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CHARACTERISATION OF THE NEUROSTEROID ANALGESIC ALPHADOLONE

A THESIS PRESENTED FOR THE DEGREE OF A DOCTOR OF PHILOSOPHY

BY

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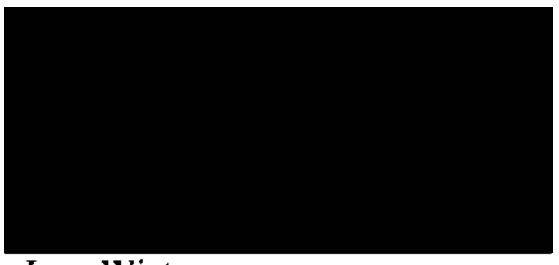
Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other institution and affirms that to the best of the candidate's knowledge, the thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.



Lara Winter

Summary

Alphadolone is a neurosteroid capable of causing antinociceptive effects in acute nociceptive models in rats by positive modulation at $GABA_A$ receptors in the spinal cord. The work described in this thesis characterised alphadolone in several different acute and chronic nociceptive models in rats.

Two chemically similar neurosteroids, alphaxalone and alphadolone were assessed for sedative (using the rotarod and activity monitor) and antinociceptive effects (using heat, electrical and mechanical nociceptive tests). Alphaxalone was twice as potent as alphadolone in causing sedation. Both neurosteroids had no effect on sensitivity to noxious heat stimulation. Alphadolone demonstrated antinociceptive effects in other acute and chronic nociceptive models in rats. Although alphadolone and alphaxalone are two neurosteroids that share almost identical chemical structures, their pharmacological properties with respect to sedation and antinociception are different. Alphadolone was also combined with several clinically available opioids in different nociceptive models in rats to further demonstrate enhanced antinociceptive effects without causing increased sedative effects.

This thesis demonstrates the potential clinical value of alphadolone, both alone and in combination with opioids, for effective pain management.

Abbreviations

5-HT	serotonin; 5-hyroxy-tryptamine
ACh	acetylcholine
ANOVA	analysis of variance
BZ	benzodiazepine
Ca^{2+}	calcium (intracellular)
cAMP	cyclic adenosine 5′-monophosphate
CCK	cholecystokinin
CD	cyclodextrin
CNS	central nervous system
COX	cyclooxygenase
EAA	excitatory amino acids
ECT	electrical current threshold
GABA	γ-aminobutyric acid
$GABA_A$ receptor	γ-aminobutyric acid type A receptor
$GABA_B$ receptor	γ-aminobutyric acid type B receptor
$GABA_C$ receptor	γ-aminobutyric acid type C receptor
GAD	glutamic acid decarboxylase
HPβCD	2-hydroxypropyl-β-cyclodextrin
Hz	hertz
IASP	International Association for the Study of Pain
IP	intraperitoneal
IT	intrathecal
IV	intravenous
kg	kilograms
mA	milliampere
µg	micrograms
mg	milligrams
µl	microlitres

ml	milliliters
mins	minutes
MMC	Monash Medical Centre
MPE	maximum possible effect
NMDA	*N*-methyl-*D*-aspartic acid
NO	nitric oxide
NSAIDs	non-steroidal anti-inflammatory drugs
PCA	patient controlled analgesia
PKA	protein kinase A
PKC	protein kinase C
pmol	pico mole
PP	paw pressure
r	response
RH	reversal of hyperalgesia
rpm	revolutions per minute
SD	standard deviation
secs	seconds
SEM	standard error of the mean
STZ	streptozotocin
TFL	tail flick latency
THIP	4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridine-3-ol
wt	weight

Definitions

analgesia	absence of pain in response to stimulation which would normally cause pain
antinociception	decreased sensation signals in the nervous system providing information regarding tissue damage
hyperalgesia	increased sensitivity to noxious stimuli
multimodal analgesia	combination of several different compounds or therapies for pain relief
neuropathic pain	persistent pain due to nervous system damage
nociception	the reception of signals in the nervous system which provide information regarding tissue damage
pain	an unpleasant sensory and emotional experience associated with actual or potential tissue damage
potentiation	an increased effect of a compound by a dose of another compound that had no effect when given alone

Symbols

%	percentage
$L_{1/2}$	lumbar vertebrae numbers 1 and 2
T_{11}	thoracic vertebrae 11
n	number of samples
r	ratio response (ECT, PP)
®	registered trademark
*	statistically significant
°C	degrees in Celsius
±	plus or minus a certain value
≤/≥	less than or equal to / greater than or equal to
</>	less than / greater than

Technical Notes

Microsoft Word and Excel 2000 were used.

Sigmaplot 2001 for Windows Version 7.0 was used for all graphs. Graphs were directly inserted into Word documents.

Reference Manager Professional Network Edition Version 9.5N was used for all references.

Version 3.06, 32 bit for Windows was used for all statistics.

Presentations and Publications

Winter L, Nadeson R, Goodchild CS. A treatise on the analgesic properties of alphadolone. March 2004, APS, Canberra, Australia.

Winter L, Kosolov A, Nadeson R, Goodchild CS. Alphadolone: A neurosteroid analgesic for diabetic neuropathic pain. December 2003, ASCEPT, Sydney, Australia.

Winter L, Nadeson R, Tucker AP, Goodchild CS. Antinociceptive properties of neurosteroids: a comparison of alphadolone and alphaxalone in potentiation of opioid antinociception. Anesth Analg. 2003 Sep;97(3):798-805.

Winter L, Nadeson R, Goodchild CS. The carrageenan inflammatory pain model. March 2003, Christchurch, New Zealand.

Winter L, Nadeson R, Goodchild CS, Han C. Alphadolone potentiation of opioid antinociception; an interaction at spinal cord $GABA_A$ receptors. August 2002, IASP, San Diego, USA

Winter L. Pain is inevitable; suffering is optional. August 2002, CSL National Sales Conference, Hamilton Island, Australia

Winter L, Nadeson R, Goodchild CS. Alphadolone potentiates opioid antinociception but not sedation. March 2002, APS, Sydney, Australia.

Awards

First prize for poster presentation:

Winter L, Nadeson R, Goodchild CS. Alphadolone potentiation of opioid antinociception but not sedation. October 2001, Monash University, Australia.

Acknowledgements

It is quite daunting writing this section of my thesis; it could only mean one thing - the end is near. It's hard to believe, but this brief section is one of the most difficult to piece together. How is it possible in so few words to thank many people who played such important roles throughout the completion of this body of work?

First and foremost is the driving force behind the Department of Anaesthesia of Monash University, Prof Goodchild. Colin you are an inspiration and a godsend to scientific research. It is tough to imagine that for a man with so little time, I never heard the word "no". I am so appreciative for the support and encouragement you have engulfed me with for the past four years.

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contributed to my work were Adam, Maureen, Li Cheng and Chunyan. Thank you for helping make my work a priority.

The Australian Pain Society and CSL Ltd. made this thesis possible by rewarding me with a scholarship. I was lucky enough to spend valuable time with people from both these associations who gave me wonderful opportunities and endless encouragement. The uniquely designed scholarship program is more beneficial than any PhD student could ask for. I hope to give back to you one day like you so willingly gave to me. A special thank you goes to Professor Maree Smith who still managed to give the most helpful and heartening advice from so far away.

To Bin, Em, Lains and Amanda - I started off as the piker and now you girls mean so much to me. The support group we formed for each other throughout our years of study became so special. It is now something that will carry on for a long time. Dano, you managed to join my support team at the worst time possible. As much as I apologise for that, I couldn't be more grateful. Through all of it you managed to stick it out and be as supportive and loving as ever.

Omi and Opi you saved my thesis! Thank you for so warmly opening up your house to me. My life was made so much easier because of your generosity. Last, and most importantly, my family - you Winters are a special bunch. You were there for every success and every defeat. You listened to never-ending ideas, complaints and presentations that you didn't even understand. I am without doubt so lucky to be part of such an encouraging, inspirational and, most of all, caring family. I only hope to make you even more proud from here.

Dedication

This work is dedicated to not one, but *all* those who so willingly and positively helped me fulfill a dream. There is no doubt in my mind that it was each and every one of you who made this thesis possible. Whether you assisted me in the nightmarish task of editing or merely made me smile with a playful joke - thank you.

Chapter 1 Introduction

1.1 Introduction

It is well established that γ-aminobutyric acid (GABA) is involved with sensory processing throughout the central nervous system and processing of nociceptive information at the level of the spinal cord (Dickenson, Chapman et al. 1997). The $GABA_A$ receptor has binding sites for GABA and other compounds including neurosteroids (Lambert, Belelli et al. 1995). Neurosteroid compounds may be useful as analgesics either alone or in combination with other drugs, i.e. opioids. Even though neurosteroids were discovered over half a century ago, their potential use in pain has only recently been shown. This thesis starts by introducing the history of pain and some of its underlying theories. It then discusses the importance of GABA transmission in nociception and its positive modulation by $GABA_A$ receptor agonists such as neurosteroids. This thesis characterises the neurosteroid analgesic alphadolone both alone and in combination with several opioids in different animal nociceptive models.

1.1.1 The History of Pain

In the 17th century René Descartes originally proposed that pain is a purely physical phenomenon; tissue injury stimulates specific nerves that transmit an impulse to the brain, causing the mind to perceive pain. Pain was thought of as being mediated by specific neural pathways as shown in the famous drawing by Descartes of a boy with his toe in a fire (Figure 1.1). This neural specificity theory became preserved in the medical texts of the 20th century and in current times can still be found in many medical texts, however more often as a starting point for an explanation of naïve modern concept and constructs.

Figure 1.1 Descartes View of Pain Transmission

This definition of pain allowed doctors in everyday medicine to describe pain in Cartesian terms - as a physical process, a sign of tissue injury. In the Cartesian view,

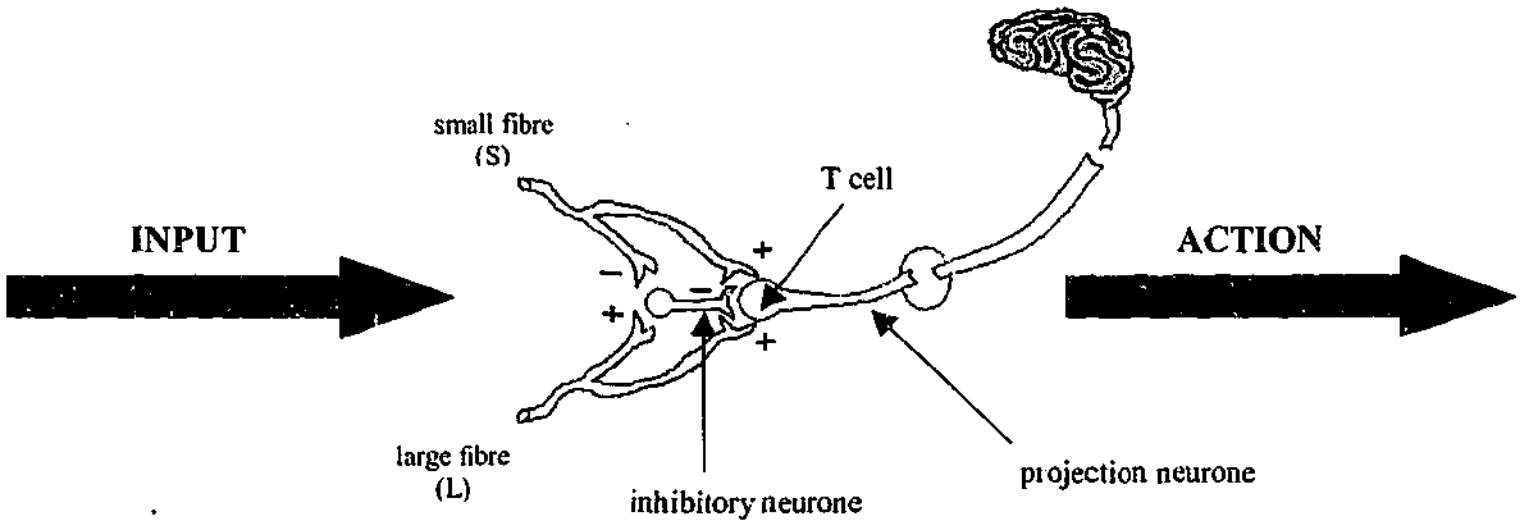
the degree of injury ought to determine the degree of pain, rather like a dial controlling volume. The limitations of this mechanistic explanation, however, have been apparent for some time, for example the classic study of men with serious battlefield injuries during the Second World War conducted by Lieutenant Colonel Henry K. Beecher. This study described men with compound fractures, gunshot wounds, torn limbs (58% in total) who reported only slight pain or no pain at all and just 27% of the men felt enough pain to request pain medication (Beecher 1946). Clearly, something was going on in their minds; Beecher thought they were overjoyed to have escaped alive from the battlefield - the joy counteracted the signals sent by their injuries. It has since been suggested that a more meaningful construct of pain is that it is multifaceted, far more complex than a one-way transmission from injury to "ouch".

In the 1960's, the study of pain was profoundly influenced by the work of Ronald Melzack and Patrick Wall. Melzack and Wall proposed that the Cartesian model be replaced with what they called the *gate-control theory of pain* (Melzack and Wall 1965). This was an extension of the growing knowledge at the time that specificity of response in the central nervous system was caused by interplay of excitatory and inhibitory processes. Melzack and Wall argued that before pain signals reach the brain they must first go through a gating mechanism in the spinal cord that determined the amount of onward transmission of incoming signals. Incoming information entering the spinal cord and signals descending from the brain interact to determine whether the "gate" to onward transmission is open or closed; the pain transmission can be blocked from reaching the higher centres necessary for perception of pain. Thus, this hypothetical gate could simply stop pain impulses from getting to the brain. The theory explained such ordinary puzzles as why rubbing a painful foot makes it feel better; the

rubbing sends signals to the spinal cord dorsal horn that close the gate to nearby pain impulses. The growing consensus that was supported by Melzack and Wall was that the gate was controlled not just by signals from sensory nerves but also by emotions and other output from the brain. A diagram for the gate-control theory is shown in Figure 1.2.

Nociceptive (small diameter fibres - S) and non-nociceptive inputs (large diameter fibres - L) converge in the spinal cord onto a common system shown as the transmission (T) cell. The relative effects of excitatory and inhibitory influences by these projections, as well as interneurone pools and fibres descending from the brain, ultimately determine whether the T cell fires to transmit information to the brain. These events are thought to occur in the superficial part of the dorsal horn of the spinal cord called the substantia gelatinosa. The interactions between these pathways are complex, involving multiple influences, chemicals and neurological processes (Millan 1999).

Figure 1.2 Gate-Control Theory
adapted from (Melzack and Wall 1965)



1.1.2 Pain and Nociception

There are many definitions describing the nature of pain. As a basic scientific definition, pain is a sensation caused by some type of noxious stimulus. From the behavioral aspect, pain is a pattern of responses that function to protect an individual from harm (Benoliel 1995). The International Association for the Study of Pain (IASP) defines pain as "an unpleasant, subjective sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Merskey 1994). However, because pain is a highly personal and subjective experience, that which is most appropriate for use in clinical practice is: "pain is whatever the experiencing person says it is, existing whenever the patient says it does" (McCaffery 1968). Certainly all these definitions describe pain; it is a multi-factorial phenomenon. Furthermore the overall pain experience in humans can be influenced by various factors such as culture, environment and physiological differences. With all the varying definitions and confounding factors, for the sake of clarity pain is generally divided into these basic components: sensation, emotion and psychosocial experience. Researchers use the term *nociception* to describe the physiological component of pain. The understanding and treatment of pain and nociception is one of the oldest challenges for physicians, scientists and philosophers.

There are several types of pain sensation. This thesis describes (a) acute nociceptive pain, (b) inflammatory pain and (c) neuropathic pain.

(a) Acute Nociceptive Pain

Acute nociceptive pain is the function of nociceptive afferent fibres in uninjured tissues. It is usually a signal of *impending* tissue damage and thus permits an individual to avoid harm. The process by which such a stimulus causes pain with no tissue injury is sometimes called physiological pain. The sensation of nociception is relayed to the brain where higher centres respond appropriately to protect the individual from further injury. The pain sensation and nociceptive behaviour do not persist after withdrawal of the stimulus.

(b) Inflammatory Pain

The nociceptive signal in inflammatory pain is caused by peripheral tissue injury and subsequent inflammation that cause persistent electrical discharges from local tissue nerve endings. This is followed by the nerve endings releasing substance P both in the spinal cord and into the tissues. In the tissues this leads to increase blood flow and the production of oedema at the site of injury. The local tissue release of bradykinin, prostaglandins, substance P and nitric oxide (NO) sensitises the local afferent terminals. This causes primary hyperalgesia i.e. increased sensitivity to stimulation at the site of an injury. Spinal cord release of substance P and excitatory amino acids (EAA) by the primary afferent discharge causes sensitisation of post-synaptic neurones. Secondary hyperalgesia follows i.e. decreased nociceptive thresholds in the undamaged tissues surrounding the area of injury (Woolf 1983). This increase in excitability of spinal neurones after peripheral injury is termed *central sensitisation*, where the enhanced

reflex excitability after peripheral tissue damage does not require ongoing peripheral neuronal input.

(c) Neuropathic Pain

Neuropathic pain syndromes are sensory disorders that result from changes due to damage of neuronal pathways, peripheral or central. Neuropathic pain in humans is characterised by (Jensen, Gottrup et al. 2001): spontaneous pains in the regions of injury in the absence of any stimulus, the sensation of pain induced by a normally non-noxious stimulus (allodynia), increased sensitivity to noxious stimuli (hyperalgesia) and the induction of abnormal activity within the sympathetic nervous system that potentiates and prolongs pathological hypersensitivity.

Multiple mechanisms are believed to contribute to the heightened pain state including:

- the recruitment of silent nociceptors that increase afferent barrage to cause sensitisation of dorsal horn neurones (Chapman, Ng et al. 1998);
- severe loss of small fibre input causing spinal reorganisation from sprouting of large fibres into superficial 'nociceptive' laminae in the dorsal horn (Woolf and Salter 2000);
- inflammation of nerve trunks that can cause ectopic nerve activity;
- increased sympathetic activity or altered brain processing that recruits brain areas that are not usually involved in pain (Casey 2000).

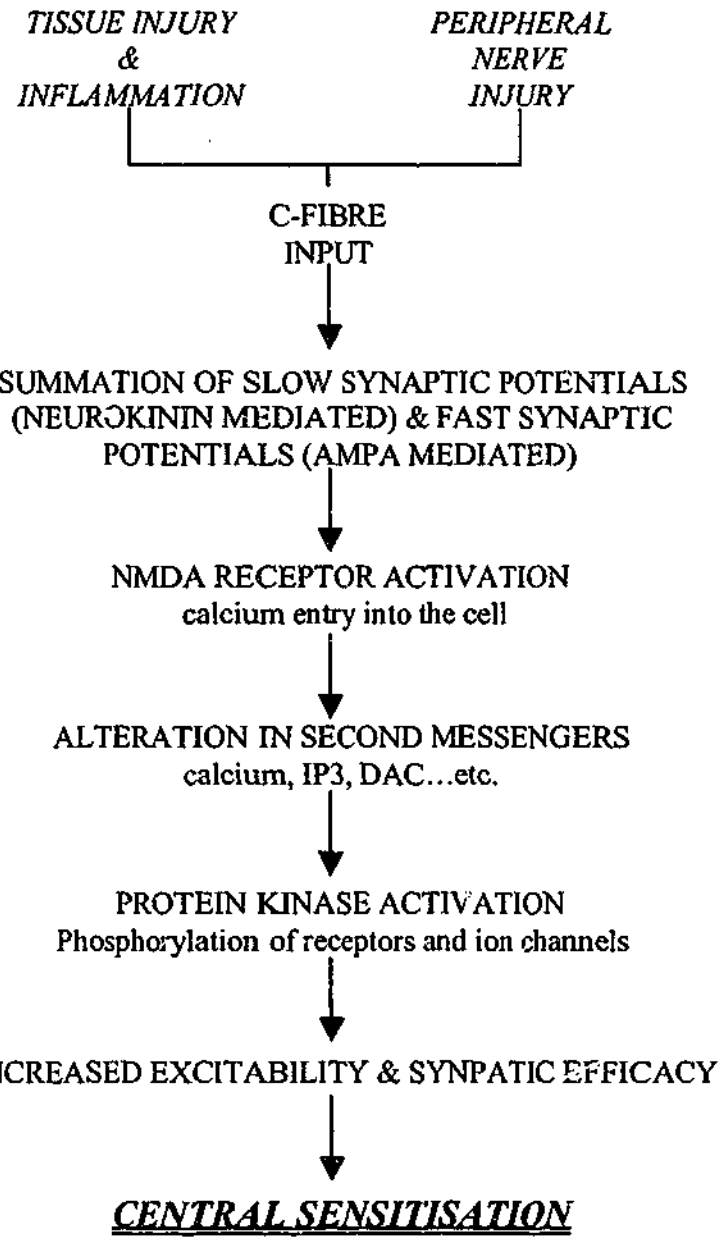
Any of these individual mechanisms or a combination of these mechanisms may account for various conditions and symptoms seen in patients complaining of

neuropathic pain. The nature of neuropathic pain is obviously severe and complex, hence it is not well understood and is difficult to treat clinically.

1.1.3 Central Sensitisation

Peripheral injuries like inflammation and neuropathies may result in long-term changes in the excitability of neurones in the dorsal horn of the spinal cord (Cesaro and Ollat 1997). The receptive fields of the dorsal horn neurones expand in size and their spontaneous activity increases. In addition, neurones become more responsive to noxious stimuli. This phenomenon is referred to as "central sensitisation" and is thought to be responsible for the persistence of pain after the removal of noxious stimuli. It is well established that central neural plasticity contributes to the development and maintenance of pathological pain (Coderre, Katz et al. 1993). Therefore central sensitisation reflects plasticity within the CNS. C-fibres have been implicated in CNS plasticity because they are associated with the release of chemical mediators like substance P (Go and Yaksh 1987) and neurokinin A (Duggan, Hope et al. 1990), which cause prolonged excitation of dorsal horn neurones. Furthermore there is evidence that has shown excitatory amino acids (EAA) to be involved in triggering nociceptor-induced neuroplasticity. Glutamate and aspartate are released in the spinal cord dorsal horn during noxious stimulation (Sorkin, Westlund et al. 1992). These central changes involve interaction of primary afferent neurotransmitters with second messengers systems including protein kinases and intracellular calcium (Ca^{2+}) (Coderre, Katz et al. 1993). N-methyl-D-aspartate (NMDA) receptor activation as well as nitric oxide (NO) have also been implicated in maintaining central sensitisation (Urban and Gebhart 1999). This cascade of events is depicted in Figure 1.3 on page 10.

Figure 1.3 Central Sensitisation
adapted from (Wall and Melzack 1989)



1.1.4 Animal Laboratory Research

In animal laboratory research in pain medicine, nociceptive behaviour in response to noxious stimuli is used as a correlate for human pain sensation. Nociception in animals is defined as "an aversive sensory experience caused by real or potential damage that provokes motor and vegetative protective reactions" (Jourdan, Ardid et al. 2001). This is usually demonstrated as avoidance behaviour and can modify the specific behaviour of the species including its social behaviour. It is these observations that are used for nociceptive measurement in animals. Animal subjects are necessary in pain research because: (1) animal experimentation permits detailed manipulation of experimental variables that are necessary to obtain an understanding of nociceptive mechanisms and analgesic therapies and (2) animal preparations can be used to model certain human pathological conditions in a controlled environment (Chapman, Casey et al. 1985). Animal nociceptive models allow the manipulation of physiological and pharmacological variables that would not be possible or ethically justifiable in humans.

There has been a vast increase in animal studies specifically for the screening of new drugs. It is widely accepted that effective drugs in animal studies will be used for further investigation with the possibility of entering clinical trials. However, the translation of animal experimentation to human application is a complicated process. Nociceptive animal models are considered in terms of 'predictiveness' of clinical applicability. In the context of drug discovery, the predictive value of an animal model in relation to humans can be defined with regard to certain criteria. Firstly, are the signs and symptoms experienced by both animal and patient similar? Although this criterion is only superficial it is a beneficial indicator of how relevant the experiment is. This

measure is termed *face validity* (Jourdan, Ardid et al. 2001). The second criterion is to analyse whether the abnormality (pain state) in the animal is produced by the same pathophysiological mechanism as in the patient; finding a treatment involves targeting the mechanisms and if they differ, the comparison of animal and human work would be irrelevant. The final criterion is 'pure empiricism' whereby we assume that if a drug works in animals then it works in humans. This rule is practical and assumes direct prediction between the two subjects. It is beneficial for initial basic research but requires further analysis in the later stages of the drug discovery program.

In the case of analgesic screening, evidence for a compound's effectiveness is often assessed using rat behavioural tests. Various methods for producing experimental nociception in rats have been described. The noxious stimuli include a variety of heat, mechanical, electrical and chemical procedures for use in animal models of acute, inflammatory and neuropathic pain.

Acute nociception in animals is often studied using simple reflexes of withdrawal or more elaborate reactions such as vocalization (Chapman, Casey et al. 1985). Tests based on the evaluation of reflex reaction include heat stimulation in the tail flick test and reflex paw withdrawal from noxious mechanical stimulation (Le Bars, Gozariu et al. 2001). Tests using electrical stimuli are based on the evaluation of vocalization behaviour (Jourdan, Ardid et al. 2001).

Several persistent inflammatory pain models exist that involve injection of different substances into the paw i.e. formalin, carrageenan or Freund's adjuvant (Hogan 2002). In each of these models inflammation appears after several hours. This inflammatory

induction period varies according to which inflammatory agent was injected. The development of hyperalgesia follows, thus mimicking clinical inflammatory pain (Mao, Price et al. 1995). The evaluation of the induced hyperalgesia is achieved by the additional application of an acute stimulation, generally using the paw pressure test (paw withdrawal from a noxious mechanical stimulus).

Several models of neuropathic pain have been developed. The application of ligatures to different levels of the sciatic nerve has been shown to induce spontaneous pain behaviours and hyperalgesia (Bennett and Xie 1988; Kim and Chung 1992). A diabetes-induced model of neuropathy has also been developed. Here spontaneous pain behaviours and hyperalgesia are caused by peripheral nerve damage following the chemical indicator of diabetes mellitus (Courteix, Eschalier et al. 1993). Like inflammation testing, the evaluation of the induced hyperalgesia is achieved by the additional application of an acute stimulus, generally the paw pressure test.

1.2 γ-Aminobutyric Acid (GABA)

Pain and nociception is associated with excessive neuronal activity that is influenced by numerous neurotransmitters and modulators. Therefore, it is expected that decreasing excitatory neurotransmitters, or increasing inhibitory neurotransmitters will control nociception. Some neurotransmitters and modulators that are used for the treatment of pain are listed: acetylcholine (ACh), cholecystokinin (CCK), 5-hydroxytryptamine (5-HT), prostaglandin, substance P, NMDA antagonists, opioids and γ-aminobutyric acid (GABA). This thesis concentrates on the inhibitory neurotransmitter GABA.

The presence of GABA in the central nervous system, and its powerful inhibitory effect on neurones was first discovered in the 1950's (Curtis, Duggan et al. 1970). Since that work was published, GABA has become widely accepted as the most ubiquitous inhibitory neurotransmitter. GABA is thought to function as an inhibitory transmitter in many different CNS pathways. Hence GABA is particularly abundant in the brain but is also found in other mammalian tissues. The widespread distribution of GABA and the fact that most neurones are sensitive to its inhibitory effect, suggest its importance to all mammals as well as insects, fish and birds. It is formed from glutamate by the action of glutamic acid decarboxylase (GAD) (Florey 1991). Thus GABA and glutamate and their respective receptors often coexist in the same neurones. Their opposing actions exemplify the concept of neuroplasticity. In many different systems these two compounds have been shown to have opposing effects, and the balance between the two determines the specificity and selectivity of the responses within a system.

Processing of nociceptive information in the spinal cord is powerfully influenced by inhibitory circuits containing GABA (Dickenson, Chapman et al. 1997). Local GABAergic inhibitory interneurones have presynaptic terminals on primary afferent fibres. They also make post-synaptic contact with interneurones or projection neurones transmitting nociceptive information in the dorsal horn (Haefely, Kulcsar et al. 1975). For this reason GABA has become a key target for investigation of pain therapies.

Three receptor subtypes have been discovered for the modulation of GABA transmission. The $GABA_A$ receptor subtype was classified as being bicuculline sensitive. A separate class of GABA receptors characterised by bicuculline resistance and activation by baclofen are called $GABA_B$ receptors (Dutar and Nicoll 1988). A third type is the $GABA_C$ receptor; also resistant to bicuculline (Johnston 1994). These latter two receptor classes will not be discussed further in this thesis.

1.2.1 $GABA_A$ Receptors

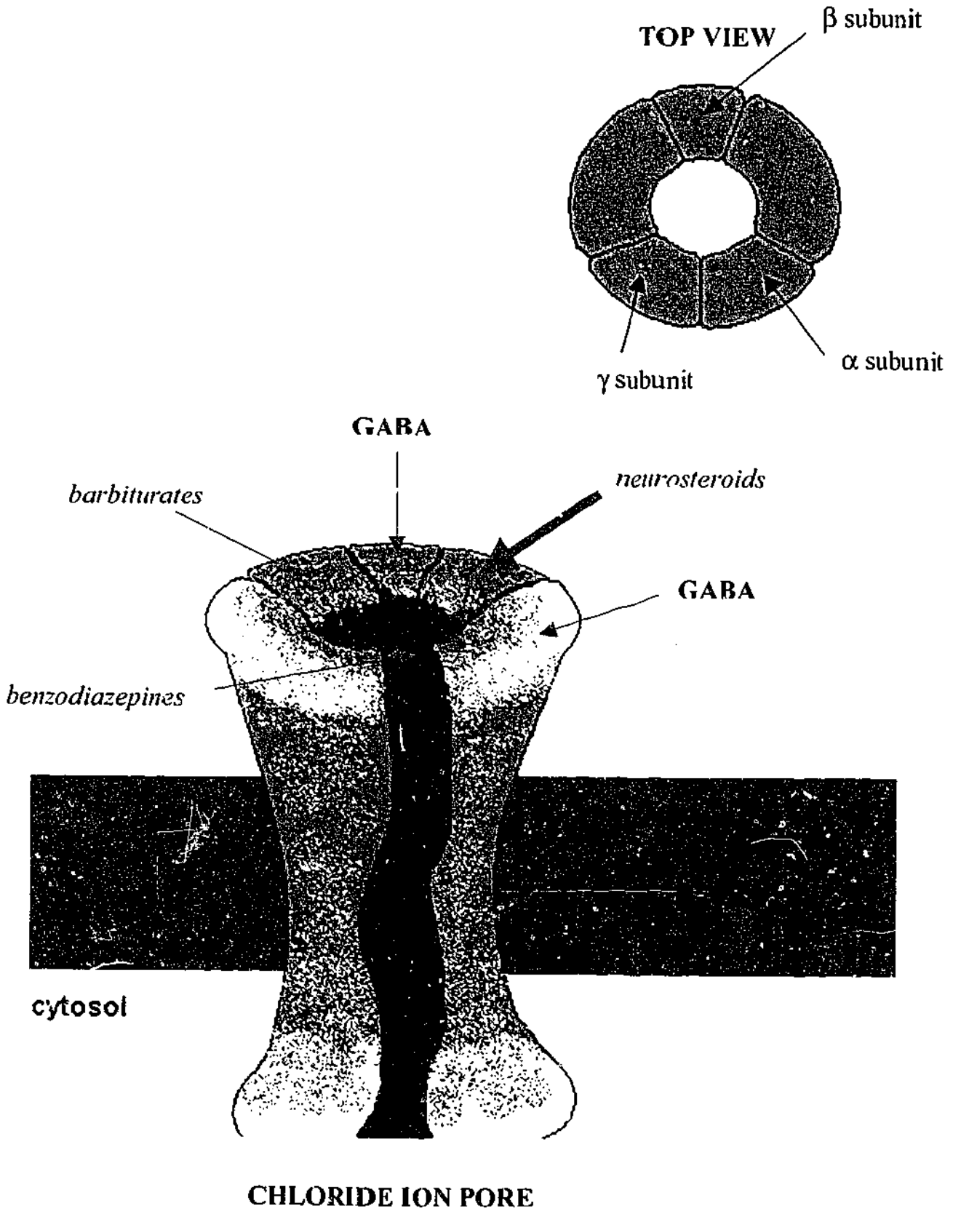
The $GABA_A$ receptor is the most important inhibitory receptor in the central nervous system (CNS) (Bormann 1988). It is present at inhibitory synapses and postsynaptically on neurones. Electrophysiological studies on the action of GABA on CNS neurones have shown that its postsynaptic inhibitory effect is mediated mainly by $GABA_A$ receptors, which are coupled directly to anion channels that cause an increase in chloride permeability of the postsynaptic membrane. This has the effect of hyperpolarizing the neurone and reducing the depolarization caused by excitatory transmitter action. The $GABA_A$ receptor is a hetero-pentamer with a central chloride channel that is gated by GABA (gamma-aminobutyric acid). The $GABA_A$ receptor

mediates fast synaptic inhibition when the binding of GABA increases the chloride-ion conductance in the post-synaptic membrane. Figure 1.4 on page 17 displays a schematic diagram of the $GABA_A$ receptor. Various selective agonists and antagonists helped define the $GABA_A$ receptor, principle examples being muscimol and bicuculline respectively.

The $GABA_A$ receptor (Figure 1.4) is composed of structurally related protein subunits. The subunits are arranged to form a central pore, which, when opened, allows for the passage of chloride ions into the postsynaptic neurone. The opening of this pore allows for the influx of chloride ions and hence its inhibitory effect. The inhibitory $GABA_A$ receptor is thought to be the target for many clinically useful drugs such as general anaesthetics, barbiturates, benzodiazepines and anti-epileptics, all of which can potentiate GABA currents. Most modulators do not bind to the GABA site, but to alternate sites on the receptor complex to induce conformational changes in the receptor, and ultimately to affect the chloride channel opening caused by GABA binding (Hevers and Luddens 1998).

The receptor itself has different subunits forming the pentameric chloride ion channel. Subunits identified to date include α, β, γ, δ, ε, π, θ and ρ; many exist in multiple isoforms (e.g. $\alpha_{1\text{-}6}$, $\beta_{1\text{-}4}$ and $\gamma_{1\text{-}4}$) (Hammond 2001). Therefore a plethora of potential subunit combinations exist. The component subunits show regional variation in the CNS (Wisden and Seeburg 1992). This prospective diversity promises the possibility of increased clinical specificity for drugs targeting different $GABA_A$ receptor subtypes throughout the CNS and the periphery.

Figure 1.4 The $GABA_A$ receptor
adapted from ($GABA_A$ receptors: www.williams.edu/imput/team.html 2003)



1.2.2 $GABA_A$ Receptors as a Target for Pain Relief

It is well established that the spinal cord is involved in nociception. Hence since the early discoveries of high concentrations of GABA in the dorsal horn, GABA has been believed to fulfill a role in the modulation of nociception (Miyata and Otsuka 1975). $GABA_A$ receptors are present in great numbers in the superficial dorsal horn further implicating GABA in nociceptive processes (Waldvogel, Faull et al. 1990). Based on numerous behavioural, anatomical and electrophysiological investigations it is now well established that GABA is involved in the modulation of noxious sensory input to spinal cord systems. GABA has also been implicated in pathological pain states such as neuropathic pain and inflammation. In these conditions GABAergic inhibition has become dysfunctional (Castro-Lopes, Tavares et al. 1993; Eaton, Plunkett et al. 1998; Ibuki, Hama et al. 1997). Evidently GABA modulation must be considered as a potential therapeutic target for pain relief.

Electrophysiological investigations have provided further evidence for a role of GABA in nociception. Spinal application of GABA has been shown to suppress the responses of dorsal horn neurones to noxious stimuli (Sawynok 1984). However, since GABA undergoes metabolic reuptake processes, the amino acid itself is not an ideal agent for spinal administration. Various behavioural pharmacological studies have provided strong evidence that activation of $GABA_A$ receptors by exogenously administered agonists can produce antinociception. Muscimol and THIP ($GABA_A$ agonists) have been shown to act at supraspinal sites to produce antinociception (Sawynok 1984). Although these experiments report antinociception they speak of supraspinal sites of action. If these drugs interact with receptors in the brain they cause other effects such as

sedation, memory disturbance and mood alteration and thus these experiments highlight the problem of obtaining specificity of responses. Experiments administering intrathecal midazolam demonstrated that this benzodiazepine is also able to positively modulate $GABA_A$ receptors and therefore altering transmission of nociceptive information at the level of the spinal cord (Goodchild and Serrao 1987; Moreau and Pieri 1988).

$GABA_A$ receptors display an extensive structural heterogeneity based on the differential assembly of their subunits (Fritschy and Mohler 1995). The subunit composition of receptor subtypes is expected to determine their physiological properties and pharmacological profiles. Hence heterogeneity in the $GABA_A$ receptor composition may provide a molecular basis for the diverse pharmacological properties associated with this receptor. There is expression of different $GABA_A$ receptors with different pharmacological effects that are localised in distinct areas of the central nervous system during development (Perez-Velazquez and Angelides 1993). Different drug classes and novel compounds can in theory target select groups in this vast array of $GABA_A$ receptors to achieve different and specific clinical effects.

1.3 Neurosteroids and Antinociception

In 1940, Selye investigated the anaesthetic properties of progesterone-derived steroids (Selye 1941). As anaesthetics, steroids were found to have a major advantage over barbiturates; they have a far greater therapeutic ratio, and thus safety margins (Stock 1973). The main hindrance for introducing them as clinical anaesthetics was that these compounds were difficult to dissolve in suitable preparations for intravenous anaesthesia. Much work eventually led to the introduction of Althesin® by Glaxo, a combination of two steroids, alphaxalone and alphadolone. In the 1980's Althesin® was introduced into clinical practice and proved to be a highly successful intravenous anaesthetic used all over the world. All the active properties of the mixture were attributed to the alphaxalone content, while alphadolone (half as potent as an anaesthetic) was present to assist in the dissolution of alphaxalone in the aqueous vehicle, Cremophor EL (Stock 1973). Eventually Althesin® was removed from clinical practice in the late 1980's due to major anaphylactoid reactions to the vehicle.

Alphaxalone was found to allosterically positively modulate the $GABA_A$ receptor (Cottrell, Lambert et al. 1987). Since these early works, it has been shown that a number of related steroids have also been shown to interact directly with a surface membrane receptor-complex to cause a rapid change in central nervous system excitability. Such steroids with physiological functions within the CNS (including alphaxalone and alphadolone) are termed *neurosteroids* (Puia, Santi et al. 1990; Purdy, Moore, Jr. et al. 1992). Many endogenous and synthetic steroids have since been shown to cause potent modulation of the $GABA_A$ receptor. Furthermore, binding studies

demonstrated that such compounds bind directly with the $GABA_A$ receptor at a novel steroid recognition site (Lambert, Belelli et al. 1995).

The neurosteroids acting on $GABA_A$ receptors were originally found to prolong GABA-mediated inhibitory post-synaptic potentials (Harrison, Majewska et al. 1987). When the properties of GABA-gated chloride ion channels were studied using patch-clamp techniques, steroid agonists were found to increase open time, numbers of channels open, and the frequency of channel opening (Barker, Harrison et al. 1987; Mistry and Cottrell 1990; Twyman and Macdonald 1992).

The therapeutic potential for neurosteroids has been described for various disorders. Neurosteroids have anticonvulsant properties in the treatment of epilepsy (Monaghan, McAuley et al. 1999). Evaluation of neurosteroids for anxiolytic activity has also been proposed. This research, although preliminary, has shown potential for utility of neurosteroids for anxiolysis compared with benzodiazepines. For example, the neurosteroid Co 2-6749 has shown great selectivity for anxiolytic-like effects compared with sedative side effects (Vanover, Rosenzweig-Lipson et al. 2000). Aside from alphaxalone, several new compounds have been evaluated as potential sedatives and hypnotics. However, tolerance at sedative doses following chronic treatment has been reported for some (Marshall, Stratton et al. 1997).

Since the discovery of neurosteroid modulation of $GABA_A$ receptors, researchers have revealed a plethora of physiological functions for neurosteroids within the CNS. However, although GABAergic involvement in pain and nociception is well established, very few neurosteroids have been tested for analgesic properties. In fact no

neurosteroid has produced analgesia without producing side effects like sedation or mood alteration. The Department of Anaesthesia from Monash University has reported analgesic properties of such compounds i.e. ORG 20380, ORG 20549 and alphadolone (Goodchild, Guo et al. 2000; Nadeson and Goodchild 2001). It is interesting to note that although alphadolone was used as a counterpart in the Althesin® mixture, it was only ever classified as an anaesthetic "that was present to improve solubility" (Stock 1973). The related compound alphaxalone, whose structure differs only in a 21-hydroxyl group, is ineffective at producing analgesia. The analgesic activity of alphadolone has been attributed to modulation of $GABA_A$ receptors in the spinal cord since antinociception is reversed by administration of the $GABA_A$ antagonist bicuculline (Nadeson and Goodchild 2001).

1.4 Multimodal Analgesia

For centuries opioids have been used for pain-relief and today they remain the mainstay for the treatment of acute and chronic severe pain (Mather 1995). Like many drugs used for severe pain, opioids are associated with adverse dose dependent side effects. With respect to opioids these include nausea and vomiting, constipation, sedation and respiratory depression (Meert 1996). The adverse pharmacological side effects associated with analgesic compounds make them less attractive for pain therapy. Therefore there is a continuous effort to discover new analgesic compounds or improved pain relief therapies. For example, the discoveries of opioid receptors in the brain and spinal cord have aided development of therapeutic targets and clinical dosing strategies for systemic and spinal delivery methods, i.e. epidural and intrathecal administration using chronic infusions with indwelling catheters, slow-release preparations and transdermal delivery systems. However no method is without potential side effects. These methods attempt to lower the dose of opioid administered thus restricting the development of adverse side effects. An important question one must pose is how to decrease the opioid dose whilst maintaining effective analgesia.

Multimodal analgesia is the term used to describe combination analgesic regimens. Such therapies use two or more compounds to produce analgesia. The aim is to use lower doses of each compound to produce fewer dose dependent adverse side effects, whilst achieving comparable or better pain relief than is possible with any single analgesic (Broadbent 2000). This major development in pain management has become more viable as different compounds acting on different pain pathways have been

identified. For instance compounds not normally regarded as analgesics have been shown to fulfill such a role i.e. midazolam and ketamine. Non-opioids such as these have been combined with various clinically used opioids to produce increased analgesic effects while reducing the incidence of adverse side effects. This leads ultimately to improving the quality of pain management (Goldstein 2002).

