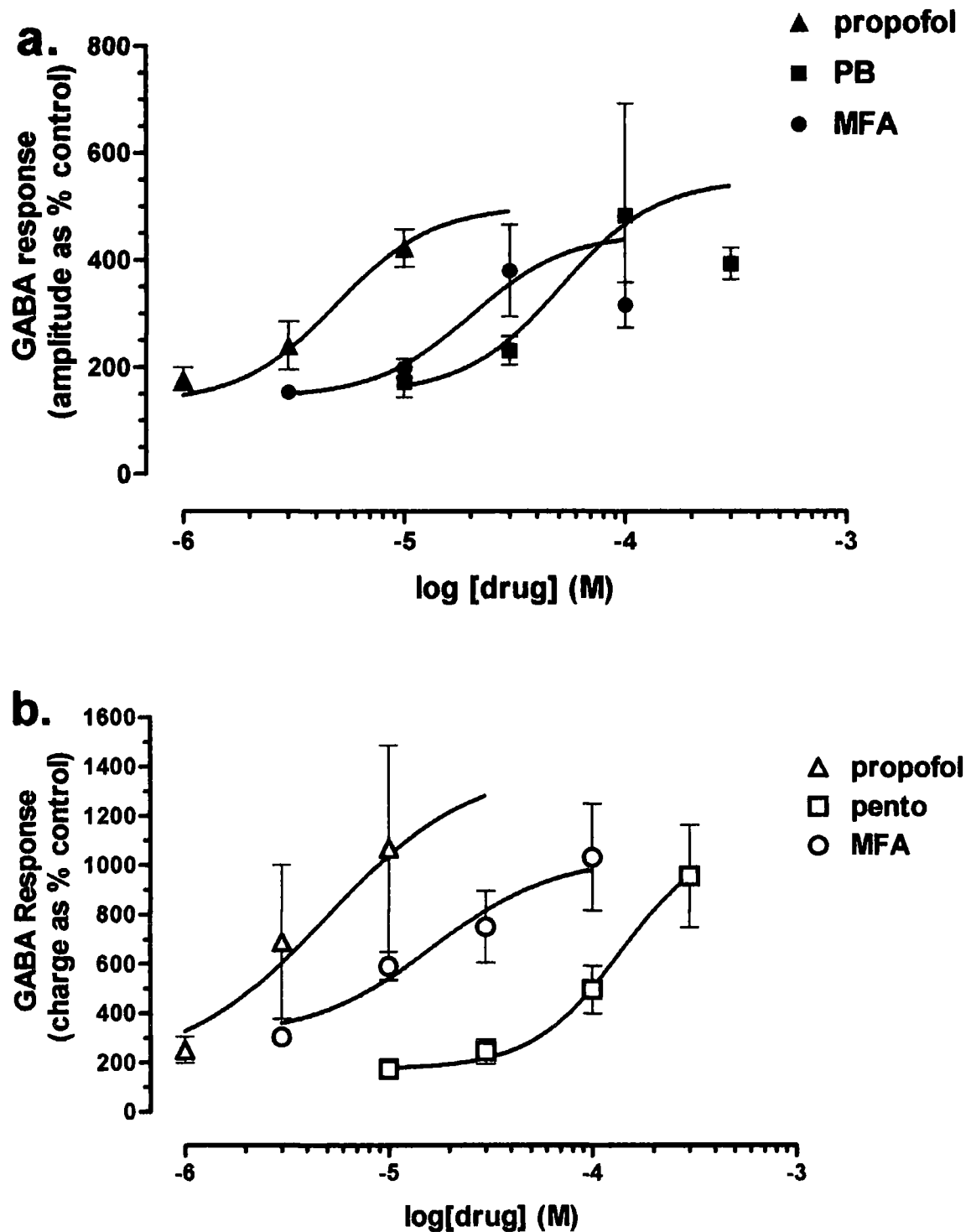
Mefenamic acid, sodium pentobarbitone and propofol (sodium pentobarbitone and propofol are shown for comparison) positively modulated GABA-evoked whole cell currents recorded from voltage-clamped hippocampal neurones. Mefenamic acid (3-100 $\mu$ M) concentration-dependently potentiated GABA-evoked response amplitude and duration. Similar observations were made for pentobarbitone (10-300 $\mu$ M) and propofol (1-10 $\mu$ M), although mefenamic acid was a more potent modulator than sodium pentobarbitone. The increase in response duration in the presence of mefenamic acid, sodium pentobarbitone and propofol was reflected in the percentage increase in the total charge passed during GABA-evoked responses. Mefenamic acid (3-100 $\mu$ M), sodium pentobarbitone (10-300 $\mu$ M) and propofol (1-10 $\mu$ M) also evoked a concentration-dependent transmembrane current. High concentrations of these drugs caused a rapid desensitisation of GABA-evoked currents, such that the response amplitude was often diminished in the continued presence of the drug (see figure 5.1).

Mefenamic acid (3-100 $\mu$ M) concentration-dependently and reversibly potentiated submaximal GABA-evoked whole-cell current amplitude with  $EC_{50}$  value of 9 $\mu$ M (5-15 $\mu$ M, n=7-25) and a mean maximal potentiation of  $337\pm 44\%$ , (n=19) with 30 $\mu$ M mefenamic acid. In contrast to experiments conducted in the vagus nerve, mefenamic acid was more potent than the general anaesthetic pentobarbitone ( $EC_{50}=64\mu$ M), but consistent with the vagus nerve data, was less potent than the general anaesthetic propofol ( $EC_{50}=6\mu$ M). However, also consistent with experiments conducted in the vagus nerve, pentobarbitone (300 $\mu$ M) evoked a higher mean maximal potentiation of GABA-activated currents ( $393\pm 30\%$ , n=3) than mefenamic acid. (see figure 5.2).

These data demonstrate that mefenamic acid, with greater potency than pentobarbitone, concentration-dependently potentiated submaximal GABA-evoked responses in hippocampal neurones. The characteristics of this potentiation were similar to those observed for sodium pentobarbitone and



**Figure 5.1: Concentration-dependent potentiation of GABA-evoked responses by mefenamic acid.** Chart recorder traces of submaximal GABA-evoked whole-cell currents concentration-dependently and reversibly potentiated by mefenamic acid (3-100 $\mu$ M), the effects of propofol (10 $\mu$ M) and sodium pentobarbitone (100 $\mu$ M) are shown for comparison. **a** and **b**: Low concentrations of mefenamic acid (3-10 $\mu$ M) evoked a marked and well maintained potentiation of submaximal GABA-evoked current amplitude and duration. **c** peak potentiations of GABA-evoked currents were achieved with 30 $\mu$ M mefenamic acid. **d**: higher concentrations of mefenamic acid (>100 $\mu$ M) evoked a biphasic effect on submaximal GABA-evoked currents with a transient potentiation, followed by a reduction of the GABA response amplitude with the duration of the response remaining prolonged; this is reversible upon continued wash. **a-d**: Mefenamic acid (3-100 $\mu$ M) evoked a concentration-dependent transmembrane current amplitude. **e** and **f**: propofol (10 $\mu$ M) and sodium pentobarbitone (100 $\mu$ M) also potentiated the GABA-evoked current amplitude and duration and evoked a transmembrane current. All cells were voltage-clamped at -60mV. Drug contact time is represented by the solid line above each trace. Recordings are taken from different cells.



**Figure 5.2: Concentration-dependent potentiation of GABA-evoked responses by mefenamic acid, sodium pentobarbitone and propofol.** Mean log concentration-effect curves for potentiation of GABA-evoked whole-cell current amplitude (a: filled symbols) and charge movement (b: open symbols) by mefenamic acid (3-100 $\mu$ M), sodium pentobarbitone (10-300 $\mu$ M) and propofol (1-10 $\mu$ M). The log<sub>10</sub> concentration of modulator is plotted on the abscissa and the response, as a percentage of the control GABA response, is plotted on the ordinate. Each data point represents the mean  $\pm$  s.e.m (indicated by the vertical lines) of 7-25, 3-14 and 6-10, experiments for mefenamic acid, sodium pentobarbitone and propofol, respectively.

propofol. Experiments were therefore conducted to further investigate the site and mechanism of action of mefenamic acid on the GABA<sub>A</sub> receptor.

### **3.ii. Determination of the effects of non-fenamate NSAIDs on GABA-gated chloride channels in voltage-clamped hippocampal neurones.**

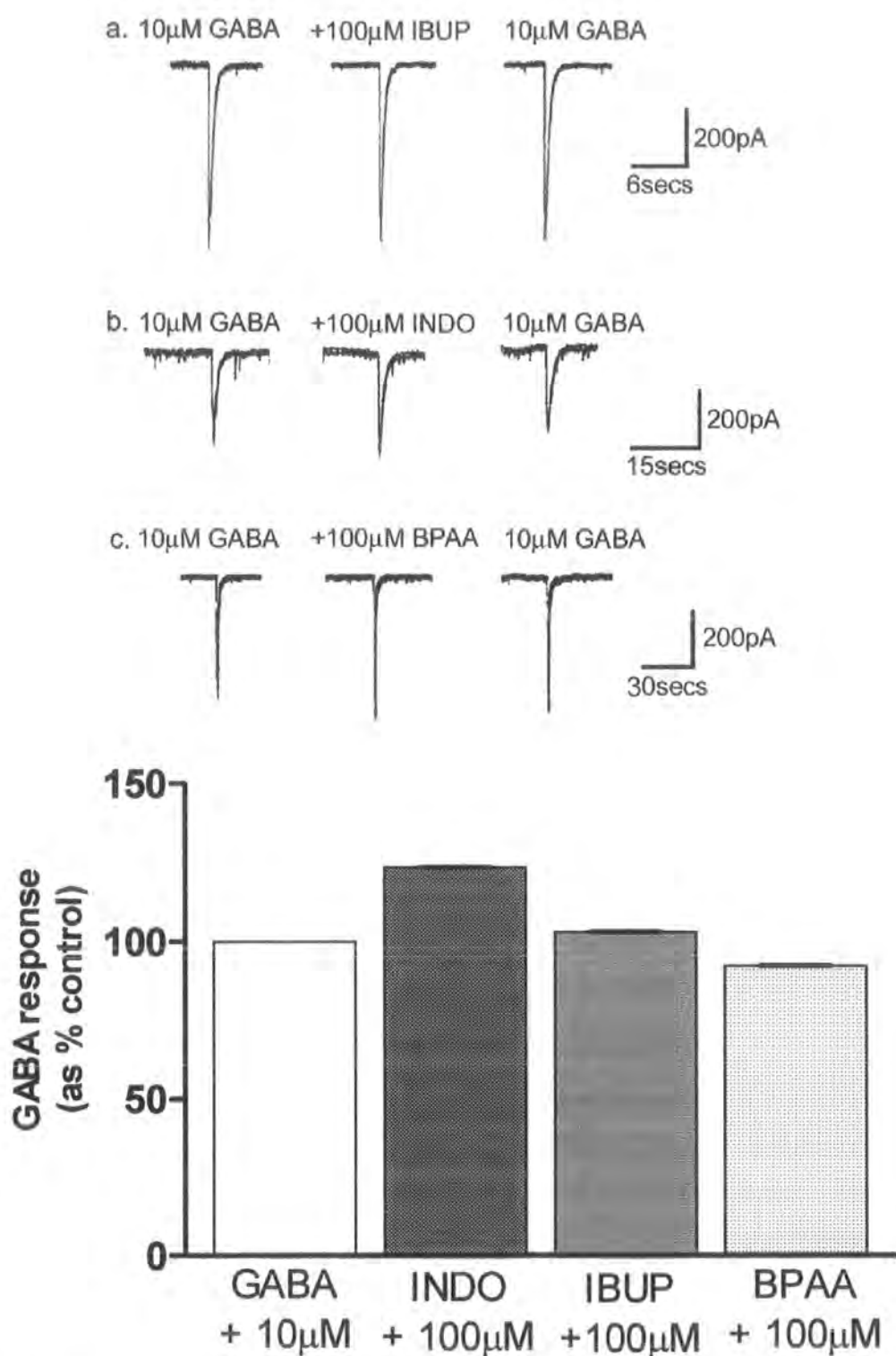
Since mefenamic acid modulated GABA-evoked responses in voltage-clamped hippocampal neurones, the effects of other, non-fenamate, NSAIDs on these responses were determined to investigate the hypothesis that this modulation was a common property of all NSAIDs, perhaps related to their ability to inhibit cyclo-oxygenase activity and prostaglandin synthesis in the cell.

Submaximal GABA-evoked responses in the presence of the non-fenamate NSAIDs, ibuprofen (100 $\mu$ M) and BPAA (100 $\mu$ M) were 103 $\pm$ 5% (n=3) and 92 $\pm$ 6% (n=5) of control, respectively. However, indomethacin (100 $\mu$ M), also a non-fenamate NSAID, did evoke a modest potentiation of GABA-mediated responses to 124 $\pm$ 15% (n=3) of control (see figure 5.3).

### **3.iii. Modulation of GABA-evoked whole cell currents recorded in voltage-clamped hippocampal neurones by fenamate NSAIDs**

Since mefenamic acid caused a significant modulation of GABA-evoked responses in voltage-clamped hippocampal neurones, the effects of other fenamate NSAIDs on these responses were determined to investigate the possibility that this modulation was a common property of fenamates.

The fenamate NSAIDs, mefenamic acid, (3-100 $\mu$ M), flufenamic (3-1000 $\mu$ M), meclofenamic (3-100 $\mu$ M) and tolfenamic (3-100 $\mu$ M), concentration-dependently potentiated submaximal GABA-evoked currents with EC<sub>50</sub> values of 9 $\mu$ M (6-15 $\mu$ M, n=7-25), 112 $\mu$ M (90 - 220  $\mu$ M, n=1-5), 8 $\mu$ M (2-21 $\mu$ M, n=1-3) and 14 $\mu$ M (5-38 $\mu$ M, n=2-3), respectively. In addition, mefenamic acid, flufenamic, meclofenamic and tolfenamic (all at  $\geq$ 3 $\mu$ M) were associated with a concurrent baseline shift (20-720pA) similar to that observed for mefenamic



**Figure 5.3: The effect of 3 non-fenamate NSAIDs on GABA-evoked whole-cell currents.** Top: shows chart recorder traces of submaximal (10 μM) GABA-evoked responses in the absence (left), presence (middle) and following wash (right) of **a**: ibuprofen (IBUP; 100 μM), **b**: indomethacin (INDO; 100 μM) and **c**: BPAA (100 μM). Bottom: the histogram shows control (10 μM) GABA-evoked responses in absence and then presence of indomethacin (100 μM), ibuprofen (100 μM) and BPAA (100 μM). Each data point is the mean ± s.e.m. (represented by the vertical bars) of 3, 3 and 5 experiments for ibuprofen, indomethacin and BPAA, respectively.



acid. Mean maximal potentiations induced by mefenamic acid (30 $\mu$ M), flufenamic (1000 $\mu$ M), meclofenamic (30 $\mu$ M) and tolfenamic (100 $\mu$ M) were 337  $\pm$ 44% (n=19), 829 $\pm$ 119% (n=2), 321  $\pm$ 83% (n=2), and 524 $\pm$ 153% (n=2), of control, respectively. In contrast, niflumic acid (10-100 $\mu$ M), concentration-dependently inhibited GABA-evoked currents with an IC<sub>50</sub> of 16 $\mu$ M and was not associated with a baseline holding current shift (see figures 5.4 and 5.5).

The above experiments determined the effects of a series of NSAIDs on GABA-evoked responses of voltage-clamped hippocampal neurones and demonstrated that modulation of GABA-evoked responses is not a general property of NSAIDs, but is particular to fenamate NSAIDs. Thus, the following series of experiments were conducted to determine the site and molecular mechanism of action of one fenamate NSAID in particular, mefenamic acid. The inhibitory effects of niflumic acid were also investigated and will be discussed later.

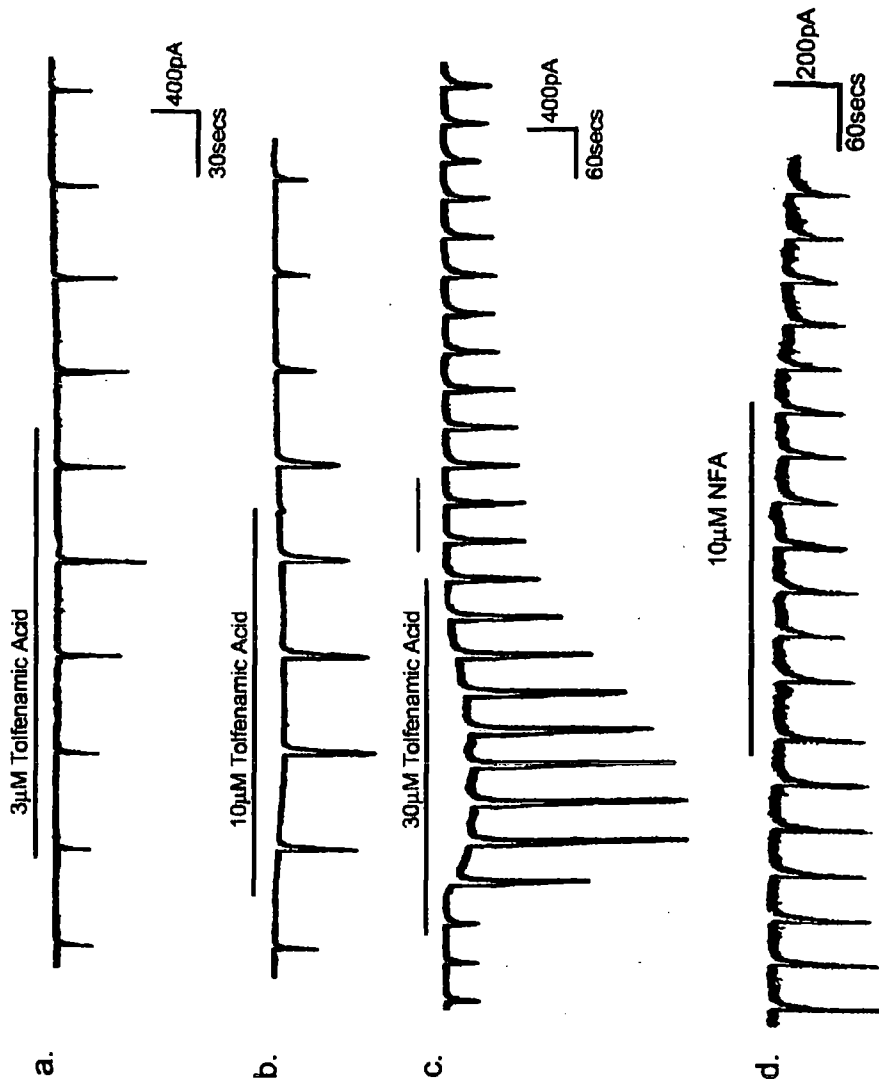
#### **3.iv. The potentiating effects of mefenamic acid are not mediated *via* the benzodiazepine site of the GABA<sub>A</sub> receptor.**

Potentiation of the GABA-evoked response by mefenamic acid may be mediated through the benzodiazepine site of the GABA<sub>A</sub> receptor. To address this hypothesis, the potentiating effects of mefenamic acid and diazepam were determined in the presence of the benzodiazepine antagonist, flumazenil.

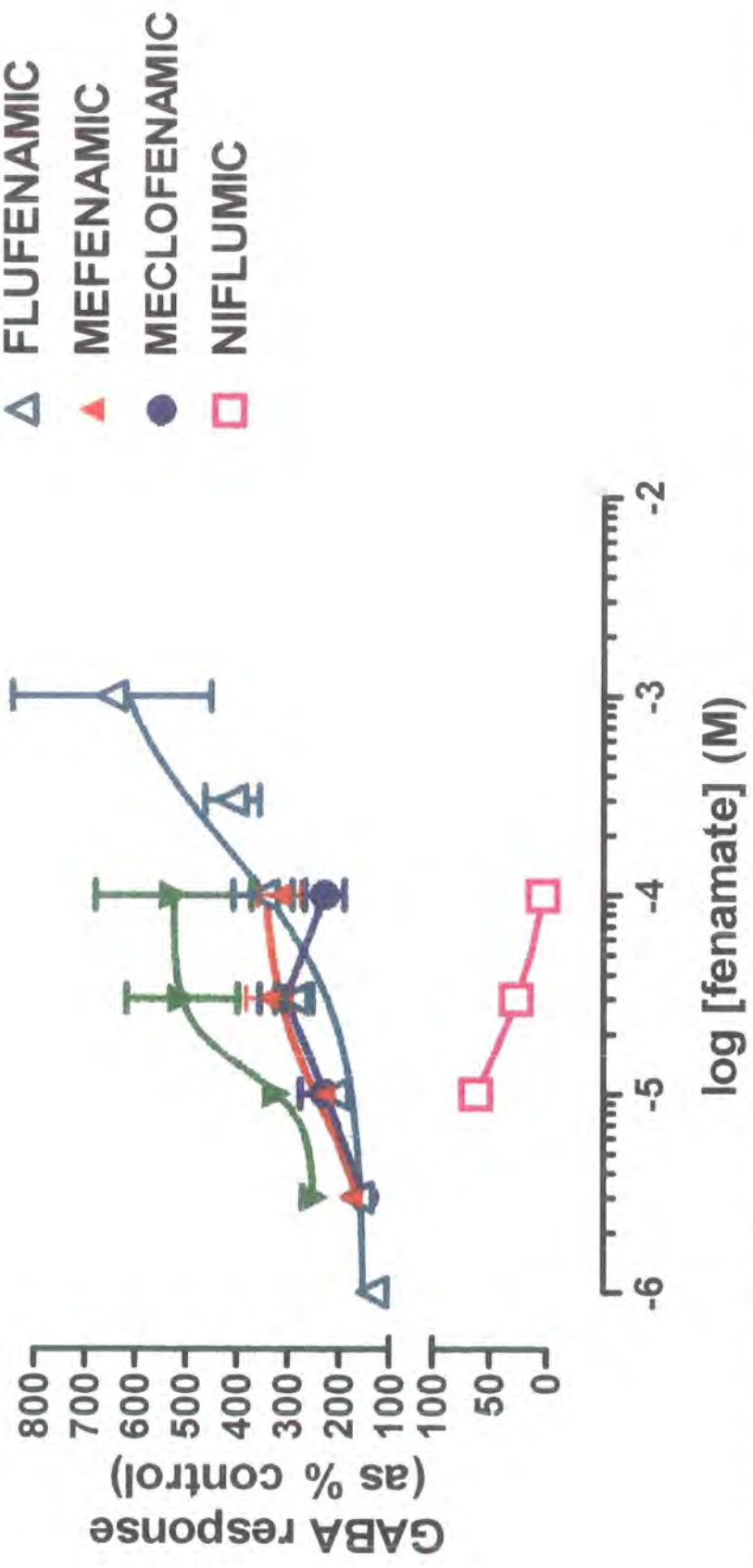
Flumazenil alone (1 $\mu$ M) was without effect on the GABA-evoked current (106% of control, n=1), but reversed the potentiation of GABA-evoked currents by diazepam (1 $\mu$ M) from 208.5 $\pm$ 8.5% (n=2) to 100% (n=2) of control. In contrast, the presence of flumazenil (1 $\mu$ M) did not inhibit the potentiating effects of mefenamic acid (10 $\mu$ M, n=2), but rather increased the potentiation evoked by mefenamic acid (10 $\mu$ M) from 173 $\pm$ 2% (n=2) to 236.5 $\pm$ 96.5% (n=2) of control (see figure 5.6). All drug effects were reversible upon wash.

