An investigation of the neuropharmacological and behavioural effects of fenamate and other NSAIDs.

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Abstract

Recent evidence has indicated that NSAIDs might have direct effects on CNS tissue in addition to their classical inhibitory action on COX enzymes. This thesis addresses this hypothesis using electrophysiological and behavioural techniques.

The effects of fenamate and other NSAIDs on native neuronal $GABA_A$, $5\text{-}HT_3$, nicotinic ACh, P2x and strychinine-sensitive glycine receptors, expressed on isolated vagus or optic nerves, was investigated using an extra-cellular recording technique. The fenamate NSAID, mefenamic acid (MFA) potentiated GABA (10μM)- evoked responses in the vagus nerve. Application of MFA also resulted in non-competitive inhibition of 5-HT- and α,βMeATP- evoked responses. Non-competitive like inhibition was also observed with flufenamic acid on DMPP- and α,βMeATP- evoked responses and with meclofenamic acid on GABA- evoked responses. Non-fenamate NSAIDs, including aspirin, did not significantly modulate the $GABA_A$, $5\text{-}HT_3$, nicotinic ACh, P2x or glycine receptors.

The cognitive and behavioural effects of fenamates and other NASIDs were then investigated. MFA (5-20mg/kg) caused a significant dose- and time-dependent enhancement in the non-spatial object discrimination working memory task when compared to saline controls. The enhancement observed with MFA was greater than that of the cognitive enhancer piracetam. This enhancement was not due to a change in non-mnemonic processes such as arousal, anxiety or locomotion. MFA also enhanced rats' performance in the spatial object location working memory task.

The fenamate NSAID, meclofenamic acid (20mg/kg) mimicked the effect of MFA, but the non-fenamate NSAIDs aspirin and ibuprofen, did not enhance object discrimination indicating that these cognitive effects are not via inhibition of COX. The $GABA_A$ receptor modulators diazepam, bicuculline and loreclezole, did not replicate the effect of MFA on object discrimination, suggesting that its effects do not depend entirely on the $GABA_A$ receptor. Scopolamine (0.25-1mg/kg) significantly impaired object discrimination in a dose-dependent manner. This action could be fully reversed by co-treatment with MFA (20mg/kg).

In the T-maze task, MFA (20mg/kg) decreased the number of arm entry errors and days taken to reach criterion. The number of arm entry errors made when a 5-minute intra-trial interval was introduced was also significantly reduced by MFA compared with saline treated animals. In the radial maze, MFA (20mg/kg) did not decrease the number of never baited arm entries to reach criterion. However MFA did significantly reduce the number of re-entry errors to baited arms, compared to controls, when an intra-trial delay (10-30 secs) was introduced. These results support the hypothesis that MFA enhances spatial working memory and that these effects are not task-specific.

Overall, the data in this thesis show that fenamate NSAIDs can directly modulate native neuronal ligand-gated ion channels and that MFA can enhance working memory in normal and scopolamine-impaired rats. These results suggest additional pharmacological potential for certain fenamate NSAIDs.

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Chapter One: General Introduction

Chapter Two: Modulation of Ligand-Gated Ion Channels By NSAIDs

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, which was carried out under the supervision of Dr. R.F. Halliwell, was undertaken in the School of Biological and Biomedical Sciences at the University of Durham, and has not previously been submitted for a higher degree.

Publications

Research during my Ph.D. has resulted in the following publications:

Papers:

Patten, D.*, Foxon, G.R.*, Martin, K.F. & Halliwell, R.F. (2001). Determination of the relative effects of propofol on native neuronal ligand-gated ion channels. *Clin. & Exp. Pharmacol. & Physiol.*, **28**, 451-458. (* joint first authors)

Tavasli, M., O'Hagan, D., Batsanov, A.S., Foxon, G.R., Halliwell, R.F., & Howard, J.A.K. (1999). The synthesis conformation and antimuscarinic properties of ketone analogues of tropane esters. *J. Chemical Society - Perkin Trans. 1*, **23**, 3455-3461.

Abstracts:

Foxon, G.R., Ennaceur, A. & Halliwell, R.F. (2000). Enhanced object recognition memory evoked by the non-steroidal ani-inflammatory drug, mefenamic acid: a study of the underlying mechanisms. *Eur. J. Neurosci.*, **12** (supple 11) p473: 216.03.

Foxon, G.R. & Halliwell, R.F. (2000). An investigation of the hypothesis that NSAIDs modulate neuronal ligand-gated ion channels. *Br. J. Pharmacol.*, **129**, U65.

Foxon, G.R., Ennaceur, A. & Halliwell, R.F. (1999). Evidence that mefenamic acid enhances object recognition memory in the rat. *Brit. Neurosci. Association*, Abstr., **Vol 15**, 54.02.

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Finally, I would like to dedicate this thesis to my parents, Brian and Carolyn, my Gran, Dina and to my brothers Duncan and Gary, who have supported me in everything that I have ever achieved and kept me smiling throughout – Thank you.

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Chapter One: General Introduction

1.1: Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) is the term used for a broad range of chemically diverse compounds (figure 1.1). They are probably the single most important group of self-prescribed pharmaceuticals and certainly the most widely consumed drugs world-wide (Mitchell & Warner, 1999). NSAIDs are taken to treat a diverse range of ailments mainly through their anti-inflammatory, anti-pyretic and analgesic properties (Orme, 1990). The most commonly used and well known NSAID is *aspirin* which has been chemically synthesised on a large scale and used clinically since 1874 (Weissmann, 1991). However it was not until the early 1970's that the mechanism of action of these drugs was elucidated.

1.2: Mechanism of action of NSAIDs

In 1971 three related papers, published in Nature, showed for the first time that the NSAIDs, aspirin and indomethacin, could inhibit prostaglandin synthesis. The first paper (Vane, 1971) reported that the prostaglandins, PGE_2 and PGF_2 obtained from guinea-pig lung could contract isolated rat stomach strips and colon and that these contractions could be blocked in a concentration-dependent manner by aspirin and indomethacin. The second paper (Smith & Willis, 1971) went on to show that aspirin and indomethacin could prevent the thrombin-induced formation of the prostaglandins PGE_2 and $PGF_{2\alpha}$, from human platelets. The third paper (Ferreira *et al.*, 1971) showed that prostaglandin release from the dog spleen, induced by intra-arterial injection of bradykinin, could be abolished when the spleen was perfused with aspirin or indomethacin. The conclusions drawn from these papers were that aspirin-like drugs

NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

Carboxylic Acids

Salicyclates

- Acetylsalicyclic Acid (Aspirin)
- Salsalate
- Diflunisal

Acetic Acids

- Indomethacin
- Sulindac
- Diclofenac

Propionic Acids

- Ibuprofen
- Naproxen
- Ketoprofen

Fenamates

- Mefenamic Acid
- Flufenamic Acid
- Niflumic Acid

Pyrazoles

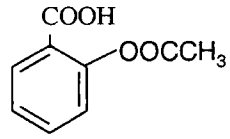
- Phenylbutazone
- Apazone
- Kebuzone

Oxicams

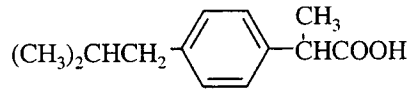
- Piroxicam
- Isoxicam
- Tenoxicam

Coxibs

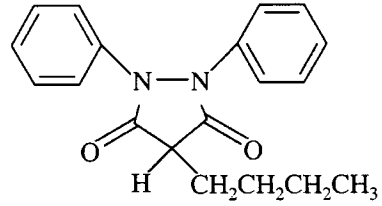
- Rofecoxib
- Celecoxib



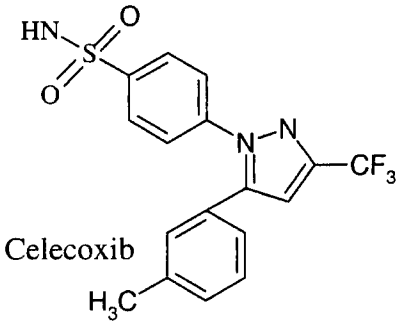
Aspirin



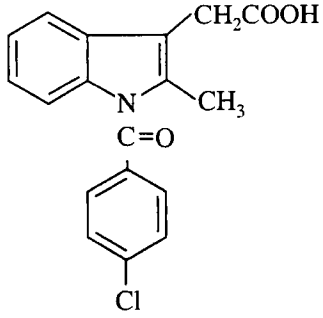
Ibuprofen



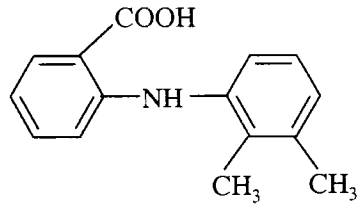
Phenylbutazone



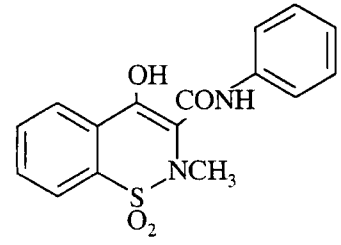
Celecoxib



Indomethacin



Mefenamic acid



Piroxicam

Figure 1.1: Illustration of the different chemical groups, classified as non-steroidal anti-inflammatory drugs. Diagram adapted from Weissmann (1991).

produced their anti-inflammatory and anti-pyretic actions by inhibiting the formation of prostaglandins since it was recognised that prostaglandin levels are elevated in inflamed tissue (Ferreira, 1972) and are found in the cerebrospinal fluid during fever (Feldberg & Gupta, 1973). However, their analgesic properties could not be explained simply by inhibition of prostaglandin synthesis, since prostaglandins elicit a pain response only when they are injected into humans at high concentrations (Ferreira, 1972). The analgesic properties of NSAIDs were only explained when it was recognised that prostaglandins potentiated the actions of other pain mediators, such as bradykinin, and induced marked hyperalgesia by sensitising sensory nerve endings (Ferreira *et al.*, 1973). The hypothesis that NSAIDs produce their analgesic actions through inhibition of peripheral prostaglandin synthesis has now been generally accepted for several years.

1.3: Prostaglandin biosynthesis

Prostaglandins are cellular mediators which are derived from arachidonic acid (van Dorp *et al.*, 1964, Bergstrom *et al.*, 1964), a twenty carbon unsaturated fatty acid (Figure 1.2). Prostaglandins are designated by a capital letter and a subscript number, the letter (A - I) refers to the type of ring substitutions and the number refers to the number of double bonds present within the chemical structure (Bergstrom *et al.*, 1968). Arachidonic acid is converted to prostaglandins via two unstable prostaglandin endoperoxides, PGG_2 and PGH_2 (Hamberg & Samuelsson, 1974). The conversion is catalysed by an enzyme known as cyclooxygenase (COX), also known as prostaglandin H_2 synthetase. The COX enzyme has a dual function: firstly, it oxidises arachidonic acid to produce the unstable intermediate PGG_2 which is then reduced to the more stable PGH_2; PGH_2 is then converted to individual prostaglandins or thromboxane A_2 via tissue specific prostaglandin synthetases or thromboxane synthetase respectively (figure 1.2; for review see Vane *et al.*, 1998).

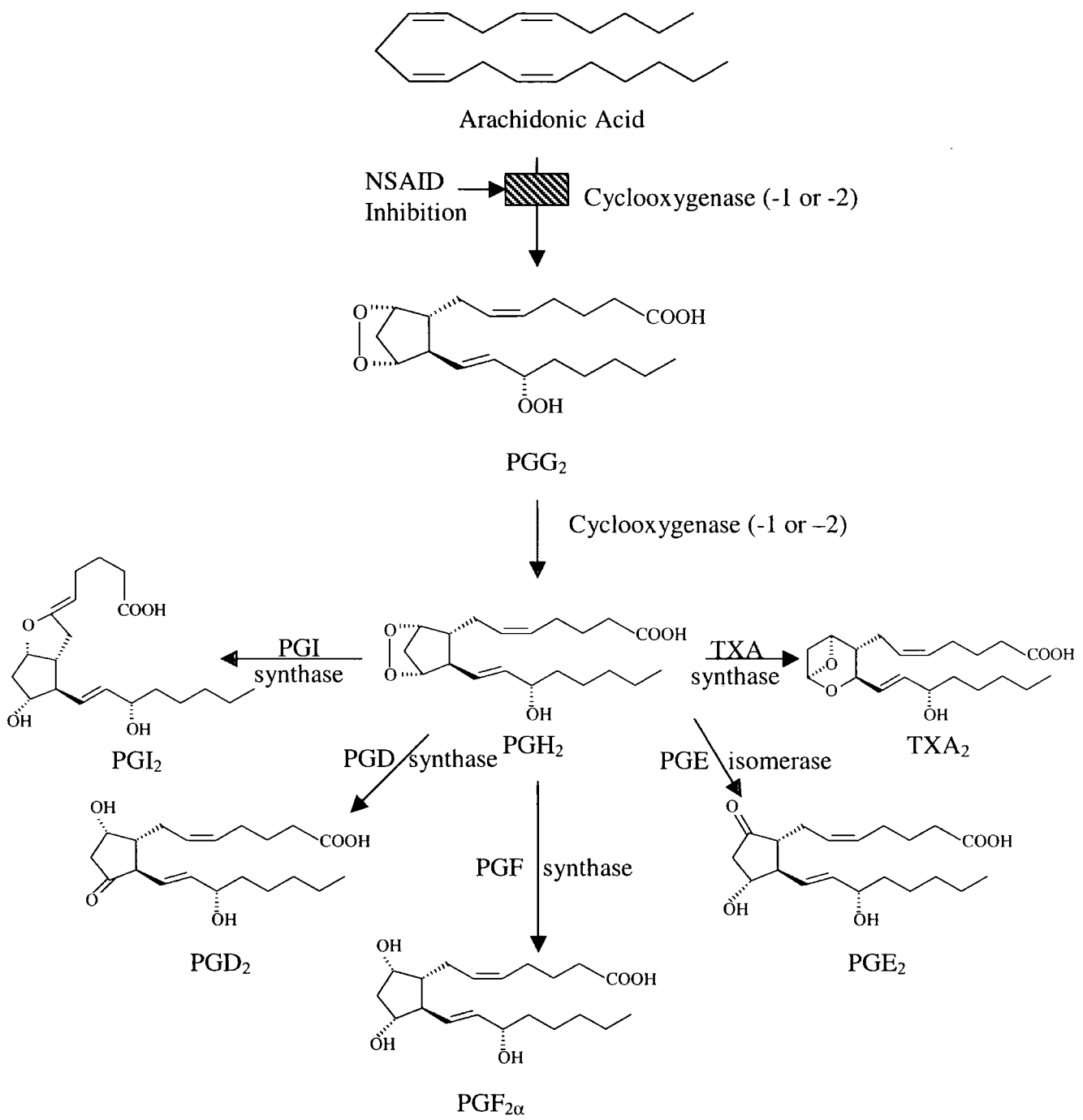


Figure 1.2: Prostaglandin synthesis pathways from arachidonic acid. Arachidonic acid is first oxidised by cyclooxygenase -1 or -2 to form the unstable PGG_2 which is then hydrolysed to form PGH_2. This compound is then converted into specific prostaglandins by specific enzymes. Thromboxane (TXA_2) and prostacyclin (PGI_2) are also formed from this pathway. Diagram adapted from Vane *et al.* (1998)

1.4: COX-1 and COX-2 isoenzymes

It was originally thought that there was only one COX enzyme, which was first purified from bovine vesicular gland in 1976 (Miyamoto *et al.*, 1976) and was subsequently cloned by three separate groups in 1988 (DeWitt & Smith 1988, Merlie *et al.*, 1988 and Yokoyama *et al.*, 1988). However, by the early 1990's immunoprecipitation experiments revealed a second COX enzyme gene (Rosen *et al.*, 1989; Holtzmann *et al.*, 1992), which was found to be an inducible gene product (Kujubu *et al.*, 1991; Xie *et al.*, 1991). This inducible gene was shown to encode a protein with COX related activities (Fletcher *et al.*, 1992, O'Banion *et al.* 1992) and has since been termed COX-2.

Structurally COX-1 and COX-2 enzyme proteins are similar, with human COX-1 and COX-2 proteins sharing 64% amino acid sequence homology (Funk *et al.*, 1991, Hla & Neilson, 1992) and accordingly, they have similar molecular weights of around 70kDa. They are both glycosylated integral membrane haemoproteins (Garavito & DeWitt, 1999), which are similar in structure, except that the COX-2 protein has a larger substrate channel and also contains a larger inhibitory pocket binding site (Kurumbail *et al.*, 1996). The COX-2 isoenzyme is located in the endoplasmic reticulum and the nuclear envelope whereas the COX-1 isoenzyme is predominantly located in the endoplasmic reticulum (Morita *et al.*, 1995).

1.5: Function and location of COX isoenzymes

The COX-1 isoenzyme protein is found within most mammalian tissue at a relatively constant level (Otto & Smith, 1995), with high levels found in the kidney (Smith & Bell, 1978), seminal vesicles (DeWitt *et al.*, 1981), platelets (Funk *et al.*, 1991), vascular endothelial cells (DeWitt *et al.*, 1983) and monocytes (O'Sullivan *et al.*, 1992). It is

thought that the main role of COX-1 is in the maintenance of normal physiology such as inhibition of gastric acid secretion in the stomach (Robert *et al.*, 1967, Cohn, 1997) and vascular homeostasis (Eliasson, 1959; Kargman *et al.*, 1996). COX-2 enzyme mRNA is found in very low levels in human body tissue under normal physiological conditions (O'Neill & Ford-Hutchinson, 1993) with the exception of the kidney (Harris *et al.*, 1994) and certain brain regions including the cortex, hypothalamus and hippocampus (Breder *et al.*, 1995, Breder & Saper, 1996) where COX-2 is constitutively expressed.

Levels of COX-2 are rapidly increased, however, in arthritic human synovial joints (Sano *et al.*, 1992; Crofford *et al.*, 1994) and in rat models of peripheral inflammation (Sano *et al.*, 1992, Kargman *et al.*, 1994). Within the CNS COX-2 levels are also increased in rat cortical neurones after normal synaptic activity (Yamagata *et al.*, 1993, Kaufmann *et al.*, 1996) and during kainic acid- induced seizures in adult rats (Tocco *et al.*, 1997). COX-2 levels are also increased in the brain immediately after ischaemic insult as detected by Northern blot analysis of COX-2 mRNA (Collaco-Moraes *et al.*, 1996). This increase in COX-2 has been shown to contribute to focal ischaemic hippocampal brain damage, as it has been shown that administration of a COX-2 selective inhibitor (NS-398) reduced the infarct volume by one third. COX-1 levels remained constant throughout the ischaemic episode (Nogawa *et al.*, 1997). COX-2 mRNA expression is also increased in the frontal cortex taken from post mortem Alzheimer's diseased human brain (Pasinetti & Aisen, 1998).

Together this evidence has led to the hypothesis that the COX-1 enzyme is involved in maintaining normal physiological function and is constitutively expressed, whilst COX-2 levels are generally only induced in disease states such as inflammation or excitotoxicity. Targeting inhibition of the COX-2 isoenzyme has therefore been

suggested as a means of gaining effective therapy whilst decreasing the adverse effects, such as gastro-intestinal irritation, associated with currently available non isoform selective NSAIDs (Vane *et al.*, 1998).

1.6: Inhibition of COX by NSAIDs

Although Vane and colleagues were the first to show that NSAIDs inhibited prostaglandin formation (Vane, 1971), it was Smith and Lands (1971) who first showed, indirectly, that the inhibition of prostaglandins by NSAIDs was due to their ability to block an (COX) enzyme. They showed that aspirin and indomethacin inhibited, in a concentration-dependent manner, the increase in oxygen consumption observed during prostaglandin formation from sheep vesicular glands, concluding that NSAIDs inhibited an oxygen dependent enzyme in the prostaglandin formation cascade.

Today NSAIDs can be classed into three groups depending on the mechanism by which they inhibit COX isoenzymes (Smith & DeWitt, 1995), thus,

Class I NSAIDs: Involves a 'simple' competitive interaction between the NSAID and arachidonic acid for the COX binding site. Three examples of competitive antagonists of COX are ibuprofen, mefenamic acid and flufenamic acid (Rome & Lands, 1975).

Class II NSAIDs: Includes indomethacin and meclofenamic acid (Laneuville *et al.*, 1995. These agents initially bind rapidly and reversibly to the COX enzyme, but after a sufficient period of time, a conformational change in the NSAID binding site on the COX enzyme occurs from which the NSAID can only slowly dissociate. These NSAIDs are said to inhibit the COX enzyme in a competitive and time-dependent reversible manner, (Kulmacz & Lands, 1985).

Class III NSAIDs: These are competitive and time-dependent but irreversible antagonists of COX. The only commonly used NSAID in this class is aspirin, which acetylates the COX enzyme to form an irreversible bond (Roth *et al.*, 1975).

1.7: Specificity of action of NSAIDs for COX1 and COX2 isoenzymes

The majority of NSAIDs available on the market are not selective for the COX-1 or COX-2 isoenzymes in humans (Cryer & Feldman, 1998). This is consistent with previous studies, which showed that NSAIDs were not particularly selective in cell lines expressing human (Gierse *et al.*, 1995, Chulada & Langenbach, 1997) or murine (Meade *et al.*, 1993, Mitchell *et al.*, 1994) COX-1 or COX-2 isoenzymes.

Recently two new NSAIDs, rofecoxib (Chan *et al.*, 1999) and celecoxib (Geis, 1999) have been shown to be relatively selective for the COX-2 isoenzyme and have been given federal drug administration (FDA) approval for the relief of osteoarthritis and management of acute pain. Clinical studies have shown that these compounds significantly reduce the gastrointestinal side effects, such as gastroduodenal ulceration, associated with the use of more traditional NSAIDs (Brooks & Day, 2000).

1.8: NSAIDs and central analgesia

Although a considerable body of literature shows that NSAIDs produce analgesia through actions in the periphery, there is now a growing body of evidence suggesting that NSAIDs may have a central as well as a peripheral analgesic action.

In 1971, Dubas and Parker reported that sub-cutaneous injection of sodium salicyclate dose-dependently suppressed the pain escape responses in rats evoked by electrical

stimulation of the hypothalamus, indicating a central analgesic action. This work was later supported by Ferreira *et al.* (1978) who showed that intracerebroventricular injection of aspirin or indomethacin dose-dependently decreased the hyperalgesia induced by injection of carrageenin into the rat hind paw. Intrathecal administration of indomethacin or diclofenac, at concentrations found in the CNS after therapeutic oral administration, inhibited the pain vocalisation response to electric shock in the arthritic rat model (Okuyama & Aihara, 1984). Malberg and Yaksh (1992a) also demonstrated that intrathecal administration of indomethacin, flurbiprofen, ketorolac, ibuprofen or aspirin dose-dependently reduced the painful responses induced by formalin injected into the hind-paw of rats at concentrations 100-1000 times lower than doses required to inhibit the response when these NSAIDs were given by intra-peritoneal injection. These studies provide evidence that NSAIDs may have a central analgesic action, although the mechanism of action remains to be determined.

One possible hypothesis is that NSAID-induced analgesia is due to inhibition of centrally formed prostaglandins. As it has been shown that intrathecal injection of $PGF_{2\alpha}$ induces hyperalgesia in rats (Yaksh, 1982). Additionally, *in vivo* formation of $PGF_{2\alpha}$, and PGE_2 in the CNS is reduced in a dose-dependent manner following subcutaneous administration of indomethacin, diclofenac or naproxen but not aspirin (Abdel-Halin *et al.*, 1978). This evidence supports the hypothesis that NSAIDs produce analgesia via inhibition of centrally formed prostaglandins.

A second hypothesis hitherto investigated was that NSAIDs increase the levels of brain endorphins. Sacerdote and colleagues (1985) for example, showed that intra-peritoneal injection of diclofenac or piriprofen into rats increased excretion of β endorphins from

the pituitary gland and elevated levels of β endorphins in the hypothalamus. The authors concluded that these effects could explain the potent analgesic properties of NSAIDs. NSAID modulation of neuronal pathways involved in nociception has also been postulated as a mechanism for their central analgesic actions. Intra-peritoneal injection of aspirin (300-400 mg/kg) dose-dependently prevented the pain-induced behaviour following intrathecal administration of substance P or capsaicin in mice (Hunskaar *et al.*, 1985), suggesting that their analgesic action could be due inhibition of a substance P sensitive mechanism. Aspirin, ketoprofen and ibuprofen can block the thermally-induced hyperalgesia caused by activation of substance P receptors (NK-1) by substance P or glutamate, NMDA and AMPA receptors by glutamate, (Malmberg & Yaksh, 1992b), which again suggests that NSAIDs may modulate neuronal transmission.

Centrally administered aspirin also dose-dependently increased the pain threshold of electrical stimulation of tooth pulp afferent fibres in monkeys (Shyu *et al.*, 1984). These analgesic properties of aspirin in monkeys were directly related to the increased level of 5-HT neuronal activity in the hypothalamus and spinal cord. Shyu and Lin (1985) additionally reported that centrally administered aspirin resulted in anti-nociception in monkeys and that it could be blocked by the 5-HT antagonist cyproheptadine. Aspirin also reduced thalamic neuronal activity associated with mechanical noxious stimuli in rats, which could be blocked by the 5-HT antagonist metergoline (Groppetti *et al.*, 1988). These studies indicate a possible interaction between NSAIDs and serotonergic neurotransmission.

In man, electroencephalogram recordings of brain electrical potentials, which could be related to pain responses associated with painful tooth stimulation, were significantly reduced in the presence of aspirin (Chen & Chapman, 1980). A reduction in neuronal

activity has also been reported in rats because intravenous injection of aspirin, (Carlsson *et al.*, 1988) indomethacin or ibuprofen (Jurna & Brune, 1990) depressed the activity of rat thalamic neurones evoked by electrical stimulation of C-fibres in the peripheral sural nerve. This depression of thalamic neuronal activity was also observed when aspirin or indomethacin were administered *via* intrathecal injection (Jurna *et al.*, 1992).

However, despite a wealth of evidence indicating that NSAIDs might have a central analgesic effect, the mechanism remains to be determined, but it could be due to inhibition of central prostaglandins, elevation of brain endorphin levels, modulation of neurotransmission, or a combination of the above mechanisms.

1.9: Neuroprotection by NSAIDs

Aspirin has been used as a prophylactic agent in the prevention of stroke in susceptible patients for several years. The stroke preventative actions are thought to be due to aspirin's ability to block platelet aggregation (Muir *et al.*, 1997). However electrophysiological studies have suggested that there may be additional neuroprotective mechanisms. For example, Grilli and colleagues (1996) have shown that aspirin can reduce glutamate induced excito-toxicity in primary rat cerebellar granule cells and hippocampal slices. Aspirin also improves neurone viability (as indicated by population spike activity) following hypoxia of hippocampal slices (Riepe *et al.*, 1997). An *in vivo* rat focal ischaemic model (achieved by ligation of the common carotid artery) has also shown that intra-peritoneal administration of aspirin (20mg/kg) can significantly reduce infarct volumes by up to 60% as measured by immunological staining (Khayyam *et al.*, 1999).

Other NSAIDs have also been shown to provide neuroprotection against ischaemic insult. Thus high concentrations of piroxicam or indomethacin reduced and delayed the

cell death of rat hippocampal CA1 neurones after global ischaemia (Sasaki *et al.*, 1988; Nakagomi *et al.*, 1989). The fenamates mefenamic acid, flufenamic acid and meclofenamic acid (all tested at 1mM) protected chick embryo retinal cells from low glucose and low oxygen induced ischaemic injury (Chen *et al.*, 1998). Ibuprofen has also been shown to protect dopaminergic neurones (the integrity of which was assessed by high affinity dopamine uptake) in embryonic rat mescencephalic cell cultures against glutamate induced- neurotoxicity (Casper *et al.*, 2000).

The increase in COX-2 levels observed during ischaemia has led to the hypothesis that NSAIDs could provide protection from ischaemic injury. The COX-2 selective inhibitor NS-398 (N-[2-(cyclohexyloxy)-4-nitrophenyl]methane-sulphonamide), given via intra-peritoneal injection, significantly reduced the ischaemic infarct in rats whose middle cerebral artery was occluded (Nogawa *et al.*, 1997). Another COX-2 selective inhibitor, SC58125 (1-[(4-methylsulfonyl)phenyl]-3-tri-fluoromethyl-5-(4-fluorophenyl)pyrazole) has been shown to be neuroprotective against global ischaemia in rats when administed by gastric lavage (Nakayama *et al.*, 1998).

These studies show that NSAIDs can be neuroprotective against a range of neurotoxic insults and that their neuroprotection may be due to inhibition of COX-2.

1.10: Modulation of seizure activity by NSAIDs

NSAIDs have been reported to modulate seizure activity *in vivo*. Indomethacin (given by intra-peritoneal injection) blocks the increase of prostaglandins evoked by pentamethylentetrazole, picrotoxin, isoniazid (Spagnuolo *et al.*, 1978) or pentetrazole (PTZ) (Steinhauer *et al.*, 1979) induced- and electrically- induced (Zatz & Roth, 1975) seizures in rats. This effect has also been observed with intra-muscular injection of

flurbiprofen and ibuprofen but not aspirin in rats (Steinhauer & Hertting, 1981) and with diclofenac, flurbiprofen and indomethacin in mice (Forstermann *et al.*, 1982). Inhibition of prostaglandin formation does not, however, prevent these seizures from occurring, indicating that prostaglandins are not important in the onset of convulsions.

Diclofenac, flurbiprofen and indomethacin have been reported to lower the LD_{50} of PTZ- induced seizures (Forstermann *et al.*, 1982) as has ibuprofen (Steinhauer & Hertting, 1981) which also decreased the time for the onset of seizures. When aspirin was administered centrally it also potentiated both PTZ and electrically- induced seizures in rats (Climax & Sewell, 1981). These studies indicate that some NSAIDs may in fact lower the convulsive threshold of convulsants.

The seizures reviewed above were reported from whole body responses. Wallenstein (1985a) used a more sensitive and objective technique to monitor brain seizures. He reported that intra-peritoneal injection of indomethacin or ibuprofen attenuated electro-cortical activity from PTZ induced seizures in rats, while injection of mefenamic acid (15mg/kg) or meclofenamic acid (15mg/kg) potentiated the excitatory effects of PTZ. Higher concentrations of mefenamic acid (150 mg/kg) or meclofenamic acid (150 mg/kg) induced concentration-dependent excitation alone. Wallenstein suggests that NSAIDs can be divided into two groups, with one group (mefenamic acid and meclofenamic acid) producing CNS excitation and the other group (indomethacin and ibuprofen) causing CNS sedation, implying that such central effects of NSAIDs are not related to modulation of cyclooxygenase activity. In a penicillin model of generalized epilepsy, mefenamic acid and ibuprofen decreased the number of penicillin-induced electro-cortically recorded seizures, while ibuprofen but not mefenamic acid delayed the onset of seizures (Wallenstein, 1987). The NSAIDs, sodium salicyclate and

phenylbutazone, converted a non-convulsive dose of pilocarpine to a convulsive one, while inducing no central effects alone. In the same study, mefenamic acid prevented the pilocarpine- induced seizures and protected the rats against the seizure- induced forebrain damage, while neither ibuprofen nor indomethacin had any effect on the seizures (Ikonomidou-Turski *et al.*, 1988). The contrasting effect observed with mefenamic acid was highlighted by Wallenstein (1991) when he showed that low doses of mefenamic acid (20 mg/kg) attenuated PTZ- induced excitation in rats, while higher doses of mefenamic acid (60 mg/kg) potentiated PTZ- induced excitation.

High doses of indomethacin have also been shown to provide protection against generalised seizures induced by PTZ, bicuculline and electroshock treatment in mice whereas aspirin was without effect (Wong, 1993). In contrast Baran and colleagues (1994) showed that indomethacin increased the mortality rate from kainic acid induced seizures in rats, while ibuprofen was without effect. Neither aspirin, ibuprofen, indomethacin, metanizole nor piroxicam affected the threshold for electro-convulsions in mice but they all, however, enhanced the protective activity of the anti-convulsant, valproate against electrically induced seizures (Kaminski *et al.*, 1998). In contrast, both the COX-2 selective inhibitor, NS-398 and the non-selective COX inhibitor, indomethacin, aggravated kainic acid- induced seizures in rats, leading to increased cell damage in the hippocampus. The seizures observed with the COX-2 inhibitor were earlier and more severe than in controls and it was observed that it did not prevent the increase in prostaglandin synthesis induced by the seizure (Baik *et al.*, 1999). However the selective COX-2 inhibitor, rofecoxib, significantly reduced the hippocampal cell damage associated with kainic acid- induced seizures in rats (Kunz & Oliw, 2001).

These studies demonstrate that NSAIDs can modify seizure activity in a range of animal models. However the data is conflicting with some studies reporting that NSAIDs potentiate seizure activity whilst others report attenuation of seizure activity. These diverse effects of NSAIDs maybe in part a result of differences in dose and route of administration. They may also stem from additional and undefined direct effects on neuronal function.

1.11: Modulation of non-neuronal ion channels by NSAIDs

NSAIDs, especially the fenamate class, have been shown to modulate a number of non-neuronal ion channels. For example, niflumic acid (Cousin & Motais, 1979) and flufenamic acid (Cousin & Motais, 1982) are both potent non-competitive and reversible inhibitors of chloride transport across human erythrocyte membranes, as measured by radio-labelled Cl^- flux.

Flufenamic acid and niflumic acid have been reported, from patch clamp studies, to be potent reversible inhibitors of Ca^{++} activated Cl^- channels expressed in *Xenopus* oocytes (White & Aylwin, 1990). In addition, Ca^{++} activated Cl^- channels recorded using patch clamp experiments on rabbit portal vein smooth muscle cells have been shown to be inhibited in a voltage-dependent manner by niflumic acid (Hogg *et al.*, 1994), flufenamic acid and mefenamic acid (Greenwood & Large, 1995). Ca^{++} activated Cl^- channel activation, evoked by caffeine or noradrenaline, is inhibited in a concentration-dependent manner by niflumic acid in rat vascular smooth muscle (Kirkup *et al.*, 1996). Flufenamic acid and niflumic acid are potent, concentration-dependent inhibitors of suproterenol- or forskolin- stimulated chloride conductance as demonstrated by from patch-clamp recordings of cultured bovine and canine tracheal epithelial cells (Chao & Mochizuki, 1992). Higher concentrations of flufenamic acid (200μM) induce a voltage-

dependent block of a Cl^- channel known as the cystic fibrosis transmembrane conductance regulator channel, when expressed in *Xenopus* oocytes (McCarty *et al.*, 1993). Together these studies show using a variety of methods, that fenamate NSAIDs are clearly able to inhibit Cl^- conductance across cell membranes through several different ion channels in an array of tissues.

Niflumic acid, flufenamic acid and mefenamic acid reversibly block Ca^{++} activated non-selective cation channels as measured by patch-clamp recordings of inside-out patches from the membranes of rat exocrine pancreas (Gogelein *et al.*, 1990). Flufenamic acid and mefenamic acid (both at 10μM) also produced rapid and reversible block of non-selective cation channels in mouse fibroblasts, while aspirin, indomethacin and ibuprofen were without effect (Jung *et al.*, 1992).

Flufenamic acid and tolfenamic acid (10-30μM) but not the non-fenamate ketoprofen, block radiolabelled calcium influx into human polymorphonuclear leukocytes (Kankaanranta & Moilanen, 1995). Tolfenamic acid has also been shown to inhibit calcimicin- induced Ca^{++} influx by 60% into human neutrophils (Kankaanranta *et al.*, 1995) and, like flufenamic acid (10-100μM), suppress the proliferation of human peripheral blood lymphocytes via inhibition of Ca^{++} influx into the cells (Kankaanranta *et al.*, 1996). The authors suggest that these mechanisms may explain an additional prostaglandin-independent mechanism of action for their anti-inflammatory actions.

Flufenamic acid and mefenamic acid have been shown from patch-clamp studies to be potent and dose-dependent activators of K^+ currents from a number of tissues, including rabbit corneal epithelium cells (Rae & Farrugia, 1992), canine circular smooth muscle

cells (Farrugia *et al.*, 1993a), human jejunum (Farrugia *et al.*, 1993b) and rabbit portal vein smooth muscle cells (Greenwood & Large, 1995), resulting in hyperpolarisation of the cells. K^+ current from slowly activating voltage-dependent human K^+ channels, expressed in *Xenopus* oocytes, is increased by low concentrations (10μM) of flufenamic acid, mefenamic acid and niflumic acid, while higher concentrations (100μM) of niflumic acid and flufenamic acid decrease the K^+ current. In contrast, even at higher concentrations mefenamic acid (100μM) caused an additional increase in K^+ conductance (Busch *et al.*, 1994). Ca^{++} activated K^+ channels, from pig coronary smooth muscle cells, are dose-dependently (50-1000μM) activated by flufenamic acid, mefenamic acid and niflumic acid. External application of the fenamates was five times more potent than internal application suggesting an external fenamate binding site (Ottolia & Toro, 1994).

These studies show that fenamate NSAIDs can modulate a number of non-neuronal ion channels in a prostaglandin- independent manner. The actions of NSAIDs on neuronal ion channels will now be reviewed.

1.12: Modulation of neuronal ion channels by NSAIDs

One of the first experiments investigating the effects of NSAIDs on neurones was by Barker and Levitan (1971), who showed that salicyclate rapidly increased the permeability of mollusan ganglion cells to K^+ and decreased the permeability to Cl^- ions in a reversible dose-dependent manner, resulting in hyperpolarisation of the ganglion. They followed this work up by investigating the ability of a range of non-narcotic analgesics to hyperpolarise mollusan neurones (Levitan & Barker, 1972) and found a

good correlation between their ability to alter membrane permeability and their analgesic action.

Salicylate has been shown to prolong the repolarisation of the action potential and at higher concentrations (≥ 1.5 mM), completely blocked nerve conduction along giant squid axons (Neto & Narahashi, 1976). The authors followed up this finding by investigating the effect of salicyclate on compound action potentials evoked in the rabbit vagus or frog sciatic nerve using the sucrose gap recording technique. They showed that salicyclate caused a concentration-dependent reduction in the amplitude of the electrically stimulated compound action potential, and that higher concentrations of salicyclate caused direct depolarisation of the nerve resulting in blockade of nerve conduction (Neto, 1980).

At the frog neuro-muscular junction, niflumic acid increased the amplitude of pre-synaptic voltage-activated K^+ currents, while decreasing the amplitude of Na^+ currents, in a concentration-dependent manner (0.1-1mM). Indomethacin (0.1mM) had no effect on these currents when tested at the neuromuscular junction, suggesting a cyclooxygenase independent mechanism of action (Miralles *et al.*, 1996). Voltage-clamp recordings from snail circumoesophageal ganglia showed that high concentrations of flufenamic acid (500μM) but not mefenamic acid, can inhibit Ca^{++} activated non-selective cation channels (Shaw *et al.*, 1995). A similar inhibitory effect has previously been observed in non-neuronal preparations (Gogelein *et al.*, 1990; Jung *et al.*, 1992).

In addition to NSAIDs being able to modulate a number of voltage-gated and Ca^{++} activated neuronal ion channels they have also been reported to modify the actions of a number of neuronal ligand-gated ion channels.

When certain NSAIDs are co-applied with fluoroquinolones it has been shown that there is a syngerstic antagonism of the $GABA_A$ receptor. Whole cell patch-clamp recordings from rat dorsal root ganglion cells showed that neither the NSAID fenbufen nor its active metabolite, biphenyl acetic acid (BPAA), had any effect on GABA-evoked currents. Application of the fluoroquinolones, ciprofloxacin or ofloxacin, resulted in a weak but concentration-dependent inhibition of GABA- evoked currents. In contrast, when BPAA or fenbufen and quinolones were applied together there was marked synergistic inhibition of GABA-evoked currents (Halliwell *et al.*, 1991). This synergistic inhibition of GABA currents by BPAA and fluoroquinolones has also been observed in patch-clamp recordings from cultured rat hippocampal neurones (Akaike *et al.*, 1991; Shirasaki *et al.*, 1991a; Halliwell *et al.*, 1995). The interaction between BPAA and ciprofloxacin appears to be selective for the $GABA_A$ receptor, since there was no effect on $5\text{-}HT_3$, nicotinic ACh, or P2x receptor- mediated responses recorded from isolated rat vagus nerve, whereas GABA induced responses were markedly inhibited by addition of BPAA and ciprofloxacin (Green & Halliwell, 1997). Also, NMDA currents recorded from rat hippocampal neurones are not inhibited by addition of fluroquinolones and BPAA (Shirasaki *et al.*, 1991b; Halliwell *et al.*, 1995).

Niflumic acid potently inhibits the anion evoked radio-labelled TBPS binding to the ion channel pore of the $GABA_A$ receptor complex from rat brain homogenates. This suggests that niflumic acid binds to the $GABA_A$ receptor complex and that it may regulate the flow of anions through the ion channel (Evoniuk & Skolnick, 1988). High concentrations of indomethacin bind to the $GABA_A$ receptor complex as indicated by [^{3}H] GABA binding assay (Wong, 1993). The author also reported that indomethacin is

a weak non-competitive inhibitor of GABA uptake in mouse cortical synaptosomes, suggesting it might prolong the inhibitory actions of GABA (Wong, 1993).

In *Xenopus* oocytes expressing rat brain $GABA_A$ receptors, fenamate NSAIDs have been shown to have a dual effect on GABA currents; they potentiate GABA currents elicited by low concentrations of GABA (10μM), but non-competitively inhibit currents elicited by high concentrations of GABA (100μM). Mefenamic acid induced the greatest potentiation of the sub-maximal GABA current by around 300%, while niflumic acid produced the greatest inhibition, reducing the GABA response to 40% of control. Thirteen other NSAIDs were also tested in this study, of which only diflunisal had similar effects to the fenamates, and only indomethacin (100μM) was shown to inhibit GABA (10μM) currents by around 40% of control (Woodward *et al.*, 1994). Mefenamic acid (3μM) has also been reported to potentiate GABA currents from human $GABA_A$ receptor subunits $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_4\beta_2\gamma_{2L}$ expressed in *Xenopus* oocytes, although the level of potentiation depended on the sub-unit composition being 325% and 160% of control GABA response, respectively (Whittemore *et al.*, 1996). It has recently been reported (Halliwell *et al.*, 1999) that modulation of human recombinant $GABA_A$ receptors expressed in *Xenopus* oocytes and HEK-293 cells is highly dependent on the β subunit. Mefenamic acid potentiated GABA-evoked currents (10μM) and directly activated $GABA_A$ receptors composed of $\alpha_1\beta_2\gamma_{2S}$ subunits, but did not potentiate or directly activate $\alpha_1\beta_1\gamma_{2S}$ receptor constructs. Furthermore GABA- evoked currents were inhibited by mefenamic acid in oocytes expressing $\alpha_1\beta_1$ receptors (Halliwell *et al.*, 1999).

Nicotinic ACh receptors expressed in *Xenopus* oocytes are differentially modulated by niflumic acid and flufenamic acid, depending on the nicotinic ACh sub-units expressed.

α3β2 nACh receptor currents are inhibited by niflumic acid and flufenamic acid with IC_{50}'s of 90μM and 260μM respectively, while α3β4 nicotinic ACh receptor mediated currents were potentiated by niflumic acid and flufenamic acid (Zwart *et al.*, 1995). These data show that the subunit composition of ligand-gated ion channels are important for modulation by fenamates.

Finally high concentrations of niflumic acid and flufenamic acid have been shown to be non-competitive inhibitors of NMDA currents (IC_{50} ~330μM) evoked in cultured mouse spinal neurones (Lerma & Del Rio, 1992).

Together these studies have shown, using a wide range of methods, that NSAIDs can modulate a number of neuronal and non-neuronal ion channels. Fenamate NSAIDs, in particular, can modulate the $GABA_A$ receptor and there is limited evidence suggesting that other ligand-gated ion channels are modulated by these and other NSAIDs.

To date there have been no studies that have looked directly at how NSAIDs modulated native neuronal ligand-gated ion channels. The first part of this study aims to investigate the effects of a range of NSAIDs on native neuronal ligand-gated ion channels.

1.13: Clinical adverse effects of NSAIDs

Clinically, NSAIDs are relatively safe medicinal compounds with few serious adverse effects, even when taken in overdose (Smolinske *et al.*, 1990). In 1999 only 4% of all cases of adverse drug effects reported to the American Association of Poisons Control Centres were associated with NSAIDs, and of these only 5% resulted in a serious or life threatening outcome (Litovitz *et al.*, 2000).

The most common unwanted effect associated with NSAID use is gastro-intestinal ulceration, mainly in the stomach (Orme, 1990). This is thought to be due to inhibition of prostaglandin production, where they prevent gastric ulceration by reducing gastric acid secretion and evoke vasodilation of the gastric mucosa (Vane & Botting, 1997).

A number of central effects from NSAID use have been reported. For example, a study from New Zealand has reported that over a third of adverse effects from NSAIDs were of a neurological origin (Clark & Ghose, 1992) including dizziness, headache, drowsiness, confusion and depression. A number of clinical case reports have also shown that fenamate NSAIDs, in particular, when taken in overdose, cause more serious adverse central effects including seizures and coma. For example a nineteen year old woman was found in a tonic-clonic epileptic state after taking an overdose of 12.5g mefenamic acid (Young, 1979). Seizures relating to mefenamic acid overdose have also been reported by Robson *et al.* (1979), Balali Mood *et al.* (1981), Frank *et al.* (1983) and Shipton & Muller, (1985). Prescott and colleagues (1981) reported that seizures occur in over a third of mefenamic acid overdoses. Notably, mefenamic acid has also been reported to cause coma in overdose (Gossinger *et al.*, 1982, Hendrickse, 1988), as has ibuprofen (Chelluri & Jastremski, 1986, Lee & Finkler, 1986).

These reports indicate that NSAIDs can enter the human CNS at clinically relevant doses and can have profound effects upon the CNS. However there have been few studies on human or animal models which have investigated the mechanisms underlying these adverse central effects.