Multimodal analgesia is already utilised in the clinic. For example, the modern strategy for the management of postoperative pain has adopted such methods. The reason for this is that it is particularly difficult to find a dose that affords a balance between effective pain control and the adverse effects (Pasero 2003). In this postoperative period patients are experiencing pain as well as the adverse effects from general anaesthetics and other drugs received during surgery. Various studies have shown an effective alternative to conventional approaches for managing postoperative pain is with multimodal regimens that include several different drugs, for example opioids plus nonsteroidal anti-inflammatory drugs (NSAIDs). They each work on different mechanisms along the pain pathway to interact positively (Kehlet 1997). Although there is continuous growth in the popularity of such therapies, various experimental works studying multimodal analgesia have often shown pain is not reduced albeit there is a beneficial decrease in the side effect profile (Choi, Kliffer et al. 2003; Keita, Benifla et al. 2003; Serralta, Bueno et al. 2002).

Another rationale for the development of multimodal therapies is that some pain symptoms are non-responsive to existing therapies and require the development of new effective treatment strategies. For example, neuropathic pain is complex and the mechanisms causing it are not well understood, making it difficult to treat clinically.

No drugs exist that restore nerve damage seen in neuropathia, but instead drugs are used to treat the associated severe pain. Opioids are one class of compounds used for neuropathic pain. However past work has suggested neuropathic pain is less responsive to opioids. Opioids may alleviate the pain but at higher doses than normal and this often leads to side effects (Jadad, Carroll et al. 1992; Portenoy, Foley et al. 1990). Some promising animal experiments have confirmed multimodal therapies can improve the effectiveness of analgesia for neuropathic pain. For example, one such study combined the anticonvulsant gabapentin (a structural analogue of GABA) and morphine to show increased effectiveness in an animal model of neuropathic pain (Matthews and Dickenson 2002). Multimodal analgesic therapies require further attention as they may hold the key to effective treatment for difficult to treat neuropathic pain.

Another issue with opioid use is the development of tolerance, where an increased dose is needed to produce a given pharmacological effect. This effect occurs readily in patients that receive continuous opioid treatment for pain conditions such as cancer or neuropathic pain. Once tolerance to the analgesic effect of the opioid is observed in humans, and in order to avoid unnecessary further development of tolerance, administration of other analgesics, perhaps targeting different receptors is advocated (Le Bars, Glowinski et al. 2000). However, it has been suggested that the concept of multimodal analgesia consisting of the simultaneous use of analgesics with a different mode of action can also counteract tolerance development (Freye and Latasch 2003).

Since the description of the gate-control theory, complex interactions between systems have been found within the CNS. The intimate interactions between different systems suggest that these neurotransmitters work in complex ways together, *rather than alone*,

in the modulation of nociception (Millan 1999). Since single analgesics have fixed-dose regimens and increased analgesia requires higher dosing, multimodal analgesia is the foundation of improved pain management where moderate doses of multiple agents will enhance the quality and magnitude of analgesia.

There is an abundance of data on existing analgesics and their mechanisms of action. However each patient with pain will not experience the same drug effect. An acceptable balance is required between therapeutic goal and side effects for each individual patient (Mather 2001). Therefore once a pain is identified, a personal pain management strategy using multimodal therapies can be developed for individual patients. Different strategies can involve a choice for route of administration of varying analgesic drugs depending on the nature and duration of pain, and prompt recognition of side effects to optimize pain management (Broadbent 2000).

In the past opioids have demonstrated positive interactions for antinociceptive effects with several compounds such as serotonin (5HT) (Crisp, Stafinsky et al. 1991), acetylcholine (ACh) (Chen and Pan 2001), NMDA antagonists like ketamine (Nadeson, Tucker et al. 2002), α_2 adrenoceptor agonists (Hylden, Thomas et al. 1991) and compounds acting at $GABA_A$ receptors like midazolam (Rattan, McDonald et al. 1991) and muscimol (Hara, Saito et al. 1999). The neurosteroid analgesic alphadolone also positively modulates $GABA_A$ receptors and thus the studies described in this thesis investigated a potential interaction between alphadolone and several opioids.

1.5 Aims of the Investigations

The studies described in this thesis sought to characterise the antinociceptive properties of alphadolone using acute, inflammatory and neuropathic rat behavioural models. The sedative effects of alphadolone were determined as an indication of adverse side effects. Sedation testing also ensured the exclusion of doses from further nociceptive testing that might have led to false reporting of antinociception. Several clinically used opioids were also tested for antinociceptive and sedative effects. These opioids were combined with alphadolone to test for increased antinociception without sedation.

These studies included an:

1. Investigation comparing the antinociceptive and sedative properties of two structurally related neurosteroids, alphadolone and alphaxalone, when administered via the intraperitoneal route.

2. Investigation testing whether alphadolone or alphaxalone could potentiate the antinociceptive effects of opioids fentanyl, morphine and oxycodone without potentiating the sedative side effects.

3. Investigation testing whether spinal cord $GABA_A$ receptors are involved in the enhancement of opioid antinociception by alphadolone.

4. Investigation using the carrageenan-induced inflammation model to determine whether alphadolone alone could reverse hyperalgesia or increase the effects of opioids fentanyl, morphine and oxycodone.

5. Investigation on diabetic neuropathic animals to determine whether alphadolone alone demonstrated any antinociceptive effects or increased the antinociceptive effects of opioids fentanyl, morphine and oxycodone.

6. Investigation using morphine tolerant animals to determine whether alphadolone could either prevent the development of morphine tolerance or restore the antinociceptive effects of morphine in tolerant animals.

Chapter 2 Methods: Screening of Novel Analgesic Compounds

2.1 Introduction

The focus of many human studies is based on actual pain resulting from real injury or disease. Experimental studies of severe chronic pains have been slow because they occur rarely in isolation; mixed and varied presentations are common. Therefore large series of patients are needed for clinical trials to have sufficient power for useful results (Richards 1992). The behavioural responses to a particular painful injury can vary markedly between individuals (McHugh and McHugh 2000). Even in healthy individuals pain thresholds and tolerance levels may vary (Richards 1992). A pain trial by Koopmans *et al.* on a large group of patients complaining of chronic lower back pain proved inconclusive due to extreme patient variation (Koopmans, Meeuwesen et al. 1996). Although low numbers for clinical studies often prove inconclusive they often occur because of highly stringent inclusion criteria and narrowly defined protocols. We therefore rely on animal models to perform screening of new potential analgesics and also for analysis of mechanisms of action. In addition, ethical approval is often difficult to achieve for pain experiments in humans. It is challenging to apply physiologically meaningful painful stimuli that do not harm the volunteer. Clinical testing of early phase compounds raises many moral questions and is often the subject of lengthy debates. However, it is a vital step for further scientific understanding and treatment of pain conditions.

Although the study of pain in animals also raises ethical and technical problems, there are good scientific and moral reasons for its continuation. *In vivo* animal studies have become an important tool in understanding pain in humans and for the development of new therapeutic strategies (Besson 1997). Animal models have been used for decades

in scientific research and their establishment over such a time ensures their protocols are reproducible not only within the same laboratory but also between different laboratories. The development of new drugs is extremely costly and the community is not prepared to fund drug development unless there is reliable evidence from animal studies of therapeutic potential (In Vivo Pharmacology Training Group 2002). Consequently, for preliminary scientific research for drug discovery, animal *in vivo* testing plays an integral part.

Animal studies are seen as a necessary step in pain research but, as with all biomedical research involving animals, they too have their limitations. The most significant limitation is whether animal models accurately mimic human disease (Hogan 2002). Pain is described subjectively in human terms as an "unpleasant sensory and emotional experience associated with actual or potential tissue damage" (Mersky 1986). Investigators can only speculate on the experience of the animal and must use various nociceptive behavioural indicators to deduce pain. For this reason there exists a broad range of measurement techniques for injury and nociception. Several acute and chronic animal pain models and protocols are described in this chapter.

Studies of nociception in conscious animals most often involve monitoring motor responses to a particular stimulus. Drugs are generally considered analgesic if there is a change in motor response following noxious stimuli. This response is commonly shown as an increase in response time or latency period. Anaesthetics and sedatives have the capability to impair motor response regardless of any effect on nociception. This may lead to false representation of antinociceptive activity (Cartmell, Gelgor et al. 1991; Plummer, Cmielewski et al. 1991). An investigator must be wary of such invalid

indicators of antinociception. It must therefore be determined for every potential therapeutic analgesic whether it changes motor responses by interfering with nociceptive pathways or by causing sedation and/or direct interference with motor coordination. Ethical implications also exist when producing motor malfunction or impairment. Such changes in motor function may render an experimental animal unable to escape noxious stimuli or indicate stress to its observer. Usually it is exactly these aforementioned physiological responses that classify the end-point of behavioural animal testing for pain. Therefore an animal may be harmed if these signs are not present to signal the end of an experiment.

2.1.1 Aim

For all experiments throughout this thesis, drugs were considered analgesic by characterising and separating antinociceptive effects from sedative effects. Only non-sedating doses were tested for antinociceptive effects. Therefore these experiments established doses below the sedative threshold in order to discover a compound's putative pain-relieving properties. The work outlined in this chapter sets the stage for a new approach for analgesic screening, where all compounds should undergo sedative testing, so these doses can henceforth be excluded from subsequent testing in nociceptive paradigms. The purpose of this chapter is to describe several rat models that were used to define the antinociceptive and sedative properties of drugs given individually and in combinations.

2.2 General Protocol

2.2.1 Ethical Guideline

Ethical guidelines were followed for the investigation of experimental pain in conscious animals (Zimmermann 1983). All work was carried out with the permission from the Monash University Standing Committee on Ethics in Animal Experimentation (MMC 2000/06, 2001/15). In order to remain in agreement with these outlined ethical guidelines, the minimum number of replicate experiments for scientific validity was performed.

2.2.2 Rat Care

For all behavioural testing male Wistar rats weighing 180-200g were used. The rats were housed 4 per cage in the Monash Medical Centre Animal House prior to experimentation. Rooms were kept at 21 ± 1°C with a 12-hour light/dark cycle (lights on at 8:00am). Food and water were provided *ad libitum*.

2.2.3 Blind Experiments

There are often conflicting reports for the findings of different investigations in scientific research (Chalmers 2001). One cause of variation is simply chance. Statistical mathematics and various calculations assess the likelihood of this explanation. Another

cause of variation is different kinds of bias and to what extent they have been avoided. Researchers should attempt to take all necessary precautions to eliminate bias. Therapeutic studies can be controlled in terms of observer bias in assessing the outcomes. The experiments performed in this thesis attempted to remove observer bias by performing experiments in a blinded manner i.e. the observer was not aware of the nature of the drug treatment whilst performing the measurements. At the start of each experimental day, drug doses were made up into syringes and labelled with a code. The meaning of the code was unknown to the investigator. At the end of each experimental day, the code was broken and the result ascribed to the treatment given.

2.2.4 Statistics Program

All statistical calculations were conducted using GraphPad Instat (Version 3.06, GraphPad Software, Inc. 2003, San Diego, USA). A value of $p \leq 0.05$ was considered statistically significant.

2.3 Acute Antinociception

2.3.1 Tail Flick Latency

Tail flick latency (TFL) is the most widely used animal test for the assessment of pain. It is considered a predictive model of acute thermal pain in humans (Archer and Harris 1965). D'Amour and co-workers first outlined this test in the early 1940's. Thermal stimulation is applied to the tail of an animal and the reaction time for the tail movement from the heat source is recorded (D'Amour and Smith 1941). This time was often referred to as 'tail flick latency'. The pain intensity induced by the heat stimuli is measured by the delay of tail withdrawal. A lengthening of the reaction time after drug administration is interpreted as an analgesic action.

For this nociceptive test the animal was placed in a custom fit Perspex restrainer and positioned on top of a commercial tail flick unit (Ugo Basile, Italy). It is known that reaction times may vary depending on which point of the tail is stimulated (Kawakita and Funakoshi 1987). Therefore a black marker was painted on the tail approximately 3cm from the tip to allow accurate placement on the heat source so comparable repeated tests could be performed. The black paint on this region also helped limit reflection and increase heat absorption from the light source (Le Bars, Gozariu et al. 2001). An infrared laser beam fixed at an intensity that would stimulate skin nociceptors was focused onto the marked part of the tail. A timer was automatically triggered simultaneously with the infrared beam in order to give accurate readings. The

rat withdrawing its tail from the heat source would break the infrared beam and automatically stop the timer.

(a) Interval Time Between Stimuli

Past research has demonstrated skin temperature returns to baseline slowly after heat stimulation (Le Bars, Gozariu et al. 2001). Previous work has shown the tail flick test is prone to habituation, which is observed as a reduction in the response to repetitive stimulation (Groves and Thompson 1970). Furthermore, this habituation increases with shortening of the intervals between stimuli (Carstens and Wilson 1993). These confounding issues were taken into consideration for the determination of a suitable time interval between consecutive readings. Other experiments most commonly test rats at 5-minute intervals (Abe, Kikuta et al. 2003; Lograsso, Nadeson et al. 2002; Zhang, Shu et al. 2001). For the experiments reported in this thesis, TFL readings were taken every 5 minutes. TFL readings using this time interval were shown to be consistently stable in normal rats (refer to Appendix A, page 264).

(b) Cut Off Time

If the rat did not remove its tail within 10 seconds the instrument would be shut off in order to prevent tissue damage. This cut off time of 10 seconds has been used by other workers (Abe, Kikuta et al. 2003; Gambhir, Mediratta et al. 2002). In experiments where maximum tail flick time was achieved (10 seconds) no damage was detected in any rat (n=45). In all of these rats, baseline TFL values had returned to normal

(previous levels) by the following day when further nociceptive testing was performed (refer to Appendix A, page 264).

(c) Nociceptive Measurement Requirements

One advantage of this behavioural method is the small inter-animal variability in reaction time measurements under a given set of controlled conditions (Le Bars, Gozariu et al. 2001). Baseline readings are most commonly between 2 and 4 seconds. Since 10 seconds is the cut-off period for an individual test, a small baseline of approximately 3 seconds allows ample opportunity for the latency time to increase for the interpretation of an analgesic action. In these experiments altering the intensity of the infrared heat source can control the reaction time. An intensity of 55 on the Ugo Basile TFL machine maintained the ideal baseline of approximately 3 seconds (refer to Appendix A, page 264). Therefore this intensity setting was selected and remained unchanged for all experiments.

A baseline consisting of three consecutive readings taken 5 minutes apart was considered stable if the difference in TFL values was 1 second or less. If rats showed a baseline that did not meet the predetermined guidelines they were immediately removed from the restrainer and left for 24 hours. Rats from previous experiments with tails that appeared damaged by visual inspection or with raised TFL thresholds (>4.0 seconds) were not used for further testing.

(d) Calculations

Once the rats were restrained, TFL readings were taken every 5 minutes until three stable baseline readings were achieved. After this point a test drug was administered and TFL readings were continually assessed at 5-minute intervals for the next 25 minutes. In experiments where two drugs were being tested in combination, the latter drug was administered 5 minutes after the first drug. The two drugs administered in combination were alphadolone and one of fentanyl, morphine or oxycodone. The time taken for the onset of antinociception caused by alphadolone was frequently slower than for each of the opioids. Therefore alphadolone was administered 5 minutes prior to the opioids in order to achieve simultaneous maximal antinociceptive effects. The time response curves for the three opioids are shown in Appendix B (page 266). The time response curves for alphadolone are shown in Chapter 3 (page 107). The protocol is outlined in Figure 2.1 on page 45. The initial three readings were averaged (*mean TFL pre-drug injection*), as well as three readings after drug administration (*mean TFL post-drug injection*), and were used to calculate the percentage of maximum possible effect (%MPE). TFL changes were standardised by calculating the %MPE as follows:

$$\%\ MPE = \frac{(mean\ TFL\ \ post - drug\ injection)\ -\ (mean\ TFL\ pre - drug\ injection)}{cut\ off\ \ time\ -\ (mean\ TFL\ pre - drug\ injection)} \ x\ 100$$

2.3.2 Electrical Current Threshold

The electrical current threshold (ECT) and TFL tests are both useful for analgesic testing in animals (Petersen-Felix and Arendt-Nielsen 2002). Some analgesics have shown different responses to these two nociceptive tests. For example, experiments on the analgesic compound midazolam demonstrated potent analgesic activity in response to electrical stimulation while the TFL test showed no such activity (Castilho, Avanzi et al. 1999; Goodchild and Serrao 1987). The use of multiple nociceptive tests rather than a single testing paradigm helps determine a comprehensive pharmacological profile for pain-relieving compounds.

Noxious heat activates a restricted group of nociceptors while electrical stimulation activates all primary afferent fibres (Chapman, Casey et al. 1985). Therefore cutaneous electrical stimulation used in conjunction with heat stimuli may be useful in differentiating innocuous neural activity from noxious effects respectively. The use of both these tests is also advantageous because they can be completed simultaneously. It is also much more difficult to produce tissue damage using electrical stimulation at intensities that examine analgesia, unlike the TFL test (Dubner 1983).

One application of ECT testing in this thesis was to determine the role of specific spinal cord systems that might be mediating the interactions of different drug combinations (Chapter 4, page 126). By testing both neck and tail ECT, intrathecal drugs can be assessed for correct insertion and maintenance within the spinal cord. Differences in neck and tail thresholds would prove an isolated spinal effect of the intrathecally-

administered drug. This use of the ECT test is described in detail in a following section (see Intrathecal Catheter Insertion, page 46).

Tail ECT testing used a pair of exposed wire electrical stimulating electrodes coated in electrode gel (Pate Reux, Hewlet Packard). They were each placed on the tail 5cm and 7cm from the base of the tail. Neck ECT testing used two needle electrodes placed in the skin at the base of the neck 1cm apart. A single half-second train of one millisecond pulses with a frequency of 50 Hz was delivered individually, in turn, to both sets of electrodes by a constant current electrical stimulator.

The electrical current threshold was defined as the minimum current necessary to cause the rat to vocalise or make a strong aversive movement. It was determined by an 'up-down' method for each skin site. The protocol for this method initially involves increasing the current in large increments until it elicits a strong aversive movement or vocalisation. When an approximate intensity range is determined the current is then increased and decreased with progressively smaller changes until the threshold is established. This method was adapted from the mathematical equation that determines a minimum value of an unknown variable (Vetterling, Teukolsky et al. 1993).

(a) Nociceptive Measurement Requirements

Only rats that achieved baseline ECT values between 0.1 and 0.5mA underwent further experimentation. A baseline, consisting of three consecutive readings taken 5 minutes apart, was considered stable if the difference in values was 0.05mA or less. If rats

showed a baseline that did not meet the predetermined guidelines they were immediately removed from the restrainer and left for 24 hours.

For experiments using ECT testing, rats were used repeatedly on consecutive days, for a maximum of 4 days. If rats demonstrated baselines that had changed significantly (a maximum of 0.3mA) from previous days testing, they were removed from further experiments because change of this magnitude may signify damage caused by the electrical stimulation on TFL testing or residual drug effects from the previous days' experiments.

(b) Calculations

Like the TFL test, ECT readings were taken every 5 minutes until three consecutive stable baseline readings were achieved. After this point a test drug was administered and ECT readings were assessed at 5-minute intervals thereafter for the next 25 minutes. In experiments where two drugs were being tested in combination, the second drug was administered 5 minutes after the first drug. The two drugs administered in combination were alphadolone and one of fentanyl, morphine or oxycodone. The time of onset for the production of antinociception by alphadolone was slower than for each of the opioids. Therefore alphadolone was administered 5 minutes prior to the opioids in order to achieve simultaneous maximal antinociceptive effects. The time response curves for the three opioids are shown in Appendix B (page 268). The time response curves for alphadolone are shown in Chapter 3 (page 107). The outline of the general protocol can be seen in Figure 2.1 on page 45. The initial three readings were averaged (*mean ECT before drug injection*) as well as three readings after drug administration

(*mean ECT after drug injection*). These changes were standardised as a ratio of control (r):

$$r = \frac{mean\ of\ three\ ECT\ readings\ after\ drug\ injection}{mean\ of\ three\ ECT\ readings\ before\ drug\ injection}$$

The equipment used for the ECT and TFL tests allowed them to be completed simultaneously. The addition of the ECT test to the nociceptive test paradigm did not affect the TFL response. When drugs were administered intrathecally ECT was additionally measured in the neck. When all three tests were performed the order of tests was TFL, tail ECT and then neck ECT, at each 5-minute time point.

2.3.3 Paw Pressure

The further addition of the paw pressure (PP) test to the TFL and ECT tests is useful in adding additional information to the profile of a novel analgesic compound. The PP test assesses noxious mechanical stimuli by pressure applied to the rat's hind paw. Randall and Selitto first described the paw pressure test: the test-paw is placed between a flat surface and a blunt pointer that is used to apply a variable force to the paw (Randall and Selitto 1957). The instrument (Analgesy-Meter; Ugo Basile) used for experiments described in this thesis exerts a force that increases at a constant rate by moving a weight attached to a pointer along a linear scale used to indicate the force applied in grams. When the nociceptive threshold is reached the rat can easily withdraw its paw from under the pointer. The operator depresses a pedal switch to start the mechanism.

When the rat withdraws its paw or struggles, the operator releases the pedal and reads the nociceptive thresholds in grams off the scale.

(a) Nociceptive Measurement Requirements

Rats that had paw pressure values between 40 and 90 grams were included in the experiment. A baseline consisting of three consecutive readings taken at 5-minute intervals was considered stable if the difference in values was 10 grams or less. If rats had a baseline that did not meet the predetermined guidelines they were left to rest for 24 hours.

(b) Calculations

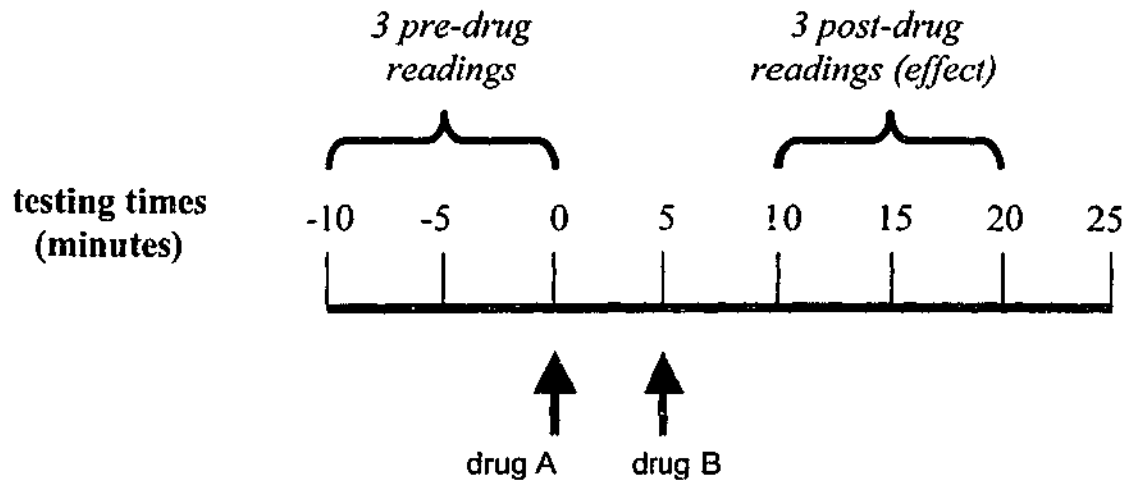
Paw pressure readings were taken at 5-minute intervals like the TFL and ECT test, to ensure all nociceptive tests were directly comparable. This was continued until three stable baseline readings were achieved. After this point a test drug was administered and readings were assessed at 5-minute intervals thereafter for the next 25 minutes. In experiments where two drugs were being tested in combination, the second drug was administered 5 minutes after the first drug. The two drugs administered in combination were alphadolone and one of fentanyl, morphine or oxycodone. The time response for the production of antinociception by alphadolone was slower than for each of the opioids. Therefore alphadolone was administered 5 minutes prior to the opioids in order to achieve simultaneous maximul antinociceptive effects. The time response curves for the three opioids are shown in Appendix B (page 270). The time response curves for alphadolone are shown in Chapter 3 (page 107). This protocol is outlined in Figure 2.1

on page 45. The initial three readings were averaged (*mean paw pressure before drug injection*) as well as three readings after drug administration (*mean paw pressure after drug injection*). These changes were standardised as a ratio of control (r):

$$r = \frac{mean\ of\ three\ paw\ pressure\ readings\ after\ drug\ injection}{mean\ of\ three\ paw\ pressure\ readings\ before\ drug\ injection}$$

The protocol shown in Figure 2.1 below was used for all three nociceptive tests: ECT, TFL and PP. Nociceptive thresholds were measured at 5-minute intervals until three stable readings were achieved. These three readings were averaged and determined as the baseline. An individual drug or drug combination was administered and thresholds were measured at 5-minute intervals for the next 25 minutes. Three readings were averaged and determined as the "effect" for the individual drug or drug combination.

Figure 2.1 Summary of the Nociceptive Paradigm



2.4 Intrathecal Catheter Insertion

In the late 1970's Wang was the first to demonstrate that drugs could produce analgesia when injected directly onto the spinal cord (Wang 1978). The localisation of drugs within the spinal cord has broadened the knowledge and direction of pain research by helping characterise receptors within the spinal cord responsible for signaling of nociception. The surgical procedure enabling intrathecal (IT) drug administration was based on work previously published (Goodchild and Serrao 1987).

(a) Catheter Implantation

A 5cm plastic tube was hand made into a catheter for direct insertion into the intrathecal space. The Portex catheter had an internal diameter of 0.28mm and an external diameter of 0.61mm. The tubing was heated to produce four swellings (shown as solid red dots in the catheter in Figure 2.2 on page 50. The two proximal swellings were used to secure the catheter to muscle near the spinal cord cavity and the exit wound. One swelling close to the distal (external) end was used for visual volume reference to facilitate accurate delivery of drug dosage. The most distal swelling was cut open to allow a tight connection with a blunt needle of a microsyringe for intrathecal drug injection. A diagram of the catheter is shown in Figure 2.2 on page 50.

(b) Anaesthesia

The rats were anaesthetised with 5% halothane in oxygen-enriched air. A Boyles Minor anaesthetic machine delivered the gas mixture to a small plastic box that housed the rat.

The anaesthetised rat was removed from the compartment and placed in a prone position on a circulating hot water blanket. During the surgical procedure, anaesthesia was maintained by an inhalation facemask that supplied 1.5-2.0% halothane in oxygen-enriched air.

(c) Surgery Technique

A 3cm long incision was made in the thoraco-lumbar region (T_{11}-L_2). Skin and muscle were reflected by blunt dissection so the left lamina of L_1 or L_2 was exposed. A dissecting microscope was used for visualisation of drilling a hole in the lamina with a ball-shaped steel drill bit (Ash RND PC 8 HP). Once the vessels overlying the dura were seen through the thinned bone, drilling was stopped and fine forceps were used to pierce the remaining bone flap and to remove excess bone fragments to expose the dura. The dura was incised with the point of a 26-gauge needle to allow for 10mm of the Portex catheter to be introduced in the subarachnoid space of the rat. The catheter tip was inserted directed caudally so it lay adjacent to caudal segments of the spinal cord as previously described (Serrao, Stubbs et al. 1989). As an indication of correct placement, cerebrospinal fluid was observed flowing out along the catheter once the tip was inserted through the dura. Bone cement (Hottinger Baldwin Messtechnik X60) was used to seal the laminectomy catheter, which ensured a secure seal for the catheter tip insertion within the hole in the lamina. The catheter was tunnelled under the skin to an exit wound at the base of the neck. The wounds were closed with interrupted 5-0 nylon (Ethilon; Ethicon) sutures. The catheter was attached to the muscle with 5-0 nylon sutures at the exit wound at the base of the neck before the skin incision was closed with interrupted 2-0 silk sutures (Mersilk; Ethicon). Following surgery, 30mg of

penicillin (benzypenicllin sodium, CSL, Parkville, AUS) and 4mg of gentamicin (Pharmacia, Auckland, NZ) were diluted in saline and administered intraperitoneally in a 0.4ml injection. At least 24 hours was allowed for recovery after surgery before nociceptive testing was conducted.

(d) Surgical Controls

After recovery from general anaesthesia rats were observed for normal behaviour and movement. If there were any signs of neurological damage, such as paralysis, rats were immediately killed by an overdose of anaesthetic (MMCB 2001/01).

An intrathecal injection of 5µl of 2% lignocaine (Xylocaine, Astra, Nth Ryde, AUS) was administered via the newly inserted catheter to verify its correct placement. The desired position of the catheter was confirmed if the tail and hind limbs of the rat became paralysed within 30 seconds of the injection of lignocaine. All rats that failed the lignocaine test were excluded from the study. After the lignocaine test the catheter was flushed with 20µl of saline. The lignocaine test was also carried out after each experiment to ensure continued correct placement of the catheter. If the rats produced a negative result, the results obtained in that session for that rat were excluded. This ensured that all the results collated for the calculation of antinociceptive effects were from experiments where the drug was injected intrathecally and not epidurally or into paraspinal tissues.

Thresholds for the ECT and TFL nociceptive tests were compared before and after surgery to assess whether there were subtle neurological changes caused by the surgery.

Three readings were averaged for each rat and all averages were compiled as the mean ± SEM. The means for before and after surgery were compared using a *t*-test. These values showed no significant changes in antinociceptive effects assessed by TFL and ECT. This suggests that the successful completion of the surgery did not cause neurological damage that would affect ECT and TFL testing. These results are shown in Figure 2.3 on page 52.

(e) Measurements for IT Antagonist Administration

ECT measurements in the neck and tail were used in conjunction when the experiment included intrathecal injection of drugs via catheters implanted in the lumbar intrathecal space. If a drug injected down the intrathecal catheter is delivered into the intrathecal space *and* its actions are confined to the most caudal segments of the lumbosacral spinal cord, then one would expect that ECT measurements in the tail would change after the drug injection but no such change would occur in the neck. This principle is used in this thesis in the following way: if an antinociceptive drug or drug combination is injected intraperitoneally then tail ECT and neck ECT values rise above baseline levels. If, however, the tail ECT values return to baseline after the injection of an *antagonist* down an intrathecal catheter without a concomitant change in neck ECT values, then it can be deduced that the antinociceptive effect caused by the intraperitoneal drug or drug combination is due to actions at the level of the spinal cord and not the brain. Any data that showed a significant decrease in neck ECT measurements, therefore representing rostral spread of the intrathecally-administered drug, were excluded before any statistical analysis was conducted.

The selective $GABA_A$ receptor antagonist bicuculline was used for intrathecal experiments to evaluate the involvement of spinal cord $GABA_A$ receptors. As a control, IT bicuculline (50pmol) was tested for effects when given alone. IT bicuculline (50pmol) caused no significant changes in TFL, tail ECT or neck ECT responses when given alone. These results are shown in Figure 2.4 on page 54.

Figure 2.2 A Diagram of the Intrathecal Catheter Surgically Implanted in Rats

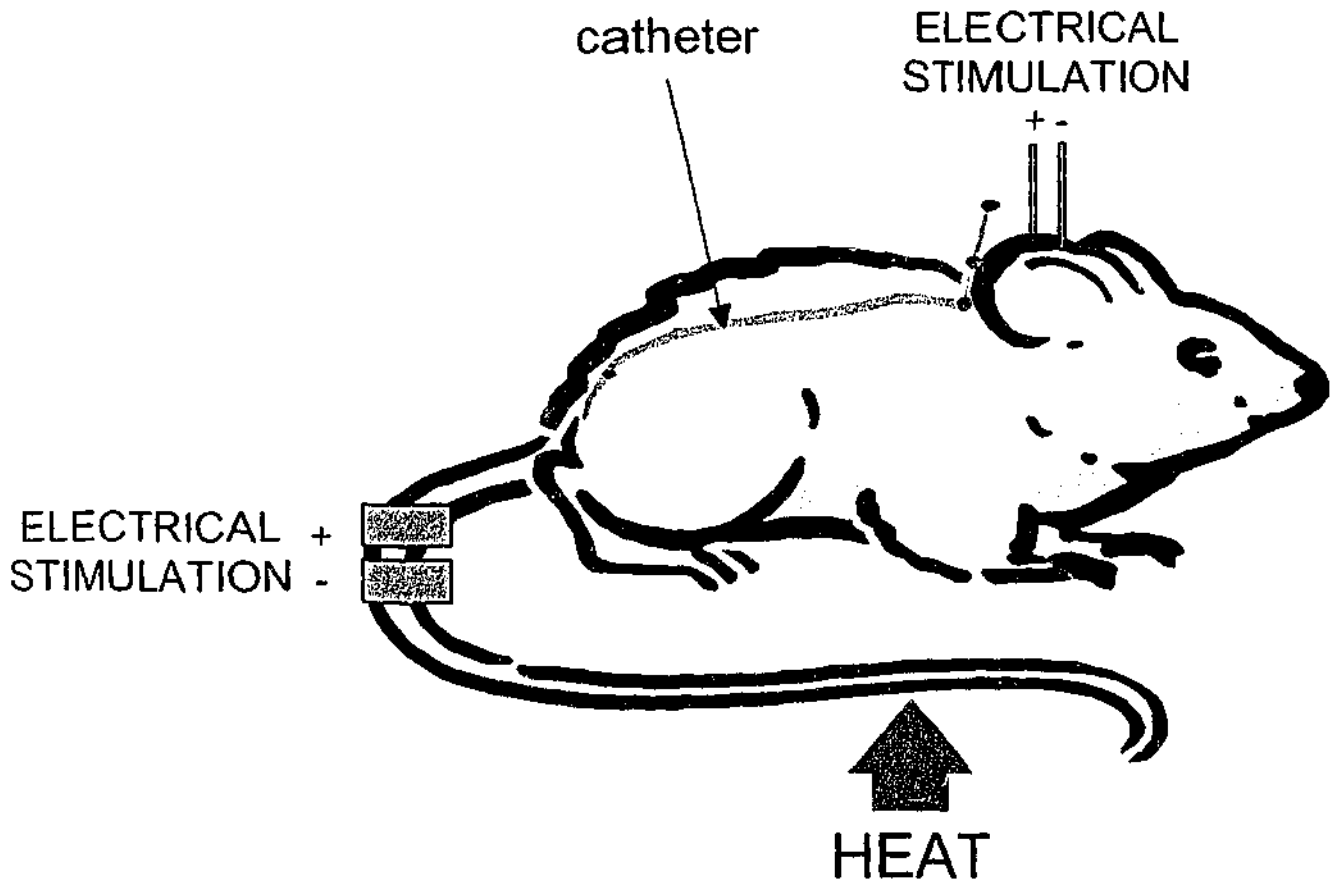


Figure 2.3

This graph shows the nociceptive thresholds for both neck and tail ECT and TFL before and after intrathecal catheter insertion. For each rat three readings were averaged and thresholds were compiled as the means ± SEM. Nociceptive thresholds showed no significant difference between values taken before and after surgery (*t*-test). Each histogram represents the mean ± SEM for 22 rats.

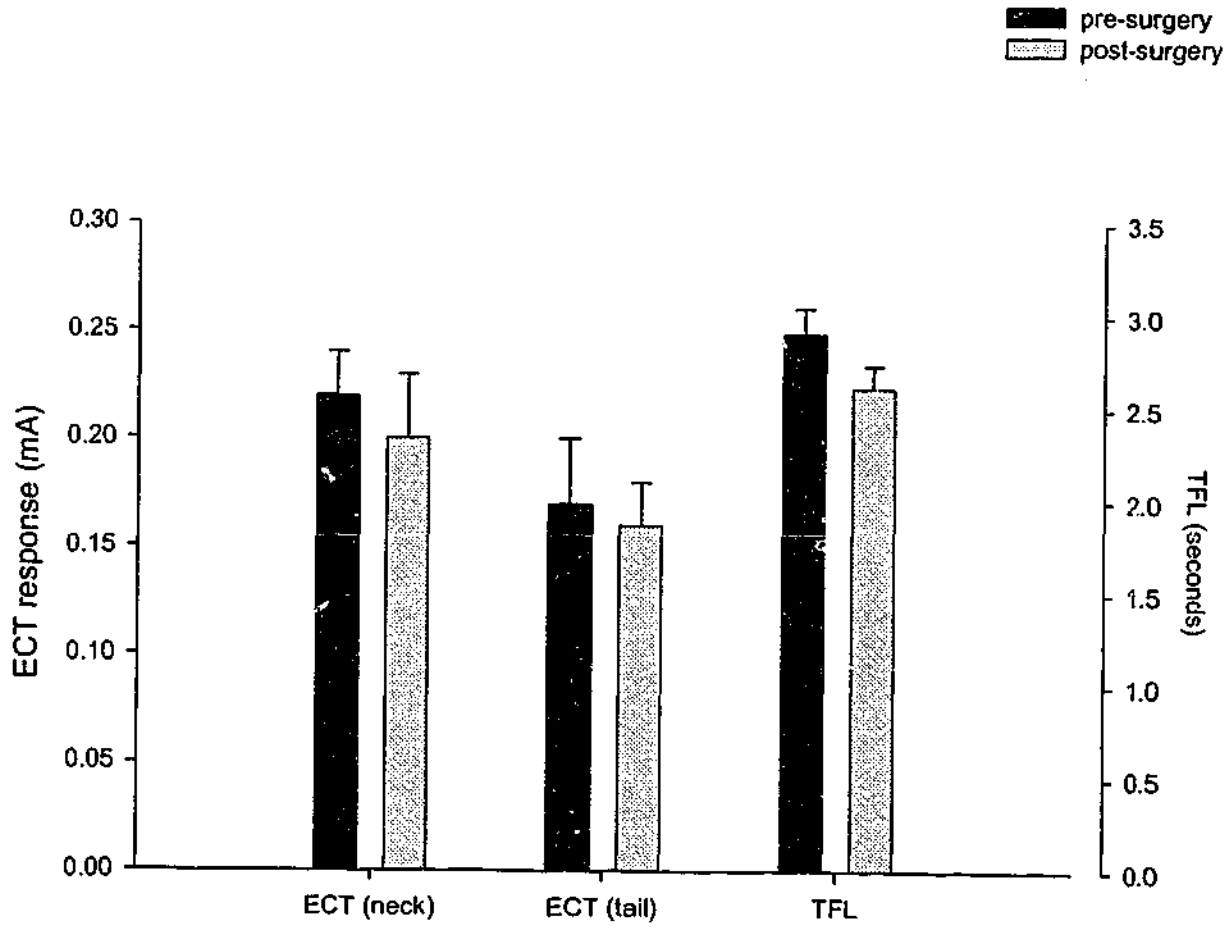


Figure 2.3 The Effects of Intrathecal Catheter Insertion on ECT and TFL

Figure 2.4

This graph shows the time response effects of IT bicuculline (50pmol) administration in rats with surgically implanted catheters. IT bicuculline caused no significant changes in ECT or TFL responses (ANOVA). Each point represents the mean ± SEM for 4 rats.

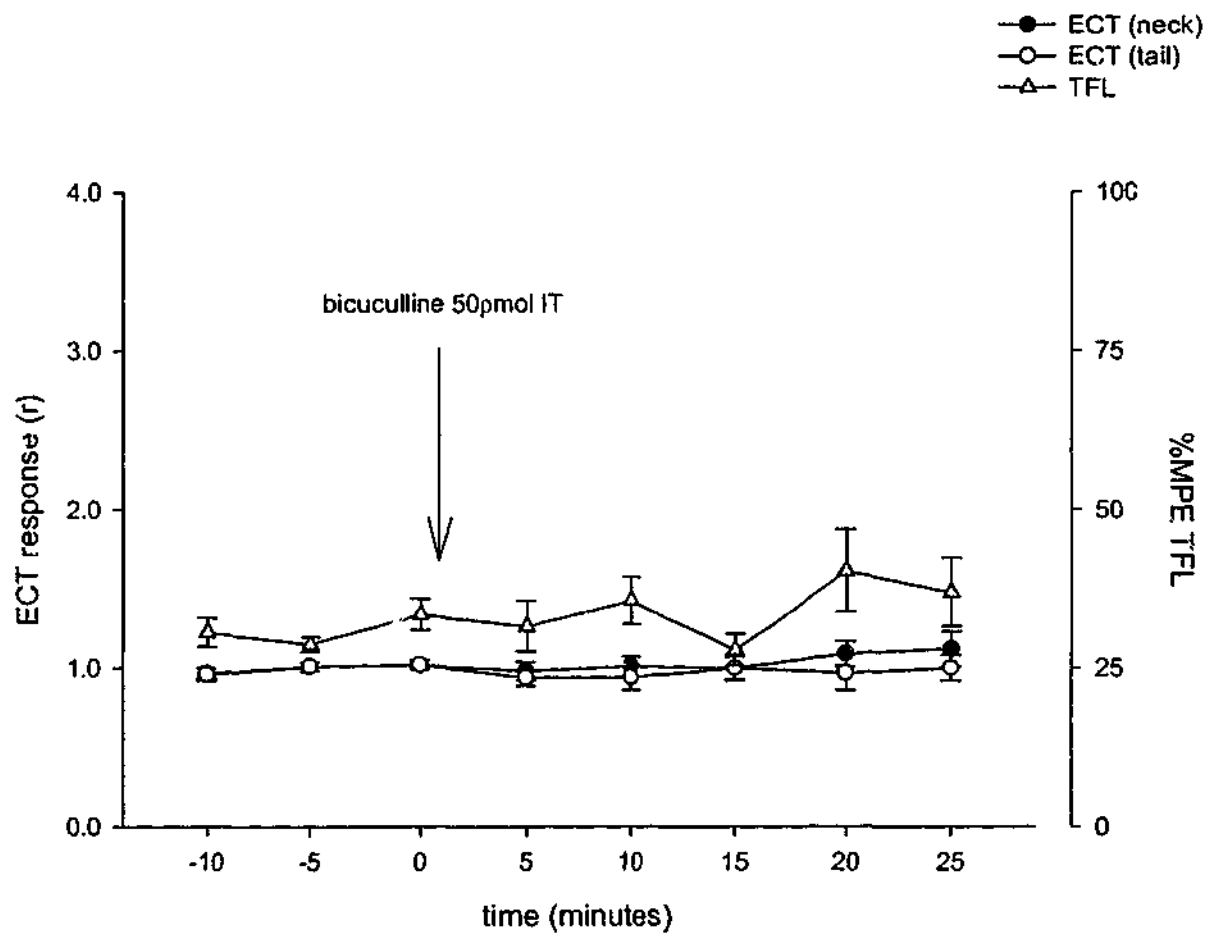


Figure 2.4 The Effects of IT Bicuculline on ECT and TFL

2.5 Induced Pain States

The acute pain tests described previously are reliable ways of mimicking acute pain episodes seen in humans (Archer and Harris 1965). However, these tests have little relevance to long-term painful conditions for which patients typically seek treatment. Comparable conditions of prolonged pain states such as inflammation and neuropathic pain have been modeled in rats.