These data may indicate that the potentiation of GABA-evoked responses by mefenamic acid is probably not mediated via the benzodiazepine site of the GABA<sub>A</sub> receptor. However, given the variable sensitivity of

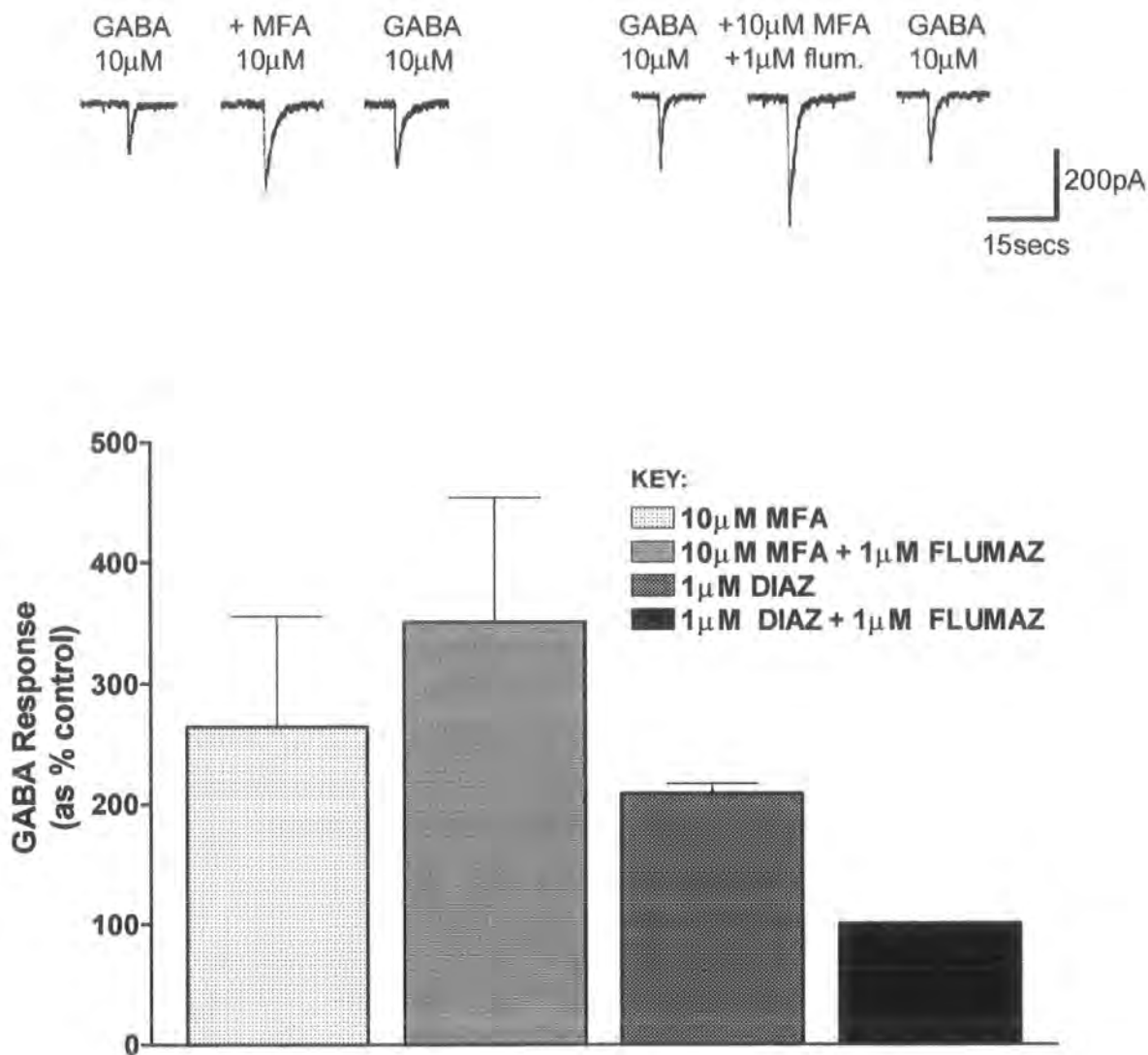




**Figure 5.4: Fenamate NSAIDs modulate GABA-evoked whole-cell currents in voltage-clamped hippocampal neurones. Chart recorder traces of submaximal GABA (10 μM)-evoked currents are shown in the absence, presence and following wash of, for example, tolifenamic acid (a: 3 μM; b: 10 μM and c: 30 μM) and niflumic acid (d: 10 μM). Drug contact time is represented by the solid bar above each trace. Tolifenamic acid concentration-dependently and reversibly potentiates submaximal GABA-evoked currents. Note the transmembrane current evoked by tolifenamic acid in traces b and c. Trace d: Niflumic acid does not evoke a transmembrane current and irreversibly inhibits the GABA-evoked current. Traces a and b are taken from one cell, traces c and d are from different cells.**



**Figure 5.5: Modulation of GABA-evoked responses by fenamate NSAIDs.** Graph shows mean log concentration-effect curves for submaximal GABA (10 $\mu$ M)-evoked whole cell responses in voltage-clamped hippocampal neurons by mefenamic acid (1-100 $\mu$ M), flufenamic acid (3-1000 $\mu$ M), meclofenamic acid (3-100 $\mu$ M), tolfenamic acid (3-100 $\mu$ M) and niflumic acid (10-100 $\mu$ M). The log<sub>10</sub> concentration of fenamate is shown on the abscissa and the GABA response, as a percentage of control, is shown on the ordinate. Each data point represents the mean $\pm$ s.e.m (represented by the vertical lines) of 7-25, 1-5, 1-3, 1-4 and 1-5 experiments for mefenamic, flufenamic, meclofenamic acid, tolfenamic acid and niflumic acid, respectively.



**Figure 5.6: Mefenamic acid-induced potentiation of GABA-evoked currents is not reversed by the benzodiazepine antagonist, flumazenil. Top:** Chart recorder traces of submaximal GABA-evoked whole-cell currents (voltage clamped at,  $V_h = -60\text{mV}$ ) potentiated by mefenamic acid ( $10\mu\text{M}$ ) are shown in the absence (left half) and presence of flumazenil ( $1\mu\text{M}$ ; right half); traces are from the same cell. **Bottom:** histogram shows control GABA ( $10\mu\text{M}$ )-evoked whole-cell currents potentiated by diazepam ( $1\mu\text{M}$ ) and mefenamic acid ( $10\mu\text{M}$ ) are shown in the absence (control) and presence of flumazenil ( $1\mu\text{M}$ ) as indicated in the key. Each data point represents the mean  $\pm$  s.e.m. (represented by the vertical lines) of 3 experiments for mefenamic acid, and the mean of 2 experiments for diazepam.

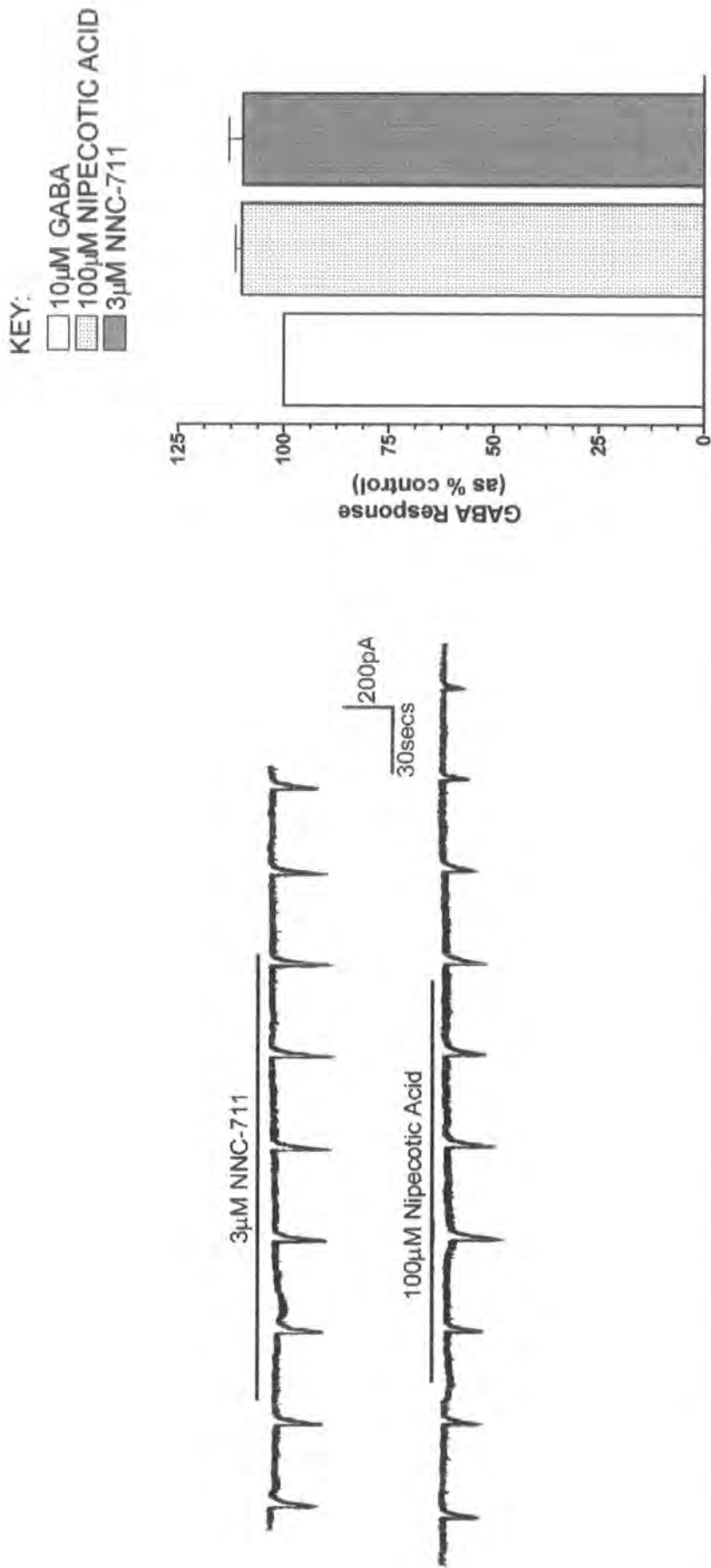
hippocampal neurones to the potentiating effects of mefenamic acid, these data do not disprove an involvement of the benzodiazepine site over the potentiating effects of mefenamic acid on the GABA<sub>A</sub> receptor.

### **3.v. The potentiating effects of mefenamic acid are not due to GABA uptake inhibition**

GABA-uptake inhibitors have been shown to have anti-convulsant properties (e.g. Suzdak *et al.*, 1992). They are thought to exert these effects by prolonging the duration of inhibitory post-synaptic potentials in neurones by elevating GABA levels at nerve terminals. In order to determine if the potentiating effects of mefenamic acid on GABA-evoked currents were due to inhibition of GABA uptake by mefenamic acid, the effects of two GABA uptake inhibitors, namely nipecotic acid and NNC-711, were examined on GABA-evoked currents. NNC-711, is a lipophilic derivative of nipecotic acid which is a selective and potent inhibitor of the GAT-1 transporter (Borden *et al.*, 1994). The classical GABA-uptake inhibitor, nipecotic acid, is a hydrophilic compound which is less potent and less selective than NNC-711 (nipecotic acid can interact with L-proline and pipecolic acid, Krosggaard-Larsen *et al.*, 1975) and in addition, can act as substrate for the GABA-transporter potentially complicating the interpretation of pharmacological data (Krosggaard-Larsen *et al.*, 1994).

Application of nipecotic acid (100 $\mu$ M) or NNC-7111 (3 $\mu$ M), at concentrations 3 fold greater than their IC<sub>50</sub> values in binding experiments (33 $\mu$ M and 1.2 $\mu$ M, respectively, Suzdak *et al.*, 1992), did not mimic the potentiating effects of mefenamic acid on GABA-evoked responses. In the presence of nipecotic acid and NNC-711, GABA currents were 111% (n=2) and 105% (n=2) of control, respectively. However, both compounds did produce a small background current (both between 30-40pA, n=2) and a concurrent increase in baseline noise levels (see figure 5.7). All drug effects were reversible upon wash.

These data suggest that the potentiating effects of mefenamic acid are not due to inhibition of GABA uptake.



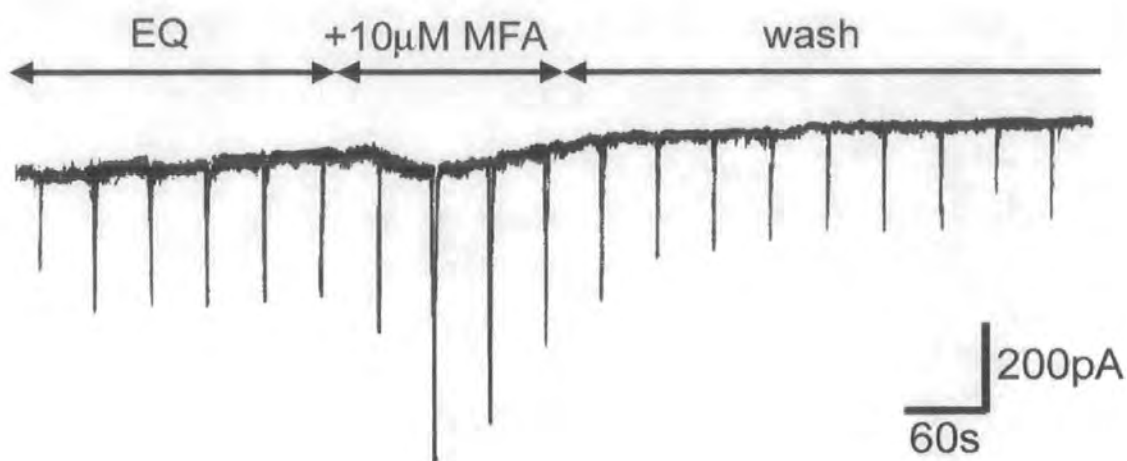
**Figure 5.7: Mefenamic acid-induced potentiation of GABA-evoked currents is not via GABA-uptake inhibition.** Left : Chart recorder traces of submaximal (10  $\mu$ M) GABA-evoked responses in the absence and presence of NNC-711 (3  $\mu$ M; top) and nipepotic acid (100  $\mu$ M; bottom) are shown. Drug contact time is represented by the solid line above each trace. Traces were recorded from one cell. **Right:** histogram shows control (10  $\mu$ M) GABA-evoked responses in the presence of nipepotic acid (100  $\mu$ M) and NNC-711 (3  $\mu$ M). Each data point is the mean  $\pm$  s.e.m. (represented by the vertical bars) of 3 and 4 experiments for nipepotic acid and NNC-711, respectively.

### **3.vi. The potentiating effects of mefenamic acid are not due to membrane perturbation**

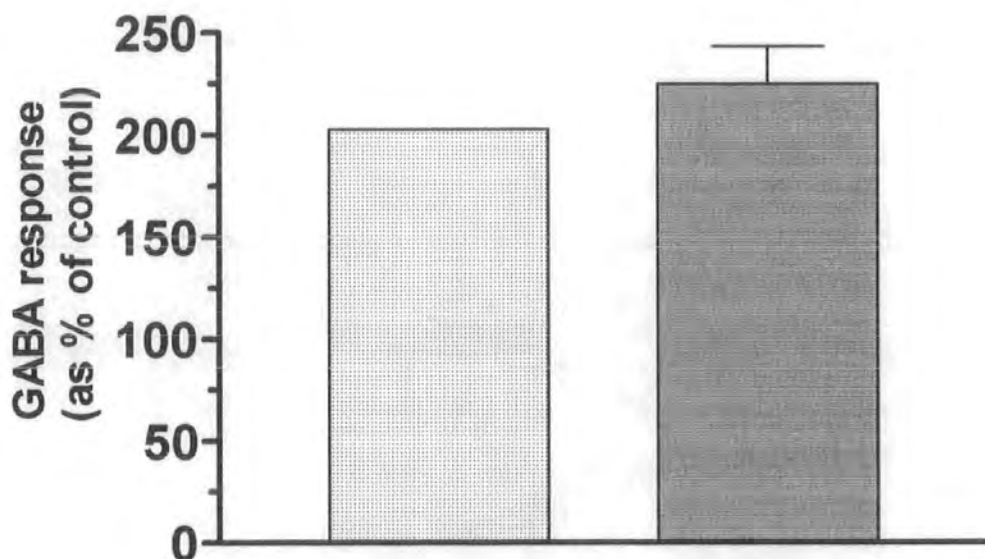
Potential of the GABA-evoked response by mefenamic acid may be due to general membrane perturbation (i.e. changes in membrane fluidity), a hypothesis investigated for the action of neuroactive steroids of the GABA<sub>A</sub> receptor (Lambert, 1990). To address this hypothesis, a high concentration of mefenamic acid (1mM) was applied, via the patch electrode, to the inside of the cell membrane. Thus, mefenamic acid at (1mM) was added to the internal solution (see Methods) and the pH of this solution was adjusted to 7.2 by addition of concentrated hydrochloric acid. The effects of intracellularly applied mefenamic acid were investigated on the GABA-activated current after the whole-cell configuration of the voltage-clamp had been obtained for a period of 71 seconds, when diffusional exchange between the recording pipette solution and the cell interior had occurred (see Methods).

The intracellular application of mefenamic acid (1mM) did not inhibit activation of a GABA-evoked response; this response was characteristic of a control GABA-evoked response in the absence of intracellularly applied mefenamic acid. For example, in cells containing mefenamic acid, 30ms pulses of GABA produced whole-cell currents of 400pA in amplitude (n=2), comparable with those GABA-evoked responses observed under normal recording conditions (see Results, section 2, page 88). Moreover, application of mefenamic acid (10 $\mu$ M) to the external membrane surface of these cells evoked potentiations of GABA currents to 203% (n=2) of control. (see figure 5.8). The enhancement of the GABA-evoked response was in keeping with those previously observed when mefenamic acid (10 $\mu$ M) was applied only externally (225 $\pm$ 18%, of control, n=25).

The finding that mefenamic acid applied intracellularly had little or no effect on the GABA-activated current, or its ability to enhance GABA responses when applied extracellularly, may be indicative of the existence of an extracellular recognition site existing for mefenamic acid. However, since mefenamic acid is lipid soluble, the final concentration of mefenamic acid achieved intracellularly may be considerably less than 1mM, if mefenamic acid



1mM MFA inside cell  
 no MFA inside cell



**Figure 5.8: The potentiating effects of mefenamic acid are not due to membrane perturbation.** Top: shows an actual chart recorder trace of GABA-evoked currents in the presence of mefenamic acid (1mM) applied intracellularly. Control GABA-evoked currents were obtained for a period of time, EQ (EQ=263 seconds), which was approximately 4 times in excess of the calculated period for diffusional exchange to occur (71 seconds, see Methods). Subsequent extracellular application of mefenamic acid (10µM; indicated by the middle arrow above the trace) potentiated the GABA-evoked response. Bottom: Histogram shows the relative potentiations of the GABA response evoked by mefenamic acid (10µM) in the presence (n=2) and absence (n=25) of intracellular mefenamic acid (1mM).

escapes across the cell membrane down its concentration gradient. These experiments cannot therefore convincingly discount a mefenamic acid-mediated membrane perturbation effect.

### **3.vii. The modulatory effects of niflumic acid and mefenamic acid are not use-dependent**

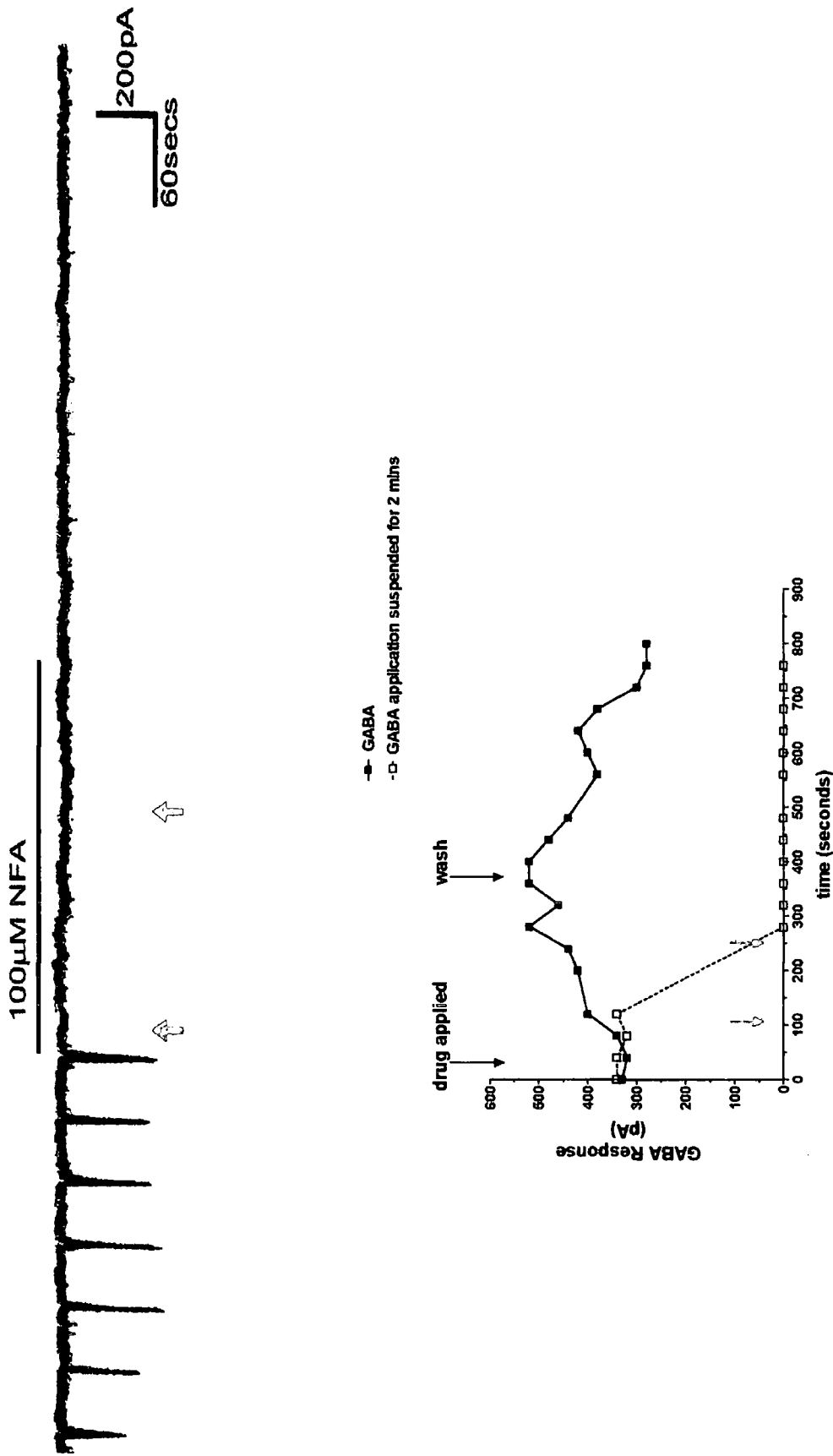
A number of drugs which modulate neuronal ligand-gated ion channels are use-dependent. Such drugs require an agonist-evoked activation of the receptor-gated channel to occur before these drugs can exert their effects. For example, at the GABA<sub>A</sub> receptor, GABA must bind to its site on the receptor to open the ion channel before picrotoxin can bind and produce its blocking effects (Akaike *et al.*, 1985). Similarly, for the most part, the NMDA-gated ionotropic glutamate receptor channel must be activated by NMDA before for MK-801 can produce its channel-blocking effects, although a small component of NMDA receptor blockade by MK-801 is by a non-use-dependent mechanism (Halliwell *et al.*, 1989). It is generally thought that such "use-dependent" drugs may have their binding sites located within the ion channel itself.