1.14: Some effects of NSAIDs on cognition

The clinical reports outlined above indicate that NSAIDs can enter the CNS and this has led investigators to address the effect of NSAIDs on cognition. Two small clinical studies have shown that NSAIDs may impair cognitive performance. A retrospective study by Goodwin and Regan (1982) investigated eight patients from their clinic for alterations in cognition after taking ibuprofen or naproxen. They found that six patients developed forgetfulness and an inability to concentrate within two months of starting NSAID treatment and that these symptoms disappeared within two weeks of discontinuing the drug. Another study (Wysenbeek *et al.*, 1988), involving naproxen, showed that four out of twelve elderly patients studied were impaired in at least one of four memory tests following a three week course of naproxen (750 mg/day).

Conversely several clinical reports provide evidence that NSAIDs can enhance cognitive function. For example, a study of elderly volunteers has shown that indomethacin can improve sensorimotor co-ordination and short-term memory but not attention (Bruce-Jones *et al.*, 1994). Epidemiological studies in the elderly have also shown that NSAIDs can be protective against cognitive decline. For example, Rozzini *et al.* (1996) looked at data from six thousand patients in a longitudinal study and found that long-term NSAID use was protective against cognitive deterioration when they assessed patients by the short portable mental status questionnaire (SPMSQ) over a three year period. Another longitudinal study of nearly three thousand patients showed that "indeterminate" (i.e. over-the-counter) but not regular prescription NSAID use had a protective effect against cognitive decline over a three year period, as determined by SPMSQ scores (Hanlon *et al.*, 1997). The same study, using an additional memory concentration test at the end of the three year period, also reported that patients who had regularly taken NSAIDs over this period performed better than those not taking NSAID's and that patients who had

taken high doses of NSAID did not perform as well as patients using low doses of NSAID. A large longitudinal study in elderly hypertensive patients showed a small but significant protection by NSAIDs against cognitive decline over a five year period, when measured by a paired associate learning test (Prince *et al.*, 1998).

These and other epidemiological studies have led Karplus and Saag (1998) to the conclusion that long-term use of low dose NSAIDs protects against cognitive decline, while higher doses of NSAID may impair memory. However there have been no experimental or animal model studies undertaken to date to address these epidemiological findings. There have also been no studies which have investigated the mechanism of action behind these cognitive effects.

1.15: Protection against Alzheimer's disease associated with the use of NSAIDs

A number of epidemiological studies have shown that treatment with NSAIDs can lead to a reduced risk of developing Alzheimer's disease. This link was first shown, indirectly, by Jenkinson *et al.* (1989) whose case control study found a reduction in the incidence of Alzheimer's disease in patients suffering from rheumatoid arthritis (for which the first line of treatment is NSAIDs) when compared to age-matched controls. A link between osteoarthritis (where the first line treatment is also NSAIDs) and a decreased risk of Alzheimer's disease was found by Breteler *et al.* (1991) who suggested that long term use of NSAIDs might cause the protective effects against Alzheimer's disease.

Since these initial reports a number of studies have addressed directly the association between Alzheimer's disease and use of NSAIDs. An Australian study by Broe *et al.* (1990) examined 400 control-matched pairs of Alzheimer's disease cases for incidence of arthritis and found a reduced odds ratio between Alzheimer's disease and arthritis. A

follow-up study with the same patients (Henderson *et al.*, 1992) found a significant inverse association between "heavy analgesic (including NSAIDs) use" and Alzheimer's disease. Using data from the Canadian study of health and ageing (McDowell *et al.*, 1994), a large case control study showed that a history of NSAID use resulted in a lower risk of developing Alzheimer's disease. A reduction in the risk of developing Alzheimer's disease in patients taking NSAIDs was also observed in a population based study of disease and disability in older people in Rotterdam, when compared to age, education and gender matched controls (Andersen *et al.*, 1995).

In 1996, McGeer and collegues collated the findings from seventeen published epidemiological studies which had investigated the possible link between NSAIDs or arthritis and Alzheimer's disease. Using statistical analysis to combine the results from these studies they found negative associations between the use of NSAIDs or arthritis and the development of Alzheimer's disease (McGeer *et al.*, 1996). The combined data also showed that NSAID users had a low odds ratio for developing Alzheimer's disease when compared to control subjects. This finding was supported by data analysed from the Baltimore longitudinal study of ageing (Stewart *et al.* 1997) in which subjects were tested on a range of neuropsychological tasks every two years. They reported that NSAIDs, with the exception of aspirin and paracetamol, were protective against Alzheimer's disease and they also found a decrease in the risk of developing Alzheimer's disease when the NSAIDs had been taken for longer than two years.

In order to control for the genetic influences possibly involved in developing Alzheimer's disease, Breitner *et al.* (1994) examined fifty elderly twin pairs (twenty six of which were monozygotic) for protection against Alzheimer's disease with NSAIDs. They discovered that anti-inflammatory drug use (including steroidal treatment and

NSAIDs) was protective against Alzheimer's disease, although when NSAID protection alone was analysed, it was not significant, probably due to the small number (six) of twins in that group. In a follow-up investigation (Breitner *et al.*, 1995), the association between NSAIDs and Alzheimer's disease in siblings whose family history showed a high risk of Alzheimer's disease was investigated. They reported that sustained use of NSAIDs resulted in a delay in the onset (by an average of eleven years) and a reduced risk of developing Alzheimer's disease.

In addition to the reduction in risk and onset of Alzheimer's disease, several studies have also shown that NSAIDs may slow the progression of cognitive decline after the onset of Alzheimer's disease. A clinical trial investigating the effect of indomethacin (150mg/day) on Alzheimer's diseased patients showed that the untreated groups performance on mini-mental state examination tests declined from baseline after six months, whereas the indomethacin group showed a slight improvement over baseline (Rogers *et al.*, 1993). However a high number of patients (20%) taking indomethacin withdrew from the trial with gastrointestinal problems. Another small double blind randomised clinical trial has shown that the NSAID, diclofenac, taken in conjunction with a gastro-protective agent, misoprostal, results in a cognitive improvement, as tested by a broad range of cognitive tests, in Alzheimer's diseased patients taking the NSAID (Scharf *et al.*, 1999).

From the epidemiological and limited clinical data presented above, there is strong evidence that NSAID use is associated with a reduced risk of developing Alzheimer's disease as well as with a delay in the onset of Alzheimer's. There is also evidence suggesting that NSAIDs may improve cognitive performance is patients suffering from Alzheimer's disease. The underlying mechanisms behind the effect of these NSAIDs are

not clearly understood and there have been no animal studies which have investigated the effects of NSAIDs on memory. The second part of this study will investigate the behavioural and cognitive effects of NSAIDs in a range of animal behavioural paradigms.

Chapter Two: Modulation of Ligand-Gated Ion Channels By NSAIDs

2.1: Introduction and rationale

The main aim of this chapter is to investigate the actions of a range of NSAIDs on several native neuronal ligand-gated ion channels. The rationale behind this is that fenamate NSAIDs have been reported to modulate rat brain $GABA_A$ receptors (Woodward *et al.*, 1994), and nicotinic ACh receptors (Zwart *et al.*, 1995) expressed in *Xenopus* oocytes. Also a preliminary study by Halliwell and Davey (1994) showed that GABA currents recorded from rat hippocampal neurones were positively modulated by mefenamic acid. The effect of non-fenamate NSAIDs on ligand-gated ion channels is still unclear; Woodward and colleagues (1994) report that indomethacin but not other non-fenamates can modulate $GABA_A$ currents in *Xenopus* oocytes and bi-phenyl acetic acid has been reported to have weak inhibitory actions at the $GABA_A$ receptors on rat hippocampal neurones (Halliwell *et al.*, 1995), and the isolated rat vagus nerve (Green & Halliwell, 1997).

The rat isolated vagus and optic nerves have proved useful preparations to investigate the mechanism, site and selectivity of compounds on a number of ion channels that are present on these nerves (Marsh, 1989).

The first part of this chapter will investigate the effects of known modulators on the $GABA_A$, 5-HT_3, nicotinic ACh (nACh) and P2x receptors present on the vagus nerve and the $GABA_A$ and glycine receptors present on the optic nerve, in order to ascertain the pharmacological validity of these preparations. The second part of this chapter will examine the actions of fenamate and non-fenamate NSAIDs on agonist evoked-responses from the isolated rat vagus and optic nerves.

2.2: Methods and Materials

2.2i: The extra-cellular recording technique

The extra-cellular "grease-gap" recording technique has been used by many investigators to investigate the pharmacology of neuronal ligand-gated ion channels. For example several studies have shown concentration-dependent depolarisations evoked by GABA (Green & Halliwell 1997; Patten *et al.*, 2001); 5-HT (Ireland & Tyers, 1987; Bley *et al.* 1994) and ACh (Marsh, 1989; Green & Halliwell 1997) in the rat isolated vagus nerve. ATP and α,β-methylene ATP also evoke depolarisations through activation of P2x receptors in the rat vagus nerve (Trezise *et al.*, 1993; Green & Halliwell, 1997) and rat sympathetic ganglia (Connolly, 1995). Concentration-dependent depolarisation responses were also evoked by glycine and GABA in the rat isolated optic nerve (Simmonds, 1983; Patten *et al.*, 2001).

The extra-cellular recording technique used in this study to investigate the potential modulation of ligand-gated ion channels by NSAIDs is based on a method devised by Green and Halliwell (1997) and is described below.

2.2ii: Animals

Male Wistar rats (250g - 400g), bred in-house and maintained under standard laboratory conditions of a light (07:00-19:00)/dark cycle with *ad libitum* access to food and water, were killed by a rising concentration of CO_2.

2.2iii: Dissection

Vagus nerve dissection:

The skin surrounding the throat area was removed exposing the thyroid glands, the sternomastoideus, posterior digastricus and sternohyoideous muscles, which were carefully removed. The vagus nerves could be seen lying adjacent to the common carotid arteries running bilateral to the trachea. Using fine forceps and small dissecting scissors, the vagus nerves were carefully separated from the carotid arteries and cut away at the nodose ganglion and at the point where they entered the thorax. The free vagus nerves were then placed in a dish of cold, oxygenated, physiologically buffered salt solution (PBS) and the connective tissue sheaths surrounding the nerves were removed using watchmaker forceps under a binocular dissecting microscope (Nikon SMZ-2B, Surrey, U.K.).

Optic nerve dissection:

Following sacrifice the cadavers were decapitated and the skin overlying the skull was removed exposing the dorsal surface of the skull, which was bisected using bone scissors. The bone on the skull was broken away with artery forceps to reveal the brain; bone around the eye orbit, above the zygomatic arch was also removed. The oculormotor muscles were then teased away, using fine forceps, to expose the optic nerves. The brain was then gently lifted caudally out of the skull cavity using a spatula, exposing the full length of the optic nerves which were cut at the optic chiasma and removed from the back of the eyeballs using small dissecting scissors. The free optic nerves were then transferred to a dish of cold oxygenated PBS.

2.2iv: Electrophysiology

Extra-Cellular Recording from the Excised Rat Vagus and Optic Nerves:

A thin seam of high vacuum silicone grease (British Drug Houses (BDH), Poole U.K.) was placed halfway across the width of a microscope slide. A T-shaped piece of one-way nappy liner (Mothercare, Herts U.K.) soaked in PBS was placed on either side of the grease seal and served to facilitate perfusion of the nerve and removal of PBS and drugs from the nerve. Freshly excised vagus or optic nerve was placed across the grease seam and another layer of grease placed across the first grease layer and nerve to create a high resistance seal around the middle portion of the nerve. The slide was then placed onto a perspex frame housed inside a Faraday cage (built in-house). Silver-silver chloride recording electrodes (RC1 electrodes, Clark Electromedical, Kent, U.K.) were positioned onto the nappy liner on either side of the grease seam, adjacent to the free ends of the nerve (figure 2.1).

PBS and drugs were dripped onto the free ends of the nerve via 21G hypodermic needles, (positioned 1cm above the slide) at a rate of 2ml per minute using a variable speed peristaltic pump (Gilson Miniplus 3, Villers le Bel, France). Before agonists were applied to the nerves, the potential difference across the grease seal was allowed to equilibrate, this generally took between twenty minutes and one hour and is thought to be due to the nerve ends sealing over and redistribution of ions across the nerve membrane (Marsh, 1989).

Agonist-evoked direct current (DC) potentials across the grease seam were recorded, at ambient room temperature (20-23°C), via the silver-silver chloride electrodes. The current was relayed through miniature coaxial cable to a Neurolog amplifier (NL 100, Digitimer, Hertfordshire, U.K.) and filter (NL 125) with signals filtered between DC

and 50Hz. The signal was then relayed to a flatbed chart recorder (Sycopel, Tyne & Wear, U.K.) where agonist-evoked changes in DC potential were detected and recorded between 0.01mV and 5.0mV. Baseline noise was generally less than 0.02mV and was caused mainly by the dripping of PBS onto the nappy liner. The noise could be reduced by a slight repositioning of the perfusion needles and/or recording electrodes.

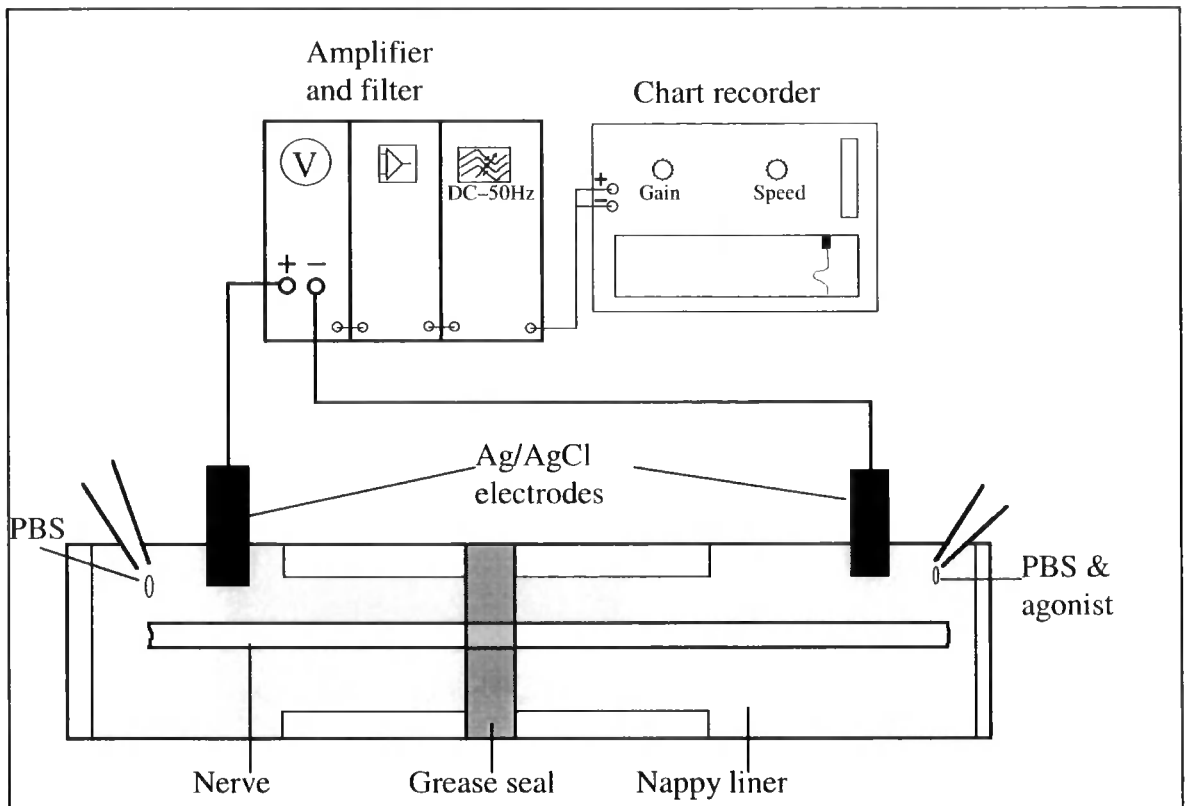


Figure 2.1: Schematic of the extra-cellular recording technique. A vagus or optic nerve was placed onto a glass slide across a grease seal barrier. The nerve was perfused with a physiologically balanced salt solution (PBS). Agonist -evoked responses were recorded extracellulary via Ag/AgCl electrodes, amplified, filtered (DC - 50Hz), then displayed and stored on a flat-bed pen chart recorder.

Experimental Protocol:

Agonists, dissolved in PBS, were applied *via* the perfusion system to one side of the nerve with PBS being applied to the other side of the nerve. Pilot studies showed that a two-minute administration of agonist elicited a clear equilibrium peak. These studies also showed a ten to fifteen minute interval between agonist application resulted in

stable and repeatable agonist responses. Concentration effect curves were constructed using a quasi-random order of agonist concentration applications.

To investigate the effect of drugs on agonist-evoked responses, the drug was added to the bath solution and continuously perfused onto both ends of the nerve for two minutes prior to agonist application (unless stated otherwise). Agonist concentrations that were approximately 50% of the maximum agonist-evoked response or 20% of the maximum agonist evoked response were then observed in the presence of drug. These sub-maximal agonist concentrations were applied to the nerve until two consecutive responses of agonist resulted in equal (± 10%) depolarisation responses. A full agonist concentration effect curve in the presence of a drug could then be obtained.

2.2vi: Data analysis

Agonist responses were measured from baseline to peak amplitude of response and expressed as the mean ± standard error of mean (s.e.m. of n experiments). In the case of concentration-effect experiments, agonist-induced depolarisations are expressed as a percentage of the maximum control agonist response.

The concentration-effect data was then plotted and fitted by a least squares fitting, non-linear regression analysis (a sigmoidal concentration response curve with a variable slope; Graphpad Prism v2.1) to the following logistic equation:

$$R = R_{max} \times \{[A]^{nH} / ([A]^{nH} + EC_{50}{}^{nH})\}$$

Where R and R_{max} represent the response evoked by the agonist concentration, [A], and a saturating concentration, respectively. EC_{50} is the concentration evoking half of the maximal response and nH is the Hill slope.

From this analysis, the mean concentration of drug that resulted in 50% of the control maximum response (EC_{50}) or the mean concentration of drug which resulted in a 50% inhibition of control agonist response (IC_{50}) and the 95% confidence intervals (95% C.I.) were obtained. The Hill slopes (± s.e.m) reported were also calculated from the curve fit. Hill slopes from concentration effect curves in the presence of varying concentration of NSAID were compared statistically using ANOVA, followed by Student Newman-Keuls *post hoc* analysis if the overall analysis was significant ($p \leq 0.05$). Care should be taken when interpreting these Hill slopes since the final concentration of agonist that reaches the receptor may be different from the concentration applied exogenously. For example, the degree of agonist uptake/metabolism within the neuronal preparation is unknown and may reduce agonist concentrations at the receptor site (Simmonds, 1990).

Where agonists are applied in the presence of a drug, the data is presented as a percentage of the control agonist response height. Concentration effect curves in the presence of a drug were presented as percentage of the control maximum agonist response.

Agonist responses in the presence of a NSAID (at a test concentration of 100μM) were analysed statistically by comparing the depolarisation responses in the absence and presence of the NSAID, with a paired Student's t-test (two-tailed). If there was a significant difference in agonist response heights, a concentration effect curve for that NSAID against sub-maximal agonist concentrations was then performed. Concentration

effect curves for the agonist in the presence of a range of concentrations of the NSAID were also constructed.

2.2vii: Drugs and solutions

PBS was made up in ultra pure double distilled deionised water (Milli-Q_{plus}, Millipore, Hertfordshire, U.K.) with the following analytical grade compounds obtained from BDH unless otherwise stated (in mM): 118 NaCl, 1.18 KH_2PO_4, 4.7 KCl, 1.18 $MgSO_4$, 2.5 $CaCl_2$, 11 glucose and 10 HEPES (Sigma, Poole, U.K.). The PBS was then titrated to pH 7.2 using 2.5M hydrochloric acid.

All drugs were supplied by Sigma unless otherwise stated. GABA and glycine were each dissolved in PBS as 1M stock solutions. 5-HT and α,β-methyleneadenosine 5'-triphosphate (α,βMeATP) were dissolved in PBS as 10mM stock solutions. 1,1-dimethyl-4-phenylpiperazinium (DMPP) was dissolved in PBS to give a 1mM stock solution. All compounds were serially diluted as required in PBS.

Bicuculline was dissolved in a small volume (~ 0.5mL) of 1mM hydrochloric acid then diluted down to 1mM stock with double distilled deionised water. Sodium pentobarbitone was made up as a 1mM stock solution in PBS. 1αH,3α,5αH-tropan-3-yl-3,5-di-chlorobenzoate (MDL 72222) was dissolved in 1M hydrochloric acid to give a 1mM stock solution. Hexamethonium was dissolved in PBS to give a 10mM stock solution. Pyridoxyalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS; Tocris, Bristol, U.K.) was made as a 10mM stock solution in double distilled deionised water. Strychnine was made as a 1mM stock in double distilled deionised water. Propofol (kindly provided by Organon Laboratories, Newhouse, Scotland) was dissolved in

absolute ethanol as a 100mM stock solution and then serially diluted in PBS (the maximal concentration of ethanol used was < 0.1%).

Mefenamic acid, acetylsalicyclic acid and indomethacin were each made up in 0.1M NaOH to give 10mM stock solutions. Niflumic acid, flufenamic acid and meclofenamic acid were each dissolved in 0.1M NaOH as a 50mM stock solutions. Ibuprofen was dissolved in 0.1M NaOH to give a 100mM stock solution. 4-biphenylacetic acid (BPAA) was made up as a 100mM stock in absolute ethanol.

2.3: Results

2.3i: Pharmacological characterisation of GABA, 5-HT, DMPP and α,βMeATP evoked responses in the isolated rat vagus nerve

GABA (3μM - 3mM), 5-HT (0.1μM - 30μM), DMPP (3μM - 1mM) and α,βMeATP (1μM - 300μM) each evoked concentration-dependent depolarisations of the rat isolated vagus nerve (figure 2.2). EC_{50}'s (95% C.I.; for n experiments) for GABA, 5-HT, DMPP and α,βMeATP were 45μM (38μM - 53μM; n=23), 1μM (0.9μM - 1.1μM; n=36), 26μM (22μM - 29μM; n=40) and 47μM (38μM - 58μM; n=19) respectively. Hill slopes for these agonists were calculated to be 1.0 ± 0.11 for GABA, 1.3 ± 0.07 for 5-HT, 1.0 ± 0.11 for α,βMeATP and 1.5 ± 0.09 for DMPP.

Concentrations of each agonist approximating their respective EC_{50}'s were used to investigate the effects of control drugs and NSAIDs. Responses to GABA (50μM), 5-HT (1μM), DMPP (30μM) and α,βMeATP (30μM) evoked depolarisations (mean ± s.e.m) of 0.60 ± 0.04mV (n=90), 0.73 ± 0.04mV (n=57), 0.52 ± 0.04mV (n=48) and 0.86 ± 0.08mV (n=20), respectively. In some experiments, designed to examine the effects of positive allosteric modulators, a concentration approximating the GABA EC_{20} of 10μM was used, which evoked a depolarisation of 0.09 ± 0.01mV.

The potencies of the four agonists tested were compared to that of a 1mM GABA response (figure 2.3). The largest depolarisation was observed with α,βMeATP (300μM) of 1.98 ± 0.24mV, this was followed by 5-HT (30μM) with 1.44 ± 0.1mV; GABA (10mM) with 1.38 ± 0.17mV and DMPP (100μM) with 0.98 ± 0.1mV.

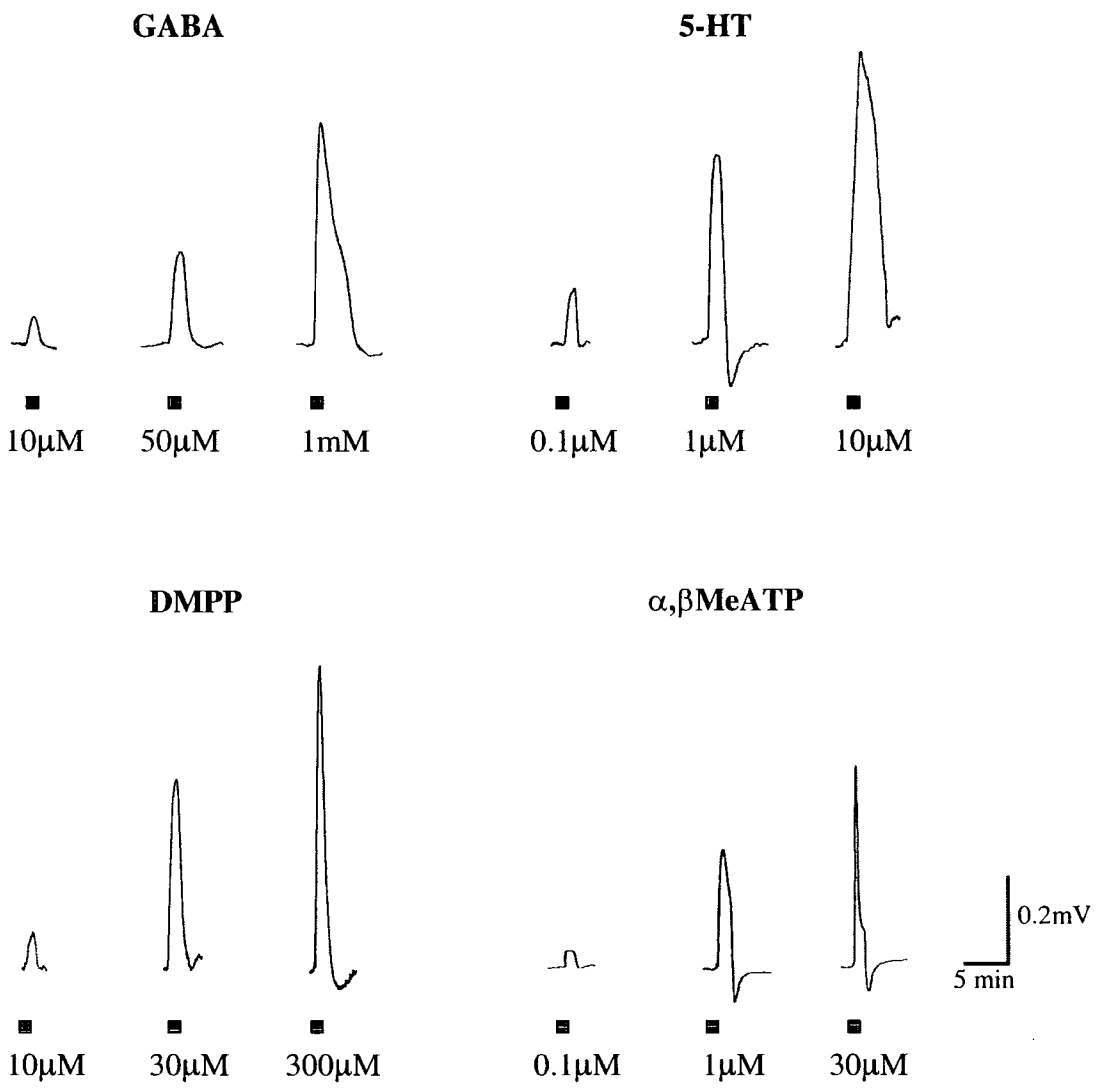


Figure 2.2 Electrophysiological recordings of GABA-, 5-HT-, DMPP- and α,βMeATP- evoked depolarisations in the rat isolated vagus nerve. The figure shows chart recorder traces of GABA-, 5-HT-, DMPP- and α,βMeATP- evoked depolarisations. The agonist applied is given above triplets of responses and the concentration of agonist is given below each individual response, the agonist contact time is represented by a solid bar beneath each trace.

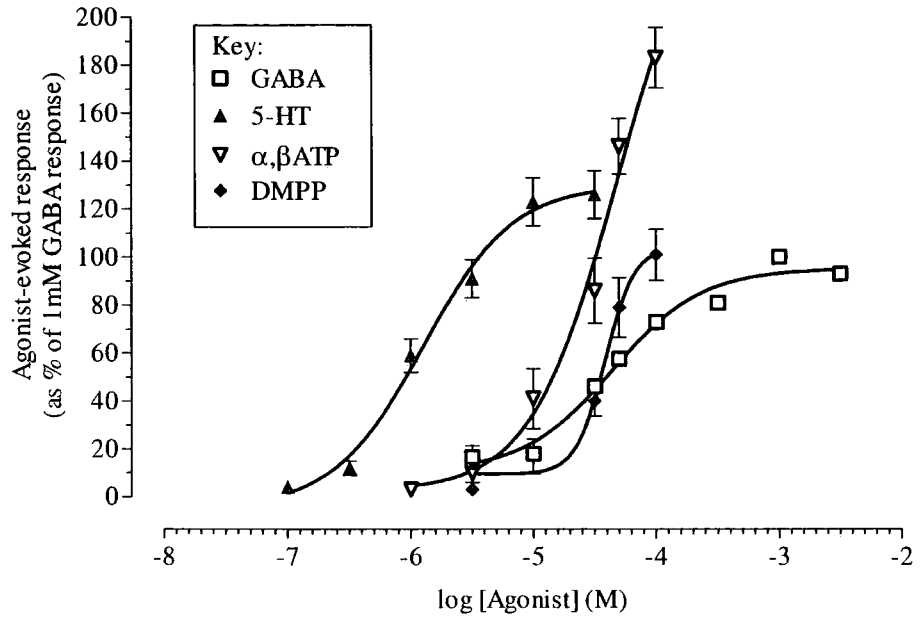


Figure 2.3: GABA, 5-HT, DMPP and α,βMeATP each evoke concentration-dependent responses in the rat isolated vagus nerve.
The figure shows log concentration response curves for GABA, 5-HT, DMPP and α,βMeATP. The $\log_{10}$ of the agonist concentration is shown on the x-axis and the agonist response, expressed as % of 1mM GABA- evoked response is shown on the y-axis. Each data point is the mean ± s.e.m of 23, 36, 40 and 19 experiments for GABA, 5-HT, DMPP and α,βMeATP respectively.

In order to establish the type of receptors mediating the depolarisations on the rat isolated vagus nerve, the selective antagonist, bicuculline, for the $GABA_A$ receptor, MDL 72222, for the $5\text{-}HT_3$ receptor, hexamethonium, for the nicotinic ACh receptor and PPADS for the $P2_x$ receptor were applied to the nerves.

Bicuculline (0.3μM - 10μM) caused a concentration-dependent inhibition of the GABA (50μM) evoked responses (figures 2.4; 2.5), with an IC_{50} of 1.4μM (1.1μM - 1.8μM; n=6). Bicuculline (10μM) resulted in complete abolition of the GABA response. The effects of bicuculline were fully reversible upon washout and responses comparable to those of control were observed after thirty minutes of drug wash out.

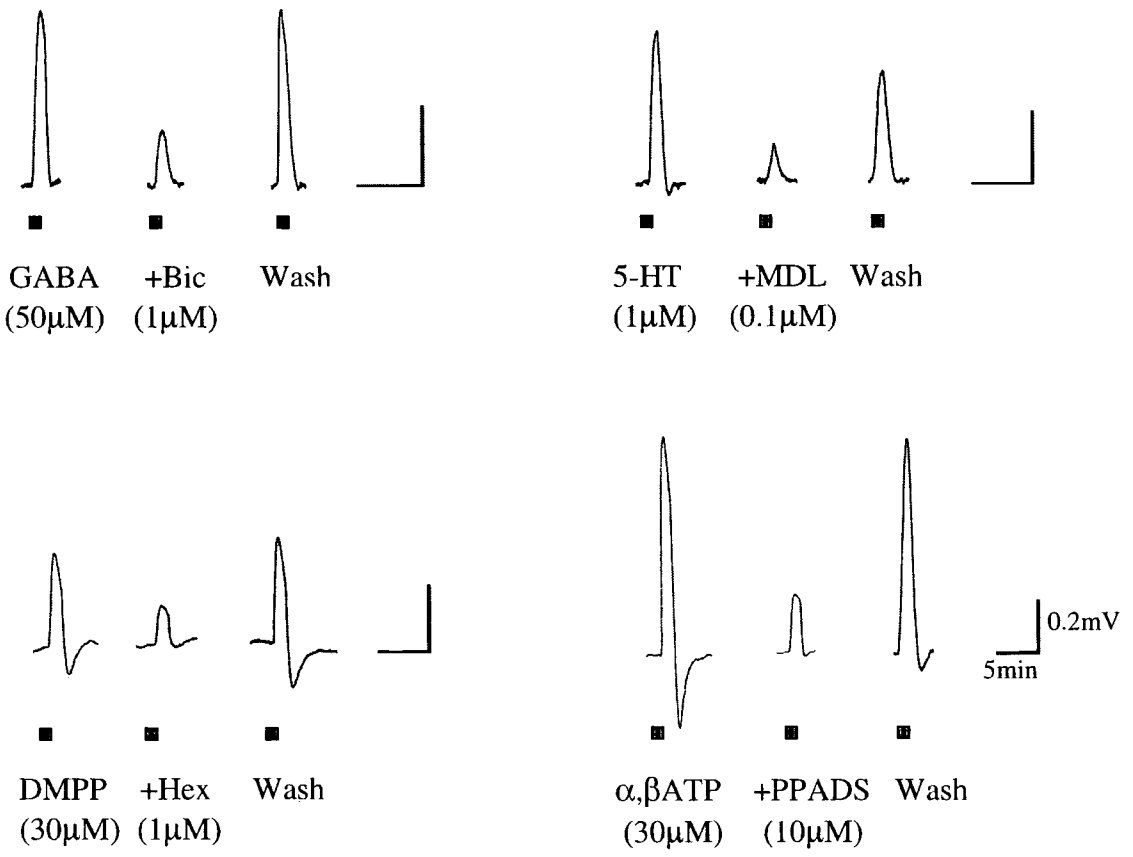


Figure 2.4: Electrophysiological recordings of GABA-, 5-HT-, DMPP- and α,βMeATP-evoked responses inhibited by antagonists of the $GABA_A$, $5\text{-}HT_3$, nACh and P2x receptors, respectively. Examples of chart recorder traces showing sub-maximal GABA-, 5-HT-, DMPP- and α,βMeATP- evoked responses in the absence and presence of bicuculline (Bic; 1μM), MDL 72222 (MDL; 0.1μM), hexamethonium (Hex; 1μM) and PPADS (10μM) respectively. The concentration of agonist and antagonist applied to the isolated rat vagus nerve is indicated below each response, the agonist contact time is represented by the solid bar beneath each response.

Application of MDL 72222 (10nM - 300nM) resulted in a concentration-dependent inhibition of 5-HT (1μM) responses (figure 2.4) with an IC_{50} of 36nM (25nM - 52nM; n=4; figure 2.5). It was observed that the maximum inhibition of the 5-HT response was 80 ± 4% of the control response with MDL 72222 (300nM). The effects of MDL 72222 were only partially reversed following extensive (≥ 90 minutes) washing.

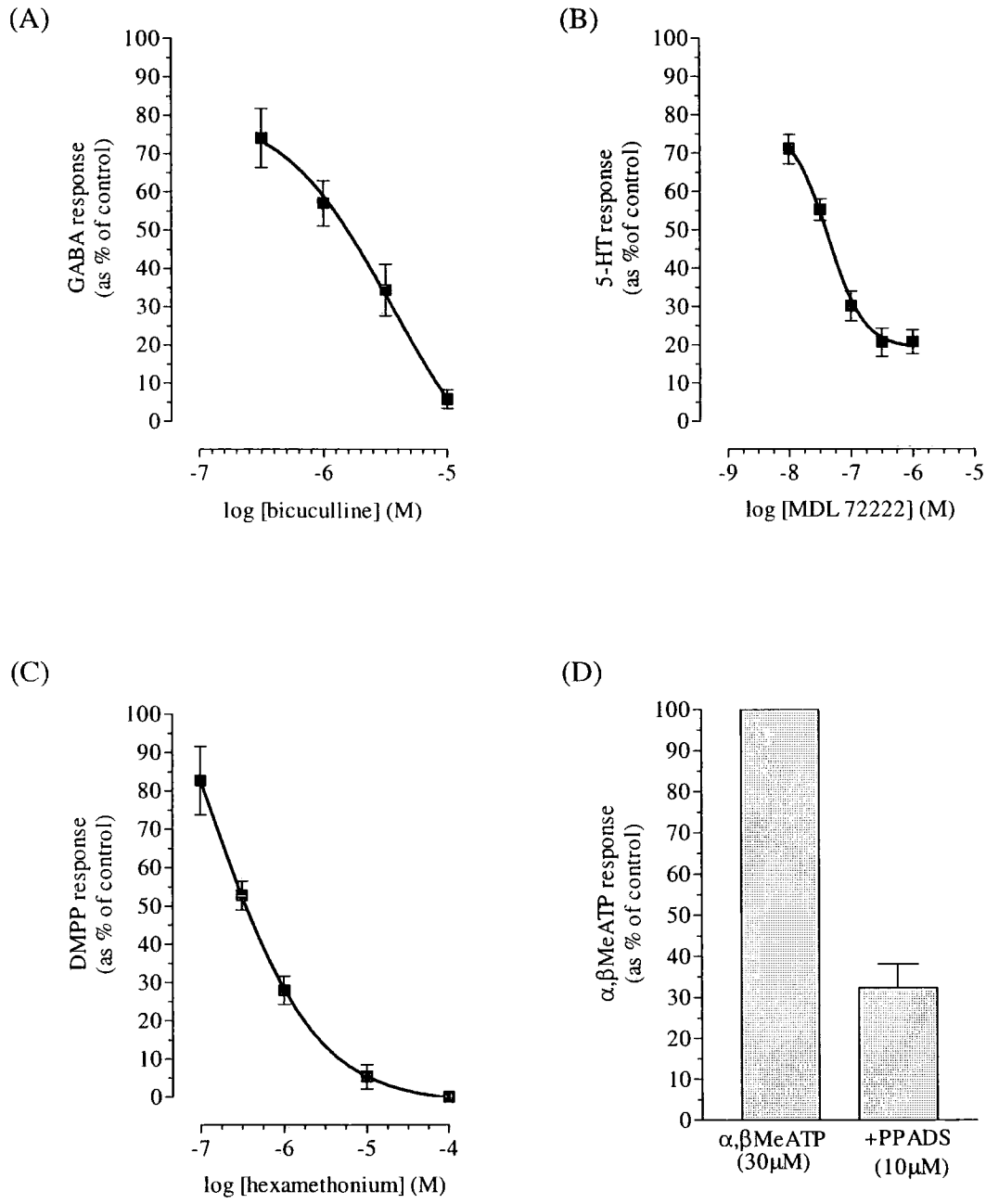


Figure 2.5: Bicuculline, MDL 72222, hexamethonium and PPADS inhibit sub-maximal GABA-, 5-HT-, DMPP-, and α,βMeATP- evoked responses, respectively. Log_{10} concentration inhibition curves for (A) bicuculline on GABA (50μM) responses; (B) MDL 72222 on 5-HT (1μM) responses; (C) Hexamethonium on DMPP (30μM) responses. The y-axis of each graph is the response, expressed as % of control, and the x-axis is log_{10} concentration of antagonist. (D) Histogram summarising the inhibitory effects of PPADS on the α,βMeATP (30μM) response. Each data point represents the mean ± s.e.m. of 4-6, 4-8, 3-7 and 3 experiments for bicuculline, MDL 72222, hexamethonium and PPADS respectively.

Addition of hexamethonium (0.1μM - 100μM) resulted in a concentration-dependent inhibition of DMPP (30μM) responses (figure 2.4) with an IC_{50} of 393nM (302nM - 511nM; n=4). Hexamethonium (100μM) completely blocked the DMPP- evoked depolarisation (figure 2.5), which was fully reversible following thirty minutes of drug wash out.

PPADS (10μM) induced a 66% ± 6% (n=3) inhibition of the α,βMeATP (30μM) response (figures 2.4; 2.5). The effect was fully reversible after extensive drug wash out (≥ 90 minutes).

The known $GABA_A$ receptor modulators, sodium pentobarbitone and propofol, were investigated for their effects on the GABA (10μM) responses in the rat isolated vagus nerve.

Sodium pentobarbitone (10μM - 300μM) concentration-dependently potentiated GABA (10μM) responses (figure 2.6), with a maximum enhancement to 345 ± 26% of control response at 300μM (n=4). The effect of pentobarbitone was fully reversible upon extensive washout (≥ 90 minutes).

Propofol (1μM - 10μM) concentration-dependently potentiated GABA (10μM) evoked responses with a maximal enhancement to 458 ± 53% (n=25) of control in the presence of 10μM propofol (figure 2.6). GABA responses were still potentiated to 210% ± 9% of control even after ninety minutes of washout of propofol (10μM).

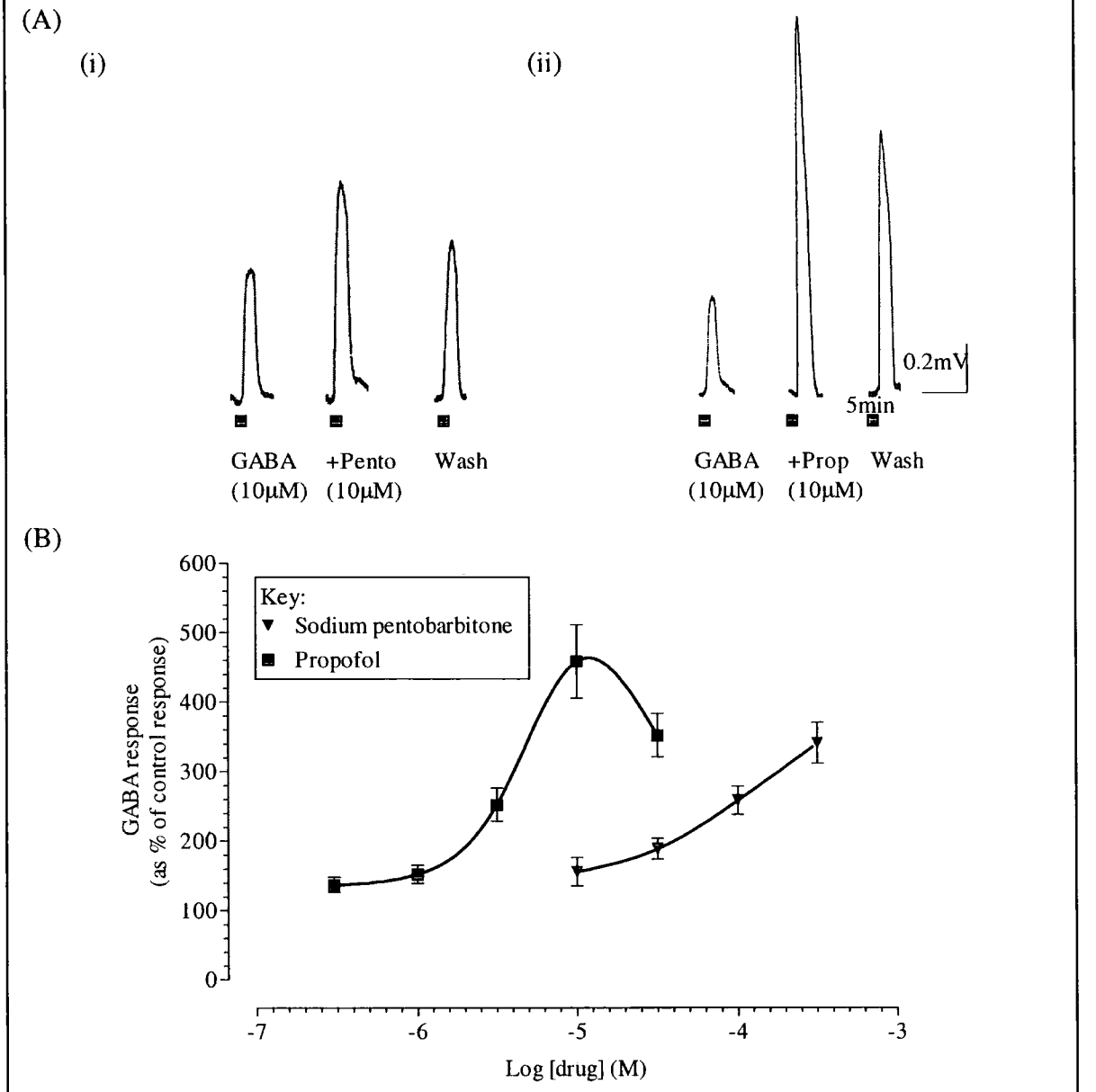


Figure 2.6: Sodium pentobarbitone and propofol potentiate sub-maximal GABA-evoked responses in the isolated rat vagus nerve. (A) Examples of chart recorder traces of GABA (10μM)-evoked responses potentiated by (i) sodium pentobarbitone (10μM) and (ii) propofol (10μM). The drug concentration applied to the nerve and agonist contact time, represented by the solid bar, are indicated below each response. (B) Log concentration effect curves for the potentiation of GABA (10μM) responses by propofol (0.3μM - 30μM) and sodium pentobarbitone (10μM - 300μM). The $\log_{10}$ of the modulator is plotted on the x-axis and the responses, as % of control, plotted on the y-axis. Each data point represents the mean ± s.e.m. of 8-12 and 4-7 experiments for propofol and sodium pentobarbitone, respectively.

These results are consistent with data from other studies using extracellular recording techniques for the activation of the $GABA_A$, $5\text{-}HT_3$, nACh and P_{2x} receptors in the rat isolated vagus nerve (e.g. Patten *et al.*, 2001; Green & Halliwell, 1997; Trezise, 1993; Ireland & Tyers, 1987).

2.3ii: Pharmacological characterisation of GABA and glycine evoked responses on the rat isolated optic nerve

In the rat isolated optic nerve, application of GABA (100μM - 30mM) or glycine (100μM - 30mM) evoked concentration-dependent depolarisations (figure 2.7) with EC_{50} values (95% C.I.) of 1mM (800μM - 2.3mM; n=6) and 3mM (2.4mM - 3.8mM; n=5) for GABA and glycine, respectively. The Hill slopes for GABA and glycine were 1.0 ± 0.10 and 1.3 ± 0.17, respectively. Sub-maximal concentrations of 1mM for GABA and glycine (approximating 50% and 25% of maximum response, respectively) were used in subsequent experiments and resulted in depolarisations of 0.35 ± 0.03mV (n=9) and 0.25 ± 0.04mV (n=7), respectively. Maximum responses to GABA (30mM) and glycine (30mM) resulted in depolarisations of 0.68 ± 0.04mV (n=9) and 0.82 ± 0.13mV (n=7), respectively.