2.5.1 Carrageenan-Induced Inflammation

The carrageenan-induced model for inflammatory pain has been developed to study the effects of drugs in a hyperalgesic pain state. The substance carrageenan when injected into live tissue induces an inflammatory response in which substance P, bradykinin, serotonin (5-HT) and prostaglandins are released. These are ultimately responsible for the hyperalgesia seen in this model (Ferreira, Zanin et al. 1978; Garry and Hargreaves 1992; Hargreaves, Dubner et al. 1988a; Ren, Williams et al. 1992). Studies in rats have shown that almost immediately after intraplantar injection of carrageenan, oedema develops, shortly followed by hyperalgesia after 3-4 hours, which returns to normal 24 hours later (Hedo, Laird et al. 1999; Kowaluk, Mikusa et al. 2000; Rygh, Svendsen et al. 2001).

This series of experiments measures paw withdrawal thresholds using the Analgesy-Meter to perform screening of analgesic drugs on the inflamed rat paw according to the Randall-Sellito test (Randall and Selitto 1957).

(a) Carrageenan Injection

Experimental inflammation was induced by subcutaneous injection of carrageenan (Sigma-Aldrich Pty. Ltd. Australia) into the hind paw. The rats were anaesthetised with halothane in oxygen-enriched air. The dose given was 100µl of a 2% carrageenan solution diluted in saline as described by other workers (Clayton, Marshall et al. 2002; Hurley, Chatterjea et al. 2002; Vachon and Moreau 2002).

(b) Development of Inflammation

An experiment was performed to measure the time required for carrageenan to produce inflammation and hyperalgesia. A group of rats (n=10) received subcutaneous injections of carrageenan and their paw pressure withdrawal thresholds were measured every 30 minutes. In parallel, another group of rats (n=7) received subcutaneous injections of vehicle control (saline) into their hind paw and paw pressure (PP) thresholds were measured every 30 minutes.

A comparison of the carrageenan treated group and the saline treated group was performed using a two-way ANOVA with a Bonferroni post-hoc test for multiple comparisons. The saline group showed no significant difference in paw withdrawal thresholds when compared to the initial baseline value (pre-saline injection). A comparison of the saline and carrageenan groups showed a significant difference in effects ($p<0.0001$, ANOVA). Within 60 minutes the carrageenan group had decreased PP thresholds that were significantly different from the saline group. The carrageenan readings remained consistently decreased for the rest of the test period. Based on

previous work (Al Arfaj, Mustafa et al. 2003; Gupta, Mazumdar et al. 2003; Zhang, Ji et al. 2002), a period of 180 minutes was selected to allow for development of inflammation in subsequent experiments before nociceptive testing began. The baseline values before carrageenan administration (time zero) were compared with PP values at 180 minutes. The carrageenan group had a decrease in baseline PP thresholds from 80.3g ± 5.5 (mean ± SEM) to 22.9g ± 3.9. This decrease is highly significant ($p<0.0001$, *t*-test) and it represents a decrease in PP values to approximately one third of the original baseline value. The results are shown in Figure 2.5 page 60.

(c) Nociceptive Measurement Requirements

Three stable consecutive readings taken at 5-minute intervals were averaged and determined as the initial baseline. Rats that showed baseline PP values between 40 and 90 grams were injected with carrageenan. The baseline was again determined after the carrageenan injection. This inflammatory baseline was also averaged for three stable readings. A stable baseline was considered as three consecutive PP readings taken at 5-minute intervals that varied no more than a maximum change of 10 grams. Thresholds that had decreased to approximately one third from the original baseline values were used for further experimentation. If the process of determining a stable inflammatory baseline in any one rat was delayed over 1 hour that experiment was terminated. Any rat that displayed bruising from the subcutaneous injection was eliminated from the study.

(d) Calculations

Individual rats varied in the absolute value for paw withdrawal thresholds measured in grams. Thus a customised formula was used to compile and standardise all rat data. The formula was designed to calculate the percentage reversal of hyperalgesia (%RH). Initial paw pressure readings were taken prior to carrageenan injection. Readings were taken until three stable baseline readings were achieved. These values were averaged and used as a baseline for normal rats (*initial baseline*). Carrageenan was injected and after 180 minutes a new baseline was established. PP values were taken until three stable readings were achieved. These values were averaged and calculated as the *inflammatory baseline*. A test drug was injected and readings were continued for the next 25 minutes. Three readings were averaged from these data (*test drug effect*) and used in the final formula. Figure 2.6 on page 61 shows the nociceptive test paradigm for the carrageenan pain model.

The PP values were transformed into the percentage of reversal of hyperalgesia, where 100% effect meant the test drug had returned PP values to initial baseline readings:

$$\%RH = \frac{(mean\ of\ test\ drug\ effect) - (mean\ of\ inflammatory\ baseline)}{(mean\ of\ initial\ baseline) - (mean\ of\ inflammatory\ baseline)} \times 100$$

Figure 2.5

Paw withdrawal thresholds are plotted against time after intraplantar carrageenan injection. These results are compared with a control group injected with saline. Paw pressure readings were taken at 30-minute intervals. The carrageenan treated group showed a significant decrease in paw pressure values compared with the saline group ($p<0.0001$, ANOVA). The PP values in the carrageenan treated group were stable with no drift from 60-270 minutes after the carrageenan injection. The saline group showed no significant change from baseline thresholds. This experiment was terminated after 270 minutes. Each point represents the mean of 7-10 rats ± SEM.

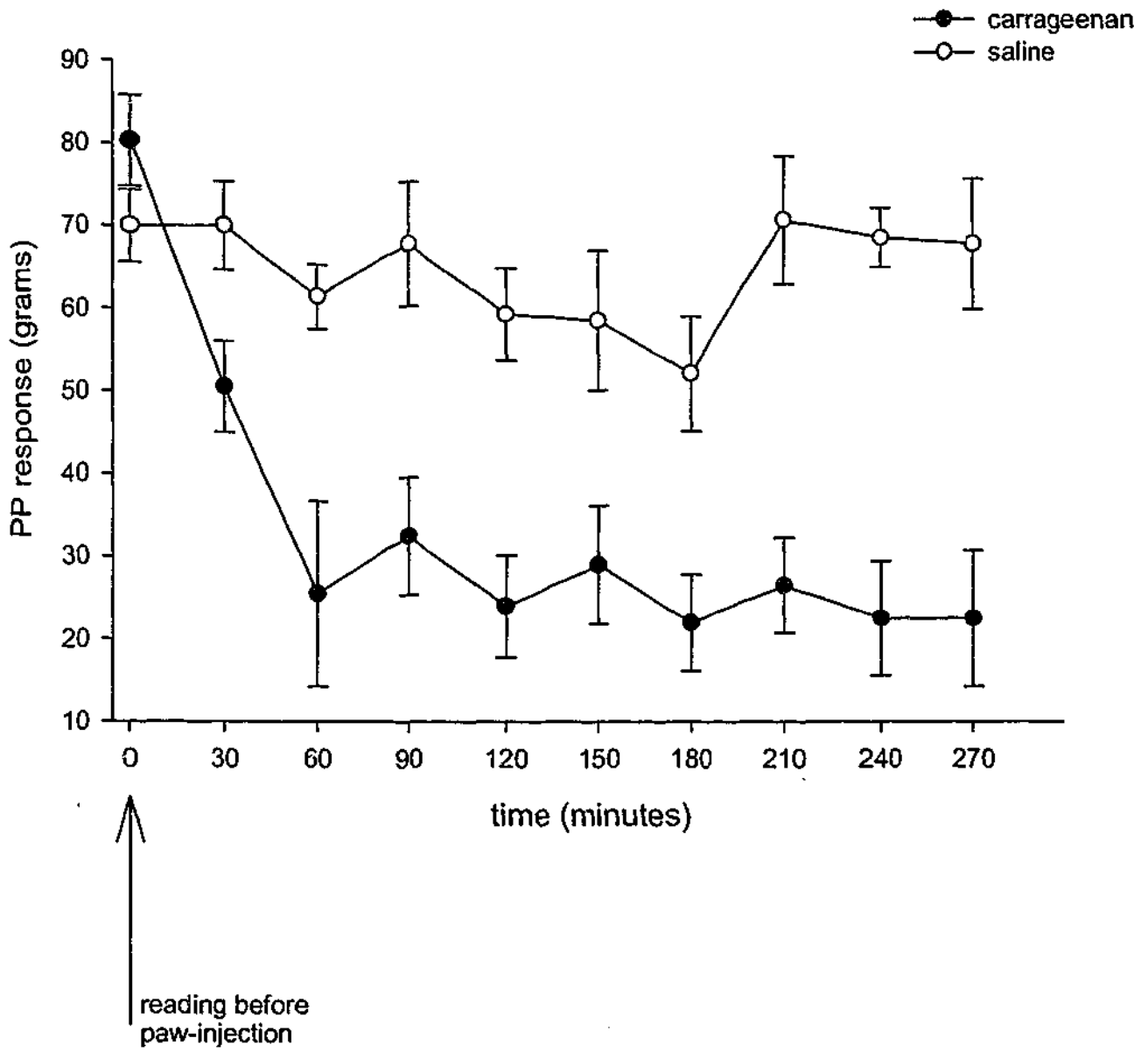
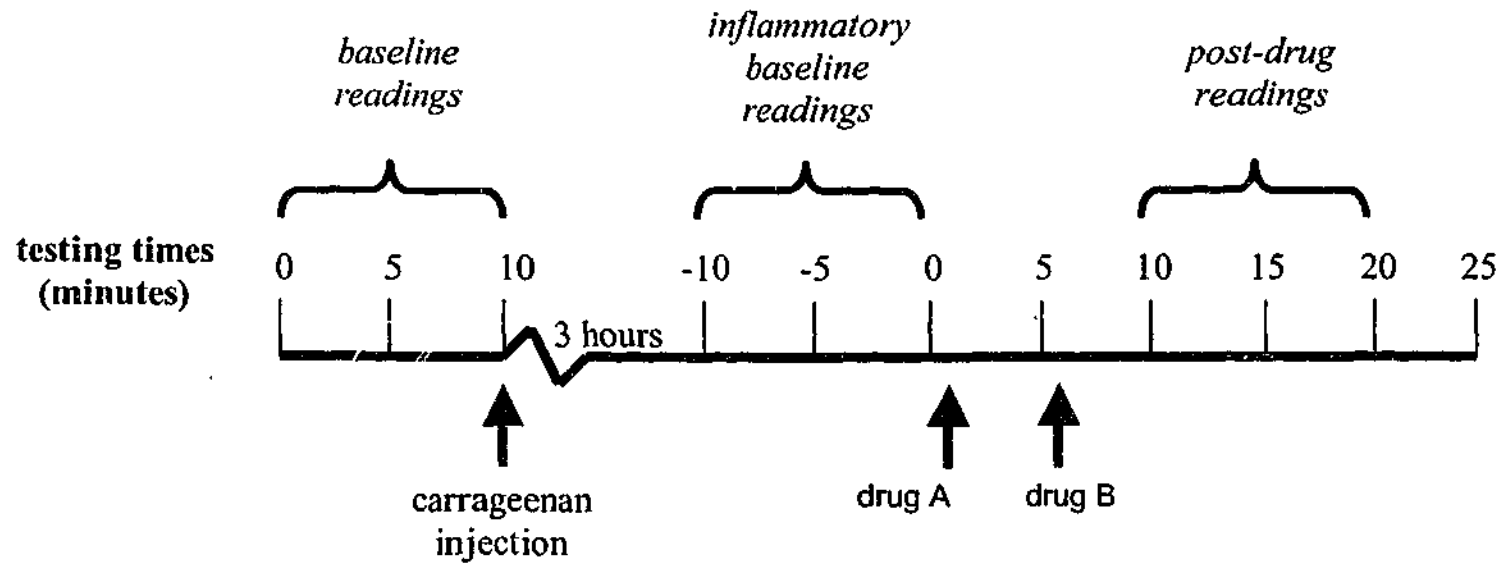


Figure 2.5 Carrageenan Time Controls

Initial baseline readings were measured at 5-minute intervals until three stable consecutive baseline readings were achieved. These readings were averaged. Rats were then injected with carrageenan and allowed to rest for 180 minutes. Paw pressure values were measured every 5 minutes until three stable readings were established and averaged, giving the inflammatory baseline threshold. A test drug was then injected and paw pressure thresholds were measured every 5 minutes for the next 25 minutes. For experiments where two drugs were tested, the second drug was injected 5 minutes after the first. Rats were only used once for carrageenan experiments.

Figure 2.6 Nociceptive Test Paradigm for Carrageenan-Induced Inflammation



2.5.2 Diabetic Neuropathy

Animal models of painful diabetic neuropathy have been used to investigate the therapeutic potential of a range of experimental agents and also to explore potential aetiological mechanisms. The induction of experimental insulin-dependent diabetes mellitus in rats causes allodynia and hyperalgesia (Courteix, Eschalier et al. 1993). Hyperalgesia was assessed using the PP test described previously (page 43).

(a) Animals

Male Wistar rats (w/t 65-80g) were injected with streptozotocin to induce diabetes. Rats were housed four per cage under standard laboratory conditions. After diabetic induction rats were monitored for 4-5 weeks until their weight range was 180-200g and hence suitable for behavioural testing.

(b) Streptozotocin Injection

Streptozotocin (STZ) is an antibiotic that is selectively toxic to pancreatic β-islet cells by activating immune mechanisms and by alkylating DNA (Simon and West 1992). β-islet cells are responsible for insulin secretion. A deficiency in insulin causes hyperglycaemia (high glucose blood levels), which occurs because the liver and skeletal muscle cannot store glycogen and the tissues are unable to take up and use glucose (Rang, Dale et al. 1996). This is the underlying process leading to diabetes.

STZ (150mg/kg total dose) (Sapphire Bioscience, Crows Nest, AUS) was dissolved in normal saline. The total dose was given over two days in 75mg/kg IP injections on consecutive days. This paradigm was based on the work from previous investigators (Zurek, Nadeson et al. 2001).

(c) Diabetes Confirmation

One week after injection of STZ, diabetes was confirmed by measurement of tail vein blood glucose levels with Ames Glucofilm test strips and a reflectance colourimeter (ACCU-CHEK Active, Roche, Mannheim, Germany). Only rats with a blood glucose reading of greater than 15mM were considered diabetic. Blood glucose levels were measured weekly for rats that remained under examination. These rats were housed for 4-5 weeks until their weight range was 180-200g.

(d) Rat Selection

Rats that displayed a blood glucose reading of less than 15mM one week after STZ injection were immediately eliminated from further experiments.

Other similar studies using this model have claimed the general health of some animals became extremely poor (Fox, Eastwood et al. 1999; Rittenhouse, Marchand et al. 1996). In such cases animals become emaciated with bloated abdomens and can even develop a bone structure so brittle that limb bones and ribs break on contact. None did so in this series of experiments. Rats that seemed to be very ill or suffering were removed from the study and killed.

Reports of mechanical hyperalgesia are fairly consistent; a number of groups have demonstrated a reduction in nociceptive thresholds of approximately 30-50% (Ahlgren and Levine 1993; Wuarin-Bierman, Zahnd et al. 1987; Zhuang, Snyder et al. 1996). In the experiments reported in this thesis, nociceptive thresholds of diabetic rats were compared with those of weight-matched controls. Normal weight-matched control rats showed an average baseline withdrawal threshold of r = 59.19 ± 1.20 (n=183) assessed by the PP test. Only diabetic rats that displayed approximately 30-50% decrease (PP readings below 30g) in nociceptive thresholds were used for further experimentation.

Rat behaviour was assessed by observation throughout the test period. As long as a healthy status was maintained, according to the guidelines listed above, rats were tested once daily for a maximum of 5 consecutive days. The numbers of rats excluded from these studies are outlined in Table 2.1 below.

Table 2.1 Summary of Rat Selection and Exclusion

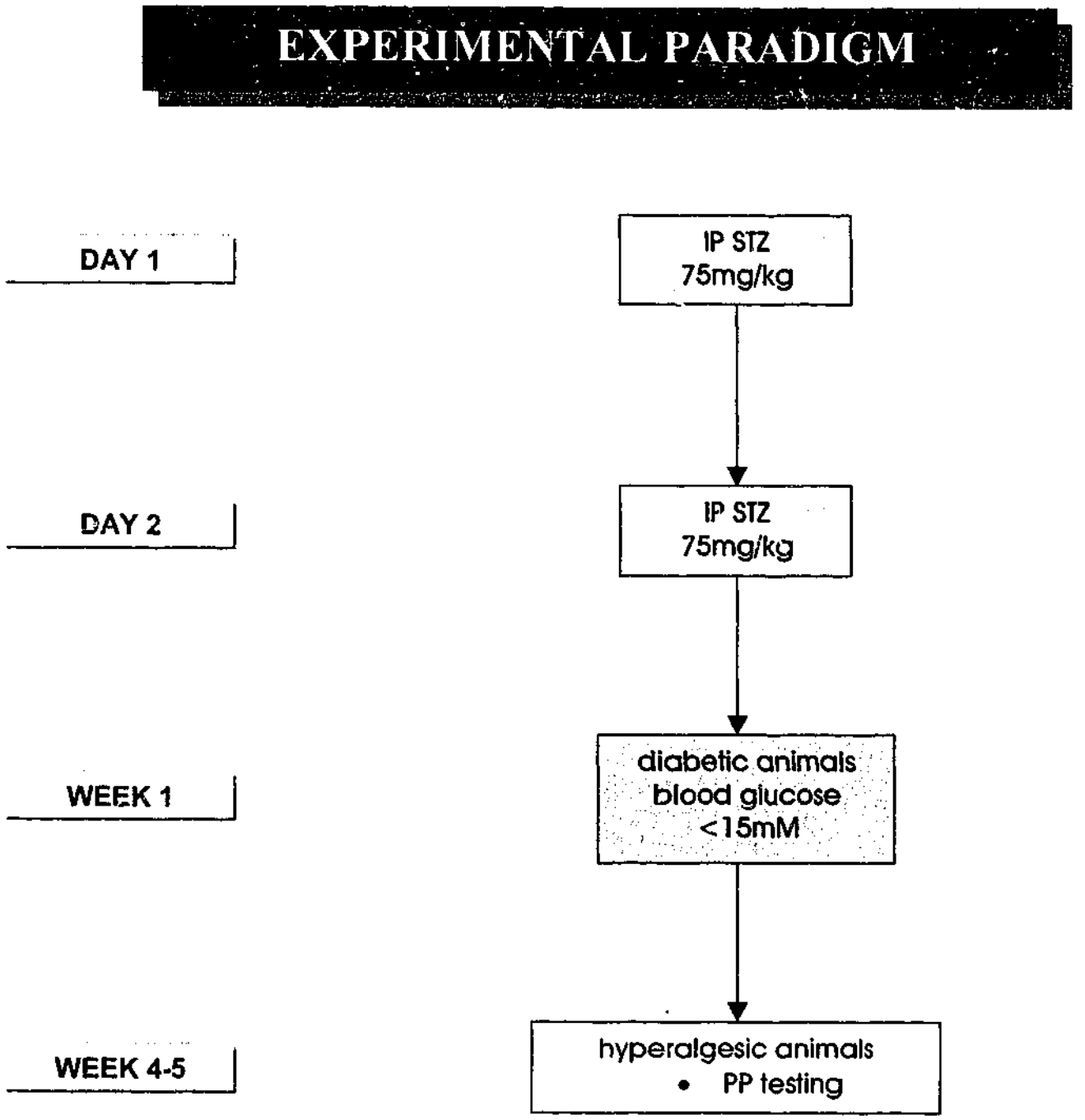
number of rats which became diabetic and were used in further experiments	73
number of rats which were not diabetic after week 1	91
number of rats that died or had to be killed because of their ill health	24
number of rats which were not found to be neuropathic by PP test.	37
total number or rats used for induction of diabetes	**225**

(e) Final Paradigm

Once diabetic rats reached a weight range of 180-200g nociceptive testing paradigms were carried out. Only rats displaying hyperalgesia assessed by the PP test were used in further experimentation. Initial baseline readings were measured at 5-minute intervals until three stable consecutive baseline readings were achieved. In determining these baselines paw pressure values were restricted to a maximum change of 10 grams in order to be included in the study. A test drug was then injected and paw pressure thresholds were measured every 5 minutes for the next 25 minutes. For experiments where two drugs were tested, the second drug was injected 5 minutes after the first. This protocol is shown in Figure 2.7 on page 66. The initial three readings were averaged (*mean paw pressure before drug injection*) as well as three readings after drug administration (*mean paw pressure after drug injection*). These changes were standardised as a ratio of control (r):

$$r = \frac{mean\ of\ three\ paw\ pressure\ readings\ after\ drug\ injection}{mean\ of\ three\ paw\ pressure\ readings\ before\ drug\ injection}$$

Figure 2.7 Diabetes Experimental Paradigm



2.5.3 Morphine Tolerance

Opioids remain the mainstay for pain management in moderate to severe pain. Therefore development of tolerance to opioids is common and often a problem in the clinic. Tolerance to opioids is characterised by shortened duration of effect and decreased intensity of analgesic and sedative effects (Bhargava 1994). Several techniques exist for the experimental induction of morphine tolerance including multiple intraperitoneal (IP) injections, subcutaneously implanted morphine-containing pellets, osmotic mini-pumps and oil in water emulsions from which morphine is released slowly (Salem and Hope 1998). This series of experiments studied morphine tolerance in rats using a slow-release morphine emulsion. Nociceptive responses to TFL were tested the day prior to emulsion administration. After the induction of tolerance nociceptive responses to TFL were reassessed.

(a) Preparation of Slow-Release Emulsion

The method of formulating morphine in a slow-release emulsion has already been described (Salem and Hope 1998). Morphine (morphine sulphate – David Bull Laboratories) 25mg/ml was diluted in normal saline and mixed with liquid paraffin oil (AJAX Chemicals, Sydney, AUS) and mannide mono-oleate (Sigma Chemical Co., St. Louis, USA) in a ratio of 8:6:1. First the two oils were mixed using a magnetic stirrer. Morphine was then dissolved in saline and this mixture was added slowly to the oil mixture to form a water/oil emulsion.

(b) Induction of Tolerance

Subcutaneous injections were given at the base of the neck using an 18-gauge needle. The total daily dose of morphine administered in the form of the slow-release emulsion was 250mg/kg. This amount was given over two days. The dosage was divided on the first day into two subcutaneous injections of 62.5mg/kg separated by 4 hours. The second day treatment consisted of a single injection of 125mg/kg. TFL testing began the following day, 20 hours after the last treatment. Experiments have shown by this time 99% of the total morphine was absorbed from the injection site (Salem and Hope 1998). The protocol for the induction of morphine tolerance is shown in Figure 2.8 on page 70.

Figure 2.8

The flow diagram represents the daily procedures for the induction and assessment of morphine tolerance. On day 1 the effects of IP morphine (6.4mg/kg) were assessed by TFL response. On day 2 rats were injected with two half doses of the morphine slow-release emulsion (2 x 62.5mg/kg). On day 3, rats were injected with a single dose of the morphine slow-release emulsion (125mg/kg). On day 4 the effects of IP morphine (6.4mg/kg) were assessed by TFL response.

EXPERIMENTAL PARADIGM

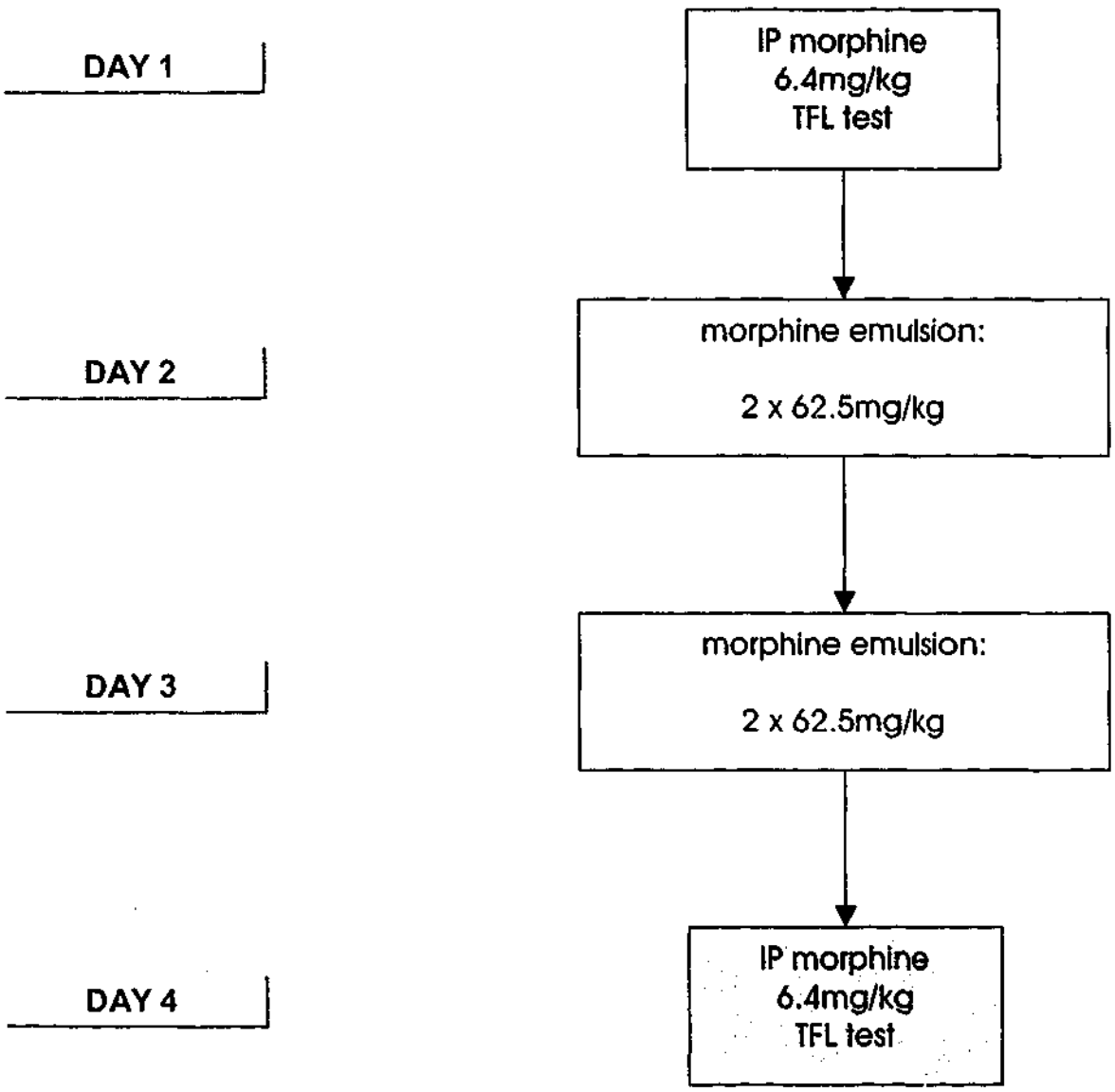


Figure 2.8 Tolerance Induction Protocol

2.6 Sedation

Locomotor activity is the most common behaviour used for the evaluation of sedative effects in the central nervous system of rats (Mead, Hargreaves et al. 1995). A more comprehensive way of measuring sedative effects is the use of two or more measures of different aspects of locomotor activity obtained in the same situation (Iverson 1977). This thesis describes two sedation tests used to investigate the pharmacological profile of different study drugs, the open-field activity monitor and rotarod. These two tests have been acknowledged as measuring different components of locomotion; the activity monitor measures spontaneous exploratory behaviour while the rotarod measures motor coordination (Della Maggiore and Ralph 2000). The use of two tests should allow better, more accurate definition of the sedative doses of compounds.

2.6.1 Activity Monitor

In 1977 Lubinsky, Dickson and Cairns were first to develop automated equipment to convert animal movement to "X" and "Y" coordinates (Ossenkopp, Kavaliers et al. 1996). The introduction of automated devices meant the removal of a visual observer. This helped decrease observer calibration errors and biases produced from fairly tedious tasks (Sanberg, Hagenmeyer et al. 1985). This also provided experiments with greater sampling capacities of the ongoing animal behaviours over time (Mullenix 1989). Since these early developments the field has exploded to introduce several different automated devices to create sensitive tools for the study of locomotor behaviour. Open-field instruments now produce an infrared photobeam array with

sources and sensors that define an X, Y and Z coordinate "map" for the containment environment. The sensors detect the presence or absence of the infrared beam (i.e. the subject) at the corresponding coordinates. These beam interruptions are relayed and interpreted by a computer to reveal many aspects of locomotion. Table 2.2 below describes the different behaviours that can be measured using the activity monitor.

Table 2.2 Behavioural Aspects Measured by the Activity Monitor

Animal Activity	Measured By
gross motor activity	distance travelled
anxiety	pattern of exploration - dark and light areas
hyper/hypoactivity	velocity determined from distance and time
stereotypical behaviour	moving time within one area
sedation	time of no movement
rearing behaviour	time spent vertically
zoning behaviour	i.e. subject stays in corners, rotational behaviour
jumping behaviour	subject leaves photobeam array for a period of time

The open-field activity monitor used for the work described in this thesis (MedAssociates Inc. Vermont, USA) is an enclosure of 16 sources and sensors of infrared beams. Interruptions of beams by subjects are electronically converted and recorded by computers. Throughout this work the monitor was only used to test for the sedative effects of drugs. Therefore the only relevant measurement is rest time. This value is automatically calculated in seconds as the amount of time the animal has spent breaking no new infrared beams. If any given dose of a test compound caused significantly increased rest time values in animals compared with the rest time values of animals given placebo, it was considered sedative. All other data collected from these experiments was considered irrelevant in determining the sedative effects of a drug. Figure 2.9 on page 76 shows an example of a subject's plot throughout a single experiment.

(a) Habituation

Repeat testing in the activity monitor has been shown to lead to a decrease in spontaneous exploratory movement. This is due to animal habituation to the experimental environment (Holson, Ali et al. 1989; Teicher, Andersen et al. 1996). This decrease in activity would be represented by an increase in rest time. Such results would be misleading and would therefore interfere with accurate sedation testing. A validation experiment was required to determine the habituation rate of rats to this novel environment. This would verify how many days the rat could be tested prior to their habituation to the activity monitor environment.

A group of 10 rats were each injected with vehicle control (saline) and tested for 20 minutes in the activity monitor. The same procedure was repeated for the next 3 consecutive days. The rest times for the rats were compiled as the mean ± SD. Results for each day were collated and compared using a one-way ANOVA with a Bonferroni post-hoc test for multiple comparisons. A value of $p \leq 0.05$ was considered statistically significant. Over the 4-day test period, days 1 and 2 showed no significant difference in rest time values. Rest time values of day 3 and 4 showed significant increases ($p<0.0001$, ANOVA) compared with day 1 and day 2. These results are shown in Figure 2.10 on page 78. The increase in rest time over the 4-day test period indicates animal habituation to the test environment. This decrease in activity could falsely be interpreted as sedation. For sedation experiments in this thesis, rats were only ever tested twice in the activity monitor and never on consecutive days.

(b) Experimental Time

Studies have shown the lighting conditions and time of day can affect the animal behavioural response in this apparatus (Ferraro, Antonakos et al. 1998; Kallman and Isaac 1975). All experiments were performed during the hours of 9.00am and 5.00pm. Rats were tested for changed activity levels in this 8-hour test period. A group of 10 rats were tested in the activity monitor between the hours of 9.00am and 12.30pm (morning). Another group of 10 rats were tested between 1.30pm and 5.00pm (afternoon). Rats were injected with placebo then placed in the box for 20 minutes. Rest time values were combined as the mean ± SD. Rest time values from the morning group and the afternoon group were compared for a statistical difference using a *t*-test. A value of $p \leq 0.05$ was considered statistically significant. Figure 2.11 on page 80 shows the rest times for the morning and afternoon groups. The rest time means of the two groups showed no significant difference. Rats were tested in the activity monitor at any time during the allocated 8-hour period.

(c) Final Paradigm

Rats are known to be nocturnal; therefore the enclosure for the activity monitor is darkened to help emulate a rat's most active time. Each experiment investigating a test drug was performed with matching vehicle control groups. The rest time values of the control group were compared with the rest time values of the test drug group. Rats were only ever tested twice and never on consecutive days. Therefore those that received placebo on the first experimental day received the test drug on the second experimental day and vice versa. Test drugs or drug combinations were injected into a rat and 5

minutes later the rat was placed in the box for a 20-minute period. Movement automatically activated the box when the subject was first placed in the enclosure. The rats' movement was directly analysed by a computer. Rest time data was given in seconds and collated for experimental results. Figure 2.12 on page 81 shows the sedative test paradigm for the activity monitor.

Figure 2.9 Activity Monitor and an Example of a Line Trace

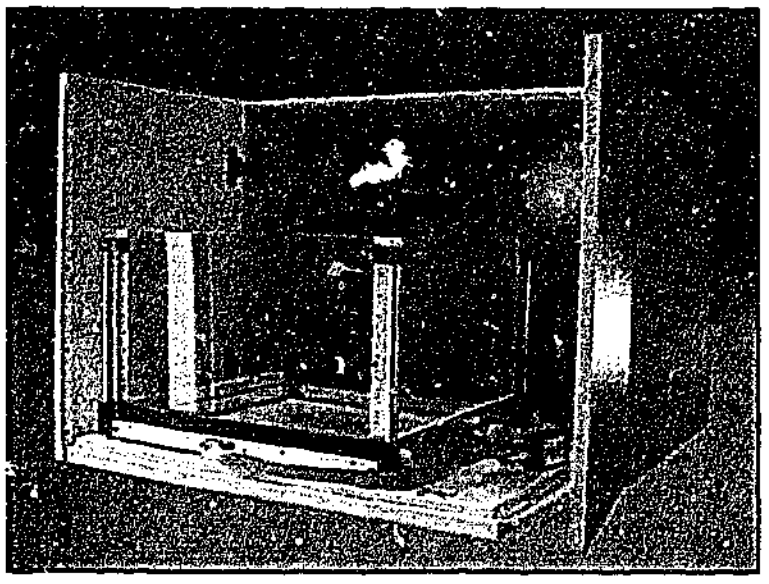
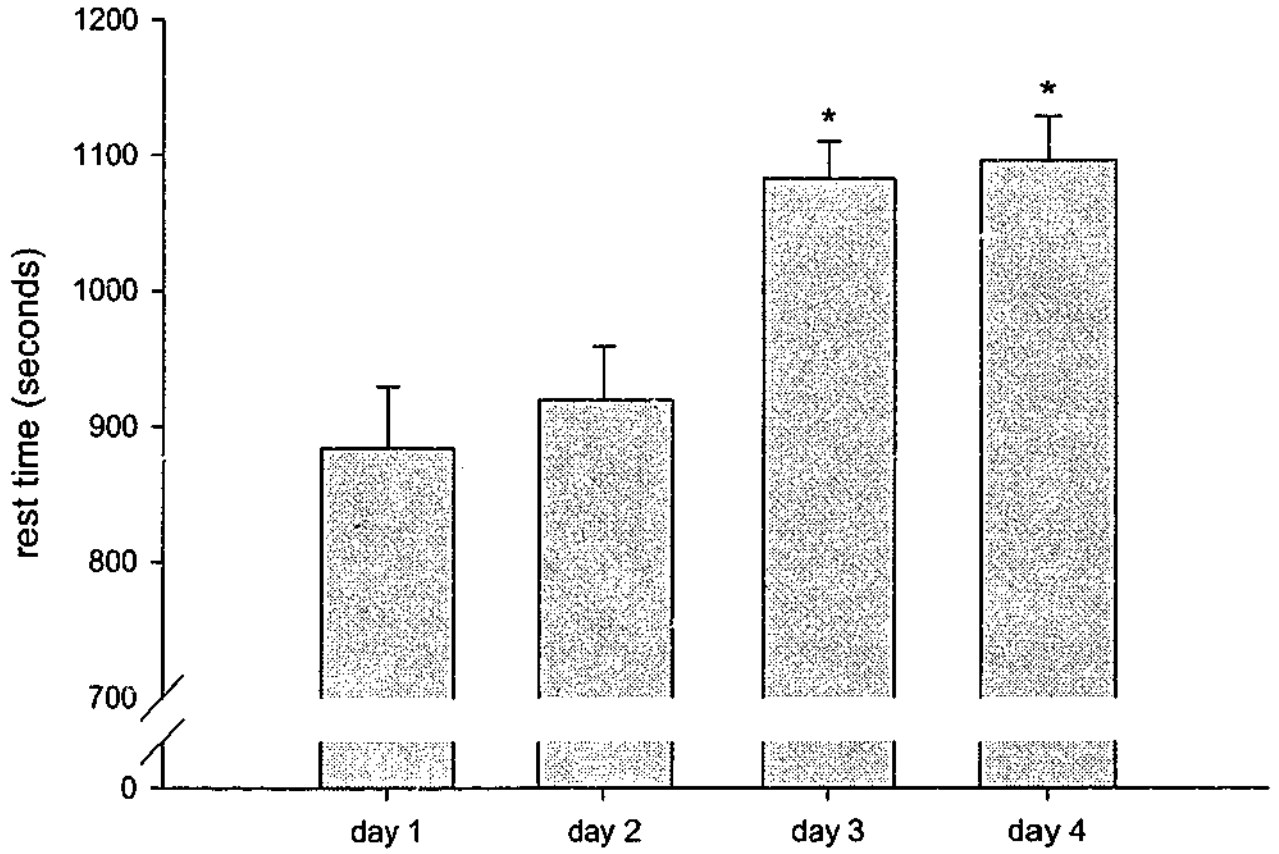


Figure 2.10

Habituation to the activity monitor was plotted as rest time in seconds. Rats treated with placebo (IP saline) were tested in the activity monitor on 4 consecutive days. There was no significant difference in rest time values for day 1 and 2. Day 3 and 4 showed a significant increase in rest times values compared with day 1 and 2 ($p<0.0001$, ANOVA). Each histogram represents the mean rest time of 10 rats ± SD.



* denotes statistical significance

Figure 2.10 Habituation to the Activity Monitor

Figure 2.11

Rats were tested in the morning and afternoon between the hours of 9am-12.30pm and 1.30-5pm. Each rat was given a placebo injection. The two groups showed no significant difference in rest time values. Rats could be tested in the activity monitor any time between 9am and 5pm. Each histogram represents the mean rest time for 10 rats ± SD.

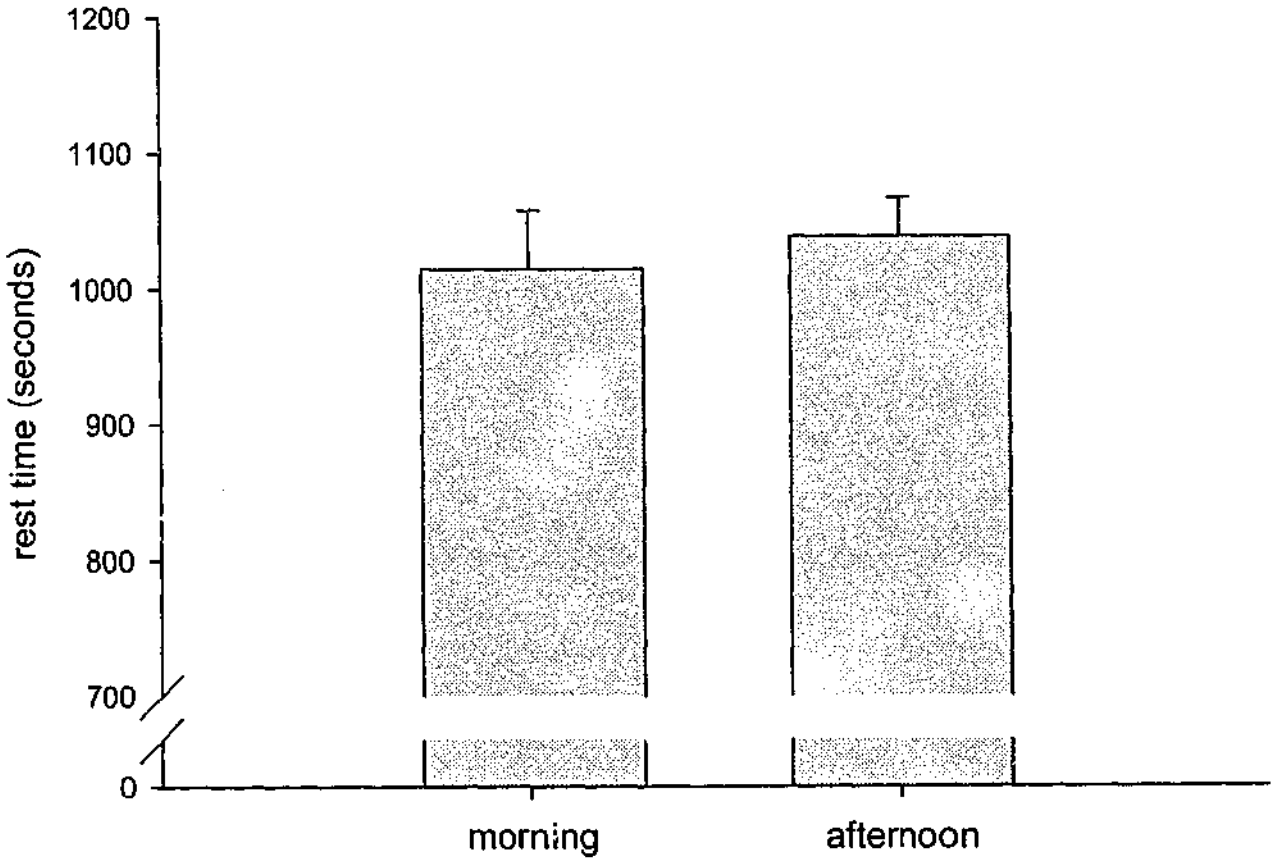
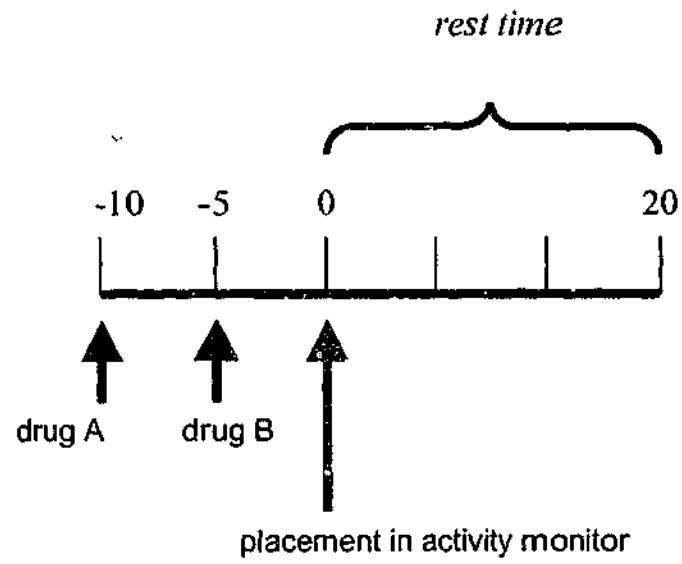


Figure 2.11 Time of Day: Effect on Rest Time

Figure 2.12 Summary of the Activity Monitor Paradigm

An individual drug or drug combination was administered. After 5 minutes rats were placed in the activity monitor and rest time was measured for the next 20 minutes.