Thus, the following experiments were conducted in order to investigate the hypothesis that mefenamic acid and niflumic acid may be exerting their modulatory effects at the GABA<sub>A</sub> receptor-gated chloride channel via a use-dependent mechanism.

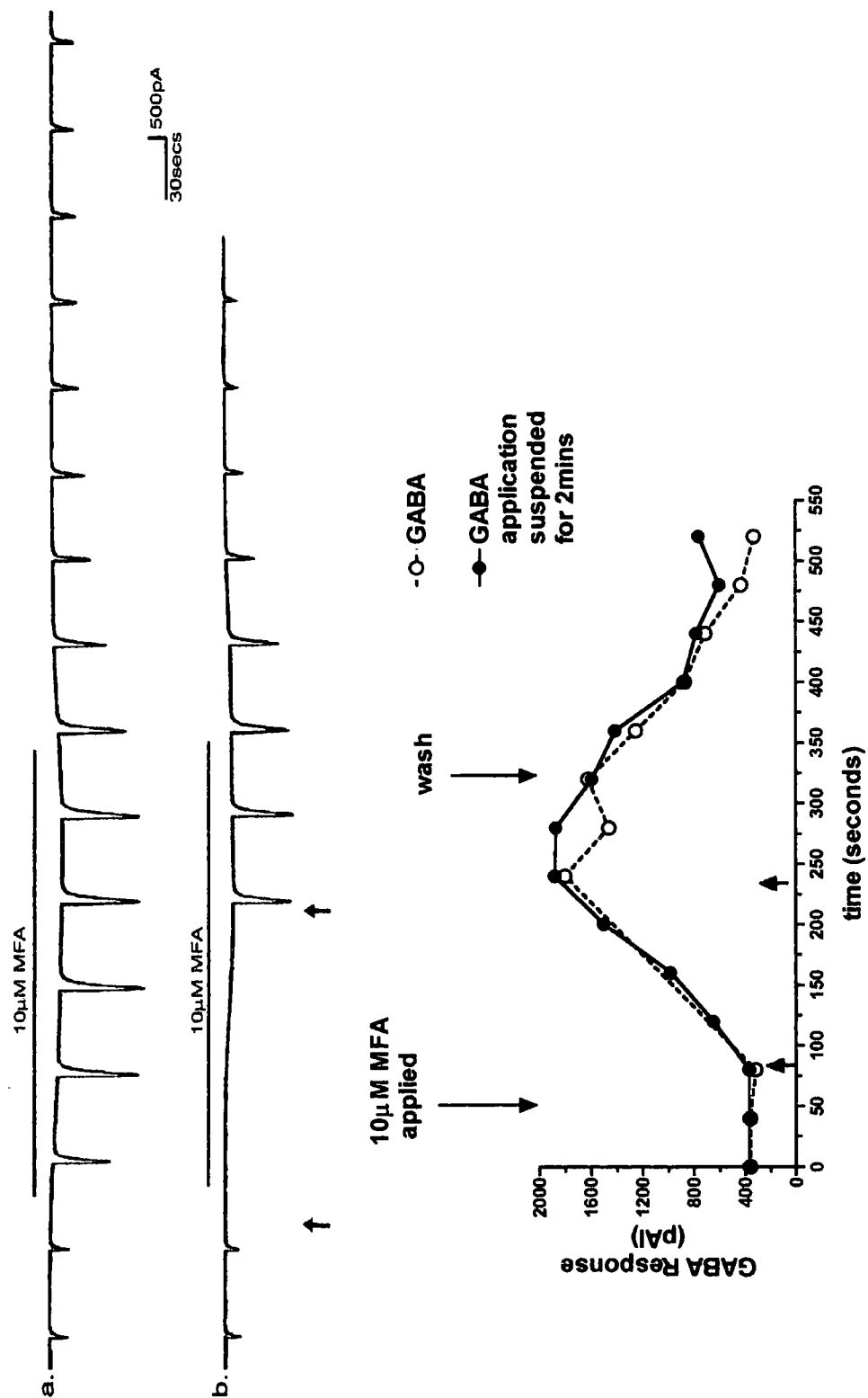
#### **3.vii. i. Procedures**

Repeated pressure application of GABA (at a rate of 0.03Hz and pulse duration of 10-100ms) was suspended during niflumic acid or mefenamic acid perfusion onto the cell, held under voltage-clamp ( $V_h = -60\text{mV}$ ) for a pre-determined period of time. This time was determined by the time taken for the same or another fenamate drug (control), to achieved its maximum effect in the same cell. GABA application was then recommenced after this interval, whilst in the continued presence of the drug.





**Figure 5.9: Inhibition of GABA-evoked currents by niflumic acid is not use-dependent.** Top: chart recorder trace showing submaximal GABA (10µM)-evoked currents in the presence of niflumic acid (100µM). The line above the trace indicates the drug contact time. The red arrows indicate the period during which GABA application was suspended whilst niflumic acid was perfused into the bath, following a pre-determined equilibrium period (the time required for another fenamate, flufenamic acid to exert its maximum effect). Bottom: graph shows GABA (10µM) response (in pA) plotted on the ordinate against time (secs) on the abscissa. The red arrows indicate the time during which GABA application was suspended. The black arrows indicate the times at which a control drug, flufenamic acid (■) and niflumic acid (□) were applied and removed (wash).



**Figure 5.10: Potentiation of GABA-evoked currents by mefenamic acid is not use-dependent.** Top: chart recorder traces, a and b, showing submaximal GABA ( $10\mu\text{M}$ )-evoked currents in the presence of mefenamic acid ( $10\mu\text{M}$ ). The line above each trace indicates the drug contact time. Trace b: the red arrows indicate the period during which GABA application was suspended whilst mefenamic acid was perfused into the bath, following a pre-determined equilibrium period (the time required for a previous application of mefenamic acid, trace a, to exert its maximum effect). Bottom: graph shows GABA ( $10\mu\text{M}$ ) response (in pA) plotted on the ordinate against time (secs) on the abscissa. The red arrows indicate the time during which GABA application was suspended. The black arrows indicate the times at which mefenamic acid ( $10\mu\text{M}$ ) were applied and removed (wash), during continuous GABA application (●) and during interrupted GABA application (○). Each data point represents the mean of 2 experiments.

### **3.vii. ii. Results**

Niflumic acid (10-100 $\mu$ M) concentration-dependently inhibited the GABA-evoked current. At higher concentrations (100 $\mu$ M) niflumic acid caused immediate inhibition of the GABA-evoked response, when GABA application was resumed (see figure 5.9).

Mefenamic acid concentration-dependently (3-10 $\mu$ M) potentiated the GABA-evoked responses. The potentiation of the GABA-evoked current by mefenamic acid occurred at the same time point as a previous application of mefenamic acid had produced a maximal potentiation. The potentiation of GABA-evoked responses from voltage-clamped rat hippocampal neurones was, therefore, not use-dependent (see figure 5.10).

### **3.viii. The potentiating effects of mefenamic acid are voltage-dependent**

Mefenamic acid may exert its modulatory effects upon the GABA<sub>A</sub> receptor by a number of mechanisms, one of which could be to open other ion channels which may add to, or take away from, the GABA-evoked current. However, in these experiments, currents were measured only from the baseline and were therefore, GABA-evoked currents. Another mechanism for the potentiating effects of mefenamic acid, if they are mediated at a binding site within the chloride channel, is that mefenamic acid could change the membrane electric field and, thereby, change the GABA<sub>A</sub> receptor chloride channel permeability. If mefenamic acid does act at a site within the channel, it may exhibit some voltage-sensitivity or may change the Cl<sup>-</sup> reversal potential.

### **3.viii. i. Procedures**

Experiments were conducted to determine if the potentiating effects of mefenamic acid and propofol (for comparative purposes) on GABA-evoked currents altered the reversal potential of the GABA-evoked current and/or were voltage-sensitive. GABA-evoked whole-cell currents were recorded over a

range of holding potentials (-100 to +40mV) in the absence and presence of mefenamic acid (3 $\mu$ M) and propofol (1 $\mu$ M).

### **3.viii. ii. Results**

Mefenamic acid potentiated the GABA-evoked currents throughout the range of holding potentials (-100 to +40mV) investigated. The  $E_{Cl}$  was not significantly different in the presence of mefenamic acid when compared to control. However, the potentiating effects of mefenamic acid did exhibit voltage-sensitivity, with those GABA responses recorded at more negative holding potentials being potentiated to a larger degree than those recorded at more positive holding potentials (see figure 5.11 and 5.12).

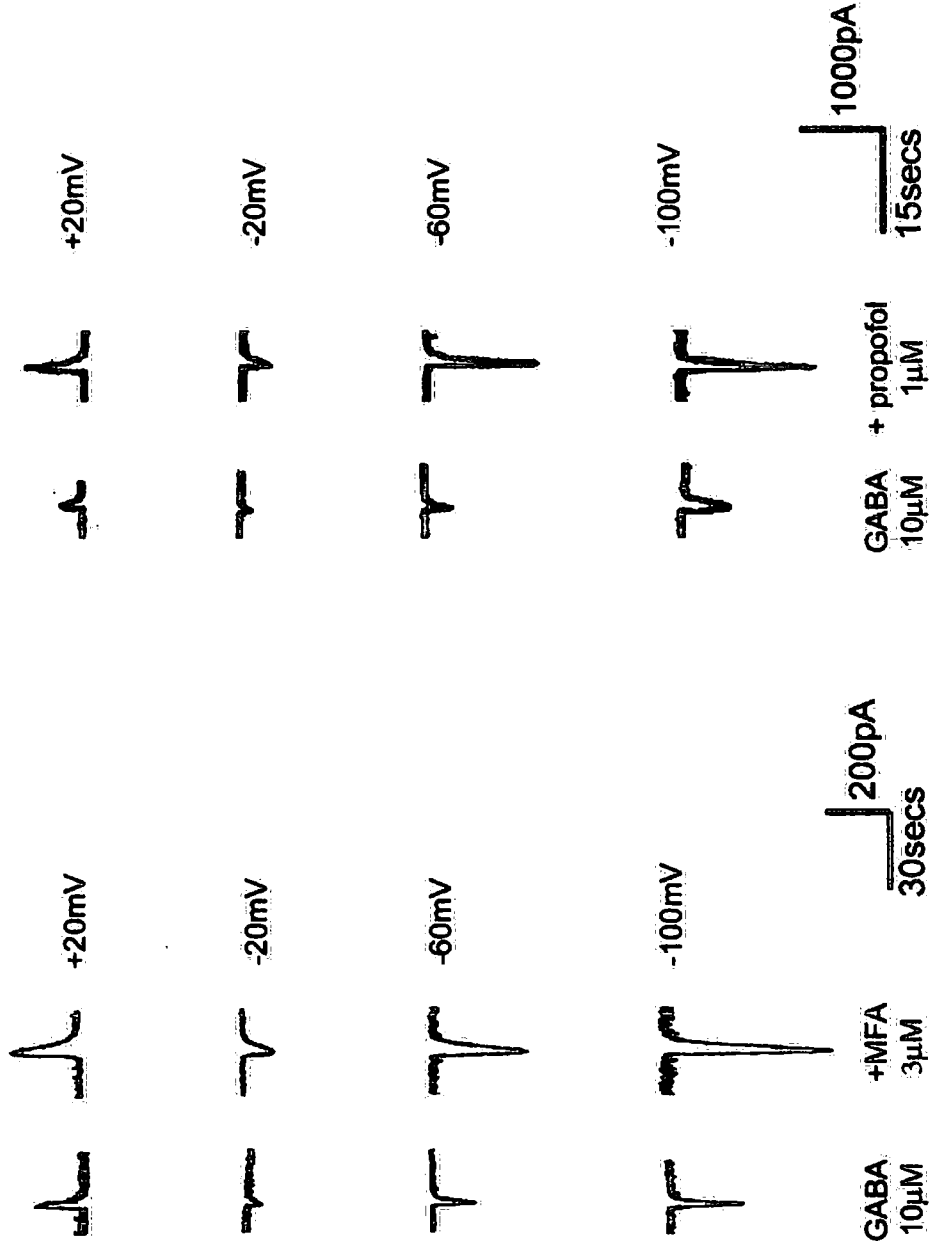
### **3.ix. Mefenamic acid directly activates the GABA<sub>A</sub> receptor**

In studies investigating the potentiating effects of mefenamic acid on GABA-evoked currents, perfusion of mefenamic acid (3-300 $\mu$ M) also resulted in the development of an inward current associated with application of the drug and also proportional to its concentration.

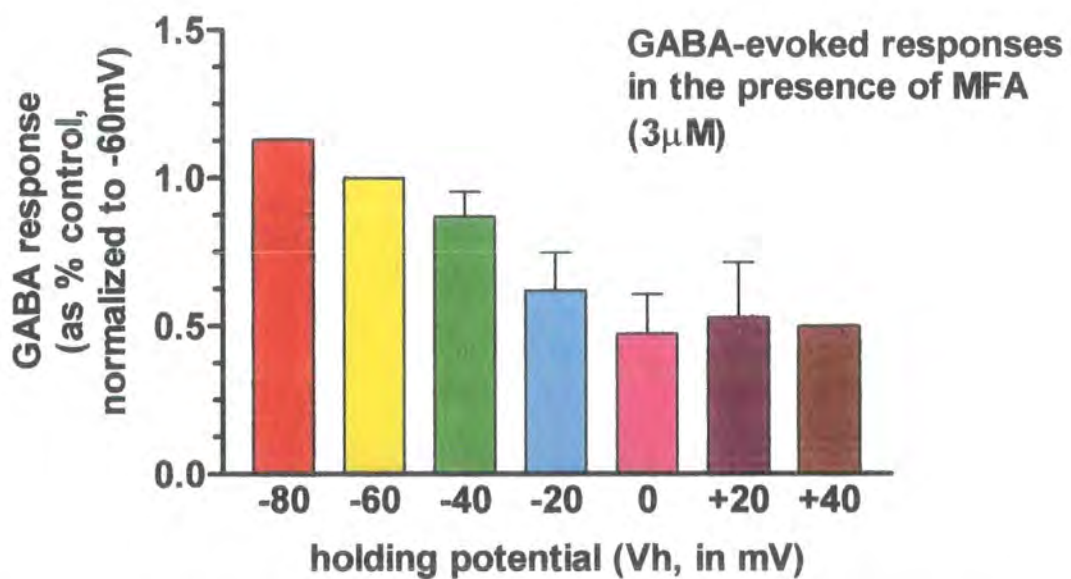
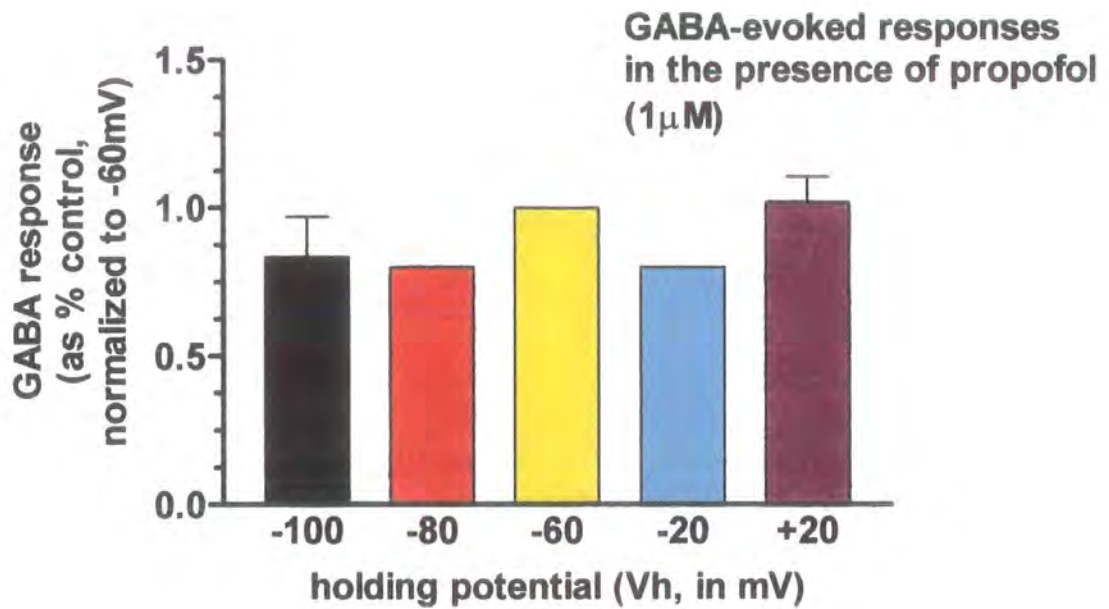
Previous studies have shown that the positive allosteric modulators of the GABA<sub>A</sub> receptor, including pentobarbitone, propofol, alphaxalone and loreclezole are also able to directly activate the GABA<sub>A</sub> receptor in the absence of GABA. The hypothesis that mefenamic acid also directly activated the GABA<sub>A</sub> receptor in voltage-clamped rat hippocampal neurones was therefore addressed.

#### **3.ix. i. Procedures**

Mefenamic acid (100 $\mu$ M), in the absence of GABA, was spritzer-applied to the cell at a duration of 20ms, once per minute. Higher concentrations of mefenamic acid (1mM), applied at this rate and duration, evoked currents which diminished in amplitude over time. The concentration of mefenamic acid and



**Figure 5.11: Potentiation of the GABA response by meprenamic acid shows voltage-sensitivity. Chart recorder traces of submaximal GABA (10 μM)-evoked responses in the absence and presence of (left) meprenamic acid (3 μM) and (right) propofol (1 μM), recorded over a range of holding potentials (given to the right of the individual currents).**



**Figure 5.12: Potentiation of the GABA response by mefenamic acid, but not propofol, shows voltage-sensitivity.** Histograms show the potentiation of submaximal GABA (10 $\mu$ M)-evoked responses by propofol (1 $\mu$ M; top) and mefenamic acid (3 $\mu$ M; bottom). Responses in the presence of drugs are expressed as the percentage of the control GABA-evoked response, normalized to a holding potential of -60mV. Each data point represents the mean $\pm$ s.e.m (represented by the vertical lines) of 2-4 and 2-3 experiments for propofol and mefenamic acid, respectively.

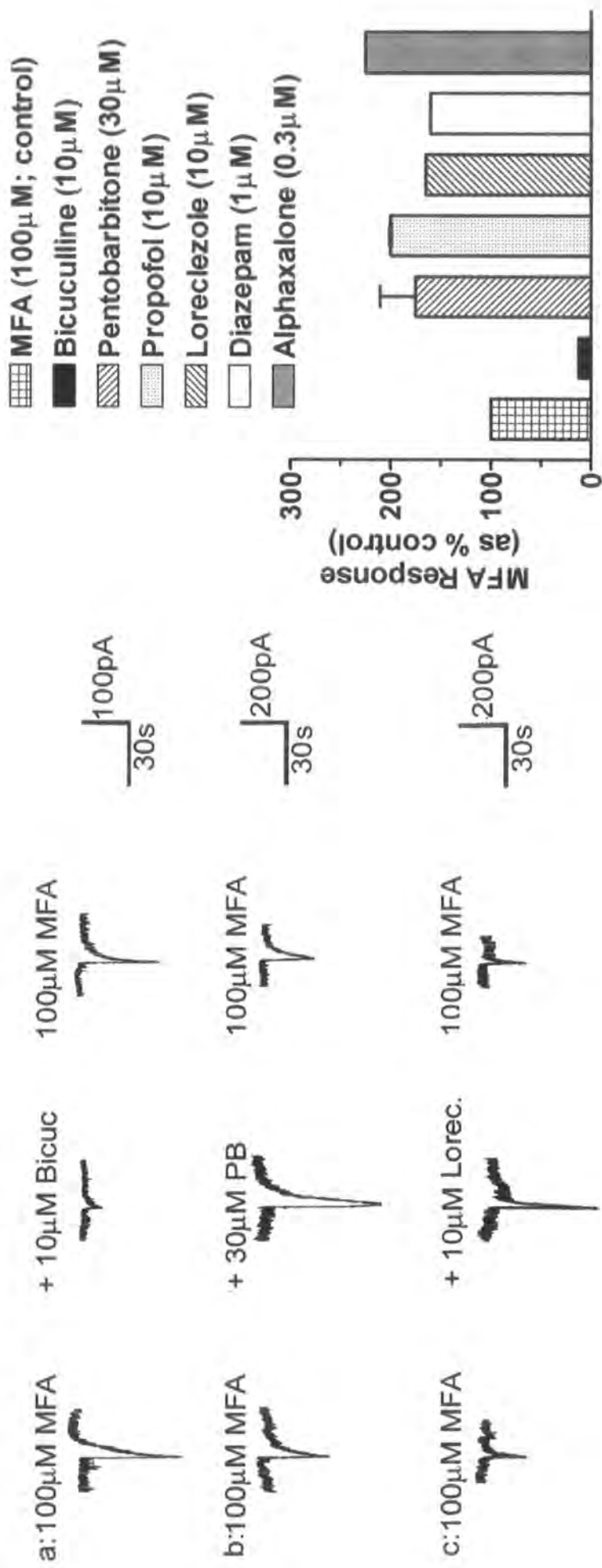
rate of application were, therefore, predetermined to minimize this current "rundown". The current-voltage relationship for mefenamic acid-activated whole-cell currents in hippocampal neurones, voltage-clamped between -100mV and +40mV was determined (using a CsCl-based internal and a normal external bathing solution as described in Methods).

### **3.ix. ii. Results - Mefenamic acid alone activates an inward current**

Repeated pressure application of mefenamic acid (1mM), in the absence of exogenously applied GABA, evoked inward currents which "ran down" over time. Repeated pressure application of mefenamic acid (100 $\mu$ M), in the absence of exogenously applied GABA, evoked inward currents which were stable over time. These currents were inhibited by bicuculline (3 $\mu$ M, n=1) to 12% of control and potentiated by sodium pentobarbitone at 30 $\mu$ M to 210 $\pm$ 8% (n=3). Mefenamic acid-induced currents were also potentiated by propofol (10 $\mu$ M) to 200% of control (n=2), by loreclezole (10 $\mu$ M) to 196% of control (n=2), by diazepam (1 $\mu$ M) to 160% of control (n=1), and by alphaxalone (0.3 $\mu$ M) to 225% of control (n=1) (figure 5.13). All drug effects on mefenamic acid-induced currents were reversible upon wash.