The selective antagonists, bicuculline for the $GABA_A$ receptor, and strychnine for the glycine receptor, were investigated for their inhibitory actions on the GABA- and glycine- evoked responses in the rat isolated optic nerve.

GABA (1mM) responses were inhibited in a concentration-dependent fashion by bicuculline (3μM - 30μM; figure 2.8) with an IC_{50} of 18μM (13μM - 24μM; n=4). The

GABA response was completely inhibited in the presence of bicuculline (100μM). The effects of bicuculline were completely reversible upon thirty minutes of drug washout.

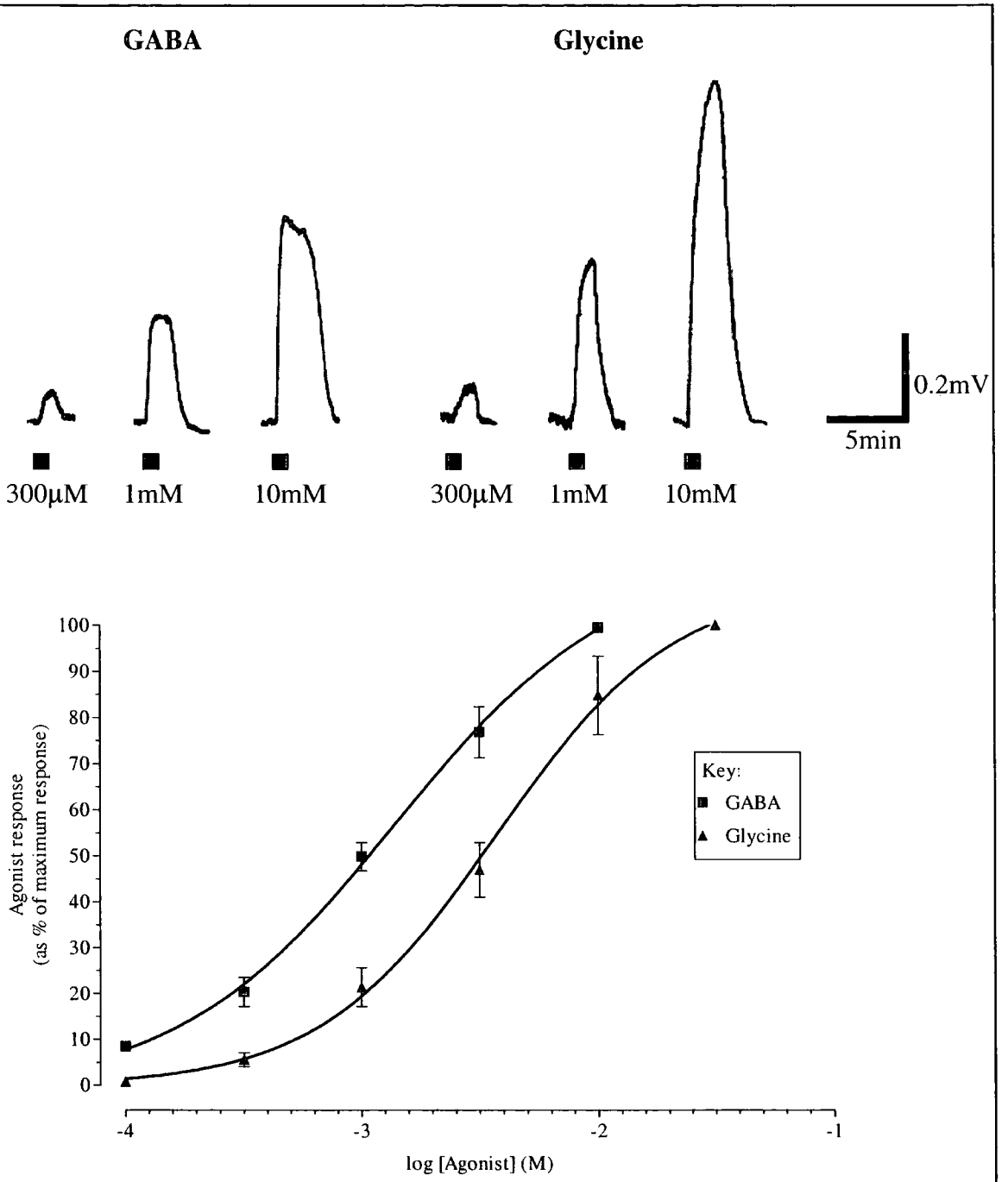


Figure 2.7: GABA and glycine each evoke concentration-dependent depolarisations in the isolated rat optic nerve. (A) Examples of chart recorder traces of GABA- (top left) and glycine- (top right) evoked responses. The agonist concentration and contact time, represented as a solid bar, is shown below each response. (B) Log concentration-effect curves for GABA and glycine. The $\log_{10}$ agonist concentration is given on the x-axis and the response, expressed as % of maximum agonist response, is given on the y-axis. Each data point is the mean ± s.e.m. of 5 experiments for GABA and glycine.

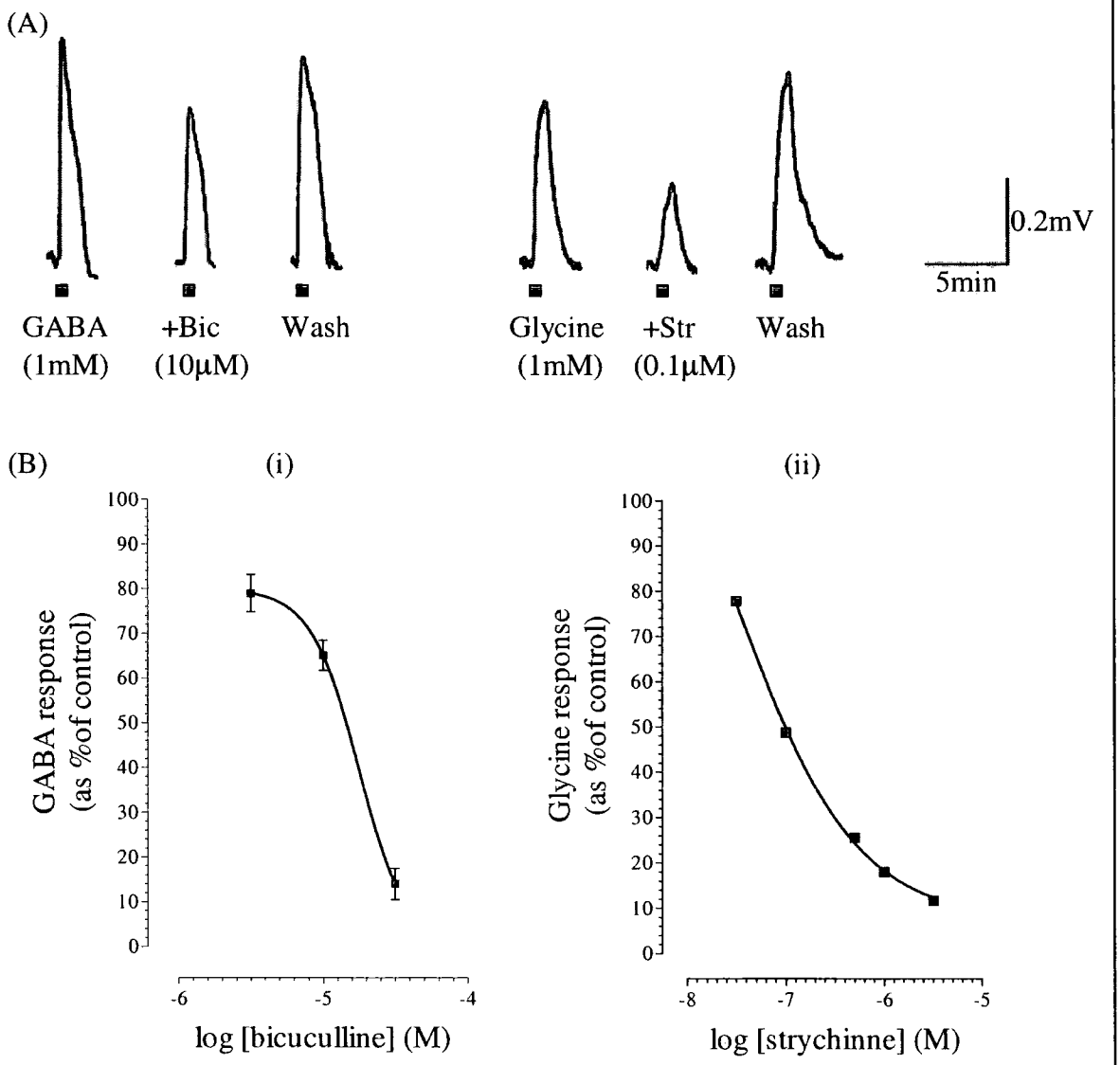


Figure 2.8: GABA and glycine- evoked responses are inhibited by bicuculline and strychnine respectively. (A) Example chart recorder traces of sub-maximal GABA- and glycine- (both 1mM) evoked responses in the absence and presence of bicuculline (10μM) and strychnine (0.1μM), respectively. The drug concentrations applied to the isolated rat optic nerve and agonist contact time, represented as a solid bar, are given below each response. (B) Log-concentration inhibition curves for (i) bicuculline on GABA (1mM) responses and (ii) strychnine on glycine (1mM) responses. The x-axis for each graph is the $\log_{10}$ concentration of the antagonist and the y-axis is the response expressed as % of control. Each data point is the mean ± s.e.m. of 4-10 and 3-4 experiments for GABA and glycine respectively.

Strychnine caused a concentration-dependent inhibition (0.01μM - 3 μM) of glycine (1mM) responses (figure 2.8) with an IC_{50} of 130nM (94nM - 179nM; n=4). The glycine response was abolished in the presence of 3μM strychnine. The effect of strychnine on glycine responses was completely reversible upon thirty minutes of drug washout.

This data is consistent with that from other studies using the extracellular recording technique for activation of the $GABA_A$ and strychnine-sensitive glycine receptors in the rat isolated optic nerve. (e.g. Patten *et al.*, 2001; Green & Halliwell, 1997; Simmonds 1983)

2.3iii: Determination of the effects of fenamate NSAIDs on $GABA_A$, 5-HT_3, nicotinic ACh and P2x receptors.

Agonist evoked- responses in the presence of fenamate NSAIDs (initially at 100μM) were compared to the control responses using a paired Student t-test (two-tailed). On the isolated rat vagus nerve, only mefenamic acid significantly enhanced the $GABA_A$ receptor- mediated response (t_5= 4.20, $p \leq 0.05$). In contrast, flufenamic acid significantly inhibited nACh (t_4= 3.58, $p \leq 0.05$) and P2x (t_6= 4.88, $p \leq 0.05$) receptor-mediated responses; meclofenamic acid significantly inhibited $GABA_A$ receptor mediated- response (t_7= 6.48; $p \leq 0.05$) and mefenamic acid significantly inhibited 5-HT_3 (t_6= 7.91, $p \leq 0.05$) and P2x (t_6= 5.80, $p \leq 0.05$) receptor mediated- responses, whereas niflumic acid did not significantly change ($p \geq 0.10$) any agonist- evoked response. Additionally the fenamates did not significantly change the GABA or glycine responses in the isolated rat optic nerve. The effect of fenamate NSAIDs (100μM) on sub-maximal agonist evoked responses from the rat isolated vagus and optic nerves are summarised in table 2.1 and table 2.2 respectively.

Table 2.1: Fenamate NSAIDs modulate agonist- evoked responses in the rat isolated vagus nerve. Table showing the agonist responses, as a % of control ± s.e.m. for GABA, 5-HT, DMPP, α,βMeATP in the isolated rat vagus nerve after application of the fenamates (at 100μM): flufenamic acid (FFA), mefenamic acid (MFA), meclofenamic acid (Meclo) and niflumic acid (NFA). Results highlighted in **bold** are responses that were significantly ($p \leq 0.05$) different from control in the presence of a fenamate NSAID. n= number of experiments, t=test statistic with degrees of freedom in subscript.

Agonist (concentration)	**Fenamate** (at 100μM)			
	FFA	Meclo	MFA	NFA
GABA (50μM)	91±9% (n=3) (t_2= 0.71)	**41±6% (n=8)** **(t_7= 6.48)**	**155±11% (n=6)** **(t_5= 4.20)**	92±6% (n=6) (t_5= 1.38)
5-HT (1μM)	78±5% (n=7) (t_6= 1.19)	82±2% (n=4) (t_3= 0.80)	**43±2% (n=11)** (t_{11}= 7.91)	106±7% (n=7) (t_6=1.4)
DMPP (30μM)	**50±9% (n=5)** **(t_4=3.59)**	77±6% (n=4) (t_3=0.19)	76±2% (n=6) (t_5=6.30)	76±5% (n=4) (t_3=2.94)
α,βMeATP (30μM)	**59±7% (n=7)** (t_6=4.88)	106±16% (n=4) (t_3=0.03)	**65±6% (n=7)** **(t_6=5.80)**	79±4% (n=4) (t_3=1.26)

Table 2.2: Modulation of agonist responses by fenamate NSAIDs in the rat isolated optic nerve. Table showing the response as % of control ± s.e.m. for GABA, and glycine depolarisations in the isolated rat optic nerve in the presence of fenamates (at 100μM): flufenamic acid, mefenamic acid, meclofenamic acid and niflumic acid. None of the fenamates significantly affected ($p \geq 0.10$) these responses. n= number of experiments. t=test statistic with degrees of freedom in subscript. Abbreviations as in table 2.1, above.

Agonist (concentration)	**Fenamate** (at 100μM)			
	FFA	Meclo	MFA	NFA
GABA (1mM)	107±5% (n=4) (t_3=2.35)	87±6% (n=4) (t_3=0.17)	98±3% (n=10) (t_9=1.41)	89±6% (n=4) (t_3=2.16)
Glycine (1mM)	97±3% (n=6) (t_5=0.78)	100±3% (n=4) (t_3=0.40)	97±1% (n=4) (t_3=1.73)	108±4% (n=7) (t_6=1.62)

2.3iv: Experiments to address the mechanisms underlying the inhibition of nicotinic acetylcholine receptors by fenamates

Flufenamic acid (30μM - 300μM), inhibited DMPP responses in a concentration-dependent manner (figure 2.9).

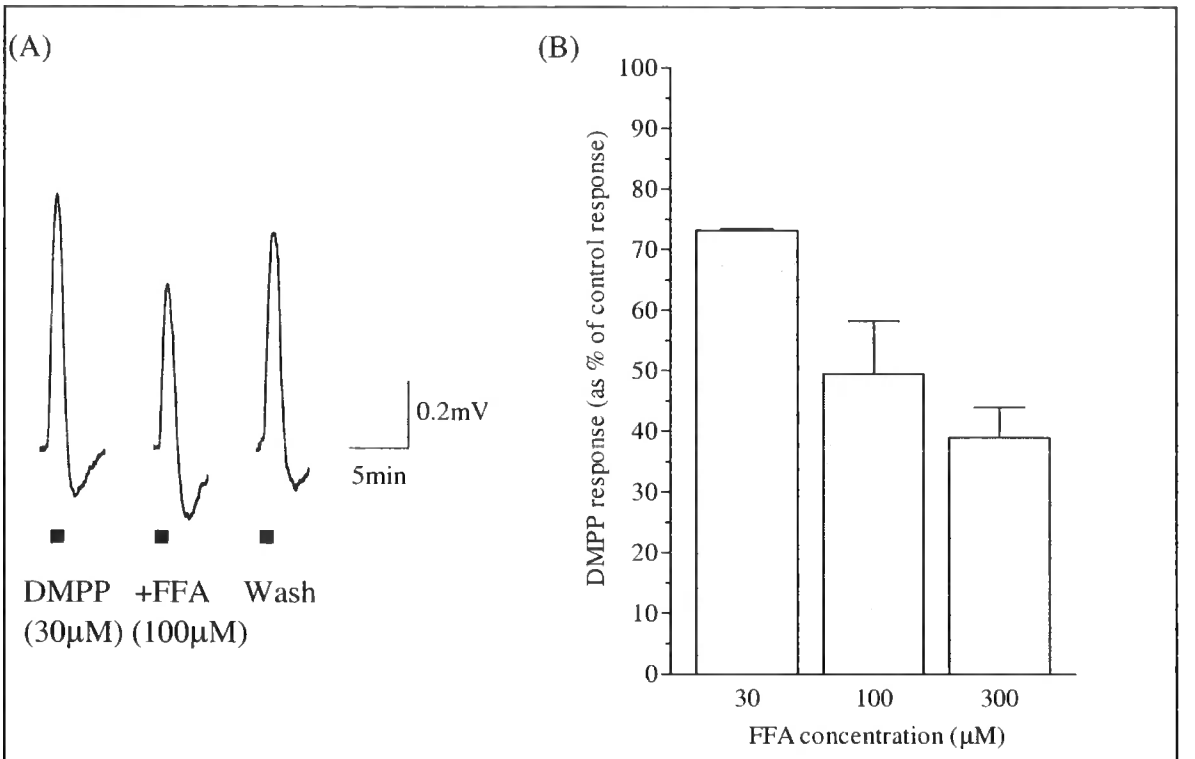


Figure 2.9: Flufenamic acid inhibits nACh-mediated responses. (A) Examples of chart recorder traces for DMPP- (30μM) evoked responses in the absence and presence of flufenamic acid (FFA; 100μM). The DMPP contact time is represented by the solid bar beneath each response. (B) Histogram showing DMPP (30μM) response, as % of control, in the presence of flufenamic acid (30μM - 300μM). Each data bar is the mean (± s.e.m.) of 3-4 experiments.

Concentration response curves to DMPP (3-300μM) were depressed and shifted to the right by increasing concentrations of flufenamic acid (30-300μM). The maximal DMPP response was decreased to 67±5%, 48±5% and 23±5% of the control maximal DMPP response in the presence of 30μM, 100μM and 300μM flufenamic acid respectively. These data are shown in figure 2.10. Hill slopes for DMPP were not significantly changed from control by flufenamic acid ($F_{3,18}$= 0.55, p≥ 0.10).

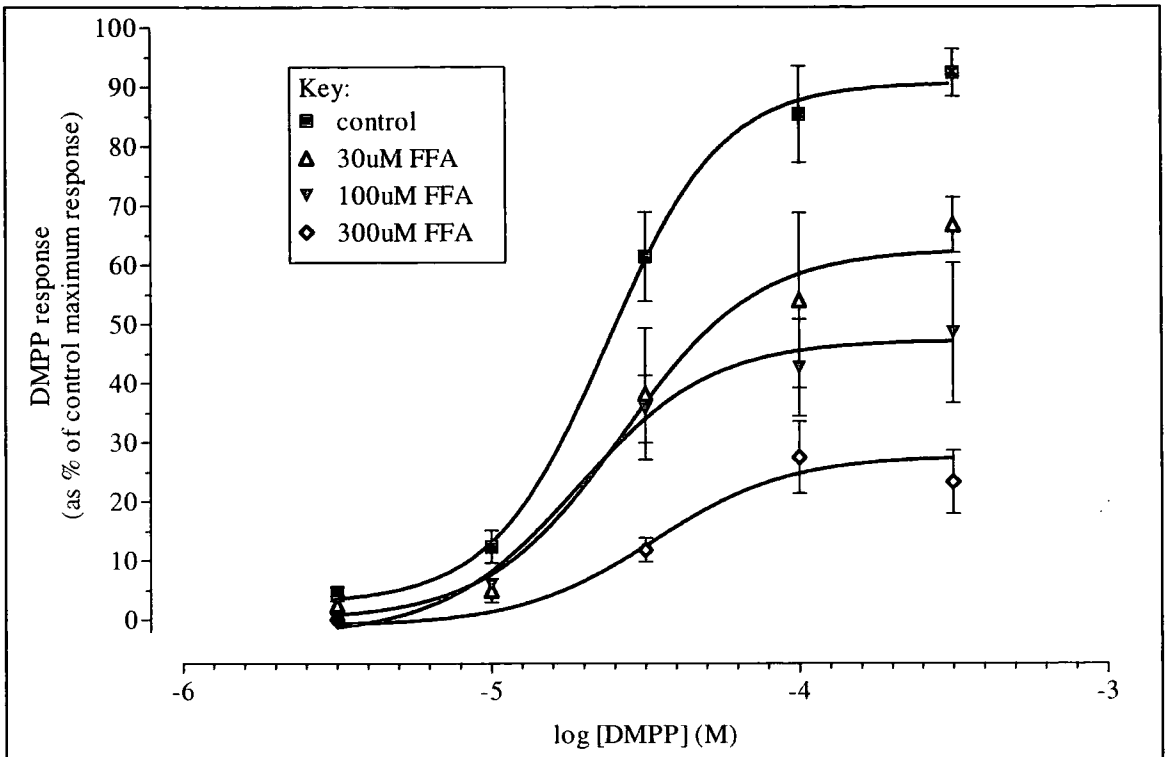


Figure 2.10: Flufenamic acid inhibits nicotinic ACh receptors in a non-competitive fashion. Graph showing log concentration-effect curves for DMPP in the absence and presence of flufenamic acid (FFA; 30μM - 300μM). The x-axis shows the $\log_{10}$ concentration of DMPP and the y-axis gives the DMPP response, represented as % of maximum control DMPP response. Each data point is the mean ± s.e.m. of 4-8 experiments.

This data suggests that flufenamic acid causes a weak non-competitive antagonism of nicotinic acetylcholine receptors.

2.3v: Experiments to address the mechanisms underlying the modulation of $GABA_A$ receptors by fenamates

GABA- evoked responses (50μM) were inhibited in a concentration dependent fashion manner by meclofenamic acid (30-300μM) as shown in figure 2.11.

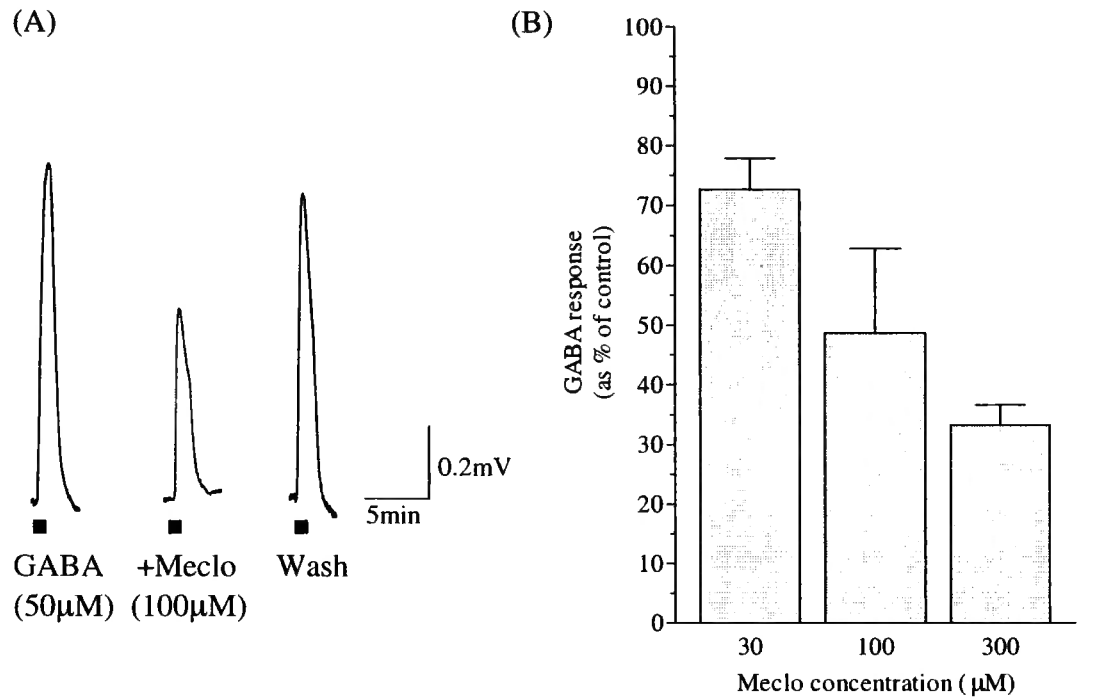


Figure 2.11: Inhibition of $GABA_A$ receptors by meclofenamic acid. (A) Examples of chart recorder traces for GABA- (50μM) evoked responses in the absence and presence of meclofenamic acid (Meclo; 100μM). (B) Histogram showing the % of GABA (50μM) control response in the presence of meclofenamic acid (30μM - 300μM). Each data bar is the mean ± s.e.m. of 3-10 experiments.

Concentration response curves to GABA (3μM –1mM) were depressed and shifted to the right by increasing concentrations of meclofenamic acid (30-300μM). The maximal GABA response was decreased to 77±7%, 55±5% and 36±4% of the control maximal GABA response in the presence of 30μM, 100μM and 300μM meclofenamic acid respectively, as shown in figure 2.12. Hill slopes were not significantly changed ($F_{3,13}$= 2.34, p≥ 0.10) by meclofenamic acid. This data indicates that meclofenamic acid causes a non-competitive like inhibition of $GABA_A$ receptors.

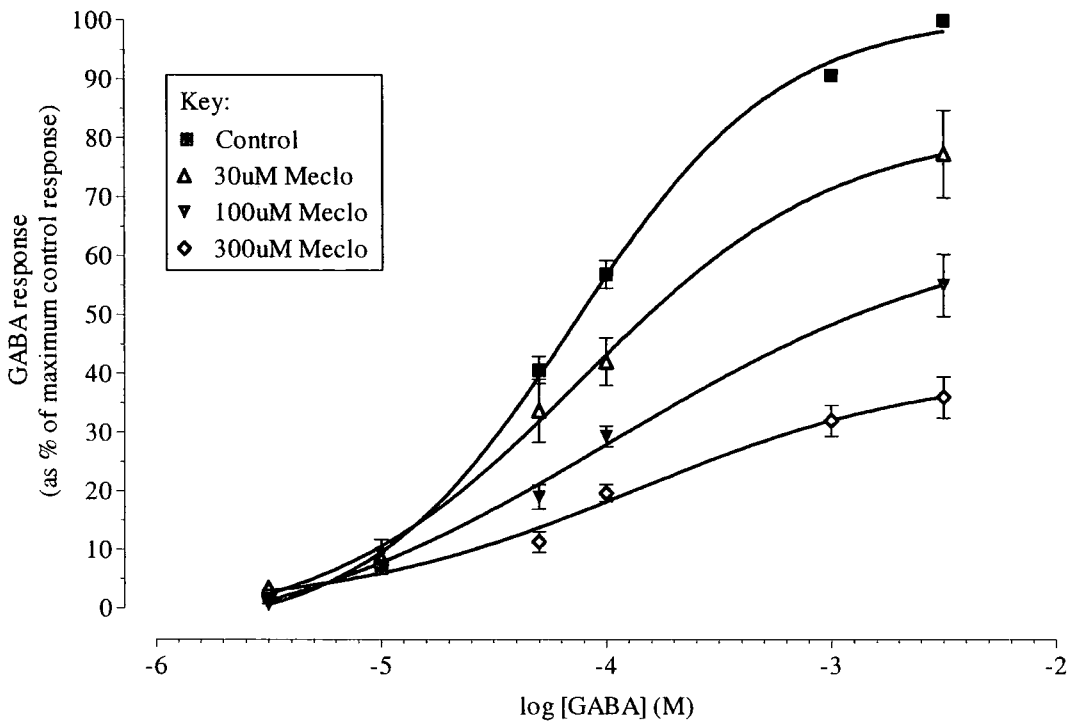


Figure 2.12: Non-competitive-like inhibition of $GABA_A$ receptors by meclofenamic acid. Graph showing log concentration-effect curves for GABA in the absence and presence of meclofenamic acid (Meclo; 30μM - 300μM). The x-axis is the log_{10} concentration of GABA and the y-axis is the GABA response represented as % of the control maximum GABA response. Each data point is the mean ± s.e.m. of 4 experiments.

In contrast to meclofenamic acid, mefenamic acid potentiated the GABA (50μM) - mediated response. Therefore, the effects of mefenamic acid on submaximal GABA (10μM) responses were further investigated. Mefenamic acid (10-100μM) concentration-dependently increased the amplitude of the GABA response with a maximum of potentiation of 153±10% (n=12) of control in the presence of mefenamic acid (30μM) as shown in figure 2.13. At mefenamic acid (100μM), the enhancement was lower than that of mefenamic acid (30μM), and was also associated with a shift in the baseline recording possibly due to direct activation of the $GABA_A$ receptor.

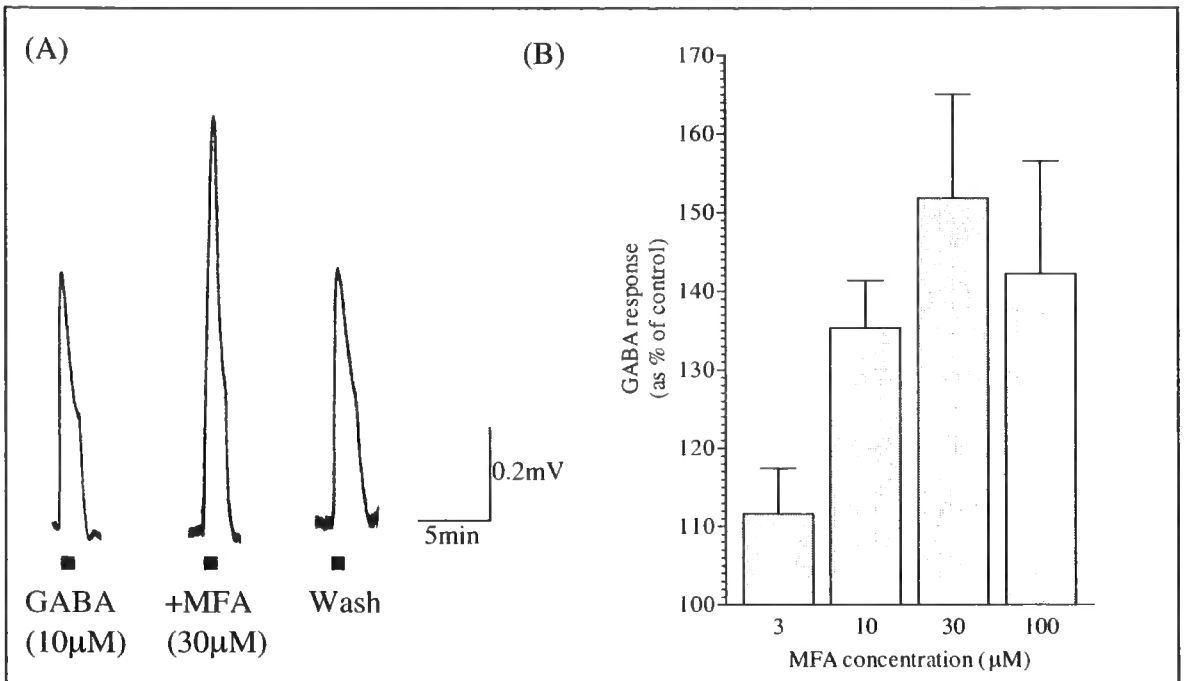


Figure 2.13: Concentration-dependent potentiation of $GABA_A$ receptor mediated responses by mefenamic acid. (A) Examples of chart recorder traces for GABA- (10μM) evoked responses in the absence and presence of mefenamic acid (MFA; 30μM). The agonist contact time is represented as a solid bar beneath each response. (B) Histogram showing the response, as a % of GABA (10μM) control, in the presence of mefenamic acid (3μM - 100μM). Each data bar is the mean ± s.e.m. of 3-5 experiments.

This data demonstrates that mefenamic acid can act as a positive modulator at the $GABA_A$ receptor.

2.3vi: Experiments to address the mechanisms underlying the inhibition of 5-HT_3 receptors by fenamates

Mefenamic acid (30μM - 300μM) inhibited 5-HT- (1μM) responses in a concentration-dependent manner (figure 2.14). Concentration effect curves to 5-HT were depressed and shifted to the right in a concentration-dependent manner by mefenamic acid (30-300μM). The maximal 5-HT response was decreased to 85±4%, 51±5% and 21±4% of control maximal 5-HT response in the presence of 30μM, 100μM and 300μM mefenamic acid, respectively, as shown in figure 2.15. Hill slopes for 5-HT in the

presence of mefenamic acid were not significantly different from the control Hill slope for 5-HT ($F_{3,22}$= 0.66, p≥ 0.10).

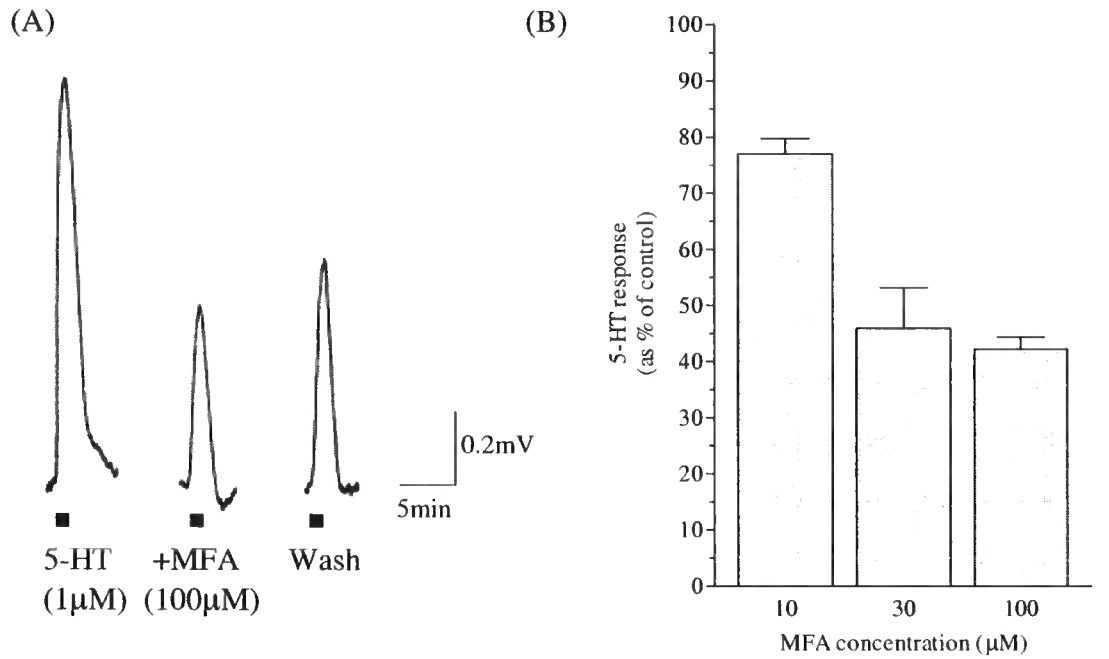
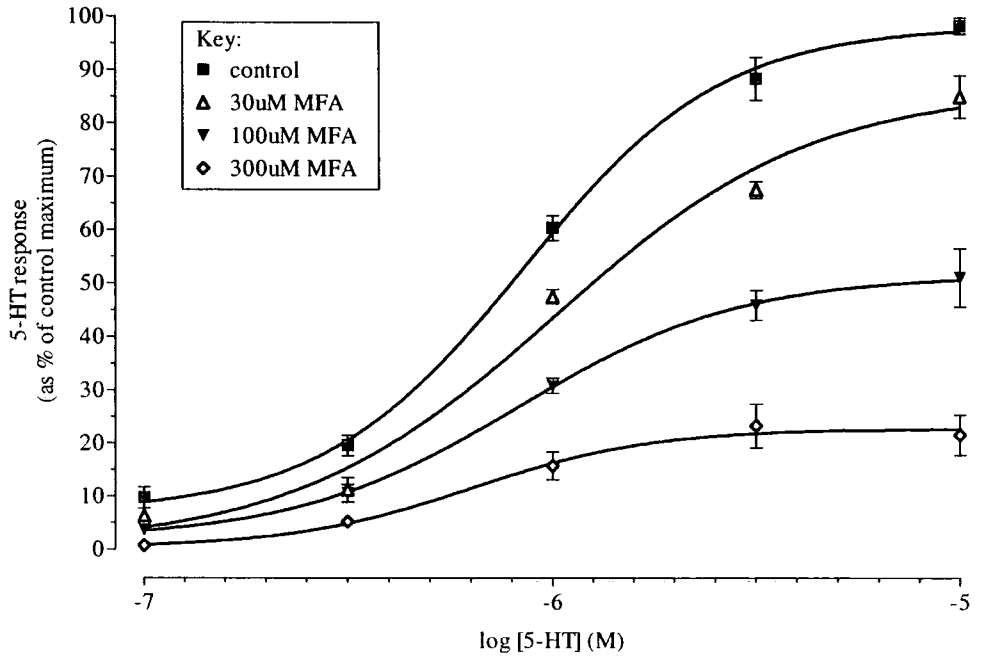


Figure 2.14: Concentration-dependent inhibition of 5-HT_3- receptor mediated responses by mefenamic acid. (A) Examples of chart recorder traces for 5-HT- (1μM) evoked responses in the absence and presence of mefenamic acid (MFA; 100μM). The agonist contact time is represented as a solid bar beneath each response. (B) Histogram showing the 5-HT (1μM) response in the presence of mefenamic acid (10μM - 100μM) as % of control. Each data bar is the mean ± s.e.m. of 7-10 experiments.

These results demonstrate that mefenamic acid causes a weak non-competitive antagonism of 5-HT_3 receptors.



2.15: Non-competitive like inhibition of 5-HT$_3$ receptors by mefenamic acid. Graph showing log concentration-effect curves for 5-HT in the absence and presence of mefenamic acid (MFA; 30μM - 300μM). The x-axis shows the $\log_{10}$ concentration of 5-HT and the y-axis gives the 5-HT response, represented as % of the control maximum 5-HT response. Each data point is the mean ± s.e.m. of 4-10 experiments.

2.3vii: Experiments to address the mechanisms underlying the inhibition of P2x receptors by fenamates

Flufenamic acid (30μM - 300μM) inhibited α,βMeATP - (30μM) responses in a concentration-dependent manner (figure 2.16). Concentration effect curves to α,βMeATP were depressed and shifted to the right in a concentration-dependent manner by flufenamic acid (30-300μM). The maximal α,βMeATP response was decreased to 73±3%, 53±4% and 39±2% of the control maximal α,βMeATP response in the presence of 30μM, 100μM and 300μM flufenamic acid, respectively. These data are shown in figure 2.17. Hill slopes for α,βMeATP in the presence of flufenamic acid were not significantly different from the control α,βMeATP Hill slope ($F_{3,28}$= 0.70, p≥ 0.10).

This data suggests that flufenamic acid inhibits P2x receptors in a non-competitive like manner.

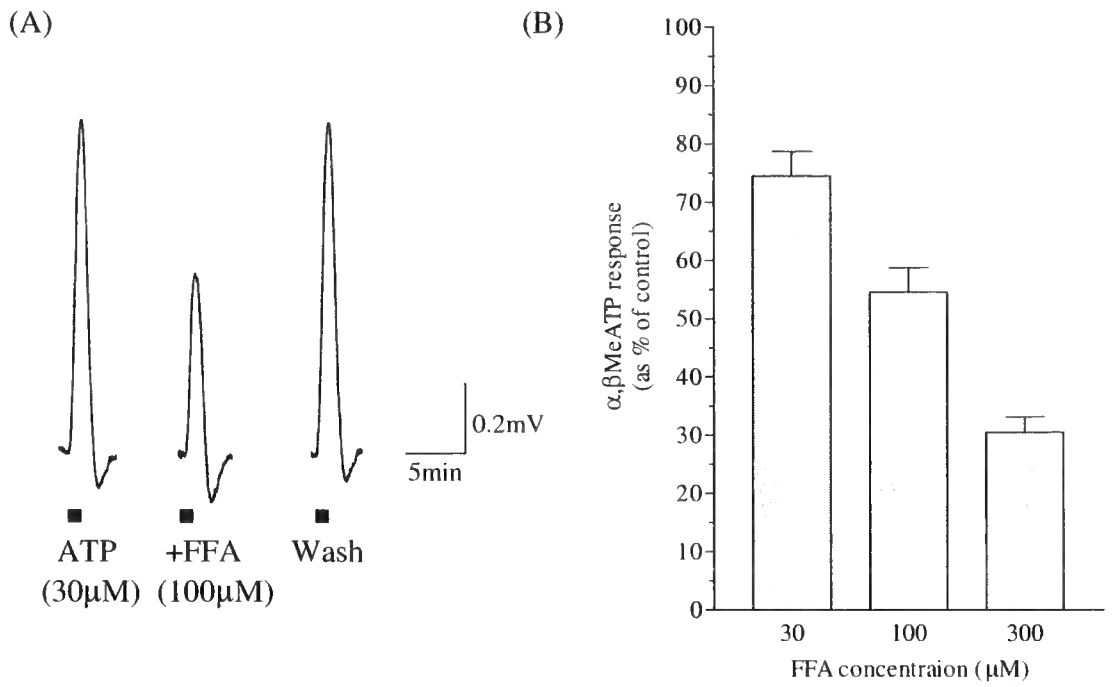


Figure 2.16: Concentration-dependent inhibition of P2x receptors by flufenamic acid. (A) Examples of chart recorder traces for α,βMeATP - (ATP; 30μM) evoked responses in the absence and presence of flufenamic acid (FFA; 100μM). The α,βMeATP and flufenamic acid concentration and agonist contact time, represented as a solid bar, is given below each response. (B) Histogram showing the response, as % of control, in the presence of flufenamic acid (30μM - 300μM). Each data bar is the mean ± s.e.m. of 4-7 experiments.

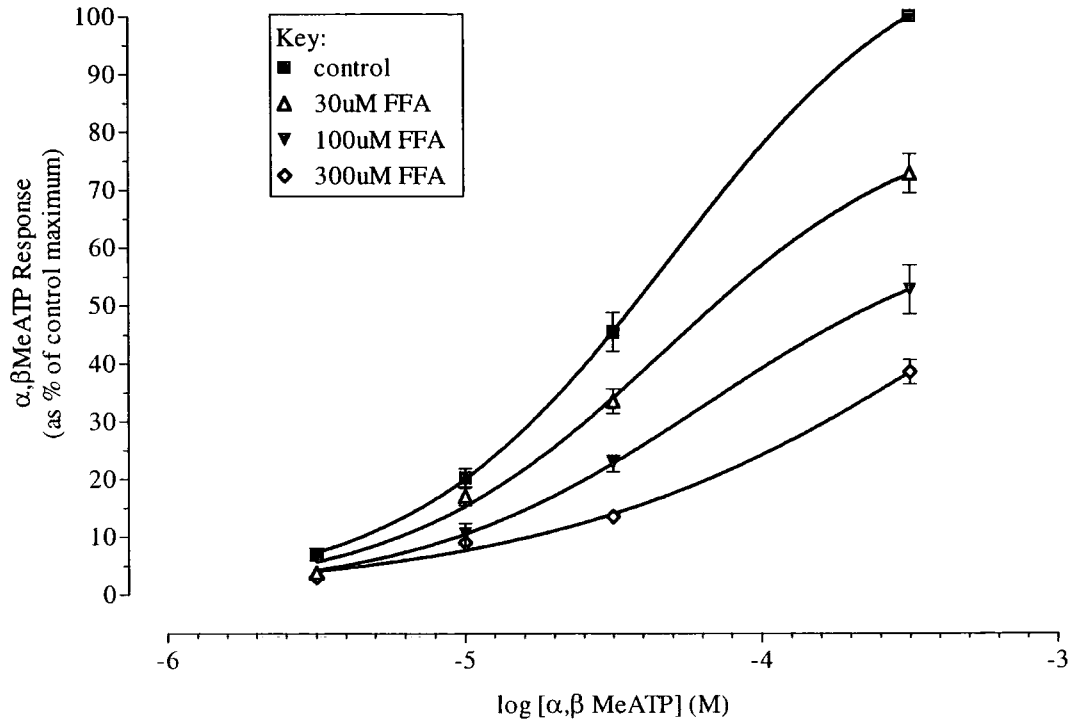


Figure 2.17: Non-competitive like inhibiton of P2x receptors by flufenamic acid. Graph showing log concentration-effect curves for α,βMeATP in the absence and presence of flufenamic acid (FFA; 30μM - 300μM). The x-axis is the $\log_{10}$ concentration of α,βMeATP and the y-axis is the α,βMeATP response represented as % of maximum control α,βMeATP response. Each point is the mean ± s.e.m. of 4 experiments.

Mefenamic acid (30μM - 300μM) inhibited α,βMeATP responses (30μM) in a concentration-dependent manner (figure 2.18). Concentration effect curves to α,βMeATP were depressed and shifted to the right in a concentration-dependent manner by mefenamic acid (30-300μM). The maximal α,βMeATP response was decreased to 68±12%, 55±5% and 52±9% of control maximal α,βMeATP response in the presence of 30μM, 100μM and 300μM flufenamic acid, respectively as shown in figure 2.19. Hill slopes for α,βMeATP in the presence of flufenamic acid were not significantly different from the control α,βMeATP Hill slope ($F_{3,28}$= 0.55, p≥ 0.10). This data indicates that flufenamic acid inhibits P2x receptors in a non-competitive like fashion.