2.6.2 Rotarod

The rotarod treadmill (7650 accelerator rotarod, Ugo Basile, Italy) consists of a 7cm diameter drum and a rotating wheel that can be made to rotate at a constant or steadily increasing speed. The rotarod test is an instrumental technique that measures the length of time a rat can remain on the rotating drum. The apparatus was developed to measure drug effects on motor coordination, balance, and motor learning of rats. The rotarod is an objective test because it doesn't require an experienced observer or a technical scoring procedure. Once the rat is placed on the rotarod apparatus the rat walks on the drum until it falls off, at which point the test is terminated. The rotarod performance time is defined as the period of time rats can remain on the rotating drum. A sedated rat walks on the drum for a shorter time compared with non-sedated controls.

Although the rotarod has been used extensively in the assessment of drug effects on motor function, no standardised protocol is described in the literature. Variations in rod measurements, rotational speed, prior training and testing time are either published in little detail or vary greatly among experiments (Bogo, Hill et al. 1981; Rozas, Guerra et al. 1997).

(a) Animal Selection Procedure

In establishing this test it was seen that most rats instinctively ran against the movement of the drum and hence stayed on it. As the drum accelerated, their grip tightened and they ran faster to stay on. However a small group of rats did not run and thus turned with the drum and immediately fell off. In order to create consistent accurate data these

rats were eliminated from testing. Any rat that successfully completed the training procedure was included in the subsequent experiments. These selection procedures were based on the work of other researchers (Cartmell, Gelgor et al. 1991).

(b) Training

The training procedure was conducted the day before the experiment. Training consisted of the rats being placed on the rotating drum at a steady low speed of 4 revolutions per minute. Every time a rat fell, it was immediately placed back on the drum until 15 minutes of continuous running had elapsed. This training procedure was repeated after the rat had been allowed to rest for at least 30 minutes. Prior to experimentation on following days, each rat was re-trained for 15 minutes of continuous running on the steadily rotating drum set at a speed of 4 revolutions per minute.

(c) Time limit

In order to prevent lengthy run times and fatigue, the introduction of a time limit for any one trial is necessary to obtain satisfactory measures of performance (Jones and Roberts 1968). Different publications have suggested various time limits for rotarod testing. For example Carlson *et al.* suggested a 3-minute cut-off time for rotarod testing (Carlson, Haskew et al. 2001) while Coughenour *et al.* employed a 60 second cut-off (Coughenour, Mclean et al. 1977). For the experiments reported in this thesis, a cut-off time was determined from a control experiment using 16 newly trained rats tested on the rotarod. Each rat was placed on the drum and the wheel accelerated from 4

revolutions per minute with a steady acceleration of 20 revolutions per minute every minute thereafter. When a rat fell from the drum their run time was recorded in seconds. This procedure was repeated for 4 consecutive days. Over this 4-day test period, not one trained rat fell from the drum before they had completed 120 seconds of running on the rotarod. Therefore 120 seconds (2 minutes) was decided as the cut-off time limit for the rotarod experiments. The data are shown in Figure 2.13 on page 86.

(d) Final Paradigm

Once trained, rats were used in one experiment per day for a maximum of 4 consecutive days. For each experiment, a rat was injected with the test drug or drug combination and 5 minutes later placed onto the rotating drum in an isolated section. At this point a timer started and the wheel accelerated from 4 revolutions per minute with a steady acceleration of 20 revolutions per minute every minute thereafter. The rotarod performance time was the time taken for the rat to fall from the wheel. Rats were removed from the drum after 2 minutes. Therefore, this was used as the control cut-off or maximum run time for the test. The rats' run times were measured on the wheel three times, at 10-minute intervals (time=5, 15, 25 minutes from test starting time). The shortest run time of the three measured during the test period was identified for each experiment. For any individual treatment, this run time value was an indication of the level of sedation. Figure 2.14 on page 87 shows the paradigm for the rotarod.

Figure 2.13

A group of 16 trained rats were tested on the rotarod on four consecutive days. Each rat was given a placebo injection. All rats demonstrated run times that were significantly greater then 120 seconds ($p \leq 0.05$, *t*-test). Therefore this was selected as the cut-off time for rotarod experiments. Each histogram represents the mean run time for 16 rats ± SD.

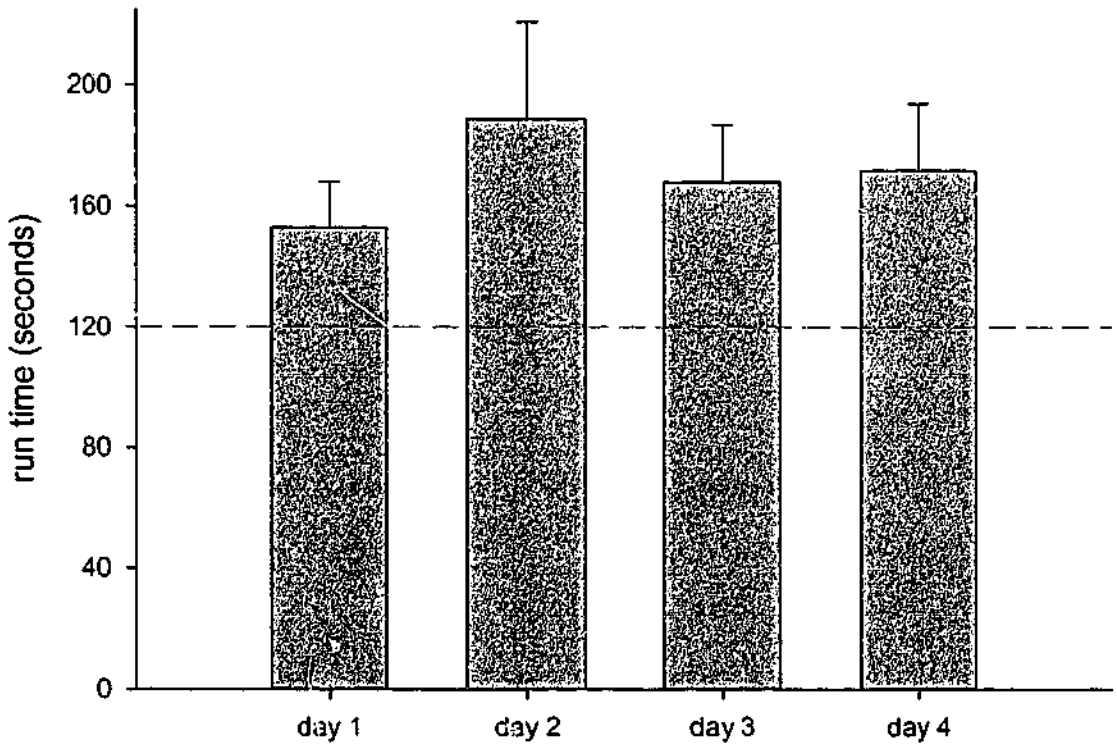
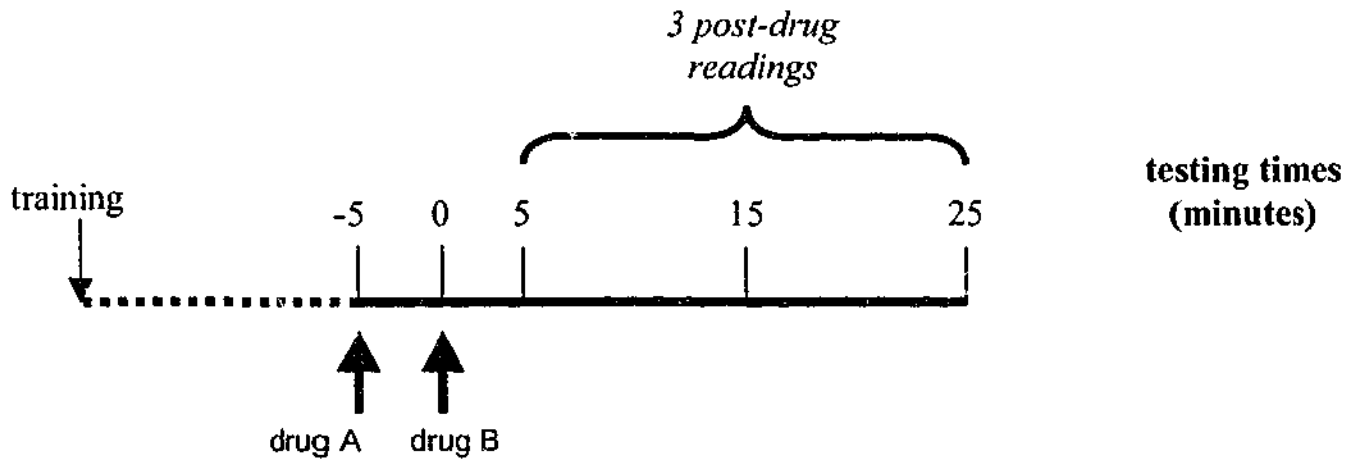


Figure 2.13 Control Cut-off Time

Figure 2.14 Rotarod Test Paradigm

Trained rats were given a test drug or drug combination. Rats were placed on the rotarod and run time was measured for 120 seconds. Rat run times were measured on the rotarod at time points 5, 15 and 25 minutes.



2.7 General Testing Paradigm

The nociceptive and sedative models described in this chapter were selected for this thesis to help achieve a better understanding of neurosteroid and opioid pharmacology and their applications in pain medicine. These different compounds were tested both individually and in combination.

One of the major aims of this thesis was to identify doses of drugs or drug combinations that can produce pain relief without sedation. It was first determined whether the individual drug in question possessed any sedative effects. A drug that impairs motor function may impair an animal's ability to respond and hence be wrongly considered to have analgesic activity (Plummer, Cmielewski et al. 1991). Sedative effects were established in the activity monitor and on the rotarod. Both tests are accurate measures for sedation and are not subject to observer bias (Mullenix 1989). Furthermore, the rotarod and activity monitor measure different aspects of sedation (Della Maggiore and Ralph 2000). Thus the use of two tests strengthens the chance of any sedative drug doses being excluded from the nociceptive testing stage.

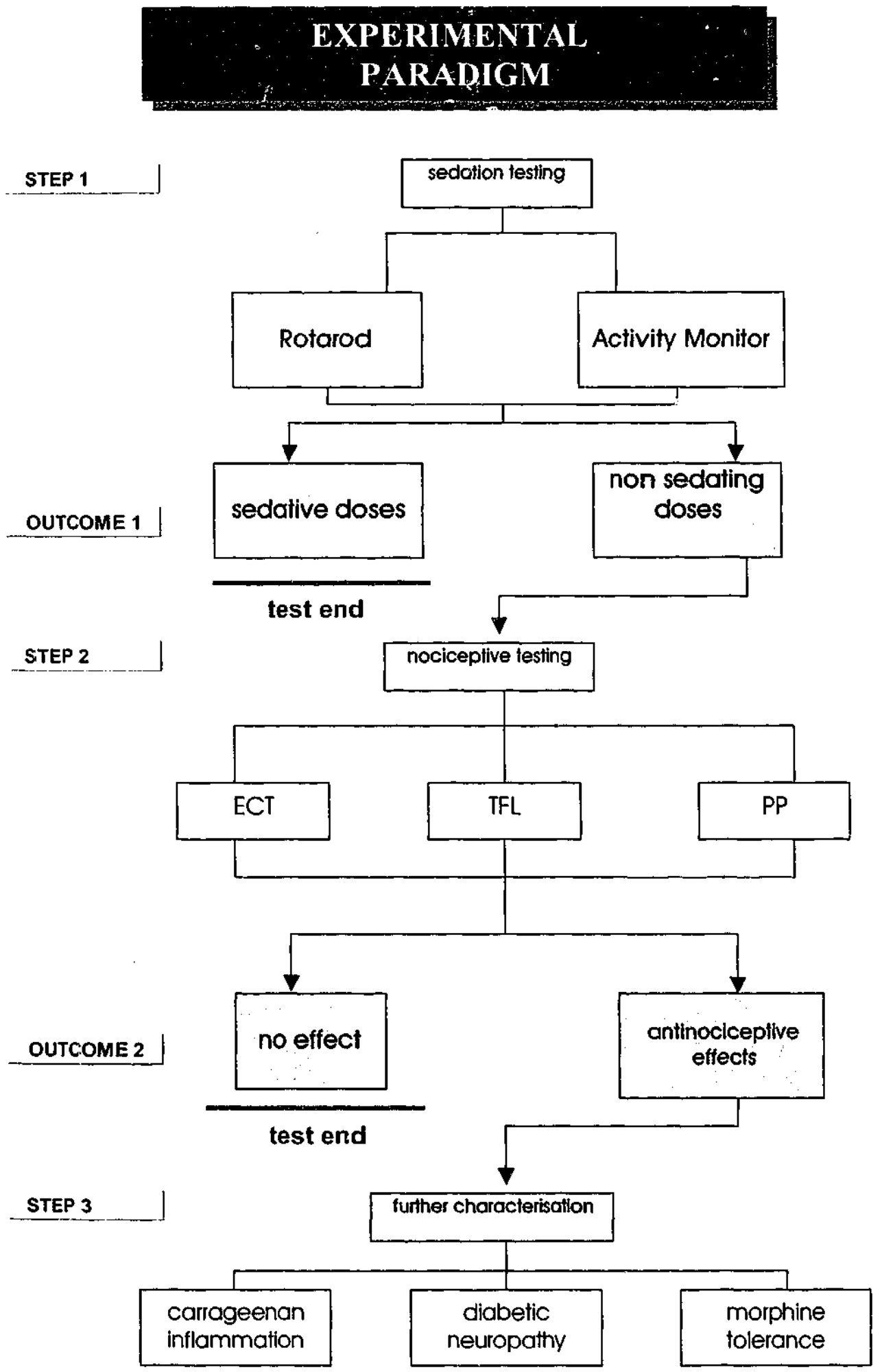
After completion of sedation testing, 4-point dose response curves for antinociceptive effects were determined using the acute nociceptive tests ECT, TFL and PP tests. The dose response curves attempted to describe both top and bottom doses for all test-drugs. Further characterisation of a drug or drug combination was completed using models of carrageenan-induced inflammation, diabetic neuropathy and morphine tolerance. This testing sequence helped provide an extensive antinociceptive and sedative profile for

the test drug or drug combinations. Figure 2.15 on page 90 shows a flow diagram of the testing procedure followed throughout this thesis.

2.7.1 Summary

Animal models play a critical role in understanding the mechanisms and treatments of human pain. There is a wide selection of animal models of painful clinical conditions. It is necessary to use a range of pain induction and assessment techniques in this research because one simple model cannot describe the very complex and multi-faceted aspects of pain and its treatment (Petersen-Felix and Arendt-Nielsen 2002). For the purpose of this thesis, the tests described in this chapter were used in conjunction with one another to help provide a thorough investigation of the pain relieving properties for individual drugs and drug combinations.

Figure 2.15 Experimental Paradigm



Chapter 3 Investigation of Two Neurosteroids; Alphadolone and Alphaxalone

3.1 Introduction

Selye was the first to demonstrate that certain pregnanes and androstanes have potent anaesthetic and anticonvulsant effects occurring within a few minutes of administration (Selye 1941). It has since been found that these steroids interact directly with a surface membrane receptor-complex to cause a rapid change in central nervous system excitability. These compounds are termed neuroactive steroids (Puia, Santi et al. 1990; Purdy, Moore, Jr. et al. 1992). *Neurosteroids* are neuroactive steroids only found within central nervous system (CNS) tissue. All endogenous neurosteroids are derivatives of the parent compound cholesterol and therefore share very similar structures (Baulieu, Robel et al. 1999). However the few slight chemical differences between compounds gives them extraordinarily diverse biological specificities. Since the discovery of their brain function activity, neurosteroids have been described as anxiolytics, hypnotics and analgesics (Gee, McCauley et al. 1995; Majewska 1992; Smith 1994). More recent work has shown that the selective properties of these compounds are due to positive modulation of $GABA_A$ receptors (Hamilton 2002; Lambert, Belelli et al. 1995; Purdy, Moore, Jr. et al. 1992). This suggests there exists a wide therapeutic potential for neurosteroids.

The discovery of fast acting steroid sedatives led to the development of CT1341 (Althesin®) as an intravenous anaesthetic. This mixture contained two neuroactive steroids, alphaxalone and alphadolone, with an aqueous vehicle, Cremophor EL (polyethoxylated castor oil surfactant). It was stated in the literature of the time that the anaesthetic component of Althesin was mainly due to alphaxalone, while alphadolone, with one-half the anaesthetic activity of alphaxalone, was present to improve solubility

(Stock 1973). Althesin® was removed from clinical practice because of major anaphylactoid reactions that were attributed to the Cremophor vehicle. The identical preparation of these two neurosteroids dissolved in the Cremophor vehicle continued to be available for veterinary use (Saffan®). Work with Saffan® revealed that intraperitoneal (IP) injections of Saffan® led to sedation and antinociception and that these properties of the mixture could be separated and ascribed to the two constituents: alphaxalone – sedation; alphadolone – antinociception (Nadeson and Goodchild 2000).

3.1.1 Aim

The series of experiments described in this chapter set out to characterise and compare in more detail the sedative and antinociceptive properties of alphaxalone and alphadolone.

3.2 Methods

Both alphaxalone and alphadolone were administered intraperitoneally (IP) in all experiments. Dose response relationships were determined for the sedative effects of the neurosteroids using the rotarod and activity monitor. Antinociception was assessed with tail flick latency (TFL), electrical current threshold (ECT) and paw pressure (PP) tests. Chapter 2 describes all methods used throughout this series of experiments. Initial experiments were performed to identify the highest doses of the two neurosteroids that did not cause sedation. Individual drug doses that were not sedative were tested for antinociceptive effects.

3.2.1 Study Drugs

The drugs used for this series of experiments were alphaxalone (Jurox, Rutherford, NSW) and alphadolone (alphadolone acetate, Jurox, Rutherford, NSW). Both neurosteroids are combined with 2-hydroxypropyl-β-cyclodextrin (HPβCD). These cyclodextrins (CD) are used as complexing agents to improve the aqueous solubility of non-polar drugs. Alphadolone was weighed out each day and dissolved in a phosphate buffer solution. Alphaxalone required the accompanying Alfaxan-CD diluent for reconstitution. The neurosteroid dose ranges for each test are given in Table 3.1 on page 95. Replicate experiments were performed for each dose in several different rats.

Table 3.1 Dose ranges tested for IP Alphadolone and IP Alphaxalone

	sedative test		antinociceptive test		
	rotarod	activity monitor	TFL	ECT	PP
alphadolone mg/kg	1-200 *n=5*	1-200 *n=5-10*	0.1-10 *n=5-8*	0.05-10 *n=5-13*	0.5-10 *n=5*
alphaxalone mg/kg	10-50 *n=5-6*	10-50 *n=5-10*	10 *n=5*	10 *n=5*	10 *n=5*

n denotes the number of replicate experiments at each dose

3.2.2 Data Analysis

Run time values for the rotarod were combined for each dose of drug and expressed as means ± SD. Each dose was compared with the control maximum run time of 120 seconds using a *t*-test. Rest time values from the activity monitor were combined for each drug dose and expressed as means ± SD. Each dose was compared statistically with the vehicle control group by means of a *t*-test. For nociceptive dose response curves, the ECT (r), PP (r), and TFL (%MPE) responses were combined for each dose of neurosteroid and plotted as means ± SEM. Statistical significance was tested with a *t*-test. Since a ratio was used for tail ECT and PP, a value of 1 indicated that the drug was ineffective. Thus using a *t*-test, if any dose variable for tail ECT and PP responses was significantly different from the value of 1, it was considered effective. TFL response is calculated as a percentage of maximum effect (%MPE). Therefore a value of zero suggests the drug treatment was ineffective. Any dose that was significantly different from zero for the %MPE for TFL responses was therefore considered effective. For all statistical comparisons, a value of $p \leq 0.05$ was considered statistically significant. For the construction of time response relationships (for ECT and PP), the

ratio response value (r) calculated at each 5-minute interval was combined for all rats. Each value was expressed as the mean ± SEM for each individual time point.

3.3 Results

3.3.1 Sedative Effects

Sedation testing assessed by the rotarod showed doses of alphadolone above 10mg/kg caused a significant decrease in rat run time responses compared with that of vehicle-treated rats. Doses of 10mg/kg caused run times that were not significantly different from vehicle-treated rats. These results are shown in Table 3.2 on page 101. Assessed by the activity monitor, doses above 60mg/kg caused a significant increase in rest time values compared with vehicle-treated rats. Doses of 60mg/kg and lower caused rest time values that were not significantly different from vehicle-treated rest time values. These results are shown in Table 3.3 on page 103.

Sedation testing using the rotarod showed doses of alphaxalone above 10mg/kg caused a significant decrease in run time responses compared with that of vehicle-treated rats. Only a dose of 10mg/kg showed run times that were not significantly different from vehicle-treated rat rest times. These results are shown in Table 3.2 on page 101. Assessed by the activity monitor, doses above 20mg/kg caused a significant decrease in rest time values compared with vehicle-treated rats; doses of 20mg/kg and lower caused rest time values that were not significantly different from vehicle-treated rats. These results are shown in Table 3.3 on page 103.

Taking these results together one can conclude that the highest non-sedating dose was 10mg/kg for both IP alphadolone and IP alphaxalone. This dose and lower doses were therefore used for further nociceptive testing.

3.3.2 Antinociceptive Effects

Figure 3.1 on page 105 shows the antinociceptive effects of alphadolone in the ECT, PP and TFL tests. All the non-sedating doses of alphadolone tested (0.05-10mg/kg) caused no significant antinociceptive effects assessed by the TFL test (*t*-test). Alphadolone caused a dose related increase in ECT response. The highest dose of 10mg/kg caused a significant rise ($p<0.0001$, *t*-test) in ECT, $r = 2.45 \pm 0.41$ (mean ± SEM). Alphadolone also showed a dose dependent relationship for PP with increasing responses. The highest dose of 10mg/kg caused a significant increase ($p<0.0001$, *t*-test) in PP thresholds, $r = 1.66 \pm 0.06$ (mean ± SEM).

The highest non-sedating dose assessed by the activity monitor and rotarod for IP alphaxalone was also 10mg/kg. This dose was tested in the three acute nociceptive tests; ECT, TFL and PP. Alphaxalone caused no significant antinociceptive effects in any of the tests (*t*-test) (see Figure 3.1 on page 105).

3.3.3 The Time Response Relationship of Intraperitoneal Alphadolone

The time of onset and duration of response for the ECT and PP antinociceptive effects were examined by plotting a time response relationship for alphadolone (see Figure 3.2 page 107). A significant increase in antinociceptive response was seen in the first 5 minutes following alphadolone administration for both ECT and PP responses. The time taken to peak effect for both tests was between 15-20 minutes post-drug

administration. This effect remained consistently elevated for the following 3 readings with no significant changes in thresholds (one-way ANOVA). Time points 15, 20 and 25 minutes post-drug administration were the three readings showing the most effective and stable time points for alphadolone effect compared with the three baseline readings. Therefore these time points were used for calculations in all further analyses.

Table 3.2

This table summarises the sedative actions of alphadolone and alphaxalone assessed by the rotarod test.

The highest non-sedating dose of alphadolone assessed by rotarod performance was 10mg/kg; all rats given this dose completed the entire test run time of 120 seconds. A dose of alphadolone 60mg/kg caused rat run times to decrease significantly ($p=0.039$, *t*-test). A dose of 200mg/kg caused maximum sedation where rats did not run at all for the entire test period.

All rats given 10mg/kg alphaxalone completed the entire test run time of 120 seconds on the rotarod. A dose of 20mg/kg alphaxalone caused a significant decrease in run time response ($p=0.025$, *t*-test). Thus any dose higher than 10mg/kg was considered sedative. A dose of 25mg/kg caused maximum sedation where rats did not run at all for the entire test period.

Table 3.2 The Effect of IP Alphadolone and IP Alphaxalone on rotarod performance

alphadolone		alphaxalone	
	run time (secs)		run time (secs)
dose (mg/kg)	mean ± SD	*dose (mg/kg)*	mean ± SD
1	111 ± 13 *n=5*	*10*	120 ± 0 *n=5*
10	120 ± 0 *n=5*	*20*	57 ± 49* *n=6*
60	41 ± 58* *n=5*	*25*	0 ± 0* *n=5*
100	11 ± 16* *n=5*	*50*	0 ± 0* *n=5*
200	0 ± 0* *n=5*		

* denotes statistical significance

n denotes the number of replicate experiments at each dose

Table 3.3

This table summarises the results of alphadolone and alphaxalone tested for sedative effects using the activity monitor.

Alphadolone 100mg/kg caused a significant increase in rest time values compared with vehicle-treated rats (p=0.018, *t*-test). This dose was considered sedative. Doses lower than 100mg/kg did not show a significant difference in the rest time values compared with vehicle-treated rats.

Doses of alphaxalone 20mg/kg and lower showed no significant difference from rest time values of vehicle-treated rats. In contrast, a dose of 25mg/kg caused a significant increase in rest time values (p=0.035, *t*-test). This dose of alphaxalone was considered sedative assessed by the activity monitor.

Table 3.3 The Effect of IP Alphadolone and IP Alphaxalone Assessed by the Activity Monitor

alphadolone		alphaxalone	
	rest time (secs)		rest time (secs)
dose (mg/kg)	mean ± SD	*dose (mg/kg)*	mean ± SD
vehicle control	930 ± 282 *n=55*	*vehicle control*	930 ± 282 *n=55*
1	866 ± 108 *n=10*	*10*	965 ± 70 *n=10*
10	928 ± 28 *n=10*	*20*	968 ± 94 *n=5*
60	965 ± 65 *n=5*	*25*	1063 ± 80* *n=5*
100	1061 ± 51* *n=5*	*50*	1200 ± 0* *n=5*
200	1139 ± 45* *n=5*		

* denotes statistical significance
n denotes the replicate number of experiments at each dose

Figure 3.1

(A) PP

This graph shows the effect of increasing doses of alphadolone on PP response in rats. Alphadolone caused a clear dose dependent antinociceptive effect in this model. Alphaxalone 10mg/kg caused no significant antinociceptive effects. Each point represents the mean for 5 rats ± SEM.

(B) ECT

This graph shows the effect of increasing doses of alphadolone on ECT response in rats. Alphadolone caused dose dependent antinociceptive effects in this model. Alphaxalone 10mg/kg caused no significant antinociceptive effects. Each point represents the mean for 5-13 rats ± SEM.

(C) TFL

This graph shows the effect of increasing doses of alphadolone on TFL response in rats. Alphadolone caused no significant antinociceptive effects in this model. Alphaxalone 10mg/kg caused no significant antinociceptive effects. Each point represents the mean for 5-8 rats ± SEM.

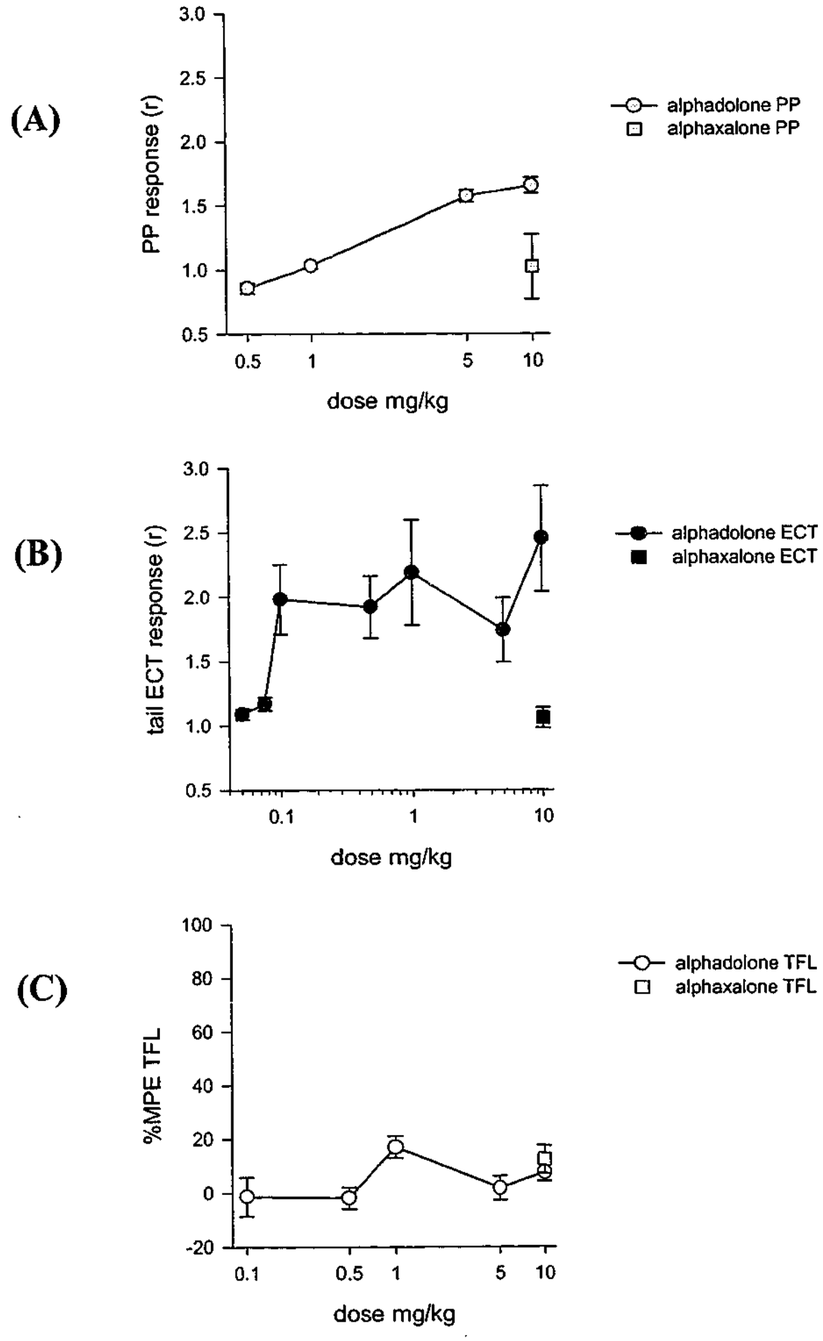


Figure 3.1 The Antinociceptive Effects of IP Alphadolone and IP Alphaxalone

Figure 3.2

(A) ECT

The changes in ECT are plotted against time, both before and after administration of increasing doses of alphadolone. Three stable baseline measurements were taken at 5-minute intervals (-10, -5, 0 minutes), after which alphadolone was administered. Low doses of alphadolone (0.05-0.075mg/kg) did not cause any significant changes from baseline values. Higher doses of alphadolone (0.1-10mg/kg) caused an increase in tail ECT that reached a peak effect within 15 minutes of administration. This effect remained at its maximum for 3 readings. Each point represents the mean for 5-10 rats ± SD.

(B) PP

Testing time is plotted against change in PP response, both before and after alphadolone administration. Three stable baseline measurements were taken at 5-minute intervals (-10, -5, 0 minutes), after which alphadolone was administered. Low doses of alphadolone (0.5, 1mg/kg) demonstrated no significant changes in PP responses. Higher doses (5, 10mg/kg) caused an increase from baseline values that peaked within 15 minutes of alphadolone administration. This effect remained elevated for 3 readings. Each point represents the mean for 5-13 rats ± SD.

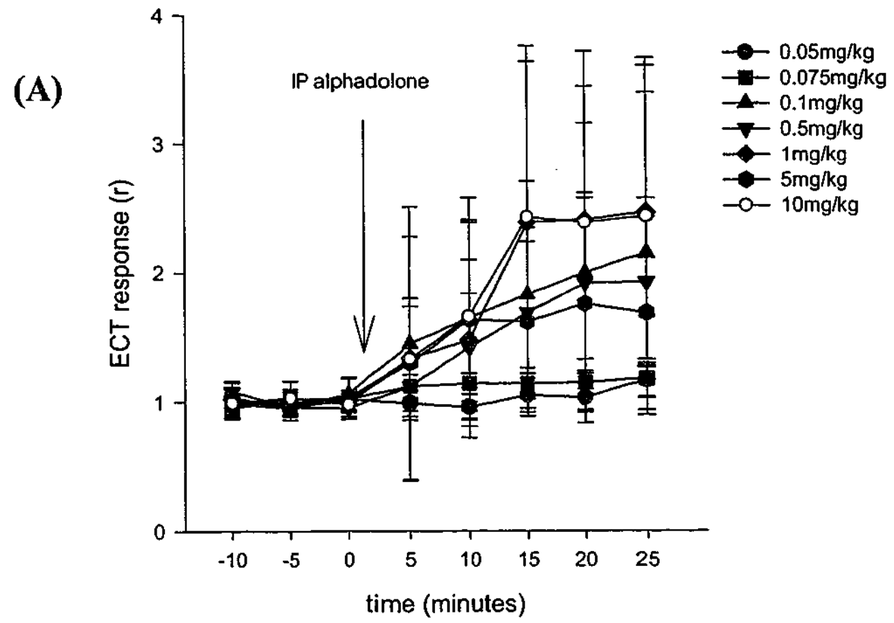


Figure 3.2 Time Response Relationship for IP Alphadolone

3.4 Discussion

It is well established that GABA is the major inhibitory neurotransmitter in the CNS (Wall and Melzack 1989). Drugs that behave as analgesics or anaesthetics can produce their effects by increasing inhibition in the CNS. Neurosteroids have been shown to selectively positively modulate $GABA_A$ receptors to increase the effects of GABA (Lambert, Belelli et al. 1995; Purdy, Moore, Jr. et al. 1992). Therefore neurosteroids may have therapeutic potential in both areas of analgesia and anaesthesia.

The experiments described in this chapter investigated the sedative and antinociceptive properties of two neurosteroids, alphadolone and alphaxalone. This series of experiments demonstrated that both alphaxalone and alphadolone cause dose related sedation revealed by rotarod performance and spontaneous movement in the activity monitor. Furthermore, these experiments highlighted the antinociceptive properties of alphadolone, in contrast to alphaxalone that caused no antinociceptive effects at non-sedating doses. Alphadolone exhibited potent antinociception assessed by both the PP and ECT tests but not the TFL test. One possibility for the difference in activities of alphadolone and alphaxalone is molecular structural differences leading to different drug-receptor interactions.

Studies have illustrated a regional difference in distribution of binding sites for various neurosteroids. Gee and co-workers used $GABA_A$ receptors isolated from the rat cortex and spinal cord to reveal differences in steroid modulation between the receptors isolated from the two abovementioned sites (Gee and Lan 1991). Specific

investigations have shown that alphadolone interacts with $GABA_A$ receptors in the spinal cord (Nadeson and Goodchild 2000). Those experiments were performed using bicuculline, a selective $GABA_A$ receptor antagonist, injected intrathecally directly onto the spinal cord. The antagonist blocked the entire antinociceptive effects of alphadolone. Binding studies have shown that alphaxalone binds to receptors in several regions in the human and rat brain (Nguyen, Sapp et al. 1995). Because the $GABA_A$ receptor complex consists of a heterogeneous group of structurally distinct subunits (Lambert, Belelli et al. 1995) it is reasonable to expect that different neurosteroid analogues possess different binding for such subunits. The binding differences of various compounds could suggest different behavioural profiles, similar to what is seen with alphaxalone and alphadolone in this chapter's experiments.

3.4.1 Intraperitoneal Alphaxalone and Intraperitoneal Alphadolone Have Sedative Activity

Since Selye first discovered fast-acting sedative neurosteroids, their development as anaesthetics was keenly followed. In the early 1980's alphaxalone was synthesised as an anaesthetic for clinical administration. Experiments in rats demonstrated that alphaxalone caused sedation by selectively enhancing the interaction of GABA with the $GABA_A$ receptor (Harrison and Simmonds 1984). Other investigators used *in vitro* experiments with voltage-clamp recordings to demonstrate a dose dependent potentiation of GABA-evoked currents resulting from alphaxalone action at $GABA_A$ receptors (Cottrell, Lambert et al. 1987).

At the time of introduction of the alphaxalone/alphadolone anaesthetic mixture (Althesin®) neurosteroids had a major advantage over other clinically used anaesthetics such as the barbiturates; they were thought to have a far greater therapeutic index, therefore superior safety for clinical practice (Davis and Pearce 1972). Althesin® was comprised of 9mg alphaxalone and 3mg alphadolone acetate. The company that distributed the mixture claimed alphadolone possessed a half of the anaesthetic potency of alphaxalone and was only included in the mixture to improve its solubility (Brogden, Speight et al. 1974). This present study confirmed that alphaxalone and alphadolone both cause sedation. In addition, when assessed for sedation in the activity monitor, alphaxalone 20mg/kg was the highest non-sedating dose while alphadolone 60mg/kg was the highest non-sedating dose. In this case it can be stated that alphaxalone displayed a greater potency of approximately 3 times than its counterpart alphadolone. Unfortunately alphadolone was not tested at 20mg/kg on the rotarod. Therefore an accurate comparison between alphaxalone and alphadolone cannot be conducted with regard to sedation testing using the rotarod.

Anaesthetics that impair motor function may pose as analgesics in pain tests. The experiments reported in this chapter identified sedative doses of the test compounds and excluded these doses from further nociceptive testing. It was important to separate the confounding sedation and motor effects of the neurosteroids from their activity as antinociceptive compounds. This step has obvious practical implications when characterising a compound for possible clinical application in pain.

Sedative effects following IP administration were investigated in detail using two well-established tests; rotarod performance and spontaneous locomotion in the open-field

activity monitor. Both these models have been shown to be accurate measures of sedation (Jones and Roberts 1968; Mullenix 1989). Both compounds showed greater sensitivity to sedation in the rotarod. This might imply that the rotarod is a more conservative measure for sedation than the activity monitor for these two neurosteroids. Some previous published work has suggested the two apparatus used in these experiments measure different properties of behaviour. The activity monitor measures unprovoked spontaneous movement, while the rotarod requires physical effort and motor coordination to remain on the revolving rod (Luesse, Schiefer et al. 2001). Other studies have shown the equivalent measures of locomotion in these two apparatus and hence their interchangeable nature (Della Maggiore and Ralph 2000). However, the experiments reported in this chapter demonstrated a difference in maximum non-sedating doses for both neurosteroids. Therefore the use of two tests increased the chance discovery of sedative drug doses and their exclusion from further nociceptive testing.

3.4.2 Different Nociceptive Tests

The results of the experiments described in this chapter showed alphadolone causes dose dependent antinociception assessed by ECT and PP responses but not TFL. Other analgesics have shown similar antinociceptive profiles whereby they have no effect on one behavioural test but display potent dose dependent effects using another. For example, reports have shown midazolam caused an increase in ECT without any effect on TFL (Edwards, Serrao et al. 1990; Yanez, Sabbe et al. 1990). Other work has suggested a TFL effect for midazolam but only at high doses (Niv, Davidovich et al.

1988). It is possible that sedative effects caused by higher doses were misinterpreted as antinociception and thus explain the conflicting reports.

Various forms of stimuli that cause withdrawal are used to indicate nociception in animal testing. The three acute pain tests used throughout these experiments activate different nociceptive systems, namely PP for mechanical, ECT for electrical and TFL for thermal. Table 3.4 on page 116 shows the advantages, disadvantages and differences between the three noxious stimuli used in the experiments reported here. The use of a variety of nociceptive tests is important for determining a comprehensive pharmacological profile for novel compounds.

Some investigators have shown different pain experiences are produced by activation of different afferent fibres. For example it is commonly regarded that C fibres principally mediate tail flick stimulation (Chakour, Gibson et al. 1996; Torebjork 1985). These fibres are implicated in producing dull, burning sensations that often result in protective reflexive behaviours like withdrawal of a limb in humans or the tail flick reflex in animals (Wall and Melzack 1989). Although TFL is most widely used for evaluation of analgesics in rats, it revealed no antinociceptive effects for alphadolone. As described earlier, alphadolone positively modulates $GABA_A$ receptors to increase the effects of GABA. Experiments have suggested that GABAergic terminals contact more Aδ fibre terminals than C fibre terminals (Bernardi, Valtschanoff et al. 1995). Therefore alphadolone may produce its antinociceptive effects by causing a greater interference with Aδ fibres than with C fibres. This is further supported by other work that showed midazolam, another known $GABA_A$ receptor positive modulator, caused only weak depression of C fibre activation but

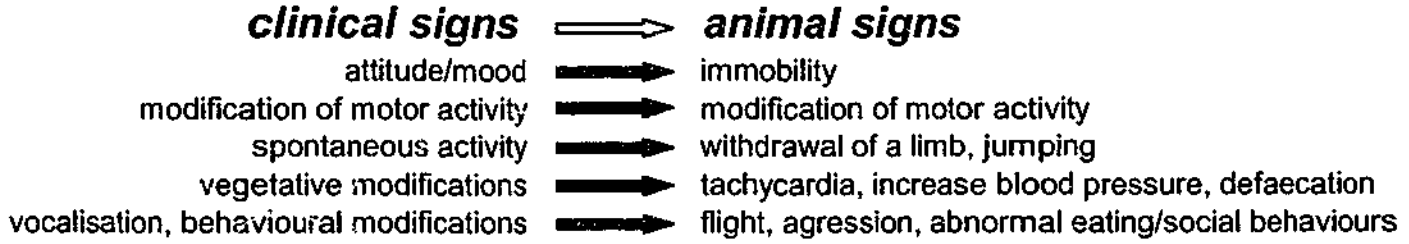
displayed a marked effect on Aδ fibre activation (Clavier, Lombard et al. 1992; Wang, Chakrabarti et al. 1992). In addition, another experiment showed bicuculline (a $GABA_A$ receptor antagonist) caused only a small C fibre-evoked response in comparison with it's Aδ fibre-evoked response (Besson and Chaouch 1987). These descriptions might help explain the lack of antinociceptive effects of alphadolone in the TFL test.