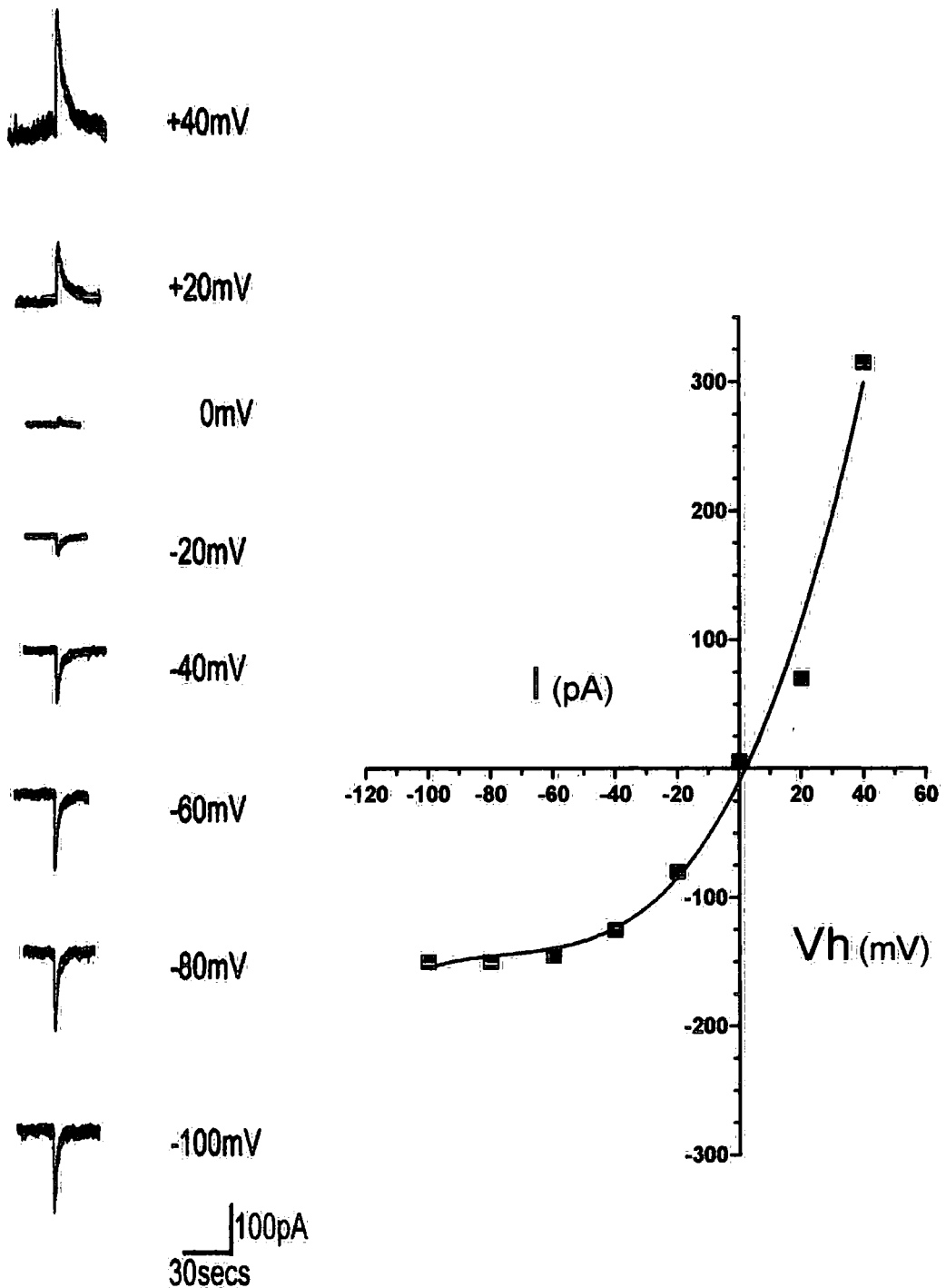
### **3.ix. iii. Current-voltage relationship of mefenamic acid-activated currents**

Mefenamic acid (100 $\mu$ M) activated a whole-cell current in hippocampal neurones voltage-clamped between -100 to +40mV. Mefenamic acid-induced currents had a reversal potential of 1.9mV (interpolated from the intercept of the curve on the x-axis). This value which was similar to the reversal potential calculated for GABA (2.3mV) with equal chloride across the membrane. The I-V plot for mefenamic acid exhibited outward rectification, also similar to that observed for GABA (see figure 5.14).



**Figure 5.13: Mefenamic acid evokes an inward current in the absence of GABA which is modulated by drugs which modulate the GABA<sub>A</sub> receptor. Left:** chart recorder traces showing mefenamic acid (100µM)-evoked currents in the absence (left) and presence (middle) and following wash (right) of bicuculline (trace b: 10µM), sodium pentobarbitone (trace a: 10µM), propofol (10µM), diazepam (1µM) and alphaxalone (0.3µM). **Right:** histogram shows control mefenamic acid (100µM)-evoked currents in the presence of bicuculline (10µM), sodium pentobarbitone (30µM), propofol (10µM), lorclezole (10µM) and alphaxalone (0.3µM). Each bar represents 1 data point for bicuculline, diazepam and alphaxalone, 2 data points for propofol and lorclezole and 3 data points for sodium pentobarbitone (mean±s.e.m, represented by the vertical line).





**Figure 5.14: The mefenamic acid-evoked whole-cell current for voltage-clamped hippocampal neurones. Left: chart recorder traces of mefenamic acid ( $100\ \mu\text{M}$ )-evoked currents recorded over a range of holding potentials (given to the right of the current). Right: graph shows the mefenamic acid-evoked current ( $I$  in  $\text{pA}$ , on the ordinate), plotted against the holding potential ( $V_h$ , in  $\text{mV}$  on the abscissa). The mefenamic acid-evoked current reversed at  $+1.9\ \text{mV}$  and showed outward rectification. Data points represent the mean of 2 experiments.**

### **3.x. Sensitivity to the modulatory effects of mefenamic acid varies across hippocampal neurones.**

Neuronal sensitivity to mefenamic acid (30 $\mu$ M) varied with peak potentiations ranging between 125% and 909%; these data may indicate a subunit selective property of mefenamic acid, which would be concomitant with the observations of Halliwell *et al.*, (in press). Loreclezole has been also been reported to be subunit selective, (Wafford *et al.*, 1994).

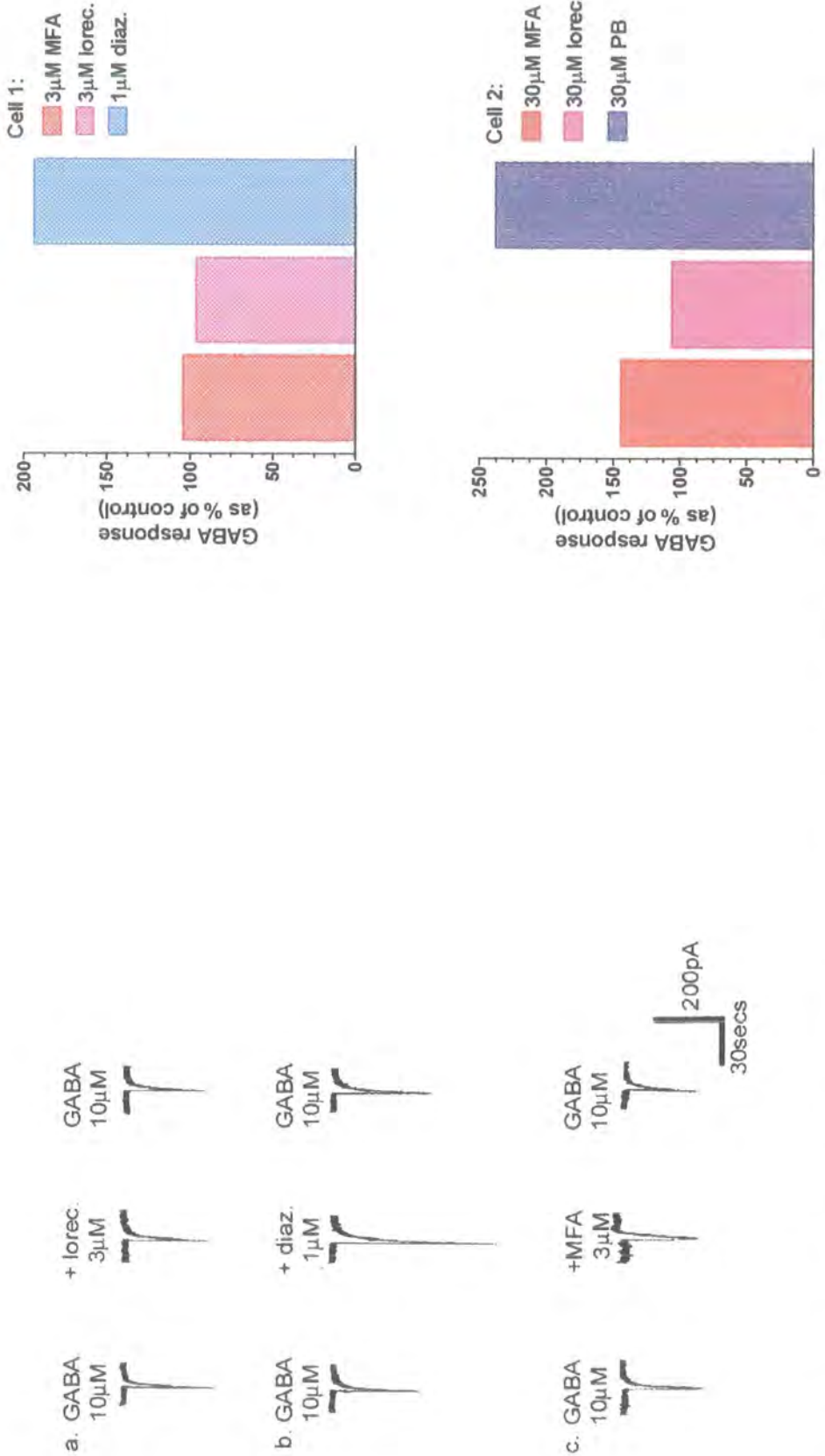
In addition, a small number of all cells tested were relatively insensitive to potentiation by mefenamic acid and loreclezole (n=2) but were potentiated, for example, by diazepam (1 $\mu$ M) to 194 % of control (n=1) or sodium pentobarbitone (30 $\mu$ M) to 238% of control (n=1). Mefenamic acid and loreclezole, therefore, have similar characteristic modulatory effects on GABA-evoked responses in hippocampal neurones (see figure 5.15).

### **3.xi. Mefenamic acid acts at or shares a recognition site with loreclezole**

The similarities between the modulatory effects of mefenamic acid and loreclezole in hippocampal neurones may suggest that these drugs are acting at a single or shared recognition site on the GABA<sub>A</sub> receptor. To address this hypothesis, the following series of experiments were carried out.

Concentrations of mefenamic acid and loreclezole which evoked maximal potentiations of (submaximal) GABA-evoked responses were previously determined in each cell. The peak potentiations evoked by mefenamic acid (10 $\mu$ M), followed by loreclezole (10 $\mu$ M) were thus determined individually and when co-applied. Peak potentiations evoked by mefenamic acid (10 $\mu$ M) and loreclezole (10 $\mu$ M) were determined individually and found to be 205% (n=2) and 277% (n=2) of control, respectively. When co applied, the peak potentiation evoked by both mefenamic acid and loreclezole, was 237% of control (n=2).

In addition, the peak potentiations evoked by mefenamic acid (10 $\mu$ M), followed by propofol (10 $\mu$ M) were determined individually and when co-applied. Peak potentiations evoked by mefenamic acid (10 $\mu$ M) and propofol (10 $\mu$ M)



**Figure 5.15: Some cells are relatively insensitive to modulation by mefenamic acid and loreclezole.** Left: shows actual chart recorder traces of submaximal GABA (10µM)-evoked currents taken from one voltage-clamped hippocampal neuron ( cell 1 ); in the absence (left-hand responses), presence (middle responses) and following wash (right-hand responses) of loreclezole (3µM; trace a); diazepam (1µM; trace b) and mefenamic acid (3µM; trace c). Note potentiation of the GABA-evoked response by diazepam, but not mefenamic acid or loreclezole. **Top Right:** histogram shows the modulation of submaximal GABA (10µM)-evoked currents in cell 1 by diazepam (1µM), but little modulation by mefenamic acid (3µM) or loreclezole (3µM). **Bottom Right:** histogram shows the modulation of submaximal GABA (10µM)-evoked currents by sodium pentobarbitone (30µM), but little modulation by mefenamic acid (30µM) or loreclezole (30µM) in cell 2. Data are taken from two different cells as shown in the key.

were determined individually and found to be 312% (n=1) and 420% (n=1) of control, respectively. When co applied, the peak potentiation evoked by both mefenamic acid and propofol, was 375% of control (n=1).

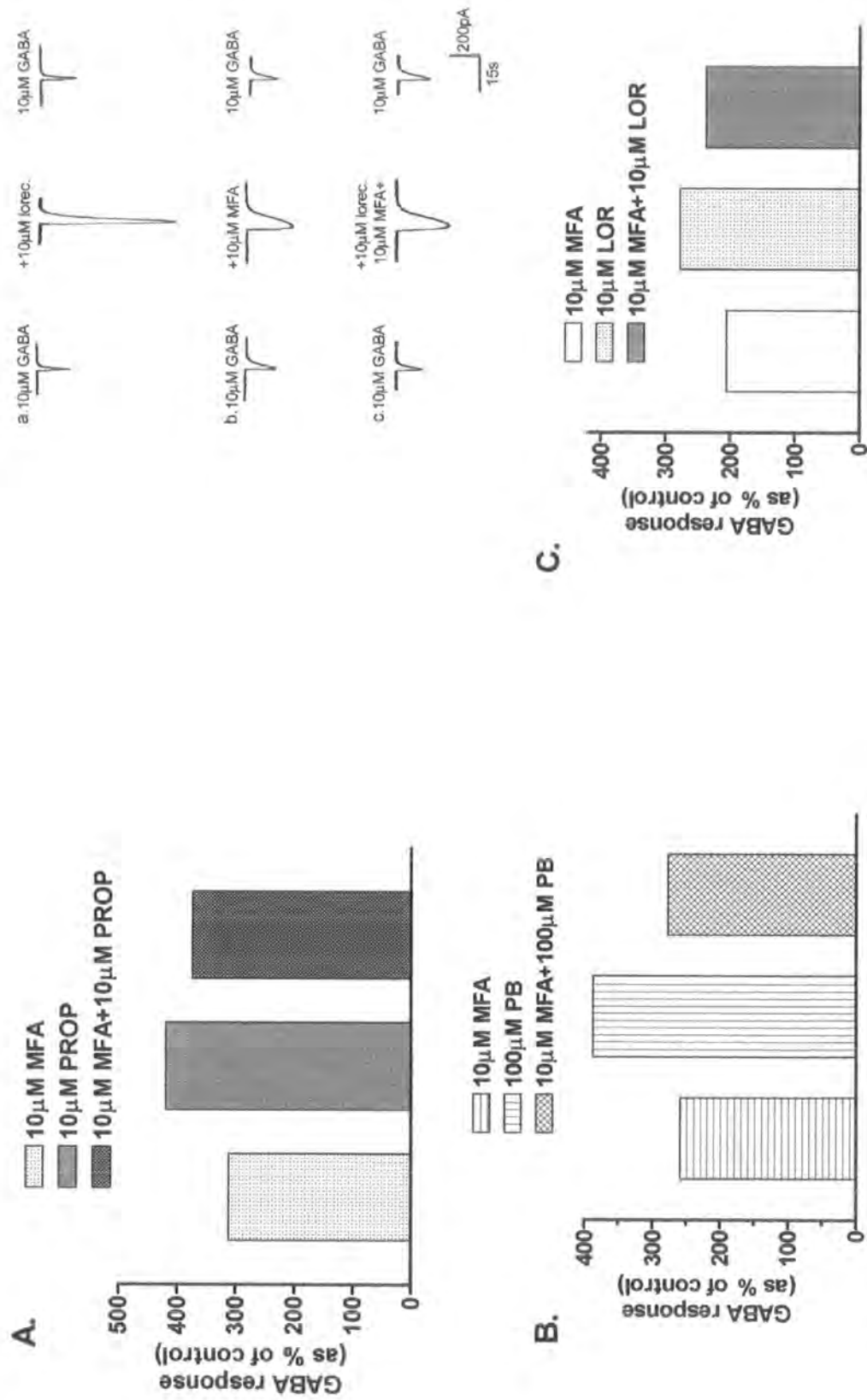
Similarly, the peak potentiations evoked by mefenamic acid (10 $\mu$ M), followed by sodium pentobarbitone (100 $\mu$ M) were determined individually and when co-applied. Peak potentiations evoked by mefenamic acid (10 $\mu$ M) and sodium pentobarbitone (100 $\mu$ M) were determined individually and found to be 259% (n=2) and 387% (n=2) of control, respectively. When co applied, the peak potentiation evoked by both mefenamic acid and sodium pentobarbitone, was 277% of control (n=2). (see figure 5.16)

The potentiating effects of mefenamic acid together with loreclezole, at concentrations which evoked maximal potentiations, were not additive when applied together. Under similar recording conditions, the potentiations evoked by mefenamic acid together with propofol or pentobarbitone, at concentrations which evoked maximal potentiations, were also not additive when co-applied. Although all drug effects were determined against submaximal GABA-evoked responses in hippocampal neurones, these data suggest that the non-additive potentiations of loreclezole, propofol and pentobarbitone together with mefenamic acid, are most likely reaching the ceiling of the available potentiation of the GABA-evoked responses in this recording system.

#### **4. Summary - modulation of GABA-evoked responses in hippocampal neurones by mefenamic acid.**

Mefenamic acid evoked a complex change in the characteristics of the GABA-evoked response recorded from rat hippocampal neurones. Submaximal GABA-evoked responses were concentration-dependently and reversibly potentiated in both amplitude and duration by mefenamic acid. The maximal potentiations evoked by mefenamic acid (30 $\mu$ M) and loreclezole (10 $\mu$ M) ranged from 125% to 909% and 149 to 349 % of control, respectively.

In addition to its potentiating effects, at concentrations  $\geq 10\mu$ M, mefenamic acid evoked a concentration-dependent inward transmembrane



**Figure 5.16: The potentiating effects of mefenamic acid together with loreclezole or propofol or pentobarbitone, at concentrations which evoked maximal potentiations, were not additive when applied together.** Histograms show the potentiation of submaximal GABA (10  $\mu$ M)-evoked responses by mefenamic acid (10  $\mu$ M), propofol (10  $\mu$ M), sodium pentobarbitone (100  $\mu$ M) and loreclezole (10  $\mu$ M) alone, and the additive potentiations of mefenamic acid (10  $\mu$ M) when co-applied with propofol (A, 10  $\mu$ M, n=1), sodium pentobarbitone (B, 100  $\mu$ M, n=2) and loreclezole (C, 10  $\mu$ M, n=2). An explanatory key is shown above each histogram. Actual chart recorder of GABA-evoked responses potentiated by loreclezole (trace a: 10  $\mu$ M) and mefenamic acid (trace b: 10  $\mu$ M) applied alone and in combination (trace c), are shown above histogram C.

current. The mefenamic acid-activated current, evoked in the absence of GABA, showed a similar current-voltage relationship and reversed at a value similar to that of GABA.

The effects of mefenamic acid were not mimicked by other non-fenamate NSAIDs, such as BPAA, indomethacin or ibuprofen, although indomethacin (100 $\mu$ M) did evoke a small potentiation of the GABA-evoked response.

GABA-evoked responses recorded from hippocampal neurones were modulated not only by mefenamic acid, but also by other fenamates. In descending order of potency, mefenamic > meclofenamic > flufenamic and tolfenamic acid, concentration-dependently and reversibly potentiated submaximal GABA-evoked currents. The potentiation of GABA-evoked responses recorded from rat hippocampal neurones by mefenamic acid was not delayed by the interruption of GABA application, suggesting that the potentiating effects of mefenamic acid are not use-dependent.

In contrast, niflumic acid concentration-dependently inhibited submaximal GABA-evoked responses. This inhibition could not be surmounted by increasing agonist exposure. Inhibition of the GABA-evoked responses by niflumic acid (100 $\mu$ M) was not delayed by the interruption of GABA application, suggesting that the inhibitory effects of niflumic acid are not use-dependent.

The potentiating effects of mefenamic acid were insensitive to the benzodiazepine antagonist, flumazenil.

The potentiating effects of mefenamic acid, loreclezole, propofol and pentobarbitone, at concentrations which evoked maximal potentiations, were not additive when co-applied.

The GABA-uptake inhibitors, nipecotic acid and NNC-711 did not mimic the effects of mefenamic acid either qualitatively or quantitatively.

Intracellular application of mefenamic acid did not cause modulation of GABA-evoked responses recorded from voltage-clamped rat hippocampal neurones.

Mefenamic acid potentiated GABA-evoked responses recorded from rat hippocampal neurones in a voltage-sensitive manner without altering the reversal potential of the GABA-evoked current.

## CHAPTER 4:

### Determination of the effects of mefenamic acid on other neuronal ligand-gated ion channels

#### **1. Introduction**

NSAIDs exert a diverse range of effects within the CNS which include analgesic effects, anti-inflammatory effects, pro- and anti-convulsant effects and neuroprotective effects. It would therefore appear unlikely that such diverse effects are mediated entirely *via* inhibition of prostaglandin synthesis. Although many studies have examined the effects fenamates on a variety of non-neuronal preparations (see introduction), there is a paucity of data describing the effects of such drugs in neuronal preparations. Recently, Lerma and Del Rio (1992) report inhibition of NMDA-mediated currents in mouse spinal neurones and Chen *et al.*, (1998) report neuroprotective effects of fenamates against the neurotoxic effects of glutamate and ischaemia.

In light of these observations and, furthermore, having determined that fenamates positively modulated GABA-mediated responses recorded from voltage-clamped rat hippocampal neurones, the action of mefenamic acid on a number of other neuronal ligand-gated ion channels was determined. Thus, the effects of mefenamic acid were investigated at another inhibitory ligand-gated ion channel, the strychnine-sensitive glycine receptor and also at the excitatory ligand-gated ion channels gated by NMDA, AMPA and kainic acid.

Additionally, the effects of propofol were also determined on glycine-evoked response in hippocampal neurones given the conflicting reports in the literature (Lambert *et al.*, 1991; Hara *et al.*, 1993; results chapter 1).

#### **2. Methods**

Hippocampal neurones held under voltage-clamp at  $-60\text{mV}$ , produced rapid inward currents in response to pressure-applied (10-100 millisecond pulses) agonists, namely, glycine, NMDA (N-methyl-D-aspartate), AMPA ( $\alpha$ -

amino-3-hydroxy-5-methyl-4-isoxazolepropionate) and kainic acid. Agonist-evoked responses increased in amplitude with increased duration of agonist exposure. Glycine-, NMDA-, AMPA- and kainic acid-evoked responses were antagonised by their respective antagonists, strychnine, (D)-AP5, CNQX and CNQX (see Results chapter 2). All drug effects were determined against submaximal agonist-evoked control responses. All responses were measured at their peak.