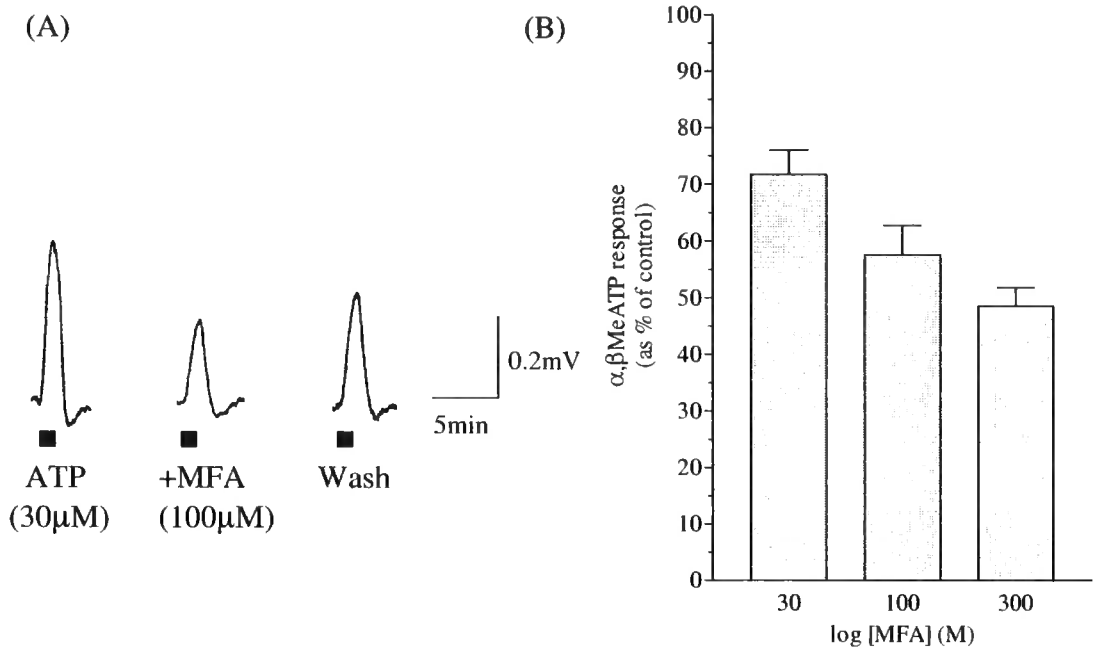


Figure 2.18: Concentration-dependent inhibition of P2x receptors by mefenamic acid. (A) Examples of chart recorder traces for α,βMeATP - (ATP; 30μM) evoked responses in the absence and presence of mefenamic acid (MFA; 100μM). The α,βMeATP and mefenamic acid concentration and the agonist contact time, represented by the solid bar, is given below each response. (B) Histogram showing the response, as % of control, in the presence of mefenamic acid (30μM - 300μM). Each data bar is the mean ± s.e.m. of 4-7 experiments.

The effects of all fenamate NSAIDs on each agonist-evoked response were poorly reversible even after prolonged (≥ two hours) washout.

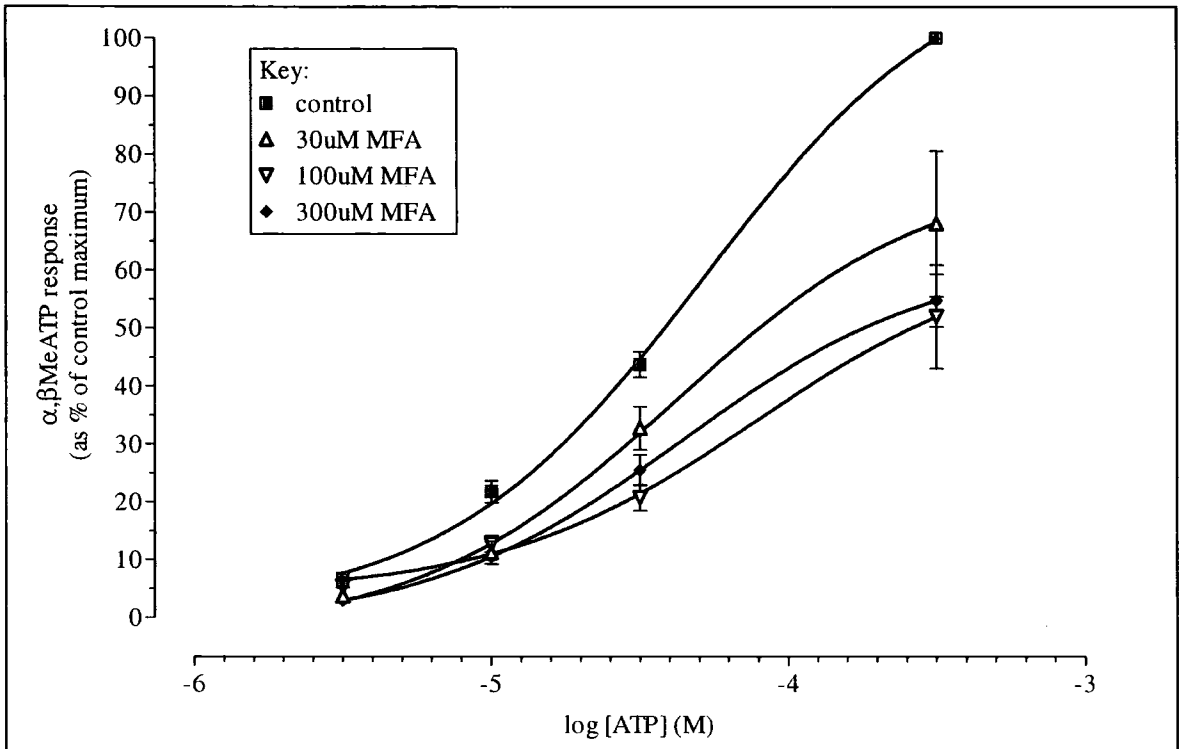


Figure 2.19: Mefenamic acid induces a non-competitive like inhibition of P2x receptors. Graph showing log concentration-effect curves for α,βMeATP in the absence and presence of mefenamic acid (MFA; 30μM - 300μM). The x-axis gives the $\log_{10}$ concentration of α,βMeATP and the y-axis shows the α,βMeATP response represented as % of maximum control α,βMeATP response. Each point is the mean ± s.e.m. of 4 experiments.

2.3viii: Effect of Non-Fenamate NSAIDs on Agonist- Evoked Responses

To investigate the hypothesis that the effects of fenamates on the ligand-gated ion channels described above were the result of COX inhibition in the nerves, the effects of non-fenamate NSAIDs on sub-maximal GABA, 5-HT, DMPP and α,βMeATP responses in the isolated rat vagus nerve and GABA and glycine responses in the isolated rat optic nerve was investigated.

Statistical analysis of agonist-evoked responses in the absence and presence of each NSAID was performed using a paired Student t-test (two-tailed). None of the non-fenamate NSAIDs had a significant effect on agonist responses in the isolated rat vagus

or optic nerves ($p \geq 0.10$). The results of these experiments are summarised in tables 3.3 and 3.4 respectively.

Table 2.3: Non-fenamate NSAIDs have little effect on agonist- evoked responses in the rat isolated vagus nerve. Table showing the agonist response, as a percentage of control ± s.e.m. for GABA, 5-HT, DMPP and α,β MeATP in the rat isolated vagus nerve after application of (at 100μM) aspirin (ASA), bi-phenyl acetic acid (BPAA), ibuprofen (IBU) and indomethacin (INDO). None of the NSAIDs significantly affected ($p \geq 0.10$) these responses. n= number of experiments. t=test statistic with degrees of freedom in subscript.

Agonist (concentration)	**Non-fenamate NSAID** (at 100μM)			
	ASA	BPAA	IBU	INDO
GABA (50μM)	111±4% (n=6) (t_5= 1.25)	76±4% (n=8) (t_7= 1.88)	88±11% (n=5) (t_4= 0.03)	77±5% (n=6) (t_5= 1.50)
5-HT (1μM)	85±1% (n=4) (t_3= 1.53)	77±6% (n=4) (t_3= 1.24)	108±4% (n=3) (t_2= 2.69)	106±4% (n=7) (t_6= 0.71)
DMPP (30μM)	77±2% (n=4) (t_3=2.94)	84±3% (n=3) (t_2= 2.51)	78±6% (n=6) (t_5= 2.54)	83±9% (n=3) (t_2= 1.61)
α,βMeATP (30μM)	117±14% (n=3) (t_2= 0.84)	77±3% (n=9) (t_8= 1.42)	84±4% (n=6) (t_5=1.15)	97±5% (n=4) (t_3= 0.92)

Table 2.4: Non-fenamate NSAIDs have little effect on agonist- evoked responses in the rat isolated optic nerve. Table showing the agonist response as a percentage of control ± s.e.m. for GABA and glycine depolarisations in the isolated rat optic nerve in the presence of (at 100μM) aspirin, bi-phenyl acetic acid, ibuprofen and indomethacin. None of the NSAIDs significantly affected ($p \geq 0.10$) these responses. n= number of experiments. t=test statistic with degrees of freedom in subscript. Abbreviations as in table 2.3, above.

Agonist (concentration)	**Non-fenamate NSAID** (at 100μM)			
	ASA	BPAA	IBU	INDO
GABA (1mM)	97±3% (n=3) (t_2= 1.0)	97±5% (n=4) (t_3= 0.92)	101±3% (n=4) (t_3= 0.00)	92±5% (n=4) (t_3= 2.22)
Glycine (1mM)	88±4% (n=6) (t_5 =2.12)	94±6% (n=6) (t_5= 1.59)	93±10% (n=5) (t_4= 0.14)	95±4% (n=4) (t_3= 1.22)

2.4: Summary

The first part of this thesis has focused on the hypothesis that NSAIDs modulate native neuronal ligand-gated ion channels.

GABA, 5-HT, DMPP and α,βMeATP all evoked concentration dependent depolarisations on the isolated rat vagus nerve, these responses could be inhibited by application of bicuculline, MDL 72222, hexamethonium and PPADs respectively. Sub-maximal GABA responses were also potentiated by sodium pentobarbitone and propofol. Together these results are consistent with the activation of $GABA_A$, $5\text{-}HT_3$, nicotinic ACh and P2x receptors, respectively. GABA and glycine each evoked concentration-dependent depolarisations on the isolated rat optic nerve, which were selectively inhibited by bicuculline and strychinine, respectively. These data are consistent with the activation of $GABA_A$ and strychinine-sensitive glycine receptors.

Certain fenamate NSAIDs significantly modulated neuronal ligand-gated ion channels. Mefenamic acid positively modulated sub-maximal GABA- evoked responses in a concentration dependent manner. Mefenamic acid also caused a non-competitive inhibition of 5-HT- and α,βMeATP- evoked responses. Non-competitive inhibition was also observed with flufenamic acid on DMPP- and α,βMeATP- evoked responses and with meclofenamic acid on GABA- evoked responses.

Application of non-fenamate NSAIDs did not significantly affect agonist-evoked responses. These data indicate that the effects observed with fenamates on neuronal ligand-gated ion channels are not due to inhibition of COX enzymes.

The fenamate effects observed on ion channel function may account for some of the "*central*" behavioural effects reported from their use in human and animal studies (see chapter one).

Chapter Three: The Effect of Mefenamic Acid on Open Field Behavioural Paradigms

3.1: Introduction

It was reported in the previous chapter that application of mefenamic acid positively modulated GABA- evoked responses and inhibited 5-HT- and α,βMeATP- evoked responses in the isolated rat vagus nerve. Considering this and other recent electrophysiological studies, which have shown that mefenamic acid can bi-directionally modulate GABA- evoked responses recorded from *Xenopus* oocytes (Woodward *et al.*, 1994; Halliwell *et al.*, 1999), it is hypothesized that mefenamic acid may have behavioural and/or cognitive effects.

There is a paucity of studies that have investigated the central effects of mefenamic acid *in vivo* and these have shown it to modulate seizure activity (Ikonomidou-Turski *et al.*,1988; Wallenstein, 1985a,b, 1991).

To date there are no studies that have investigated the effect of fenamate NSAIDs on behaviour and cognition. The aim of this chapter was therefore to investigate the hypothesis that mefenamic acid modulates behaviour.

3.2: Methods and Materials

3.2i: The object recognition task

Introduction:

The object discrimination task was developed by Ennaceur and Delacour (1988), to investigate object recognition memory in the rat. The method utilises the rat's spontaneous exploration of a new object, does not require the learning of a rule and does not require food deprivation to investigate the effects of drugs or lesions on working memory, which has been defined by Olton *et al.* (1979) as "memory that is specific for the completion of a single task". A number of studies have shown that this method is a valid paradigm for the investigation of drug treatments. For example, the muscarinic acetylcholine antagonist, scopolamine (Ennaceur and Meliani 1992a) and the histamine H_3 agonists, methylhistamine and imetit (Blandina *et al.*, 1996), have been shown to cause concentration dependent impairments in object discrimination. In contrast, injections of piracetam (Ennaceur *et al.*, 1989), apamin (Deschaux *et al.*, 1997) or nicotine (Puma *et al.*, 1999) have all been shown to enhance object discrimination in this task. A modified and revised version of the object discrimination task is described below.

Apparatus:

The testing arena consisted of a 85cm x 85cm x 50cm aluminium box (built in-house) with the floor covered in sawdust. The arena was lit (approx. 40 Lux) by a 60w angle poised lamp. The objects were made of glass, plastic or clay and weighted so they could not be moved by the animals. All objects existed in triplicate and had no apparent ethological significance for rodents and had never been associated with re-enforcement. After each trial, the objects were cleaned with ethanol and the sawdust moved around the arena to prevent build up of odours in certain areas.

Animals:

Male Lister hooded rats (250 - 350g; Charles River U.K.) were housed in pairs with *ad libitum* access to food and water throughout the study. Animals were maintained on a twelve hour light (07:00 - 19:00)/ twelve hour dark cycle and were tested during the light phase. The ambient room temperature was 23±1°C.

Experimental protocol:

Prior to testing, rats were habituated to the arena and testing room by placing them in the arena and allowed to explore freely for five minutes each day for three days. Experimental sessions comprised of two three-minute phases, a 'sample' and 'choice' phase, which were separated by an intra-trial interval.

Two identical objects (defined as, A1 and A2) were placed about ten centimetres from the far corners of the arena (see figure 3.1). A rat was then placed into the arena, for the sample phase, at the centre of the near wall, with its head facing the near wall and was given three minutes to explore the objects. Exploration was defined as directing the nose at a distance of less than two centimetres from the object and/or touching the object with its nose. Turning around or sitting on the object was not considered exploratory behaviour. After three minutes the rat was removed from the arena and placed back in to its holding cage. During this intra-trial interval, the two objects were removed from the arena and replaced with two new objects, one familiar (A3) and one novel (B) in the same location as the previous objects. After the intra-trial interval the rat was re-introduced into the arena, again at the centre of the near wall, for the choice phase, and given three minutes to explore the objects. The rat's exploration time of each object during the sample phase and choice phase was individually recorded. The objects used

as the familiar and novel objects were counter-balanced between animals and the position of the objects was randomly alternated during the choice phase to prevent any place preference.

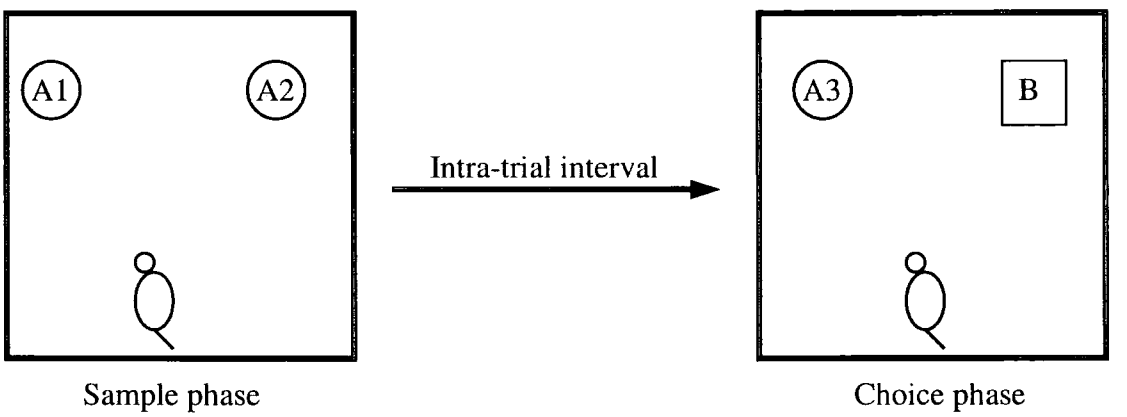


Figure 3.1: Schematic diagram of the object discrimination task. Diagram showing the apparatus used in the object discrimination task. During the sample phase the rat was presented with two identical objects (*circles*) and give three minutes to explore. The rat was then removed from the arena for a pre-determined intra-trial interval and then returned to the arena, for the choice phase, where it was presented with a familiar object (*circle*) and a novel object (*square*) and again given three minutes to explore.

Data analysis:

The time spent exploring each of the objects in the sample and choice phases was individually recorded enabling further analysis of the data that is described in table 3.1.

Table 3.1: The measures and data analysis applied in the object discrimination task.

A1, A2	Time (secs) exploring A1 and A2 objects, respectively, in the sample phase
A3, B	Time (secs) exploring A3 and B objects, respectively, in the choice phase
e1	Total exploration time (secs) in sample phase (A1 + A2)
e2	Total exploration time (secs) in choice phase (A3 + B)
d1	Discrimination time (secs) between objects (B - A3) in the choice phase

Investigation of side and object preferences:

For all experiments, analysis of the times spent exploring the two pairs of objects in the sample phase was analysed, with an un-paired Student t-test, to investigate for object preferences. None of the animals showed a preference for either pair of objects used in the experiments ($p \geq 0.10$). Analysis of side preferences within the arena was also analysed for each experiment using a paired Students t-test. None of the experiments revealed a significant side preference ($p \geq 0.10$) for any experiment.

e1, e2 and d1 values were analysed with either the un-paired Student t-test (two-tailed) to compare two groups, or one-way ANOVA to compare the differences between three or more groups. When an overall ANOVA proved significant ($p \leq 0.05$), the means of each group were compared with Student-Newman-Keuls *post hoc* analysis. All statistical tests were performed using INSTAT V2.05a (Graphpad software, San Diego, USA). A two-way ANOVA was performed on results with two variable factors, followed by Student-Newman-Keuls *post hoc* analysis if results were significant ($p \leq 0.05$), using SPSS v10.0.

3.2ii: The object location task

Introduction:

A modified version the object discrimination, known as the object location task, was developed by Ennaceur and Meliani (1992b) to investigate spatial memory in the rat. This test has been shown to be sensitive to electrolytic lesions of the medial septum (Ennaceur & Meliani, 1992b) and neurotoxic lesions of the fornix and cingulate (Ennaceur *et al.* 1997). A revised version of this task is described below.

Apparatus:

The testing arena and objects, which exisited in quadruplicate was described above (pg 64).

Animals:

Male Lister hooded rats (250 - 350g; Charles River U.K.) were housed and maintained as described above (pg 65).

Experiment protocol:

Prior to testing, rats were habituated to the arena and testing room as described earlier (pg 66). Experimental sessions comprised of two three-minute phases, a sample and choice phase, which were separated by an intra-trial interval.

Two identical objects (A1 and A2) are placed about ten centimetres from the rear corners of the arena (figure 3.2). A rat was then placed into the arena, for the sample phase, at the near corner of the arena, with its head facing the near wall, to allow an approximate equal distance between each of the objects and the rat and given three minutes to explore the objects. Exploration of an object was defined of pg 65. After three minutes the rat was removed from the arena and placed in its holding cage. During this intra-trial interval the two objects were removed and replaced with two objects identical to those in the sample phase, one object (A3) was placed in the same location as in the sample phase, the other object (A4) was located in a new position within the arena (figure 3.2). The rat's exploration time of each object during the sample phase and choice phase was individually recorded. The position of the objects in the arena for the sample and choice phases was randomly alternated.

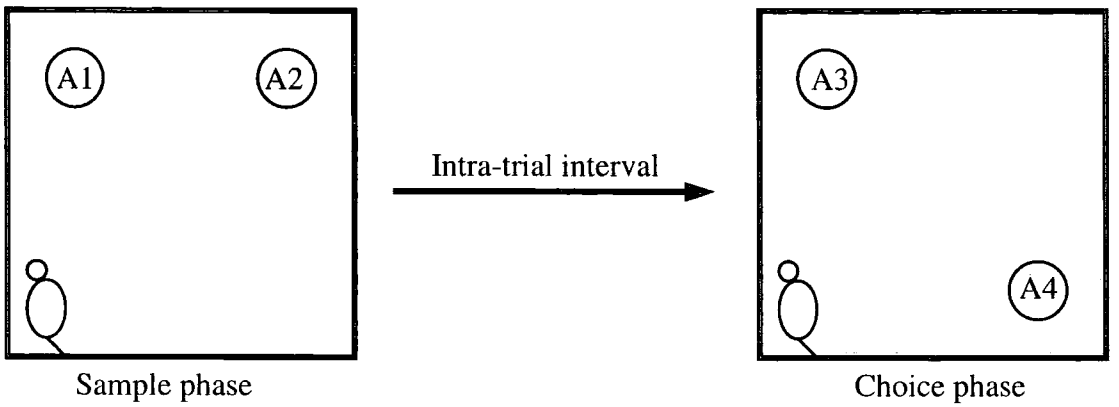


Figure 3.2: Schematic diagram of the object location task. Diagram showing the apparatus used in the object location task. During the sample phase the rat was presented with two identical objects (*circles* A1 and A2) and given three minutes to explore. The rat was then removed from the arena for a pre-determined intra-trial interval and then returned to the arena, for the choice phase, where it was then presented with two identical objects (circles A3 and A4) with one of the objects moved to a new location. The rat was given three minutes to explore.

Data analysis:

The time spent exploring each of the objects in the sample and choice phases was individually recorded enabling further analysis of the data that is described in table 3.2.

Table 3.2: The measures and data analysis applied in the object location task.

A1, A2	Time (secs) exploring A1 and A2 objects respectively in the sample phase
A3, A4	Time (secs) exploring A3 and A4 objects respectively in the choice phase
e1	Total exploration time (secs) in sample phase (A1' + A'2)
e2	Total exploration time (secs) in choice phase (A3 + A4)
d1	Discrimination time (secs) between locations (A4 - A3) in the choice phase

e1, e2 and d1 values were analysed with either the un-paired Student t-test (two-tailed) to compare two groups or one-way ANOVA to compare the differences between three or more groups. When the overall ANOVA proved significant ($p \leq 0.05$), the means of each group were compared using the Student-Newman-Keuls *post hoc* analysis. All above statistical tests were performed using INSTAT V2.05a (Graphpad software).

3.2iii: Open field tests

Introduction:

The open field paradigm is a widely used test that has been used to investigate the effects of drugs on anxiety, defined as "persistent and recognisably irrational fears of a circumscribed objects or situations" (File, 1995), and locomotion. For example, anxiety and locomotion in the open field are sensitive to the effects of the $GABA_A$ receptor modulator, diazepam (Bodnoff *et al.*, 1989), the $GABA_A$ receptor antagonist, bicuculline (Car *et al.*, 1998), the 5-HT_3 antagonist, ondansteron (Rex *et al.*, 1998) and the NMDA channel blocker, MK-801 (Jessa *et al.*, 1996).

Rat's were tested in open field tests to gain insight into the effects of mefenamic acid on anxiety and locomotor activity since both of these factors can lead to changes in object discrimination or object location results (Buhot *et al.*, 1989; Besheer & Bevins, 2000).

Anxiety Testing

Animals:

Male Lister hooded rats (250 - 350g; Charles River U.K.) were housed and maintained as described above (pg 65). They were handled by the investigator for five days prior to testing but were not placed in the testing room until the day of testing.

Apparatus:

An 85 x 85 x 50cm aluminium arena (built in-house) with the floor covered in sawdust was used as the open field. An unmoveable object was placed in the middle of the arena. A video camera (Sanyo, Herts, U.K.) was set-up above the arena, which was connected to a T.V. monitor in an adjoining room.

Experimental protocol:

Rats were given an intra-peritoneal injection of either saline or mefenamic acid (20mg/kg) thirty minutes prior to testing. They were then taken individually into the testing room, placed in the corner of the arena and given three minutes to explore the arena and the object. The amount of time taken for the rat to approach the object and the amount of time spent exploring the object was recorded via the T.V. monitor in the adjoining room.

Data analysis:

The amount of time taken to approach the object (secs) and the amount of time spent exploring the object (secs) were analysed with an un-paired Students t-test (two-tailed), $p \leq 0.05$ was considered significant.

Locomotor Test

Animals:

Male Lister hooded rats (250 - 350g; Charles River U.K.) were housed and maintained as described above (pg 65). They were handled daily by the investigator and habituated to the testing room and arena by placing them in the arena and allowing them to explore for five minutes a day for three days.

Apparatus:

The test arena is described previously on pg 70. The T.V. monitor was spilt into twenty equal sized squares by placing a cotton thread grid over the monitor screen.

Experimental protocol:

Thirty minutes prior to testing, the rats were given an intra-peritoneal injection of either saline or mefenamic acid (20mg/kg). They were then individually placed into the arena for three minutes and the number of squares crossed, as observed on the monitor screen, was counted. A square was considered to have been crossed when both the front and hind paws crossed the dividing line.

Data analysis:

The number of squares crossed for each group was analysed with an un-paired Student t-test (two-tailed), and $p \leq 0.05$ was considered significant.

3.2iv: Drugs and drug administration

Mefenamic acid (Sigma) was dissolved in 0.05M NaOH and made up with double distilled water to a dose of 10mg/ml. Saline was made up as a 0.9% solution in double distilled water. Drugs were given via an intra-peritoneal injection, with 0.2-1.0mls given per injection, thirty minutes prior to testing (unless otherwise stated).

3.3: Results

3.3i: The effect of mefenamic acid on behaviour:

The aim of the first experiment was to observe the gross behavioural effects of mefenamic acid.

Treatments and testing:

Twenty-four rats were divided into one of three groups. Each group received an intra-peritoneal injection of mefenamic acid at either 20mg/kg (n=8), 40mg/kg (n=8) or 60mg/kg (n=8). The behaviour of rats in their holding cage was monitored for thirty minutes after injection of mefenamic acid.

Results:

Rats injected with mefenamic acid (5mg/kg – 20mg/kg) did not show any *unusual* behavioural effects compared to saline controls: they maintained normal posture and showed exploratory behaviour. However injection of mefenamic acid (40mg/kg) resulted in all rats having myoclonus and head bobbing within five minutes of injection. Five of the eight rats were also apparently sedated, with the animals being motionless and bodies carried low or flattened against the cage floor. They remained in this state for thirty minutes post-injection and therefore were not tested further. Injection of mefenamic acid (60mg/kg) resulted in heavy sedation in all rats within five minutes of injection: they were motionless and flattened against the floor, when lifted by their torso they hung motionless until returned to their cage. These animals also made "swimming-like" movements when they tried to move around their cage. Six of the rats also had severe whole body jerks, which lasted for up to fifteen seconds per episode, but these were not apparent twenty minutes post-injection. All rats were still heavily sedated

thirty minutes after injection and could not therefore be tested in the object discrimination task.

Conclusion:

High doses of mefenamic acid (40mg/kg and 60mg/kg) result in "*central*" behavioural effects such as sedation and seizures.

3.3ii: The effect of mefenamic acid on anxiety

To investigate whether mefenamic acid acts as an anxiolytic or anxiogenic agent, its effect on anxiety in the open field task was investigated (see pg 69 for further discussion of this).

Treatments and testing:

Sixteen non-habituated rats were randomly divided into two groups. One group was given an intra-peritoneal injection of saline (n=8) the other group an injection of mefenamic acid (20mg/kg; n=8), thirty minutes prior to testing in the open field.

Results:

There was no significant difference between saline and mefenamic acid treated groups in the time taken to approach an object in the open field (t_{14}= 0.08, p≥ 0.10) as shown in figure 3.3A. The amount of time spent exploring the object was also not significantly different between groups (t_{14}= 0.41, p≥ 0.10) as shown in figure 3.3B.

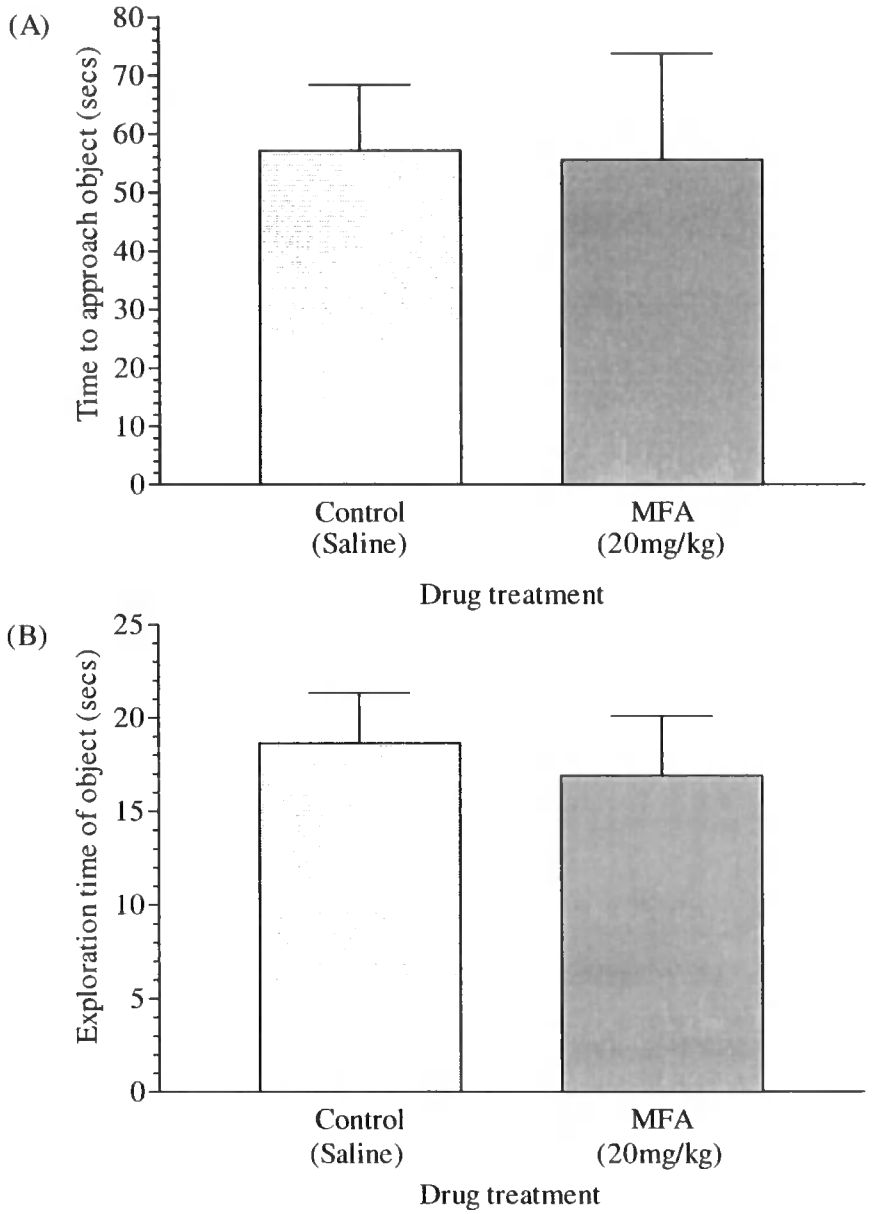


Figure 3.3: Mefenamic acid does not affect the time taken to approach a novel object or the duration of exploration of a novel object in the open field. (A) The histogram shows the time (secs) taken to approach an object in the open field on the y-axis, for saline and mefenamic acid (MFA) treated groups. (B) The histogram shows the exploration time (secs) of an object in the open field on the y-axis for saline and MFA treated groups. n=8 for each group. Each bar represents the mean ± s.e.m.

Conclusion:

Mefenamic acid does not change the "anxiety state" of the rat.

3.3iii: The effect of mefenamic acid on gross locomotor activity

Treatments and testing:

Sixteen non-habituated rats were randomly divided into two groups. One group was given an intra-peritoneal injection of saline (n=8) the other group an injection of mefenamic acid (20mg/kg; n=8), thirty minutes prior to testing in the open field. The number of squares crossed in the open field over a three-minute interval was then observed.

Results:

There was no significant difference between the saline and mefenamic acid treated groups in the number of squares crossed in the open field (t_{14}= 0.84, p≥ 0.10), as shown in figure 3.4.

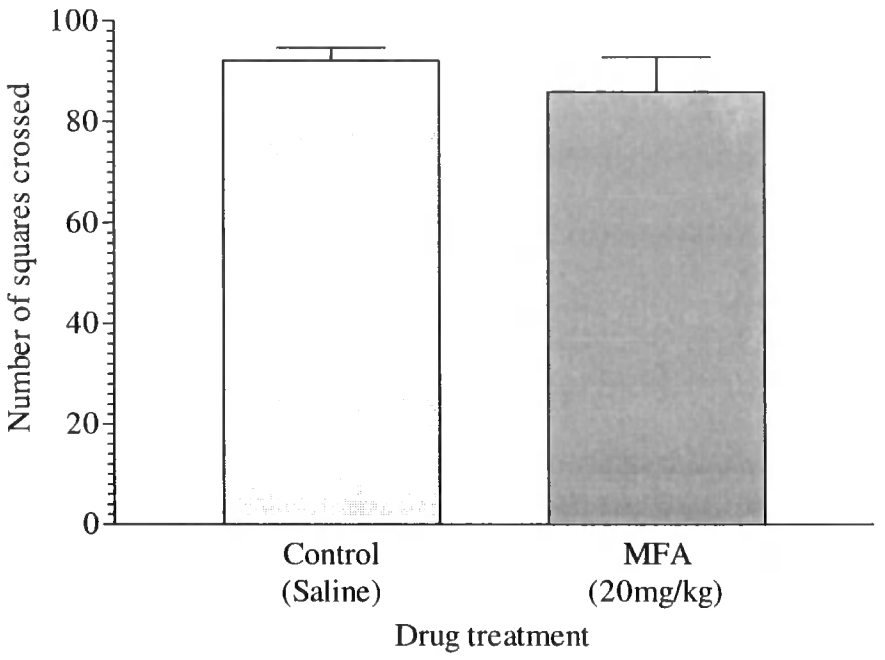


Figure 3.4: Mefenamic acid does not affect the number of squares crossed in the open field. The histogram shows the number of squares crossed in the open field on the y-axis for saline and mefenamic acid (MFA; 20mg/kg) treated groups. MFA did not significantly change in the number of lines crossed within an open field during a three-minute session when compared to saline controls. Each bar represents the mean ± s.e.m.

Conclusion:

Mefenamic acid did not affect gross motor activity in the open field.

3.3iv: Investigation of the effects of mefenamic acid on arousal

Another possible behavioural effect of mefenamic acid is that it can affect arousal, defined as "a state of alertness and high responsiveness to stimuli" (Uttal, 1978). In order to investigate this, the effect of mefenamic acid on object exploration times when the two objects in the choice phase are either both identical or both different to the objects in the sample phase was undertaken.

Treatment and testing:

Thirty-two rats were randomly allocated to one of four groups. Two groups were given an intra-peritoneal injection of saline (n=8 for each group), and two groups were given an intra-peritoneal injection of mefenamic acid (20mg/kg; n=8 for each group), thirty minutes prior to testing in the object discrimination task. For this experiment, one saline and one mefenamic treated group were exposed to two identical objects in the sample phase and re-exposed to the same objects during the choice phase. The second saline and mefenamic treated groups were exposed an identical set of objects during the sample phase but during the choice phase they were presented with two new identical objects but different to those presented in the sample phase.

Results:

When the two objects in the choice phase are identical to those in the sample phase (figure 3.5A) the total exploration time during the sample (t_{14}= 0.66, p≥ 0.10) and choice phases (t_{14} =0.15, p≥ 0.10) are not significantly different between groups. Comparison between e1 and e2 with a paired Student t-test revealed no significant difference between saline (t_7= 1.10, p≥ 0.10) or mefenamic acid (t_{15}= 1.83, p≥ 0.10) treated groups.

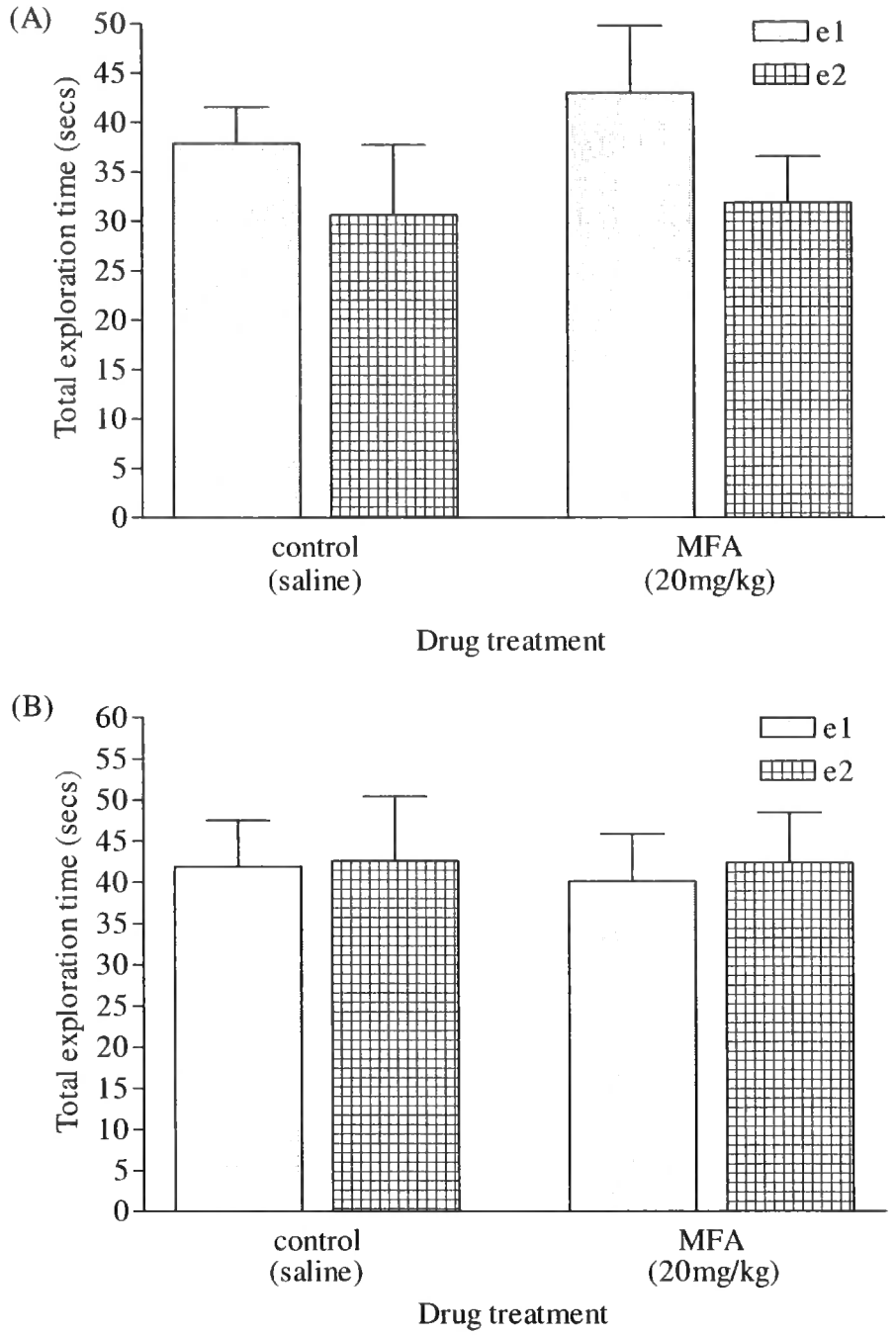


Figure 3.5: Exploration times are not affected by mefenamic acid when the two objects in the choice phase are either identical to or novel to those in the sample phase. (A) The histogram shows the total exploration time during the sample (e1) and choice phase (e2) on the y-axis for saline and mefenamic acid (MFA; 20mg/kg) groups when both objects in e2 were identical to those in e1. The total exploration times during e1 and e2 were not significantly different between groups. (B) The histogram shows the total exploration time during the sample (e1) and choice phase (e2) on the y-axis for saline and mefenamic acid (MFA; 20mg/kg) groups when both objects in e2 were different to those in e1. The total exploration times during e1 and e2 were not significantly different between groups. Each data bar represents the mean ± s.e.m. n=8 for each group.

When the two objects in the choice phase are different to those in the sample phase, the total exploration times during the sample (t_{14} =0.22, p≥ 0.10) and choice phases (t_{14} =0.02, p≥ 0.10) are not significantly different between saline and mefenamic acid treated groups (figure 3.5B). Comparison between e1 and e2 with a paired Student t-test revealed no significant difference between saline (t_7= 0.14, p≥ 0.10) or mefenamic acid (t_7= 0.43, p≥ 0.10) treated groups.

Conclusion:

The exploration times of animals pre-treated with mefenamic acid during the choice phase when the objects were either identical or different to those in the sample phase were not significantly different from control. These data suggest that mefenamic acid does not enhance non-specific exploratory behaviour.

3.3v: Behavioural measures in the object discrimination task

To establish the behavioural parameters of the object discrimination task, the total exploration time and discrimination of objects over a range of intra-trial intervals was compared.

Testing and treatments:

Forty-two rats were randomly allocated to six different groups and tested in the object discrimination task. Each group was tested with either a one-minute (n=7), fifteen-minute (n=7), thirty-minute (n=7), sixty-minute (n=7), four-hour (n=7) or twenty-four hour (n=7) intra-trial interval.

Investigation of the effect of the intra-trial interval on object exploration:

The total exploration time of the two objects in the sample phase (e1) was not significantly different across intra-trial intervals ($F_{5,36}$= 0.95, p≥ 0.10). The total object exploration time during the choice phase (e2) was also not significantly different across intra-trial intervals ($F_{5,36}$= 1.69, p≥ 0.10) as shown in figure 3.6.

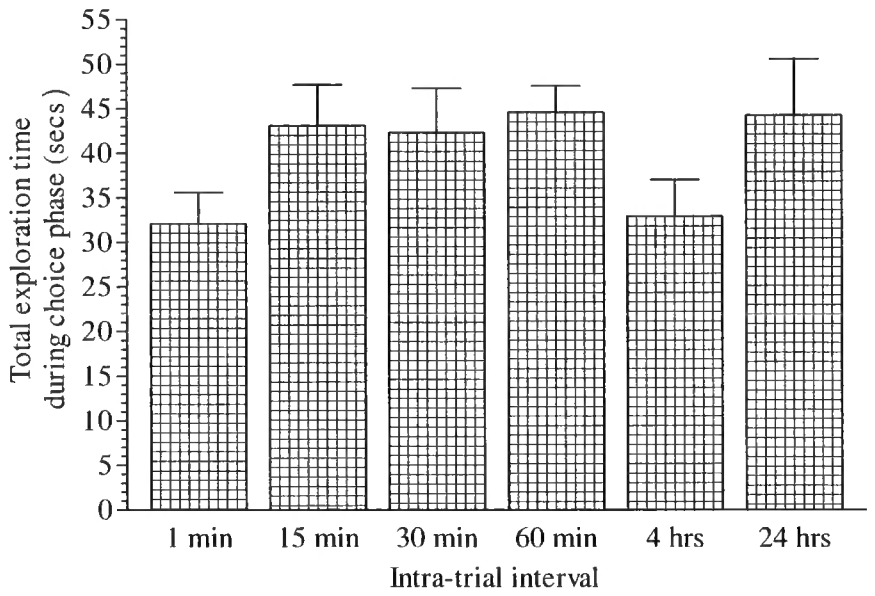


Figure 3.6: The effect of the intra-trial interval on exploration activity during the choice phase. The histogram shows the total exploration time (secs) of objects during the choice phase (e2) on the y-axis against the intra-trial interval (ITI) on the x-axis. The data shows that increasing the ITI does not result in a significant change (p≥ 0.10) in the exploration of objects during e2. Each bar represents the mean ± s.e.m., n=7 for all groups.

The effect of the intra-trial interval on object discrimination:

The groups mean exploration time of novel and familiar objects for each intra-trial interval is shown in table 3.3, where it can be seen that there is a significant difference in the discrimination between objects with a one-minute, fifteen-minute, thirty-minute and sixty-minute intra-trial interval. However, rats did not discriminate between the familiar and the novel object with a four or twenty-four hour intra-trial interval.

Table 3.3: Exploration times for familiar and novel objects during the choice phase. Table showing that as the intra-trial interval (ITI) is increased the difference in the exploration time between the novel and the familiar object is decreased. Comparison of the exploration of each object for each ITI (n=7) is analysed with a paired Student t-test (two-tailed).

ITI	Exploration (secs) of familiar object (mean ± s.e.m.)	Exploration (secs) of novel object (mean ± s.e.m.)	p
1 min	6.8±1.2	25.3±2.9	≤ 0.001, $t_7=5.7$
15 min	9.7±1.9	33.2±3.9	≤ 0.001, $t_7=5.7$
30 min	13.6±2.7	28.6±2.9	≤ 0.001, $t_7=5.7$
60 min	15.9 ± 1.6	28.7 ± 2.3	≤ 0.05, $t_7=4.8$
4 hrs	13.6 ± 2.5	19.3 ± 2.2	≥ 0.05, $t_7=2.2$
24 hrs	19.3 ± 3.2	25.0 ± 4.6	≥ 0.05, $t_7=1.2$

The level of discrimination (d1) between novel and familiar objects for each intra-trial interval are shown in figure 3.7, where it can be seen that d1 decreases as the intra-trial interval was increased. A one-way ANOVA revealed significant differences between groups ($F_{5,36}= 4.23$, $p\leq 0.01$). A *post hoc* analysis showed significant differences ($p\leq 0.01$) between the fifteen-minutes intra-trial interval group compared to the groups tested with four hour and twenty-four hour intra-trial intervals. Comparison of d1 for linear progression between intra-trial intervals revealed a significant linear trend ($F_{5,36}= 17.41$, $p\leq 0.001$) between groups.

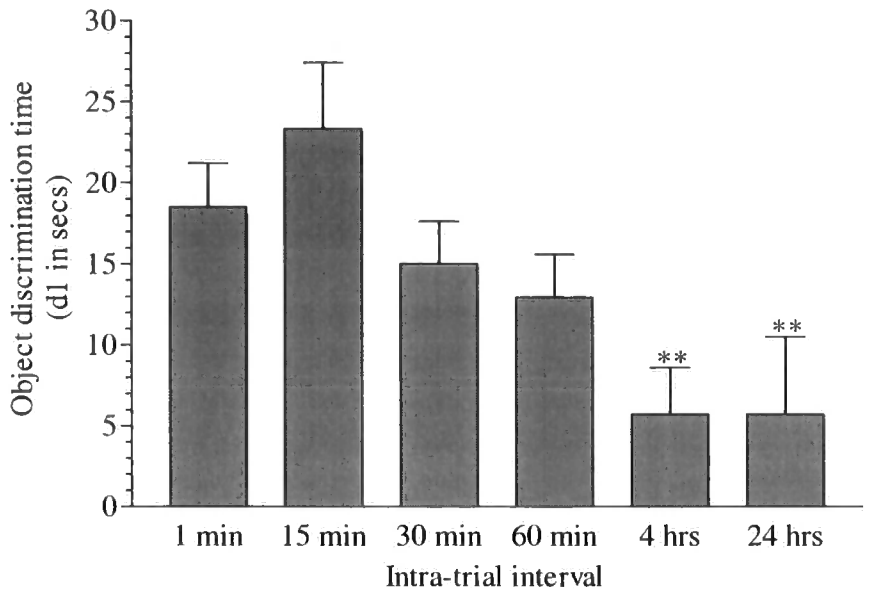


Figure 3.7: The effect of the intra-trial interval on object discrimination. The histogram shows the object discrimination time (d1) on the y-axis against the intra-trial interval (ITI) on the x-axis. The data shows that changing the ITI results in a significant change in d1 ($F_{5,36}$= 4.23, p≤ 0.01,), ** p≤ 0.01 when compared to 15 minute ITI. Each data bar represents the mean ± s.e.m. n=7 or each intra-trial interval.