In nature nociceptive reflexes and related behavioural patterns have a protective function. These behaviours can be studied in the laboratory setting to gain information about nociceptive mechanisms (Chapman, Casey et al. 1985). However, it has been suggested that pain and the tail flick reflex are not one and the same phenomenon (McGrath 1981). Pain sensation or experience should not be inferred from reflexes alone. This point can be exemplified in humans when reflex behaviour, such as the knee jerk reflex is totally disassociated from pain sensation. The effects drugs have on these reflexes might have very little to do with their analgesic power. For example, changes in reflex activity can often result from alterations in motor and sensory processing rather than interruptions of pain pathways (Willer, Boureau et al. 1979). Therefore the study of reflex responses such as tail flick can only provide a guide to nociception and the antinociceptive effects of drugs. On the other hand, the heat threshold for C polymodal nociceptors in a wide variety of animals is 48°C. This corresponds well to the threshold at which withdrawal in the TFL test occurs.

Clinical signs that correspond to the perception of uncomfortable sensations in animals are listed in Figure 3.3 on page 114. However not all reflexes seen in animals are always found in humans (Le Bars, Gozariu et al. 2001). This can make it difficult to

draw parallel conclusions from experimental observations to the clinic. No human analogy to the tail flick response can be found, and human withdrawal latency when a finger is placed on a heated plate is vastly different from that of animals (Chapman, Casey et al. 1985). It is quite difficult to relate simple reflex models for nociception in animals to the complex nature of the pain experience in humans.

Figure 3.3 Clinical Signs Interpreted in Animals
adapted from (Jourdan, Ardid et al. 2001)



The paw pressure test is also a measure of reflex behaviour, where limb-withdrawal is used as an end-point for an experiment. Therefore the reflex withdrawal action witnessed in this test might also be an invalid indication of pain sensation (Le Bars, Gozariu et al. 2001). Another drawback of this animal pain model is that its technique simultaneously stimulates both cutaneous and deep sensory receptors (Dubner 1992). This means noxious stimuli aren't activating a restricted group of nociceptors. The mechanism of a particular drug that causes antinociception in this animal model cannot be entirely defined by this acute test alone without further research.

ECT testing is beneficial as it has a rapid onset and termination and the testing procedure is non-invasive. However, the individual use of the ECT test also cannot entirely define an analgesic drug's mechanism of action. This is because electrical

stimulation of the skin appears to bypass specific receptor activation and instead activates all primary afferent fibres including large diameter non-nociceptor fibres (Dubner 1992; Vierck, Jr. and Cooper 1984).

Although electrical stimulation activates all primary afferent fibres, nociceptive information in this test is primarily conducted along Aδ fibres (Chakour, Gibson et al. 1996). In agreement with earlier arguments, the electrical activation of Aδ fibres might explain the potent antinociceptive effects caused by alphadolone revealed by this test. These fibres are associated with sharp and pricking pain sensations. Unlike the true pain sensation experienced from noxious heat stimuli, the ECT test does not mimic a natural pain stimulus seen in humans.

Thus any of the nociceptive tests described in this chapter, if used alone, would be unreliable and incapable of determining fully the nociceptive profile of a drug. However, their collective use provides a much more reliable and comprehensive profile for the antinociceptive actions of a drug.

Table 3.4 Selection of an Appropriate Noxious Stimulus

Requirements	Thermal	Electrical	Mechanical
Variable natural quantified stimulus	++	-	+
Rapid onset and termination	+	++	++
Levels comparable between species	++	-	+
Stimulation activates restricted group of nociceptors	++	-	-
Stimulation activates only nociceptors in the pain sensitivity range	++	-	-
Stimulation does not produce tissue damage	+/-	++	+/-

++ = Fully satisfactory; + = minimally satisfactory; - = unsatisfactory

Taken from (Dubner 1985).

3.4.3 Intraperitoneal Alphaxalone and Intraperitoneal Alphadolone Display Different Activities

Experiments on compounds shown to possess antinociceptive properties such as midazolam, muscimol and serotonin (5HT) have revealed that their effects are mediated by $GABA_A$ receptors. Such conclusions were drawn by using the selective $GABA_A$ receptor antagonist bicuculline given intrathecally directly onto the lumbosacral spinal cord (Edwards, Serrao et al. 1990; Nadeson, Guo et al. 1996). Evidence for neurosteroid modulation of $GABA_A$ receptors implies the potential capacity for neurosteroids as analgesics. However few studies have searched exclusively for analgesic properties of neurosteroids. Apart from alphadolone and alphaxalone only progesterone has been studied for antinociceptive effects. A study reported progesterone produced an antinociceptive effect in the tail flick test that was reversed by bicuculline (Gambhir, Mediratta et al. 2002). Nadeson and Goodchild instigated some preliminary investigations studying the antinociceptive effects of alphadolone and alphaxalone. This work showed that a single dose of alphadolone (750mg/kg administered intragastrically) caused an increase in the ECT response that was reversed by intrathecal bicuculline (Nadeson and Goodchild 2001). This same dose of alphadolone had no effect on TFL response. Alphaxalone showed no antinociceptive effects assessed by either acute pain test. The experiments shown in this chapter are consistent with results from this earlier work. Alphadolone caused dose dependent antinociceptive effects in the ECT, but not the TFL test at doses below those that caused sedation. Furthermore, a non-sedating dose of alphaxalone caused no such antinociception.

The main conclusion from this series of experiments is that alphadolone and alphaxalone possess different properties with respect to antinociception. Upon intravenous (IV) administration both compounds behave as anaesthetics, differing only in potency (Brogden, Speight et al. 1974). Their structures are shown in Figure 3.4 on page 121. The pronounced pharmacological differences seen between these almost identical neurosteroids may be due to structural differences, although pharmacokinetic reasons cannot be ruled out entirely.

When Selye first described fast-acting sedative steroids, he tested several different compounds to establish some general structure-activity rules. Preliminary work was able to show the anaesthetic potency of some steroids, namely progesterone and deoxycorticosterone acetate (Selye 1941). More recent work has attempted to describe further a structure-activity relationship for neurosteroid compounds. Published work has indicated all active sedative compounds possess a 5α- or 5β-reduced pregnane skeleton with a α-hydroxyl at the C3 position of the steroid A ring and a keto group at either C20 of the pregnane steroid side chain or C17 of the androstane ring system (Harrison, Majewska et al. 1987; Lambert, Belelli et al. 1995). Alphadolone and alphaxalone are pregnanediones and possess the structural attributes listed above. The only structural difference between these two compounds is alphadolone, administered as alphadolone acetate, possesses an acetoxy methyl at the C20 position whilst alphaxalone bears a methyl group at the same position.

Neurosteroids undergo metabolism via the liver and they are therefore likely to undergo structural changes at this point (Stock 1973). Some authors claim reductive

metabolism is often required to release the active neurosteroid compound (Hamilton 2002), while other investigators have suggested it is difficult to decipher whether neurosteroid effects are due to parent steroids or their metabolites (Rupprecht, Hauser et al. 1996). These confounding factors suggest one should consider the metabolic implications that might produce the different activities of alphaxalone and alphadolone. A paper by Nadeson and Goodchild showed the different effects of intravenous (IV) alphadolone compared with IP and intragastric administration (Nadeson and Goodchild 2001). Alphadolone caused potent anaesthesia in rats when given IV. However alphadolone caused no sedative effects when given via IP and intragastric routes. Other experiments showed alphaxalone caused sedation and anaesthesia when administered IV and IP. (Britton, McLeod et al. 1992; Hill-Venning, Peters et al. 1996). This might indicate that metabolism of alphadolone occurs when it is presented to the liver, prior to entering the systemic circulation. This could inactivate its anaesthetic properties whilst still allowing potent antinociception to occur by the action of a metabolite.

Because their structures are closely related, the metabolic pathways of alphadolone and alphaxalone would be quite similar. Both compounds possess a hydroxyl group at the C3 position that is capable of conjugation (Mensah-Nyagan, Do-Rego et al. 1999). However alphadolone acetate possesses an acid labile acetate at the C20 position, which would almost certainly be hydrolysed in the stomach to reveal a C21 primary hydroxyl group (-OH) (Baulieu, Robel et al. 1999). This primary alcohol could be readily conjugated in the liver. The C20 methyl group belonging to alphaxalone *cannot* undergo this transformation (Holly, Trafford et al. 1981). The possible

conjugations occurring at the C20 position of alphadolone are shown in Figure 3.4 on page 121.

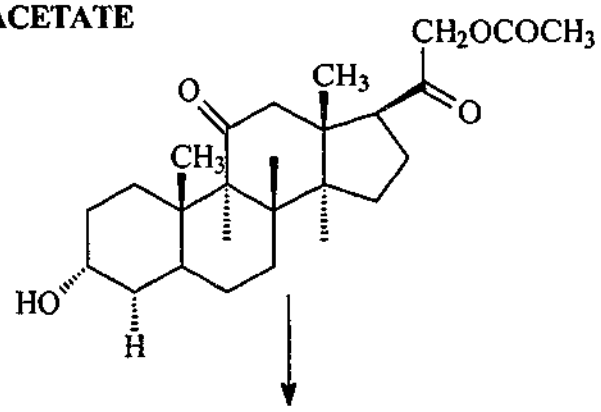
Glucuronides and sulphates have been described as conjugates for neurosteroid excretion (Holly, Trafford et al. 1981). It is a common occurrence for active drugs to undergo these conjugations to be inactivated. It is rare that compounds require conjugation to become active, although this is the case with morphine and its glucuronide conjugates. One morphine metabolite, M6G is a more potent analgesic, whilst another metabolite, M3G, does not bind to opioid receptors at all (Frances, Gout et al. 1992; Gong, Hedner et al. 1991; Milne, McLean et al. 1997). Sulphate conjugation may also play a part in the differences seen between the two neurosteroids. Alphadolone and alphaxalone can both become sulphated at the C3 position, yet only alphadolone can gain a sulphate group at the C20 poistion. A report confirms that both unsulphated and sulphated neurosteroids can modulate $GABA_A$ receptors. Those reports go on to suggest an entirely different binding site on the $GABA_A$ receptor for sulphated neurosteroids (Park-Chung, Malayev et al. 1999). The very separate activities of alphadolone and alphaxalone might be due to their different metabolic pathways and the conjugated compounds they produce.

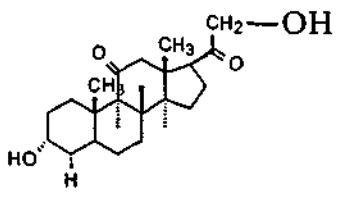
Preliminary clinical tests for alphadolone's analgesic activity have been performed. Those studies have shown that although alphadolone was administered in the form of alphadolone acetate, it was rapidly hydrolysed to native alphadolone (Sear and Sanders 1984). Other human trials with alphadolone revealed no parent compound in blood samples but discovered significant amounts of alphadolone-glucuronide (Goodchild, Robinson et al. 2001). This may indicate that the compound causing analgesic effects

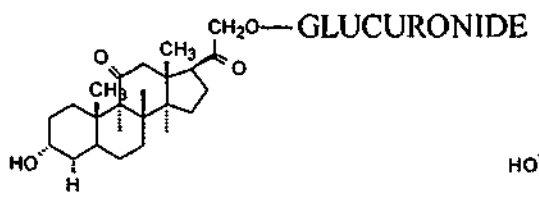
is the conjugated alphadolone-glucuronide. Investigations of this compound and other analogues would be useful in further characterising the activities of neurosteroids.

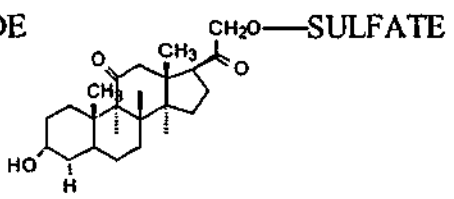
Figure 3.4 Structures of Alphadolone and Alphaxalone

ALPHADOLONE-ACETATE

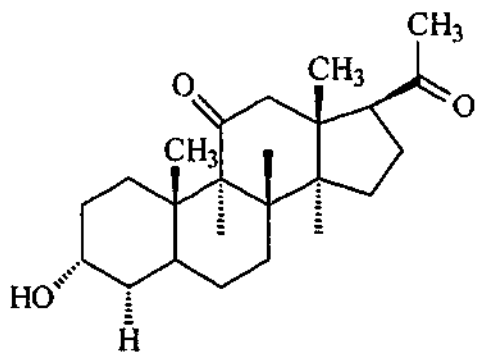








ALPHAXALONE



3.4.4 Summary

Both neurosteroids tested in this chapter demonstrated their clinical potential. Although the combination mixture Althesin® was once used clinically in anaesthesia, its resurrection has not been successful. However, its counterpart Saffan® does remain in practice for animal use. Although their structures are similar, alphadolone and alphaxalone display obvious differences in pharmacological activity. A structure-activity study would expose important aspects of neurosteroid chemistry with therapeutic potential. This directs future thought to the discovery of new analgesics or other clinically useful compounds.

Chapter 4 An Interaction Between Opioids and Alphadolone

4.1 Introduction

For centuries opioids have been used as treatments for various acute and chronic pain states. While they are used most widely and are effective in many pain conditions, opioids have a number of well-known adverse side effects such as sedation, nausea, vomiting, and respiratory depression. The use of opioids in some chronic pain states has been controversial. Studies have shown opioids may alleviate the pain but at higher doses than normal (Jadad, Carroll et al. 1992; Portenoy, Foley et al. 1990), which promotes the use of higher opioid dosing leading to dose dependent side effects such as sedation. The use of higher doses also induces the development of opioid tolerance, where a particular dose does not provide the same amount of analgesia as it did initially. Furthermore, the development of opioid tolerance and dependence may result in altered pain sensitivity, and subsequent response to additional opioids (Doverty, White et al. 2001). Some have speculated that processes involved in the development of opioid tolerance in spinal cord dorsal horn neurones are the same as those associated with the development of central hyperalgesia following tissue injury and inflammation (Mao, Price et al. 1995). Thus there is a growing consensus that the disadvantages associated with opioids far outweigh their therapeutic advantage. Therefore the discovery of new and effective therapies is required for the management and treatment of severe pain.

A major advance in pain management is the concept of *multimodal analgesia:* the use of combinations of drugs acting via different mechanisms to provide effective analgesia. Multimodal analgesia suggests the use of lower doses of individual drugs in combination, to reach the same level of pain relief that might have been difficult to

achieve with higher doses of individual analgesics. The aim in using lower doses is to decrease the incidence of dose dependent side effects while maintaining the quality of analgesia. Therapies developed from this concept of multimodal analgesia have increased the diversity and quality of pain management as well as personalised the treatment for each individual patient.

The characterisation of links between opioid and non-opioid systems within the central nervous system (CNS) has enabled the development of some effective multimodal therapies. For instance, recent studies have investigated the interaction between opioid and GABAergic systems. Experiments showed intrathecal (IT) midazolam, a $GABA_A$ receptor positive modulator, potentiated the tail flick latency antinociceptive effects of morphine (Rattan, McDonald et al. 1991; Yanez, Sabbe et al. 1990). Alphadolone, like midazolam, also positively modulates the action of GABA at spinal cord $GABA_A$ receptors to cause inhibition of neuronal responses and antinociception (Nadeson and Goodchild 2000). Since the antinociceptive profiles of midazolam and alphadolone are similar, alphadolone may also demonstrate an interaction with opioids. (Harrison, Majewska et al. 1987; Harrison, Vicini et al. 1987; Mistry and Cottrell 1990)

Past experiments demonstrating the ability of midazolam to increase opioid antinociception have suggested this interaction occurred in the spinal cord. Since midazolam was injected intrathecally in these experiments, it was likely, although not proved, that a spinal cord interaction occurred. The experiments reported in this chapter test for an interaction between alphadolone and opioids. It has been established previously that alphadolone activates $GABA_A$ receptors in the spinal cord to produce

antinociception; therefore any interaction with opioids might also occur within the spinal cord.

Several experiments have tested for spinal receptor involvement by investigating the effects of intrathecally-administered drugs. Various investigators using the tail flick test have injected intrathecal agonists and antagonists and measured their antinociceptive effects (Holtman, Jr., Jing et al. 2003; Taira, Nakakimura et al. 2000; Yoon, Choi et al. 2003). To date, Goodchild and his co-workers have ensured this style of experiment determines accurate placement of the intrathecally-administered drug. This group performs TFL testing in parallel with ECT testing. ECT values are determined at both neck and tail sites. After a drug is administered intrathecally, its correct placement is determined by a change in tail ECT values but not in the neck (Boulter, Serrao et al. 1991; Goodchild, Guo et al. 1996; Lograsso, Nadeson et al. 2002; Nadeson, Tucker et al. 2002; Serrao, Stubbs et al. 1989).

4.1.1 Aim

The experiments described in this chapter investigated interactions between two neurosteroids, alphadolone and alphaxalone and three opioids: fentanyl, morphine and oxycodone. Drug combinations were assessed for increased antinociceptive effects. An increase in antinociceptive effects might be paralleled by an increase in sedative effects. Therefore drug combinations were assessed for increased sedative effects as well.

The experiments described in this chapter also investigated whether the enhancement of opioid antinociception by neurosteroids involved spinal cord $GABA_A$ receptors. Rats with intrathecal catheters were tested for antinociception using ECT measured at both the neck and tail. The administration of IP agonists (opioids and neurosteroids) would be expected to cause an increase in ECT values at both the neck and tail. A $GABA_A$ receptor selective antagonist, bicuculline, was administered intrathecally to test the involvement of spinal cord $GABA_A$ receptors. If spinal cord $GABA_A$ receptors were involved one would expect reversal of the antinociception observed in the tail but not the neck, since the $GABA_A$ antagonist would have been delivered intrathecally to act at the most caudal segments of the spinal cord responsible for tail innervation.

4.2 Methods

Experiments were performed in male Wistar rats. All opioids and neurosteroids were administered IP. Opioids alone and in combination with alphadolone were assessed for antinociceptive effects using the PP and TFL tests. Combinations of opioids and alphaxalone were assessed for antinociceptive effects with the TFL test. Sedative effects were measured with the activity monitor and rotarod. Increased antinociceptive effects were tested with IT bicuculline (selective $GABA_A$ receptor antagonist) and assessed by TFL response. The confinement of IT bicuculline to the spinal cord was determined using the ECT test measured at the neck and tail. All experiments were performed in a blinded manner. All experimental procedures and data transformation are described in Chapter 2.

4.2.1 Study Drugs

Alphadolone (alphadolone acetate, Jurox, Rutherford, NSW) and alphaxalone (Jurox, Rutherford, NSW) were combined with a complexing agent, 2-hydroxypropyl-β-cyclodextrin (HPβCD) to create a powder more soluble in aqueous solution. Alphadolone was weighed out fresh each day and dissolved in a phosphate buffer solution. Alphaxalone was delivered as a known weight of powder in a vial (10mg/ml), requiring the accompanying Alfaxan-CD diluent for reconstitution. Fentanyl (fentanyl citrate USP – David Bull Laboratories), morphine (morphine sulphate – David Bull Laboratories) and oxycodone (oxycodone hydrochloride tablets – Endone, Boots Healthcare) were diluted in normal saline fresh each day for IP administration.

All doses in mg/kg were made in a volume of 1ml calculated for a 200g rat. The average weight of rats was 180-200g so each syringe was slightly adjusted in volume, and therefore dose, to allow for the small weight differences between rats so rats received accurate doses of drug tailored to their specific weight.

A stock solution of bicuculline (bicuculline methiodide, Sigma Chemical Co., St. Louis, USA) was prepared; powder was weighed out and diluted in normal saline to be stored in the freezer. Bicuculline (50pmol) was administered intrathecally (IT) via a surgically implanted catheter. A volume of 5µl was injected IT for each rat.

4.2.2 Experimental Paradigm

The test paradigm used for this chapter is outlined in Figure 4.1 on page 133. The range of doses of each drug used is given in Table 4.1 on page 134. Replicate experiments were performed for each dose in several different rats. All experiments in this series were performed in a blinded manner.

(a) Opioids Alone: Sedation and Antinociception

Opioid sedative effects were determined using the rotarod and the activity monitor. Only non-sedating doses were used for further nociceptive testing. Dose response relationships for antinociceptive effects were measured for all opioids using PP and TFL tests.

(b) Opioids in Combination with Alphadolone: Antinociception

A dose of alphadolone that caused no antinociception when given alone was selected and combined with increasing doses of each opioid. For PP testing, a dose of alphadolone 1mg/kg was used for combination experiments, as it was the highest dose that caused no significant antinociceptive effects when given alone (refer to Data Analysis, page 135). For TFL testing a dose of alphadolone 10mg/kg was used in combination experiments, also being the highest non-sedating dose that caused no significant antinociceptive effects in the TFL test when given alone (refer to Data Analysis, page 135).

(c) Opioids in Combination with Alphaxalone: Antinociception

Alphaxalone at a dose of 10mg/kg, which has no antinociceptive effects when given alone, was tested in combination with a high and low dose of each opioid. This measured any increased antinociceptive effects assessed with the TFL test.

(d) Experiments on Rats with IT Catheters

Rats with implanted intrathecal catheters (n=80) were used for intrathecal antagonist experiments (refer to Chapter 2, page 46). A dose of each opioid in combination with alphadolone (10mg/kg), which caused an increase in antinociceptive effects compared with the antinociceptive effects of that opioid dose given alone, was selected for these experiments (fentanyl 5µg/kg, morphine 1.6mg/kg and oxycodone 0.25mg/kg). In these

experiments, after the administration of alphadolone and opioid, bicuculline (50pmol), a selective $GABA_A$ receptor antagonist was administered via the intrathecal route to investigate the involvement of spinal cord $GABA_A$ receptors in enhanced opioid antinociception by alphadolone. The antinociceptive tests performed in these experiments involved measurement of neck and tail ECT responses, as well as TFL testing. Accurate confinement of IT bicuculline to the spinal cord was determined using ECT assessed at neck and tail sites. A reversal of the ECT response at the tail but *not* the neck would indicate (1) confinement of IT bicuculline within the spinal cord without any rostral spread and (2) the involvement of $GABA_A$ receptors in the observed antinociceptive response. The completion of further experiments were required using a lower dose of alphadolone (0.1mg/kg) with IT bicuculline in order to tease out the involvement of spinal $GABA_A$ receptors by showing a clear reversal of tail threshold rises following drug administration.

(e) Opioids in Combination with Alphadolone: Sedation

Combinations of doses of alphadolone and opioids that caused increased antinociceptive effects, compared with the effect of either drug alone, were tested for a concurrent increase in sedative effects assessed with the rotarod and activity monitor.

Figure 4.1 Experimental Paradigm

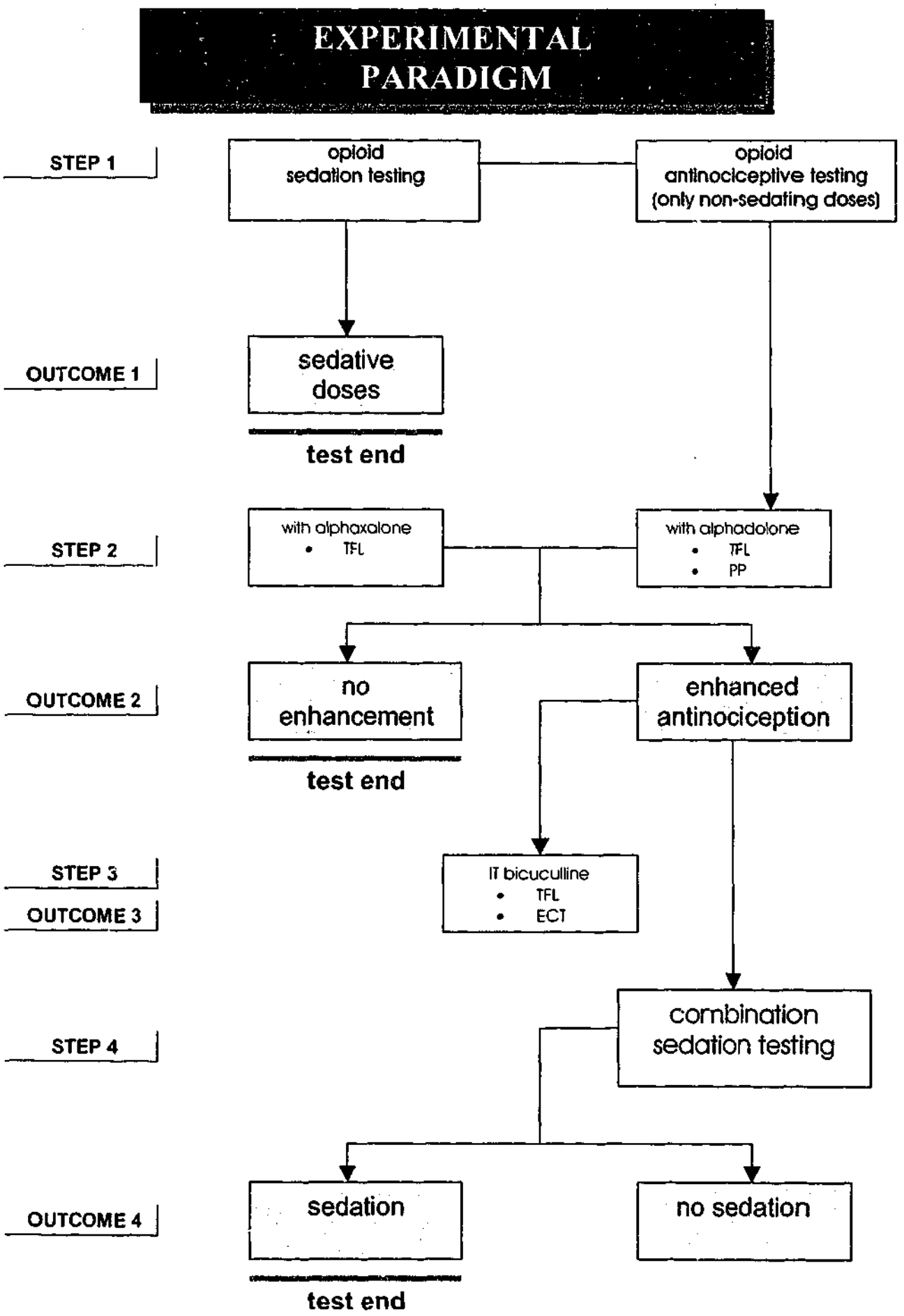


Table 4.1 Dose Ranges of IP Opioids Given Alone and in Combination with IP Neurosteroids for Antinociception and Sedation

Behaviour Model	Neurosteroid In combination	Fentanyl (µg/kg)	Morphine (mg/kg)	Oxycodone (mg/kg)
Rotarod	none	2.5-80 *n=5-8*	0.4-12.8 *n=5-8*	0.125-2 *n=5-8*
Activity Monitor	none	2.5-80 *n=5-10*	0.4-12.8 *n=5-8*	0.125-2 *n=5*
PP	alphadolone 1mg/kg	2.5-20 *n=5-8*	0.4-3.2 *n=7-11*	0.125-1 *n=5-12*
TFL	alphadolone 10mg/kg	2.5-40 *n=6-21*	0.2-3.2 *n=6-16*	0.0625-1 *n=5-12*
TFL	alphaxalone 10mg/kg	5 & 20 *n=5*	0.8 & 1.6 *n=5*	0.25 & 0.5 *n=5*
ECT & TFL (IT bicuculline)	alphadolone 10mg/kg	5 *n=9*	1.6 *n=12*	0.25 *n=10*
ECT & TFL (IT bicuculline)	alphadolone 0.1mg/kg	5 *n=5*	1.6 *n=5*	0.25 *n=5*
Rotarod	alphadolone 10mg/kg	5 *n=5*	3.2 *n=5*	0.25 *n=5*
Activity Monitor	alphadolone 10mg/kg	5 *n=5*	3.2 *n=5*	0.25 *n=5*

n denotes number of replicate experiments at each dose

4.2.3 Data Analysis

Run time values for the rotarod were combined for each dose of drug when given alone and in drug combinations and expressed as means ± SD. These data were compared with the control maximum run time of 120 seconds using a *t*-test. Rest time results from the activity monitor were combined for each drug dose given alone or drug combination and expressed as means ± SD. Each group was compared statistically with a matched vehicle-control group by means of a *t*-test.

Doses of neurosteroids that caused no antinociceptive effects when given alone in TFL and PP tests were determined from previous work described in Chapter 3 (page 105). That dose for PP response was determined statistically by comparison with the value 1 (*t*-test) and in a similar manner; the dose for TFL response was determined statistically by comparison with the value of zero (*t*-test). For nociceptive dose response curves, the PP responses (r) and TFL values (%MPE) were combined for each dose of drug or drug combination and plotted as means ± SEM. The responses for each of the opioid doses were compared for antinociceptive effects with the value 1 and zero for PP and TFL respectively, using a *t*-test. The responses to each opioid dose given alone were compared with the same opioid dose given in combination with the neurosteroid, again using a *t*-test.

For the effect of IT bicuculline on the changes in nociceptive thresholds caused by drug combinations, responses for TFL (%MPE) were combined and plotted as means ± SEM. The TFL response after IT bicuculline administration was compared with the TFL response of the respective opioid dose when given alone, using a *t*-test for

statistical testing. This showed whether the effect of alphadolone, which is capable of reversal by IT bicuculline, was responsible for increasing opioid antinociception. Following the administration of IT bicuculline, the response of the combination of alphadolone and opioids should be reduced to the level of antinociception caused by the respective opioids when given alone.

For all statistical comparisons, a value of $p \leq 0.05$ was considered statistically significant.

4.3 Results

4.3.1 The Sedative Properties of Intraperitoneal Opioids

Each opioid caused dose dependent sedative effects assessed by the rotarod. Sedative doses caused a significant decrease in run time values compared with that of vehicle-treated rats (normal run time responses of 120 seconds). All opioids caused dose dependent sedative effects assessed by the activity monitor. Sedative doses caused a significant increase in rest time values compared with that of matched vehicle-treated rats. All sedative doses were excluded from further nociceptive testing. These results are shown in Table 4.2 on page 143.

4.3.2 The Antinociceptive Properties of Intraperitoneal Opioids Assessed by Paw Pressure Response: Alone and in Combination with Alphadolone

These results are shown in Figure 4.2 on page 145. All opioids were tested at non-sedating doses. Morphine 3.2mg/kg caused significant antinociceptive effects assessed by PP response (p=0.0078, *t*-test). Oxycodone 1mg/kg also caused significant antinociceptive effects assessed by PP response (p=0.003, *t*-test), however fentanyl caused no significant antinociceptive effects (*t*-test). All three opioids were tested in combination with alphadolone (1mg/kg). This dose of alphadolone caused no significant antinociceptive effects when given alone (refer to Chapter 3, page 105). The

addition of 1mg/kg alphadolone had no affect on any of the three opioids in this pain model (*t*-test).

4.3.3 The Antinociceptive Properties of Intraperitoneal Opioids Assessed by Tail Flick Latency: Alone and in Combination with Intraperitoneal Alphadolone

These results are shown in Figure 4.3 on page 147. All opioids showed dose dependent antinociceptive effects assessed by the TFL test for the non-sedating doses tested. The dose of alphadolone (10mg/kg) chosen for combination studies was shown previously not to affect TFL thresholds (Chapter 3, page 105).

The addition of alphadolone 10mg/kg to fentanyl 5µg/kg significantly elevated the TFL response compared with fentanyl 5µg/kg alone (p=0.0043, *t*-test). All other responses to combinations of fentanyl and alphadolone were not significantly different to the TFL responses to the same fentanyl doses given alone.

The addition of alphadolone 10mg/kg to morphine 1.6 and 3.2mg/kg significantly elevated the TFL response compared with these doses of morphine given alone (p=0.05 and p=0.025, *t*-test). All other responses to combinations of morphine and alphadolone were not significantly different to those obtained with the same morphine doses given alone.

The addition of alphadolone 10mg/kg to oxycodone 0.25mg/kg significantly elevated the TFL response compared with oxycodone 0.25mg/kg alone (p=0.04, *t*-test). All other dose combinations led to antinociceptive effects that were not significantly different to those obtained with the same oxycodone doses given alone.

4.3.4 The Antinociceptive Properties of Intraperitoneal Opioids Assessed by Tail Flick Latency: Alone and in Combination with Intraperitoneal Alphaxalone

Opioids were tested in combination with the maximum non-sedating dose of alphaxalone (10mg/kg). This dose of alphaxalone had no significant antinociceptive effects assessed by TFL responses (Chapter 3, page 105). Neither a low nor high dose of each opioid co-administered with alphaxalone caused a significant increase in antinociceptive effects (*t*-test). These results are shown in Figure 4.4 on page 149.

4.3.5 Intrathecal Bicuculline Reversal of Potentiated Antinociceptive Effects: Alphadolone and Opioids

These results are seen in Figure 4.5 on page 151. The increased antinociceptive effects produced by the co-administration of each dose of opioid (fentanyl 5µg/kg, morphine 1.6mg/kg and oxycodone 0.25mg/kg) with alphadolone 10mg/kg were entirely reversed by IT bicuculline (50pmol), i.e. the TFL responses for the drug combinations were blocked such that they were not significantly different from the TFL response of each

opioid given alone (*t*-test). Using the same dose combinations, ECT neck and tail measurements were taken. All neck and tail ECT values were significantly increased after the co-administration of alphadolone and opioids (*t*-test). It was expected that IT bicuculline would cause a change in tail ECT but not affect neck ECT values. Neither the neck or tail ECT responses were reversed by IT bicuculline at the doses recorded.

Experiments with a lower dose of alphadolone (0.1mg/kg) were tested with IT bicuculline. These results are seen in Figure 4.6 on page 153. No increased antinociception was observed for the TFL response. The administration of alphadolone and opioids caused an increase in ECT neck and tail values. Furthermore, the neck ECT response was not reversed by IT bicuculline at the doses recorded. However the increase in tail ECT was reversed entirely (*t*-test).

All neck ECT data from these animals are tabulated in Appendix C (page 271) and show that the thresholds after IT bicuculline did not change.

4.3.6 The Sedative Properties of Intraperitoneal Opioids in Combination with Intraperitoneal Alphadolone

The co-administration of alphadolone with opioids, which demonstrated a potentiation of antinociceptive activity, was assessed for a concurrent increase in sedative effects using the rotarod and activity monitor. Both the rotarod and activity monitor showed all test-drug combinations caused no significant sedation. The drug combinations assessed by the rotarod all displayed run time responses that were no different to that of vehicle-

treated rats (run time responses of 120 seconds). All test-drug combinations assessed by the activity monitor also caused no significant increases in rest time values compared with matched vehicle-treated rats. These results are shown in Table 4.3 on page 155.

Table 4.2

These tables summarise the sedative action of opioids assessed by the rotarod and activity monitor.

(A) Rotarod

Run time values are shown in seconds as means ± SD for each dose. All opioids caused dose dependent sedative effects assessed by the rotarod. Run time values of test-rats were compared with vehicle-treated rats with a control run time value of 120 seconds. Doses that caused a significant decrease in run time values were considered sedative ($p \leq 0.05$, *t*-test).

(B) Activity Monitor

Rest time values are shown in seconds as means ± SD for each dose. All opioids showed dose dependent sedative effects assessed by the activity monitor. Rest time values were compared with vehicle-treated rats with a control rest time value of 960 seconds ± 45 (*n*=80). Doses that caused a significant increase in rest time values were considered sedative ($p \leq 0.05$, *t*-test).

Table 4.2 The Sedative Properties of IP Opioids

(A)

fentanyl		morphine		oxycodone	
runtime (secs)		runtime (secs)		runtime (secs)	
dose (μg/kg)	mean ± SD	*dose (mg/kg)*	mean ± SD	*dose (mg/kg)*	mean ± SD
2.5	120 ± 0 *n=5*	*0.4*	120 ± 0 *n=5*	*0.125*	120 ± 0 *n=5*
5	120 ± 0 *n=5*	*0.8*	120 ± 0 *n=5*	*0.25*	120 ± 0 *n=5*
20	112 ± 25 *n=8*	*1.6*	111 ± 17 *n=8*	*0.5*	105 ± 31 *n=8*
40	105 ± 42 *n=8*	*3.2*	40 *n=8*	*1*	110 ± 17 *n=8*
80	110 ± 28 *n=8*	*6.4*	77 ± 37* *n=8*	*2*	49 ± 40* *n=8*
		12.8	48 ± 51* *n=8*		

* denotes statistical significance
n denotes number of replicate experiments at each dose

(B)

fentanyl		morphine		oxycodone	
rest time (secs)		rest time (secs)		rest time (secs)	
dose (μg/kg)	mean ± SD	*dose (mg/kg)*	mean ± SD	*dose (mg/kg)*	mean ± SD
vehicle control	960 ± 45	*vehicle control*	960 ± 45	*vehicle control*	960 ± 45
2.5	815 ± 22 *n=5*	*0.4*	800 ± 40 *n=5*	*0.125*	773 ± 58 *n=5*
5	851 ± 76 *n=5*	*0.8*	912 ± 67 *n=5*	*0.25*	856 ± 78 *n=5*
20	861 ± 59 *n=8*	*1.6*	908 ± 49 *n=5*	*0.5*	952 ± 38 *n=5*
40	1097 ± 108* *n=10*	*3.2*	961 ± 82 *n=5*	*1*	1016 ± 112 *n=5*
80	1114 ± 57* *n=10*	*6.4*	1026 ± 54 *n=8*	*2*	1117 ± 56* *n=5*
		12.8	1091 ± 136* *n=8*		

* denotes statistical significance
n denotes number of replicate experiments at each dose

Figure 4.2

(A) Fentanyl

This graph shows the antinociceptive effect of increasing doses of fentanyl on PP response in rats. Fentanyl alone caused no significant antinociceptive effects (*t*-test). In addition, none of these effects were significantly increased by the addition of alphadolone 1mg/kg to any of the doses (*t*-test). Each point represents means for 5-8 rats ± SEM.

(B) Morphine

This graph shows the antinociceptive effect of increasing doses of morphine on PP response in rats. Morphine 3.2mg/kg alone caused significant antinociceptive effects (p=0.0078, *t*-test). In combination with alphadolone 1mg/kg, none of these effects were significantly increased at any dose of morphine (*t*-test). Each point represents means for 7-11 rats ± SEM.

(C) Oxycodone

This graph shows the antinociceptive effect of increasing doses of oxycodone on PP response in rats. Oxycodone 1mg/kg given alone caused significant antinociceptive effects (p=0.003, *t*-test). In combination with alphadolone 1mg/kg, none of the effects were significantly increased at any dose of oxycodone (*t*-test). Each point represents means for 5-12 rats ± SEM.

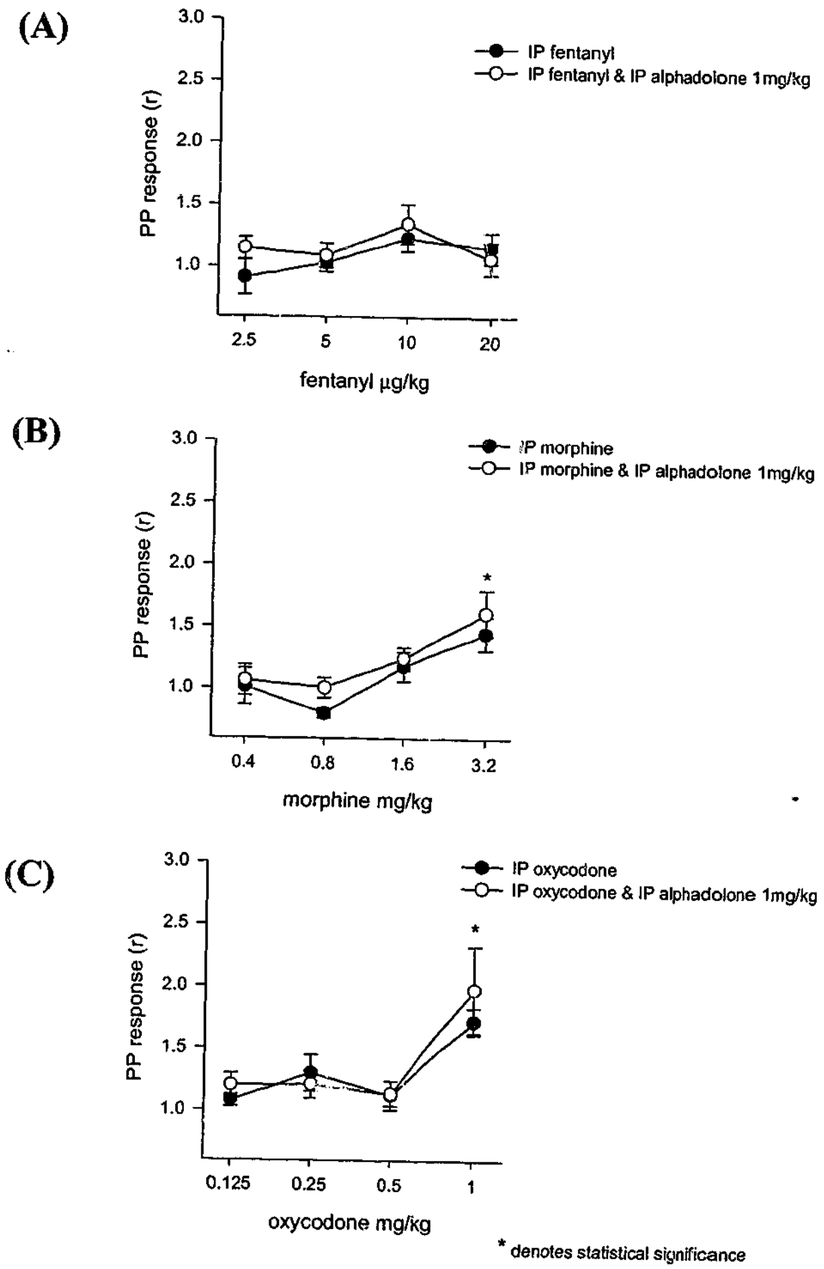


Figure 4.2 The Antinociceptive Properties of IP Opioids Assessed by PP Response: Dose Response Curves for the Opioid Given Alone and in Combination with Alphadolone

Figure 4.3

(A) Fentanyl

This graph shows the antinociceptive effect of increasing doses of fentanyl assessed by TFL response. Fentanyl alone caused dose dependent antinociceptive effects. In combination with alphadolone 10mg/kg, the TFL response to fentanyl was significantly increased at a dose of 5µg/kg (p=0.0043, *t*-test). The antinociceptive responses following other dose combinations were not significantly different from those caused by the same fentanyl doses given alone. Each point represents means for 6-21 rats ± SEM.

(B) Morphine

This graph shows the antinociceptive effect of increasing doses of morphine on TFL response. Morphine alone caused dose dependent antinociceptive effects. In combination with alphadolone 10mg/kg, the TFL response to morphine was significantly increased at doses of 1.6 and 3.2mg/kg (p=0.05 and p=0.025, *t*-test). Each point represents means for 6-16 rats ± SEM.