### **3. Results**

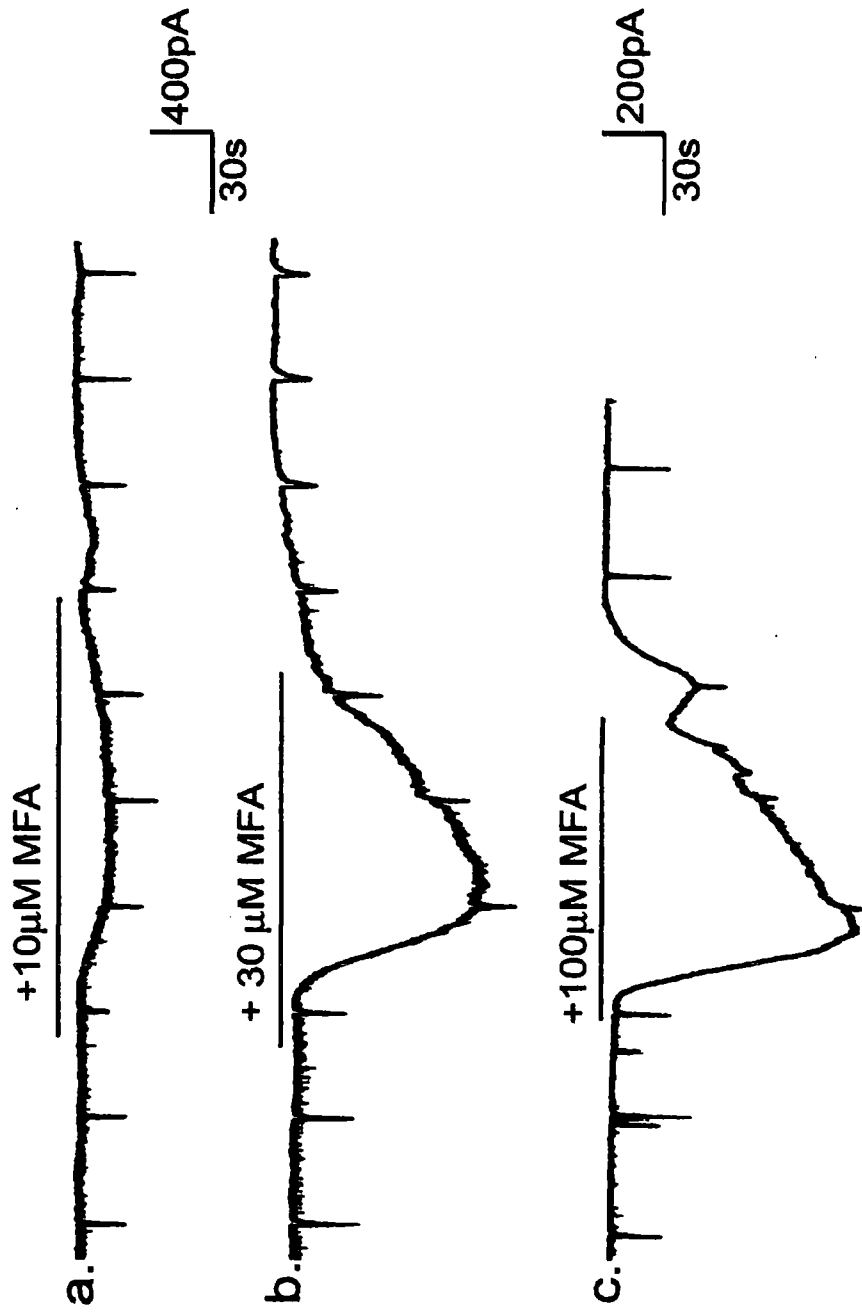
#### **3.i. Glycine**

In voltage-clamped rat hippocampal neurones, mefenamic acid (10-30 $\mu$ M), had little effect on submaximal glycine-evoked responses (105 $\pm$ 6%, n=3 and 101 $\pm$ 10 %, n=3 of control, respectively). However, at these concentrations, mefenamic acid did evoke a large transmembrane current (80-1600pA). A higher concentration of mefenamic acid (100 $\mu$ M) evoked a large transmembrane current and inhibited the glycine-evoked current to 71% of control (n=1; see figures 6.1 and 6.2). Additionally, propofol (3,10 $\mu$ M), also had little effect on submaximal glycine-evoked responses (105 $\pm$ 6%, n=3 and 101 $\pm$ 10 %, n=3 of control, respectively). Propofol (3-10 $\mu$ M) also evoked a transmembrane current ranging between 160-960pA (see figure 6.3).

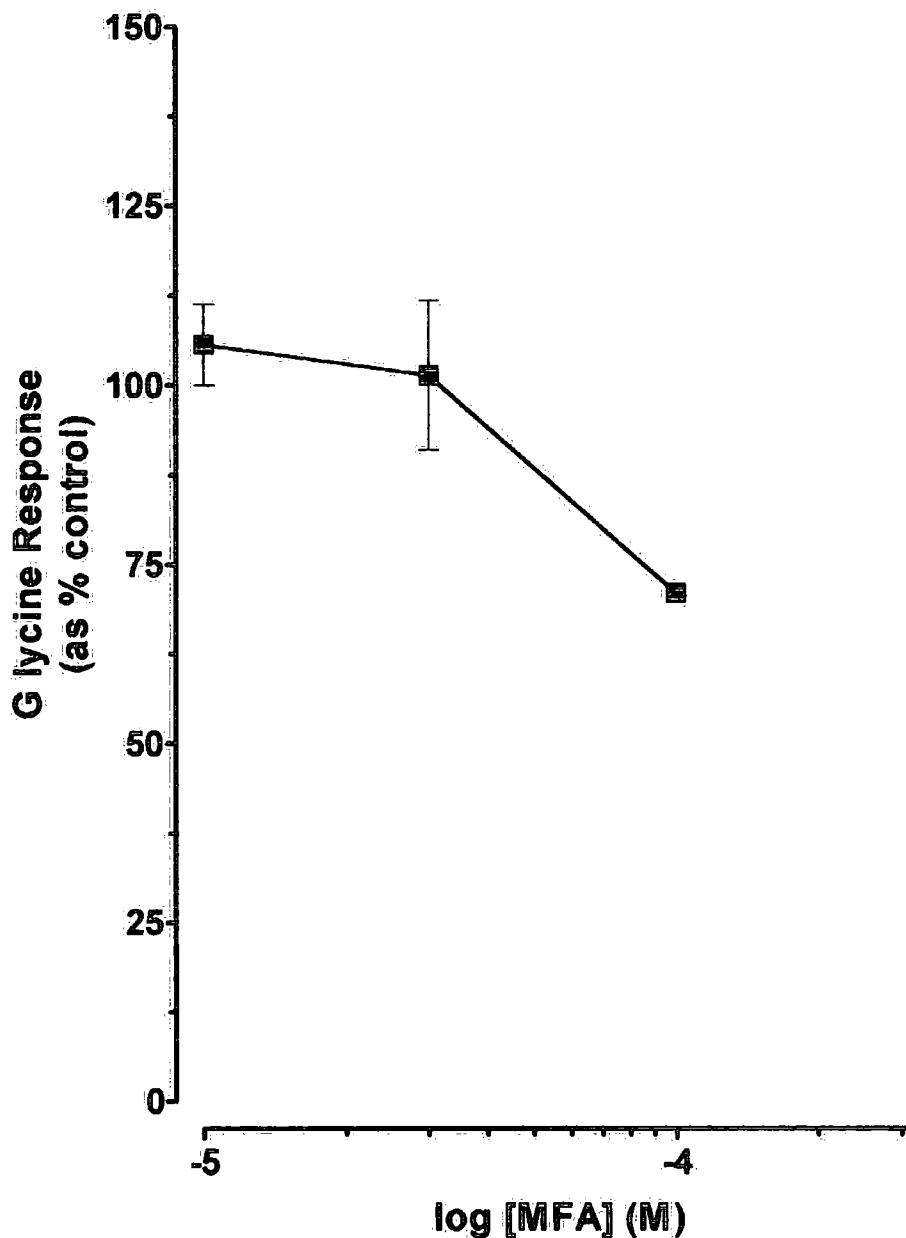
#### **3.ii. NMDA**

In voltage-clamped rat hippocampal neurones, mefenamic acid (30, 100 and 300 $\mu$ M) had little effect on submaximal NMDA-evoked responses (101 $\pm$ 7%, n=4, 101 $\pm$ 4 %, n=4 and 98 $\pm$ 5 %, n=3 of control, respectively). However, at these concentrations, mefenamic acid did evoke a transmembrane current. Mefenamic acid (1000 $\mu$ M) inhibited the NMDA-evoked response to 26 $\pm$ 2% of control, (n=3). The activation of the mefenamic acid-evoked transmembrane current could be inhibited by the competitive GABA<sub>A</sub> receptor antagonist, bicuculline (3-20 $\mu$ M). In some instances the mefenamic acid-evoked current

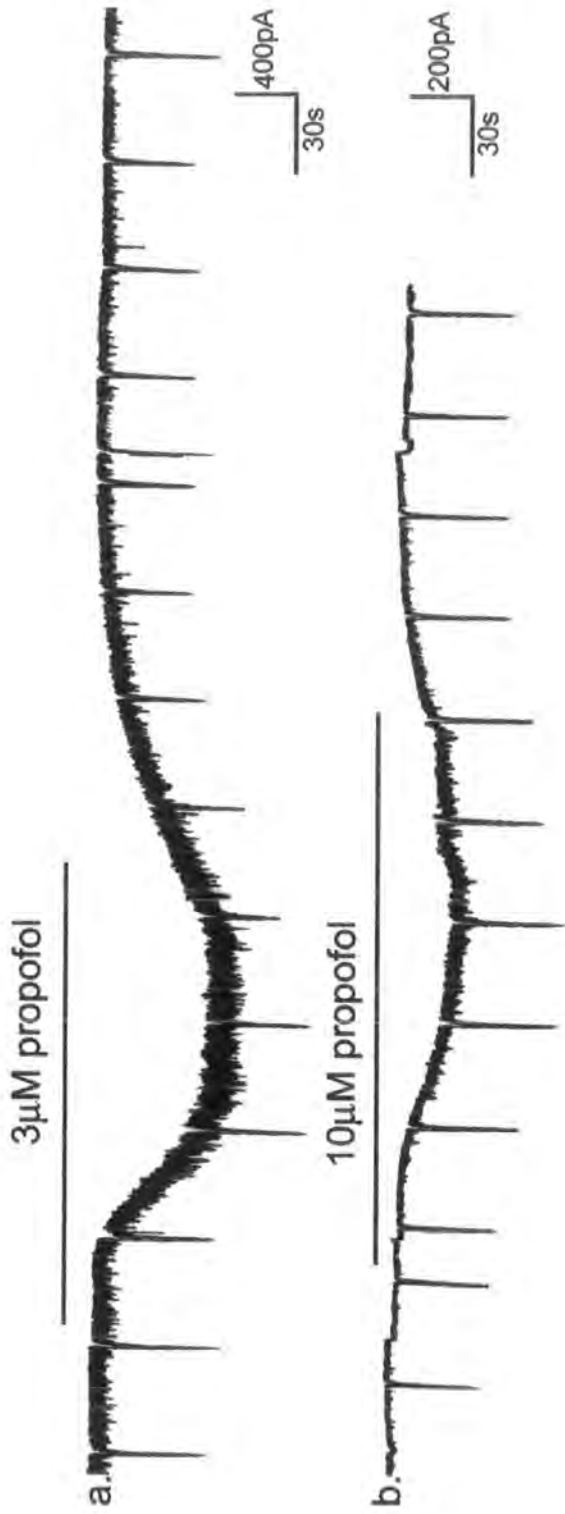




**Figure 6.1: The effect of mefenamic acid on submaximal glycine-evoked currents in whole-cell voltage-clamped hippocampal neurones.** The figure shows actual chart recorder traces of glycine ( $100\mu\text{M}$ )-evoked whole-cell currents recorded from hippocampal neurones in the absence, presence of (a:  $10\mu\text{M}$ , b:  $30\mu\text{M}$  and c:  $100\mu\text{M}$ ) mefenamic acid, and following washout. Note that perfusion of mefenamic acid is associated with a shift in baseline (holding current), which is concentration-dependent. Mefenamic acid at  $10\mu\text{M}$  and  $30\mu\text{M}$ , had little effect on the glycine current, but at  $100\mu\text{M}$ , mefenamic acid causes an apparent inhibition of the glycine response which reverses upon wash. Recording (a) is from one neurone and recordings (b) and (c) are from a second neurone. The drug contact time is represented by the horizontal line above each recording.



**Figure 6.2:** The effect of mefenamic acid on glycine-evoked whole-cell currents recorded in voltage-clamped hippocampal neurones. Graph shows mean log concentration-effect curves for the action of mefenamic acid (10-100 $\mu$ M) on glycine (100 $\mu$ M)-evoked whole-cell currents. The log<sub>10</sub> concentration of mefenamic acid is plotted on the abscissa and the response, as percentage of control agonist-evoked response, is plotted on the ordinate. Each data point represents the mean  $\pm$  s.e.m. (represented by the vertical bars) of 1-3 experiments.



**Figure 6.3: The effect of propofol on submaximal glycine-evoked whole-cell currents.** The figure shows actual chart recorder traces of glycine ( $100\mu\text{M}$ )-evoked whole-cell currents recorded from hippocampal neurones in the presence of  $3\mu\text{M}$  (a) and  $10\mu\text{M}$  (b) propofol. Note that the perfusion of propofol is associated with a shift in the baseline (holding) current, but has little or no effect on the glycine currents. Recordings a and b are taken from different neurones. Drug contact time is represented by the horizontal line above each trace.

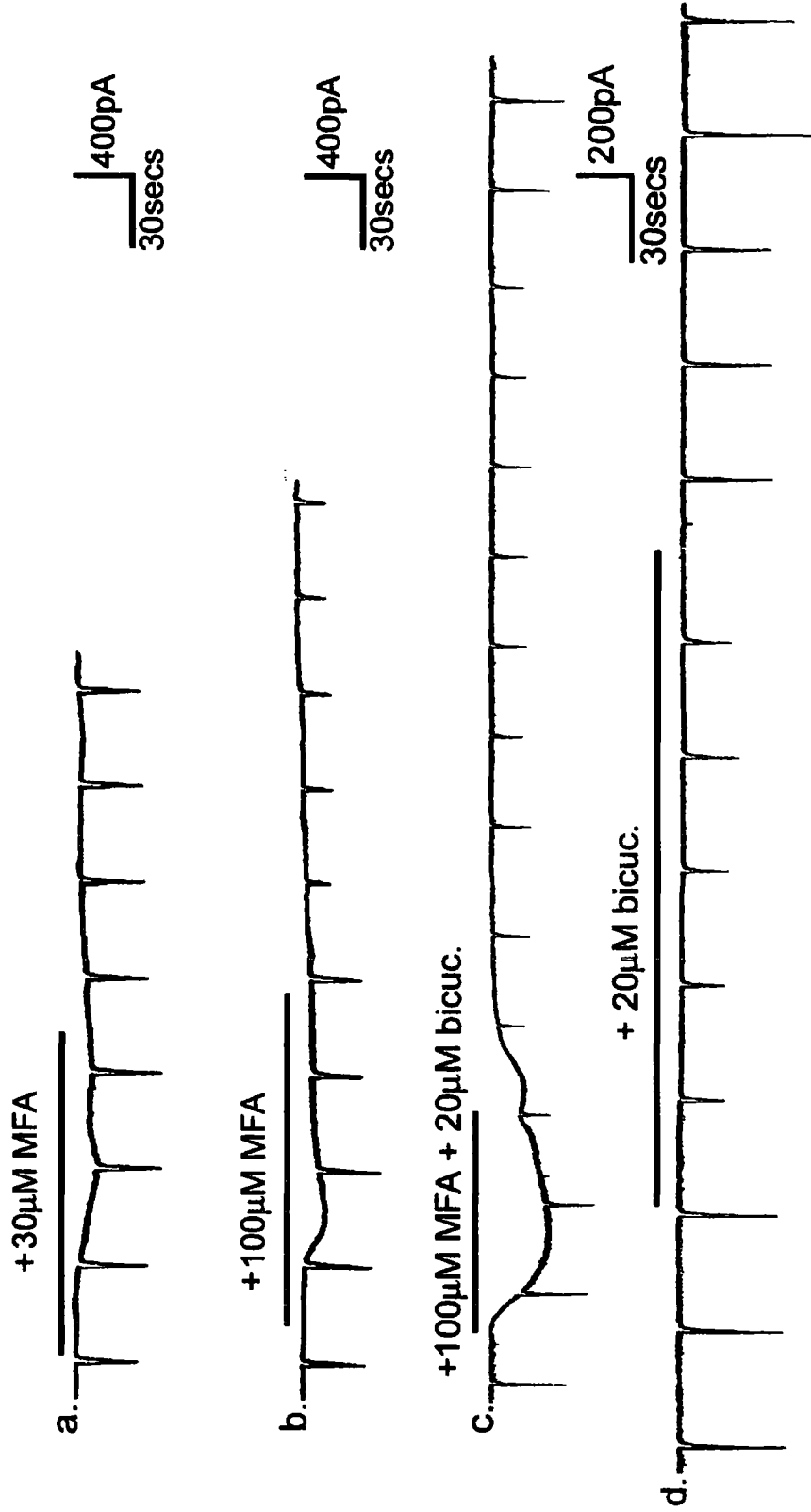
could not be inhibited by low concentrations of bicuculline ( $3\mu\text{M}$ ), in these cases higher concentrations of bicuculline ( $10\text{-}20\mu\text{M}$ ) were employed to block/inhibit the mefenamic acid-evoked transmembrane current. In the presence of bicuculline ( $3\text{-}20\mu\text{M}$ ), mefenamic acid ( $100\mu\text{M}$ ) inhibited the NMDA-evoked response to  $82\pm 19\%$  of control ( $n=3$ ), compared to  $101\pm 4$  ( $n=4$ ), in the absence of bicuculline. In the presence of bicuculline ( $3\text{-}20\mu\text{M}$ ), mefenamic acid ( $300\mu\text{M}$ ) inhibited the NMDA-evoked response to  $43\pm 11\%$  of control ( $n=3$ ), compared to  $98\pm 5$  ( $n=3$ ), in the absence of bicuculline. The NMDA-evoked current was also inhibited by bicuculline ( $20\mu\text{M}$ ) alone to  $49\%$  of control ( $n=1$ ). (Figures 6.4 and 6.5).

### 3.iii. AMPA

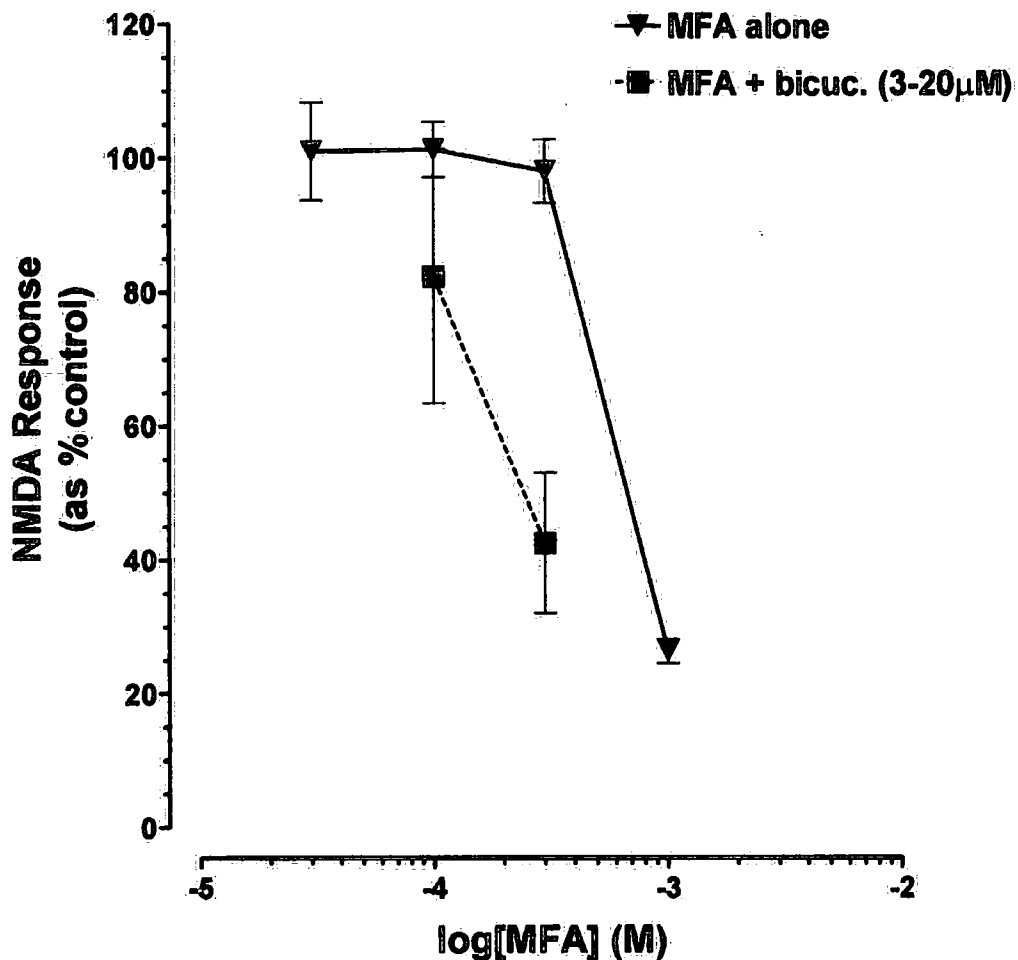
In voltage-clamped rat hippocampal neurones, mefenamic acid ( $10\text{-}30\mu\text{M}$ ) had little effect on submaximal ( $100\mu\text{M}$ ) AMPA-evoked responses although at these concentrations ( $84\pm 5\%$ ,  $n=4$ , and  $90\pm 15\%$ ,  $n=3$  of control, respectively). However, at these concentrations mefenamic acid did evoke a transmembrane current ( $200$  to  $1800\text{pA}$ ). Higher concentrations of mefenamic acid ( $100$ ,  $300$  and  $1000\mu\text{M}$ ) evoked large transmembrane currents. At these concentrations, mefenamic acid caused an inhibition of these AMPA-evoked responses to  $66\pm 5\%$ ,  $n=4$ ;  $75\%$ ,  $n=2$  and  $57\pm 3\%$ ,  $n=3$  of control, respectively (figures 6.6 and 6.7).