Conclusion:

The results of these studies demonstrate that rodents are able to discriminate between objects and that such discrimination is sensitive to intra-trial intervals. This finding is consistent with the hypothesis that object discrimination over time involves working memory (Ennaceur & Delacour, 1988; Ennaceur & Meliani, 1992a; Bartolini *et al.*, 1996). Therefore, the following series of experiments used this task to address the effect of mefenamic acid on working memory.

3.3vi: The effect of drug solvents on object discrimination

In order to test the hypothesis that the drug solvents used in subsequent experiments can influence object discrimination. The effect of NaOH, ethanol and cyclodextrin on rats' performance in the object discrimination task was investigated.

Treatments and testing:

Thirty-two rats were randomly allocated to one of four different groups. Each group received an intra-peritoneal injection of either saline (n=8), NaOH (0.1M; n=8), cyclodextrin (10%; n=8) or ethanol (10%; n=8), thirty minutes prior to testing. Each group of rats were then tested in the object discrimination task with a fifteen-minute intra-trial interval.

The effect of solvents on total exploration:

The overall exploration of objects was not significantly different between groups in e1 ($F_{3,28}$= 0.42, p≥ 0.10) or e2 ($F_{3,28}$= 0.86, p≥ 0.10), as shown in figure 3.8.

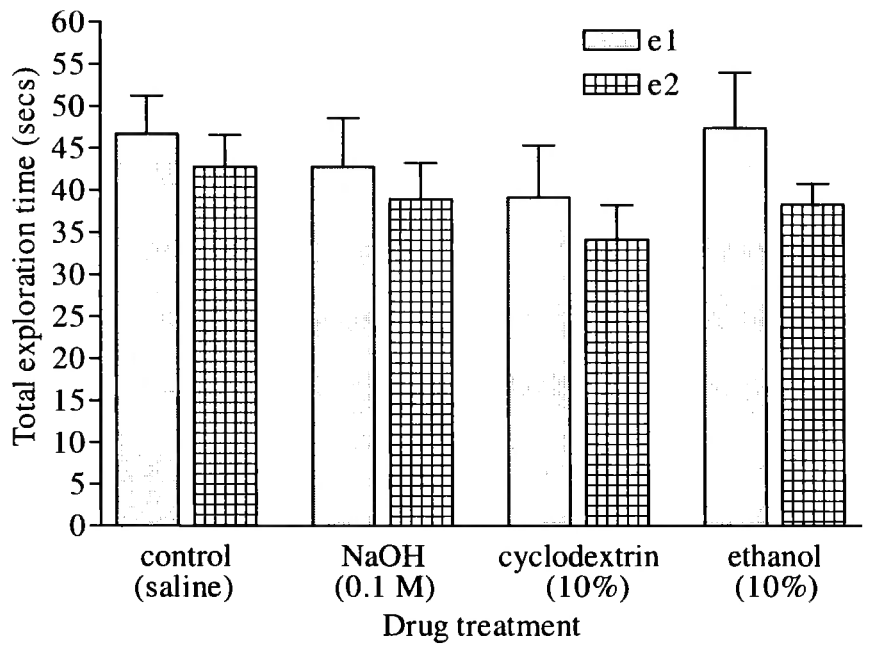


Figure 3.8: The drug solvents do not influence total exploration times. The histogram shows the total exploration time (secs), on the y-axis, for the objects in the sample (e1) and choice (e2) phases for control, NaOH, cyclodextrin and ethanol groups. There was no significant difference between groups for e1 or e2. Each data bar represents the mean ± s.e.m. n=8 for each group.

The effect of drug solvents on object discrimination:

The discrimination time (d1) was not significantly different across saline and solvent groups ($F_{3,28}$= 0.70, p≥ 0.10). These data are shown in figure 3.9.

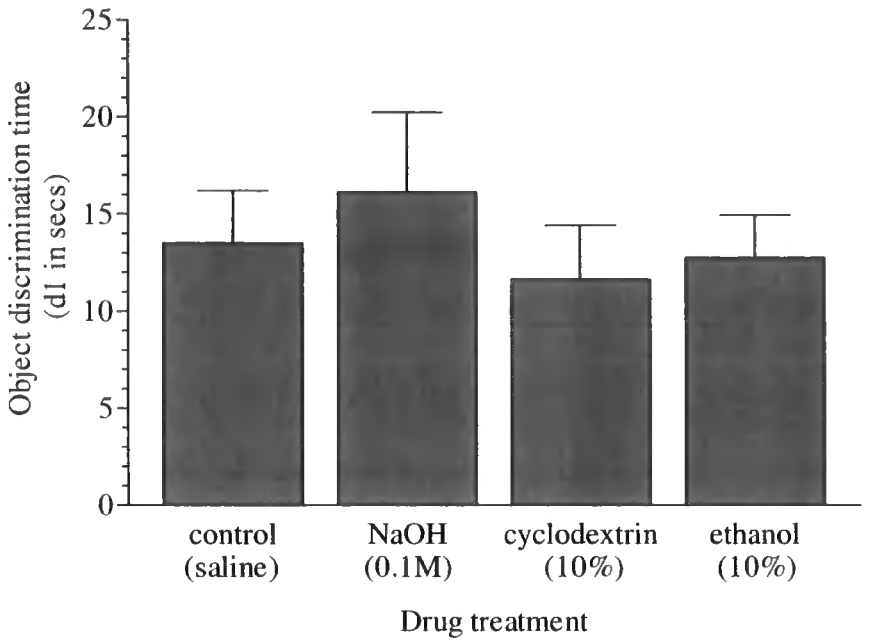


Figure 3.9: The drug solvents do not influence object discrimination. The histogram shows the object discrimination time (d1), on the y-axis, for control, NaOH, cyclodextrin and ethanol treatment groups. None of the drug solvents significantly affected d1. Each data bar represents the mean ± s.e.m. n=8 for each drug group.

Conclusion:

None of the drug solvents used in this study significantly affected object discrimination.

3.3vii: The effect of mefenamic acid on object discrimination

Treatment and testing:

Thirty-two rats were randomly allocated to one of four groups, each group received an intra-peritoneal injection of either saline (n=8) or mefenamic acid at 5mg/kg (n=8), 10mg/kg (n=8) or 20mg/kg (n=8), thirty minutes prior to testing. Each group of rats was then tested in the object discrimination task with a fifteen-minute intra-trial interval.

The effect of mefenamic acid on object exploration times:

The overall exploration of objects was not significantly different between groups during the sample phase ($F_{3,28}$= 2.17, p≥ 0.10). There was, however, a significant difference in

object exploration between groups during the choice phase ($F_{3,28}$= 7.58, p≤ 0.001). This data is shown in figure 3.10. A *post hoc* analysis showed significant differences between the saline treated group when compared to mefenamic acid at 10mg/kg and 20mg/kg (p≤ 0.05); and between the mefenamic acid (5mg/kg) group compared to the mefenamic acid at 10mg/kg and 20mg/kg (p≤ 0.05). There was no significant difference between saline and mefenamic acid (5mg/kg) or between mefenamic acid (10mg/kg) and mefenamic acid (20mg/kg) treated groups.

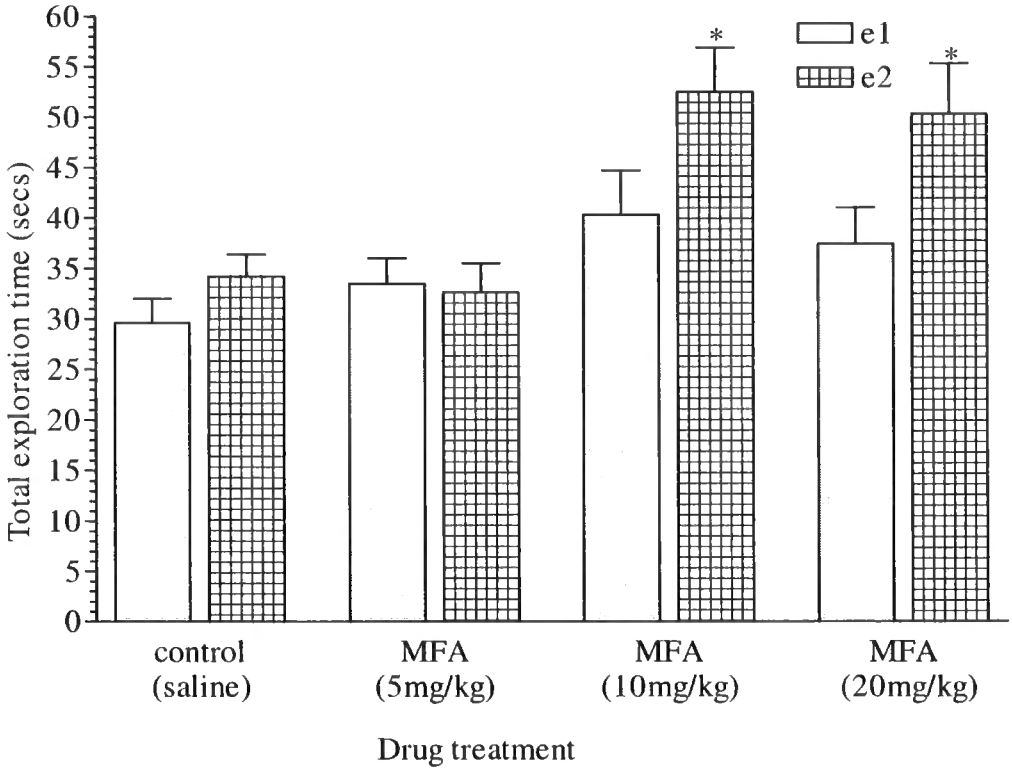


Figure 3.10: Mefenamic acid increases total exploration time during the choice but not the sample phase. The histogram shows the total exploration time (secs) on the y-axis for objects in the sample (e1) and choice (e2) phases, against the dose of mefenamic acid (MFA) on the x-axis. This data shows that e1 is not increased by MFA, whereas e2 is significantly increased by MFA ($F_{3,28}$=7.58, p≤ 0.001), * p≤ 0.05 when compared to control. Each data bar represents the mean ± s.e.m. n=8 for each drug group.

The effect of mefenamic acid on object discrimination:

The discrimination time (d1) between objects in the choice phase was significantly increased by mefenamic acid ($F_{3,28}$= 4.90, p≤ 0.01) and this data is shown in figure 3.11.

A *post-hoc* analysis showed significant differences between the saline group compared to mefenamic acid at 10mg/kg ($p \leq 0.05$) and 20mg/kg ($p \leq 0.01$); and between the mefenamic acid (5mg/kg) compared to mefenamic acid at 10mg/kg ($p \leq 0.05$) and 20mg/kg ($p \leq 0.01$). d1 values were also analysed for a linear trend between groups and were shown to be significant $p \leq 0.001$ ($F=18.47$).

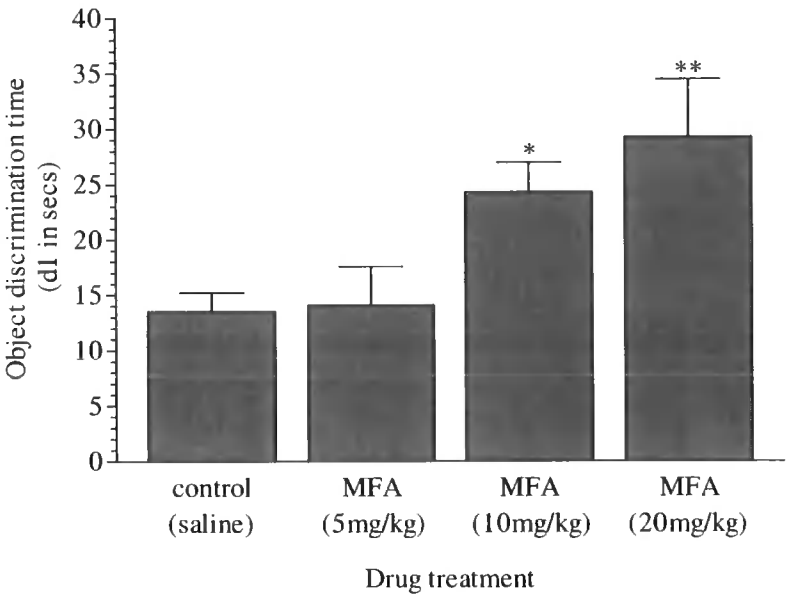


Figure 3.11: Mefenamic acid significantly increases object discrimination. The histogram shows the object discrimination time (secs) on the y-axis for control and mefenamic acid (MFA) at 5, 10 & 20mg/kg groups. MFA treatment at 10 and 20mg/kg significantly increased object discrimination when compared to controls ($F_{3,28}= 4.90$, $p \leq 0.05$), **$p \leq 0.01$, * $p \leq 0.05$ compared to control. Each data bar represents the mean ± s.e.m., n=8 for each group.

Conclusion:

Mefenamic acid (at 10mg/kg and 20mg/kg) enhanced object discrimination with a fifteen-minute intra-trial interval.

3.3viii: Is the effect of mefenamic acid time-dependent

Mefenamic acid increased object discrimination with a fifteen-minute intra-trial interval. The effect of mefenamic acid on object discrimination over a range of intra-trial intervals was therefore investigated to determine if its effect was time-dependent.

Treatments and testing:

Forty-eight rats were randomly allocated to six different groups. Three groups were given an intra-peritoneal injection of saline and three groups were given an intra-peritoneal injection of mefenamic acid (20mg/kg), thirty minutes prior to testing in the object discrimination task. Three intra-trial intervals were used in the experiment (a fifteen-minute, thirty-minute and sixty-minute interval) with one saline group (n=8) and one mefenamic treated group (n=8) tested for each intra-trial interval.

The effect of mefenamic acid on object exploration with longer intra-trial intervals:

A two-way ANOVA of the total exploration time during the sample phase revealed no significant difference between groups ($F_{5,42}$= 130, $p \geq 0.10$). Total exploration time during the choice phase was also not significantly different between groups ($F_{5,42}$= 1.25, $p \geq 0.10$), as shown in figure 3.12.

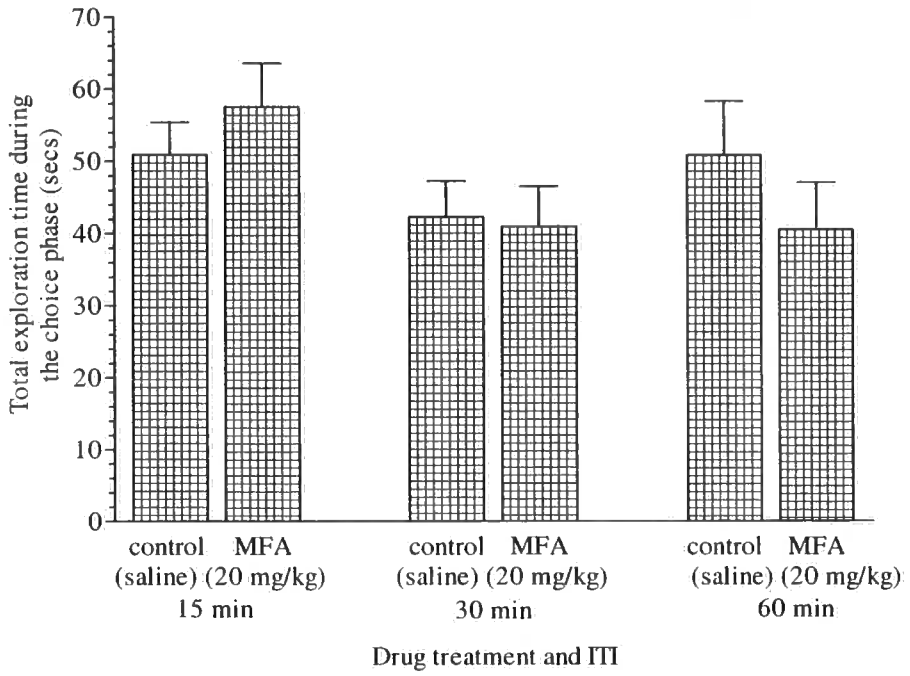


Figure 3.12: Mefenamic acid does not affect total exploration times over a range of intra-trial intervals. The histogram shows the total exploration time during the choice phase (e2) on the y-axis for control and mefenamic acid (MFA; 20mg/kg) groups with 15, 30 and 60 minute intra-trial intervals (ITI). There was no significant difference between groups for exploration of objects during e2. Each data bar represents the mean ± s.e.m. n=8 for each group.

Effect of mefenamic acid on object discrimination with increasing intra-trial intervals:

There was an overall significant difference in discrimination times between treatment and time groups ($F_{5,38}$= 2.42, p≤ 0.05) when analysed with an ANOVA (two-way). There was a significant difference between drug groups ($F_{1,46}$= 5.70, p≤ 0.05). The difference between time groups, however, was not quite significant ($F_{2,45}$= 2.60, p= 0.08). A *post hoc* analysis for mefenamic acid treated groups at each intra-trial interval, revealed a significant difference at fifteen and thirty minute intra-trial intervals when compared to saline controls (p≤0.05). There was no interaction between drug and time groups ($F_{2,45}$= 0.71, p≥ 0.10). The results are illustrated in figure 3.13.

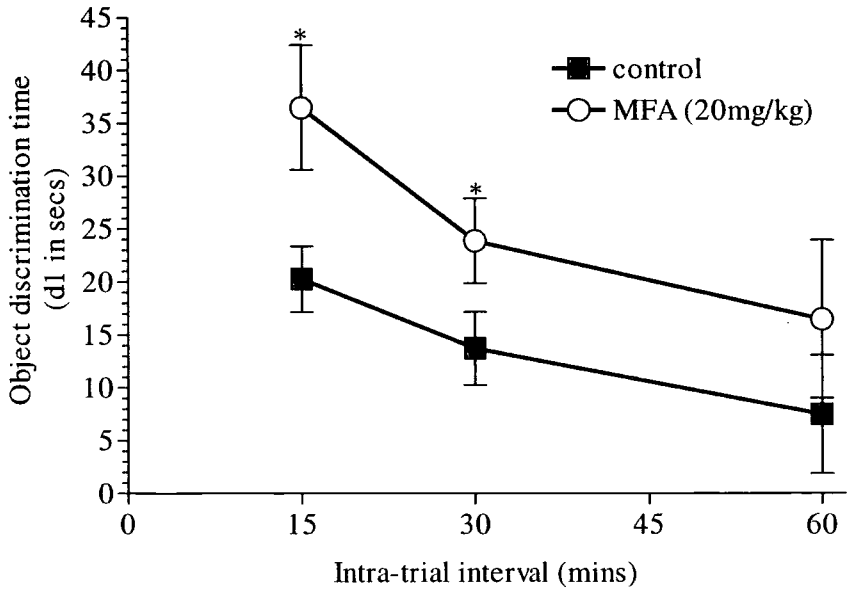


Figure 3.13: Mefenamic acid increases object discrimination compared to control over a range of intra-trial intervals. The graph shows the object discrimination time (d1) on the y-axis against the intra-trial interval (ITI) on the x-axis for saline and mefenamic acid (MFA; 20mg/kg) groups. It can be seen that d1 for the MFA groups are significantly greater than control groups for the 15 and 30 minute ITIs. *p≤ 0.05 when compared to control for that ITI. The lines joining each data point are for visual purposes only. Each data point represents the mean ± s.e.m. n=8 for each group.

Conclusion:

The results show that object discrimination is increased by pre-treatment with mefenamic acid and that the effect diminishes with longer intra-trial intervals.

3.3ix: A comparison of mefenamic acid with piracetam

A range of other compounds have also been reported to enhance working memory in normal animals including the nootropic, piracetam, (Bartus *et al.*, 1981; Nalini *et al.*, 1992; Christoffersen *et al.*, 1998). It was therefore of importance to compare the efficacy of mefenamic acid with that of piracetam.

Treatments and testing:

Thirty-six rats were randomly allocated to different group treatments; they received an intra-peritoneal injection of saline (n=12), mefenamic acid (20mg/kg; n=12), or piracetam (400mg/kg; n=12), thirty minutes prior to testing. Each group of rats were then tested in the object discrimination task with a fifteen-minute intra-trial interval.

The effect of piracetam on total exploration times:

The overall exploration of objects was not significantly different between groups in either the sample phase ($F_{2,33}$= 0.38, p≥ 0.10) or the choice phase ($F_{2,33}$= 0.32, P ≥ 0.10) of the test. (Figure 3.14).

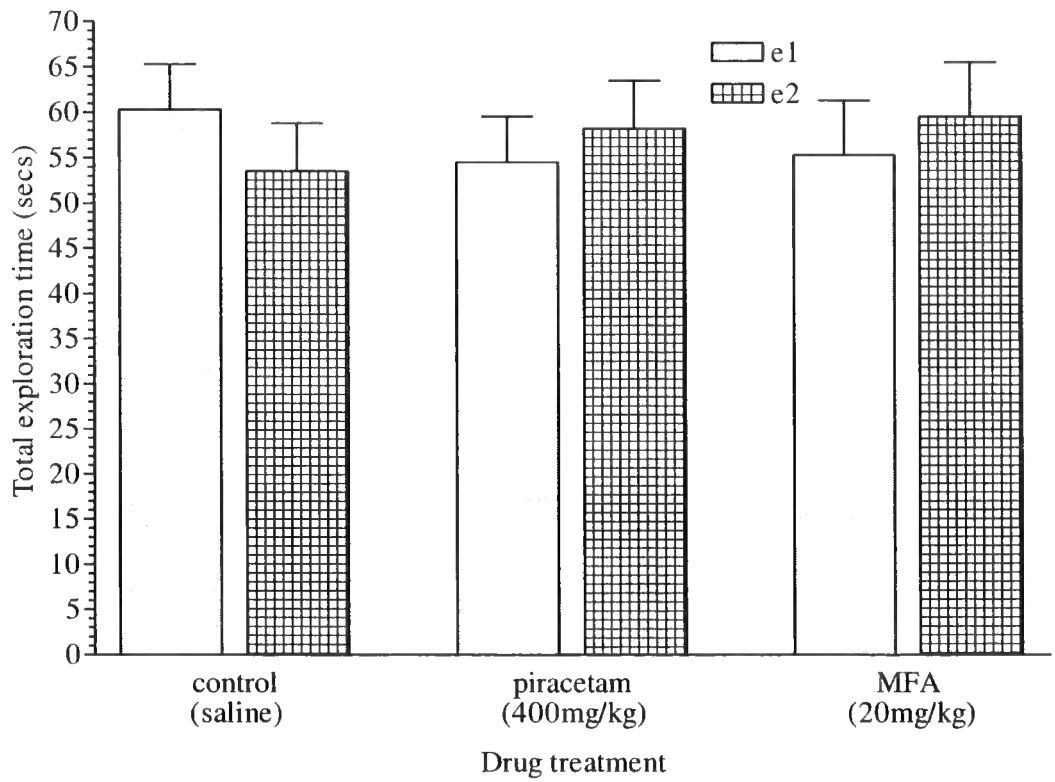


Figure 3.14: Total exploration times are not increased by mefenamic acid or piracetam. The histogram shows the total exploration time (secs) on the y-axis, of the objects in the sample (e1) and choice phase (e2) for control, piracetam (400mg/kg) and mefenamic acid (MFA; 20mg/kg) groups. The total exploration times during e1 and e2 were not significantly different between groups. Each data bar represents the mean ± s.e.m. of n=12 for all groups.

The effect of piracetam on object discrimination:

The object discrimination time (d1) was significantly different between groups ($F_{2,33}$= 4.60, p≤ 0.05) as shown in figure 3.15. A *post-hoc* analysis showed significant differences (p≤ 0.05) between the saline controls when compared to the mefenamic acid (20mg/kg) group. There was no significant difference (p≥ 0.10) between saline and piracetam groups or between the piracetam and mefenamic treated groups.

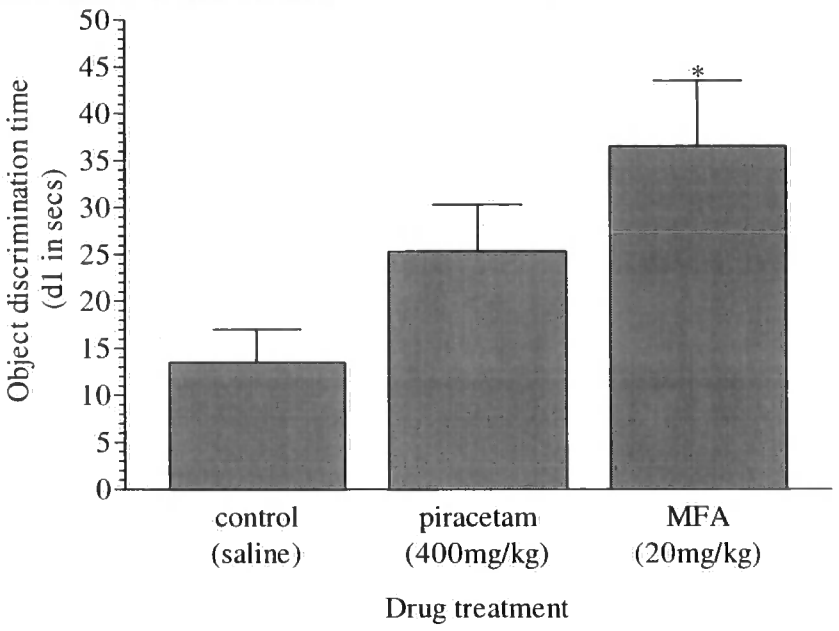


Figure 3.15: The effect of mefenamic acid and piracetam on object discrimination. The histogram shows the object discrimination time (d1) on the y-axis for saline, piracetam (400mg/kg) and mefenamic acid (MFA; 20mg/kg) groups. MFA but not piracetam significantly increased d1 ($F_{2,33}$= 4.60, p≤ 0.05), * p≤ 0.05 when compared to saline control. Each data bar represents the mean ± s.e.m. n=12 for each group.

Conclusion:

The results showed that animals treated with mefenamic acid discriminated between novel and familiar objects to a higher degree than saline or piracetam treated rats.

3.3x: The affects of chronic treatment with mefenamic acid on object discrimination

Clinically, NSAIDs are used long-term for the treatment of rheumatoid and osteo-arthritis (Orme, 1990). The affects of long-term, daily treatment with mefenamic acid on rats behaviour in the object discrimination task was therefore investigated.

Treatments and testing:

Twelve rats that had been used in a previous series of experiments requiring repeated mefenamic acid treatment were utilized in this study: six of the rats had been given daily intra-peritoneal injections of saline for the previous twenty-five days. A second group of six rats had been given daily intra-peritoneal injections of mefenamic acid (20mg/kg) for the previous twenty-five days. On the day of testing the saline treated rats were given an intra-peritoneal injection of saline and the mefenamic acid treated rats given an intra-peritoneal injection of mefenamic acid (20mg/kg), thirty minutes prior to testing in the object discrimination task.

The effect of chronic mefenamic acid treatment on object exploration:

The overall exploration of objects was not significantly different between groups in either the sample phase (t_{10}= 0.74, $p \geq 0.10$) or choice phases (t_{10}= 0.63, $p \geq 0.10$), as shown in figure 3.16.

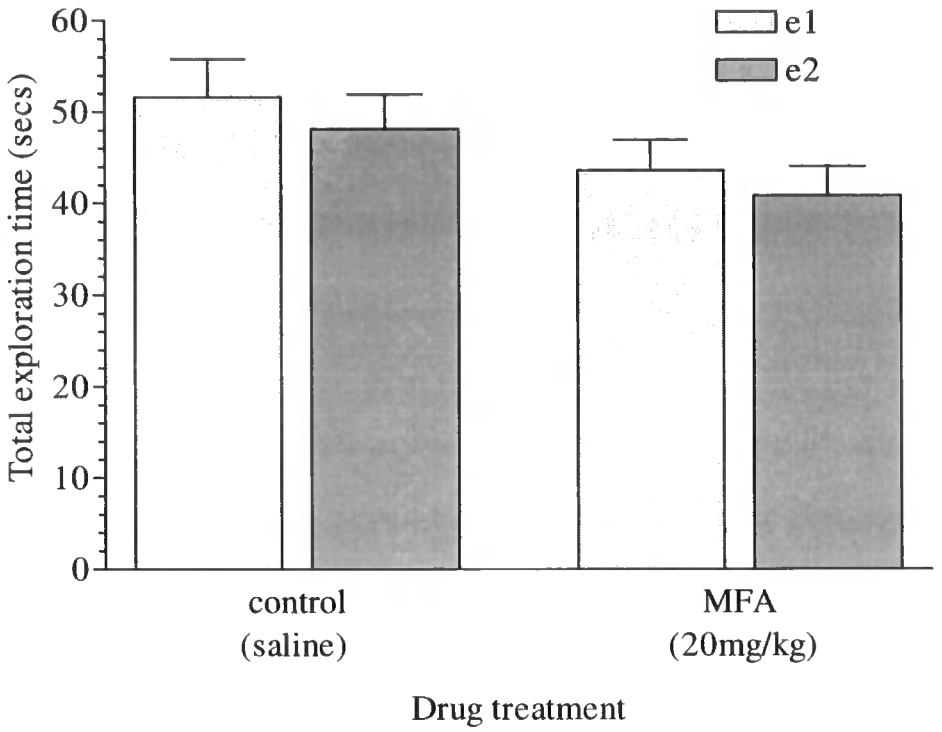


Figure 3.16: Chronic exposure to mefenamic acid does not affect total exploration times. The histogram shows the total object exploration time during the sample (e1) and choice (e2) phases on the y-axis for saline and chronic mefenamic acid (MFA; 20mg/kg) treated groups. Chronic exposure to MFA does not affect exploration times during the sample (e1) or choice phases (e2) when compared to saline controls. Each bar represents the mean±s.e.m. n=6 for each group.

The effect of chronic mefenamic acid treatment on object discrimination:

The discrimination time during the choice phase was significantly different between saline and rats chronically treated with mefenamic acid (t_{10}= 2.28, p≤ 0.05). This data is shown in figure 3.17.

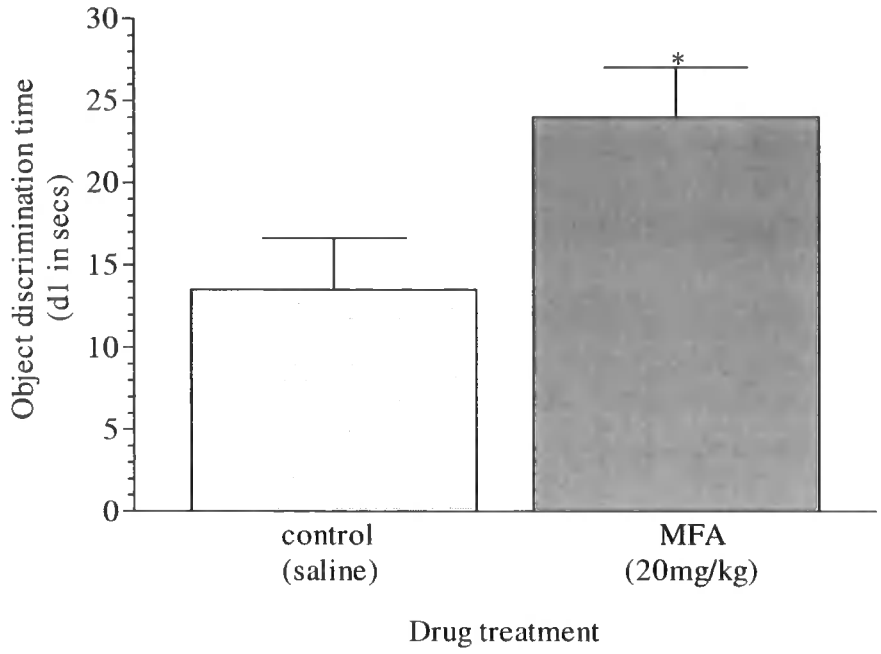


Figure 3.17: The effect of chronic treatment with mefenamic acid on object discrimination. The histogram shows the object discrimination time (d1) on the y-axis for saline and chronic mefenamic acid (20mg/kg; MFA) groups. Chronic MFA treatment results in a significant increase in d1, * $p \leq 0.05$. Each bar represents the mean±s.e.m.

Conclusion:

Chronic exposure to mefenamic acid results in increased object discrimination.

3.3xi: Experiment to investigate the stage of the mnemonic process modulated by mefenamic acid

The data obtained in this study suggest that mefenamic acid enhances object recognition memory. The aim of this experiment is to determine the stage of the mnemonic process (acquisition, storage or retrieval) that is modulated by mefenamic acid.

Treatment and testing:

Thirty-two rats were randomly allocated to four groups. Two groups were given an intra-peritoneal injection of either saline (n=8) or mefenamic acid (20mg/kg; n=8) thirty-minutes prior to testing in the object recognition task. Two other groups were

given an intra-peritoneal injection of either saline (n=8) or mefenamic acid (20mg/kg; n=8) immediately after the sample phase of the object recognition task. All groups were tested with a thirty-minute intra-trial interval.

The effect of mefenamic acid injected after the sample phase on total exploration times: The overall exploration of objects was not significantly different between groups in either the sample phase ($F_{3,28}$= 0.03, p≥ 0.10) or choice phase ($F_{3,28}$= 2.52, p= 0.08), as shown in figure 3.18.

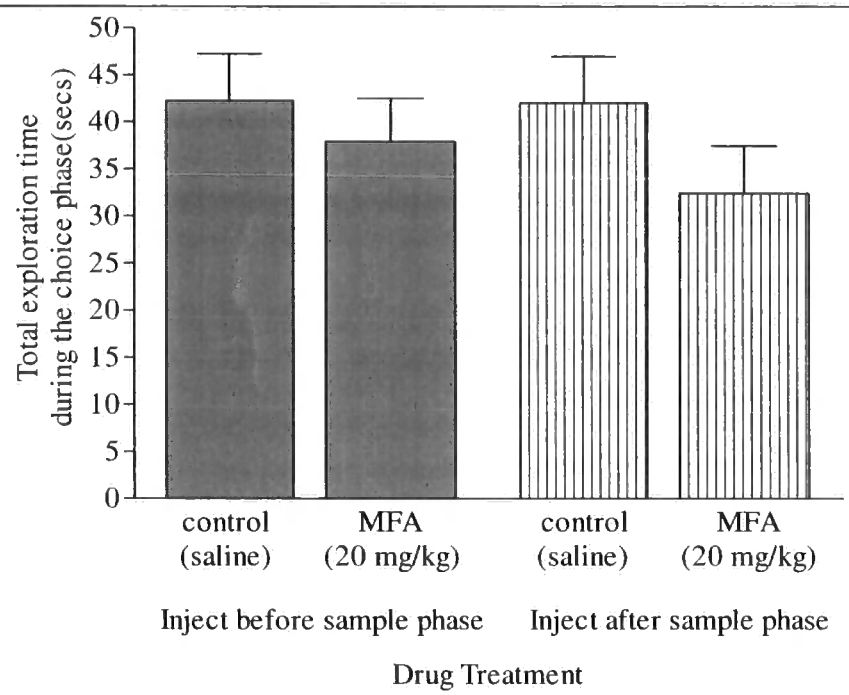


Figure 3.18: The effect of mefenamic acid on total exploration times when injected before and after the sample phase. The histogram shows the total exploration time during the choice phase (e2) for saline and mefenamic acid (MFA; 20mg/kg) groups injected before and after the sample phase. MFA did not significantly affect e2 when injected before or after the sample phase. Each data bar represents the mean ± s.e.m. n=8 for each group.

The effect of injecting mefenamic acid after the sample phase on object discrimination: The discrimination time between objects during the choice phase was significantly different across groups ($F_{3,28}$= 4.13, p≤ 0.05). A *post hoc* analysis revealed a significant

difference between mefenamic acid (injected before the sample phase) compared to saline (injected before the sample phase), saline (injected after the sample phase) and mefenamic acid (injected after the sample phase) ($p \leq 0.05$). These data are shown in figure 3.19.

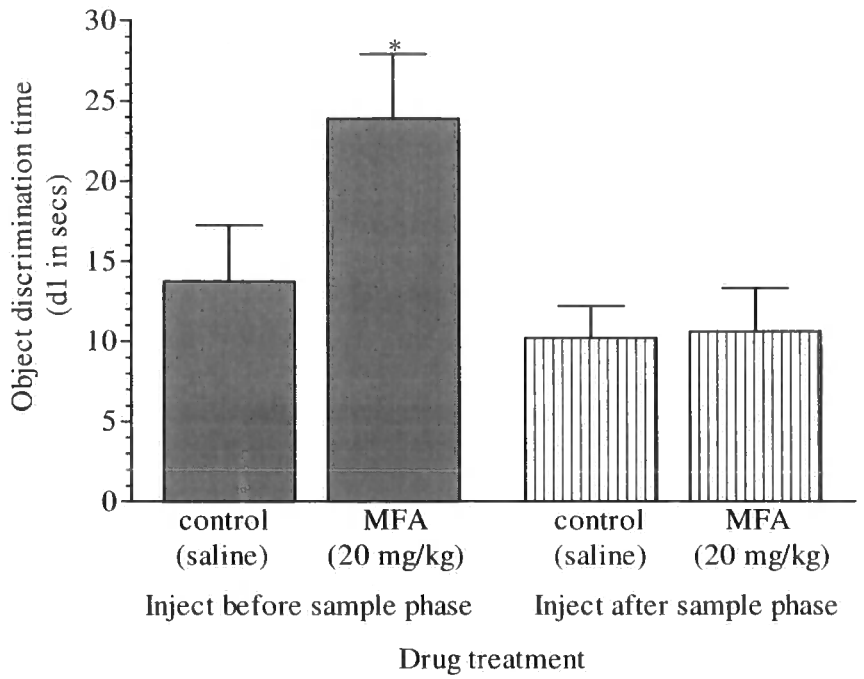


Figure 3.19: Mefenamic acid does not increase object discrimination when injected immediately after the sample phase. The histogram shows the object discrimination time (d1) on the y-axis for control and mefenamic acid (MFA; 20mg/kg) groups injected, 30 minutes before or immediately after the sample phase. MFA significantly increased d1 when injected prior to the sample phase but not when injected after the sample phase with a 30 minute intra-trial interval ($F_{3,28}= 4.13$, $p \leq 0.05$), * $p \leq 0.05$ compared to all other groups. Each data bar represents the mean ± s.e.m. n=8 for each group.

Conclusion:

Mefenamic acid only enhanced object discrimination when injected prior to the sample phase suggesting the hypothesis that mefenamic acid modulates the acquisition but not the retention or retrieval of information.

3.3xii: Behavioural parameters of the object location task

A previous study has reported that the object discrimination task, a non-spatial working memory task, uses different brain processes to those used in the object location task, a

spatial working memory task (Ennaceur & Meliani 1992b). It has been shown, above, that increasing the intra-trial interval causes a decrease in object discrimination. The following experiment addresses the effect of changes of intra-trial interval on rats' performance in the object location task.

Treatment and testing:

Thirty rats were randomly allocated into one of five groups. Each group was tested in the object location task with either a one-minute (n=6), fifteen-minute (n=6), sixty-minute (n=6), four hour (n=6) or twenty-four hour (n=6) intra-trial interval.

The effect of increasing the intra-trial interval on total exploration times:

The overall exploration of objects was not significantly different between groups in either the sample phase ($F_{4,25}$= 2.58, p≥ 0.10) or the choice phase ($F_{4,25}$= 0.22, p≥ 0.10). These data are shown in figure 3.20.

The effect of increasing the intra-trial interval on spatial discrimination:

The rats exploration time of moved and un-moved objects, during the choice phase (e2), for each intra-trial interval is shown in table 3.4, where it can be seen that there is a significant difference between exploration times of each object with a one-minute and fifteen-minute intra-trial interval, but not with a sixty-minute, four-hour or twenty-four hour intra-trial interval.

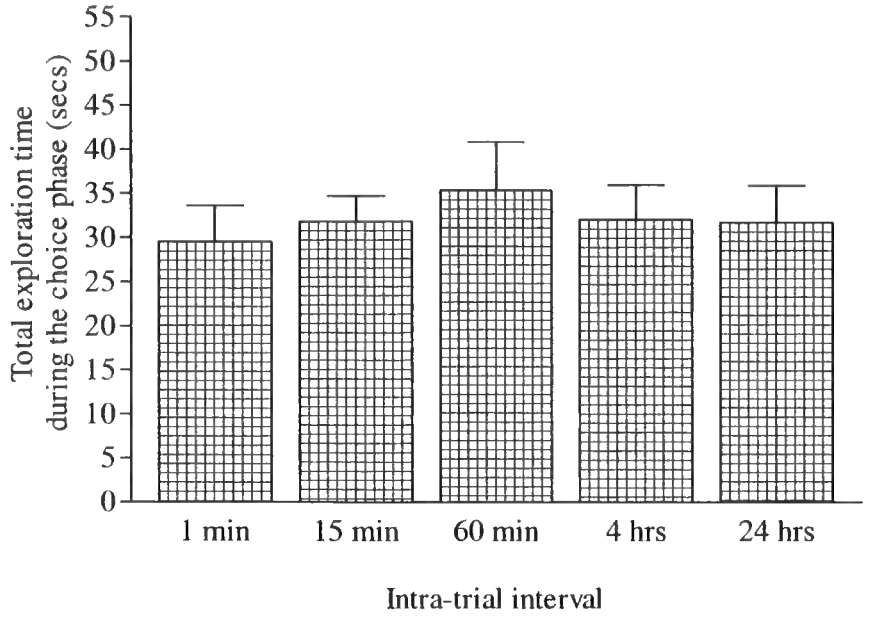


Figure 3.20: The total exploration times are not affected by changing intra-trial intervals in the object location task. The histogram shows the total exploration time during the choice phase (e2) on the y-axis against the intra-trial interval on the x-axis. e2 is not significantly affected by changing intra-trial intervals. Each data bar represents the mean ± s.e.m. n=6 for each delay.

Table 3.4: Effect of intra-trial interval on discrimination between a moved and un-moved object. Table showing that as the intra-trial interval (ITI) is increased the difference in the exploration time between moved and un-moved objects is decreased. Comparison of the exploration of each object for each ITI (n=6 for each ITI) is analysed with a paired Student t-test (two-tailed).

ITI (time)	exploration time (secs) of un-moved object (mean ± s.e.m.)	exploration time (secs) of moved object (mean ± s.e.m.)	p
1 min	7.8 ± 1.3	21.7 ± 3.9	$t_4=3.29, \leq 0.05$
15 min	7.2 ± 1.4	19.6 ± 3.3	$t_4=4.19, \leq 0.01$
60 min	15.0 ± 1.5	20.3 ± 4.6	$t_4=1.32, \geq 0.10$
4 hrs	15.2 ±2.6	16.8 ±2.4	$t_4=0.54, \geq 0.10$
24 hrs	15.0 ±1.7	16.7 ± 3.1	$t_4=0.60, \geq 0.10$

There is an overall significant difference between the discrimination times (d1) across intra-trial intervals ($F_{5,25}$= 2.82, p≤ 0.05), but *post-hoc* analysis showed no significant difference between comparison of individual intra-trial intervals (figure 3.21).

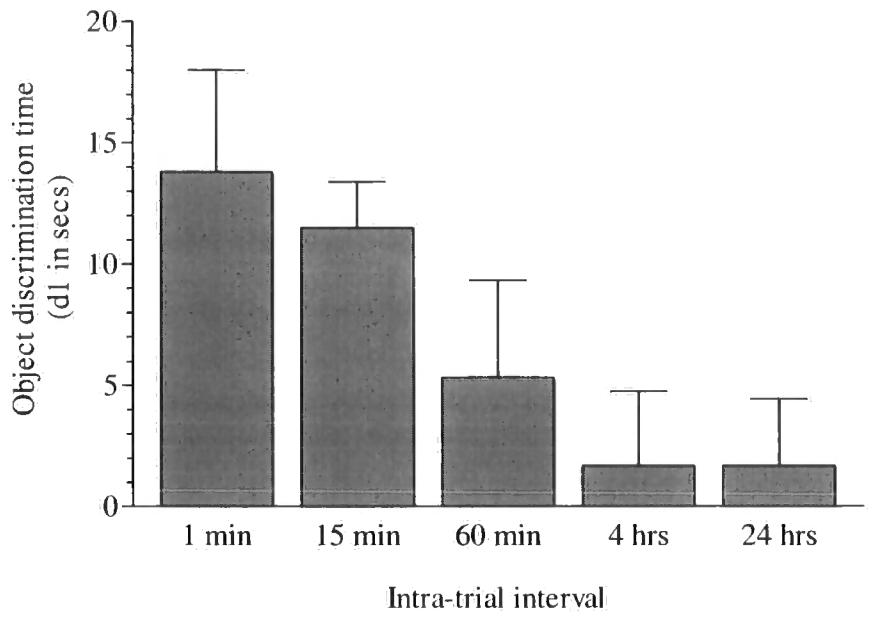


Figure 3.21: Increasing the intra-trial interval leads to a decrease in discrimination between moved and un-moved objects. The histogram shows the discrimination time between the moved and the un-moved object (d1) on the y-axis, against the intra-trial interval on the x-axis. It can be seen that as the intra-trial interval is increased, d1 is decreased. Each data bar is the mean ± s.e.m. n=6 for each intra-trial interval.

Conclusion:

The results of these studies demonstrate that rodents are able to discriminate between object location and that such discrimination is sensitive to intra-trial intervals. This finding is consistent with that of Ennaceur and Meliani, (1992b). Therefore, the following experiment addresses the effect of mefenamic acid on spatial working memory, using the object location task.