(C) Oxycodone

This graph shows the antinociceptive effect of increasing doses of oxycodone on TFL response. Oxycodone alone caused dose dependent antinociceptive effects. In combination with alphadolone 10mg/kg, the TFL response to oxycodone was significantly increased at a dose of 0.25mg/kg (p=0.04, *t*-test). Each point represents means for 5-12 rats ± SEM.

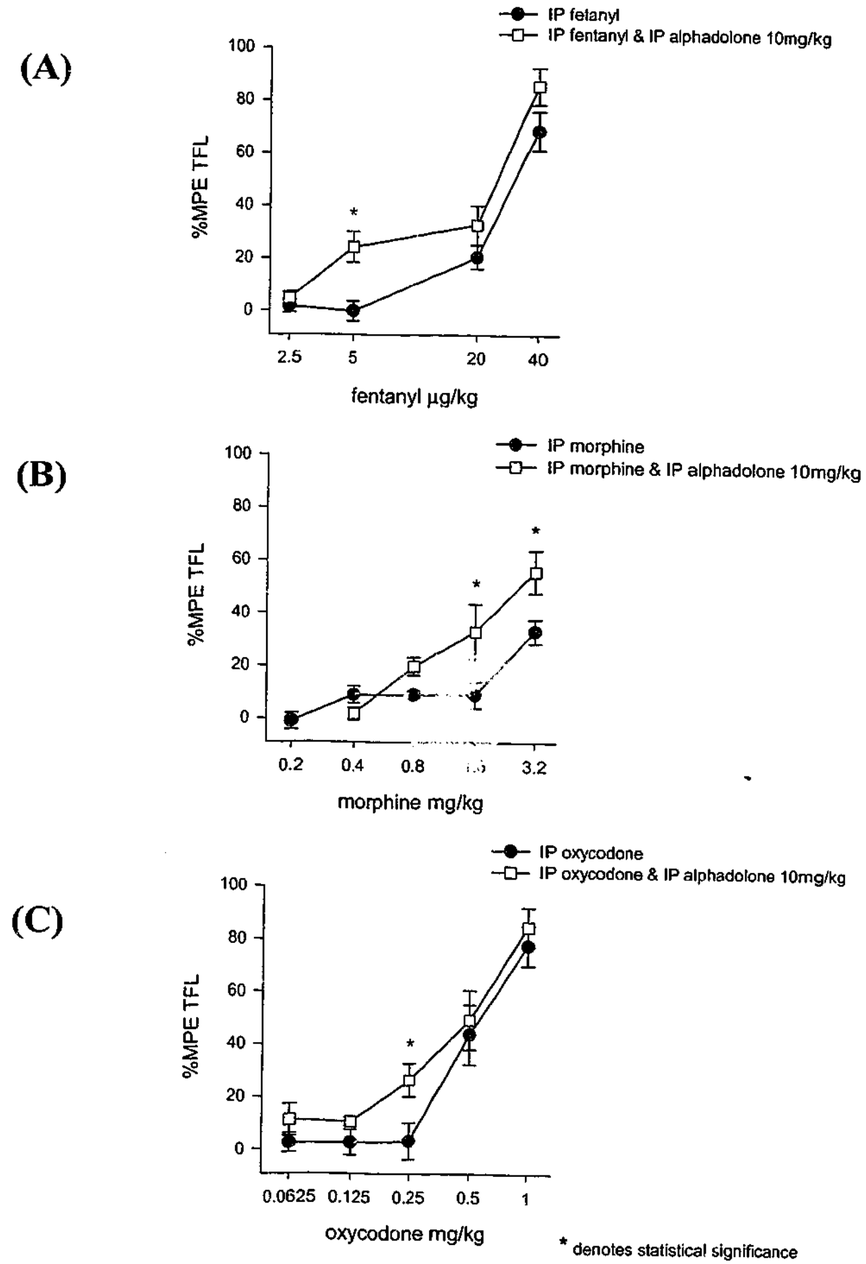


Figure 4.3 The Antinociceptive Effects of IP Opioids on TFL: Dose Response Curves for Opioids Given Alone and in Combination with IP Alphadolone

Figure 4.4

(A) Fentanyl

This bar graph shows the antinociceptive effect of fentanyl 5µg/kg and 20µg/kg alone and in the presence of 10mg/kg alphaxalone assessed by TFL response. There was no significant increase in antinociceptive effects at either dose of fentanyl co-administered with alphaxalone (*t*-test). Each histogram represents means for 5 rats ± SEM.

(B) Morphine

This bar graph shows the antinociceptive effect of morphine 1.6mg/kg and 3.2mg/kg alone and in the presence of 10mg/kg alphaxalone assessed by TFL response. There was no significant increase in antinociceptive effects at either dose of morphine co-administered with alphaxalone (*t*-test). Each histogram represents means for 5 rats ± SEM.

(C) Oxycodone

This bar graph shows the antinociceptive effect of oxycodone 0.25mg/kg and 0.5mg/kg alone and in the presence of 10mg/kg alphaxalone assessed by TFL response. There was no significant increase in antinociceptive effects at either dose of oxycodone co-administered with alphaxalone (*t*-test). Each histogram represents means for 5 rats ± SEM.

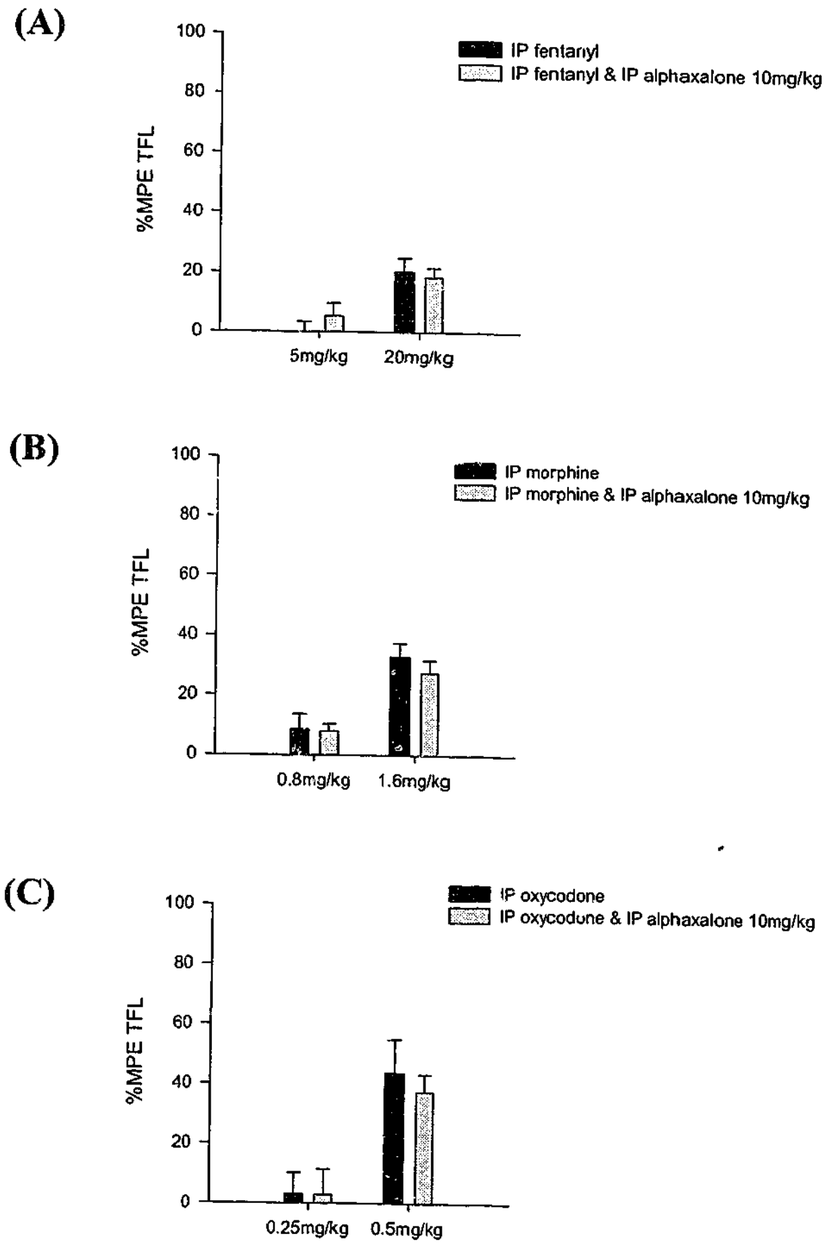


Figure 4.4 The Antinociceptive Effects of IP Opioids Assessed with TFL: Alone and in Combination with IP Alphaxalone

Figure 4.5

(A) Fentanyl and Alphadolone

After IT bicuculline administration, the *increased* TFL response for the combined effects of alphadolone (10mg/kg) and fentanyl (5µg/kg) was reversed; the TFL response was not significantly different compared with fentanyl given alone (*t*-test). The tail ECT response for the combined effects of alphadolone and fentanyl was not reversed by IT bicuculline. Each histogram represents means for 9 rats ± SEM.

(B) Morphine and Alphadolone

After IT bicuculline administration, the *increased* TFL response for the combined effects of alphadolone (10mg/kg) and morphine (1.6mg/kg) was reversed; the TFL response was not significantly different compared with morphine given alone (*t*-test). The tail ECT response for the combined effects of alphadolone and morphine was not reversed by IT bicuculline. Each histogram represents means for 12 rats ± SEM.

(C) Oxycodone and Alphadolone

After IT bicuculline administration, the *increased* TFL response for the combined effects of alphadolone (10mg/kg) and oxycodone (0.25mg/kg) was reversed; the TFL response was not significantly different compared with oxycodone given alone (*t*-test). The tail ECT response for the combined effects of alphadolone and oxycodone was not reversed by IT bicuculline. Each histogram represents means for 10 rats ± SEM.

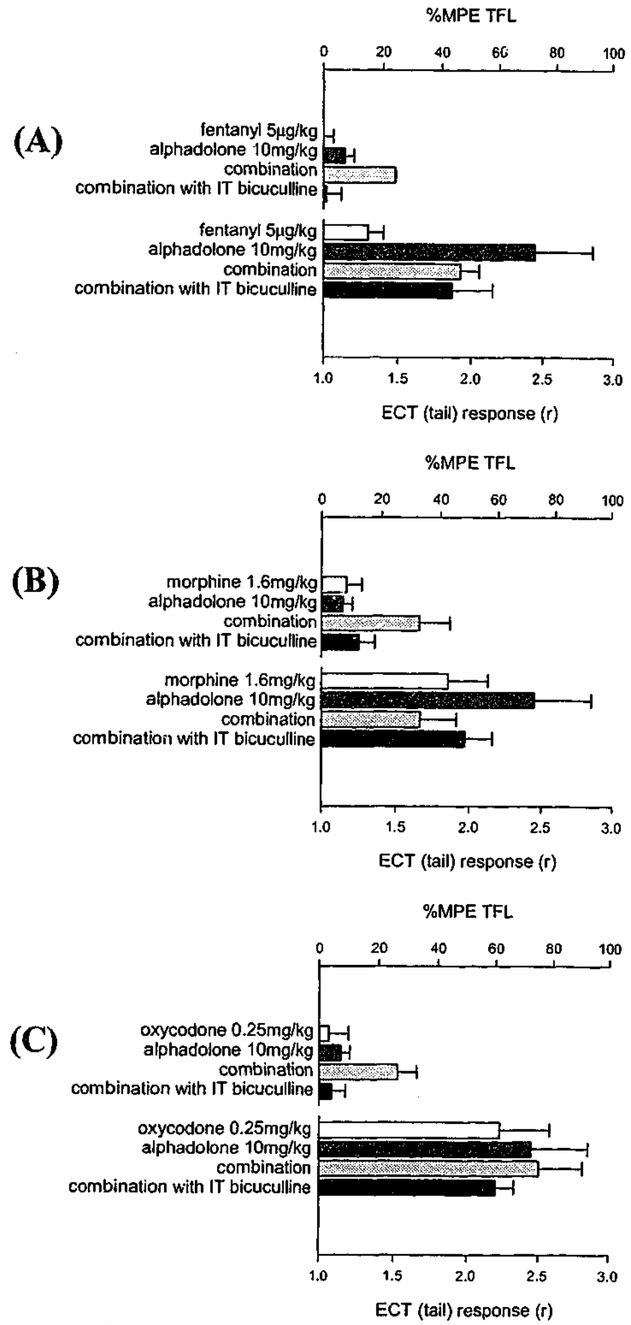


Figure 4.5 Testing for Reversal by IT Bicuculline; Antinociceptive Effects Caused by Combinations of IP Opioids and IP Alphadolone 10mg/kg

Figure 4.6

(A) Fentanyl and Alphadolone

There was no increased effect for the TFL response for the combined effects of alphadolone (0.1mg/kg) and fentanyl (5µg/kg). The increased tail ECT response for the combined effects of alphadolone and fentanyl was reversed by IT bicuculline. Each histogram represents means for 5 rats ± SEM.

(B) Morphine and Alphadolone

There was no increased effect for the TFL response for the combined effects of alphadolone (0.1mg/kg) and morphine (1.6mg/kg). The increased tail ECT response for the combined effects of alphadolone and morphine was reversed by IT bicuculline. Each histogram represents means for 5 rats ± SEM.

(C) Oxycodone and Alphadolone

There was no increased effect for the TFL response for the combined effects of alphadolone (0.1mg/kg) and oxycodone (0.25mg/kg). The increased tail ECT response for the combined effects of alphadolone and oxycodone was reversed by IT bicuculline. Each histogram represents means for 5 rats ± SEM.

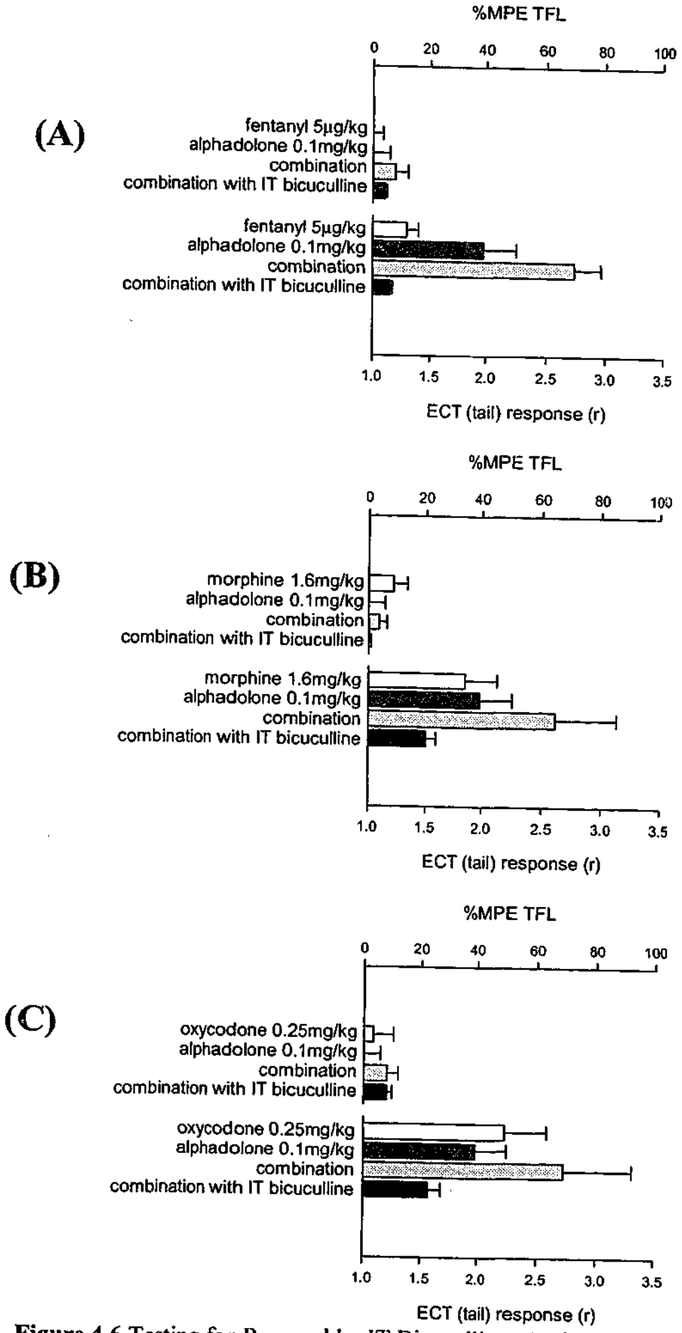


Figure 4.6 Testing for Reversal by IT Bicuculline; Antinociceptive Effects Caused by Combinations of IP Opioids and IP Alphadolone 0.1mg/kg

Table 4.3

(A) Rotarod

This table summarises the investigation of sedative effects for the combination of opioids fentanyl 5µg/kg, morphine 3.2mg/kg and oxycodone 0.25mg/kg with alphadolone 10mg/kg assessed by the rotarod. There was no significant difference in run time values for test-drug groups compared with that of vehicle-treated (120 seconds) (*t*-test).

(B) Activity Monitor

This table summarises the investigation of sedative effects for the combination of opioids fentanyl 5µg/kg, morphine 3.2mg/kg and oxycodone 0.25mg/kg with alphadolone 10mg/kg assessed by the activity monitor. There was no significant difference in rest time values for test-drug groups compared with that of vehicle-treated (*t*-test).

Table 4.3 The Sedative Properties of IP Opioids in Combination with IP Alphadolone

(A)

drug dose	fentanyl 5µg/kg runtime mean (secs) ± SD	morphine 3.2mg/kg runtime mean (secs) ± SD	oxycodone 0.25mg/kg runtime mean (secs) ± SD
co-administered with alphadolone 10mg/kg	102 ± 25 *n=10*	114 ± 19 *n=10*	116 ± 12 *n=10*

n denotes number of replicate experiments at each dose

(B)

drug dose	fentanyl 5µg/kg rest time mean (secs) ± SD	morphine 3.2mg/kg rest time mean (secs) ± SD	oxycodone 0.25mg/kg rest time mean (secs) ± SD
control rest time	936 ± 18	1064 ± 22	1064 ± 22
co-administered with alphadolone 10mg/kg	889 ± 83 *n=5*	1062 ± 18 *n=5*	932 ± 172 *n=5*

n denotes number of replicate experiments at each dose

4.4 Discussion

Experiments reported in this chapter showed a dose of alphadolone, which had no antinociceptive effect when given alone, was able to enhance opioid antinociception assessed by the TFL test. By contrast the PP test showed no such interaction. Furthermore no increased antinociception assessed by TFL was observed when alphaxalone was co-administered with opioids. Alphadolone enhanced opioid antinociception, which was reversed by IT bicuculline, a selective $GABA_A$ receptor antagonist. The increased antinociceptive effects for co-administered alphadolone and opioids occurred without a concomitant increase in sedative effects.

4.4.1 Multimodal Analgesia

It is now accepted that pain is not the simple activation of a single specific isolated signaling system, rather it is the product of a number of pathways in parallel that are subject to a series of controls acting in the context of a whole integrated nervous system (Wall and Melzack 1989). It has become more relevant to combine different compounds acting on several of these mechanisms to provide effective analgesia with minimal side effects. This approach has been developed as a result of a better understanding of the complex pathophysiology of pain. The reduction of adverse side effects by decreasing the fractional dosage of component analgesics, particularly opioids, has improved the quality of analgesia by the use of a more balanced approach to pain management.

Different investigators have studied the interactions of opioids with various drugs such as local anaesthetics, NSAIDs, NMDA antagonists and α_2-adrenoceptor agonists. More recently a link between opioids and drugs acting at $GABA_A$ receptors has been investigated. Some studies with the benzodiazepine midazolam have shown a potentiation of opioid antinociceptive effects; intrathecal midazolam caused an increase in antinociceptive effects of several opioids assessed by both the hot-plate and TFL tests in the rat (Luger, Hayashi et al. 1995; Rattan, McDonald et al. 1991; Yanez, Sabbe et al. 1990). Another known $GABA_A$ receptor agonist, muscimol, when injected intrathecally, was also able to increase the antinociceptive effects of morphine assessed by the TFL test (Hara, Saito et al. 1999). However intrathecal muscimol causes paralysis (Nakamura, Kakinohana et al. 2002) and therefore these results need to be interpreted with caution. These experiments demonstrated the potential for multimodal therapies that combine opioids and drugs acting at $GABA_A$ receptors. As some neurosteroids have been shown to positively modulate $GABA_A$ receptors in the CNS (Lambert, Belelli et al. 1995), it is possible an interaction may exist between neurosteroids and opioids. There exist no published literature on investigations studying increased opioid antinociception caused by neurosteroid analgesics. However some neurosteroids have been shown to affect opioid tolerance. For example several pre-existing endogenous neurosteroids like allopregnanolone, pregnenolone sulphate or dehydroepiandrosterone sulphate have been shown to prevent morphine tolerance (Reddy and Kulkarni 1997). Such neurosteroids have not been implicated in analgesia but rather behavioural activities such as mood alteration, learning and memory (Barrot, Vallee et al. 1999; Rouge-Pont, Mayo et al. 2002). These neurosteroids caused no antinociceptive effects alone but when co-administered with morphine they caused

normal TFL responses to opioid administration after the induction of tolerance to the opioid. The positive interaction of these neurosteroids and morphine suggests the possible role of neurosteroids and opioids in the development of new multimodal therapies.

Recent studies discovered the neurosteroid alphadolone caused antinociception in rats (Nadeson and Goodchild 2001). This work instigated a pilot clinical study using patients receiving morphine via a patient-controlled analgesia machine. Of these patients a group received alphadolone whilst another group received placebo. The alphadolone-treated group showed a reduced morphine requirement after surgery as well as improved pain scores even in the presence of lower morphine use (Goodchild, Robinson et al. 2001). Although this work could not conclude a direct interaction between morphine and alphadolone it demonstrated a morphine-sparing effect. The improved pain relief might be indicative of an interaction between alphadolone and morphine. The experiments described in this chapter provide more evidence for this interaction between opioids and neurosteroids.

4.4.2 Alphadolone Enhancement of Opioid Antinociception: a Spinally Mediated Effect

The term *potentiation* refers to the "combination of an ineffective dose of one drug with an effective dose of another drug to create a greater effect then either drug given alone" (Dickenson and Sullivan 1993). An ineffective dose of alphadolone i.e. a dose that caused no effect when given alone was selected for the combination experiments.

Previous experiments showed that non-sedating doses of alphadolone caused no antinociceptive effects assessed by TFL response (Chapter 3, page 105). The highest non-sedating dose of alphadolone 10mg/kg was used to maximize the chances of observing any positive interaction between alphadolone and the opioids. Any significant increase in opioid antinociception due to the co-administration of alphadolone can be considered potentiation since alphadolone has no effect on TFL measurements when given alone. Thus for the TFL test, alphadolone was shown to potentiate the antinociceptive effects of all three opioids. These effects were only seen for low opioid doses. Alphadolone might only display its capacity for opioid potentiation at these low doses, because higher doses of opioids when given alone were capable of producing their own potent antinociceptive effects.

Past experiments have shown alphadolone interacts with spinal cord $GABA_A$ receptors even when given by a non-spinal route (Nadeson and Goodchild 2000). Experiments described in this chapter investigated whether the potentiation that occurred between alphadolone and opioids was mediated by spinal cord $GABA_A$ receptors. Antinociceptive effects were measured using the TFL test. A dose of each opioid that caused no antinociception when given alone, but caused increased antinociception when combined with alphadolone 10mg/kg, was selected for testing with IT bicuculline. Assessed by TFL, the administration of IT bicuculline completed reversed the potentiated antinociceptive effects for all three opioids. Therefore the potentiation of opioid antinociception by alphadolone must involve an interaction with spinal cord $GABA_A$ receptors.

4.4.3 Segmental Effects in Nociceptive Testing

Alphadolone causes antinociceptive effects that can be assessed by ECT. These experiments are shown in Chapter 3 (page 105). Past experiments have shown that the administration of a single dose of alphadolone via a non-spinal route caused an increase in ECT response that was entirely reversed by IT bicuculline (Nadeson and Goodchild 2000). The experiments involving IT bicuculline described in this chapter measured ECT at both the neck and tail of every rat. The ECT measurements were completed in parallel with TFL measurements at each test point. The primary function of the ECT test in this series of experiments was to determine whether drug interactions occurred in the spinal cord. If the co-administration of IP alphadolone 10mg/kg and each opioid causes an increase in ECT values, *and* this is due to actions at the level of the spinal cord, then the administration of IT bicuculline would be expected to reverse these antinociceptive effects but only in tail ECT and not in the neck. This would occur because IT bicuculline as a selective $GABA_A$ receptor antagonist was injected onto, and *restricted* to acting on the most caudal segments of the spinal cord responsible for tail innervation. Unfortunately these experiments with alphadolone 10mg/kg did not produce the expected results after IT bicuculline administration. Although IT bicuculline was capable of reversing antinociceptive effects assessed by TFL response, tail ECT remained unaffected.

The difference between the effect of bicuculline on the combined ECT and TFL responses needs discussion. Clearly the dose of opioids used in these experiments caused little effect on TFL response when given alone, and it was established in an earlier chapter (refer to Chapter 3, page 105) that alphadolone alone caused no effect on

TFL. Reversal of the reported increased opioid/alphadolone effect on TFL by bicuculline suggests that spinal cord $GABA_A$ receptors are involved with this effect. On the other hand, with respect to the ECT test, both opioids and alphadolone when given alone caused significant antinociceptive effects at the doses used in those combination experiments. Firstly, this means that only an additive effect (rather than clear potentiation) could be shown with the ECT test. Secondly, since opioids themselves have an action when given alone, the ECT effect of these compounds would not be expected to be reversed by a selective $GABA_A$ receptor antagonist. It was intended to use the differential effect of IT bicuculline on tail ECT compared with neck to prove a spinal cord $GABA_A$ mechanism for these interactions, like others have done so in the past (Crawford, Jensen et al. 1993; Nadeson, Guo et al. 1996). However, the increase in TFL response occurred at doses of alphadolone and opioids that caused supramaximal ECT effects. Therefore these same experiments were repeated using a lower dose of alphadolone (0.1mg/kg). When bicuculline was administered in these experiments, the tail ECT response was reduced to the value produced by the administration of opioid alone, whereas the neck ECT response remained unchanged. Thus there was an additive effect between the antinociception caused by alphadolone and each opioid, and bicuculline was capable of reversing the neurosteroid component of this additive effect. Using a lower dose of alphadolone 0.1mg/kg, such a reversal *was* seen for the tail ECT additive effects, suggesting that spinal cord $GABA_A$ receptors are involved.

The measurement of neck ECT values was used to determine any rostral spread of the intrathecally administered drug. Any experiments that showed an animal to have decreased neck ECT values after IT bicuculline were excluded from collated data. Neck

ECT data from all rats are tabulated in Appendix C (page 271) and show that the thresholds after IT bicuculline did not change.

4.4.4 Antinociception Without Sedation

The dose combinations of alphadolone and opioids that displayed enhanced antinociception were tested for sedative effects using the rotarod and activity monitor. Both behavioural models showed no significant sedative effects. Therefore this suspected spinal cord interaction between alphadolone and opioids may be a potential way to target nociceptive pathways without causing sedative side effects. In clinical medicine, drugs are administered epidurally or intrathecally to reduce sedative side effects. The rationale for their spinal administration is that these drugs will interfere selectively with nociceptive pathways within the spinal cord itself without affecting brain centres associated with sedation (Goodchild and Gent 1992). The positive interaction between opioids and alphadolone was mediated by spinal cord receptors even though both drugs were administered via a non-spinal route. This selective targeting of spinal cord receptors might help decrease the sedative side effects most commonly associated with opioids.

4.4.5 Different Nociceptive Tests

Enhanced opioid antinociception by co-administration of alphadolone was observed for TFL and ECT tests. No interaction was seen for antinociception assessed with PP. It is possible the different results produced by these two tests might be due to differences in

the afferent-pathways activated. While the TFL test assesses the effects of thermal stimulation, PP thresholds measure mechanical nociception. It is not uncommon for analgesics to have different antinociceptive profiles whereby they have no effect on one behavioural test but show potent dose dependent effects using another (Appendix D on page 272 shows the different dose response curves for each opioid measured by ECT and TFL response). For example, reports have shown midazolam causes an increase in ECT but has no effect on TFL (Edwards, Serrao et al. 1990; Yanez, Sabbe et al. 1990). The mechanistic differences between the nociceptive models were discussed in detail in Chapter 3 (page 105).

Another explanation for the different antinociceptive effects observed by TFL and PP might be due to the different potencies that drugs possess for different nociceptive tests. Assessed by PP, the opioids morphine and oxycodone only showed antinociceptive effects at the highest dose tested (3.2mg/kg and 1mg/kg respectively) whilst fentanyl showed no effects at all. These doses are quite different to those demonstrated to be effective using the TFL test. The different results seen in TFL and PP models could be related to the doses of drugs used. The opioids might display more potent antinociceptive effects assessed by TFL response as opposed to PP. However, these experiments ensured only non-sedating doses were tested. This might have restricted the antinociceptive effects observed by PP measurement.

Potentiation has been defined as the "combination of an ineffective dose of one drug with an effective dose of another drug to create a greater effect then either drug given alone" (Dickenson and Sullivan 1993). Therefore when assessing potentiation, a dose of alphadolone that had no effect when given alone was required for co-administration

with opioids. No dose of alphadolone causes antinociceptive effects on TFL response (Chapter 3, page 105). This enabled the highest non-sedating dose of 10mg/kg to be used for combination testing. According to the definition of potentiation described above, no dose of alphadolone assessed by PP or ECT could be used to investigate true potentiation with opioids because both tests demonstrated antinociceptive effects for alphadolone. These tests might only be used to demonstrate an *increased* effect between opioids and alphadolone. Since alphadolone did cause dose dependent antinociceptive effects assessed by PP, an ineffective dose of 1mg/kg was used for combination testing with opioids to determine any increased antinociceptive effects. Alphadolone did not increase the opioid antinociceptive effects assessed by PP. It is possible the use of a lower dose of alphadolone (1mg/kg) for the PP experiments may have restricted the chances of observing any increased opioid antinociception.

4.4.6 An Interaction Between the GABAergic and Opioid Systems

The results from this chapter show spinal cord $GABA_A$ receptors are involved in the interaction between alphadolone and opioids causing enhanced antinociception in the TFL test, as well as the ECT test. Several possible mechanisms have been described whereby $GABA_A$ receptor agonists might enhance the analgesic activity of opioids. Previous work analysing the interaction between morphine and midazolam suggested an acute intraperitoneal injection of morphine increased the activity of glutamate decarboxylase (GAD) (Kuriyama and Yoneda 1978). This enzyme is responsible for the production of GABA. As midazolam increases the efficacy of GABA, it is plausible that the morphine-induced increase in GABA content may promote the increased antinociceptive interaction between morphine and midazolam. Like midazolam,

alphadolone is able to increase the efficacy of GABA by modulating $GABA_A$ receptors. If opioids promote GAD activity to increase GABA production, alphadolone can utilize these greater concentrations of GABA in modulating $GABA_A$ receptors. Therefore, the combination of alphadolone and opioids could enhance their individual antinociceptive effects.

Another possible mechanism has been described for the interaction between opioids and GABA systems. A variety of different hormones, neurotransmitters and cytokines regulate the cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) signalling cascade. A μ-opioid is one such neurotransmitter that can interact with this pathway. The activation of the μ-opioid receptor has been shown to decrease the activity of the enzyme adenyl cyclase and therefore decrease cAMP formation (Carter and Medzihradsky 1993). This results in decreased protein kinase activity. Protein kinase activity has been known to decrease $GABA_A$ receptor-mediated responses in most cell types by reducing its channel opening frequency (Porter, Twyman et al. 1990). If protein kinase activity is decreased, $GABA_A$ receptor mediated-responses can be increased. This sequence of events was demonstrated experimentally with morphine and muscimol (Hara, Saito et al. 1999). Similar to muscimol, alphadolone selectively modulates $GABA_A$ receptors. The experiments seen in this chapter demonstrated an interaction between the opioids and alphadolone to produce increased antinociceptive effects. It is possible the opioids inhibit the cAMP/PKA-signaling cascade, which in turn increases the $GABA_A$ receptor-mediated response of alphadolone.

4.4.7 Alphaxalone Does Not Enhance Opioid Antinociception

Preliminary experiments in rats determined antinociceptive and sedative effects for the anaesthetic Saffan® (Nadeson and Goodchild 2000). This anaesthetic is comprised of a neurosteroid mixture of alphaxalone and alphadolone. The two neurosteroids were compared for antinociceptive and sedative effects in Chapter 3. At non-sedating doses alphadolone caused dose dependent antinociceptive effects whilst alphaxalone did not. Potentiation of antinociception is not always associated with drugs that maintain individual antinociceptive effects. Thus, although alphaxalone caused no individual antinociceptive effects it was still investigated for a possible interaction with opioids to increase antinociception.

The experiments described in this chapter showed alphadolone enhanced opioid antinociception, and that this interaction was mediated by $GABA_A$ receptors in the spinal cord, but alphaxalone showed no such interaction. The $GABA_A$ receptor complex consists of a heterogeneous group of structurally distinct subunits (Gee, McCauley et al. 1995). Therefore it is reasonable to expect that different neurosteroid analogues possess different binding for such subunits. On the one hand, investigations have shown that alphadolone activates $GABA_A$ receptors in the spinal cord (Nadeson and Goodchild 2000), and on the other, binding studies have shown that alphaxalone binds to several regions in the human and rat brain (Nguyen, Sapp et al. 1995). Therefore it might be suggested that alphaxalone interacts with different receptors to alphadolone receptors; ones that are not capable of increasing opioid antinociception.

Another explanation for alphaxalone's lack of antinociceptive effect might be related to the potency of the drugs tested for this interaction. Alphaxalone was co-administered with a low dose of opioid that when given alone caused no antinociceptive effects assessed by TFL. Alphaxalone caused no increase in opioid antinociceptive effects. A higher dose of each opioid was tested to increase the chances of observing an interaction with alphaxalone. The dose selected was sub-maximal to allow for the observation of antinociceptive effects that are capable of further enhancement. Alphaxalone still caused no change in opioid antinociception. The potency of alphaxalone may not have been great enough at the dose tested, however only non-sedating doses were used and the maximum non-sedating dose was given – 10mg/kg alphaxalone did not potentiate opioid antinociception.

4.4.8 Summary

The experiments described in this chapter used non-sedating doses of opioids fentanyl, morphine and oxycodone in combination with two neurosteroids, alphadolone and alphaxalone, to investigate increased antinociceptive effects assessed by TFL, ECT and PP tests. Alphadolone was able to enhance the antinociceptive effects of all three opioids assessed by TFL response, but not PP. The observed positive interaction was mediated by spinal cord $GABA_A$ receptors even though a non-spinal route of administration was used for both agonists. Furthermore, the increase in opioid antinociception was not associated with any sedative effects. Finally, at a non-sedating dose alphaxalone did not cause any change in opioid antinociception, thus highlighting another difference in activity between these structurally related neurosteroids.

Chapter 5 An Interaction Between Alphadolone and Opioids in the Carrageenan-Induced Paw Inflammation Model

5.1 Introduction

Hyperalgesia is defined as an exaggerated nociceptive response to noxious stimulation (Mao, Price et al. 1995). Hyperalgesia associated with inflammation is caused by a peripheral injury and is attributed to sensitisation of receptors at the site of injury and sensitisation of neurones in the spinal cord (Traub 1996).

The series of experiments described in this chapter uses a substance called carrageenan to create an inflammatory pain state in rats. The model of carrageenan-induced inflammatory pain has been developed to study the ramifications of hyperalgesia and also the usefulness of drugs in this painful state. Carrageenan, a seaweed extract, induces an inflammatory response when injected into a rat hind paw. This response is mediated by substance P, bradykinin, serotonin (5-HT) and prostaglandins being released into the tissues which sensitise nociceptors, thus leading to hyperalgesia (Ferreira, Zanin et al. 1978; Garry and Hargreaves 1992; Hargreaves, Dubner et al. 1988a; Ren, Williams et al. 1992). Studies in rats have shown that almost immediately after intraplantar injection of carrageenan, oedema develops. This is followed with hyperalgesia by 3-4 hours, which resolves 24 hours later (Kowaluk, Mikusa et al. 2000; Rygh, Svendsen et al. 2001).

Ongoing peripheral input is not required after the initial carrageenan injection which is enough to cause spinal cord neurone receptive fields to expand (Woolf 1995). This in turn increases responsiveness to stimuli so rats' response thresholds to nociceptive stimuli are therefore decreased (Dubner 1992). This carrageenan model is of interest in pain medicine research because it mimics hyperalgesia typical of some pain states

experienced in humans (Jourdan, Ardid et al. 2001). For example chronic musculoskeletal pain is a major clinical problem and data suggest carrageenan injected into the knee joint or gastrocnemius muscle of rats could be used to model this type of acute inflammation (Radhakrishnan, Moore et al. 2003). Pleurisy is an inflammation of the pleura and carrageenan is used to induce this state in animal models to assess possible treatments (Cuzzocrea, McDonald et al. 2000). Other studies have suggested that experimental work using the carrageenan-induced inflammatory model may increase our understanding of the neural mechanisms and treatment for low back pain and sciatica (Hu and Xing 1998). Therefore drugs that prove effective in this experimental model of carrageenan-induced inflammation can be translated to use in patients.

It was first shown in 1986 that GABA attenuated the hypcralgesic response produced by carrageenan injection in the hind paw in rats (Bhattacharya and Sarkar 1986). Therefore positive modulation of $GABA_A$ receptors might play a role in reversing hyperalgesia in carrageenan-induced inflammation. Alphadolone has been shown to produce antinociceptive effects in acute pain tests by an interaction with $GABA_A$ receptors in the spinal cord (Nadeson and Goodchild 2000). Therefore alphadolone might be effective in reversing hyperalgesia in carrageenan-induced inflammation.

Previous studies have shown each of μ- (fentanyl), δ- (DPDPE) and κ-selective (U69593) opioids as well as morphine to reverse hyperalgesia caused by the administration of carrageenan (Herrero and Solano 1999; Marsh, Dickenson et al. 1999; Ossipov, Kovelowski et al. 1995; Ren, Williams et al. 1992). Although opioids are effective for the treatment of inflammatory pain, they are associated with well-known

dose dependent adverse side effects such as nausea, sedation and respiratory depression. Furthermore, after persistent usage, the development of tolerance to opioids can occur, where the same level of analgesia requires higher dosing. In an attempt to reduce dose dependent adverse side effects or the development of tolerance, other non-opioid drugs may be combined with low doses of opioids to produce equally effective analgesia or that of a greater magnitude.

Some combination therapies have been tested in experimental inflammation. Studies have shown a positive interaction between opioids and α_2-adrenergic agonists occurs, and that the efficacy of both are enhanced during inflammation (Herrero and Solano 1999; Mansikka, Zhou et al. 2002). Furthermore, several researchers have demonstrated the sensitisation of neurones occurring as a result of inflammation is linked to NMDA receptors (Meller, Cummings et al. 1994; Svendsen, Rygh et al. 1999; Urban and Gebhart 1999). Therefore several opioids have been tested in combination with a variety of NMDA receptor antagonists and successfully reversed hyperalgesia caused by inflammation (Dickenson 1997; Ma, Allchorne et al. 1998; Yamamoto, Shimoyama et al. 1993). Those rat studies suggest the utility of the combination drug regimen approach.

Previous studies have also shown a positive interaction for antinociceptive effects between drugs acting at $GABA_A$ receptors such as midazolam and muscimol, and opioid analgesics such as fentanyl and morphine (Hara, Saito et al. 1999; Rattan, McDonald et al. 1991; Yanez, Sabbe et al. 1990). Neurosteroids have many possible therapeutic roles (i.e. as anxiolytics, sedatives and hypnotics) by virtue of positive modulation of $GABA_A$ receptors in the CNS (Brot, Akwa et al. 1997; Hu, Zorumski et

al. 1993; Lambert, Belelli et al. 1995; Reith and Sillar 1997). Recent animal studies demonstrated the neurosteroid alphadolone causes potent antinociceptive effects (Nadeson and Goodchild 2000) mediated by $GABA_A$ receptors in the spinal cord (Nadeson and Goodchild 2001). Additional pilot clinical studies proved alphadolone also caused analgesia in humans (Goodchild, Robinson et al. 2001). Furthermore alphadolone increases opioid antinociception in an acute model of thermal pain (TFL) (refer to Chapter 4, page 147). If such an effect exists in acute pain, such an interaction might exist between opioids and alphadolone in a model of carrageenan-induced inflammation.

5.1.1 Aim

This series of experiments administered carrageenan in rats to induce an inflammatory pain state. Once hyperalgesia was confirmed, experiments were performed to investigate the antinociceptive properties of alphadolone in this model of pain, both alone and in combination with opioids.

5.2 Methods

Carrageenan was injected into the hind paw of rats to cause inflammation. Nociceptive thresholds were measured using paw pressure (PP) withdrawal latencies. Alphadolone and three opioids (fentanyl, morphine and oxycodone) were administered via the intraperitoneal (IP) route. All experiments were performed in a blinded manner. All experimental procedures and data transformation are described in Chapter 2 (page 55).

5.2.1 Study Drugs

Alphadolone (alphadolone acetate, Jurox, Rutherford, NSW) was combined with a complexing agent, 2-hydroxypropyl-β-cyclodextrin (HPβCD) to create a powder more soluble in aqueous solution. Alphadolone was weighed out fresh each day and dissolved in a phosphate buffer solution. Fentanyl (fentanyl citrate USP – David Bull Laboratories), morphine (morphine sulphate – David Bull Laboratories) and oxycodone (oxycodone hydrochloride tablets – Endone, Boots Healthcare) were diluted in normal saline fresh each day for IP administration.

All doses in mg/kg were made in a volume of 1ml calculated for a 200g rat. The average weight of rats was 180-200g so each syringe was slightly adjusted in volume, and therefore dose, to allow for the small weight differences between rats to allow accurate dosing of drug tailored to their specific weight.

5.2.2 Experimental Paradigm

Inflammation and hyperalgesia were produced in the right hind paw of male Wistar rats (wt 180-200g) by carrageenan injections, as described in Chapter 2 (page 56). Nociceptive responses were assessed by measurement of paw withdrawal thresholds and drug effects quantified as a percentage reversal of hyperalgesia (%RH) calculated as shown in Chapter 2 (page 58). The range of doses of each drug used is given in Table 5.1. Replicate experiments were performed for each dose. The administration of carrageenan meant rats could not be used for multiple experiments.