### 3.iv. Kainate

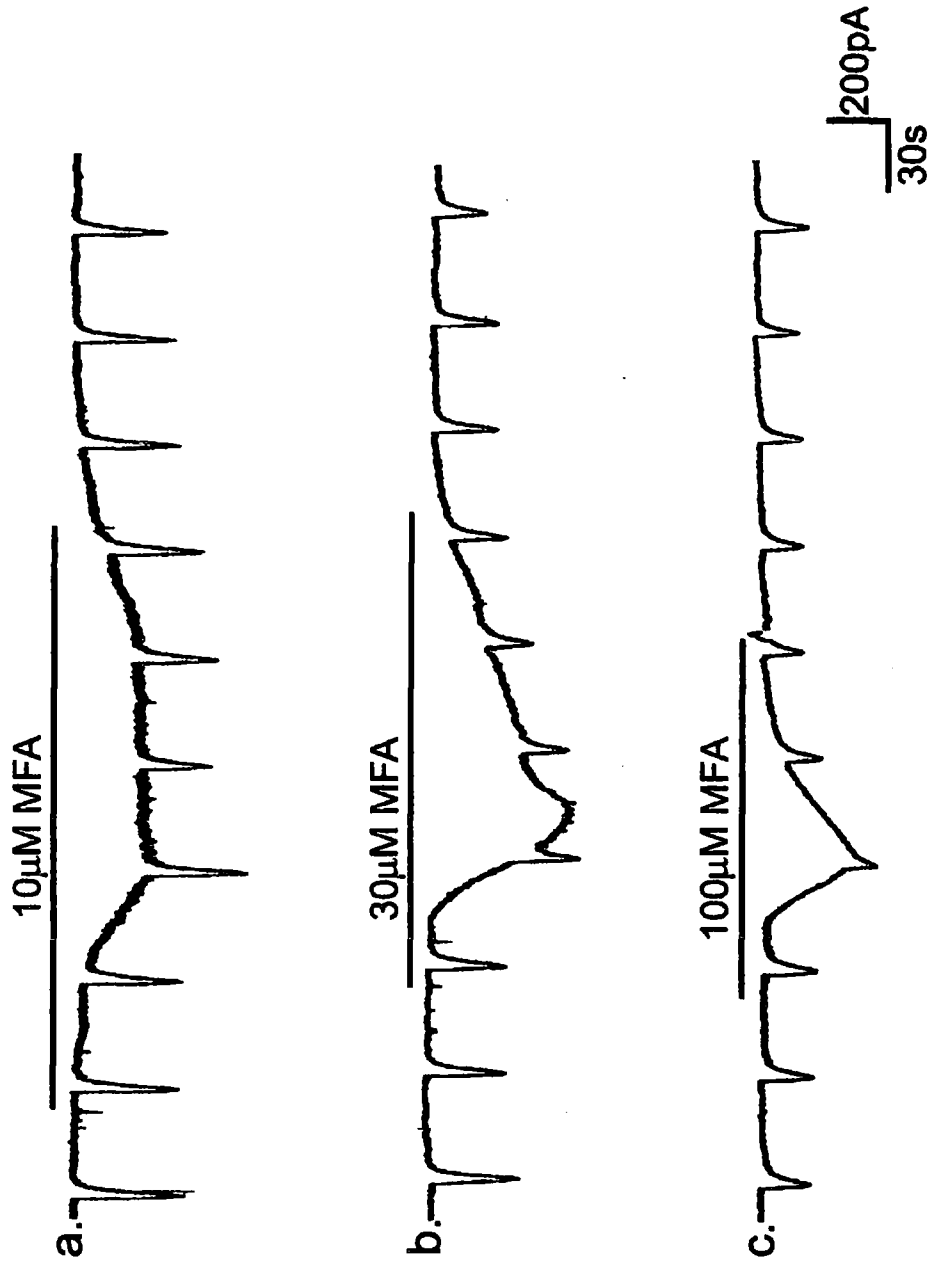
In voltage-clamped rat hippocampal neurones, mefenamic acid ( $10$ ,  $30\mu\text{M}$ ) had little effect on submaximal kainic acid-evoked responses ( $93\%$ ,  $n=2$  and  $75\pm 15\%$ ,  $n=4$  of control, respectively). At  $100\mu\text{M}$  mefenamic acid inhibited the kainic acid-evoked response to  $21\pm 10\%$  of control,  $n=3$ . However, mefenamic acid ( $10\text{-}100\mu\text{M}$ ) did evoke a transmembrane current ( $190\text{pA}\text{-}3487\text{pA}$ ) This transmembrane current could be inhibited by the competitive  $\text{GABA}_A$  receptor antagonist, bicuculline ( $3\text{-}10\mu\text{M}$ ). In the presence of bicuculline



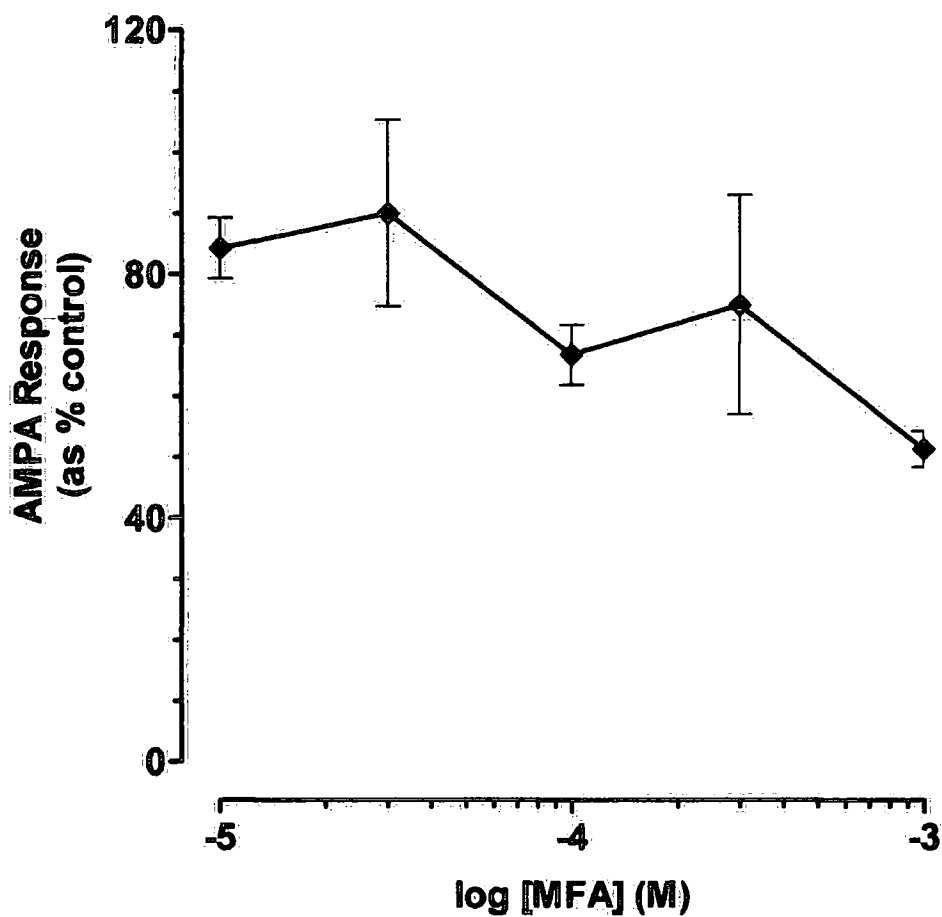
**Figure 6.4:** The effects of mefenamic acid on submaximal NMDA-evoked currents in whole-cell voltage-clamped hippocampal neurons: chart recorder traces of NMDA (100µM)-evoked currents in the presence of mefenamic acid. Drug contact time is represented by the solid line above the trace. Traces a and b: mefenamic acid (30-100µM) evokes a concentration-dependent transmembrane current and a modest inhibition of NMDA-evoked currents. Trace c: A transmembrane current is evoked in the presence of mefenamic acid (100µM) and bicuculline (20µM) and an inhibition of the NMDA-evoked current is also seen. Trace d: The NMDA-evoked current is inhibited by bicuculline (20µM) alone. Recordings a and b are taken from one neurone; recordings c and d are taken from a second neurone.



**Figure 6.5: The effect of mefenamic acid on NMDA-evoked whole-cell currents recorded in voltage-clamped hippocampal neurones. Graph shows: mean log concentration-effect curves for action of mefenamic acid (30-1000 μM) on NMDA (100 μM)-evoked whole-cell currents in the absence (▲) and presence of bicuculline (■; 3-20 μM). The log<sub>10</sub> concentration of mefenamic acid is plotted on the abscissa and the response, as percentage of control agonist-evoked response, is plotted on the ordinate. Each data point represents the mean ± s.e.m. (represented by the vertical lines) of 2-4 experiments.**



**Figure 6.6:** The effect of mefenamic acid on submaximal AMPA-evoked currents in whole-cell voltage-clamped hippocampal neurones. The figure shows actual chart recorder traces of AMPA (100 μM)-evoked whole-cell currents recorded from hippocampal neurones in the absence, presence of (a: 10 μM, b: 30 μM and c: 100 μM) mefenamic acid and following washout. Note that perfusion of mefenamic acid is associated with a shift in the baseline (holding) current. Mefenamic acid at 10 μM has little effect on the AMPA-evoked current, but with 30 μM and 100 μM, there is an apparent inhibition of the AMPA response which reverses upon wash. Drug contact time is represented by the solid line above each trace. Recordings are taken from the same cell.



**Figure 6.7: The effect of mefenamic acid on AMPA-evoked whole-cell currents recorded in voltage-clamped hippocampal neurones. Graph shows: mean log concentration-effect curves for action of mefenamic acid (10-1000 $\mu$ M) on AMPA (100 $\mu$ M)-evoked whole-cell currents. The  $\log_{10}$  concentration of mefenamic acid is plotted on the abscissa and the response, as percentage of control agonist-evoked response, is plotted on the ordinate. Each data point represents the mean  $\pm$  s.e.m. (represented by the vertical lines) of 2-4 experiments.**

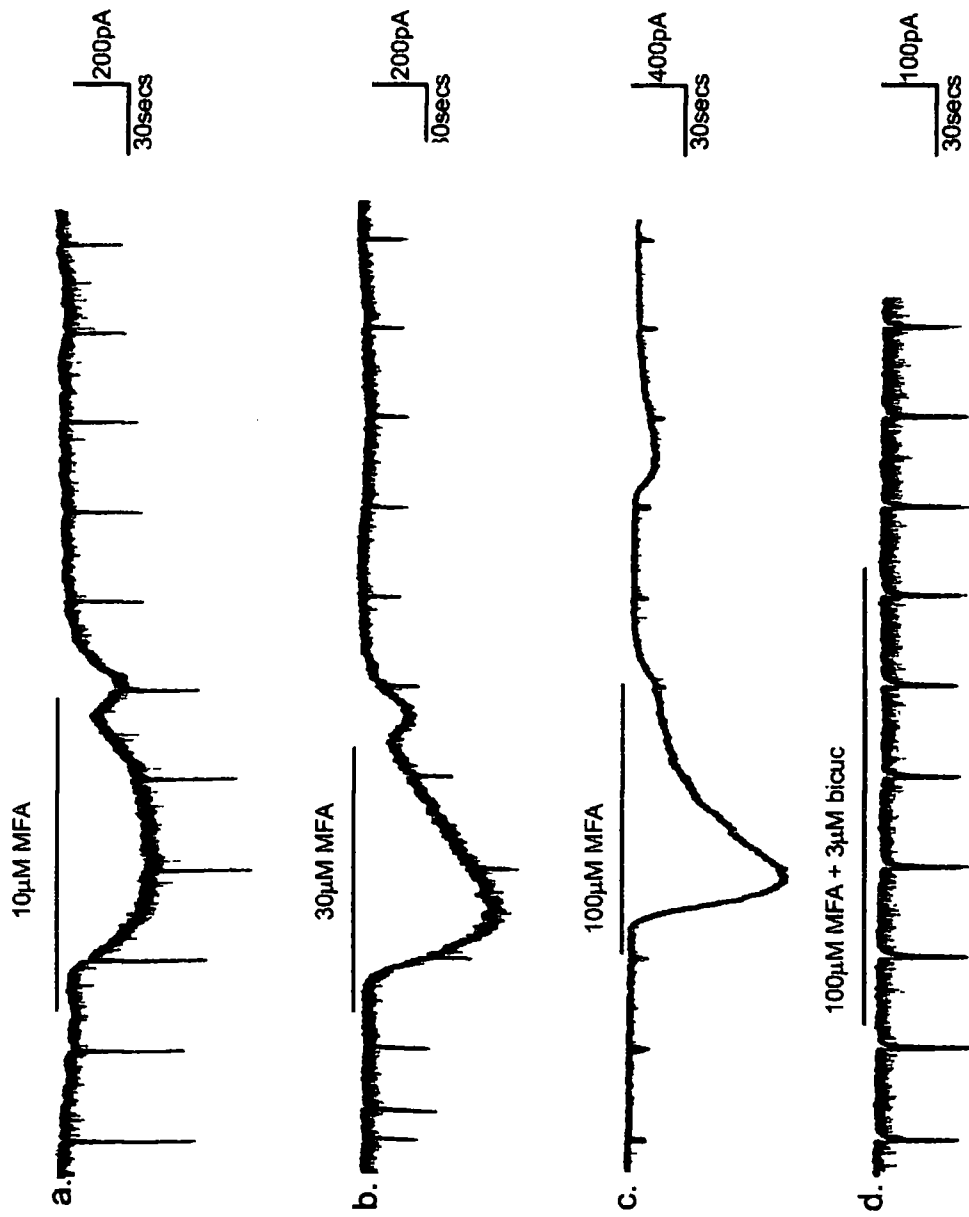


and mefenamic acid (30 $\mu$ M), the kainic acid-evoked current was 101 % (n=2). In the presence of bicuculline and mefenamic acid (100 $\mu$ M), the kainic acid-evoked current was 78% (n=2) (Figures 6.8 and 6.9).

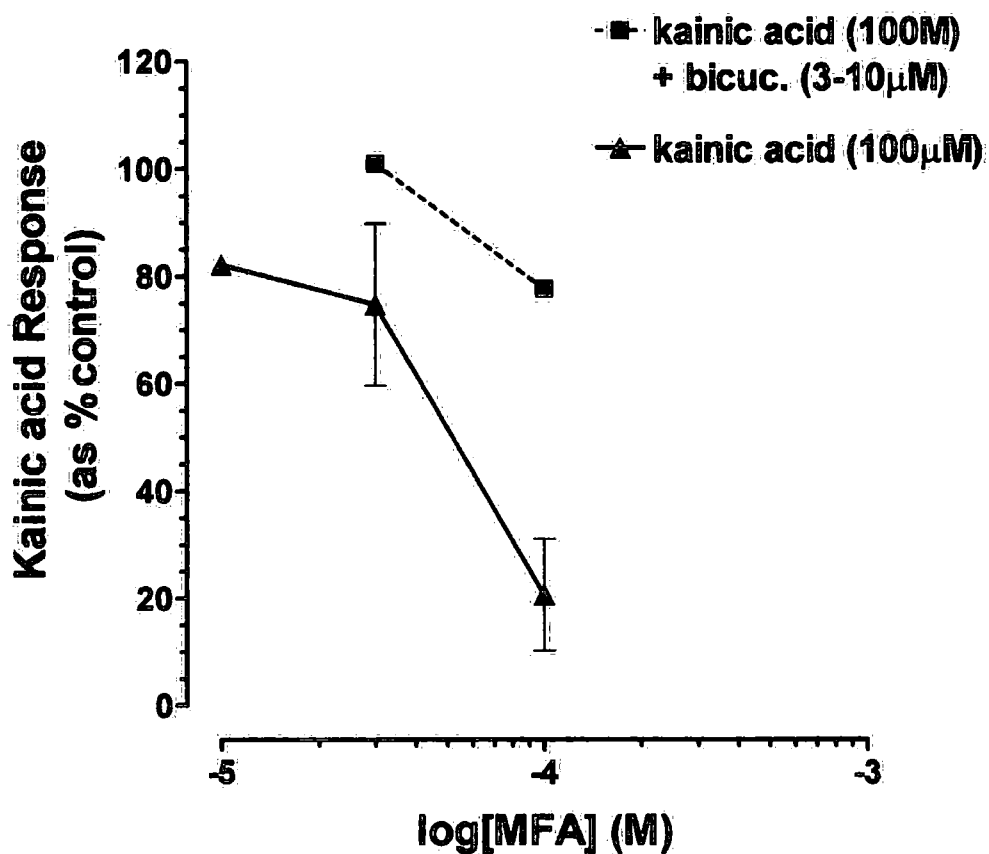
#### **4. Summary- Specificity of action of mefenamic acid**

No previous study has examined the selectivity of action of mefenamic acid on the major inhibitory and excitatory neuronal ligand gated ion channels. In this study, concentrations of mefenamic acid which evoked maximal potentiations of GABA-evoked currents had little or no effect on glycine-, NMDA-, AMPA- or kainic acid -evoked currents in voltage-clamped rat hippocampal neurones.

However, higher concentrations of mefenamic acid ( $\geq$ 100-1000 $\mu$ M) evoked a large transmembrane current and concentration-dependent inhibition of all these receptor-mediated response. Co-application of bicuculline (3-10 $\mu$ M), with higher concentrations of mefenamic acid, reduced or abolished this transmembrane current. In the presence of concentrations of bicuculline which blocked the mefenamic acid-induced transmembrane current, kainic acid-evoked currents were not inhibited by mefenamic acid. In contrast, in the presence of bicuculline, inhibition of the NMDA-evoked currents by mefenamic acid was greater.



**Figure 6.8:** The effects of mefenamic acid on submaximal kainic acid-evoked currents in whole-cell voltage-clamped hippocampal neurones. Chart recorder traces of kainic acid (100 $\mu$ M)-evoked currents in the presence of mefenamic acid. Drug contact time is represented by the solid line above each trace. Trace a: mefenamic acid (10 $\mu$ M) evokes a transmembrane current and has little effect on the amplitude of the kainic acid-evoked current. Traces b and c: mefenamic acid (30-100 $\mu$ M) evokes a transmembrane current and inhibits the amplitude of the kainic acid-evoked current. Trace d: in the presence of 100 $\mu$ M mefenamic acid and 3 $\mu$ M bicuculline, no transmembrane current is evoked and there is little effect on kainic acid-evoked current. Recordings a and b are taken from the same cell, recordings c and d are taken from different cells.



**Figure 6.9:** The effect of mefenamic acid on kainic acid-evoked whole-cell currents recorded in voltage-clamped hippocampal neurones. Graph shows: mean log concentration-effect curves for action of mefenamic acid (10-100μM) on kainic acid (100μM)-evoked whole-cell currents, in the absence (▲) and presence of bicuculline (3-10μM, ■). The  $\log_{10}$  concentration of mefenamic acid is plotted on the abscissa and the response, as percentage of control agonist-evoked response, is plotted on the ordinate. Each data point represents the mean  $\pm$  s.e.m. (represented by the vertical lines) of 2-4 experiments for mefenamic acid alone and the mean of 2 experiments for mefenamic acid in the presence of bicuculline.

## General Discussion

The experiments described in this thesis set out to investigate the hypothesis that NSAIDs could affect neuronal function by directly modulating ligand-gated ion channel function. The rationale behind this hypothesis was based upon a number of observations. Briefly these were, firstly, that NSAIDs can produce analgesic effects even when administered directly into the CNS of rodents. Secondly, in humans, NSAIDs induce complex behavioral effects especially when taken in overdose and this appeared to be particularly true for the fenamate NSAID, mefenamic acid. Thirdly, two studies had demonstrated that the fenamate, mefenamic acid could modulate the function of GABA<sub>A</sub> receptors.

Initial investigations were therefore undertaken using a simple extracellular recording technique to record agonist-evoked responses from rat isolated vagus and optic nerves. To validate the use of the vagus nerve as a suitable preparation to investigate the possible effects of NSAIDs, the modulatory effects of several well characterized drugs were first investigated. Propofol, in particular, produced a marked potentiation of GABA-evoked responses of the vagus nerve. Since the selectivity of propofol on neuronal ligand-gated ion channels was unknown at the time of this study, it was investigated using the vagus and optic nerves.

### 1. The selectivity of action of propofol

The data presented in this thesis indicate that, at clinically relevant concentrations, propofol markedly potentiates GABA<sub>A</sub>-mediated responses, and in contrast, has little or no effect on 5-HT<sub>3</sub>-, nACh- or P<sub>2x</sub>-mediated responses of the rat vagus nerve. At these concentrations, propofol also produced marked potentiations of GABA<sub>A</sub>-mediated responses and modest potentiations of glycine-mediated responses of the rat optic nerve. However, higher concentrations of propofol (up to 100μM) inhibited 5-HT<sub>3</sub>-, nACh- or P<sub>2x</sub>-mediated responses of the rat vagus nerve.

These data suggest that, at low concentrations, the actions of propofol in the vagus and optic nerves are most likely to be mediated at the GABA<sub>A</sub> receptor. If propofol is able to potentiate GABA-mediated responses in human brain at these low concentrations (which are probably close to those achieved during the induction and maintenance of anaesthesia), then the actions of propofol at human GABA<sub>A</sub> receptors in the CNS might be a significant contributing factor for the sedative and anaesthetic properties of propofol.

Although inhibition of the 5-HT<sub>3</sub> and nACh receptor channel may contribute towards the anaesthetic properties of propofol, the concentrations of propofol which have inhibitory effects at these receptors in native neurones are probably outside the therapeutic dose range (Machu and Harris, 1994; Barann *et al.*, 1993, 1998; Watchtel and Wegrzynowicz, 1992; Dilger *et al.*, 1994, 1995 and Furuya *et al.*, 1999). Nonetheless, the small inhibitory actions of propofol (at higher concentrations) at 5-HT<sub>3</sub> and nACh receptors cannot be dismissed and may contribute to the anaesthetic actions and/or side effects observed with propofol-induced anaesthesia. Notably, Violet *et al.*, (1997) have recently reported using *Xenopus* oocytes, that propofol inhibits neuronal-type recombinant nicotinic acetylcholine receptors with a greater potency than it does muscle-type recombinant nicotinic acetylcholine receptors. Further studies investigating the inhibitory actions of propofol on neuronal-type nicotinic acetylcholine receptors may elucidate contribution of these receptors to propofol-induced sedation or anaesthesia.

Administration of 5-HT<sub>3</sub> receptor antagonists do not produce drowsiness, motor impairment or cognitive deficits (Costall *et al.*, 1990). Additionally, a scarcity of 5-HT<sub>3</sub> receptors in higher brain structures (Tyers, 1991; Tecott *et al.*, 1993) has also been reported. These observations, together with the lack of modulation of homomeric murine 5-HT<sub>3</sub> receptors expressed in *Xenopus* oocytes by propofol, led Machu and Harris (1994) to the conclusion that the 5-HT<sub>3</sub> receptor does not play a major role in anaesthesia. Antagonists of the 5-HT<sub>3</sub> receptor, such as ondansetron, are however known to produce anti-emetic effects (Leeser and Lip, 1991). The anti-emetic properties of propofol reported

by Borgeat *et al.*, (1992, 1994a) might be attributed to inhibition of 5-HT<sub>3</sub> receptors, although other investigators have suggested that this is not the case since the inhibitory effects of propofol on 5-HT<sub>3</sub> receptors occurs at concentrations outside the therapeutic range (Appadu and Lambert, 1996). At present, the clinical relevance of the inhibitory effects of propofol at neuronal 5-HT<sub>3</sub> receptors remains unclear.

Similarly, concentrations of propofol which inhibit P<sub>2x</sub> receptor-mediated responses of the vagus nerve are also outside the therapeutic range. Furuya *et al.*, (1999) have demonstrated that propofol, even at concentrations outside the therapeutic range, did not affect ATP-evoked responses on neuronal P<sub>2x</sub> purinoceptors in a rat pheochromocytoma cell line.

Propofol produced a marked potentiation of GABA-evoked responses and a modest potentiation of glycine-evoked responses in the rat optic nerve. At the time of this study there were 2 conflicting electrophysiological reports in the literature. Hales and Lambert, (1991) demonstrated that propofol produced a modest potentiation of glycine currents in murine spinal neurones. In contrast, Hara *et al.*, (1994) demonstrated that propofol did not modulate glycine-evoked currents in primary dissociated hippocampal neurones. The actions of propofol were therefore examined on single cultured rat hippocampal neurones, maintained in culture, using the patch-clamp recording technique.

Notably, although propofol has been shown to potentiate glycine-evoked responses in murine spinal neurones (Hales and Lambert, 1991), in the present study propofol activated a transmembrane current, but did not modulate the glycine-evoked response in voltage-clamped rat hippocampal neurones maintained in culture. Similar observations were made by Hara *et al.*, (1994) also using voltage-clamped acutely dissociated rat hippocampal neurones. The lack of potentiation of native glycine receptors in hippocampal neurones compared to the modest potentiations observed in native murine spinal neurones (Hales and Lambert, 1991), suggests that differences may exist

between central and spinal glycine receptors. Consistent with this observation, the  $\alpha$  and  $\beta$  subunits comprising glycine receptors exhibit distinctive expression patterns within the central nervous system (Malosio *et al.*, 1991). Additionally, and similar to the GABA<sub>A</sub> receptor, a number of receptor isoforms for the glycine receptor have been identified ( $\alpha$ 1-4) and shown to impart different biophysical and pharmacological properties upon the receptor (for review see Kuhse *et al.*, 1995).

Notwithstanding, recent electrophysiological studies using recombinant glycine receptors expressed in *Xenopus* oocytes have shown that glycine receptor subunit composition had little effect on the modulatory effects of propofol (Pistis *et al.*, 1997). In this study propofol potentiated homomeric glycine receptors composed of human  $\alpha$ 1 subunits with an EC<sub>50</sub> of 16 $\mu$ M and also potentiated hetero-oligomeric glycine receptors composed of human  $\alpha$ 1 and rat  $\beta$  subunits with an EC<sub>50</sub> of 27 $\mu$ M. Similar studies by Mascia *et al.*, (1996a,b) have demonstrated that propofol potentiated human homomeric  $\alpha$ 1,  $\alpha$ 2 and mutant  $\alpha$ (A52S; mutation that alters the ability of glycine to activate the receptor) receptors expressed in *Xenopus* oocytes to an equal extent.

Behavioural evidence for the action of propofol at the strychnine-sensitive glycine receptor is conflicting. Some studies have demonstrated anticonvulsant effects of propofol (Al-Muhandis *et al.*, 1991) and others have demonstrated pro-convulsant effects (Dolin *et al.*, 1992, Bansinath *et al.*, 1995) in mice challenged with i.v. strychnine.