3.3xiii: The effect of mefenamic acid on spatial discrimination

It has been shown above that mefenamic acid enhanced object discrimination in the non-spatial object discrimination working memory task. The aim of this experiment, using the object location task, is to test the hypothesis that mefenamic acid can enhance spatial working memory.

Treatments and testing:

Sixteen rats were randomly allocated to one of two different treatment groups. Each group was given an intra-peritoneal injection of either saline (n=8) or mefenamic acid (20mg/kg; n=8), thirty minutes prior to testing in the object location task.

The effect of mefenamic acid on total exploration times:

The overall exploration of objects was not significantly different between groups in either the sample phase (t_{14}= 0.16, p≥ 0.10) or the choice phase (t_{14}= 1.18, p≥ 0.10) of the object location task (Figure 3.22).

The effect of mefenamic acid on spatial discrimination:

The discrimination between the moved and un-moved objects in the choice phase (d1) was significantly increased by mefenamic acid when compared to saline controls (t_{14}= 2.16, p≤ 0.05). These data are shown in figure 3.23.

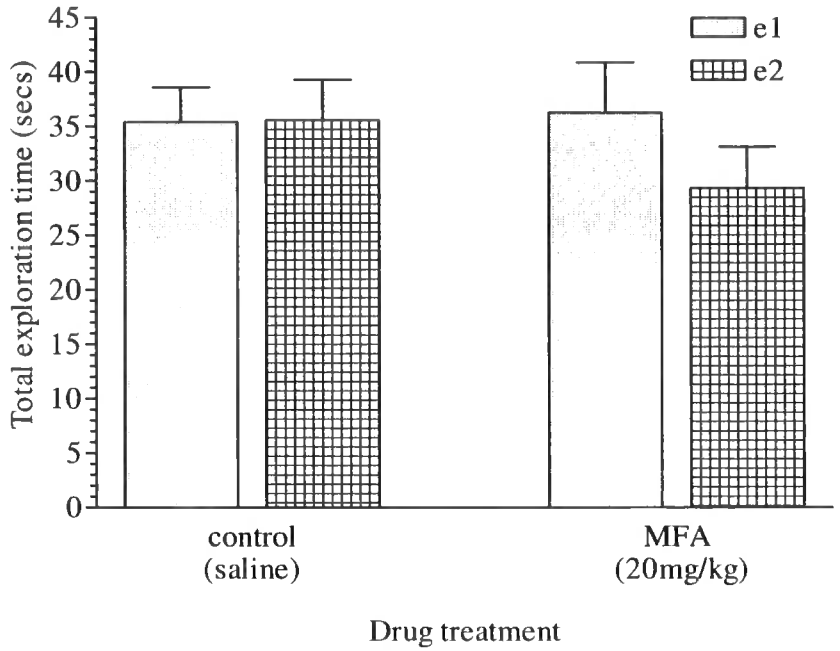


Figure 3.22: Mefenamic acid does not affect total exploration time in the object location task. The histogram shows the total exploration time during the sample (e1) and choice (e2) phases on the y-axis for saline and mefenamic acid (MFA; 20mg/kg) treatment groups. MFA does not significantly affect the total exploration times during e1 or e2. Each data bar represents the mean ± s.e.m. n=8 for each group.

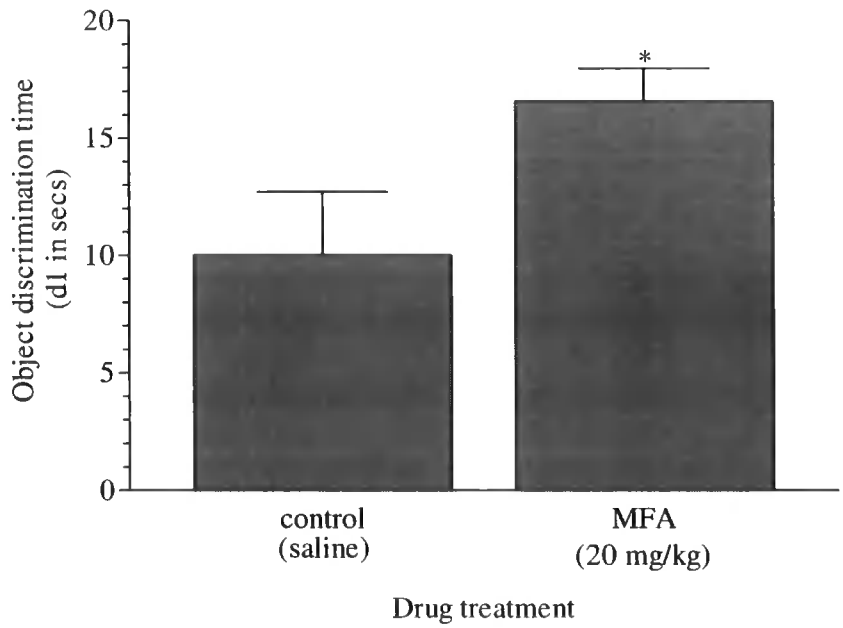


Figure 3.23: Mefenamic acid increases spatial discrimination in the object location task. The histogram shows the discrimination time (d1) on the y-axis. for a fixed and re-located object for saline and mefenamic acid (MFA; 20mg/kg) treatment groups. MFA significantly increased d1 when compared to control, * $p \leq 0.05$. Each data bar represents mean ± s.e.m. n=8 for each group.

Conclusion:

Mefenamic acid increased discrimination between a fixed and re-located object in the object location task. This finding is consistent with the results from the object discrimination task. Together, these data suggest that mefenamic can modulate both spatial and non-spatial working memory.

3.4: Summary

The experiments from this section have shown that high doses of mefenamic acid (> 20mg/kg) result in sedation and seizure-activity in the rats, while animals treated with lower doses of mefenamic acid (≤ 20mg/kg) show normal posture and exploratory activity.

Non-mnemonic processes such as anxiety, motor activity or arousal are not modulated by mefenamic acid.

The ability of rats to make a discrimination between novel and familiar objects is sensitive to intra-trial interval and this is consistent with previous studies (Ennaceur & Delacour, 1988; Ennaceur & Meliani, 1992a; Bartolini *et al.*, 1996). These data therefore provided a basis for the investigation of drug actions on object discrimination and object recognition memory.

Mefenamic acid increased object discrimination in a dose- and time- dependent manner, indicating that it can enhance non-spatial working memory. The increase in object discrimination was only observed when mefenamic acid was injected before the sample

phase, suggesting that it enhanced the acquisition or consolidation of information, but not the storage or retrieval of information.

The object discrimination task was designed to investigate non-spatial working memory. The object location task was utilised to investigate the effect of mefenamic acid on spatial working memory, as it is thought that the two forms of memory use different neuronal networks (Steckler *et al.*, 1998b). It was shown that object location is sensitive to intra-trial intervals, which is consistent with a previous report (Ennaceur & Meliani, 1991b). Animals treated with mefenamic acid increased object discrimination in the task compared to saline treated controls, indicating that mefenamic acid enhanced spatial working memory.

These experiments have shown that mefenamic acid enhanced both non-spatial and spatial working memory in normal rats and that the enhancement is not due to non-mnemonic processes such as arousal, anxiety or changes in locomotor activity.

Chapter Four: Mechanisms Underlying the Behavioural Effects of Mefenamic Acid

4.1: Introduction

The aim of the experiments described in this chapter was to determine possible mechanisms underlying the enhancement in object discrimination observed with mefenamic acid. The first group of experiments investigated the effects of several fenamate and non-fenamate NSAIDs to determine if the effect was a group (fenamate) or class (NSAID) effect.

A second potential mechanism for the behavioural effect of mefenamic acid could be due to modulation of the $GABA_A$ receptor as described in this thesis and by others (Woodward *et al.*, 1994; Halliwell *et al.*, 1999). Therefore to investigate this hypothesis, the effect of a range of $GABA_A$ receptor modulators on object discrimination was determined.

Additionally, epidemiological evidence has suggested that NSAIDs delay the onset and slow the development of Alzheimer's disease, and possibly improve memory function in Alzheimer's diseased patients (see chapter one). To investigate the effects of mefenamic acid in cognitively impaired animals, rats were treated with the muscarinic acetylcholine antagonist, scopolamine. This compound has been shown to impair performance in a range of behavioural tasks in both animal (Stevens, 1981; Dunnett, 1985; Beninger *et al.*, 1986; Huston & Aggleton, 1987) and human (Drachman, 1977; Preston *et al.*, 1989; Flicker *et al.*, 1990) studies. The effects of scopolamine in the object discrimination task were first determined. The effect of mefenamic acid was then investigated on the actions of scopolamine in the object discrimination task.

4.2: Methods and Materials

4.2i: The object discrimination task

The apparatus, protocol and statistical analysis for the object discrimination task have been described previously (chapter three, pgs 64 - 67).

4.2ii: Drugs and drug administration

All drugs were supplied by Sigma (Poole, UK) unless otherwise stated. Scopolamine (0.5mg/ml), piracetam (200mg/ml) and bicuculline (0.5mg/ml) were dissolved in double distilled water. All NSAIDs tested were initially dissolved in 0.05M NaOH and made up with double distilled water to a dose of 10mg/ml. Diazepam was dissolved in a 10% ethanol solution and administered at a dose of 0.5mg/ml. Loreclezole (kindly donated by Jannsen Pharmaceuticals, Belgium) was dissolved in a 10% cyclodextrin and 0.1M tartaric acid solution at a dose of 2mg/ml. All drugs were given via an intra-peritoneal injection, with 0.2-1.0mls given per injection, thirty minutes prior to testing.

4.3: Results

4.3i: Is enhanced object discrimination a fenamate group effect?

To observe whether the enhancement in object discrimination observed with mefenamic acid is a fenamate group effect, the actions of flufenamic acid, meclofenamic acid, niflumic acid and tolfenamic acid on object discrimination was investigated.

Treatment and testing:

Forty-eight rats were randomly allocated to six different groups. Each group received either an intra-peritoneal injection of saline (n=8), mefenamic acid (20mg/kg; n=8),

flufenamic acid (20mg/kg; n=8), meclofenamic acid (20mg/kg; n=8), niflumic acid (20mg/kg; n=8) or tolfenamic acid (20mg/kg; n=8), thirty minutes prior to testing. Each group of rats were then tested in the object discrimination task with a fifteen-minute intra-trial interval.

The effects of fenamates on total exploration times

The overall exploration of objects during the sample phase (e1) was not significantly different between fenamate groups ($F_{5,42}$= 1.60, p≥ 0.10). There was, however, a significant difference between groups in overall exploration of objects during the choice phase (e2) ($F_{5,42}$= 9.86, p≤ 0.001), shown in figure 4.1. A *post hoc* analysis showed significant differences between the mefenamic acid treated group compared to saline, flufenamic acid, niflumic acid and tolfenamic acid treated groups (p≤ 0.05); the meclofenamic acid treated group compared to saline, niflumic acid and tolfenamic acid treated groups (p≤ 0.05).

The effects of fenamates on object discrimination:

The object discrimination time (d1) was significantly different between groups ($F_{5,42}$= 4.23, p≤ 0.01). This data is shown in figure 4.2. A *post-hoc* analysis revealed significant differences between the mefenamic acid treated group compared to saline, niflumic acid and tolfenamic acid treated groups (p≤ 0.05), and between meclofenamic acid compared to saline, niflumic acid and tolfenamic acid treated groups (p≤ 0.05).

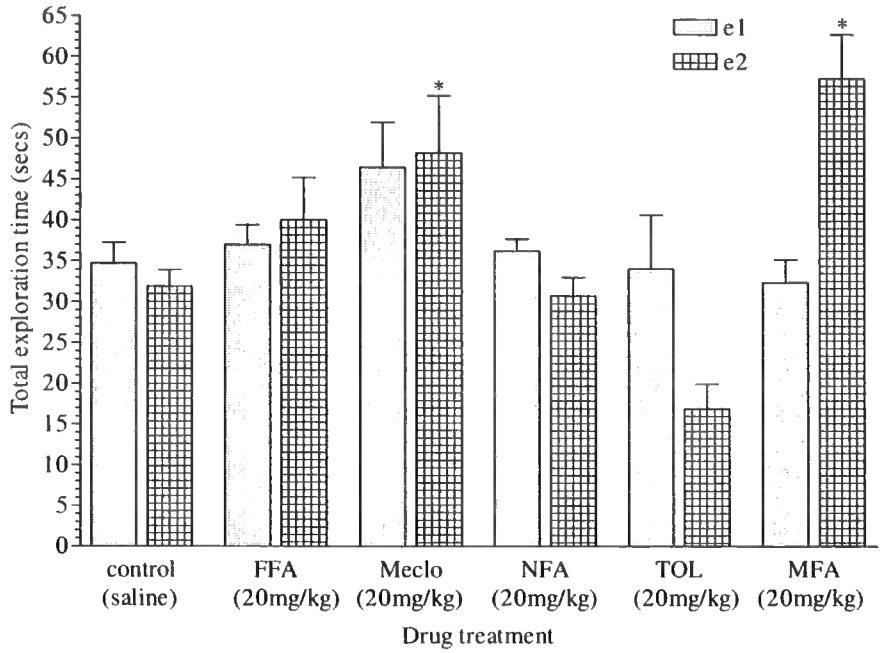


Figure 4.1: The affects of a range of fenamate NSAIDs on total exploration time during the sample and choice phases. The histogram shows the total exploration time (secs) on the y-axis, for objects in the sample (e1) and choice (e2) phases, for control, flufenamic acid (FFA), meclofenamic acid (Meclo), niflumic acid (NFA), tolfenamic acid (TOL) and mefenamic acid (MFA) groups. e1 was not significantly affected by fenamate NSAIDs. e2, however, was significantly affected by MFA and Meclo. * $p \leq 0.05$ when compared to control. Each data bar represents the mean ± s.e.m. n=8 for each group.

Conclusion:

This experiment has shown that the fenamate, meclofenamic acid can mimic the effect of mefenamic acid in the object discrimination task. However the fenamates, niflumic acid, flufenamic acid and tolfenamic acid did not replicate the effects of mefenamic acid. These data, therefore, suggest that the behavioural effects of mefenamic acid are not entirely a fenamate group effect.

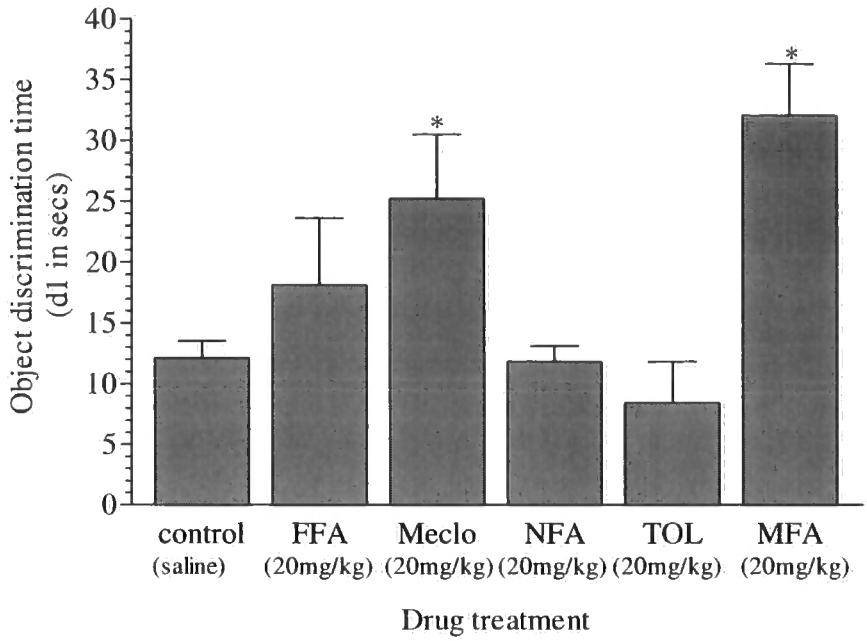


Figure 4.2: Fenamate NSAIDs modulate object discrimination. Histogram showing that the fenamates, meclofenamic acid (Meclo) and mefenamic acid (MFA), significantly modulate object discrimination in the choice phase (d1), whereas flufenamic acid (FFA), niflumic acid (NFA) and tolfenamic acid (TOL) do not modulate d1. * $p \leq 0.05$ when compared to control. Each data bar represents the mean ± s.e.m. n= 8 for each group.

4.3ii: Is enhancement of object discrimination a NSAID class effect?

Treatments and testing:

Twenty-four rats were randomly allocated to one of three different groups. Each group received an intra-peritoneal injection of saline (n=8), aspirin (20mg/kg; n=8), or ibuprofen (20mg/kg; n=8), thirty minutes prior to testing. Each group of rats were then tested in the object discrimination task with a fifteen-minute intra-trial interval.

The effects of non-fenamate NSAIDs on total exploration:

The overall exploration of objects was not significantly different between groups in e1 ($F_{2,21}= 0.77$, $p \geq 0.10$) or e2 ($F_{2,21}= 0.98$, $P \geq 0.10$) of the test. (Figure 4.3).

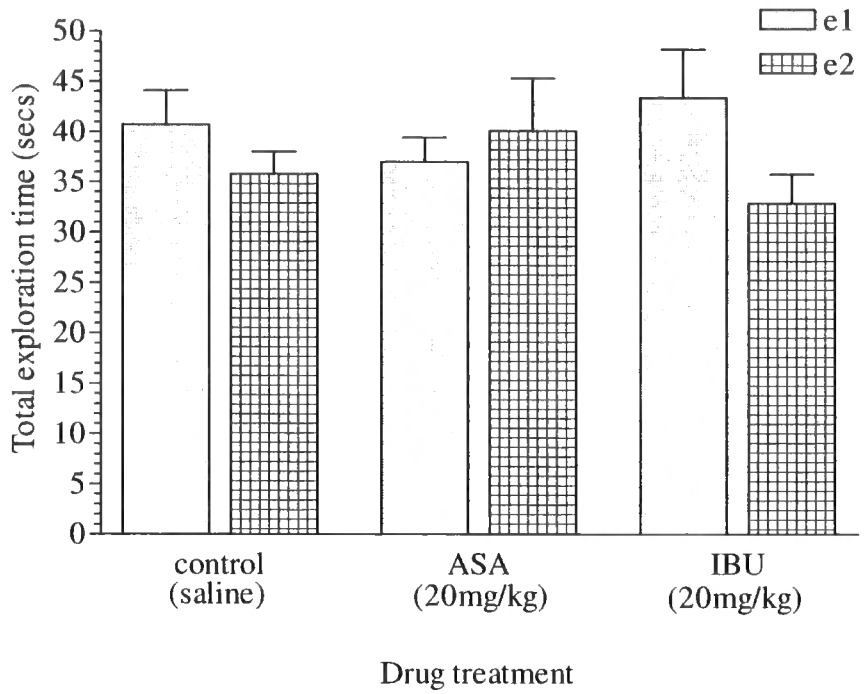


Figure 4.3: Non-fenamate NSAIDs do not affect total exploration times. The histogram shows the total exploration time (secs) on the y-axis, for the objects in the sample (e1) and choice (e2) phases, for aspirin (ASA) or ibuprofen (IBU). Each data bar represents the mean ± s.e.m. n=8 for each group.

The effect of non-fenamate NSAIDs on object discrimination:

The object discrimination times (d1) were not significantly affected by non-fenamate NSAIDs ($F_{2,21}$= 0.33, p≥ 0.10). These data are shown in figure 4.4.

Conclusion:

The non-fenamate NSAIDs aspirin and ibuprofen did not affect object discrimination, indicating that the enhancement observed with fenamate NSAIDs may not be due to inhibition of cyclooxygenases.

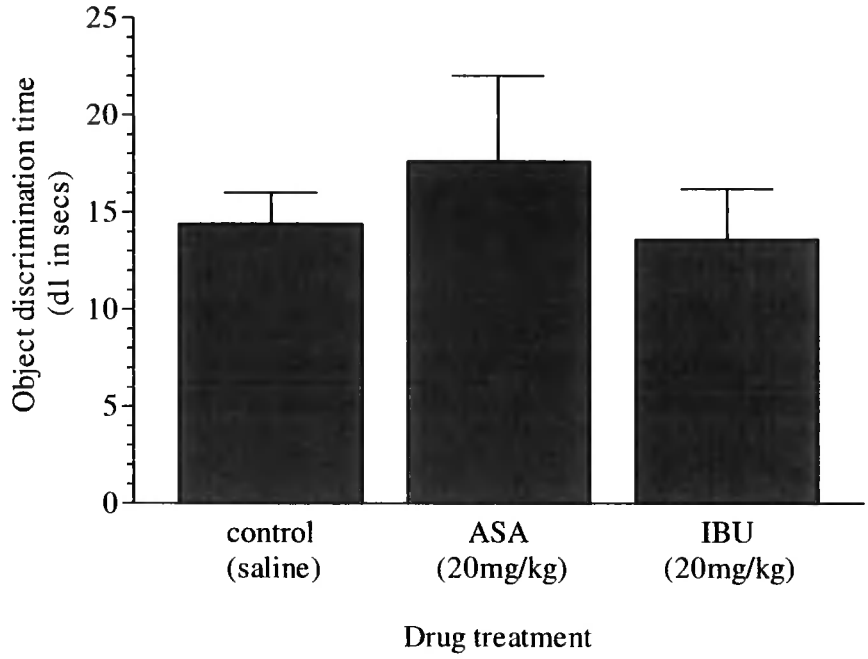


Figure 4.4: The non-fenamate NSAIDs do not affect object discrimination. The histogram shows the object discrimination time (d1) on the y-axis for control, aspirin (ASA) and ibuprofen (IBU) groups. Each data bar represents the mean ± s.e.m. n=8 for each group.

4.3iii: The effect of $GABA_A$ receptor modulators on object discrimination

In order to test the hypothesis that the behavioural effects of mefenamic acid is via modulation of the $GABA_A$ receptor, the effect of, bicuculline a $GABA_A$ receptor antagonist; diazepam a positive allosteric modulator of the $GABA_A$ receptor and loreclezole, another positive allosteric modulator of the $GABA_A$ receptor, reported to bind to the same site on the $GABA_A$ receptor complex as mefenamic acid (Halliwell *et al.*, 1999), were investigated in the object discrimination task.

Treatments and testing:

Thirty-two rats were randomly allocated to one of four different groups. Each group received an intra-peritoneal injection of either saline (n=8), bicuculline (1mg/kg; n=8), diazepam (2mg/kg; n=8) or loreclezole (10mg/kg; n=8), thirty minutes prior to testing.

Each group of rats were then tested in the object discrimination task with a fifteen-minute intra-trial interval. It was noted that two of the diazepam treated rats were sedated and did not show exploratory activity when placed in the arena; these two animals were excluded from the experiment.

The effect of $GABA_A$ modulators on total exploration times:

The overall exploration of objects was not significantly different between drug groups during e1 ($F_{3,26}$= 1.95, p≥ 0.10) or e2 ($F_{3,26}$= 1.25, p≥ 0.10). These data are shown in figure 4.5.

The effects of $GABA_A$ modulators on object discrimination:

The object discrimination times (d1) was not significantly different between groups ($F_{3,26}$= 1.44, p≥ 0.10), as shown in figure 4.6.

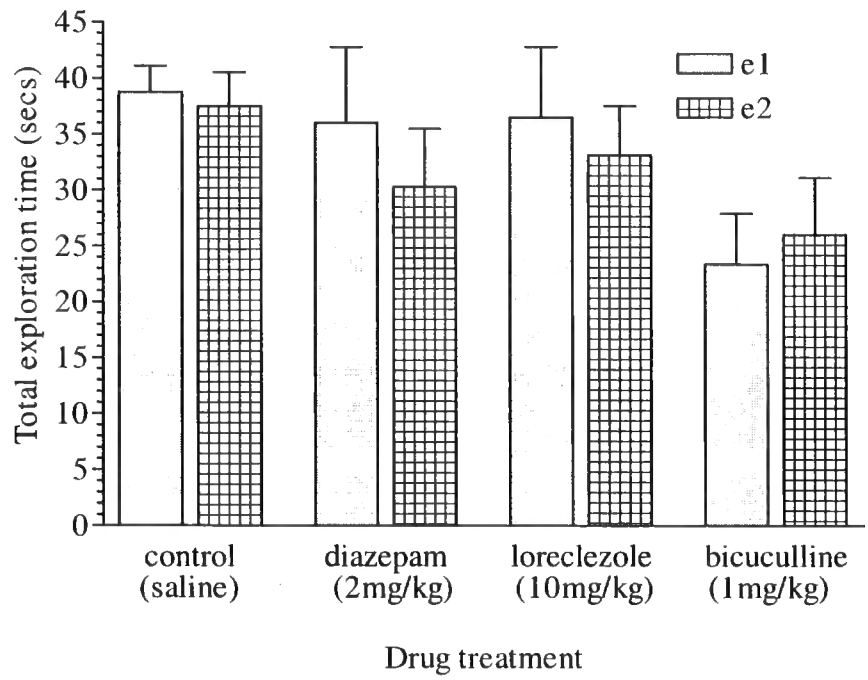


Figure 4.5: $GABA_A$ receptor modulators do not effect total exploration times. The histogram shows the total exploration time (secs), on the y-axis, for the objects in the sample (e1) and choice (e2) phases for control, diazepam, loreclezole and bicuculline treatment groups. Each data bar represents the mean ± s.e.m. n=6 for diazepam, n= 8 for control, loreclezole and bicuculline treated groups.

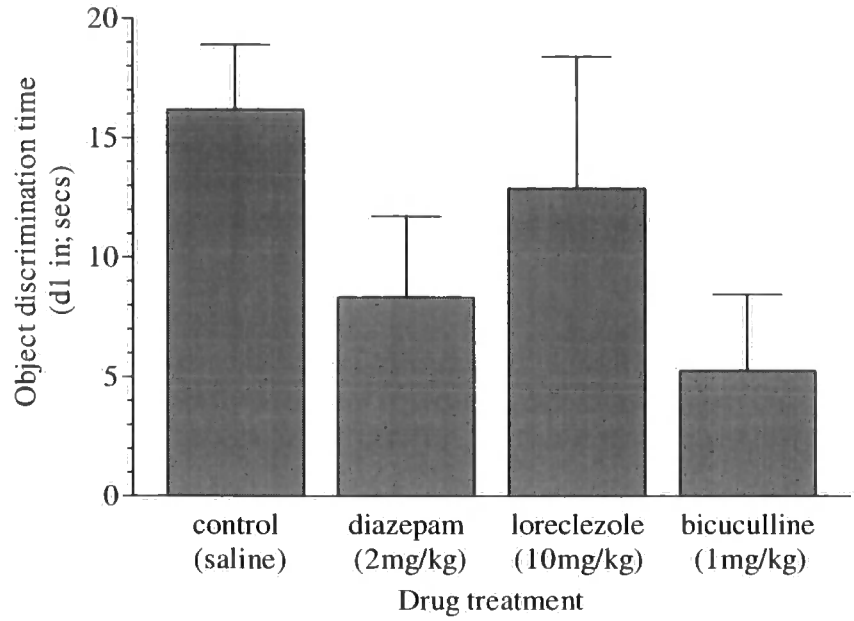


Figure 4.6: $GABA_A$ receptor modulators do not significantly modulate object discrimination. The histogram shows the object discrimination time (d1), on the y-axis for control, diazepam, loreclezole and bicuculline groups. There was no significant difference in d1 between groups. Each data bar represents the mean ± s.e.m. n=6 for diazepam, n= 8 for control, loreclezole and bicuculline treated groups.

Conclusion:

None of the $GABA_A$ receptor modulators tested significantly affected object discrimination by rats, although object discrimination was impaired in both diazepam and bicuculline treated groups when compared to saline controls. These data suggest that the increase in object discrimination observed with mefenamic acid may not depend upon modulation of the $GABA_A$ receptor.

4.3iv: The effect of scopolamine on object discrimination

Treatments and testing:

Thirty-two rats were randomly allocated to one of four different groups. Each group received an intra-peritoneal injection of either saline (n=8) or scopolamine at 0.25mg/kg (n=8), 0.5mg/kg (n=8) or 1mg/kg (n=8), thirty minutes prior to testing. Each group of

rats were then tested in the object discrimination task with a fifteen-minute intra-trial interval.

The effect of scopolamine on total object exploration:

Overall exploration times during e1 were significantly reduced ($F_{3,28}$= 7.57, p≤ 0.001) by scopolamine. A *post-hoc* analysis revealed significant differences between saline compared to scopolamine at 0.5 mg/kg and 1 mg/kg (p≤ 0.001). There was no significant difference between control and 0.25 mg/kg scopolamine (figure 4.7). Total exploration time during e2 was also significantly different between groups ($F_{3,28}$= 9.09, p≤ 0.001). A *post-hoc* analysis showed significant differences between saline compared to scopolamine at 0.5 mg/kg and 1 mg/kg (p≤ 0.05). There was a significant difference between scopolamine (0.25 mg/kg) compared to scopolamine at 1 mg/kg (p≤ 0.01). There was no significant difference (p≥ 0.10) between control and scopolamine (0.25 mg/kg).

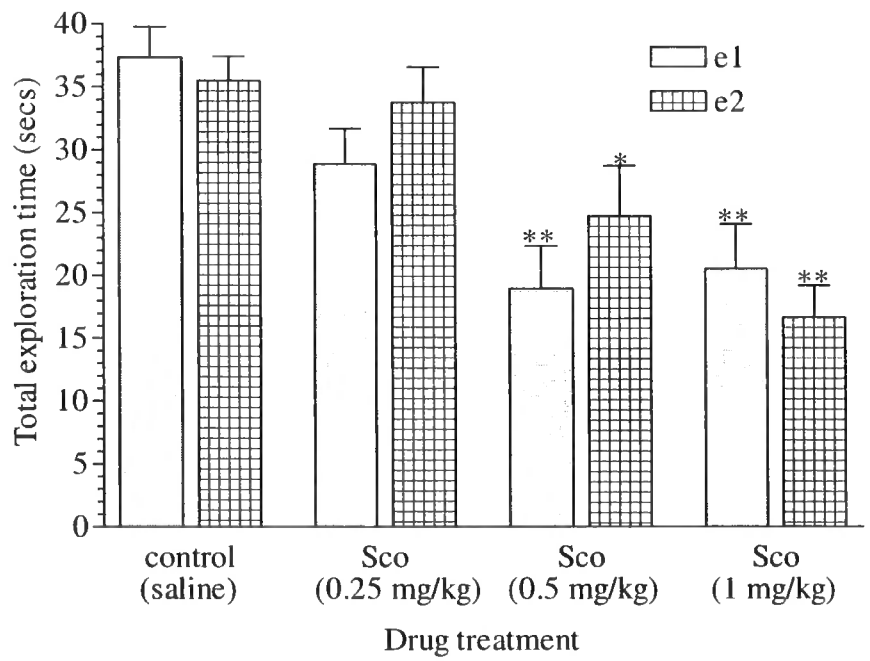


Figure 4.7: The effect of scopolamine on total exploration times.
The histogram shows the total exploration time (secs), on the y-axis, for the objects in the sample (e1) and choice (e2) phases for control and scopolamine (Sco) at 0.25, 0.5 and 1mg/kg. e1 was significantly decreased by Sco ($F_{3,28}$= 7.57, p≤ 0.001), e2 was also significantly decreased by Sco ($F_{3,28}$= 9.09, p≤ 0.001). ** p≤ 0.01, * p≤ 0.05, compared to control. Each data bar represents the mean ± s.e.m. n=8 for each group.

The effect of scopolamine on object discrimination:

The object discrimination (d1) was significantly different between groups ($F_{3,28}$= 14.0, p≤ 0.0001). A *post hoc* analysis showed significant differences between saline compared to scopolamine at 0.25mg/kg, 0.5mg/kg and 1mg/kg (p≤ 0.01). There was no significant difference between the scopolamine-treated groups (figure 4.8).

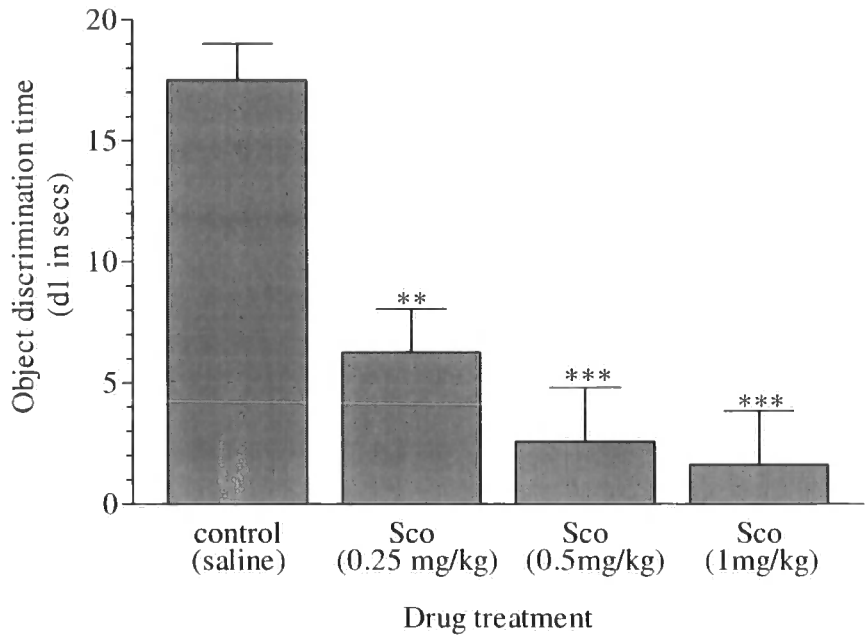


Figure 4.8: Scopolamine significantly reduces object discrimination time. The histogram shows the object discrimination time (d1) on the y-axis for control, and scopolamine (Sco) at 0.25, 0.5 and 1mg/kg. Sco significantly reduces d1 ($F_{3,28}$= 14.0, p≤ 0.0001), *** p≤ 0.001, ** p≤ 0.01 compared to control. Each data bar represents the mean ± s.e.m. n=8 for each group.

Conclusion:

Scopolamine significantly impairs object discrimination. Higher doses of scopolamine (0.5 mg/kg, 1 mg/kg) also reduced the total exploration times during the sample and choice phases suggesting an impairment in gross motor activity.

4.3v: The affect of mefenamic acid on scopolamine-induced impairment on object discrimination

Treatment and testing:

Thirty-two rats were randomly allocated to one of four different groups. Each group received an intra-peritoneal injection of saline (n=8) or a combined injection of mefenamic acid (20mg/kg) plus scopolamine at 0.25mg/kg (n=8), 0.5mg/kg (n=8) or 1mg/kg (n=8), thirty minutes prior to testing. Each group of rats was then tested in the object discrimination task with a fifteen-minute intra-trial interval.

The effect of combined mefenamic acid and scopolamine on total exploration:

There was a significant difference between groups treated with either saline, or scopolamine plus mefenamic acid in total exploration time during the sample phase (e1) ($F_{3,28}$= 4.37 p≤ 0.01). A *post-hoc* analysis revealed a significant difference between saline controls compared to mefenamic acid plus scopolamine at 0.5mg/kg and 1mg/kg (p≤ 0.05). There was no significant difference between mefenamic acid plus scopolamine (0.25 mg/kg) treated group compared to control or between scopolamine plus mefenamic acid-treated groups. These data are shown in figure 4.9.

The total exploration time during the choice phase (e2) was significantly different between groups ($F_{3,28}$= 6.12, p≤ 0.01), with *post-hoc* analysis showing a significant difference between saline compared to mefenamic acid plus scopolamine at 1mg/kg (p ≤ 0.01).

The effect of combined scopolamine and mefenamic acid treatment on object discrimination:

The discrimination times between the two objects in the choice phase (d1) was significantly different between groups ($F_{3,28}$= 7.50, p≤ 0.001). A *post-hoc* analysis showed significant differences between mefenamic acid plus scopolamine (1mg/kg) compared to saline controls (p≤ 0.001), and mefenamic acid plus scopolamine at 0.25mg/kg and 0.5mg/kg treated groups (p≤ 0.01). These data are shown in figure 4.10.

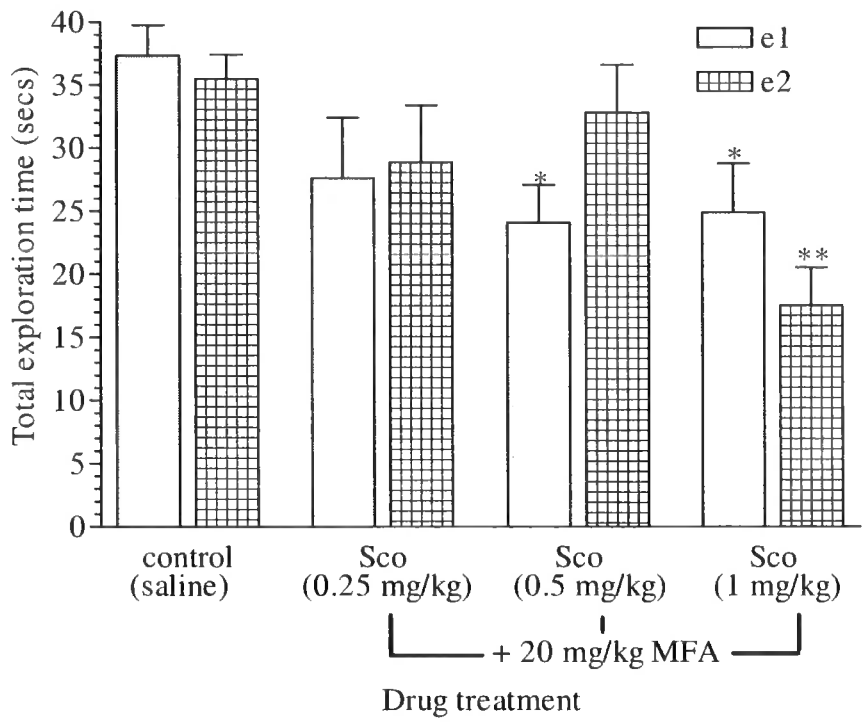


Figure 4.9: The effect of combined scopolamine and mefenamic acid treatment on total exploration times. The histogram shows the total exploration time (secs), on the y-axis, for the objects in the sample (e1) and choice (e2) phases for control and combined scopolamine (Sco) plus mefenamic acid (MFA) groups. Both e1 ($F_{3,28}$= 4.37, p≤ 0.01) and e2 ($F_{3,28}$= 6.12, p ≤ 0.01) were significantly different between groups, ** p≤ 0.01; * p≤ 0.05 compared to control. Each data bar represents the mean ± s.e.m. n=8 for each group.

When d1 data for scopolamine alone is compared to the respective d1 for mefenamic acid plus scopolamine data using an un-paired student t-test, significant differences were found between: scopolamine alone (0.25 mg/kg) compared to scopolamine (0.25

mg/kg) plus mefenamic acid (t_{14}=2.32, p≤ 0.05), and between scopolamine alone (0.5 mg/kg) compared to scopolamine (0.5 mg/kg) plus mefenamic acid (t_{14}=3.31, p≤ 0.01). There was no significant difference between scopolamine alone (1mg/kg) compared to scopolamine (1mg/kg) plus mefenamic acid (t_{14}= 0.68, p≥ 0.10).

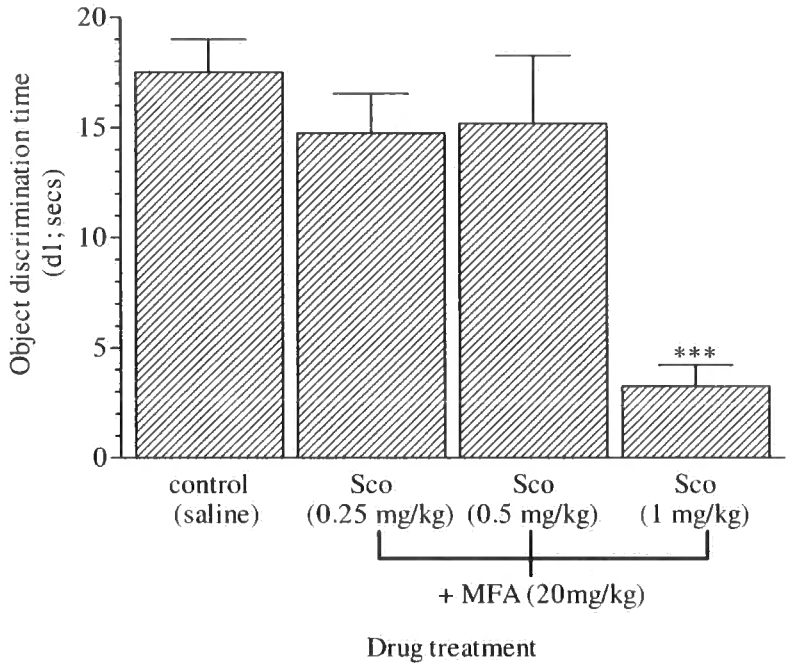


Figure 4.10: Mefenamic acid reverses the scopolamine-induced impairment in the object discrimination task. The histogram shows the object discrimination time (d1), on the y-axis for control and scopolamine (Sco) at 0.25, 0.5 and 1mg/kg in combination with mefenamic acid (MFA). The discrimination times between objects is significantly different between groups treated with Sco plus MFA ($F_{3,28}$= 7.50, p≤ 0.001), *** p≤ 0.001 when compared to control. Each data bar represents the mean ± s.e.m. n=8 for each group.

Conclusion:

This experiment has shown that mefenamic acid can reverse a scopolamine- induced impairment in object discrimination.

4.4: Summary

The experiments reported above, have shown that two fenamates (mefenamic acid and meclofenamic acid) were associated with an increase in object discrimination, but the fenamates, flufenamic acid, niflumic acid and tolfenamic acid did not increase object discrimination. These data suggest that the enhancement observed with mefenamic acid is not entirely a fenamate group effect.

The non-fenamate NSAIDs, ibuprofen and aspirin, did not significantly affect object discrimination, suggesting that the effects observed with fenamates is not a NSAID class effect .

Similarly, none of the $GABA_A$ receptor modulators tested in this study were able to mimic the effect observed with mefenamic acid, suggesting that the increase in object discrimination may not depend entirely on the $GABA_A$ receptor.

Application of scopolamine impaired rats' performance in the object discrimination task. Mefenamic acid, wholly or partially reversed the scopolamine-induced impairment, indicating that mefenamic acid might improve the performance of cognitively-impaired rats.

Chapter Five: Investigation of the Effect of Mefenamic Acid on the T-maze and Radial Maze Tasks

5.1: Introduction

The experiments reported in previous chapters have established that mefenamic acid increases discrimination between objects in both the object discrimination and object location tasks. To test the hypothesis that the effects of mefenamic acid are not task specific and moreover that it enhances working memory, the effects of mefenamic acid were investigated in the T-maze and radial maze. Both of these tasks are well characterised working memory paradigms (Olton & Samuelson, 1976; Stevens, 1981; Steckler *et al.*, 1998a)

5.2: Methods

5.2i: Non-matching-to-sample T-maze paradigm

The delayed non-matching-to-sample T-maze paradigm is a spatial memory paradigm, which requires the learning of a rule to gain food reinforcement. It has been used previously to investigate the central effects of drugs on spatial working memory. For example, Ennaceur and Delacour (1987) showed that intra-peritoneal injection of choline could enhance performance in this task. Similarly, aged rats treated with the cholinesterase inhibitor, physostigmine, perform significantly better than age match controls in the delayed non-match-to-sample T-maze task (Ohta *et al.*, 1991). Tacrine, a clinically used cholinesterase inhibitor, can reverse a scopolamine-induced impairment in the T-maze (M'Harzi *et al.*, 1997) while the selective muscarinic M1 acetylcholine partial agonist, sabcomeline can improve performance in the T-maze task (Hatcher *et*

al., 1998). Therefore the effects of mefenamic acid on working memory were investigated in the non-matching-to-sample T-maze task.

Animals:

Male Lister hooded rats (250-300g, Charles river, U.K.) were housed in pairs under a 12 hour light (07:00-19:00)/ 12 hour dark cycle, and were tested, at a regular time, in the light phase. The rats had *ad libitum* access to water but were placed on a controlled food diet so that their body weight was maintained at approximately 85% of normal at the time of testing. Their weight was monitored regularly, to ensure that their weight did not fall below 85% of normal.

Apparatus:

A matt black painted wooden T-maze was constructed (in house). Each arm of the T-maze was 50cm long by 30cm wide. At the end of each arm was a 2cm diameter food cup. The stem of the T-maze was 50cm long by 30cm wide with a guillotine door located 20cm away from the beginning of the stem. 30cm high plywood walls enclosed the T-maze. A wooden barrier was also used to block one arm of the T-maze during the sample phase. The maze was cleaned with ethanol between trials to eliminate odour cues.

Habituation:

Rats were habituated to the T-maze by being allowed to explore the maze for three minutes per day for five days. During habituation sessions, food pellets were placed around the maze; the number of pellets was reduced daily until they were only placed in the food wells. After habituation the rats moved freely around the maze without freezing, defecation or urination and thereafter testing in the T-maze began.

Acquisition of non-matching-to-sample T-maze task:

Rats were injected with saline or mefenamic acid (20mg/kg) thirty minutes prior to daily testing. The rats were tested for ten trials each day in the T-maze until they reached a criterion level of no more than three errors in total over three consecutive days. Each trial consisted of a sample and a choice phase. During the sample phase, the rat was introduced into the stem of the T-maze, with one of the arms blocked by a wooden barrier, forcing the rat to enter the open arm of the maze (figure 5.1). After the rat had visited the open arm it was removed and placed into the start box for ten seconds. At the end of the delay access to both arms was free and two food pellets (2 x 45mg; Noyes INC. New Hampshire, USA) were placed only in the food cup of the previously closed arm. The start box was then opened for the choice phase. If the rat chose the previously un-visited arm it was allowed to eat the food pellets before being removed and returned to its holding cage. If the arm visited during the sample phase was chosen, the rat was confined to the arm for ten seconds before being removed and returned to its home cage. Rats were tested in groups of four, so the inter-trial delay was approximately three minutes. Each arm was blocked for five out of the ten sample phases each day in a pseudo-random order. This procedure was repeated daily until animals reached criterion. If rats had not reached criterion by twenty days of testing they were removed from further study.