(a) Alphadolone Dose Response Relationship

A dose response curve was constructed for alphadolone administered alone. A dose of alphadolone (0.1mg/kg) that caused no effect on PP response when given alone was selected and combined with increasing doses of each opioid (refer to Data Analysis, page 176).

(b) Opioid Dose Response Relationships: Alone and in Combination with Alphadolone

Dose response relationships were measured for all opioids given alone. These experiments were repeated with a dose of alphadolone (0.1mg/kg) co-administered with each opioid as shown in the paradigm for combination experiments (refer to Chapter 2, page 61).

Table 5.1 Ranges of Drugs used in Dose Response Study

	Alphadolone (mg/kg)	Fentanyl (µg/kg)	Morphine (mg/kg)	Oxycodone (mg/kg)
administered alone	0.01-10.0 *n=8-13*	1.25-20 *n=5-11*	0.2-3.2 *n=5-10*	0.0625-0.25 *n=5-12*
co-administered with 0.1mg/kg alphadolone		1.25-10 *n=4-6*	0.2-1.6 *n=5-9*	0.0625-0.25 *n=5-10*

n denotes number of replicate experiments at each dose

5.2.3 Data Analysis

For each drug dose or drug combination the values for percentage reversal of hyperalgesia (%RH) were combined and plotted as means ± SEM as described in Chapter 2 (page 58). A dose of alphadolone that caused no effect on PP response when given alone was determined by comparing its effect (%RH) to zero using a *t*-test. All the %RH responses depicted by the dose response curves for each opioid in the presence of alphadolone were compared with the dose response curves for the respective opioid given alone using a two-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test for multiple comparisons. For all statistical comparisons, a value of $p \leq 0.05$ was considered statistically significant.

5.3 Results

5.3.1 The Antinociceptive Properties of Intraperitoneal Alphadolone

In carrageenan-induced inflamed paws, alphadolone caused dose dependent antinociceptive effects (refer to Figure 5.1, page 179). The highest non-sedating dose 10mg/kg caused a reversal of hyperalgesia of 60.03% ± 19.3 (n=12). A dose of 0.1mg/kg caused no significant effects (t-test) compared with zero (13.4% ± 7.5, n=13). The dose of 0.1mg/kg that caused no reversal of hyperalgesia when given alone was selected for further combination experiments with opioids.

5.3.2 Opioid Antinociception: Alone and in Combination with Alphadolone

The dose response relationships for each opioid given alone and in combination with alphadolone 0.1mg/kg are shown in Figure 5.2 on page 181. All three opioids given alone caused significant dose related reversal of hyperalgesia in this inflammatory pain model. Only in combination with fentanyl did the addition of alphadolone 0.1mg/kg increase the %RH (p=0.019, ANOVA). The dose related reversal of hyperalgesia effects for morphine and oxycodone were no different in the presence of alphadolone compared with the effects caused by the respective opioid when given alone.

Figure 5.1

This figure shows the effect of increasing doses of alphadolone on PP in rats with an inflamed hind paw. Alphadolone caused a clear dose dependent effect in this model measured by an increase in percentage reversal of hyperalgesia (%RH, calculated as shown in Chapter 2, page 58). A dose of 0.1mg/kg did not significantly affect the PP response (*t*-test). This dose was used for combination testing with opioids. Each point represents the mean for 8-13 rats ± SEM.

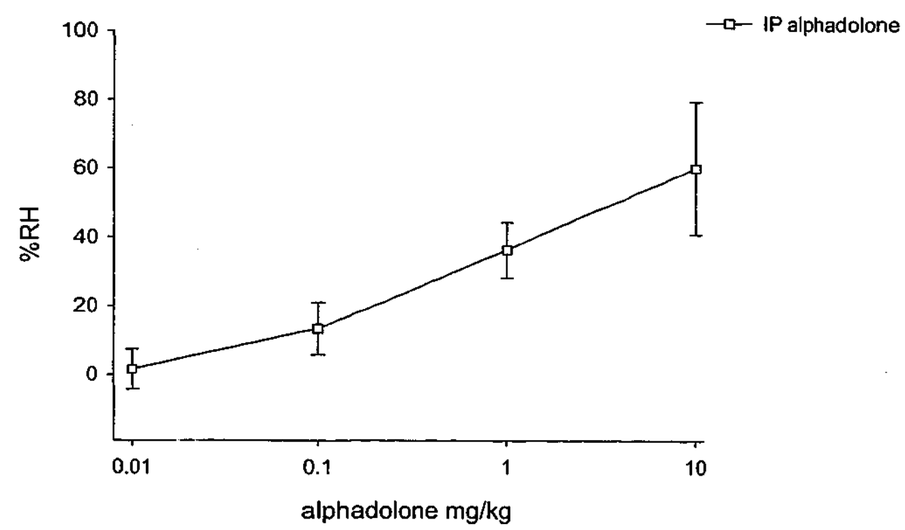


Figure 5.1 IP Alphadolone Dose Response Effect in Carrageenan-Induced Inflammation

Figure 5.2

(A) Fentanyl

Fentanyl alone caused a significant increase in reversal of carrageenan-induced hyperalgesia. The highest dose tested, 20µg/kg, caused 56.54% ± 9.54 (mean ± SEM) reversal of hyperalgesia. In combination with alphadolone (0.1mg/kg), the effect of fentanyl was significantly increased (p=0.019, ANOVA). Each point represents the mean for 4-10 rats ± SEM.

(B) Morphine

Morphine alone caused a significant dose dependent reversal of carrageenan-induced hyperalgesia. In combination with alphadolone 0.1mg/kg, the effect of morphine was not significantly different (ANOVA). Each point represents the mean for 4-12 rats ± SEM.

(C) Oxycodone

Oxycodone alone caused a significant dose dependent reversal of carrageenan-induced hyperalgesia. In combination with alphadolone 0.1mg/kg, the effect of oxycodone was not significantly different (ANOVA). Each point represents the mean for 4-11 rats ± SEM.

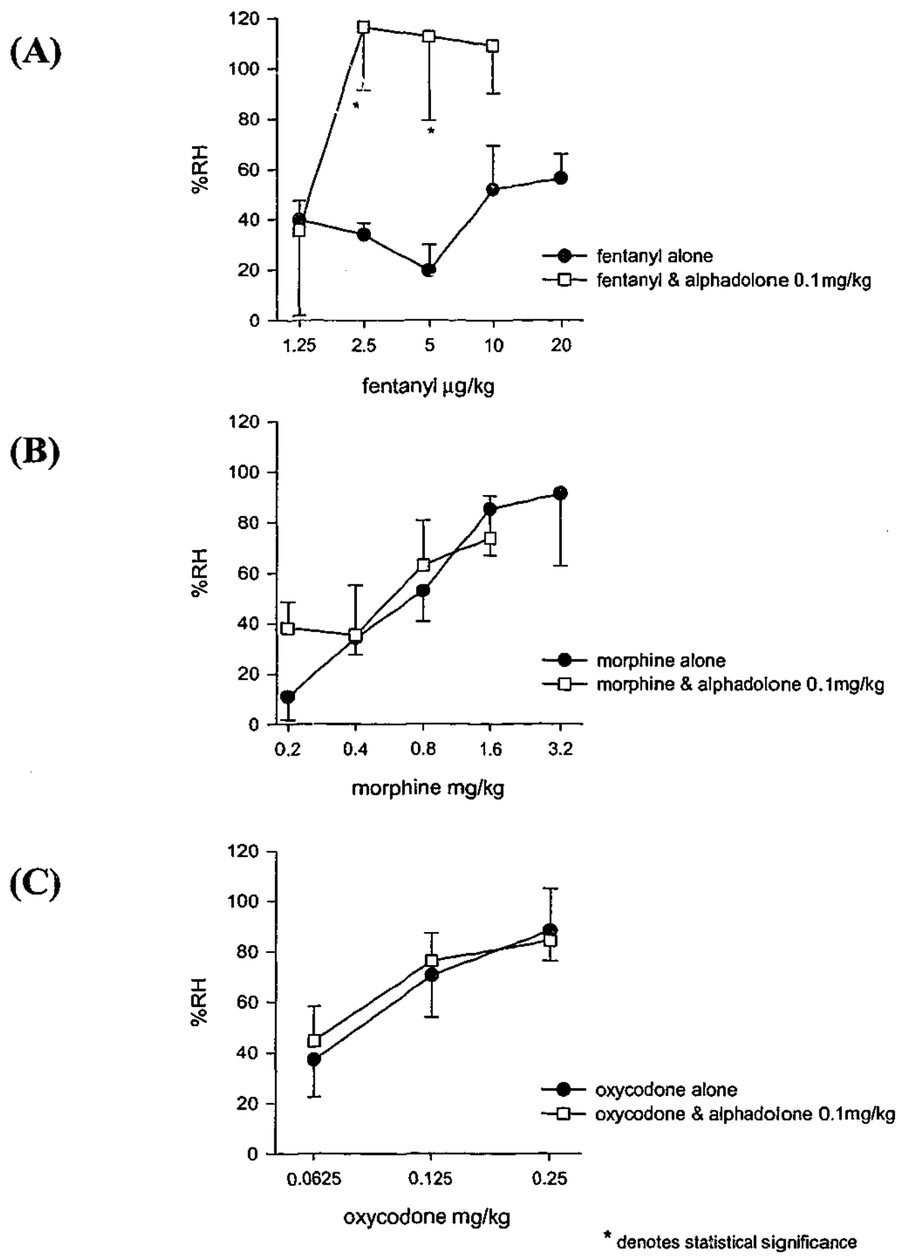


Figure 5.2 IP Opioid Dose Response Effect in Carrageenan-Induced Inflammation: Alone and in the Presence of Alphadolone

5.4 Discussion

This series of experiments showed that non-sedating doses of alphadolone and all three opioids fentanyl, morphine and oxycodone given alone could reverse hyperalgesia in a model of inflammatory pain. Furthermore, when a dose of alphadolone that was small enough *not* to cause reversal of hyperalgesia in this model when given alone, was co-administered with fentanyl, an increase in reversal of hyperalgesia was observed. This effect was not seen with either morphine or oxycodone when administered in combination with alphadolone.

5.4.1 The Carrageenan-Induced Model of Inflammatory Pain

The testing of potential analgesics in established or on-going pain, rather than in acute or physiological pain, is more effective at predicting the human pain experience (Petersen-Felix and Arendt-Nielsen 2002). Persistent pain is often characterised by hyperalgesia; defined as increased sensitivity to painful stimuli. Hyperalgesia can be produced reliably in rats by the administration of carrageenan into the hind paw. Thus potential drug candidates for common pain conditions are appropriate for testing in this model since hyperalgesia is a common feature of post-operative pain, colitis, and inflammation of the joints (Kim and Berstad 1992; Strober 1985). Although experiments described in previous chapters demonstrate alphadolone's antinociceptive activity in other acute pain tests, it is this test model that may provide more predictive results for alphadolone's analgesic activity in clinical pain.

5.4.2 Alphadolone Reverses Hyperalgesia in a Model of Carrageenan-Induced Inflammation

It is well documented that the amino acid GABA, acting as an inhibitory neurotransmitter, can contribute to the down regulation of neuronal excitability seen in central sensitisation (Kaneko and Hammond 1997; Malcangio and Bowery 1996; Sivilotti and Woolf 1994). Therefore positive modulation of $GABA_A$ receptors that increase GABA activity might play a role in reversing hyperalgesia in carrageenan-induced inflammation. Alphadolone positively modulates $GABA_A$ receptors (Nadeson and Goodchild 2000) and results of experiments described in this chapter have demonstrated its effectiveness in reversing hyperalgesia in carrageenan-induced inflammation.

To date, published works only describe the $GABA_A$ receptor agonist diazepam to be effective in reducing hyperalgesia caused by carrageenan-induced inflammation. Intraperitoneal administration of the benzodiazepine diazepam has been shown to reduce the volume of acute inflammatory paw edema in rats as a response to carrageenan administration (Lazzarini, Malucelli et al. 2001). These experiments should be analysed with caution as diazepam causes potent sedation and these experiments used high doses without testing for sedation. However, investigations have suggested that these effects were not mediated by $GABA_A$ receptors in the CNS, but rather by another recognition site in the periphery (Torres, Nardi et al. 1999). Based on this discovery one might suggest the anti-hyperalgesic effects of alphadolone reported in this chapter are due to actions in the periphery. However, the antinociceptive effects of alphadolone in other pain models have been entirely reversed by IT bicuculline

(Nadeson and Goodchild 2000). Those results suggest that the likely mechanism for the antinociceptive effects of alphadolone is positive modulation of $GABA_A$ receptors in the spinal cord. However, a peripheral action cannot be ruled out in the explanation for these results without experiments with intrathecal bicuculline such as those described in the previous chapter.

Past experiments have suggested that spinal cord GABAergic effects are enhanced in inflammatory hyperalgesia: Nahin and Hylden used immunohistochemistry studies in rats with peripheral inflammation to detect an increase in spinal cord glutamate decarboxylase (GAD), the GABA-synthesizing enzyme (Nahin and Hylden 1991). It was suspected the increased presence of GABA would cause a greater inhibitory effect in this state of inflammation. Other work supported this theory by demonstrating a selective unilateral increase in spinal GABA-immunoreactive cells after an injection of complete Freund's adjuvant in the ipsilateral limb (Castro-Lopes, Tavares et al. 1992). Such changes in GABA concentrations were specifically related to the spinal nerves that innervated the hind limbs of the ipsilateral side. All of those observations are supported by findings of the experiments described in this chapter. One may compare alphadolone's effects in the PP test in rats with normal uninflamed paws (refer to Figure 3.1, page 105) and carrageenan-induced inflamed paws (refer to Figure 5.1, page 179). In both experiments alphadolone was administered via the IP route and the PP test was used to test withdrawal latencies. This chapter showed that a dose of alphadolone 1mg/kg reversed hyperalgesia 36.2% ± 8.09 (mean ± SEM) in rats with an inflamed hind paw. By comparison, this same dose of alphadolone caused *no* antinociceptive effects in rats with normal uninflamed paws, r = 1.03 ± 0.02 (Chapter 3, page 105). The effects of alphadolone in inflammatory pain shown in this chapter were

greater compared with acute nociceptive testing using the same measuring tool. This drug that targets spinal $GABA_A$ receptors behaves as predicted by previously published work that indicates that drugs acting at GABAergic systems are more effective antinociceptive agents in inflammatory pain states.

5.4.3 Opioids have Antinociceptive Activity in a Model of Carrageenan-Induced Inflammation

Many investigators have shown that CNS neurones are more responsive to opioids following the production of central sensitisation compared with acute nociception (Woolf and Wall 1986). One study using carrageenan-induced inflammation showed morphine administration resulted in significantly greater increases in paw withdrawal latencies in the inflamed (38-139%) compared with the contralateral, saline-treated paws (4-19%) (Joris, Costello et al. 1990). Another study by Kayser and Guilbaud used an arthritis model to show increased morphine antinociceptive effects. That study showed morphine raised the vocalization threshold of rats and that this effect occurred at lower doses in the arthritic rats compared with normal rats (maximum vocalization threshold, % of control, following 1 mg/kg morphine 225.70 ± 10.21 in arthritic rats versus 140.75 ± 6.87 in normal rats) (Kayser and Guilbaud 1983). Finally, a study involving localized inflammation caused by administering Freund's complete adjuvant to one joint also demonstrated greater opioid antinociception at lower doses in the inflamed condition compared with control rats with no inflammation (Stein, Millan et al. 1988). The exact mechanism of enhanced opioid activity in inflammation is not understood.

The experiments reported in this chapter showed all three opioids fentanyl; morphine; and oxycodone reversed hyperalgesia in this model of inflammation. Furthermore the opioid-induced increase in paw withdrawal latencies was greater in rats with inflamed paws than normal rats with no inflamed paw. Data for paw withdrawal thresholds in normal rats shown in Chapter 4 (page 145) was compared with results from the experiments reported in this chapter. In normal rats without inflamed paws, fentanyl showed no significant antinociceptive effects assessed by PP. In carrageenan-induced inflammation fentanyl showed significant effects for the reversal of hyperalgesia. In normal uninflamed paws morphine 3.2mg/kg was the only dose to show significant antinociceptive effects with a PP response (r) of 1.45 ± 0.13 (mean ± SEM). In carrageenan-induced inflammation morphine displayed dose dependent effects with a value of 91.36% ± 28.68 for the reversal of hyperalgesia at a dose of 3.2mg/kg. In normal uninflamed paws oxycodone 1mg/kg was the only dose that caused significant antinociceptive effects with a PP response (r) of 1.72 ± 0.11 (mean ± SEM). In carrageenan-induced inflammation oxycodone produced significant dose dependent effects with a value of 88.12% ± 11.76 for the reversal of hyperalgesia at a dose of 1mg/kg. These results are all consistent with the notion of increased opioid sensitivity in inflammatory pain reported in the literature.

There are several possible explanations for the increased activity of opioids in carrageenan-induced inflammation. The increased effect of opioids following peripheral inflammation has been attributed to additional actions of these drugs at opioid receptors in the periphery which become functional within inflamed tissue (Hargreaves, Dubner et al. 1988b). Hyperalgesia associated with inflammation occurs

due to plasticity in both the periphery and CNS. Stein *et al.* demonstrated peripheral and central sites of action for opioids in inflammatory pain and that opioid responses were modified in inflamed paws compared with normal uninflamed paws (Stein, Millan et al. 1988). This work offered the explanation that an enhanced response to opioid antinociception in inflammation might be due to the synergism of peripheral and central actions.

The enhanced opioid effect in inflammatory pain might also be due to changes in activity of other centrally acting agonists that affect opioid function. For example Hylden *et al.* showed the involvement of noradrenergic mechanisms by attenuating morphine antinociception in carrageenan-induced inflammation with an α_2-adrenoceptor antagonist. During inflammation, dorsal spinal cord noradrenaline levels and turnover are increased (Weil-Fugazza, Godefroy et al. 1986). Therefore, in the presence of increased noradrenergic activity morphine effects might be enhanced. Other studies have suggested the increase in opioid analgesia in inflammation could be due to enhanced activity of serotonergic systems. In normal rats, the participation of serotonergic mechanisms in morphine analgesia is well documented (Crisp, Stafinsky et al. 1991; Goodchild, Guo et al. 1997; Kuraishi, Harada et al. 1983). Studies have also shown increases in levels of serotonin and its precursors in the brain and spinal cord in arthritic rats compared with those of normal rats (Godefroy, Matson et al. 1990; Weil-Fugazza, Godefroy et al. 1979).

5.4.4 Alphadolone Increases the Antinociceptive Activity of Fentanyl, but not Morphine or Oxycodone, in a Model of Carrageenan-Induced Inflammation

The experiments reported in this chapter show reversal of hyperalgesia effects by fentanyl were significantly increased with the co-administration of a dose of alphadolone (0.1mg/kg) that caused no effect when given alone. A low dose of 2.5µg/kg fentanyl produced little effect on its own. However, when the same dose was combined with alphadolone, the hyperalgesic effect produced by carrageenan-induced inflammation was totally reversed. This meant rats displayed normal paw pressure thresholds despite having a paw inflamed by carrageenan injection. In contrast, morphine and oxycodone showed no increased effects when given with alphadolone. In fact the dose response curves of these opioids given alone were coincident with the curve for the drug combinations with alphadolone.

One obvious difference between morphine and oxycodone compared with fentanyl is that fentanyl is highly selective for µ-opioid receptors (Leysen and Gommeren 1982; Villiger, Ray et al. 1983). Morphine and oxycodone are not as selective. They have mixed actions at receptors throughout the CNS and periphery (Davis, Varga et al. 2003; Reisine 1995). Furthermore, past experiments have shown the antinociceptive effects of IP alphadolone assessed by the ECT test were entirely reversed with intrathecal injections of the $GABA_A$ receptor antagonist bicuculline (Nadeson and Goodchild 2000). This suggests that although alphadolone was given via a non-spinal route its effects are via spinal cord $GABA_A$ receptors. Other work that involved combining intrathecal midazolam, a positive modulator of $GABA_A$ receptors, and fentanyl

demonstrated their interaction was also at the level of the spinal cord (Serrao, Stubbs et al. 1989; Wakita 1992). The interaction between fentanyl and alphadolone seen in these experiments may well have occurred similarly at the level of the spinal cord even though both drugs were given via the intraperitoneal route. At first thought one might feel this should be investigated by the administration of selective intrathecal antagonists using rats with chronically implanted intrathecal catheters, as described in Chapter 2 on page 46. However, once a drug is injected intrathecally, handling or moving the rat will cause rostral spread of the drug so that it acts at receptors throughout the entire length of the spinal cord and even in the brain. Neck and tail ECT data, as described in Chapter 2 on page 49, would both be affected by the intrathecal drug and thus offer no information on spinal cord involvement. Throughout this chapter the paw pressure model is used for measuring withdrawal latencies of rats. This behavioural test requires rat handling at each test point. Therefore the measurement of paw pressure thresholds is not amenable for proving spinal cord effects by selective reversal by intrathecal drugs in rats with intrathecal catheters.

The co-administration of alphadolone with morphine and oxycodone did not produce an increased effect for the reversal of hyperalgesia. It was unexpected to see such contrasting results for morphine and oxycodone compared with fentanyl. In addition, the curves of both morphine and oxycodone alone compared with their co-administration with alphadolone were coincident. Therefore the dose of alphadolone 0.1mg/kg that had no antinociceptive effect when given alone also has no effect on the antinociception caused by any dose of morphine or oxycodone in this model of carrageenan-induced inflammation. The different effects caused by the opioids might be explained by their different affinities for opioid receptors, or perhaps differences in

the activation of various opioid receptors throughout the CNS and periphery. As mentioned earlier, fentanyl is a potent μ-opioid agonist (Leysen and Gommeren 1982; Villiger, Ray et al. 1983), whereas morphine and oxycodone are not as selective and have mixed actions (Davis, Varga et al. 2003; Reisine 1995). It was discussed earlier that opioid activity is enhanced in inflammation compared with acute nociception. More specific studies investigated the effects of different opioid receptor selective agonists after inflammation. One study showed after carrageenan-induced inflammation that μ-, δ- and κ-selective opioids administered via an intrathecal injection all exhibited increased antinociceptive potencies but that this increased effectiveness was especially marked for the μ-opioid (Stanfa, Sullivan et al. 1992). Another study analyzed specific immunoreactivities for μ-, δ- and κ-opioid receptors within the rat dorsal root ganglia after carrageenan-induced inflammation (Ji, Zhang et al. 1995). This study also found all three subtypes were involved in the response to carrageenan-induced inflammation but went on to suggest a marked up regulation in μ-opioid receptors after inflammation with a distinct down regulation in δ- and κ-opioid receptors. Therefore the μ-opioid receptor within the spinal cord is upregulated in carrageenan-induced inflammation. From this it could be an expected result to see different effects for a potent μ-opioid agonist compared with other opioids with less affinity for this same receptor.

It is possible the interaction between alphadolone and morphine or oxycodone, compared with fentanyl, is absent due to differential effects of opioid receptors in the spinal cord compared to the brain or periphery. Opioid receptors are involved with antinociception in all three regions (Mather 1995; Yaksh 1997). However, alphadolone causes antinociception by acting at spinal cord $GABA_A$ receptors (Nadeson and Goodchild 2000). It could be suggested that in order for an interaction to occur between

alphadolone and another drug, both drugs must interact with spinal cord receptors. There is some evidence to suggest κ-opioids are inactive when administered intrathecally in this model of inflammation. An experiment by Hylden *et al.* tested intrathecally-administered opioids for activity in the carrageenan model of inflammation using noxious heat as a stimulus (Hylden, Thomas et al. 1991). Results showed dose response effects for intrathecal μ- and δ-opioid agonists. A selective κ-opioid receptor agonist (U-50,488H) administered intrathecally showed no activity in this assay. By contrast, systemic administration of this same agonist U-50,488H did produce significant elevations of paw withdrawal latencies in inflamed paws. Both oxycodone and morphine have been associated with κ-opioid activation. Experiments with selective opioid antagonists have shown oxycodone's antinociceptive effects are mediated by κ-opioid receptors (Ross and Smith 1997). In addition, work with intrathecal morphine has shown an interaction with spinal cord κ-opioid receptors even though its supraspinal effects are well established as μ-opioid-mediated (Goodchild, Nadeson et al. 2004). However there is a published report that suggested the increase in morphine potency produced by carrageenan-induced inflammation is modulated by δ-opioid receptors (Ossipov, Kovelowski et al. 1995).

There is some further evidence to suggest that activation of the κ-opioid receptor suppresses the analgesic effects of μ-opioid agonists. Several experiments have shown the analgesic effects of morphine were inhibited by intrathecally-administered dynorphin, a selective κ-opioid receptor agonist, using the tail flick test (Fujimoto and Holmes 1990; Schmauss and Herz 1987). If morphine and oxycodone do display a greater affinity for κ-opioid receptors it might cancel out their effects as μ-opioid agonists.

It is possible in this model of carrageenan-induced inflammation, morphine and oxycodone target spinal κ-opioid receptors (with greater affinity than spinal μ-opioid receptors). The inactivity of spinal κ-opioid receptors may explain the absent interaction of alphadolone with opioids morphine and oxycodone. The reduction in hyperalgesia in this model of inflammation for morphine and oxycodone administered alone may be due to actions at systemic or peripheral receptor sites.

5.4.5 Summary

This series of experiments used carrageenan-induced inflammation to produce hyperalgesia in rats. Alphadolone and all the three opioids tested, fentanyl, morphine and oxycodone caused reversal of hyperalgesia. The effects of alphadolone and all three opioids were enhanced in this model of inflammation compared with th. effects as antinociceptive agents in the absence of inflammation. In combination with a dose of alphadolone that caused no reversal of hyperalgesia when given alone, the effect of fentanyl was increased, while oxycodone and morphine showed no change. It is suggested that this interaction with alphadolone occurs at the level of the spinal cord and furthermore, this interaction might only be associated with spinal cord μ-opioid receptors. The results with morphine and oxycodone suggest that these opioids might target different receptors in this model of inflammation that are not associated with $GABA_A$ receptors in the spinal cord.

Chapter 6 The Antinociceptive Effects of Alphadolone and Opioids in a Model for Diabetes-Induced Neuropathic Pain

6.1 Introduction

Neuropathic pain refers to a group of pain conditions characterised by lesion or dysfunction of the normal sensory pathways (Wall and Melzack 1989). The mechanisms underlying neuropathic pain states are poorly understood but those that have been hypothesised are explained briefly. Sensitised nociceptors with the additional recruitment of silent nociceptors can increase afferent barrage to cause sensitisation of dorsal horn neurones (Chapman, Ng et al. 1998). Central sensitisation may also be caused by a severe loss of small fiber input. This causes spinal reorganisation from sprouting of large fibers into superficial 'nociceptive' laminae in the dorsal horn (Woolf and Salter 2000). Another source for central sensitisation is inflammation of nerve trunks that can cause ectopic nerve activity. Finally, increased sympathetic activity or even altered brain processing that recruits brain areas not usually involved in pain can also contribute to central sensitisation (Casey 2000). Any of these individual mechanisms or a combination of mechanisms may account for various conditions and symptoms seen in patients complaining of neuropathic pain. The severe and complex nature of neuropathic pain makes it difficult to treat clinically.

Neuropathic pain is defined as pain consequent upon damage to the peripheral or central nervous system (CNS). It may produce severe and intractable pain in the absence of any apparent damage to the tissues innervated by the injured nerve (Kapur 2003). It is a common problem that may arise from a variety of different disease processes such as diabetes, immune deficiencies or malignant disorders (Hansson 2002). Over 100 million people worldwide have diabetes mellitus, of which 60% develop diabetic neuropathy (Spruce, Potter et al. 2003). Development of experimental

rat models that mimic these disease states has been useful for studying the mechanisms and possible treatments. Diabetic neuropathy has been studied in the rat using the diabetes-induced model (intraperitoneal administration of streptozotocin) created by Courteix and co-workers (Courteix, Eschalier et al. 1993). This model reliably produces diabetes in rats and signs of neuropathy develop rapidly. The mechanisms for streptozotocin (STZ) producing diabetes in rats are described in Chapter 2 (page 62). Although the pathogenesis behind diabetic neuropathy is not entirely understood, there is an emerging consensus that persistent neuropathic pain results from damage to and subsequent morphological changes of either the central and/or peripheral nervous system caused by damage to small blood vessels (Attal and Bouhassira 1999; Bennett 1998) and rapid changes in metabolic control (i.e. glucose levels) (Ziegler, Mayer et al. 1988). The subsequent neuroplasticity changes associated with diabetic neuropathy include spinal neuronal rewiring (Boulton 1992), persistent nerve stimulation (Bennett 1998) and ectopic electrical impulses/hyperexcitability (Scadding 1981). The resultant effect is the production of both allodynia and hyperalgesia. The rat diabetic model for neuropathic pain seems to mimick faithfully the diabetic neuropathy seen in humans in that it is also characterised by hyperalgesia and allodynia (Courteix, Eschalier et al. 1993). Some analgesic agents that demonstrated antinociceptive efficacy in diabetic rats have also shown a degree of efficacy during clinical trials in diabetic patients (Backonja, Beydoun et al. 1998; Harati, Gooch et al. 2000; Oskarsson, Ljunggren et al. 1997). Therefore the diabetic rat model is effective for studying clinical neuropathic pain syndromes and its use has been prompted to screen potential agents for treating painful diabetic neuropathy.

Specific treatments, none of which restore nerve function, have already been designed and tried based on current understanding of the different mechanisms that underlie diabetic neuropathic pain (Spruce, Potter et al. 2003). Furthermore most of those treatments are associated with significant side effects. Treatments currently include tricyclic antidepressants, sodium channel blockers, NMDA receptor blockers, gabapentin and opioids. The precise mechanisms by which compounds cause analgesia in diabetic neuropathic pain are controversial although there is a general consensus (Rang, Dale et al. 1996): (1) tricyclic antidepressants are thought to cause analgesia by inhibiting noradrenaline and 5-HT reuptake; (2) sodium channel blockers decrease the occurrence of action potentials that contribute to the hyperexcitable state; (3) NMDA receptor blockers decrease the actions of excitatory amino acids; (4) gabapentin is an anticonvulsant that increases the actions of inhibitory amino acids; (5) finally, opioids interact with opioid receptors. The response of neuropathic pain to opioid treatment has been particularly controversial. While a previous study has suggested neuropathic pain is non-responsive to opioids (Arner and Meyerson 1988), others have shown opioids may alleviate neuropathic pain but at higher doses than normal, indicating a shift of the dose-response curve to the right (Jadad, Carroll et al. 1992; Portenoy, Foley et al. 1990). It is well established that higher opioid doses are associated with dose dependent adverse effects such as sedation or mood alteration, which is a particularly difficult problem in elderly patients with diabetic neuropathic pain. In terms of treating diabetic neuropathic patients, rather than causing true antinociception by affecting afferent pain pathways, the higher opioid doses may appear to produce analgesia by affecting higher CNS functions such as attention or level of consciousness. Therefore there is a need for novel therapies in this area.

Rat models have demonstrated a role for $GABA_A$ receptor modulation in neuropathic pain. Therefore $GABA_A$ receptor agonists may also be useful in diabetic neuropathic pain. However as of yet no published studies have tested $GABA_A$ receptor agonists in the STZ-induced diabetic model for neuropathic pain. Previous studies using the spinal nerve ligation model have shown spinal administration of the selective $GABA_A$ receptor antagonist bicuculline leads to tactile allodynia-like behaviour similar to that seen in neuropathic rats (Kontinen and Dickenson 2000). Muscimol, which positively modulates $GABA_A$ receptors, was also tested in the spinal nerve ligation model in rats (Sokal and Chapman 2003). Following that injury, the administration of intrathecal muscimol caused a decrease in electrically evoked responses of spinal neurones. The benzodiazepine midazolam, another positive modulator of $GABA_A$ receptors was also tested in the spinal nerve ligation injury model in rats (Kontinen and Dickenson 2000). In those experiments midazolam caused a decrease in electrically evoked responses assessed with von Frey hairs. Both midazolam and muscimol have a similar mechanism of action to alphadolone i.e. an interaction with $GABA_A$ receptors to cause an increase in GABA effect. Although the experiments described above used a different model of neuropathic pain, they do support the notion of alphadolone's ability to alleviate neuropathic pain in diabetic rats. The $GABA_A$ receptor could be a useful target for new therapies in neuropathic pain. Unlike existing treatments for diabetic neuropathic pain, alphadolone produces potent antinociception at doses that do not cause sedation. This suggests a promising treatment for severe diabetic pain without the associated unpleasant side effects.

6.1.1 Aim

This study set out to investigate the effectiveness of alphadolone and three opioids: fentanyl, morphine and oxycodone given alone at non-sedating doses to rats with diabetic neuropathy. A dose of alphadolone that did not produce any antinociception on its own was tested in combination with each opioid to determine if there were any positive interactions between the two classes of drugs in this model of diabetic neuropathic pain.

6.2 Methods

Streptozotocin (STZ) was injected via the intraperitoneal (IP) route to induce diabetes in rats. The protocol is outlined in Chapter 2 (page 66). Diabetic rats' nociceptive thresholds were measured using paw pressure (PP) withdrawal latencies. Alphadolone and three opioids (fentanyl, morphine and oxycodone) were administered via the IP route to test for antinociceptive effects in rats with diabetic neuropathy. All experiments were performed in a blinded manner. All experimental procedures and data transformation are described in Chapter 2 (page 62).

6.2.1 Study Drugs

Alphadolone (alphadolone acetate, Jurox, Rutherford, NSW) was combined with a complexing agent, 2-hydroxypropyl-β-cyclodextrin (HPβCD) to create a powder more soluble in aqueous solution. Alphadolone was weighed out fresh each day and dissolved in a phosphate buffer solution. Fentanyl (fentanyl citrate USP – David Bull Laboratories), morphine (morphine sulphate – David Bull Laboratories) and oxycodone (oxycodone hydrochloride tablets – Endone, Boots Healthcare) were diluted in normal saline fresh each day for IP administration.

All doses in mg/kg were made in a volume of 1ml calculated for a 200g rat. The average weight of rats was 180-200g so each syringe was slightly adjusted in volume, and therefore dose, to allow for the small weight differences between rats so each rat received accurate doses of drug tailored to its specific weight.

6.2.2 Experimental Paradigm

After diabetes induction, reduced nociceptive thresholds were measured in the right hind paw of male Wistar rats (wt 180-200g). After test-drug administration, PP thresholds were measured as a ratio response (r) of original PP thresholds. The range of doses of each drug used is given in Table 6.1 on page 202. Replicate experiments were performed for each dose. All rats were only used once daily for multiple experiments over a 5-day test period.

(a) Alphadolone Dose Response Relationship

A dose response curve was constructed for alphadolone given alone. A dose of alphadolone 1mg/kg was used for combination experiments, as it was the highest dose that caused no significant antinociceptive effect when given alone (refer to Data Analysis, page 202).

(b) Opioid Dose Response Relationships: Alone and in Combination with Alphadolone

Dose response relationships were measured for all opioids given alone. These experiments were repeated with a dose of alphadolone (1mg/kg) co-administered with each opioid.

Table 6.1 Ranges of Drugs used in Dose Response Study

	Alphadolone (mg/kg)	Fentanyl (µg/kg)	Morphine (mg/kg)	Oxycodone (mg/kg)
administered alone	0.5-10 *n=5-19*	2.5-20 *n=5-12*	0.4-3.2 *n=5-13*	0.125-1 *n=6-10*
co-administered with 1mg/kg alphadolone		2.5-10 *n=7-10*	0.8-3.2 *n=11-15*	0.125-1 *n=7-11*

n denotes number of replicate experiments at each dose

6.2.3 Data Analysis

For each drug dose or drug combination the values for the paw pressure ratio response (r) were combined and plotted as means ± SEM as described in Chapter 2 (page 65). A dose of alphadolone that had no effect when given alone was determined by comparing its effect (r) to the value 1 using a *t*-test. The effects of each opioid were determined by comparing their paw pressure withdrawal thresholds (r) to the value 1 using a *t*-test. The dose response curves for the opioids in the presence of alphadolone were compared with the dose response curves for the respective opioid alone using a two-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test for multiple comparisons. For all statistical comparisons, a value of $p \leq 0.05$ was considered statistically significant. In addition, the statistical calculation for oxycodone when given alone compared with its co-administration with alphadolone, was calculated differently to the other two opioids. The lowest doses of oxycodone (0.125, 0.25 & 0.5mg/kg) showed no effect both when given alone as well as in the presence of alphadolone. Therefore only doses of 0.5mg/kg and higher were used to

perform the statistical comparison of oxycodone alone and in combination with alphadolone. The inclusion of many doses that have no effect, or are at the bottom of the dose response curve, may skew statistical results to show no difference in effect when in actual fact a true effect may be present.

6.3 Results

6.3.1 The Antinociceptive Properties of Alphadolone

The dose response curve for alphadolone given alone is shown in Figure 6.1 on page 206. In diabetic neuropathic rats, alphadolone caused dose dependent antinociceptive effects assessed by increased PP thresholds. A dose of 1mg/kg (1.2 ± 0.11, n=19) caused no significant antinociceptive effects (t-test, response compared with 1, i.e. no response). This dose of alphadolone (1mg/kg), which had no effect when given alone, was selected for further combination experiments with opioids.

6.3.2 Opioid Antinociception: Alone and in Combination with Alphadolone

The dose response curves for the antinociceptive effects of the opioids are shown in Figure 6.2 on page 208. Fentanyl, morphine and oxycodone caused no significant antinociceptive effects when tested alone (t-test, compared with the value 1). Each dose of opioid was tested when co-administered with the dose of alphadolone (1mg/kg) that had no effect when given alone. The antinociceptive effect of fentanyl was significantly increased by co-administration of alphadolone ($p<0.0001$, ANOVA). The antinociceptive effects of morphine and oxycodone were also significantly increased by co-administration of alphadolone (respectively $p=0.0013$ & $p=0.0041$, ANOVA).

Figure 6.1

This figure shows the antinociceptive effect of increasing doses of alphadolone in rats with diabetic neuropathy assessed by PP (PP responses were calculated as described in Chapter 2, page 42). Alphadolone caused a dose dependent antinociceptive effect in this model. A dose of 1mg/kg did not significantly affect the PP response (*t*-test, compared to the value 1). This dose was selected for further combination testing with opioids. Each point represents the mean for 5-19 rats ± SEM.

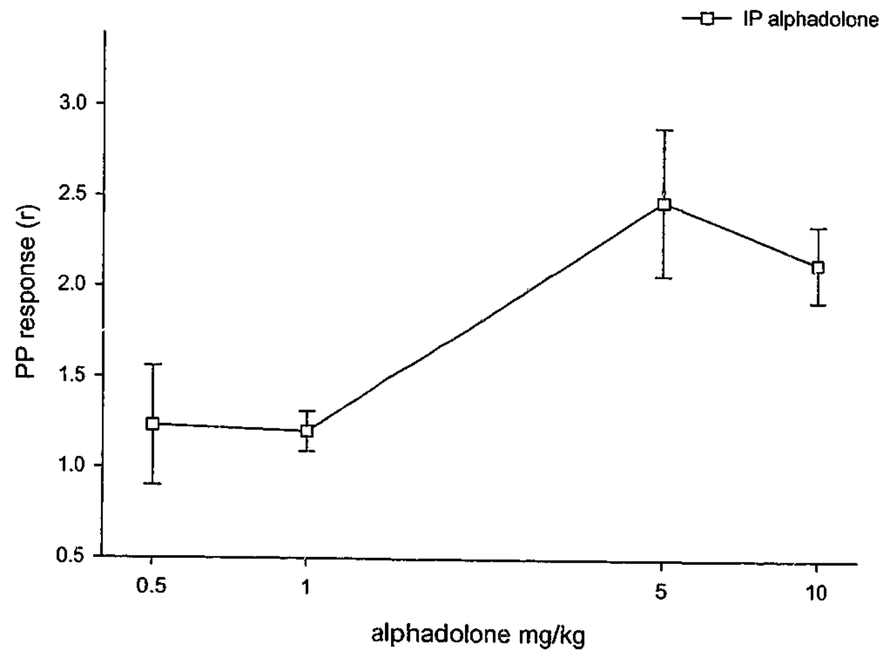


Figure 6.1 The Dose Response Relationship for IP Alphadolone in Diabetic Neuropathic Rats Assessed by PP

Figure 6.2

(A) Fentanyl

Fentanyl alone caused no significant antinociceptive effect (*t*-test, compared with the value 1). The addition of alphadolone (1mg/kg) significantly increased the antinociceptive effects of fentanyl ($p<0.0001$, ANOVA). Each point represents the mean for 5-12 rats ± SEM.

(B) Morphine

Morphine alone caused no significant antinociceptive effect (*t*-test, compared with the value 1). The addition of alphadolone (1mg/kg) significantly increased the antinociceptive effects of morphine ($p=0.0013$, ANOVA). Each point represents the mean for 5-15 rats ± SEM.

(C) Oxycodone

Oxycodone alone caused no significant antinociceptive effect (*t*-test, compared with the value 1). The addition of alphadolone (1mg/kg) significantly increased the antinociceptive effects of oxycodone ($p=0.0041$, ANOVA). Each point represents the mean for 6-11 rats ± SEM.