The modulatory effects of propofol on glycine receptors in spinal preparations and on recombinant glycine receptors are modest compared to its effects at the native and recombinant GABA<sub>A</sub> receptor receptors (Hales and Lambert, 1991; Mascia *et al.*, 1996ab; Pistis *et al.*, 1997). Nonetheless, a role for the strychnine-sensitive glycine receptor in the anaesthetic/anticonvulsant actions of propofol cannot be discounted and warrants further investigation in native neurones. Future electrophysiological studies examining the modulatory effects of propofol on glycine-evoked responses in spinal neuronal preparations

versus brain neurone preparations might indicate the relative importance of the effects of propofol on native glycine receptors within the CNS.

Similar to, though less potent than propofol, the NSAID, mefenamic acid also induced a modest and concentration-dependent potentiation of GABA-evoked responses of the rat vagus nerve. However, this modulation was less than that reported by Woodward *et al.*, (1994) using *Xenopus* oocytes and Halliwell *et al.*, (1994) recording from cultured rat hippocampal neurones. In order to investigate the site and molecular mechanism of the interaction between mefenamic acid and the GABA<sub>A</sub> receptor, further investigations were therefore carried out on single cultured rat hippocampal neurones using the more powerful patch-clamp recording technique.

## **2. Mefenamic acid potentiates GABA-evoked responses in voltage-clamped rat hippocampal neurones**

Mefenamic acid potentiated GABA-evoked responses in voltage-clamped rat hippocampal neurones in a complex manner. The degree of potentiation observed in this study was similar to the levels of potentiation reported by Halliwell *et al.*, (1994; 218% with 10 $\mu$ M mefenamic acid) and Woodward *et al.*, (1994;  $\approx$ 300% with 30 $\mu$ M mefenamic acid). More recent patch-clamp studies using human recombinant  $\alpha$ 1 $\beta$ 2 $\gamma$ 2s GABA<sub>A</sub> receptors expressed in *Xenopus* oocytes report potentiations of 355% with 30 $\mu$ M mefenamic acid (Halliwell *et al.*, in press) and 350% with 3 $\mu$ M mefenamic acid (Whittemore *et al.*, 1996).

Moreover, the data presented in this thesis demonstrates several characteristic features of the modulatory effects of mefenamic acid. These are:

1. Mefenamic acid potentiated GABA-evoked responses in both amplitude and duration.
2. Mefenamic acid evoked a transmembrane current in addition to its potentiating effects on GABA-evoked responses.



3. The modulatory effects of mefenamic acid were biphasic with low concentrations potentiating GABA-evoked responses, and higher concentrations, evoking a transient potentiation, followed by an inhibition of the GABA-evoked response.
4. The modulatory effects of high concentrations of mefenamic acid on GABA-evoked responses required long periods of washing to return to control.
5. Hippocampal neurones varied in their sensitivity to the potentiating effects of mefenamic acid.

In this study, mefenamic acid (and propofol and sodium pentobarbitone) potentiated GABA-evoked responses in both amplitude and duration. Single-channel studies have revealed that propofol (Hales and Lambert, 1991; Orser *et al.*, 1994) potentiated GABA-evoked responses by increasing the probability of the channel being in the open state, whereas pentobarbitone increased the duration of the mean open channel state (Study and Barker, 1981). The similarities between the GABA response characteristics for the potentiating effects of propofol, pentobarbitone and mefenamic acid suggest a similar mechanism of action for these drugs. Future single channel studies will allow this hypothesis to be tested.

The observation that high concentrations of mefenamic acid required long periods of washing may be attributed to a number of factors. It is possible that the prolonged effect of mefenamic acid may be due to slow removal of the drug by the perfusion system. However, the rapid reversal of the modulatory effects of high concentrations of other drugs, such as diazepam or bicuculline, suggest that this is not the case. Another possibility is that mefenamic acid is highly lipophilic and having dissolved in the cell membrane, dissociates into aqueous solutions relatively slowly. Alternatively, mefenamic acid may bind to a site on the GABA<sub>A</sub> receptor, for example within the ionophore, from which its subsequent removal is difficult. These hypotheses will be addressed later.

### 3. Inhibition of GABA-evoked responses by niflumic acid in hippocampal neurones

Submaximal GABA-evoked responses recorded from voltage-clamped rat hippocampal neurones were concentration-dependently inhibited by niflumic acid. High concentrations of niflumic acid (100 $\mu$ M) caused inhibition of the GABA-evoked response which could not be reversed even with extended washout of the drug (up to 4 times normal washout period) and could not be surmounted by increasing the agonist exposure time. Interestingly, in a binding study using whole rat brain homogenates, Evonuk & Skolnick, (1988) demonstrated that niflumate inhibited anion-enhanced [ $^{35}$ S]TPBS binding ( $IC_{50} \approx 100\mu$ M for 100mM Cl<sup>-</sup>), but not GABA-modulated radio-ligand binding to benzodiazepine receptors, and suggested that niflumate binds within or close to an anion binding site within the channel pore of neuronal GABA<sub>A</sub> receptors.

In contrast to the data presented here, Woodward *et al.*, (1994) demonstrated that niflumic acid evoked a modest potentiation of submaximal GABA-evoked responses recorded from *Xenopus* oocytes injected with rat poly<sup>+</sup>A RNA. Although the effects of fenamates on maximal GABA-evoked responses were not determined in this study, Woodward *et al.*, (1994) demonstrated that, in descending order of potency, niflumic acid (with an  $IC_{50}$  value of 7 $\mu$ M), meclofenamic acid, flufenamic acid and mefenamic acid ( $IC_{50}=33\mu$ M) acid all caused a non-competitive inhibition of maximal GABA-evoked responses. Shirasaki *et al.*, (1991) have also reported that mefenamic acid (100 $\mu$ M) inhibited  $EC_{60/70}$  GABA-evoked responses in acutely dissociated rat hippocampal neurones by ~ 40%. Most recently, Halliwell *et al.*, (in press) report that mefenamic acid (100 $\mu$ M) inhibited submaximal GABA-evoked currents in human  $\alpha_1\beta_1$  receptor constructs, expressed in *Xenopus* oocytes and in HEK-293 cells. Woodward and colleagues (1994) suggest that in *Xenopus* oocytes injected with rat poly<sup>+</sup>A RNA, agonist concentration determines the development of a potentiating or inhibitory effect by fenamates, whereby low concentrations of GABA (10-100 $\mu$ M;  $EC_{10}$ - $EC_{50}$ ) are potentiated by mefenamic acid (0.3-30 $\mu$ M) and higher concentrations of GABA (>100-3000 $\mu$ M; approximating  $EC_{50}$ - $EC_{100}$ ) are inhibited by higher concentrations of mefenamic

acid ( $>30\mu\text{M}$ ). The data of Woodward *et al.*, (1994) are therefore consistent with those of Shirasaki *et al.*, (1991).

In the present study, high concentrations ( $100\mu\text{M}$ ) of mefenamic acid evoked a transient potentiation, followed by an inhibition of the GABA-evoked current. One explanation for this observation may be attributed to the directional perfusion system employed in these experiments. Given the volume of solution in the culture dish ( $\approx 2\text{mls}$ ), the initial concentration of mefenamic acid which bathes the cell will be lower than  $100\mu\text{M}$ , which may explain the transient potentiation of the GABA-evoked response. With continued perfusion, the concentration of mefenamic acid which surrounds the cell approaches  $100\mu\text{M}$ , and at this point the inhibitory effects of mefenamic acid are observed. The biphasic modulatory effects of mefenamic acid are similar to those reported for high concentrations of pentobarbitone (e.g. Akaike *et al.*, 1987, Peters *et al.*, 1988).

In this study, the reduction in the GABA response amplitude observed with higher concentrations of mefenamic acid may be attributed to receptor desensitization (partly mediated by the direct activation of the  $\text{GABA}_A$  receptor by mefenamic acid) and/or *via* the inhibitory site for mefenamic acid on the  $\beta_1$ -subunit reported by Halliwell *et al.*, (in press). Experiments investigating the effects of mefenamic acid (and other fenamates) on maximally-evoked GABA currents in voltage-clamped hippocampal neurones may further characterize the inhibitory effects of high concentrations of mefenamic acid observed in native neurones.

#### **4. Fenamate Structure-activity relationships at native $\text{GABA}_A$ receptors**

The data in this study show that GABA-evoked currents recorded from hippocampal neurones were modulated by a range of fenamates. Similar observations were made by Woodward *et al.*, (1994) who examined the effects of fenamates on GABA-evoked responses in *Xenopus* oocytes. In the present study, the descending order of potency for the potentiating effects of fenamates was: mefenamic acid  $>$  meclofenamic acid  $>$  tolfenamic acid  $>$  flufenamic acid.

Woodward *et al.*, (1994) report a similar potency sequence for potentiation of GABA-evoked responses in *Xenopus* oocytes: mefenamic acid > meclofenamic acid > flufenamic acid > niflumic acid.

Fenamates, most commonly mefenamic acid, flufenamic acid, meclofenamic acid, tolfenamic acid and niflumic acid all belong to a group of compounds derived from the synthetic compound N-phenylanthranilic acid (PPA). Crystallographic and theoretical studies performed by Dhanaraj and Vijayan, (1988) demonstrated that fenamates are comprised of two, 6-membered rings which are linked by an imino bridge. For most fenamates, the A-ring (see Introduction, figure 1.1) carboxyl group is coplanar with the imino bridge and is stabilized by an internal hydrogen bond. Rotation of the B-ring is possible, but is limited by the steric hindrance occurring between the A-ring hydrogen *ortho* to the imino linkage and the substituted R1 and R2 groups on the B-ring, such that the 2 rings have non-planar orientations. This appears to be especially true for mefenamic acid (R1 and R2=CH<sub>3</sub>) and meclofenamic acid (R1=Cl<sup>-</sup>; R2=CH<sub>3</sub>) which have relatively bulky R1 and R2 groups compared to flufenamic acid (R1=H; R2=CF<sub>3</sub>). In the case of niflumic acid, replacement of a carbon atom with a nitrogen on the B-ring, results in a loss of steric hindrance enabling the molecule to adopt an almost planar conformation. This difference in structural conformation might account for the difference in activity of niflumic acid, compared to the other fenamates examined in this thesis.

The ability of fenamates to potentiate or inhibit GABA-mediated responses being dependent upon the conformation of the molecule was also suggested by Woodward *et al.*, (1994). Planar conformations, such as niflumic acid, were effective inhibitors and non-planar conformations, such as mefenamic acid, meclofenamic acid, flufenamic acid and tolfenamic acid, were found to be effective modulators; the degree of this modulation depended on phenyl-ring substitutions at the R2 group on the B-ring.

These data suggest that modulation of GABA-evoked responses is a feature common to fenamate NSAIDs. Moreover, the differences in potency and efficacy of the fenamates examined in this study, which may be governed by the phenyl-ring substitutions at the R2 group, are also commensurate with the existence of a specific binding site for fenamates on native GABA<sub>A</sub> receptors.

These data may also provide a template for the design and development of new subunit selective modulators of the GABA<sub>A</sub> receptor .

## **5. Possible sites and molecular mechanism(s) of action of mefenamic acid at native GABA<sub>A</sub> receptors**

### *5.i. The role of the "Benzodiazepine site" for the modulation of GABA currents by mefenamic acid.*

In native GABA<sub>A</sub> receptors, the potentiating effects of MFA were insensitive to the benzodiazepine antagonist, flumazenil. The potentiation of GABA responses by mefenamic acid recorded in *Xenopus* oocytes expressing rat cortex mRNA was also not affected by flumazenil (Woodward *et al.*, 1994). Additionally, mefenamic acid evoked similar levels of potentiation in human recombinant receptors expressing either  $\alpha_1\beta_2$  (benzodiazepine insensitive) or  $\alpha_1\beta_2\gamma_2s$  subunit combinations in *Xenopus* oocytes (Halliwell *et al.*, in press).

These data therefore suggest that fenamates do not exert their primary effect through benzodiazepine binding sites although, an involvement of this site cannot be completely discounted since mefenamic acid induced-potentiations in native neurones was greater in the presence of flumazenil. Consistent with this possibility, maximal enhancement of GABA responses by mefenamic acid in  $\alpha_1\beta_2$  receptors expressed in *Xenopus* oocytes was greater than the enhancement recorded from  $\alpha_1\beta_2\gamma_2$  receptors in oocytes (Halliwell *et al.*, in press). Similar to mefenamic acid, a number of allosteric modulators of the GABA<sub>A</sub> receptor such as propofol, sodium pentobarbitone, alphaxalone, loreclezole and etomidate have also been shown to mediate their GABA-potentiating effects at sites distinct from the benzodiazepine site (e.g. Hales and Lambert, 1991; Prince and Simmonds, 1992; Cottrell *et al.*, 1987; Wafford *et al.*, 1994; Uchida *et al.*, 1995).

### *5.ii. Possible mechanisms of action of mefenamic acid on neuronal GABA<sub>A</sub> receptors*

There are a number of possible mechanism by which the potentiating effects of mefenamic acid (and by inference, the other fenamates which produce potentiating effects) at the GABA<sub>A</sub> receptor could be mediated. For example, increased availability of GABA either due to a decrease in GABA uptake or an increase in GABA release from cells in and around the neuron under voltage-clamp: an enhancement of GABA receptor function mediated at the chloride channel: an allosteric modulatory site or possibly *via* changes in receptor phosphorylation. Other mechanisms also include the possibility that mefenamic acid may exert its effects by disrupting neuronal membranes or through mechanisms related to prostaglandin synthesis inhibition.

### *5.iii. Potentiation of GABA currents by fenamates and GABA-re-uptake inhibition.*

GABA uptake inhibitors are thought to prolong the duration of inhibitory post-synaptic potentials in neurones by elevating GABA levels at nerve terminals (Krosgaard-Larsen *et al.*, 1984). The GABA-uptake inhibitors, nipecotic acid and NNC-711 did not mimic the effects of mefenamic acid either qualitatively or quantitatively. These data suggest that mefenamic acid does not mediate its GABA-potentiating effects by altering GABA-uptake mechanisms in this system. Similar to mefenamic acid, the potentiating actions of propofol, alphaxalone and sodium pentobarbitone are not due to inhibition of GABA-uptake (Lambert *et al.*, 1991; Cottrell *et al.*, 1987; Jessel and Richards, 1977).

### *5.v. Mefenamic acid-induced modulation of GABA currents and inhibition of prostaglandin synthesis.*

Even at high concentrations (relative to those of mefenamic acid for modulation of the GABA current), the non-fenamate NSAIDs, ibuprofen and BPAA did not modulate GABA-evoked responses in voltage-clamped rat hippocampal neurones, although, indomethacin did produce a small

potentiation of the GABA-evoked response. Shirasaki *et al.*, (1991) also examined the actions of different classes of NSAIDs (up to 100 $\mu$ M) on GABA-evoked responses in voltage-clamped acutely dissociated rat hippocampal neurones and found that only mefenamic acid inhibited the GABA-evoked response. In contrast, Halliwell *et al.*, (1994) reported that mefenamic acid potentiated GABA-evoked responses in voltage-clamped hippocampal neurones in culture, and that another NSAID, BPAA, did not mimic the effects of mefenamic acid. Most recently, Halliwell *et al.*, (in press) have also shown that indomethacin (100 $\mu$ M) and ibuprofen (100 $\mu$ M) had little effect on GABA-evoked responses recorded from *Xenopus* oocytes expressing human  $\alpha_1\beta_2\gamma_{2s}$  subunits.

Inhibition of prostaglandin synthesis is thought to be a property of all NSAIDs, but there appears to be no direct relationship between the GABA-potentiating effects of the NSAIDs examined here and their ability to inhibit prostaglandin synthesis. For example, indomethacin is a more potent prostaglandin synthesis inhibitor than mefenamic acid, but has only modest GABA-potentiating ability in rat hippocampal neurones. Moreover, the other prostaglandin synthesis inhibitors tested, i.e. BPAA and ibuprofen, did not potentiate that GABA-evoked response at all. Nonetheless, the modest potentiation of GABA currents by indomethacin observed in this study and the similar modest potentiations of GABA responses by diflusalin, indomethacin, ibuprofen, naproxen and piroxicam in *Xenopus* oocytes (Woodward *et al.*, 1994) suggest that NSAIDs might indirectly modulate GABA-mediated responses by a number of other mechanisms which do not involve inhibition of prostaglandin synthesis. These are discussed below.

##### *5.v. Modulation of GABA currents by membrane perturbation*

In this study, only extracellular application of mefenamic acid resulted in modulation of GABA-mediated responses. Intracellular application, *via* the patch electrode, had no appreciable effects on the GABA currents or on their potentiation by extracellular application of mefenamic acid. Since only

extracellular application of fenamates causes any appreciable potentiation of GABA-evoked responses, it is probable that such an action is not due to changes in membrane fluidity, but rather that a membrane-bound recognition site exists for mefenamic acid (and, by inference, other fenamates) on the GABA<sub>A</sub> receptor. Furthermore, the short latency of action and rapid reversibility of action upon washout of mefenamic acid (3-30 $\mu$ M) are consistent with a specific interaction at a recognition site. Consistent with the data presented in this thesis, Woodward *et al.*, (1994) reported that intracellular application of mefenamic acid in *Xenopus* oocytes did not modulate GABA currents.

#### 5.vi. Modulation of GABA currents by changes in receptor protein phosphorylation

GABA<sub>A</sub> receptor subunits are known to contain several potential sites for protein phosphorylation by protein kinases and compounds which increase protein phosphorylation can modulate GABA<sub>A</sub> receptor function (for review see Moss and Smart, 1996). Tyrosine kinases are of particular interest here since tyrosine phosphorylation is known to increase GABA<sub>A</sub> receptor function in native neurones by increasing the probability of channel opening and mean channel open time (Moss *et al.*, 1995). A recent study has demonstrated that native GABA<sub>A</sub> receptors of cultured spinal dorsal horn neurones are phosphorylated and modulated *in situ* by endogenous protein tyrosine kinase (PTK; Wan *et al.*, 1997). These authors also report that in these cells, exogenously applied PTK increased control GABA currents by almost 2-fold, but that the time course for this was 10 minutes. In HEK cells expressing combinations of rat  $\alpha$ 1 $\beta$ 2 $\gamma$ 2 GABA<sub>A</sub> receptor subunits, the presence of the  $\beta$ 2 subunit imparted sensitivity of functional GABA<sub>A</sub> receptors to phosphorylation (Wan *et al.*, 1997). The importance of the  $\beta$ -subunit for phosphorylation is intriguing given the  $\beta$ -dependence for modulatory effects by mefenamic acid. However, the slow latency of action of PTK for potentiating GABA, compared to the rapid potentiating effects of mefenamic acid applied extracellularly and, moreover, the lack of modulation by intracellular application of mefenamic acid,



suggests that the potentiating effects of mefenamic acid are not mediated by changes in phosphorylation of the GABA<sub>A</sub> receptor. Similar conclusions were made by Woodward *et al.*, (1994) using *Xenopus* oocytes expressing rat GABA<sub>A</sub> receptors.

The effects of mefenamic acid are therefore similar to those of certain positive allosteric modulators of the GABA<sub>A</sub> receptor, such as propofol, alphaxalone and etomidate, which are also only effective when applied extracellularly (Hales and Lambert, 1991; Lambert *et al.*, 1990, Belleli *et al.*, 1997).

#### *5.vii. Use- and Voltage-dependence of the effects of mefenamic acid and niflumic acid on GABA<sub>A</sub> receptors.*

The effects of niflumic acid were examined and it was found that at 100µM, niflumic acid caused rapid inhibition of the GABA-evoked response which could not be reversed even with extended washout of the drug and could not be surmounted by increasing agonist exposure time. The inhibitory effects of niflumic acid were, therefore, not use-dependent but were consistent with niflumic acid producing a non-competitive inhibition of GABA-mediated responses. The non-competitive inhibition of the GABA-mediated current might occur, possibly via a channel-blocking mechanism. Consistent with these data, Evonuk & Skolnick, (1988) have suggested that niflumate binds with high affinity at or near an anion binding site within the channel pore of neuronal GABA<sub>A</sub> receptors.

There are no known positive modulators of the GABA<sub>A</sub> receptor which exhibit use-dependent potentiation. The potentiation of GABA-evoked responses recorded from rat hippocampal neurones by mefenamic acid was not use-dependent.

It is generally thought that "use-dependent" drugs, such as picrotoxin (Akaike *et al.*, 1985) and MK-801 (Halliwell *et al.*, 1989), have their binding sites located within the ion channel itself. The lack of use-dependency of niflumic

acid and mefenamic acid, therefore, provide no support of a fenamate binding site located within the chloride channel of the GABA<sub>A</sub> receptor .

Mefenamic acid potentiated GABA-evoked responses recorded from rat hippocampal neurones in a voltage-sensitive manner without altering the reversal potential of the GABA -evoked current. The relative potentiation at -100mV was approximately three-fold greater than at +20 mV. Consistent with these data, Halliwell *et al.*, (in press) revealed a marked voltage-dependence of the potentiating effects of mefenamic acid on GABA currents, with no associated change in reversal potential, in  $\alpha_1\beta_2$ - containing GABA<sub>A</sub> receptors expressed in *Xenopus* oocytes and in  $\alpha_1\beta_3\gamma_{2s}$  and  $\alpha_1\beta_3$ -containing GABA<sub>A</sub> receptors expressed in HEK-293 cells; a similar degree of voltage-sensitivity was also observed for loreclezole in HEK-293 cells in this study. Woodward *et al.*, (1994) showed the potentiating effects of mefenamic acid were voltage-independent in rat brain GABA<sub>A</sub> receptors expressed in *Xenopus* oocytes, with no changes in the GABA reversal potential. Additionally, although Woodward *et al.*, (1994) report that there was no change in the levels of rectification, close inspection of Woodward's data reveals that potentiations evoked by mefenamic acid (5 $\mu$ M) were 1.4 fold greater at -120mV ( $\approx$ 233%) than at +10mV ( $\approx$ 167%).

For comparative purposes, the potentiating effects of propofol were investigated and found to be voltage-independent and there was no change in GABA reversal potential. These findings are in agreement with Hales and Lambert, (1991) using bovine chromaffin cells, Hara *et al.*, (1994) using rat hippocampal neurones and Orser *et al.*, (1994) utilizing murine hippocampal neurones.

Peters *et al.*, (1988) and Cottrell *et al.*, (1987) have reported voltage-independent potentiation of GABA currents by sodium pentobarbitone and alphaxalone, respectively. Inspection of these data also reveals that potentiation of the GABA current by sodium pentobarbitone and alphaxalone was not associated with a change in reversal potential. However, the magnitude of those potentiations evoked by sodium pentobarbitone (Peters *et al.*, 1988), but not alphaxalone (Cottrell *et al.*, 1987), at hyperpolarising potentials is

approximately 1.7 times greater than that induced at depolarising potentials. Recently, Leidenheimer *et al.*, (1998), using *Xenopus* oocytes expressing human  $\alpha_1\beta_2\gamma_{2L}$  GABA<sub>A</sub> receptor subunit cDNAs, have demonstrated that potentiation of GABA-evoked responses by diazepam, pentobarbital and THDOC is not associated with a change in reversal potential, but is at least 2 fold greater at negative holding potentials compared to positive holding potentials. Notably, potentiation of the GABA response by THDOC was ~ 5 times greater at a holding potential of -60mV compared to +60mV. In conclusion, with the possible exceptions of propofol and alphaxalone, potentiation of GABA-mediated responses by a number of chemically diverse compounds, including mefenamic acid, may be dependent on membrane holding potential.