Delayed non-matching-to-sample testing:

The rats were trained in the T-maze, as described above, until they had reached criterion (defined above), after which they were not tested in the maze again until all the animals had reached criterion, to reduce over-training of the task.

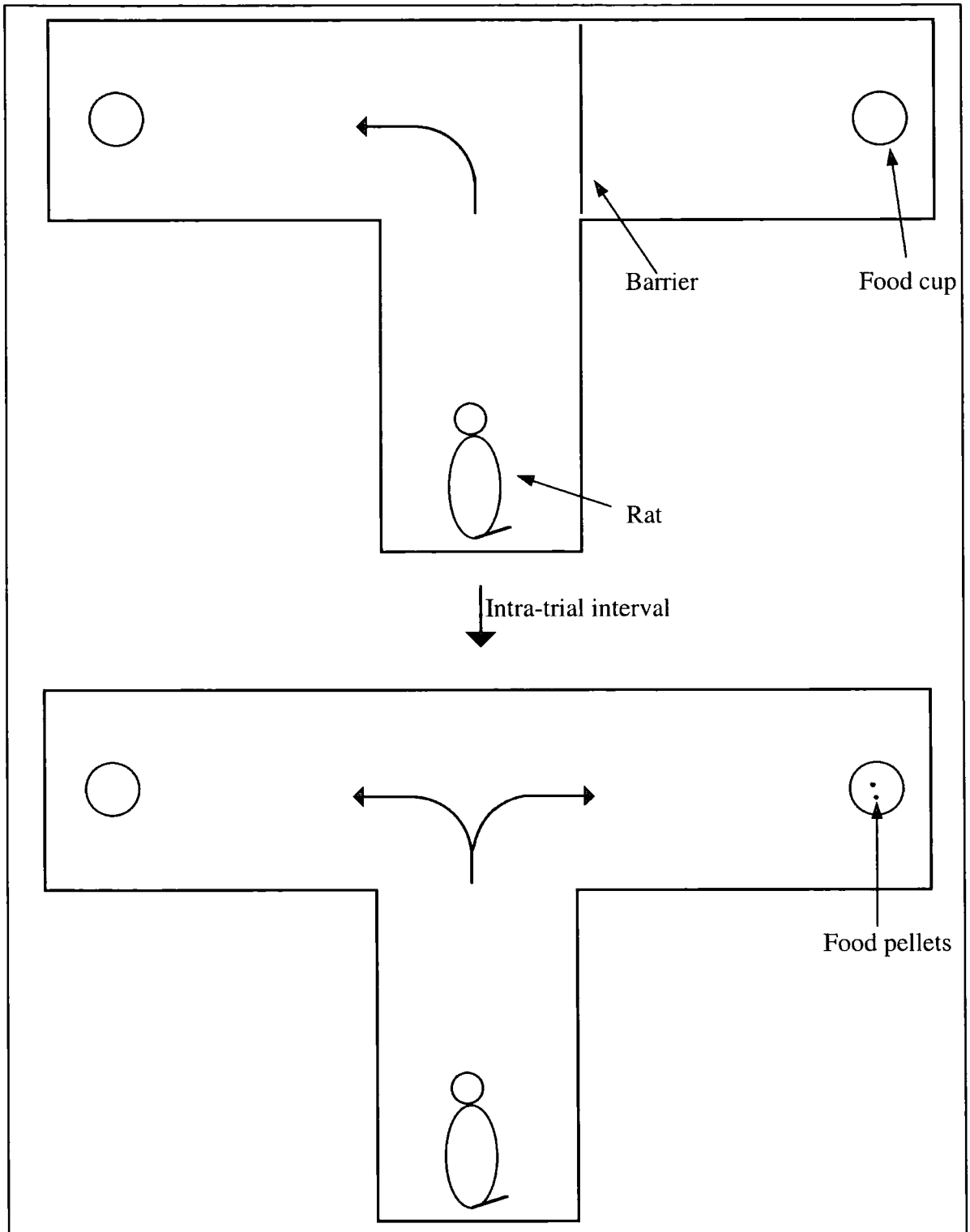


Figure 5.1: Schematic diagram of the T-maze non-matching-to-sample task. The diagram above is an illustration of the non-matching to sample T-maze task. The rat was introduced into the stem of the T-maze, with one of the arms blocked by a wooden barrier, thereby forcing the rat to enter the open arm of the maze. The rat was then removed from the open arm for the intra-trial interval. At the end of the delay, access to both arms was free and two food pellets were placed only in the food cup of the previously closed arm. If the rat chose the previously un-entered arm it was allowed to eat the food pellets before being removed. If the arm visited during the sample phase was chosen, the rat was confined to the arm for ten seconds before being removed.

Once all rats had reached criterion, they were tested in the T-maze with no intra-trial delay to ensure that they were all performing from the same baseline. The following day the rats were injected with either saline, piracetam (400mg/kg) or mefenamic acid (20 mg/kg) thirty minutes prior to testing, and again tested in the T-maze with a five minute intra-trial delay between the sample and choice phases. During the intra-trial interval, animals were returned to their home cage. Each animal was tested for ten trials. Three days later the group previously injected with saline was injected with mefenamic acid and the group injected with mefenamic acid was now injected with saline in a drug cross-over experiment. The rats were tested again in the T-maze for ten trials per day with a five-minute intra-trial interval.

Data analysis:

To investigate the effect of mefenamic acid (20mg/kg) on T-maze acquisition, the number of errors made and the number of days taken to reach criterion were recorded and calculated for each rat. The data was then analysed using the unpaired Student t-test (two-tailed), and $p \leq 0.05$ was considered significant.

To investigate the effect of piracetam (400mg/kg) and mefenamic acid (20mg/kg) on working memory, the number of errors made during the ten trials with a five-minute intra-trial interval was recorded. This data was then analysed using a one-way ANOVA followed by a Student-Newman-Keuls *post hoc* analysis. $p \leq 0.05$, was considered significant.

The data for saline and mefenamic acid treated groups that were reversed and re-tested in the T-maze were analysed with a paired Student t-test to compare the number of errors within groups. Results were considered statistically significant when $p \leq 0.05$.

5.2ii: The radial maze task

The radial maze, a widely used paradigm used to investigate spatial and reference memory, was first described in its modern form by Olton and Samuelson (1976). Pharmacological experiments have shown that centrally acting drugs can alter performance in the radial maze task. For example, scopolamine results in a concentration-dependent deficit in radial maze performance (Stevens, 1981; Watts *et al.*, 1981) while physostigmine can improve radial maze performance (Ennaceur, 1998). In contrast, the NMDA antagonist, MK-801, impairs performance in the radial maze (Wozniak *et al.*, 1990) and this impairment is reversed by nicotine (Levin *et al.*, 1998) or histamine (Chen *et al.*, 1999). Therefore, to address the effects of mefenamic acid on reference and working memory, the radial maze was utilised in the following experiments.

Animals:

Male Lister hooded rats (250-300g, Charles river U.K.) were housed and maintained as described previously (pg 120).

Apparatus:

A radial maze was manufactured in-house. The central platform (40cm in diameter) was made from vinyl coated wood and eight arms (85cm long x 10cm wide), which extended from this central platform (figure 5.2). A 2 x 0.5cm food cup was fixed to the end of each arm. A 1cm wooden rim surrounded the arms to prevent the animals from falling off the maze. The rim however gradually increased in height at 10cm from the central platform until it was at a height of 10cm at the central platform. This was to prevent the animals from jumping from arm-to-arm without entering the central platform. Wooden barriers were also placed at the entry to each arm (10cm in height). Each of these barriers was connected by a piece of string and a pulley so that the

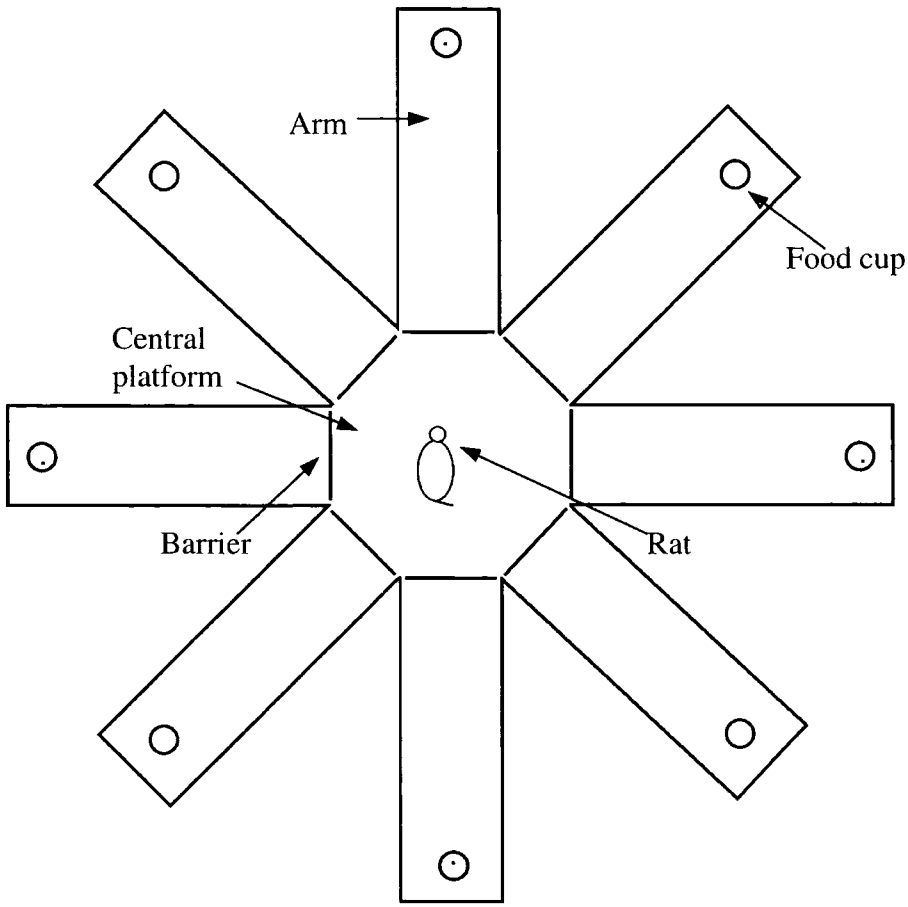


Figure 5.2: A schematic diagram of the radial maze. The diagram above is an illustration of the radial maze. During a trial the rat was placed in the central platform with all barriers down. All the barriers were then opened simultaneously and the rat allowed to select an arm to enter. Once an arm had been selected the barriers to the other seven arms were closed. When the rat returned to the central platform the barrier to the visited arm was also closed. The barriers were then all opened again and the rat allowed a free choice to select an arm. This procedure was repeated until the rat had entered the four arms containing food pellets or until ten minutes had elapsed.

investigator, who remained in the same place in the laboratory throughout testing, could raise one or all of the barriers at any time. The entire maze was positioned 70cm off the floor, with several large extra-maze cues positioned around the room. The room was lit by fluorescent tubes. The maze was cleaned with ethanol solution between trials to remove any potential intra-maze cues.

Habituation:

Rats were given four, five-minute habituation sessions (one session per day) to the laboratory and radial maze. During these sessions the barriers were open and food pellets were scattered around the maze. The amount of food pellets placed on the maze was reduced for each habituation session, until they were placed just in the food cups at the ends of each arm. After these habituation sessions there was little defecation, urination or freezing from the rats.

Reference memory testing:

On day five, testing in the radial maze began. Rats were injected daily with either saline or mefenamic acid (20mg/kg) thirty minutes prior to testing. For each rat, four of the arms were baited with a food pellet (1x45mg, Noyes) while the other four arms remained un-baited. The particular pattern of baited/un-baited arms differed between rats, but remained constant for each rat throughout the experiment. The rat was placed in the central platform with all barriers down. All the barriers were then opened at the same time and the rat allowed to select an arm to enter. Once an arm had been selected the barriers to the other seven arms were closed. When the rat returned to the central platform the barrier to the visited arm was also closed. The barriers were then all opened again and the rat allowed a free choice to select an arm. This procedure was repeated until the rat had entered the four arms containing food pellets or until ten minutes had elapsed. An arm was recorded as entered when the all four paws of the animal crossed

the entrance of the arm; the order of entries into arms was also recorded. This procedure was repeated daily until the rats had reached a criterion of no more than three entry errors (entries into arms without a food pellet) over three days or until twenty days of testing had occurred.

Working memory task:

Working memory errors were defined as the number of re-entries into previously visited arms. The criterion was no more than one working memory error over five days of testing was reached. Once a rat had reached criterion they were given an intra-peritoneal injection of either saline or mefenamic acid (20mg/kg), thirty minutes prior to testing in the radial maze. An intra-trial delay (0sec-30sec) was introduced between arm entries. During the delay the rat was confined within the central platform. Animals were removed from the maze when they had entered the four baited arms or after ten minutes had elapsed. The drug groups were then reversed and the experiment repeated for a drug *cross-over* experiment.

Data analysis:

To investigate the effect of mefenamic acid on reference memory the number of entries into never baited arms (considered reference memory errors) for each group was recorded and the number days to criterion was also calculated. An un-paired student t-test (two-tailed) was performed on both the number of reference memory errors and the number of days to criterion. A value of $p \leq 0.05$ was considered significant.

A one-way ANOVA (repeated measures) was performed on the number of working memory errors made for saline and mefenamic acid (20mg/kg) treated groups across the four intra-trial delays (0, 10, 20 & 30 secs) followed by Student-Newman-Keuls *post hoc* analysis.

5.3: Results

5.3i: Acquisition of non-matching-to-sample T-maze task

Treatments and testing:

Twelve rats were randomly allocated to one of two treatment groups, with one group given daily intra-peritoneal injections of saline (n=6), and the second group injected daily with mefenamic acid (20mg/kg; n=6), thirty minutes prior to testing in the T-maze. Each day these groups were given ten trials in the non-matching-to-sample T-maze task until a criterion of no more than five errors over three consecutive days was reached.

Results:

Mefenamic acid treated rats (n=6) made significantly fewer errors compared to saline controls to reach criterion when analysed with an un-paired Student t-test (t_{10}= 2.63, $p \leq$ 0.05). These data are shown in figure 5.3A. Mefenamic acid treated rats also took fewer days to reach criterion than saline controls (figure 5.3B) although this difference was not quite significant (t_{10}= 1.93, p= 0.08).

Conclusion:

Mefenamic acid treated rats were faster than saline controls in the acquisition of the non-matching to sample T-maze task.

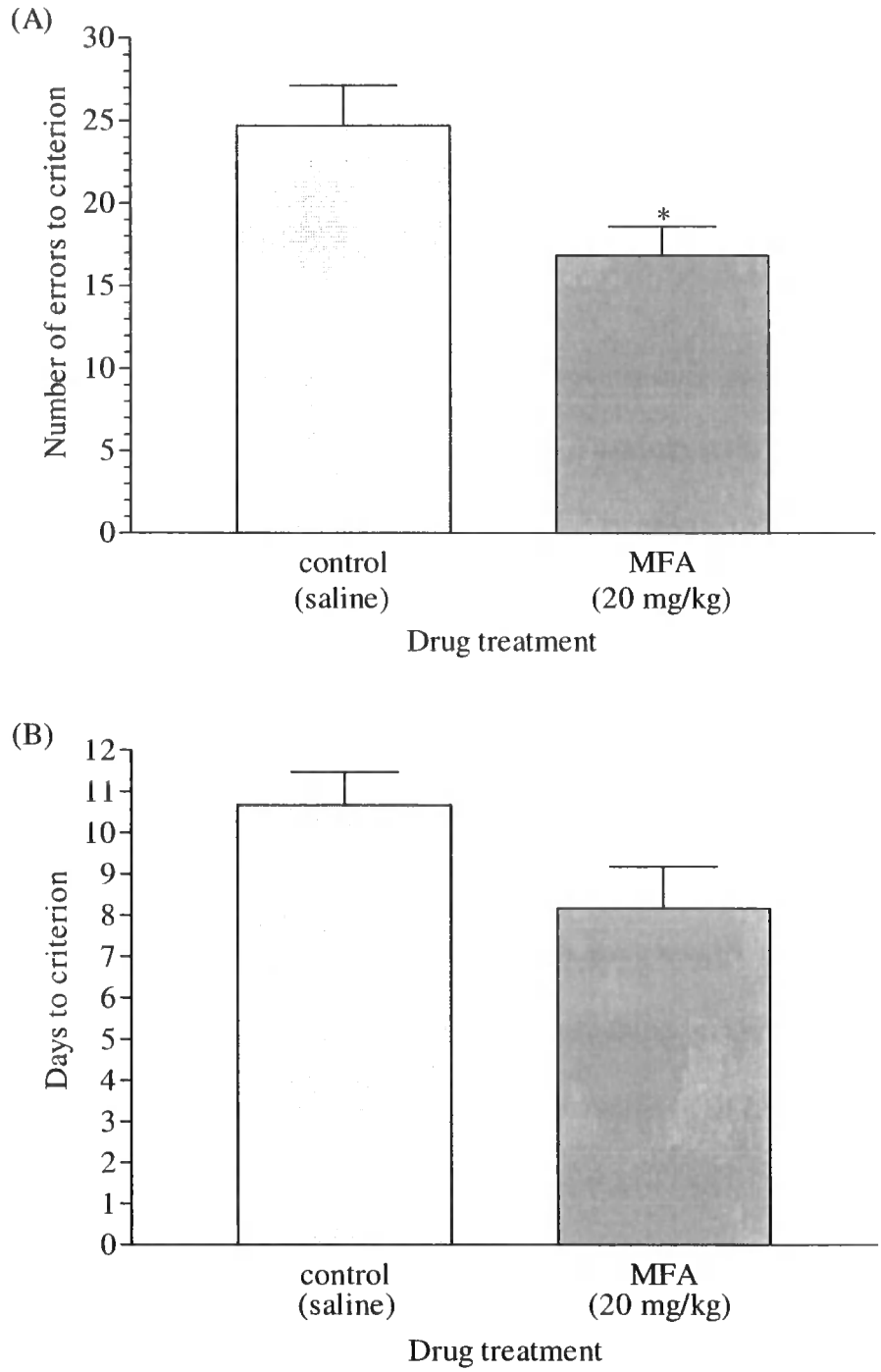


Figure 5.3: Mefenamic acid decreases errors to criterion in the non-matching-to-sample T-maze task. (A) The histogram shows the number of errors taken to reach criterion on the y-axis for saline and mefenamic acid (MFA) groups. MFA caused a significant decrease in the number of errors to reach criterion compared to saline controls. (B) The histogram shows the number of days taken to reach criterion for saline and MFA groups. * $p \leq 0.05$ n=6 for each group. Each bar represents the mean ± s.e.m.

5.3ii: The effect of intra-trial delay in the non-matching-to-sample task

Treatment and testing:

Forty-nine rats that had been trained to criterion in the T-maze non-matching-to-sample task were randomly allocated to one of seven different groups. Each group was allocated an intra-trial interval of either: ten seconds (n=7), twenty seconds (n=7), thirty seconds (n=7), sixty seconds (n=7), a hundred and twenty seconds (n=7), three hundred seconds (n=7) or six hundred seconds (n=7). Each group was given ten trials in the T-maze with their respective intra-trial interval.

Results:

There was a significant difference in the number of errors between groups when analysed by one-way ANOVA ($F_{6,42}$=7.93, $p \leq 0.0001$). A *post hoc* analysis revealed significant differences between the six hundred second interval compared to the ten second, twenty second, thirty second and sixty second interval groups ($p \leq 0.01$); the three hundred second interval was significantly different to the ten, twenty, thirty and sixty second interval groups ($p \leq 0.05$) and the ten second interval group was significantly different when compared to the two minute interval ($p \leq 0.05$). This data is shown in figure 5.4.

Conclusion:

The non-matching-to-sample T-maze task is sensitive to intra-trial delays.

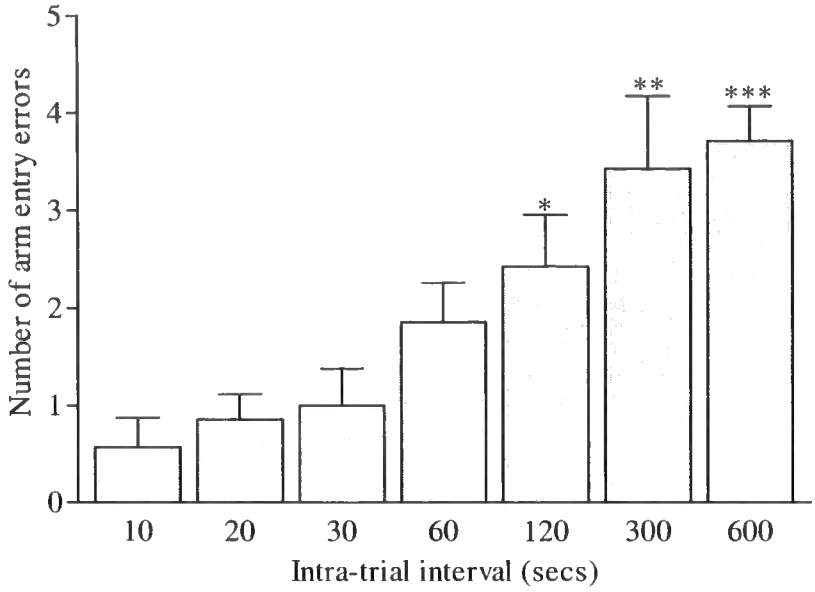


Figure 5.4: Increasing intra-trial intervals is associated with a significant increase in the number of errors in delayed non-matching-to-sample T-maze task. The above histogram shows the number of arm entry errors on the y-axis against the inter-trial interval, on the x-axis. The number of arm entry errors is significantly increased as the intra-trial interval is increased ($F_{6,42}$=7.93, p≤ 0.0001), *** p≤ 0.001, ** p≤ 0.01, * p≤ 0.05 when compared to the ten second interval. n=7 for each group. Each bar represents the mean ± s.e.m.

5.3iii: The effect of mefenamic acid and piracetam on the number of errors made with a five minute intra-trial interval

The effect of mefenamic acid in the non-matching-to-sample T-maze task with a five minute intra-trial interval was investigated in order to test the hypothesis that mefenamic acid can reduce the number of working memory errors. Additionally the effect of mefenamic acid will be compared to that of the cognitive enhancer piracetam

Treatments and testing:

Twenty-four rats that had been trained to criterion in the non-matching-to-sample T-maze task were randomly allocated to one of three groups. One group was given an intra-peritoneal (i.p.) injection of saline (n=8), a second group was given an injection (i.p.) of piracetam (400mg/kg; n=8) and a third group injected (i.p.) with mefenamic

acid (20mg/kg; n=8). Each group was then tested twice in the T-maze, with a zero second intra-trial interval and a five-minute intra-trial interval. The mefenamic acid treatment and saline treatment groups were then reversed so that the animals previously given mefenamic acid now received saline and the group previously given saline now received mefenamic acid, in a drug *cross-over* experiment. The experiment was then repeated with a five-minute intra-trial interval.

The effect of mefenamic acid and piracetam on the number of errors made with a five-minute intra-trial interval:

There was no significant difference in the number of errors made between groups when there was no intra-trial interval ($F_{2,15}$= 0.09, p≥ 0.10). When a five minute intra-trial delay was introduced (figure 5.5) there was a significant difference between groups ($F_{2,15}$= 6.78, p≤ 0.01). A *post hoc* analysis showed a significant difference between control compared to the mefenamic acid treated group (p≤ 0.05), there was no significant difference between piracetam and control.

When the mefenamic acid and saline treated groups were reversed, mefenamic acid still caused a significant decrease in the number of errors when compared to their own saline control data using a paired Student t-test (t_{15} =2.32, p≤ 0.05). These data are shown in figure 5.6.

Conclusion:

Mefenamic acid treated rats' made fewer errors than saline controls in a non-matching-to-sample T-maze task when a five-minute intra-trial interval was imposed.

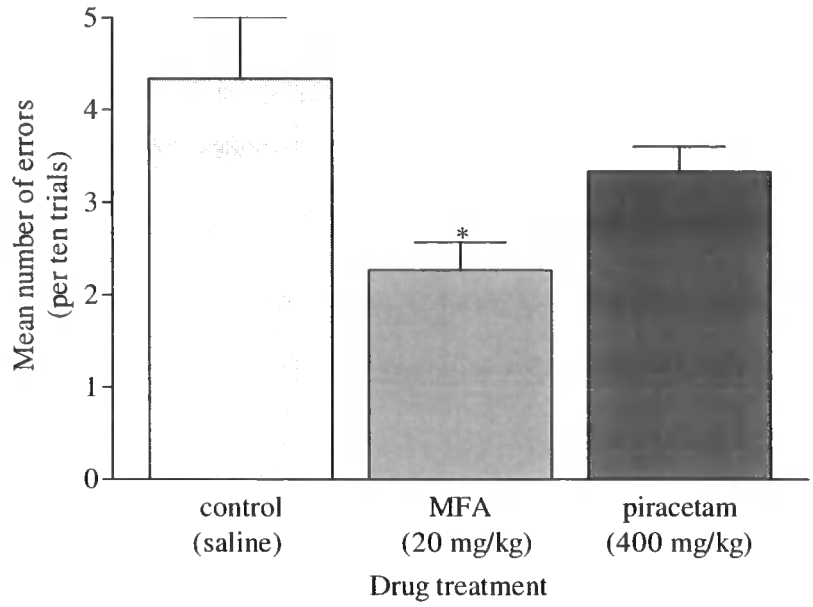


Figure 5.5: Mefenamic acid decreases errors in the delayed non-matching-to-sample T-maze task with a five minute intra-trial interval. The histogram shows the number of arm entry errors on the y-axis for control, mefenamic acid (MFA) and piracetam groups. The number of arm entry errors for the MFA group are significantly decreased compared to saline controls, with a five minute intra-trial interval ($F_{2,15}$ =6.78, p≤ 0.01), * p≤ 0.05 compared to control. n=8 for each group. Each bar represents the mean ± s.e.m.

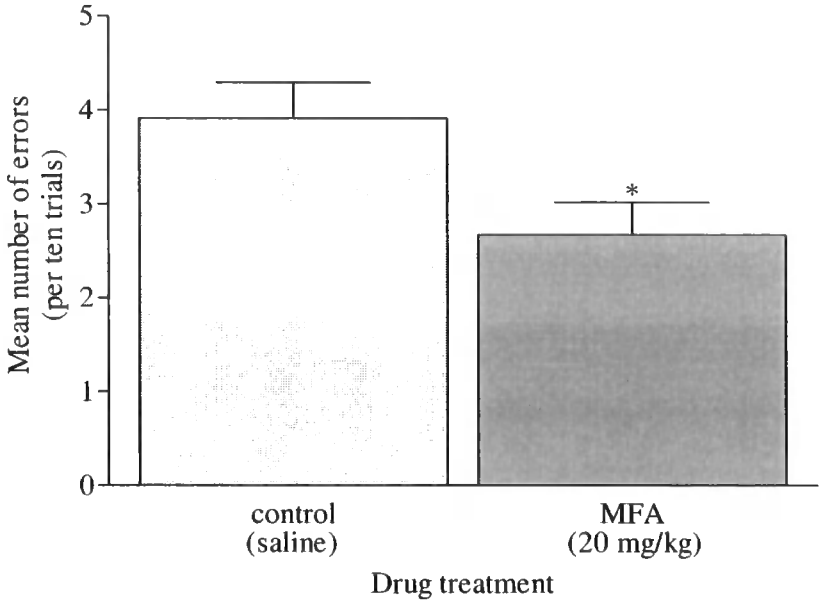


Figure 5.6: Mefenamic acid decreases errors in the delayed non-matching to sample T-maze task with a five minute intra-trial interval when compared to its own saline control in a drug *cross-over* experiment. The histogram shows the number of arm entry errors on the y-axis for saline and mefenamic acid (MFA) groups. The number of errors made with a five-minute intra-trial interval is decreased by MFA when compared to each rats' own saline treated control. * p≤ 0.05. n=8 for each group. Each bar represents the mean ± s.e.m.

5.3iv: The effect of mefenamic acid on reference memory in the radial maze task

The previous studies have investigated the effect of mefenamic acid on working memory. This experiment aims to investigate the effect of mefenamic acid on reference memory using the radial maze task.

Treatments and testing:

Sixteen rats were randomly allocated into one of two drug treatment groups. One group was given a daily intra-peritoneal injection of saline (n=8), and the second group was given a daily injection of mefenamic acid (20mg/kg; n=8). Drugs were administered thirty minutes prior to testing in the radial maze. In order to assess reference memory, animals were tested daily in the radial maze until they achieved a criterion of no more than three entries into never baited arms; the number re-entry errors into baited arms was also recorded until the criterion had been reached to determine the effect of mefenamic acid on the acquisition of the radial maze task.

Effect of mefenamic acid on reference memory:

Mefenamic acid did not significantly decrease the number of entry errors into never baited arms when compared to saline controls using an un-paired Student t-test (t_{14}= 1.49, p≥ 0.10) as shown in figure 5.7A. The number of days taken to reach criterion was also not significantly different between groups (t_{16} =1.34, p≥ 0.10) as shown in figure 5.7B.

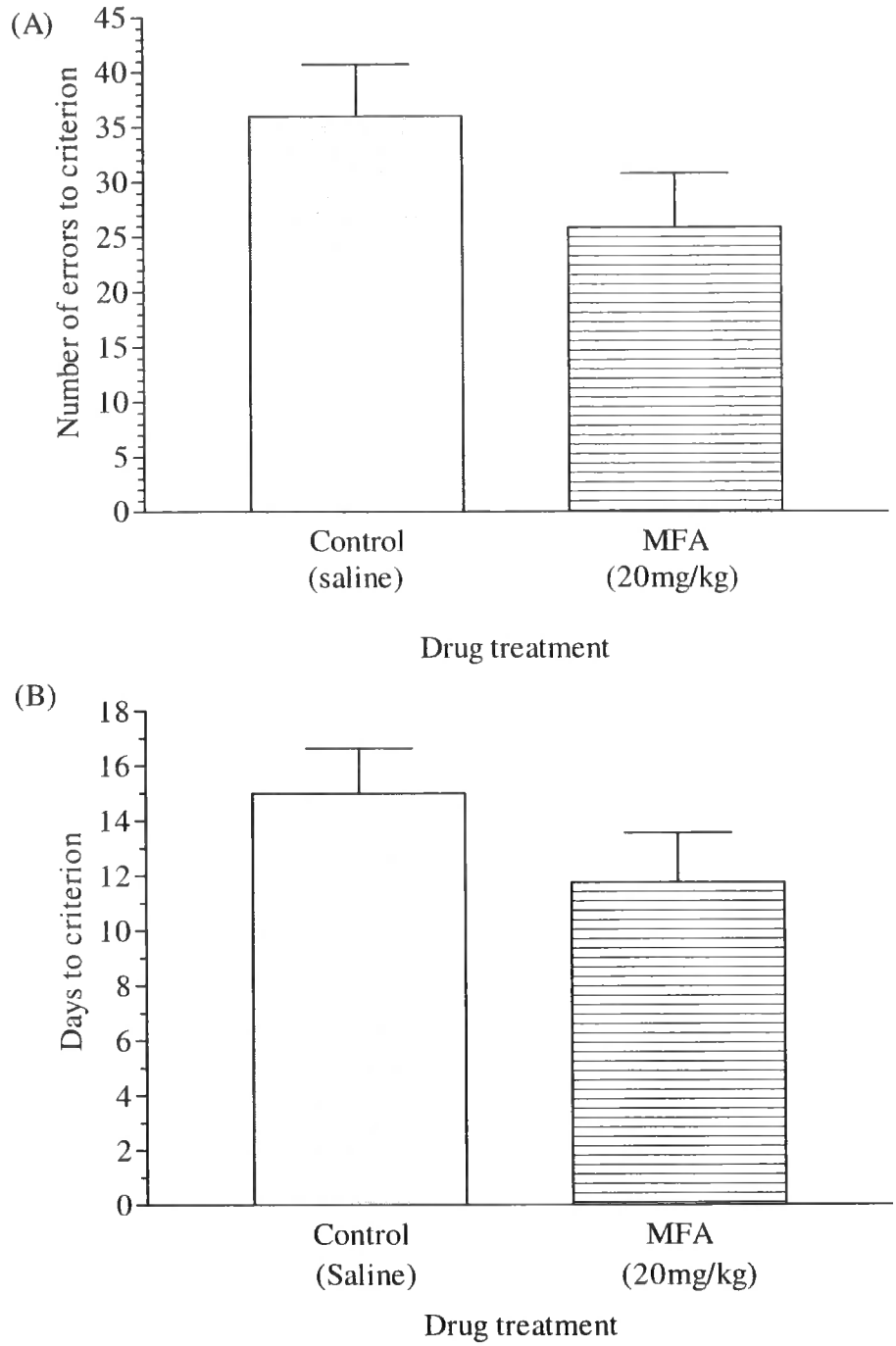


Figure 5.7: The effect of mefenamic acid on the number of entries into never baited arms and the number of days taken to reach criterion in the radial maze. (A) The histogram shows the number of never baited arm entries to criterion on the y-axis for control and mefenamic acid (MFA) groups. (B) The histogram shows the number of days taken to reach criterion on the y-axis for control and MFA groups. Each bar is the mean ± s.e.m. n=8 for each group.

Conclusion:

Mefenamic acid had no effect on the acquisition of the radial maze task.

5.3v: The effect of increasing the intra-trial interval on working memory errors in the radial maze task

Data obtained from the object discrimination and T-maze tasks suggested that mefenamic acid enhances non-spatial working memory. The following experiment therefore investigated the hypothesis that mefenamic acid could also enhance spatial working memory.

Treatment and testing:

Sixteen rats that had reached criterion of no more than one re-entry error over five days in the radial maze were randomly divided into one of two groups. One group was given an intra-peritoneal injection of saline (n=8), the second group was injected with mefenamic acid (20mg/kg; n=8). Drugs were administered thirty minutes before testing in the radial maze task. Each group of rats was then tested in the radial maze with four intra-trial intervals (a 0, 10, 20 and 30 second interval). Each rat was tested for ten trials a day over four days, with one intra-trial interval tested per day.

Results:

Analysis of the errors made between groups across intra-trial intervals with a two-way ANOVA (repeated measures) revealed a significant difference ($F_{3,28}$= 5.36, p≤ 0.01). A *post hoc* analysis revealed a significant difference between the thirty second delay compared to a twenty, ten and zero second delay (p≤0.05) for saline rats. In contrast there was no significant difference across intra-trial delays for mefenamic acid treated rats. The difference between drug groups was not quite significant ($F_{1,14}$= 3.54, p= 0.08). However there was a significant interaction between time and drug groups ($F_{3,28}$= 3.61, p≤ 0.05), as shown in figure 5.8.

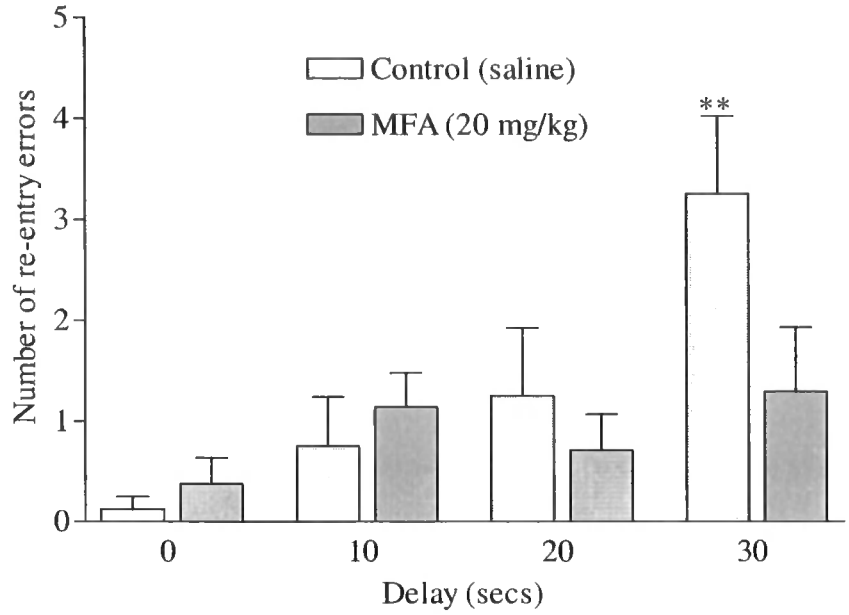


Figure 5.8: Increasing the intra-trial interval increases the number of re-entry errors made in the radial maze for saline but not mefenamic acid treated groups. The histogram shows the number of re-entry errors on the y-axis plotted against the intra-trial interval on the x-axis. The number of re-entry errors is significantly increased as the intra-trial delay is increased for saline ($F_{3,28}$= 5.36, p≤ 0.01), but not mefenamic acid (MFA) treated groups ** p≤ 0.01 compared to zero second. n=8 for each group. Each bar represents the mean ± s.e.m.

Conclusion:

In contrast to saline treated controls, rats treated with mefenamic acid made no more re-entry errors with a 10, 20 or 30 second intra-trial interval imposed than made with a zero second intra-trial interval.

5.4: Summary

The results presented above show that mefenamic acid enhanced rats performance in the in the T-maze and radial maze tasks. In the T-maze task mefenamic acid treated rats made fewer errors and took fewer days to reach criterion, suggesting they learned the task more quickly than saline treated controls. When a five-minute delay was introduced

between the sample and choice phases of the T-maze, mefenamic acid treated rats made fewer errors than controls and piracetam treated rats.

In the radial maze task, mefenamic acid treated rats were not significantly different from saline controls in the number of never baited arm entries made until criterion. When delays between baited arm entries were imposed there was a time-dependent increase in the number of re-entry errors for saline but not mefenamic acid treated rats.

Chapter Six: General Discussion

The aim of this thesis was to investigate the hypothesis that NSAIDs have direct effects on neuronal ion channels and thereby behavioural actions. This thesis has focussed mainly on fenamate NSAIDs. The rationale behind this study was reports that NSAIDs can produce analgesic effects even when injected directly into the CNS; that NSAIDs can modulate a number of ion channels in peripheral tissues and that NSAIDs can modulate central ligand-gated ion channel function when these ion channels are expressed in *Xenopus* oocytes.

6.1: Modulation of neuronal ligand-gated ion channels by NSAIDs

Initial experiments were undertaken to validate the use of the extra-cellular recording technique to record from ligand-gated ion channels expressed in isolated rat vagus and optic nerves as suitable preparations to investigate the possible effects of NSAIDs. A number of well characterised drugs were first investigated for their modulatory effects on these ligand-gated ion channels located extra-synaptically on the rat vagus and optic nerves.

Characterisation of agonist-evoked responses in the isolated rat vagus and optic nerves:

GABA, 5-HT, DMPP and α,βMeATP each evoked concentration-dependent depolarisations in the isolated rat vagus nerve. The GABA EC_{50} was calculated to be 45μM, which is consistent with GABA EC_{50} values, recorded from isolated rat vagus nerves reported by others of 28μM (Ireland & Tyers, 1987), 69μM (Green & Halliwell, 1997) and 34μM (Patten *et al.* 2001). The EC_{50} for 5-HT of 1μM found here is comparable to EC_{50}'s for 5-HT reported by others of 0.8μM (Green & Halliwell, 1997; Patten *et al.*, 2001), 0.63μM (Newberry *et al.*, 1992) and 0.48μM (Ireland & Tyers, 1987). EC_{50} values of 33μM (Patten *et al.*, 2001) and 35μM (Ireland & Tyers, 1987;

Green & Halliwell, 1997), for DMPP responses evoked from the isolated rat vagus nerve are similar to the EC_{50} of 26μM reported in this study. The EC_{50} for α,βMeATP of 47μM is in agreement with that of Patten *et al.* (2001), Green & Halliwell (1997) and Trezise *et al.* (1993) who report EC_{50}'s of 48μM, 25μM and 26μM respectively in the isolated rat vagus nerve.

GABA and glycine both evoked- concentration dependent depolarisations in the isolated rat optic nerve with EC_{50}'s of 1mM and 3mM, respectively. This data is consistent with that of Patten (2000), who report EC_{50}'s for GABA and glycine of 1.6mM and 2.6mM respectively and Green & Halliwell (1997) who report EC_{50} values of 1.1mM and 1.7mM for GABA and glycine, respectively. An interpolated EC_{50} for glycine of ≈1.4mM from the work of Simmonds (1983) on the rat isolated optic nerve is also consistent with the EC_{50} for glycine found in this study.

It was noted that high concentrations of 5-HT and α,βMeATP evoked a small after-hyperpolarising response upon washout. This observation has also been reported previously for 5-HT- (Azami *et al.*, 1985; Round & Wallis, 1986; Rhodes *et al.*, 1992) and α,βMeATP- (Trezise *et al.*, 1993; Connolly, 1995) evoked responses in vagal neurones, and is thought to be due to a direct hyperpolarizing effect following depolarisation by the agonist. Concentrations of DMPP greater than 300μM sometimes evoked responses that were very large (≥ 5mV) relative to maximal responses evoked by other agonists. The concentration that evoked a maximum response also varied greatly (100μM to greater than 1mM). For this reason DMPP- evoked responses were normalised to the response evoked by DMPP (300μM). Other investigators have also limited the DMPP response to 300μM (Patten *et al.*, 2001; Green & Halliwell 1997).

Sub-maximal GABA-, 5-HT-, DMPP- and α,βMeATP- evoked responses were inhibited by bicuculline, MDL 72222, hexamethonium and PPADs, respectively. GABA- evoked responses were inhibited in a concentration-dependent manner by the classical $GABA_A$ receptor antagonist, bicuculline (Curtis *et al.*, 1970) resulting in an IC_{50} of 1.4μM, this is in agreement with the IC_{50} for bicuculline of 1.2μM reported by Green and Halliwell (1997).

Both sodium pentobarbitone and propofol potentiated sub-maximal GABA-evoked responses in the isolated rat vagus nerve. Sodium pentobarbitione (10μM), a well-characterised positive modulator of the $GABA_A$ receptor (Study & Barker, 1981; Sieghart, 1995), resulted in an enhancement of 155% of the control GABA response. This is consistent with that of Patten *et al.* (2001) who reported a potentiation of 170% by sodium pentobarbitone (10μM).

Propofol was twice as potent as sodium pentobarbitone at potentiating GABA responses, and enhanced the GABA response more than four-fold. This is comparable to the 360% potentiation reported by Patten *et al.* (2001). The bell shaped curve of potentiation observed with propofol in this thesis, where higher concentrations of propofol (> 10μM) resulted in a lower level of enhancement of GABA- evoked responses has also been observed by others in the isolated rat vagus nerve (Patten *et al.*, 2001), rat hippocampal (Hara *et al.*, 1994) and rat cortical neurones (Hales & Lambert, 1991). This reduced level of potentiation may be due to receptor desensitisation; since it has been reported from patch-clamp recordings of mouse hippocampal neurones that propofol at these higher concentrations can also directly activate the $GABA_A$ receptor and induce receptor desensitisation (Orser *et al.*, 1994). Together these data are consistent with the activation of $GABA_A$ receptors in the vagus nerve.

MDL 72222, a potent and selective 5-HT_3 antagonist (Fozard, 1984), inhibited 5-HT responses in a concentration-dependent manner resulting in an IC_{50} of 36nM, this is consistent with concentration-dependent inhibition of 5-HT- evoked responses by MDL 72222 on vagal neurones reported by Azami *et al.* (1985) and Ireland and Tyers (1987). It was noted in this study that the maximum inhibition by MDL 72222 was 80% of the control 5-HT response. This effect has also been observed by Azami *et al.* (1985) who showed that MDL 72222 maximally inhibited 5-HT responses to 68% of the control 5-HT response. Another 5-HT_3 selective antagonist, ondansteron, has been reported to only partially inhibit 5-HT evoked responses in vagus nerves (Rhodes *et al.*, 1992; Bley *et al.*, 1994). Both these authors suggest that the remaining 5-HT response could be due to activation of 5-HT_4 receptors, since the remaining 5-HT response could be inhibited by the 5-HT_4 antagonists, tropistetron (Rhodes *et al.*, 1992) and GR113808 (Bley *et al.*, 1994). This data is consistent with 5-HT activating a 5-HT_3 receptor-mediated response in the vagus nerve.

Sub-maximal DMPP- evoked responses were inhibited in a concentration-dependent manner by hexamethonium. This is in agreement with previous extra-cellular recording studies from the vagus nerve reporting inhibition of DMPP responses by hexamethonium (Ireland & Tyers, 1987; Green & Halliwell, 1997), and is therefore consistent with the activation of nicotinic ACh receptors in the vagus nerve.

The selective P2x antagonist, PPADs (10μM), inhibited sub-maximal α,βMeATP-evoked responses to 44% of control response. This observation is consistent with those of Connolly (1995) and Green and Halliwell (1997) who report that PPADs (10μM) inhibited α,βMeATP responses to 43% and 21% of control, respectively in the isolated

rat vagus nerve. This data is consistent with α,βMeATP activating P2x receptors in the vagus nerve.

GABA- and glycine- evoked responses in the isolated rat optic nerve were inhibited in a concentration-dependent manner by bicuculline and strychnine, respectively. Complete inhibition of GABA was observed with bicuculline (100μM), which is consistent with that reported by Green and Halliwell (1997). Complete inhibition of the glycine response by strychnine was observed at 3μM. This data is comparable to the strychinine inhibition of glycine responses in the rat optic nerve reported by Green and Halliwell (1997) and Simmonds (1983). These data are consistent with GABA and glycine activating $GABA_A$ and strychnine-sensitive glycine receptors, respectively, in the rat optic nerve.

Together these experiments have shown that the pharmacology of the extra-synaptic GABA-, 5-HT-, DMPP- and α,βMeATP- evoked responses in the isolated rat vagus nerve are consistent with the activation of $GABA_A$, $5\text{-}HT_3$, nicotinic ACh and P2x receptors respectively. Similarly, the pharmacology of GABA- and glycine- evoked responses in the isolated rat optic nerve are consistent with the activation of $GABA_A$ and strychnine-sensitive glycine receptors respectively. This data supports the use of vagus and optic nerves as appropriate preparations to investigate the effect of NSAID modulation on neuronal ligand-gated ion channel function.

Modulation of neuronal ligand-gated ion channels by fenamate NSAIDs:

The effect of fenamate NSAIDs on sub-maximal $GABA_A$, $5\text{-}HT_3$, nicotinic ACh and P2x receptor mediated responses in the isolated rat vagus nerve was therefore determined. Flufenamic acid inhibited the DMPP and α,βMeATP responses in a

concentration-dependent manner. Zwart and colleagues (1995) have previously reported that flufenamic acid inhibited recombinant nicotinic acetylcholine $\alpha_3\beta_2$ receptors expressed in *Xenopus* oocytes with an IC_{50} of 90μM. The reduction of the maximum DMPP response and non-parallel shift of the concentration-effect curve by flufenamic acid, observed in this present study, indicates that the inhibition is not due to a simple competitive antagonism. This conclusion has also been proposed by Zwart *et al.* (1995) who observed that the acetylcholine concentration response curves were inhibited in a non-competitive fashion.

Flufenamic acid (100μM) did not significantly modulate sub-maximal GABA or 5-HT responses in the isolated rat vagus nerve. A modest potentiation of sub-maximal $GABA_A$ receptor mediated responses by flufenamic acid has been reported previously from patch-clamp recordings of recombinant rat cortical $GABA_A$ receptors expressed in *Xenopus* oocytes (Woodward *et al.*, 1994) and rat hippocampal neurones (Patten, 2000). These contrasting findings could be due to the different preparations and recording techniques used or possibly due to differences in the subunit composition of the expressed $GABA_A$ receptor in the vagus nerve (discussed later).

In this study meclofenamic acid significantly inhibited sub-maximal GABA- evoked responses in the isolated rat vagus nerve. In contrast, Woodward *et al.* (1994) and Patten (2000) report that sub-maximal GABA responses are potentiated by meclofenamic acid. These conflicting findings could again be due to the different preparations and recording techniques used in each study. It is also possible that the effects observed could be due to the agonist concentration used. Thus Woodward and colleagues (1994) report that the agonist concentration critically determines the development of a potentiating or inhibitory effect on GABA responses by fenamates,

whereby low concentrations of GABA (EC_{10} - EC_{50}) are potentiated by fenamates and higher concentration of GABA (EC_{50} - EC_{100}) are inhibited by fenamates. Maximum control GABA responses could not be obtained in the presence of meclofenamic acid, in this thesis, which suggests that the inhibition is not competitive. This is in agreement with Woodward *et al.* (1994) who reported that meclofenamic acid inhibited maximal GABA currents recorded from *Xenopus* oocytes.

In contrast to meclofenamic acid, mefenamic acid enhanced GABA- evoked responses. The level of potentiation by mefenamic acid is comparable to that reported by Patten (2000) who also recorded from the isolated rat vagus nerve. A greater degree of potentiation of GABA- mediated responses by mefenamic acid has been reported in rat hippocampal neurones (Halliwell & Davey, 1994; Patten, 2000). Mefenamic acid has also been reported to enhance GABA- evoked currents recorded from recombinant rat cortical $GABA_A$ receptors (Woodward *et al.*, 1994) and human $\alpha_1\beta_2\gamma_{2s}$ $GABA_A$ receptors (Halliwell *et al.*, 1999) expressed in *Xenopus* oocytes and HEK-293 cells. The variation in the level of enhancement by mefenamic acid could be due to the different preparations and recording techniques used or the subunit composition of $GABA_A$ subunits expressed in different preparations.

It was observed in this study that higher ($\geq$ 100μM) concentrations of mefenamic acid alone resulted in a shift in the baseline DC potential suggesting activation of a membrane current. Consistent with this mefenamic acid ($\geq$ 10μM) induced a whole-cell current in the absence of GABA in rat hippocampal neurones and this current could be potentiated by sodium pentobarbitone and inhibited by bicuculline (Patten, 2000). These data are consistent with direct activation of $GABA_A$ receptors by mefenamic acid. It was observed in this study that these high concentrations of mefenamic acid initially

enhanced GABA responses but this was followed by inhibition of the GABA response. This biphasic effect of mefenamic acid on GABA responses has also been observed by Patten (2000) who reported that low concentrations of mefenamic acid enhanced GABA- evoked responses and higher concentrations of mefenamic acid evoked a transient potentiation followed by an inhibition of the GABA evoked response recorded from rat hippocampal neurones. It is possible that the inhibition of GABA responses observed with high concentrations of mefenamic acid is due to desensitisation of the $GABA_A$ receptor (partly mediated by the direct activation of the $GABA_A$ receptor by mefenamic acid) and/or *via* the activation of the inhibitory site for mefenamic acid on the β_1 subunit containing $GABA_A$ receptor reported by Halliwell and colleagues (1999). In the present study there was a large degree of variation in the GABA- evoked responses to mefenamic acid. This observation has also been reported by Patten (2000) who found that some rat hippocampal neurones were insensitive to mefenamic acid although the same neurones could be potentiated by sodium pentobarbitone and diazepam. The level of variation could be due to the subunit composition of the expressed $GABA_A$ receptor. For example, Whittemore and colleagues (1996) reported that $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus* oocytes were potentiated three-fold while $\beta_2\gamma_{2L}$ $GABA_A$ receptors were only enhanced by 160% of control by mefenamic acid (3μM). More recently Halliwell and colleagues (1999) have shown that *Xenopus* oocytes expressing human $\alpha_1\beta_{2/3}\gamma_{2s}$ $GABA_A$ receptor subunits were potentiated by mefenamic acid whereas those containing $\alpha_1\beta_1\gamma_{2s}$ were not modulated by mefenamic acid. Moreover $\alpha_1\beta_1$ containing receptors were inhibited by mefenamic acid showing that the subunit composition of the $GABA_A$ receptor is critical for the type of modulation observed.

In addition to its effect on the $GABA_A$ receptor this study has shown for the first time that 5-HT_3 and P2x receptor mediated responses are inhibited by mefenamic acid in a concentration-dependent and non-competitive-like manner.

Niflumic acid (100μM) had a slight, but not significant, inhibitory effect on $GABA_A$, 5-HT_3, nicotinic ACh and P2x receptor mediated responses in the isolated rat vagus nerve. In agreement, Patten (2000) has shown that niflumic acid inhibits sub-maximal GABA responses in rat hippocampal neurones, and Zwart and colleagues (1995) report that high concentrations of niflumic acid (300μM) produce a non-competitive inhibition of acetylcholine currents recorded from $\alpha_3\beta_2$ nicotinic acetylcholine receptors expressed in *Xenopus* oocytes.

$GABA_A$ and glycine receptor mediated responses from the rat isolated optic nerve were not significantly changed by the fenamate NSAIDs tested in this study. In agreement with these findings, Patten (2000) showed that glycine responses from rat hippocampal neurones were not affected by mefenamic acid (30μM).

The finding that neither meclofenamic acid nor mefenamic acid modulated the GABA response in the optic nerve is surprising since both of these fenamates significantly affected the GABA- evoked responses recorded from the isolated rat vagus nerve. One explanation for the contrasting result could be due to the fact that higher concentrations of agonist and antagonist are required to initiate a response in the optic nerve. The EC_{50} for GABA was twenty-two fold higher in the optic nerve, while the IC_{50} for bicuculline was thirteen fold greater in the optic nerve. Higher concentrations of mefenamic acid and meclofenamic acid may have modulated the GABA response, although they could not be tested in this study due to the insolubility of fenamates at higher concentrations.

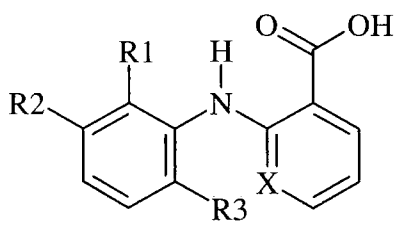
Higher concentrations of drugs required to produce a response in the rat optic nerve compared to the vagus nerve have also been reported by Green and Halliwell (1997) who found that the antagonist effects of ciprofloxacin and BPAA are forty-eight times more potent against GABA- evoked responses in the vagus nerve compared with the optic nerve. A possible explanation for the higher concentrations of drugs required to initiate a response is that they may have to diffuse further into the tissue to reach the receptor sites and that there are fewer $GABA_A$ receptors on the optic nerve (Simmonds, 1983).

After application of fenamate NSAIDs, re-establishment of control agonist responses took a considerable time (≥ ninety minutes). This could be due to slow removal of the fenamate by the perfusion system although this seems unlikely since the effects of other drugs such as bicuculline, sodium pentobarbitone and hexamethonium were quickly reversible (< thirty minutes to recovery). Another reason is that fenamates are highly lipophilic (Woodward *et al.*, 1994) and will therefore readily dissolve into the nerve membrane and then slowly dissociate back into the aqueous solution. The lengthy washout period for fenamate NSAIDs has also been reported by others (Woodward *et al.*, 1994; Halliwell *et al.*, 1999; Patten, 2000).

Structure-activity relationship of fenamate NSAIDs:

The data from this study show that a range of fenamate NSAIDs modulated agonist-evoked responses. Fenamates NSAIDs are all derived from the synthetic compound N-phenylanthranilic acid. Crystallographic and theoretical studies have shown that fenamates are comprised of two 6-membered rings linked by an imino bridge (Dhanaraj & Vijayan, 1988). For most fenamates, the A-ring (figure 6.1) carboxyl ring is coplanar with the imino bridge and is stabilised by an internal hydrogen bond. Rotation of the B-ring is possible, but is limited by steric hinderance 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 two rings have non-planar orientations. Indeed it has been proposed that mefenamic acid in its lowest energy conformation favours a right-angled conformation with the planes of the phenyl rings in an orthogonal conformation (Halliwell *et al.*, 1999). In the case of niflumic acid, replacement of a carbon atom with a nitrogen atom on the A-ring, results in a loss of steric hindrance enabling the molecule to adopt an almost planar conformation. The differences in structural conformation between fenamates, especially the phenyl ring substitutions at the R1 and R2 groups, might account for the differences observed in their efficacy and potency to modulate neuronal ligand-gated ion channels.



Fenamate	R1	R2	R3	X
Flufenamic acid	H	CF_3	H	C
Meclofenamic acid	Cl	CH_3	Cl	C
Mefenamic acid	CH_3	CH_3	H	C
Niflumic acid	H	CH_3	H	N
Tolfenamic acid	Cl	CH_3	H	C

Figure 6.1: Chemical structure of fenamate NSAIDs

Modulation of neuronal ligand-gated ion channels by non-fenamate NSAIDs:

None of the non-fenamate NSAIDs tested in this study resulted in significant affects on $GABA_A$, 5-HT_3, nicotinic ACh, or P2x receptor mediated responses in the isolated rat vagus nerve or $GABA_A$ and glycine receptor mediated responses recorded from the isolated rat optic nerve. Woodward and co-workers (1994) reported that non-fenamate NSAIDs have little effect on GABA responses recorded from *Xenopus* oocytes expressing rat cortical $GABA_A$ receptors. Similarly, it has been reported that ibuprofen has little effect of GABA- evoked currents recorded from human recombinant $GABA_A$ receptors (Halliwell *et al.*, 1999) or rat hippocampal neurones (Shirasaki *et al.*, 1991a;

Patten, 2000). Moreover, Squires and Saederup (1993) demonstrated that neither ibuprofen nor indomethacin affected [^{35}S] t-butylbicyclophosphorothionate (TBPS) binding to the $GABA_A$ receptor in rat brain membranes.

In agreement with results from this thesis, Shirasaki and colleagues (1991a) have shown that indomethacin does not modulate sub-maximal GABA- evoked currents recorded from rat hippocampal neurones. Indomethacin also does not to modulate GABA currents evoked from human $\alpha_1\beta_2\gamma_{2s}$ $GABA_A$ receptors expressed in *Xenopus* oocytes (Halliwell *et al.*, 1999).

The acetic acid, BPAA (100μM), resulted in a weak antagonism of the $GABA_A$ receptor, which is consistent with the work of Green and Halliwell (1997) who reported a 20% reduction of GABA responses recorded from the isolated rat vagus nerve. These data are also consistent with that of Halliwell *et al.* (1991) who showed a small inhibition of GABA- evoked currents recorded from rat dorsal root ganglion cells by BPAA.

Effect of NSAIDs on ionotropic glutamate receptors:

A major class of ligand-gated ion channels in the central nervous system that were not investigated in this study are the ionotropic NMDA (N-methyl-D-aspartate), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) and kainate glutamate receptors. Patten (2000) reported that sub-maximal NMDA-, AMPA- or kainate- evoked currents recorded from rat hippocampal neurones are not modulated by mefenamic acid (30μM). Higher concentrations of mefenamic acid (1mM) however have been shown to completely block NMDA- but not kainic acid- evoked currents in salamander retina cells (Chen *et al.*, 1998). High concentrations of niflumic acid (IC_{50} = 353μM) and flufenamic acid (IC_{50} > 200μM) have also been shown to inhibit NMDA- but not

AMPA- or kainic- evoked currents from rat spinal cord neurones (Lerma & Del Rio, 1991). Together these studies suggest that ionotropic glutamate receptors are not a neuronal target for the NSAIDs investigated.

The electrophysiological experiments in this thesis have shown that certain fenamate NSAIDs can modulate native neuronal $GABA_A$, $5\text{-}HT_3$ and P2x ligand-gated ion channels. It has previously been shown that modulation of neuronal $GABA_A$ (Yonkov & Georgiev, 1981; Sieghart 1995; Steckler *et al.*, 1999c) and $5\text{-}HT_3$ receptors (Pitsikas *et al.*, 1993; Steckler *et al.*, 1999c) result in "*central*" behavioural and cognitive effects. This, therefore, leads to the hypothesis that fenamates have behavioural and/or cognitive effects.

6.2: Behavioural consequences of fenamates

Mefenamic acid, at doses up to 20mg/kg, did not result in any obvious behavioural changes in the rat. However higher doses of mefenamic acid (40mg/kg and 60mg/kg) induced sedative or seizure-like effects. An action consistent with these observations was reported by Wallenstein (1991) who stated that "mefenamic acid (60mg/kg) had an excitatory behavioural effect producing body jerks, ataxia and upright convulsions while mefenamic acid (20mg/kg) did not result in any seizure activity". Minimally these behavioural effects with higher doses of mefenamic acid are consistent with this drug entering the central nervous system (discussed later).

Considering the gross "*central*" behavioural effects observed with mefenamic acid, the effects of mefenamic acid on a range of behavioural tasks was investigated. Mefenamic acid (20mg/kg) did not change the gross locomotor activity of rodents in the open field. This is consistent with the results of Wallenstein (1985b) who reported that mefenamic acid (15mg/kg) did not change locomotor activity in the open field.

Electrophysiological studies from this thesis and from those reported by others (Woodward *et al.*, 1994; Halliwell *et al.*, 1999; Patten 2000) have shown that mefenamic acid enhanced $GABA_A$ receptor-mediated responses. Given that positive modulation of the $GABA_A$ receptor results in an anxiolytic effect (Costa *et al.*, 1975; Sieghart 1995), the hypothesis that mefenamic acid acts as an anxiolytic agent was investigated. The results, from the open field anxiety test, showed that mefenamic acid did not significantly affect the time taken to approach or the amount of time spent exploring this object in the middle of the open field. Previous studies by Jessa and colleagues (1996) and more recently by Siemiatkowski *et al.* (2000) have shown that rats treated with anxyolytic agents such as diazepam will spend longer exploring the centre of an open field compared to control rats. These data, therefore, suggest that mefenamic acid does not affect the anxiety state of the rat and hence does not act as an anxiolytic drug.

The hypothesis that mefenamic acid can modify arousal, defined as "a state of alertness and high responsiveness to stimuli" (Uttal, 1978), was then investigated. In order to test this hypothesis, rats were exposed to two identical objects in the sample phase and then during the choice phase they were either re-exposed to the same objects or exposed to two new (novel) objects. The results, from these experiments showed that there was no difference in the exploration of the objects between saline or mefenamic acid treated rats for either condition, which indicates that mefenamic acid does not affect arousal. A similar test was also used by Christoffersen and colleagues (1998a) to observe the effect of piracetam on arousal. These authors showed that rats treated with piracetam spent the same amount of time exploring a novel object in a familiar environment as control saline treated rats, and concluded that piracetam does not affect arousal.

Validity of object discrimination task:

The object discrimination task, the paradigm primarily used in this study, is a two trial task that relies on the rats' spontaneous exploration of novelty, it does not depend on the retention of a rule and is not influenced by changes in responsivity to a reward. These factors suggest that the motivational state of the rat is more similar to those under which human memory is usually measured (Ennaceur & Delacour, 1988; Ennaceur & Meliani, 1992b). These advantages make the task well suited for neuropharmacological investigations in the rat (Ennaceur & Delacour, 1988; Ennaceur *et al.*, 1989; Blandina *et al.*, 1996; Deschaux *et al.*, 1997; Puma *et al.*, 1999).

The results obtained from the object discrimination task were compared with results reported by others in order to validate the task under the experimental conditions used by the investigator. Increasing the intra-trial interval did not change the overall total exploration time of objects during the sample or choice phases but did result in a time-dependent decrease in the ability of the rats to discriminate between the novel and familiar objects. Rats discriminated between novel and familiar objects with delays of up to sixty minutes but did not discriminate between such objects with longer intra-trial intervals. This observation is consistent with previous studies (Ennaceur & Delacour, 1988; Ennaceur & Meliani, 1992a,b; Bartolini *et al.*, 1996). It was also noted that the discrimination between objects was greater with a fifteen-minute delay when compared to a one-minute delay. This has also been observed by Ennaceur and Meliani (1992b). One possible reason for this could be that with a one-minute delay the rats are distracted for a greater portion of the intra-trial interval (by removing them from, and then replacing them back, into the arena) than rats tested with a fifteen-minute delay. Consistent with this Ennaceur and Meliani (1992b) have reported that rats who are distracted during the intra-trial interval, by placing them in an arena with a movable

object, perform significantly worse than rats who are not distracted during the fifteen minute intra-trial interval.

These control behavioural experiments have shown that the data obtained and recorded in this thesis are comparable with those of others. The effect of drug treatments on object discrimination was therefore investigated.

Enhanced object discrimination associated with mefenamic acid:

When mefenamic acid (5mg/kg - 20mg/kg) was tested in the object discrimination task, with a fifteen-minute intra-trial interval, there was no change in the exploration of objects during the sample phase. There was, however, a significant increase in the exploration of objects during the choice phase (with 10mg/kg and 20mg/kg mefenamic acid) and further analysis revealed that there was a significant dose-dependent increase in object discrimination. An increase in the discrimination between novel and familiar objects has been used as an index of an improvement in non-spatial working memory (Ennaceur *et al.*, 1989; Blandina *et al.*, 1996; Deschaux *et al.*, 1997; Puma *et al.*, 1999). For example, nicotine has been shown to enhance object discrimination with a twenty-four hour delay (Puma *et al.*, 1999) and the cholinesterase inhibitor, metrifonate, enhanced object discrimination in aged rats with a sixty minute intra-trial interval (Scali *et al.*, 1997). The observation in this thesis that mefenamic acid was associated with a significant increase in object discrimination suggested that it may enhance working memory. Consistent with this hypothesis, mefenamic acid enhanced object discrimination only with delays of up to sixty minutes. The observation that the actions of mefenamic acid are time-dependent suggests that enhancement is upon mnemonic processes.

Recognition memory in the rat can be divided into three stages: acquisition/encoding, storage and retrieval (Steckler *et al.*, 1998a). In order to investigate which stage was affected by mefenamic acid its effects on object discrimination when injected after the sample phase were determined. The results of this experiment showed that there was no change from control in object discrimination. This suggests that mefenamic acid does not affect the storage or retrieval processes. Notably, an enhancement in object discrimination has also been observed with the Ca^{++} activated K^{+} blocker, apamin, when injected before but not after the sample phase (Deschaux *et al.*, 1997). The authors concluded that apamin improves acquisition of information but has no effects on memory processes that occur after acquisition.

The enhancement of object recognition memory observed with mefenamic acid was compared to that of the nootropic agent, piracetam, a putative cognitive enhancer (Giurgea, 1973; Giurgea & Salama, 1977; Nicolaus, 1982). Thus piracetam has been shown to enhance memory in normal mice (Mondadori & Petschke, 1987) and rats (Bartus *et al.*, 1981; Nalini *et al.*, 1992) in the passive avoidance test and the delayed-matching-to-position task (Christoffersen *et al.*, 1998b). The results from the experiments presented in this thesis showed that piracetam did not significantly modulate total exploration times or object discrimination with a fifteen-minute intra-trial interval. This data is consistent with that of Ennaceur and Delacour (1989) who reported that piracetam did not enhance object discrimination at a sixty-minute delay, and with that of Ennaceur and colleagues (1989) who report that piracetam had no effect with at a one-minute delay. However, these authors did report that piracetam increased object discrimination with a twenty-four hour interval. The lack of enhancement observed with piracetam may be the result of utilizing normal rats. Indeed significant

cognitive effects with piracetam are reported in humans or animals that are senescent or suffering from brain pathologies (Christoffersen *et al.*, 1998b).

The differing behavioural effects of piracetam and mefenamic acid can be explained from their differing pharmacological properties. Although the mechanisms underlying the effects of piracetam which result in cognitive enhancement are not currently clear. Thus, unlike fenamates, piracetam does not modulate ACh, 5-HT or GABA neurotransmission (Poschel *et al.*, 1983; Pugsley *et al.*, 1983). And in agreement with fenamates, piracetam does not modulate NMDA and kainic glutamate receptors (Bering & Muller, 1985; Copani *et al.*, 1989). However, piracetam has been shown to increase choline uptake, in rat hippocampal synaptosomes, although at concentrations higher than those required to have a positive effect on memory (Shih & Pugsley, 1985). The effect of fenamates on choline re-uptake is currently unknown.

One possible mechanism for the cognitive enhancement of nootropics is through their facilitation of long-term potentiation (LTP), a synapse-specific enhancement of excitatory post-synaptic responses that has been proposed as a mechanism of memory (Bliss & Collingridge, 1993). Satoh and colleagues (1986) have shown that the nootropic, aniracetam, augmented LTP in guinea-pig hippocampal slices. It is thought that the augmention of LTP by nootropics is *via* enhancement of AMPA sensitive glutamate receptors (Copani *et al.*, 1989), which facilitate the process of LTP by activating NMDA glutamate receptors (Bliss & Collingridge, 1993). This hypothesis for cognitive enhancement is unlikely to account for the effects observed with fenamates, as patch clamp-recording experiments of rat hippocampal neurones have shown that neither AMPA- or NMDA- evoked currents are modulated by fenamates at low

concentrations (Patten, 2000). A possible future experiment however might address the effect of fenamates on the formation of LTP in hippocampal slices.

Another hypothesis for the memory enhancing effects of piracetam is that it enhances cognition by modulation of steroid receptors. It has been shown that piracetam does not enhance performance of adrenalectomized rats in the passive avoidance test (Mondadori *et al.*, 1989), and that mineralocortcoid or glucocorticoid antagonists block the facilitating effects of piracetam in rats (Mondadori *et al.*, 1990) and chicks (Loscertales *et al.*, 1998) tested in the passive avoidance test. The effects of fenamates on steroidal receptors is at present unknown and may be one possible mechanism for their cognitive enhancing effects.

Effect of mefenamic acid in the object location task:

The object discrimination task is thought to involve non-spatial working memory (Ennaceur & Delacour, 1988). To investigate the effect of mefenamic acid on spatial working memory, the object location task (Ennaceur & Meliani, 1991b) was utilised, since it has been proposed by Steckler and colleagues (1998b) that recognition memory involves two parallel distributed neuronal networks. Non-spatial recognition memory incorporates the cortical association areas, the rhinal cortex, mediodorsal thalamic nucleus and orbital frontal pre-frontal cortical areas. Whereas spatial memory includes the hippocampus, anterior thalamic nucleus and prelimbic prefrontal cortical areas.

Firstly the effect of increasing the intra-trial interval was investigated to test the validity of the task under the laboratory conditions adopted in this study. Consistent with a previous study (Ennaceur & Meliani 1991b) rats' were able to discriminate between the fixed and re-located objects with a one-minute and fifteen- minute delay but not at

delays of sixty minutes or more. These data are therefore consistent with the hypothesis that this spatial variant of the object discrimination task involves spatial working memory.

The effect of mefenamic acid on object location with a fifteen-minute intra-trial interval was then investigated. The results of this study showed that mefenamic acid did not increase total exploration time during the sample or choice phases, but significantly increased the discrimination time between the fixed and re-located objects. These data indicate that mefenamic acid enhances both non-spatial and spatial working memory, possibly *via* a mechanism common to both spatial and non-spatial networks.

Pharmacologcial mechanisms underlying the behavioural and cognitive effects of fenamates:

The effect of other fenamates in the object discrimination task was investigated to determine if the response observed with mefenamic was a fenamate group effect. Only one of fenamate, namely meclofenamic acid significantly increased the total exploration time during the choice phase and increased the object discrimination time. In contrast tolfenamic acid, flufenamic acid and niflumic acid were without effect. These results show that enhancement in object discrimination can be replicated by another fenamate but is not a fenamate group effect. The differences observed between fenamates may be due to their chemical structure, since both mefenamic acid and meclofenamic acid have relatively bulky R1 and R2 groups (figure 6.1) compared to other fenamates, this makes rotation of the B-ring difficult due to steric hinderance occurring between the A-ring hydrogen *ortho* to the imino linkage and the substituted R1 and R2 groups on the B-ring, thus enabling the two rings of these compounds to have non-planar orientations (Dhanaraj & Vijayan, 1988). It is feasible that the significant increase in object

discrimination observed with mefenamic acid and meclofenamic acid result from their non-planar orientation.

The non-fenamate NSAIDs, aspirin and ibuprofen, did not affect total exploration times or object discrimination when compared to saline controls. This finding suggests that the enhancement in object discrimination is not a general NSAID class effect. The finding that non-fenamate NSAIDs had no effect on object discrimination therefore suggest that enhancement in object discrimination is not due to inhibition of cyclo-oxygenase enzymes, a defining property common to all NSAIDs. Moreover aspirin, ibuprofen and mefenamic acid have a similar potency at inhibiting both COX-1 and COX-2 isoenzymes when assayed from human blood samples (Cryer & Feldman, 1998), which is consistent with the hypothesis that the cognitive effects associated with mefenamic acid is through a mechanism other than inhibition of cyclooxygenase.

An alternative hypothesis for the distinct behavioural effects observed with certain fenamates is that NSAIDs (that do not have behavioural effects) do not cross the blood-brain barrier. At therapeutic doses mefenamic acid peak plasma concentrations have been reported to be in the range of 4μM - 40μM, with half bound to plasma proteins (Flower, 1974; Glazko, 1966; Court & Volans, 1984). Unfortunately at the time of writing there is no published data on the concentrations of fenamates which enter the CNS. However, fenamate NSAIDs are highly lipophilic compounds (Woodward *et al.*, 1994), which suggests that they are able to cross the blood-brain barrier. Conversely other NSAIDs have been reported to cross the blood brain barrier at clinically relevant concentrations: Bannwarth and colleagues (1989) for example, reported that apart from the salicyclates the pyrazoles, acetic acids, and propionic acids NSAIDs are able to cross the blood brain barrier in humans and are controlled by simple physio-chemical

factors. Oxicam NSAIDs are also freely available to pass into the brains of rats after systemic administration (Jolliet *et al.*, 1997). These data suggest that apart from the salicyclates, NSAIDs can enter the CNS at therapeutically relevant concentrations, and thereby have the ability to modulate behavioural mechanisms.

A possible mechanism underlying the increase in object discrimination observed with mefenamic acid is due to its modulation of the $GABA_A$ receptor. Thus it has been shown in this thesis that mefenamic acid positively modulated $GABA_A$ receptor-evoked responses in mammalian neurones, and it has previously been reported that mefenamic acid can positively and negatively modulate the $GABA_A$ receptor depending on the concentration of GABA (Woodward *et al.*, 1994). Additionally the subunit composition of the $GABA_A$ receptor also critically determine the (positive or negative) effects of mefenamic acid at $GABA_A$ receptors (Halliwell *et al.*, 1999). Indeed it has been proposed that GABA is one of the major neurotransmitters involved in both spatial and non-spatial recognition memory (Steckler *et al.*, 1998c) and a number of GABA modulators have been shown to modulate working memory (described below). For this reason the effect of positive and negative modulators of the $GABA_A$ receptor were investigated in the object discrimination task and to determine if they could mimic the effects of mefenamic acid.

The anti-convulsant compound, loreclezole, did not significantly enhance or impair object discrimination. Consistent with this finding, loreclezole did not modulate rats working memory when tested in the passive avoidance test (Raffa *et al.*, 1990). However at higher does (≥20mg/kg) loreclezole has been shown to reduce rats locomotor activity (Green *et al.*, 1996).

Diazepam impaired object discrimination when compared to the saline controls. This data is consistent with the work of Longone and colleagues (1996) who reported that diazepam impaired rats' performance in the object recognition task with a thirty-minute delay. Diazepam has also been shown to impair rodents performance in a number of other working memory tasks including the non-matching-to-sample task (although this was associated with a decrease in gross behavioural activity relating to sedation, Kalynchuk & Beck, 1992); the passive avoidance test (Venault *et al.*, 1986); the radial maze (Pilcher & Sessions, 1999) and the Morris water maze in a dose-dependent manner (McNamara & Skelton, 1991).

The $GABA_A$ receptor antagonist, bicuculline impaired rats' performance in the object discrimination task. This is consistent with the work of Zarrindast and colleagues (1998) who reported that bicuculline impaired rats performance in the passive avoidance task and with that of Chrobak and Napler (1991) who reported that intra-septal infusion of bicuculline into rats impaired performance in the delayed non-matching-to-position radial maze task. Notwithstanding it has also been reported that bicuculline can enhance working memory. For example, it was first shown to improve retention in rats tested in a two-way avoidance task (Yonkov & Georgiev, 1981), and later to enhance performance in mice tested in the passive avoidance (Brioni & McGaugh, 1988) and visual discrimination tasks (Castellano & Pavone, 1988). Other reports have shown that bicuculline does not affect working memory. For example, Car and colleagues (1998) reported that bicuculline (0.25mg/kg) had no effect on rats' performance in the object discrimination task. It also has no effect on working memory in the delayed-matching-to-sample task when given to rats by intra peritoneal injection (Harper, 2000) or infused into the prefrontal cortex (Herremans *et al.*, 1996). The varying effects of bicuculline in memory tasks are thought to be due to the dose employed and may also be time related

(Nabeshima *et al.*, 1988; Brioni, 1993). Notwithstanding, the $GABA_A$ receptor antagonist, bicuculline, did not mimic the effects of mefenamic acid in the object discrimination task.

The results from this study therefore indicate that the behavioural effects of mefenamic acid are not dependent upon positive modulation of benzodiazepine receptors, competitive antagonism of the $GABA_A$ receptor or activation of the $GABA_A$ receptor complex through the fenamate/loreclezole binding site, since neither the positive allosteric modulator diazepam, the competitive antagonist bicuculline, nor the novel modulator loreclezole, a compound thought to bind to the same site on the $GABA_A$ receptor as fenamates (Halliwell *et al.*, 1999) replicate the effects of mefenamic acid.

These findings, however, cannot exclude the possibility that the effects of mefenamic acid is due, in part, to modulation of the $GABA_A$ receptor. Thus several reports have shown that inhibition of the $GABA_A$ receptor, by bicuculline, enhanced working memory in normal animals (Yonkov & Georgiev, 1981; Brioni & McGaugh, 1988; Castellano & Pavone, 1988; Chrobak & Napler, 1991). It has also been reported that benzodiazepine inverse agonists can enhance working memory in normal animals. For example, sub-cutaneous injection of 3-carboxyl-β-carboline in mice enhanced their performance in a passive avoidance task, when injected before but not after the first trial (Venault *et al.*, 1986), an enhancement was also observed with 3-carboxyl-β-carboline when infused into the nucleus basalis magnocellularis of rats and tested in a two-trial recognition task (Mayo *et al.*, 1992). Flumazenil, a partial inverse benzodiazepine agonist, has been shown to enhance normal rats performance in the passive avoidance task (File & Pellow, 1988) and the operant delayed-matching-to-position task (Cole & Hillmann, 1994). This leads to the hypothesis that benzodiazepine inverse agonists

might mimic the effects of mefenamic acid in the object discrimination task. Future experiments might address this hypothesis.

Considering the electrophysiological results that mefenamic acid can non-competitively inhibit 5-HT_3 receptor- evoked responses it is possible that the behavioural effects of mefenamic acid is *via* inhibition of the 5-HT_3 receptor. Indeed previous behavioural experiments have shown that 5-HT_3 antagonists can enhance cognition. For example the 5-HT_3 antagonist, itasteron has been reported to improve working memory in aged rats in the Morris water maze (Pitsikas *et al.*, 1993) and multiple choice avoidance task (Pitsikas & Borsini, 1996). The 5-HT_3 antagonists ondansteron and tropisetron have enhanced performance of normal rats and reversed the amnesic effect of the 5-HT_3 agonist p-chloroamphetamine in a conditioned response task (Hong & Meneses, 1996). A future experiment to investigate the role of 5-HT_3 receptor in enhanced object discrimination would be to determine if 5-HT_3 antagonists could replicate the effects of mefenamic acid in the object discrimination task.

Mefenamic acid also non-competitively inhibited P2x receptors in this study. Only one study to date has investigated the effects of purines on working memory where it was shown that AIT-082, a P1 receptor antagonist, enhanced memory in memory-deficient and young mice (Rathbone *et al.*, 1998). It is therefore possible that the effect of mefenamic acid could be *via* inhibition of ATP neurotransmission, although more work would be required to fully determine the level of purinoceptors involvement in working memory.

It has been reported that compounds that inhibit selective potassium channels result in cognitive enhancement. For example, inhibition of slow activating voltage-gated K^+

channels, by linopirdine, has been shown to improve performance of rats tested in the passive avoidance task (Cook *et al.*, 1990). Likewise aged rats treated with linopirdine showed improved performance in the Morris water maze (Baxter *et al.*, 1994). It is thought that the cognitive effects of linopirdine is *via* inhibition of the slow activating voltage-gated K^+ channels which decreases the sub-threshold electrical excitability of neurones in response to synaptic inputs (Wang & McKinnon, 1995). Recently fenamate NSAIDs, but not indomethacin, have been reported, from whole-cell patch-clamp current recordings, to inhibit in a concentration-dependent manner neuronal voltage-gated K^+ channels expressed in Chinese hamster ovary cells (Lee & Wang, 1999). It is therefore postulated that the cognitive enhancing effects of fenamates is *via* inhibition of these neuronal voltage-gated K^+ channels.

Possible mechanisms underlying the reversal of scopolamine-induced impairment in object discrimination observed with mefenamii acid:

The muscarinic antagonist, scopolamine, has been shown to induce memory impairments in a large variety of tasks including the radial maze (Stevens, 1981; Watts *et al.*, 1981), T-maze (Beninger *et al.*, 1986; Moran, 1993), both non-spatial (Markowska & Wenk, 1991; Ravel *et al.*, 1992) and spatial (Dunnett, 1985; Cole *et al.*, 1994; Kirkby *et al.*, 1996) delayed-matching-to-position tasks and in non-matching-to-position tasks (Huston & Aggleton, 1987; Deacon, 1991; Buxton *et al.*, 1994). The results from this present study showed that scopolamine reduced object discrimination in a concentration-dependent manner. This observation is in agreement is in agreement with reports from others (Ennaceur & Meliani, 1991a; Vannucchi *et al.*, 1997; Woolley *et al.*, 2000). It is possible that the scopolamine-induced impairments were due to a decrease in gross motor activity rather than an amnesic effect. However, scopolamine (0.25mg/kg) did not significantly reduce total exploration times but did result in a

significant impairment in object discrimination. Moreover, Buhot and colleagues (1989) have shown that scopolamine (1mg/kg) affects object discrimination more than locomotor activity by observing the number of squares crossed during the object discrimination task.

Mefenamic acid completely reversed the impairment induced by scopolamine at low doses (0.25-0.5mg/kg) but not at the highest dose tested (1mg/kg). The observation that mefenamic acid can reverse a scopolamine induced impairment leads to the hypothesis that mefenamic acid can modulate central cholinergic systems.

The cholinergic system has been known to be critically involved in processes of cognition for many years (Bartus *et al.*, 1982). Cholinergic neurones form an important component of both non-spatial (Spencer *et al.*, 1985) and spatial (Beninger *et al.*, 1986) working memory. It is feasible that the behavioural effects of mefenamic acid are due to a positive modulation of nicotinic or muscarinic acetylcholine receptors.

The role of nicotinic acetylcholine receptors in working memory is still unclear; administarion of nicotine to rats has been shown to improve performance in the radial maze (Levin *et al.*, 1995) and the object discrimination task (Puma *et al.*, 1999), but impair performance in delayed matching-to-sample tasks (Sahgal *et al.*, 1990; Dunnett & Martel, 1990). However, nicotine does not affect performance in delayed matching- (Bushnell *et al.*, 1995) and non-matching-to sample tasks (Widzowski *et al.*, 1994). In contrast, antagonism of nicotinic acetylcholine receptors results in an impairment of performance in both matching- (Andrews *et al.*, 1994; Bushnell *et al.*, 1995) and non-matching-to-sample (Deacon *et al.*, 1991; Ruotsalainen *et al.*, 1997) working memory paradigms. Given that the results presented in this thesis, that fenamates have little

effect on native neuronal nicotinic acetylcholine receptor- evoked responses, and that fenamates non-competitively inhibit the α3β2 nicotinic ACh receptor- evoked currents expressed in *Xenopus* oocytes (Zwart *et al.*, 1995) (β2 being the predominant nicotinic β subunit in the brain, (Paterson & Nordberg, 2000)), it seems unlikely that the behavioural effects observed with fenamates is *via* modulation of nicotinic acetylcholine receptors.

It has been widely reported that antagonism of muscarininc acetylcholine receptors impairs working memory (see above). Few studies have investigated the effects of non-specific muscarinic agonists on working memory tasks and these results indicate that they do not modulate working memory. For example Sahgal and colleagues (1990) showed that oxotremorine had no effect on rats tested in delayed (non-)matching-to-sample tasks, and Leanza *et al.* (1996) reported that arecoline could not ameliorate the effects of scopolamine in rats tested in a delayed matching to sample paradigm. However selective agonists of M_1 receptors do have cognitive enhancing properities; the M_1 agonist, N-[2-(1-azabicyclo[3,3,0]octan-5-yl)ethyl]2-nitro aniline fumarate (SK-946), can reverse the impairment of scopolamine in rats tested in the passive avoidance and Morris water maze tasks (Suzuki *et al.*, 1998). Another selective M_1 agonist, L-687,306 can reverse a scopolamine-induced impairment in rats tested in a delayed matching-to-sample task (Dawson & Iversen, 1993); AF150(S) (1-methyl-piperidine-4-spiro-(2'-methylthiazoline)), a partial M_1 agonist, has been shown to reverse an ethylcholine aziridinium (which induces a cholinergic hypofunction in the hippocampus (Fisher & Hanin, 1986)) induced cognitive impairment in the passive avoidance, radial and Morris water maze tasks (Brandeis *et al.*, 1995). To date no study has investigated the effects of fenamates on muscarinic acetylcholine receptors. Therefore it remains to

be determined if the behavioural effects of fenamates are *via* modulation of M_1 acetylcholine receptor activation.

Another possible mechanism for the behavioural effects of fenamate NSAIDs is that they could indirectly modulate cholinergic neurotransmission. For example, it has been shown (by double labelling light microscopic experiments) that 5-HT neurones innervate cholinergic basal forebrain (Jones & Cuello, 1989; Khateb *et al.*, 1993) and hippocampal neurones (Milner & Veznedaroglu, 1993), and behavioural experiments have shown that 5-HT_{1A} agonist 8-OH-DPAT (8-hydroxy-2-(di-*n*-propylamino)tetralin-*p*-chloroamphetamine) and parital agonist, ipspirone, attenuated a scopolamine induced impairment in rats tested in a operant delayed matching to position task (Cole *et al.*, 1994); a reversal of a scopolamine-induced impairment in object discrimination has also been shown with 5-HT_6 antagonist Ro-046790 (4-amino-N-(2,6-bis-methyl-amino-pyrimidin-4-yl)-benzene sulphonamide) (Woolley *et al.*, 2000) and the 5-HT_3 receptor antagonist itasetron has been shown to improve working memory in scopolamine impaired rats in the passive avoidance task (Brambilla *et al.*, 1993) and the Morris water maze (Pitsikas *et al.*, 1994). This leads to the hypothesis that the behavioural effects of mefenamic acid may be partially due to modulation of the serotonergic neurones which innervate the cholinergic pathways involved in working memory.

In addition, it has been shown that some of the effects of GABAergic activation are mediated *via* interactions with cholinergic neurones involved in working memory. For example intra-septal infusions of the $GABA_A$ agonist, muscimol, impaired performance in the T-maze (Naghara & McGaugh, 1992). Muscimol also impairs rat performance in the passive avoidance task when injected into the basal forebrain (Nabeshima *et al.*, 1988). It has also been shown that $GABA_A$ receptors are localized on cholinergic

neurones projecting to the cortex (Zaborszky *et al.*, 1986). It is therefore possible that the behavioural effects of mefenamic acid is through modulation of GABAeric inputs to cholinergic pathways involved in working memory.

Modulation of T-maze and radial maze performance by mefenamic acid:

An alternative hypothesis addressed in this study was that the effect of mefenamic acid was due to a particular aspect of the open field test and therefore task specific. In order to investigate this, the effect of mefenamic acid in two widely used working memory paradigms, namely the non-matching-to-sample T-maze and the radial maze tasks was investigated. These tasks also enabled the investigation of the effect of mefenamic acid on reference memory.

For a rat to complete a trial in the T-maze task, successfully, it must be able to discriminate and remember places in the environment (the goal arms) and that discrimination, based on reference memory, in turn has to be integrated with working memory of the place for food re-reinforcement in the previous trial (Brito & Thomas, 1981). Mefenamic acid treated rats made fewer errors and took fewer days to reach criterion when compared with saline treated rats. This information indicates that mefenamic acid rats learned the T-maze task faster than saline controls and therefore enhanced reference memory. Once all rats had reached criterion they were then tested in the T-maze with a five-minute intra-trial delay to investigate the effect of mefenamic acid on working memory. Mefenamic acid treated rats made significantly fewer errors than control rats which indicates that mefenamic acid enhanced working memory.

Mefenamic acid did not significantly change the number of entries into never baited arms to criterion in the radial maze, a measure of reference memory (Olton &

Samuelson, 1976). The number of re-entry errors into baited arms was, however, significantly decreased by mefenamic acid (a measure of working memory (Olton & Samuelson, 1976)) with a thirty-second intra-trial interval. This data indicates that mefenamic acid does not modulate reference memory but does enhance working memory when measured in the radial maze.

Mefenamic acid enhanced rats performance in all four working memory paradigms, namely the object discrimination, object location, non-matching-to-sample T-maze and radial maze tasks, utilised within this thesis. These data indicate that the effect of mefenamic acid is not task specific and provide further evidence in support of the hypothesis that mefenamic acid enhances working memory. The observation that mefenamic acid apparently enhanced rats' reference memory in the T-maze but not the radial maze may be due to the differences between the mazes. For example it has been suggested that rats in the T-maze use ego-centric cues (left/right turns) to solve the task whereas in the radial maze they use extra-centric spatial cues (Olton, 1982). There is also a difference in the mnemonic load needed to complete each task successfully; in the T-maze they need to learn to enter the previously un-entered arm, whereas in the radial maze there was an increased mnemonic load as they had to remember which four of the eight arms were never baited. These two factors may account for the differing reference memory results. In order to clarify the effect of mefenamic acid on reference memory its effect on an alternative reference memory tasks such as the Morris water maze (Morris, 1984) will need to be investigated.

Final remarks

The data obtained from electrophysiological experiments has shown that fenamate NSAIDs can modulate native neuronal ligand-gated ion channels. Modulation of

neuronal $GABA_A$ and $5\text{-}HT_3$ receptors could contribute to the behavioural effects of fenamates such as such as seizures and convulsions observed after overdose (Young, 1979; Robson *et al.*, 1979; Balali Mood *et al.*, 1981; Prescot *et al.*, 1981; Gossinger *et al.*, 1982; Frank *et al.*, 1983; Shipton & Miller, 1985; Hendrickse, 1988; Clark & Ghose, 1992). This modulation may also contribute to the modulation of seizure activity observed with NSAIDs in animal models (Wallenstein, 1985a,b; 1987; 1991; Ikononudou-Turski *et al.*, 1988).

The data therefore raised the hypothesis that fenamates may have other and additional behavioural and/or cognitive effects. The second major series of experiments undertaken in this thesis addressed this hypothesis.

These behavioural results have shown that mefenamic acid can increase both object discrimination and object location when compared to saline controls. This increase in object discrimination is not due to an increase in arousal, gross motor activity or an anxiolytic effect by mefenamic acid. One other fenamate NSAID, namely meclofenamic acid, but not non-fenamate NSAIDs were able to mimic the effect of mefenamic acid indicating that its effect is not a general NSAID effect. These results therefore lead to the conclusion that the behavioural effects are not due to cyclooxygenase inhibition in the brain. The actions of fenamates were not replicated by positive or negative modulators of the $GABA_A$ receptor. These data indicate that the action of mefenamic acid are not entirely dependent on modulation of the $GABA_A$ receptor. Mefenamic acid was also shown to reverse a scopolamine-induced impairment in object discrimination, indicating that mefenamic acid might modulate "cholinergic mechanisms" involved in working memory. Mefenamic acid enhanced working memory in both the T-maze and

radial maze tasks indicating that the behavioural effects of mefenamic acid are not task specific.

Speculatively, the chemical structure of fenamates may serve as a template for the design of novel compounds to help treat dementia such as Alzheimer's disease. Notably a recent study has shown that intracerebroventricular administration of β amyloid (1-40) impaired rats' performance in the object discrimination task with a five-minute intra-trial interval (Nag *et al.*, 2001). Given the epidemiological evidence that NSAIDs can delay the onset on Alzheimer's disease and the evidence from this thesis that fenamates can improve working memory, the effect of fenamates on the actions of β amyloid (1-40) in the object discrimination task might be determined to help elucidate the beneficial effects of NSAIDs in the treatment of Alzheimer's disease.

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