One possible explanation may be attributed to the outward rectification of the GABA current at positive holding potentials (Leidenheimer *et al.*, 1998). At negative holding potentials, there is a greater scope for potentiation of the GABA-evoked current which may explain the larger mefenamic acid-induced potentiations at negative versus positive holding potentials. However, in the present study the concentrations of GABA, mefenamic acid (and propofol) were submaximal and the voltage-dependence of mefenamic acid may not be explained by this hypothesis. Moreover, this hypothesis does not explain the different degrees of voltage-sensitivity between different modulators or the absence of voltage-sensitivity observed in this study for propofol, and in other studies for alphaxalone (Peters *et al.*, 1989).

Additionally, the differences in hydrophobicity between propofol, alphaxalone, sodium pentobarbitone, loreclezole and mefenamic acid might contribute to their different voltage-sensitivities. However at pH 7.2, there is little correlation between the relative hydrophobicity of these compounds and the level of voltage-sensitivity. For example, at pH 7.2, mefenamic acid is 99.94% in anionic form and loreclezole is uncharged (Halliwell *et al.*, in press), sodium pentobarbitone is 25% in anionic form (Robertson *et al.*, 1989) and diazepam (pKa=3.4; Merck Index, 1998) is 99.99% in anionic form (calculated using the Henderson-Hasselbalch equation). However, propofol differs from the other

modulators in that it is a liquid compound with high lipophilicity (the octane/water partition coefficient is 5000, Merck Index, 1998). This lack of voltage-sensitivity of propofol may be attributed to its marked hydrophobicity.

Mefenamic acid, loreclezole and etomidate exhibit  $\beta 2/3$ -subunit dependency for potentiation which is dependent upon the presence of the N290 residue. This residue resides in the TM2 region of the  $\beta 2/3$  subunit, which forms part of channel wall and it is conceivable that this site may contribute to the binding site and/or transductional mechanism, including the voltage-dependent potentiating effects of these drugs (Halliwell *et al.*, in press; Wafford *et al.*, 1994; Belleli *et al.*, 1997). On the basis of crystallographic and theoretical studies, Dhanaraj and Vijayan (1988) hypothesise that a potential binding site for fenamates may contain hydrophobic and hydrophilic regions. This molecular structure might be consistent with a binding site located in a channel ionophore. Although, it is presently unknown if N290 in the  $\beta 2/3$  subunit of the GABA<sub>A</sub> receptor is luminal facing, Xu and Akabas, (1996) have demonstrated that an equivalent N290 residue in  $\alpha 1$  subunits of the GABA<sub>A</sub> receptor is not luminal facing.

With regard to propofol, Krasowski *et al.*, (1998) have reported that a point-mutation in the TM3 region of the  $\beta 1$ -subunit (M286W) of human recombinant GABA<sub>A</sub> receptors expressed in HEK cells together with  $\alpha 1$  and  $\alpha 1\gamma 2s$  subunits, abolished the potentiation of GABA responses by propofol, without affecting the ability of propofol to directly activate the GABA<sub>A</sub> receptor. Conversely, a mutation in the TM2 region of the  $\beta 1$  subunit (S265I) produced normal potentiation, but reduced direct activation by propofol. Birnir *et al.*, (1997) have also reported the existence of a residue in TM2 in the  $\beta 1$  subunit which determines GABA-modulatory effects and GABA-mimetic effects of sodium pentobarbitone. The  $\beta$ -subunit is therefore critically important for the modulatory and GABA-mimetic effects of a range of modulators. The  $\beta 1$  subunit is important for the modulation and direct activation of the GABA<sub>A</sub> receptor by propofol, whereas the  $\beta 2/3$  subunit mediate the potentiating and direct activation effects of mefenamic acid, loreclezole and etomidate. However, since diazepam, a drug which is not  $\beta$ -subunit dependent, also exhibits a degree of

voltage-sensitivity (Leidenheimer *et al.*, 1998), the importance of the  $\beta$ -subunit in mediating the voltage-sensitivity of these modulators remains unclear.

Potentialiation of GABA currents by mefenamic acid might arise from changes in the chloride channel gating characteristics, such as increases in single channel conductance, probability of channel opening or increases in mean channel open time. An investigation of the modulation of GABA single channel properties by mefenamic acid would further elucidate the mechanism of action of mefenamic acid.

#### *5.viii. Neuronal sensitivity to mefenamic acid varies across cultured hippocampal neurones - similarities to loreclezole?*

In native neurones, a small number of cells were insensitive to modulation by mefenamic acid and loreclezole, but **were** modulated by other allosteric modulators such as diazepam, or sodium pentobarbitone. Additionally, the maximal potentiations evoked by mefenamic acid (30 $\mu$ M) and loreclezole (10 $\mu$ M) ranged from 125% to 909% and 149 to 349 % of control, respectively. This variation in sensitivity may reflect some degree of subunit selectivity.

In support of this suggestion, mefenamic acid differentially modulated human recombinant GABA<sub>A</sub> receptors expressed in *Xenopus* oocytes and HEK cells, depending on the presence of specific  $\beta$ -subunits (Halliwell *et al.*, in press). Similar observations have been made for loreclezole in *Xenopus* oocytes expressing recombinant GABA<sub>A</sub> receptors (Wafford *et al.*, 1994). Mefenamic acid potentiated GABA-evoked currents and directly activated GABA<sub>A</sub> receptors composed of  $\alpha_1\beta_{2/3}\gamma_{2s}$  receptor constructs, but did not potentiate or directly activate  $\alpha_1\beta_1\gamma_{2s}$  receptor constructs. Moreover, inhibition of GABA-currents by mefenamic acid was observed for  $\alpha_1\beta_1$  receptor constructs (Halliwell, *op. cit.*). Mutation studies also showed that the modulatory effects of mefenamic acid were dependent on the asparagine residue (N290) in the predicted second transmembrane domain of  $\beta_2$  or  $\beta_3$  subunits (Halliwell *et al.*,

in press); similar observations have been made for loreclezole (Wingrove *et al.*, 1994) and etomidate (Belleli *et al.*, 1997).

Additionally, Whittemore *et al.*, (1996) have demonstrated that mefenamic acid (and loreclezole) induced a strong modulation of  $\beta_2\gamma_{2L}$  receptors expressed in *Xenopus* oocytes, but that there was little difference between modulation of  $\alpha_1\beta_2\gamma_{2L}$  and  $\alpha_4\beta_2\gamma_{2L}$  receptors, indicating that the presence of the  $\alpha$ -subunit was not critical for mefenamic acid-induced potentiation of GABA-evoked currents in these receptors.

In light of these observations, the overall potentiating effects of mefenamic acid on native GABA<sub>A</sub> receptors of rat hippocampal neurones suggests that these receptors were predominantly  $\beta_{2/3}$ -containing receptors.

Interestingly, Zwart *et al.*, (1995) have reported an inhibition of acetylcholine-evoked currents on  $\alpha_3\beta_2$  nicotinic receptor constructs, but a potentiation with  $\alpha_3\beta_4$  receptor constructs by mefenamic acid. However, little sequence homology exists between nicotinic acetylcholine  $\beta_2$  and  $\beta_4$  receptor subunits compared to GABA<sub>A</sub>  $\beta_1$  and  $\beta_{2/3}$  receptor subunits, suggesting that mefenamic acid-evoked effects at nicotinic receptor constructs probably occur by different mechanisms or site(s) to those at GABA<sub>A</sub> receptor constructs (Halliwell *et al.*, in press).

No other study has demonstrated the effects of mefenamic acid on GABA<sub>A</sub> receptors in native neurones, although similar observations have been made for loreclezole. Indeed, Kapur and Macdonald (1996) have demonstrated that only 50% of whole-cell voltage-clamped dentate granule cells (acutely dissociated from neonatal rats) were insensitive to modulation by loreclezole and suggested that these were  $\beta_1$ -containing receptors. In agreement, Xue *et al.*, (1996) in a binding study have demonstrated a greater potency of loreclezole and a higher GABA sensitivity in the cerebellum and thalamus, where there is a relatively higher expression of  $\beta_2$  and  $\beta_3$  subunits compared to other areas of the CNS.

The variation in sensitivity of native neuronal GABA<sub>A</sub> receptors to modulation by mefenamic acid and loreclezole might be, in part, explained by

the reported  $\beta$ -subunit dependency for mefenamic acid and loreclezole observed in recombinant GABA<sub>A</sub> receptors (Halliwell *et al.*, in press; Wafford *et al.*, 1994). Similar to loreclezole (Kapur and Macdonald, 1996), the ability of mefenamic acid to discriminate between  $\beta$ 1 and  $\beta$ 2/3 containing GABA<sub>A</sub> receptors might be exploited to identify the presence of specific  $\beta$ -subunits in native receptors.

In conclusion, the  $\beta_{2/3}$ -subunit-dependency for potentiation of the GABA<sub>A</sub> receptor is a common feature of mefenamic acid, loreclezole and etomidate which distinguishes the actions of these modulators from those of propofol, pentobarbitone and alphaxalone. These observations also lend credence to the hypothesis that a distinct site exists for these modulators on the GABA<sub>A</sub> receptor.

#### *5.ix. Overlapping sites and/or transduction mechanisms for mefenamic acid and loreclezole?*

The potentiating effects of mefenamic acid and loreclezole, at concentrations which evoked maximal potentiations, were not additive when applied together. Maximal potentiations of mefenamic acid together with loreclezole were also recently found to be non-additive in HEK cells expressing  $\alpha$ 1 $\beta$ 3 $\gamma$ 2s GABA<sub>A</sub> receptor subunits (Halliwell *et al.*, in press). These authors suggest that mefenamic acid and loreclezole, may act at the same or partly overlapping sites, or share the same allosteric mechanism for potentiation.

However, experiments reported in this thesis showed that the potentiating effects of mefenamic acid together with propofol or pentobarbitone, at concentrations which evoked maximal potentiations, were also not additive when applied together. Although all drug effects were determined against submaximal GABA-evoked responses in hippocampal neurones, these data suggest that the non-additive potentiations with loreclezole, propofol and pentobarbitone together with mefenamic acid, are simply a ceiling effect in this recording system. Further experiments are therefore required to determine if

mefenamic acid shares a binding site with other known modulators on the GABA<sub>A</sub> receptor.

Woodward *et al.*, (1994) have demonstrated that the modulatory effects of mefenamic acid together with pentobarbitone or  $\delta$ -hexachlorohexane on GABA responses in oocytes expressing rat cortex mRNA, were additive, suggesting different sites of action for these drugs. Similarly, Wafford *et al.*, (1994) demonstrated that loreclezole-induced potentiation of GABA responses was additive to sodium pentobarbitone- and 3 $\alpha$ -OH-DHP-induced potentiation, again suggesting different sites of action for these drugs.

Woodward *et al.*, (1994) reported that the steroid antagonist, 5 $\beta$ -pregnan-3 $\beta$ -ol-20-one (3 $\beta$ -OH-DHP) accelerated the response decay rate of the GABA current in the presence of mefenamic acid, but had little effect on the response amplitude. No study to-date has demonstrated a synergistic effect of fenamates and steroids on GABA response decay rate in native neurones. Woodward *et al.*, (1994) have suggested that the modulatory effects of fenamates on GABA<sub>A</sub> receptor may be further modulated by endogenous steroids *in vivo*.

Further combination experiments with mefenamic acid and other modulators using a "fast step" perfusion system, would allow a range of agonists and modulator concentrations to be applied to the same cell. The results of such experiments may provide additional information regarding the site and mechanism of action of mefenamic acid.

#### 5.x. Evidence for direct activation of the GABA<sub>A</sub> receptor by mefenamic acid.

Mefenamic acid, like pentobarbitone, alphaxalone and propofol, evoked a transmembrane current, at concentrations higher than those required for potentiation of GABA-evoked responses. The mefenamic acid current was modulated by propofol, pentobarbitone, alphaxalone, diazepam and loreclezole and was inhibited by bicuculline, suggesting that the current was mediated by activation of the GABA<sub>A</sub> receptor-gated chloride channel. Additionally, the I-V characteristics of the mefenamic acid-induced current were similar to those



observed for GABA in voltage-clamped rat hippocampal neurones. These data indicate that mefenamic acid, in common with other positive allosteric modulators of the GABA<sub>A</sub> receptor, can directly activate the GABA<sub>A</sub> chloride channel and are consistent with the observations of Halliwell *et al.*, (1994 and in press, 1999).

Recently, Halliwell and colleagues (in press) demonstrated that spontaneously gated chloride channels formed by homomeric  $\beta_3$  or  $\beta_1$ , receptors expressed in HEK cells, were directly activated or inhibited by MFA, respectively. The authors suggest that the binding sites for the direct activation and inhibition effects must be largely contained within the  $\beta_3$  and  $\beta_1$  subunits, respectively. In addition, in the  $\beta_3$  homomers, pentobarbitone (1mM)-induced currents were inhibited by mefenamic acid (100 $\mu$ M), suggesting that these drugs may share an overlapping site or the same allosteric mechanism for direct activation or alternatively, that binding of one drug to its site hinders access of the other drug to its respective binding site (Halliwell *et al.*, op. cit.). These data are however consistent with direct activation/ modulation of the GABA-gated chloride channel by mefenamic acid.

## **6. Effects of mefenamic acid on other ligand-gated ion channels.**

No previous study has examined the selectivity of action of mefenamic acid on the major inhibitory and excitatory neuronal ligand gated ion channels. In this study, lower concentrations ( $\leq 100\mu$ M) of mefenamic acid, which evoked maximal potentiations of GABA-evoked currents, had little effect on glycine-, NMDA-, AMPA- or kainic acid-evoked responses in voltage-clamped rat hippocampal neurones.

Higher concentrations of mefenamic acid ( $\geq 100-1000\mu$ M), however, evoked a large transmembrane current and concentration-dependent inhibition of all the ligand-gated ion channels examined in this thesis.

In the presence of concentrations of bicuculline which blocked the mefenamic acid-induced transmembrane current, kainic acid-evoked currents were not greatly inhibited by mefenamic acid. These data suggest that the

inhibitory effects of mefenamic acid at kainate receptors, at least, may be mediated through changes in chloride permeability. In contrast, the experiments reported in this thesis indicate that the inhibition of NMDA-evoked currents in hippocampal neurones by mefenamic acid is greater in the presence of bicuculline.

Recently, Chen *et al.*, (1998) have reported inhibition of NMDA-gated ionotropic glutamate receptors of salamander retinal neurones with high concentrations of mefenamic acid (1mM). In contrast to the data presented here, however, Chen *et al.*, (1998) do not observe inhibition of kainate-mediated responses by mefenamic acid and do not report activation of a transmembrane current by mefenamic acid. The absence of the development of a transmembrane current may be due to the use of the salamander retinal ganglion since retinal GABA<sub>A</sub> receptors have distinct pharmacology governed by the presence of  $\rho$ -subunits. In light of the observations made in this study that the inhibition of kainic acid-evoked currents by mefenamic acid is reduced when the mefenamic acid-evoked transmembrane current is abolished or reduced, the absence of a mefenamic acid-evoked transmembrane current in salamander retinal neurones may explain the lack of inhibition of kainate-mediated currents in this preparation. However, the inhibition of NMDA-gated currents by mefenamic acid in salamander retinal neurones suggests that a distinct mechanism exists for inhibition of NMDA-mediated currents.

Lerma and Del Rio (1991) also report inhibition of NMDA-gated currents in rat spinal cord neurones by flufenamic acid with IC<sub>50</sub> values of  $\approx 350\mu\text{M}$ . In contrast to this study, but in agreement with Chen *et al.*, (1998), these authors report that flufenamic acid and niflumic acid (1mM) had no effect on baseline holding currents. Notably, chloride ion substitution experiments by Lerma and Del Rio (1991) indicated that the antagonism of the NMDA-currents was not mediated via an interaction with chloride permeation or a change of the chloride equilibrium potential. Moreover, these authors report that spermine was not required for inhibition of the NMDA-evoked current and suggest that flufenamic acid and niflumic acid act as inverse agonists at the polyamine site of the NMDA receptors.

The data reported in this thesis also support different mechanisms of inhibition for kainic acid and NMDA-evoked currents by mefenamic acid. However, further experiments are required to characterise the inhibitory effects of mefenamic acid on NMDA-gated glutamate receptors. The effects of blockade of the mefenamic acid-induced transmembrane currents by bicuculline on the inhibition of glycine- or AMPA-evoked currents evoked by mefenamic acid was not determined. These experiments would indicate if the inhibitory effects of mefenamic acid at strychnine-sensitive glycine receptors, and AMPA- and NMDA-gated ionotropic glutamate receptors is attributable to changes in chloride permeation or due to a direct effect of mefenamic acid at these receptor sites. Additionally, the absence of direct effects on fenamates on spinal neurones and salamander ganglion neurones warrants further investigation given the large transmembrane currents observed in voltage-clamped rat hippocampal neurones with such high concentrations of mefenamic acid.

## **7. Pharmacological Implications-a mefenamic acid binding site exists on the GABA<sub>A</sub> receptor?**

The experiments described in this thesis add to the understanding of the pharmacology of native GABA<sub>A</sub> receptors and to the understanding of NSAID pharmacology. They are supportive of a central role of action for NSAIDs, in particular fenamates and more specifically, the experiments reported in this thesis support a selective allosteric interaction of mefenamic acid with native GABA<sub>A</sub> receptors, which is not related to cyclo-oxygenase inhibition. Additionally, these data indicate that the interaction of mefenamic acid with the GABA<sub>A</sub> receptor is not unlike that observed for a number of other positive allosteric modulators of the GABA<sub>A</sub> receptor.

## **8. Neurophysiological and Clinical relevance of the action of mefenamic acid at the GABA<sub>A</sub> receptor.**

Although it is clear from the data reported in this thesis that mefenamic acid selectively modulates the GABA<sub>A</sub> receptor, other mechanisms may contribute to the clinically observed effects of mefenamic acid overdose. These will be addressed first.

NSAID inhibition of prostaglandin synthesis may lead to accumulation of arachidonic acid, which in turn induces a prolonged inhibition of glutamate uptake in glial cells (Barbour *et al.*, 1989; Volterra *et al.*, 1992) which may subsequently lead to neuronal excitation. This may be a mechanism by which mefenamic acid and other NSAIDs produce excitatory effects, however it does not explain CNS depression (e.g. coma) also observed with mefenamic acid overdose.

Leukotrienes and their metabolites may also accumulate following NSAID inhibition of prostaglandin synthesis. Leukotrienes are thought to be involved in the function of leukocytes and endothelial cells (Cronstein and Weismann, 1995). Although, Baran *et al.*, (1994) have reported that a lipoxygenase inhibitor, ebselen, had no effect on kainic acid-induced seizures in rats, at present there is no data reported on the effects of leukotrienes on GABA<sub>A</sub> receptor function. Voltage-clamp studies investigating the action of leukotrienes on GABA-evoked responses (and other agonist-evoked responses) in rat hippocampal neurones would determine if leukotrienes can modulate neuronal ion channels function.

A direct relationship between the analgesic effects of certain NSAIDs and central serotonergic pathways has been reported (e.g. Shyu *et al.*, 1984), with NSAIDs reducing brain stem levels of serotonin and 5-hydroxyindoleacetic acid (5-HIAA) (reviewed by Cashman, 1996). However, no study to-date has reported the effects of mefenamic acid on 5-HT turnover. Since decreased levels of 5-HT are associated with convulsant effects (e.g. Dailey *et al.*, 1994), it is conceivable that the convulsant effects of mefenamic acid in overdose might, in part, be attributed to reduced serotonin levels. Although this mechanism may contribute to the pro-convulsant properties of mefenamic acid in overdose, it

does not explain the complex behavioral effects of mefenamic acid overdose observed in humans or the anti-convulsant effects of mefenamic acid in rodents.

Interestingly, a study by Dailey *et al.*, (1994) who demonstrated that loreclezole, like certain other anticonvulsant drugs, caused elevations in serotonin release in CNS. Loreclezole has, however, been proposed to mediate its anti-convulsant effects by potentiating the inhibitory actions of GABA in the CNS (Wafford *et al.*, 1994). Given the pharmacological similarities reported in this thesis and by Halliwell *et al.*, (in press) of mefenamic acid and loreclezole, it will be of interest to investigate the effects of mefenamic acid on serotonergic function in the CNS to further characterise the mechanism of action of mefenamic acid in the CNS.

At therapeutic doses of mefenamic acid, peak plasma concentrations have been reported to be within the range of 4-40 $\mu$ M, with 50% bound to plasma proteins (Flower, 1974; Glazco, 1966; Court and Volans, 1984). Such concentrations of mefenamic acid are sufficient to modulate GABA<sub>A</sub> receptor function in periphery. However, no study to date has determined the concentrations of mefenamic acid which can access the CNS. Indeed, given the ability of mefenamic acid to modulate GABA<sub>A</sub> receptors at lower concentrations than those reported in overdose, it would be of interest to determine the concentrations of mefenamic acid capable of penetrating the blood-brain-barrier. In light of this information, it would be of further interest to examine the behavioral effects of mefenamic acid at concentrations within the therapeutic range, since it conceivable that at such concentrations, mefenamic acid may modulate synaptic inhibition to produce anxiolytic and/or sedative effects.

The complex modulation of GABA-gated chloride channels by mefenamic acid observed in rat neurones and in human recombinant GABA<sub>A</sub> receptors (Halliwell *et al.*, in press) might contribute to the complex behavioral effects seen in humans following mefenamic acid overdose. Mefenamic acid overdose is associated with a high incidence of seizures and coma. Several studies investigating the actions of fenamates on chemically- and electrically-induced seizures in rodents support a bi-directional mechanism of action of

fenamates which is distinct from other NSAIDs whereby low doses of fenamates had anti-convulsant effects and high doses had pro-convulsant effects (Wallenstein, 1985ab, 1987, 1991; Ikonomidou-Turski *et al.*, 1988).

In the main, reported cases of mefenamic acid overdose in humans are associated with convulsive seizures (Shipton and Müller, 1985, Young *et al.*, 1979, Balali-Mood *et al.*, 1981, Gossinger *et al.*, 1982; Turnbull *et al.*, 1988). For example, Balali-Mood *et al.*, (1981) studied 54 patients who had overdosed on mefenamic acid and reported a concentration-dependent relationship between plasma concentrations of mefenamic acid and CNS toxicity. Fewer cases of sedation or coma have been reported. However, in some studies, symptoms of coma or depressed levels of consciousness have also been reported with mefenamic acid plasma concentrations ranging from 21mg/L (Gossinger *et al.*, 1982) to 46mg/L (Turnbull *et al.*, 1988) to 462mg/L (Hendrikse, 1988). Intriguingly, Gossinger *et al.*, (1982) report one case of coma followed by a grand mal convulsion with a mefenamic acid plasma concentration as low as 21mg/L.

NSAIDs, including fenamates have been shown to lower the incidence and delay the onset of Alzheimer's disease (McGeer *et al.*, 1996) and to be neuroprotective against ischaemia (Chen *et al.*, 1998). These effects have previously been attributed to inhibition of prostaglandin synthesis (McGreer *et al.*, 1996; Nogawa *et al.*, 1997). The interactions of mefenamic acid and other fenamates at the GABA<sub>A</sub> receptor may shed new light on the neuroprotective effects of these drugs. Speculatively, the chemical structure of fenamates may serve as a template for the design of novel anti-epileptic and/or neuroprotective drugs

Several studies have shown a lack of correlation between the analgesic properties of NSAIDs and their ability to inhibit prostaglandin synthesis (e.g. McCormack, 1994). The action of fenamates of neuronal GABA<sub>A</sub> receptors is supportive of a central role of action for NSAIDs, in addition to their ability to inhibit COX-enzymes. In light of these data, investigation of the actions of NSAIDs on other neuronal ligand and perhaps voltage-gated ion channels may further elucidate the central analgesia actions of NSAIDs.

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