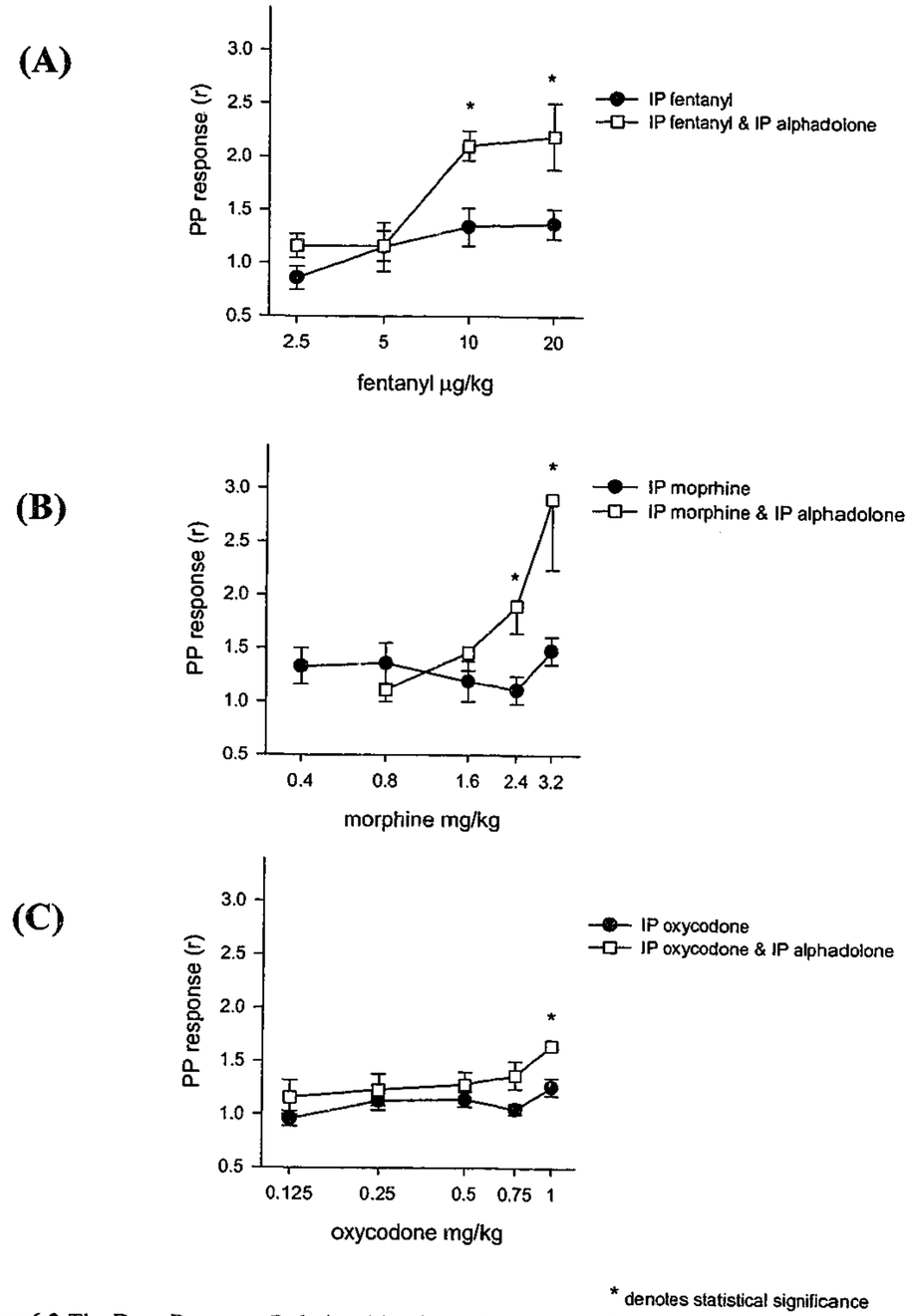


Figure 6.2 The Dose Response Relationships for IP Opioids in Diabetic Neuropathic Rats Assessed with PP: Alone and in the Presence of Alphadolone

6.4 Discussion

The doses of alphadolone and opioids that were tested for antinociceptive effects in diabetic neuropathy were non-sedating as determined from experiments described in previous chapters. This series of experiments showed dose dependent antinociceptive effects for alphadolone against hyperalgesia caused by diabetic neuropathy. At non-sedating doses the opioids fentanyl, morphine and oxycodone caused no antinociceptive effects. However, a dose of alphadolone that caused no antinociception in this model when given alone increased the antinociceptive effects of all three opioids.

6.4.1 Rat Models of Neuropathic Pain

The development of experimental rat models for neuropathic pain has been valuable in characterising mechanisms and the discovery of new effective treatments for clinical neuropathic pain syndromes. Table 6.2 on page 210 represents an overview of some existing neuropathic pain models. Bennett and Xie first described a model that involves placing ligatures around the sciatic nerve (Bennett and Xie 1988). This model causes rats to develop mechanical allodynia and thermal hyperalgesia. Kim and Chung developed a model that involves a tight ligation of L5 and L6 spinal nerves (Kim and Chung 1992). This procedure results in the development of mechanical allodynia and, to a lesser extent, thermal hyperalgesia. Another technique for producing neuropathic pain was first described by Seltzer *et al*. This technique produces hyperalgesia and allodynia by ligating the dorsal one-half to one-third of the sciatic nerve (Seltzer,

Dubner et al. 1990). Others have induced neuropathy by ligating, severing, or crushing a part of or the entire sciatic nerve, resulting in development of thermal cold allodynia (Attal, Filliatreau et al. 1994).

Table 6.2 Experimental Rat Models for Neuropathic Pain

MODEL	NERVE LESION	PHYSIOLOGICAL EFFECTS	BEHAVIOURAL EFFECTS	INVESTIGATED THERAPIES
CHRONIC CONSTRICTION INJURY	-sciatic nerve constriction	-allodynia -hyperalgesia	-abnormal posturing -guarding of the affected hindlimb -elongation of nails due to lack of attention to and use	-opioids[1] -α_2-adrenergic agonists[2] -COX inhibitors[3]
SELECTIVE LUMBAR NEURECTOMY	-spinal nerve ligation (L5 + L6)	-allodynia -less hyperalgesia	-ventroflexion of toes and foot eversion -guarding of affected hindlimb	-opioids[4] -α_2-adrenergic agonists[5] -COX inhibitors[6] -NMDA antagonists[7] -cholinergics[8] -antiepileptics[9]
PARTIAL NERVE LIGATION	-partial sciatic ligation	-allodynia -hyperalgesia	-abnormal posturing -guarding of affected hindlimb -hypolocomotion	-nicotinic agonists[10] -antiepileptics[11]
SCIATIC NERVE SECTION	-crushing/severing of limb	-allodynia	-loss of motor function -autotomy	-opioids[12] -α_2-adrenergic agonists[12]

[1] (Desmeules, Kayser et al. 1993)
[2] (Levy, Leiphart et al. 1994)
[3] (Parris, Janicki et al. 1996)
[4] (Bian, Nichols et al. 1995)
[5] (Yaksh, Pogrel et al. 1995)
[6] (Lashbrook, Ossipov et al. 1999)
[7] (Nichols, Lopez et al. 1997)
[8] (Hwang, Hwang et al. 2000)
[9] (Chapman, Suzuki et al. 1998)
[10] (Kesingland, Gentry et al. 2000)
[11] (Pan, Eisenach et al. 1999)
[12] (Przewlocka, Mika et al. 1999)

The streptozotocin-induced diabetic model has been used extensively for the study of underlying mechanisms in the development and diagnosis of diabetic neuropathy. (Hounsom and Tomlinson 1997). Hyperalgesia and allodynia have both been observed following STZ treatment in rats. However, various groups have reported conflicting results for altered pain thresholds. Reports of changes in thermal nociceptive thresholds in rats have been extremely variable. Hyperalgesia was observed in some studies by a decrease in nociceptive thresholds (Courteix, Eschalier et al. 1993; Lee and McCarty 1990), while other studies showed no change (Raz, Hasdai et al. 1988). In contrast to both of these findings, some studies have even shown loss of thermal sensation (Apfel, Arezzo et al. 1994; Pertovaara, Wei et al. 2001). With respect to mechanical hyperalgesia most groups report a fairly consistent reduction in nociceptive thresholds (approximately 30 to 40%) (Ahlgren and Levine 1993; Wuarin-Bierman, Zahnd et al. 1987; Zhuang, Snyder et al. 1996). Therefore, the experiments reported in this chapter used the PP test as a measurement of mechanical hyperalgesia and included only rats that displayed an approximate 30-40% decrease in nociceptive thresholds after diabetes induction. The number of rats excluded from experiments by these selection criteria is shown in Chapter 2, page 64.

Other than mechanical hyperalgesia, there are other reliable and consistent ways to measure the nociceptive changes caused by STZ-induced diabetes in rats. Some studies with diabetic rats measure increased sensitivity to noxious chemical stimuli. For example, after formalin injection experiments have shown increased nocifensive behaviour and decreased withdrawal thresholds assessed with the PP test (Cesena and Calcutt 1999; Kamei, Hitosugi et al. 1993). More recently studies have focussed on tactile allodynia in diabetic rats; stimulation of the plantar surface of the hind paw with

von Frey filaments (Calcutt and Chaplan 1997; Karadag, Ulugol et al. 2003; Lynch, III, Jarvis et al. 1999). It has been argued that this measure of neuropathic pain is more representative of the cutaneous hyperaesthesia reported by neuropathic patients (Fox, Eastwood et al. 1999). However it is still not possible to quantify in animals the spontaneous pain that dominates the human condition. Therefore the majority of experimental studies continue measuring defined behavioural responses to an added stimulus.

This model of STZ-induced diabetes is used not only to study underlying processes but also to evaluate potential therapies for neuropathic pain. The different drugs used in an effort to manage clinical diabetic neuropathy have been tricyclic antidepressants (amitriptyline), selective serotonin reuptake inhibitors (fluoxetine), ion channel blockers (gabapentin), NMDA antagonists (dextromethorphan), $GABA_B$ receptor agonist (baclofen), capsacin and opioids (oxycodone, tramadol) (Sindrup and Jensen 1999). None of these treatments are entirely effective against diabetic neuropathic pain. However, due to the lack of a new efficient therapy or drugs with better tolerability they remain at the forefront for treatment of this type of neuropathic pain.

6.4.2 The Effects of Alphadolone

In neuropathic pain the central nervous system (CNS) exists in a state of heightened neuronal activity (Hansson 2002). Among other possibilities this may be caused by a loss of normal inhibitory controls as seen by a reduction in local GABAergic influences (Kapur 2003). In an attempt to resume the normal state of balance, the CNS may benefit from an increase in inhibitory neuronal activity. GABA is the most ubiquitous

and abundant inhibitory neurotransmitter in the CNS and can contribute to the down regulation of neuronal excitability (Kontinen, Stanfa et al. 2001). Studies have shown that alphadolone positively modulates $GABA_A$ receptors to increase GABA activity. Therefore alphadolone could have an influence in alleviating neuropathic pain. The results in this chapter support this statement, showing alphadolone has a dose-related antinociceptive effect in neuropathic rats.

Past work has shown other positive $GABA_A$ receptor modulators to be effective in neuropathic pain. Both muscimol and midazolam, which positively modulate $GABA_A$ receptors, caused antinociception in the spinal nerve ligation model in rats (Kontinen and Dickenson 2000; Sokal and Chapman 2003). Both midazolam and muscimol have a similar mechanism of action to alphadolone whereby their modulation of $GABA_A$ receptors causes an increase in GABA activity. The past experiments described by Kontinen and Dickenson tested midazolam in a spinal nerve ligation in rats to show that it reduced spontaneous nerve firing significantly more in the spinal nerve ligation model compared with the non-operated or sham controls. The other experiments described by Sokal and Chapman with muscimol also produced significantly greater inhibition of evoked neuronal responses compared to neuronal responses in neuropathic rats compared with control rats. This suggests that positive $GABA_A$ receptor modulators are not only capable of reducing neuropathic pain but show greater antinociceptive effects in this type of pain compared to that of acute pain. The results seen in Chapter 3 (page 105) show the highest test-dose of alphadolone 10mg/kg in normal rats caused a PP response (r) of 1.66 ± 0.06. The results presented in this chapter showed 10mg/kg of alphadolone in diabetic rats caused a PP response (r) of

2.14 ± 0.21. These experiments demonstrate the antinociceptive effects of alphadolone were significantly higher in diabetic rats compared with normal rats (p=0.047, *t*-test).

Alphadolone positively modulates $GABA_A$ receptors to increase GABA activity. Therefore the increased antinociceptive effects of alphadolone in the rat diabetic model could be a result of enhanced GABA transmission. Castro-Lopes *et al.* showed a significant fall in GABA occurred after a sciatic nerve transection (Castro-Lopes, Tavares et al. 1993). A decrease in GABA concentration is one possible explanation for the excitable state of the CNS in neuropathic pain. The same group went on to demonstrate that $GABA_A$ receptor binding was substantially enhanced following a neurectomy (Castro-Lopes, Malcangio et al. 1995), even though GABA levels are reduced. It is difficult to understand why a decrease in endogenous GABA might cause an up regulation of $GABA_A$ receptors. One might suggest that this effect causes an increase in the efficacy of GABA binding in a system of diminished GABA concentrations.

Kontinen *et al.* studied the possible changes in GABAergic and glycinergic inhibitory activity in the spinal nerve ligation model of neuropathic pain. This work compared the effects of the $GABA_A$ receptor antagonist, bicuculline, and the glycine-receptor antagonist, strychnine, in neuropathic rats with their effects in sham-operated and non-operated control rats (Kontinen, Stanfa et al. 2001). Bicuculline produced dose-related hyperalgesia activity in the spinal nerve ligation group but not in the control groups. The glycine receptor antagonist strychnine did not have a statistically significant effect on any of the groups. The results support the idea of an increased GABAergic inhibitory tone in the spinal cord of neuropathic rats, possibly as compensation for

increased excitability after nerve injury. These theories could explain the increased antinociceptive effects of alphadolone in a model of diabetic neuropathy compared with its effects in normal rats.

6.4.3 The Effects of Opioids

For some time there has been an ongoing dispute concerning opioid sensitivity in neuropathic pain. Some published work suggests neuropathic pain is non-responsive to opioids (Arner and Meyerson 1988). Other studies have shown opioids may alleviate neuropathic pain but at higher doses than normal, thus indicating a shift of the dose-response curve to the right (Jadad, Carroll et al. 1992; Portenoy, Foley et al. 1990). More recent evidence has stated that opioids are effective in only certain types of neuropathic pain (Sindrup and Jensen 1999). The experiments described in this chapter showed that all three opioids; fentanyl, morphine and oxycodone caused no significant antinociceptive effects in this model of neuropathic pain. The use of low doses may be responsible for this poor activity but only non-sedating doses were tested for antinociceptive effects. Such non-sedating doses were established in earlier experiments (refer to Chapter 4, page 143).

Nociceptive testing in acute pain models showed opioids produce dose dependent antinociceptive effects at non-sedating doses (chapter 4, page 145). By contrast, the same doses of opioids produced no effects in diabetic neuropathic rats. The induction of diabetes clearly altered opioid antinociceptive effects. Few explanations have been proposed for the mechanisms by which diabetes alters opioid potency. Chen and co-workers used autoradiography studies to determine changes in the functional μ-opioid

receptors in the spinal dorsal horn in diabetic rats. A reduction in the number of functional μ-opioid receptors in the spinal cord dorsal horn of diabetic rats has been shown (Chen, Sweigart et al. 2002). This reduction constitutes one of the mechanisms underlying the reduced analgesic effect of μ-opioids in diabetic neuropathic pain. Other work suggested decreased opioid activity might be related to serotonin levels. Normal rats have been used to demonstrate a link between the opioid and serotonergic systems (Crisp, Stafinsky et al. 1991; Goodchild, Guo et al. 1997; Kuraishi, Harada et al. 1983). More importantly, however, Suh *et al.* used diabetic rats to investigate such an interaction. This work showed that a decrease in morphine activity was tied to a decrease in the release of serotonin from the bulbospinal pathways (Suh, Song et al. 1996). The decrease in serotonin release was correlated with the reduction of antinociception produced by injection of morphine assessed by the tail-flick assay. The authors concluded that the decrease in morphine activity in streptozotocin-induced diabetic rats might be, at least partly, due to the decrease of serotonin release from the spinal cord. Other experiments investigating activity of different opioid receptors suggested STZ-induced diabetes caused a decreased response to μ-opioid receptor-mediated antinociception, but normal responsiveness to drugs acting at δ- and κ-opioid receptors was preserved (Kamei, Ohhashi et al. 1992). Previous work by Ross and Smith showed that the antinociceptive effects of intracerebroventricular oxycodone were selectively mediated by κ-opioid receptors (Ross and Smith 1997). Therefore the observations from this chapter seem to be inconsistent with past findings. The work reported in this chapter demonstrated decreased antinociceptive effects for oxycodone in the diabetic model compared with normal rats. More recently, Courteix *et al.* suggested altered pharmacokinetics of opioids might be responsible for their decreased activity in the STZ-induced diabetic model. Those data indicated kinetic alterations for

morphine led to an increase of its total clearance and volume of distribution in comparison with healthy rats (Courteix, Bourget et al. 1998). This could lead to the reduction of morphine levels in the central nervous system for a given dose.

6.4.4 The Effects of Opioids in Combination with Alphadolone

In the clinic, high opioid doses are required for treating neuropathic pain and adverse side effects occur in these patients. The use of multimodal analgesia can lead to a lowering of doses of drugs to reach the same level of pain relief that might be achieved with high doses of individual analgesics given alone. The aim in using lower doses is to decrease the incidence of opioid dose dependent side effects.

Matthews and Dickenson studied one such multimodal therapy in the Kim and Chung rodent model of neuropathy. The effects of systemic morphine and gabapentin, both alone and in combination, were investigated using electrophysiological techniques to record evoked dorsal horn neuronal responses (Matthews and Dickenson 2002). Gabapentin is a structural analogue of GABA. Although its exact mechanism of action is unknown it does cause an increase in the effects of GABA (Gee, Brown et al. 1996). These experiments showed that after spinal nerve ligation, the inhibitory effects of systemic morphine on evoked dorsal horn neuronal responses were reduced compared with control, whereas the effectiveness of systemic gabapentin is enhanced. In combination with low-dose gabapentin, significant improvement in the effectiveness of morphine was observed, demonstrating a clinical potential for the use of morphine and gabapentin in combination for the treatment of neuropathic pain. Those results support the work seen in this chapter. In a model of neuropathic pain, opioid effectiveness is

decreased whereas the effectiveness of a GABA positive modulator, alphadolone, is increased. Furthermore, their combination improves the effectiveness of morphine.

No clinical or scientific research has investigated an interaction between opioids and $GABA_A$ receptor agonists in STZ-induced diabetic neuropathy or any rat model of neuropathic pain. However, some work has demonstrated a positive interaction between opioids and $GABA_A$ receptor agonists in acute pain. Selective $GABA_A$ receptor agonists, midazolam and muscimol have shown positive interactions when combined with opioids assessed by different acute nociceptive tests like the tail-flick and tail-immersion tests (Hara, Saito et al. 1999; Luger, Hayashi et al. 1995).

The experiments reported in this chapter indicate the potential for compounds interacting with $GABA_A$ receptors, like alphadolone, to be combined with opioids for an improved treatment for neuropathic pain. A dose of alphadolone (1mg/kg) that had no antinociceptive effect when given alone was able to significantly increase the antinociceptive profile for morphine, fentanyl and oxycodone assessed with the PP test.

The question one must pose is how this interaction occurs if there is a decrease in opioid activity. It is established that alphadolone antinociception, assessed by ECT, is mediated by spinal cord $GABA_A$ receptors (Nadeson and Goodchild 2000). It might be that the interaction between opioids and alphadolone seen in this chapter is also mediated by spinal cord $GABA_A$ receptors. This is similar to the findings from Chapter 4 (page 151) for acute pain assessed by TFL. It would be interesting to test intrathecal antagonists in the work outlined in this chapter to help delineate which spinal cord receptors mediate the interaction between opioids and alphadolone. Because this paw

pressure model of nociception requires rat handling at each test point, it is not compatible with intrathecal drug administration, and subsequent confinement of that drug to caudal segments of the spinal cord.

It is important to investigate any combination therapies that display increased antinociception, for a parallel increase in dose dependent adverse side effects. Experiments reported in an earlier chapter (Chapter 4, page 123) studied the antinociceptive and sedative effects of co-administered alphadolone and opioids in normal rats. Although there was an increase in antinociception following co-administration of the two drugs, it was not matched by an increase in sedation. Neuropathic pain changes many functions of the nervous system. Therefore it is important to consider sedative effects in rats with *this* particular model of neuropathic pain. Past reports have described a decrease in spinal cord μ-opioid receptors, and hence a decrease in opioid antinociception in diabetic neuropathic rats (Chen, Sweigart et al. 2002). Since this down regulation is specific to the spinal cord, opioids may still produce potent sedation caused by modulation of other opioid receptors (i.e. supraspinal). However, it was not possible to complete sedation studies for this series of experiments. The rats with STZ diabetes are unwell and the condition of some rats became extreme such that they were excluded from the study. Those rats that remained in the study were in a less severe state but nonetheless chronically ill. The ill health of the diabetic rats meant they were too fragile for rotarod testing. Furthermore, indicative of their generally poor condition, past studies have shown the spontaneous movement of diabetic rats was greatly reduced (Courteix, Eschalier et al. 1993; Fox, Eastwood et al. 1999; Shimomura, Shimizu et al. 1990). Those studies all measured spontaneous movement using the activity monitor. The use of either the rotarod or the acitivty

monitor was therefore not appropriate for testing sedative effects of drugs using diabetic rats.

6.4.5 Summary

A better understanding of neuropathic pain mechanisms would aid the discovery of a rationally founded, effective treatment for diabetic neuropathic pain. The experiments described in this chapter demonstrate a potential for neurosteroid analgesics to alleviate diabetic neuropathic pain. The exact mechanism by which alphadolone causes antinociception in this model has not been thoroughly described but is most likely to involve a positive modulation of $GABA_A$ receptors that are up regulated due to a decrease in GABA.

Chapter 7 The Effects of Neurosteroids on Morphine Tolerance

7.1 Introduction

The use of opioid medications for analgesia is associated with concerns about the potential for the development of physical dependence and tolerance (Chevlen 2003). Several definitions exist for different aspects of opioid tolerance. For example tolerance may develop in street addicts who require increasing doses of opioids to obtain the same effect. A patient becoming less affected by unwanted side effects of opioid treatments is another way to define tolerance. With respect to the work in this chapter *receptor tolerance* is described by subsequent administration of a drug at a particular dose that does not provide the same amount of analgesia as it did initially. This chapter focuses on the development of receptor tolerance to morphine, resulting in the reduction of antinociceptive effects of morphine in rats given repeated doses.

Morphine-induced tolerance may be caused by changes in the number of opioid receptors or the quantity of opioid peptides, cyclic AMP or G-proteins (Tejwani, Rattan et al. 1993). Although the exact mechanisms of these changes are not fully characterised, it has been suggested that tolerance can be considered to be an adaptive process similar to other experience-dependent changes in the brain, such as neural development involved in learning and memory (Trujillo 2002). Tolerance to opioids cannot be explained on the basis of altered metabolism or disposition of the drug (Johnson and Fleming 1989). Rather, tolerance must be the consequence of changes in some aspect of the function of the cells upon which morphine acts. There are several reports suggesting that tolerance to opioid antinociception is a spinal cord phenomenon that is closely associated with spinal sensitisation (Grisel, Watkins et al. 1996; Gutstein and Trujillo 1993; Laurido, Hernandez et al. 1996; Mao, Price et al. 1995; Mayer, Mao

et al. 1999). Central sensitisation processes that play important roles in the development of tolerance involve increased concentrations of intracellular protein kinase C in neurones of the spinal cord, the production of nitric oxide and the activation of NMDA receptors (Chevlen 2003; Trujillo 2002). This has led many workers to investigate the role of NMDA antagonists in the prevention of opioid tolerance (Allen and Dykstra 2000; Houghton, Parsons et al. 2001; Kozela, Pilc et al. 2003). The beneficial use of NMDA antagonists with opioids may be two-fold as NMDA antagonists have also been found useful in potentiating opioid analgesia (Dambisya and Lee 1994; Nadeson, Tucker et al. 2002). This potentiation effect suggests the combination of an opioid with another drug that is effective in the control of spinal cord sensitisation may help lower the dose of morphine required for treatment and ultimately decrease the production of opioid tolerance. Although NMDA receptor modulation shows such potential in animal research, drugs acting at these receptors have not entered widespread clinical practice due to their adverse side effect profile including psychomimetic symptoms, nausea and sedation (Ikonomidou and Turski 2002).

Several research groups have shown GABA's involvement in central sensitisation (Kaneko and Hammond 1997; Malcangio and Bowery 1996; Sivilotti and Woolf 1994). The role of GABA and positive modulators of the $GABA_A$ receptor have been implicated in the down regulation of central sensitisation. The neurosteroid alphadolone characterised throughout this thesis has been shown to positively modulate spinal cord $GABA_A$ receptors. Earlier chapters have shown alphadolone to interact with opioids in acute models of nociception and inflammation. Furthermore alphadolone is associated with few adverse side effects. For these reasons, it is possible that alphadolone may have some function in morphine tolerance.

7.1.1 Aim

The experiments described in this chapter sought to establish whether alphadolone could prevent the development of morphine tolerance. For comparison, another neurosteroid, alphaxalone was also tested for the prevention of morphine tolerance. Both neurosteroids have previously been shown to cause sedative effects so they were tested for such activity on the rotarod in this model of morphine tolerance. This study also investigated whether alphadolone could restore morphine antinociception lost after the production of tolerance.

7.2 Methods

All rats were initially injected with intraperitoneal (IP) morphine 6.4mg/kg and assessed for antinociceptive effects using the TFL test. A dose of morphine 6.4mg/kg was selected because it caused a near maximum antinociceptive effect (%MPE) according to the dose response curve for TFL shown in Appendix D (page 272). Morphine tolerance was then induced by means of a slow release emulsion as described in Chapter 2 (page 67). Subsequent nociceptive effects of IP morphine 6.4mg/kg were assessed with the TFL test. All experiments were done in an operator-blinded manner in order to avoid observer bias.

The first series of experiments studied the antinociceptive effects of morphine tolerant rats that received three different treatments during tolerance induction. Each group received the morphine slow release emulsion co-administered with either (1) vehicle-control (n=28), (2) subcutaneous alphadolone emulsion (250mg/kg; n=21) or (3) subcutaneous alphaxalone emulsion (80mg/kg; n=14). Different doses were selected for the neurosteroids based on earlier reports of differing potencies; alphadolone was reported to be half as potent a sedative compared with alphaxalone (Stock 1973). Thus the doses of neurosteroids were chosen to closely match for sedative effects. The neurosteroid emulsions at those doses were tested for sedative effects using the rotarod. In the second series of experiments, rats with *established morphine tolerance* were tested for antinociceptive effects of IP morphine (6.4mg/kg) when co-administered with either IP alphadolone (10mg/kg) or IP alphaxalone (10mg/kg).

7.2.1 Study Drugs

The drugs used for this series of experiments were alphaxalone (Jurox, Rutherford, NSW) and alphadolone (alphadolone acetate, Jurox, Rutherford, NSW). Both neurosteroids are combined with 2-hydroxypropyl-β-cyclodextrin (HPβCD). These cyclodextrins (CD) are used as complexing agents to improve the aqueous solubility of non-polar drugs. Alphadolone was dissolved in a phosphate buffer solution. Alphaxalone was delivered as a known weight of powder in a vial (10mg/ml), requiring the accompanying Alfaxan-CD diluent for reconstitution. The neurosteroids were used in slow-release emulsions that were made according to the same protocol outlined for the morphine emulsion in Chapter 2 (page 67). Morphine (morphine sulphate – David Bull Laboratories) was diluted in normal saline fresh each day for IP administration.

All doses of morphine for IP administration were given in a fixed concentration calculated so that a 200g rat would receive a 1ml IP injection. The average weight of rats was 180-200g. The volume of IP injection was slightly adjusted to allow for the small weight differences between rats so rats received accurate doses of drug tailored to their specific weight. Each experiment ran for 4 days.

7.2.2 Experimental Paradigm

(a) Prevention of Morphine Tolerance

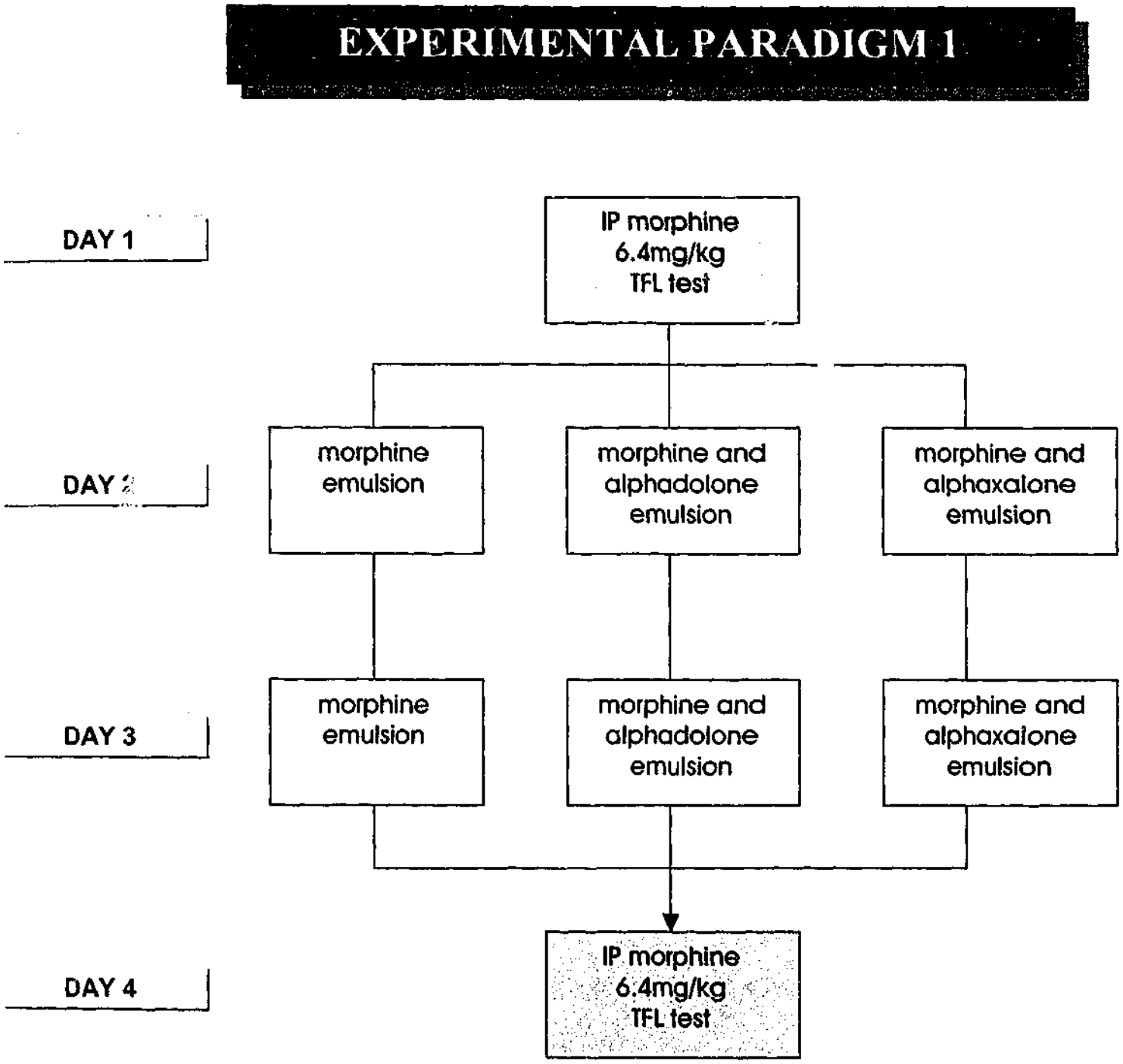
Each rat was initially tested with IP morphine 6.4mg/kg for antinociceptive effects assessed by TFL response. The rats were divided into three treatment groups where each group received the morphine slow release emulsion co-administered with either (1) vehicle control, (2) alphadolone (250mg/kg) or (3) alphaxalone (80mg/kg) each suspended in emulsions. After two days of receiving treatment, all three groups were then tested with IP morphine 6.4mg/kg for antinociceptive effects assessed by the TFL test. These groups and the protocol for their emulsions are shown in Table 7.1 below and Figure 7.1 on page 229.

Table 7.1 Emulsion Formulations of Treatment Groups

	vehicle-control emulsion	alphadolone emulsion	alphaxalone emulsion
drug dose	n/a	250mg/kg	80mg/kg
number of replicate experiments	28	21	14
phosphate buffer	8ml	8ml	8ml
liquid paraffin oil	6ml	6ml	6ml
mannide monooleate	1ml	1ml	1ml

The entire experiment was performed over a 4-day period. The protocol is outlined in Figure 7.1 on page 229.

Figure 7.1 Experimental Paradigm 1: The Effects of Neurosteroids on Morphine Tolerance Prevention



(b) Sedative Effects of Neurosteroid Emulsions

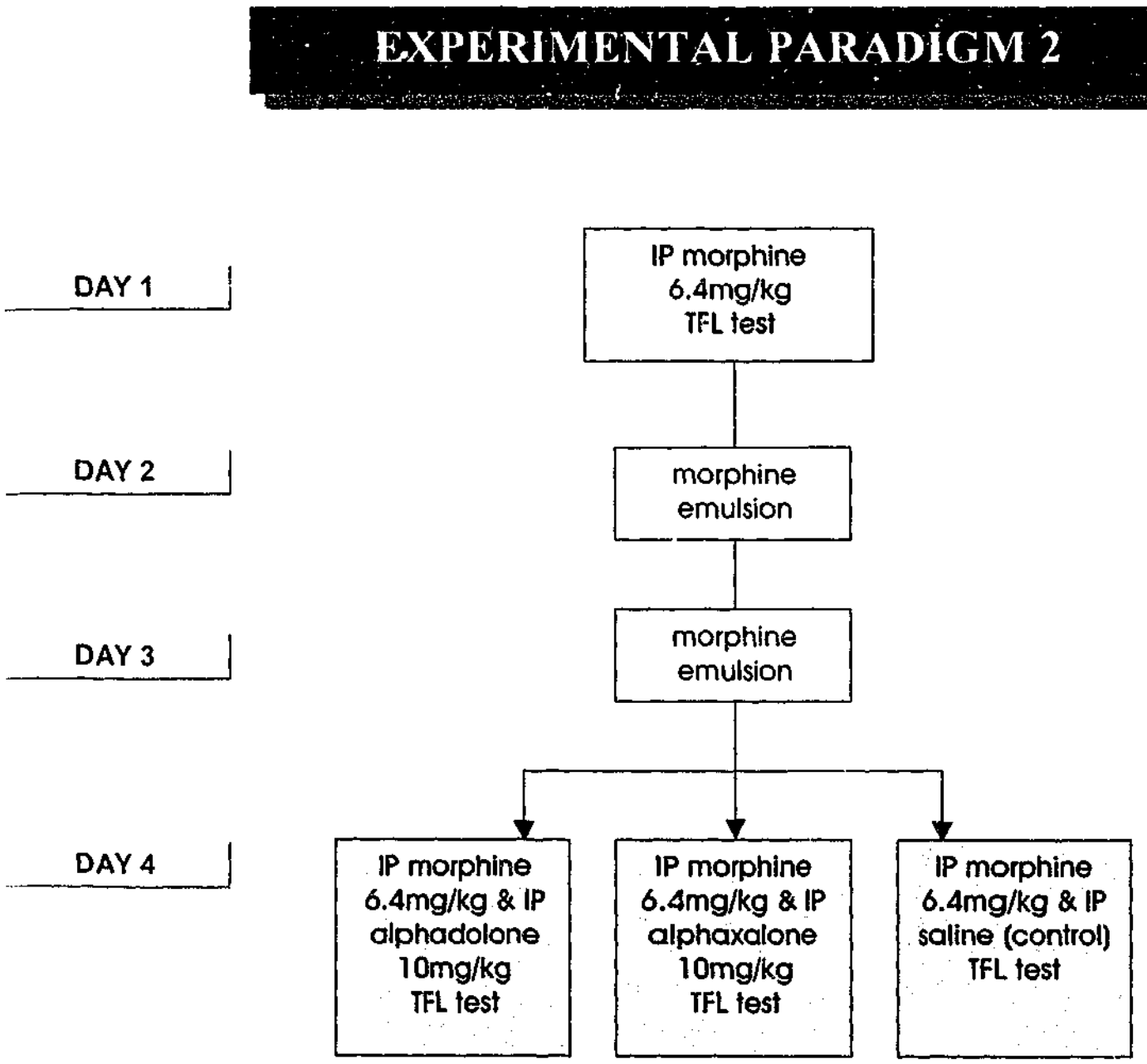
The alphadolone and alphaxalone emulsions were tested for sedative effects. Naive rats were trained and tested on the rotarod according to the protocol described in Chapter 2 (page 83). After this conditioning period the run time for each rat on the rotarod was tested at time zero (before subcutaneous neurosteroid emulsion administration), and at one-hour intervals thereafter for the following 8 hours. Each rat received a 1ml subcutaneous injection of either alphadolone (250mg/kg, n=8) or alphaxalone (80mg/kg, n=8) in a slow-release emulsion. This exact process was repeated on the following day to mirror the protocol used for administration of the neurosteroid emulsions in combination with the morphine emulsion.

(c) Antinociceptive Effects of Co-administered Neurosteroids and Morphine in Morphine Tolerant Rats

Further experiments tested whether the co-administration of IP alphadolone or alphaxalone with IP morphine could restore the diminished antinociceptive effects of morphine after tolerance had already occurred. All rats were first tested with IP morphine 6.4mg/kg for antinociceptive effects assessed by TFL response. Morphine tolerance was then induced over a two-day period by the administration of a morphine slow release emulsion as described in Chapter 2 on page 70. Once tolerance was established, the rats were tested for antinociceptive effects with IP morphine 6.4mg/kg in combination with IP alphadolone (10mg/kg) or IP alphaxalone (10mg/kg) using the TFL test. The observer was blinded with half the rats in vehicle-control groups

receiving only IP morphine 6.4mg/kg. The experimental paradigm is outlined in Figure 7.2 below.

Figure 7.2 Experimental Paradigm 2: The Effects of Co-administered IP Neurosteroid and IP Morphine on Morphine Tolerance



7.2.3 Data Analysis

For each treatment group the TFL values (%MPE) were combined to calculate means ± SEM. All groups were compared for statistical significance before and after tolerance induction using a two-way analysis of variance (ANOVA), with a Bonferroni post hoc test for multiple comparisons. Run time results for the rotarod were combined for each dose of drug and expressed as means ± SD. Each dose was compared with the control maximum run time of 120 seconds using a *t*-test. For all statistical comparisons, a value of $p \leq 0.05$ was considered statistically significant.

7.3 Results

7.3.1 Co-Administration of Neurosteroids with Morphine Reduces the Development of Morphine Tolerance

The subcutaneous morphine emulsions containing either alphadolone or alphaxalone were both able to partially prevent the development of morphine tolerance. These results are shown in Figure 7.3 on page 237. Prior to the induction of morphine tolerance, IP morphine (6.4mg/kg) caused potent antinociceptive effects assessed by TFL response in all rats (89.6% ± 2.4, n=63). Rats that received an emulsion containing vehicle (control) in conjunction with the morphine emulsion showed a significant reduction in TFL response at the end of the tolerance induction protocol (17.4% ± 3.9, n=28) i.e. tolerance to IP morphine had developed. Rats treated with the emulsion of alphadolone co-administered with the morphine emulsion showed a significant increase in TFL response (51.8% ± 6.7, n=21) compared with the morphine tolerant vehicle-control group. The other group of rats that received an emulsion of alphaxalone co-administered with the morphine emulsion also showed a significant increase in TFL response (43.1% ± 8.3, n=14) compared with the morphine tolerant vehicle-control group.

7.3.2 The Sedative Effects of Subcutaneous Alphadolone and Alphaxalone Emulsions

Groups of rats that received subcutaneous injections of slow release emulsions containing either alphadolone (250mg/kg, n=8) or alphaxalone (80mg/kg, n=8) were assessed for sedative effects using the rotarod test. These results are shown in Figure 7.4 on page 239. Alphadolone caused no sedative effects assessed by the rotarod at any time point tested. By contrast alphaxalone caused significant sedative effects for the initial four hours after its administration (p=0.01, ANOVA). Neither neurosteroid displayed any residual sedative effects after 8 hours.

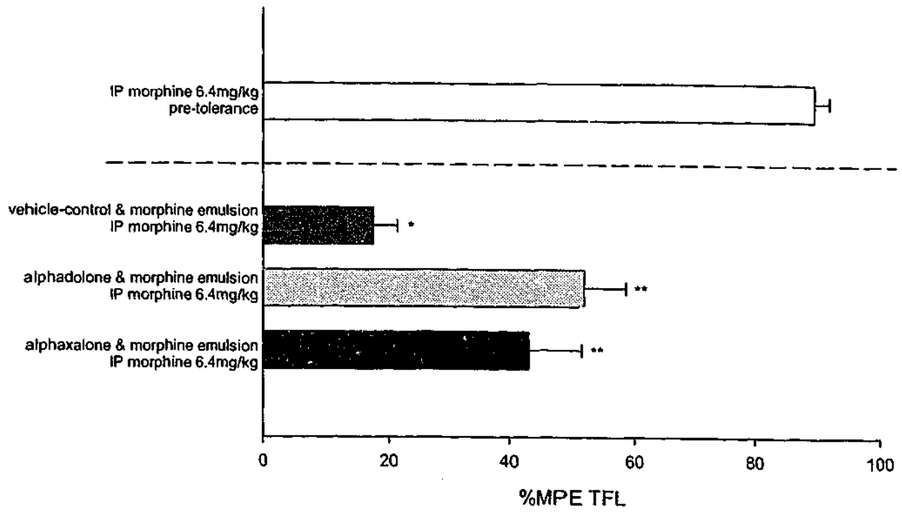
7.3.3 The Effects of Co-administered Neurosteroids and Morphine on Morphine Tolerant Rats

These results are also shown in Figure 7.5 on page 241. Initially, prior to morphine emulsion treatment to induce tolerance, all rats given IP morphine 6.4mg/kg displayed antinociceptive effects assessed by TFL (76.0% ± 3.6, n=39). After tolerance induction, rats given IP morphine 6.4mg/kg showed a significant reduction in TFL responses (23.4% ± 7.6, n=11) i.e. tolerance to IP morphine had developed. The TFL responses of the group of rats given a combination of IP alphadolone 10mg/kg and IP morphine 6.4mg/kg were the same as the responses to morphine before tolerance induction (78.6% ± 9.8, n=9) i.e. a normal response to IP morphine was produced in

rats that were tolerant to morphine. By contrast, rats given IP alphaxalone 10mg/kg in combination with IP morphine 6.4mg/kg after tolerance induction showed TFL responses (19.4% ± 10.7, n=10) that were not significantly different to the responses of rats receiving IP morphine alone i.e. tolerance to IP morphine was present.

Figure 7.3

This figure shows the TFL response to IP morphine 6.4mg/kg (n=63) before induction of morphine tolerance by subcutaneous injections of the morphine emulsion. All rats were given the morphine slow release emulsion in combir ation with an emulsion made with either vehicle-control (n=28), alphadolone (250mg/kg, n=21) or alphaxalone (80mg/kg, n=14). The figure shows the TFL response to IP morphine 6.4mg/kg for each group after their respective 2-day emulsion treatments. Significant tolerance to IP morphine occurred in all groups, i.e. the TFL response to IP morphine on day 4 was significantly lower than the TFL response to the same dose of morphine before the emulsion treatment. Both alphadolone- and alphaxalone-treated groups significantly attenuated the tolerance such that the TFL responses to morphine after the emulsion treatment were greater than the vehicle-control group. Each histogram represents means ± SEM.

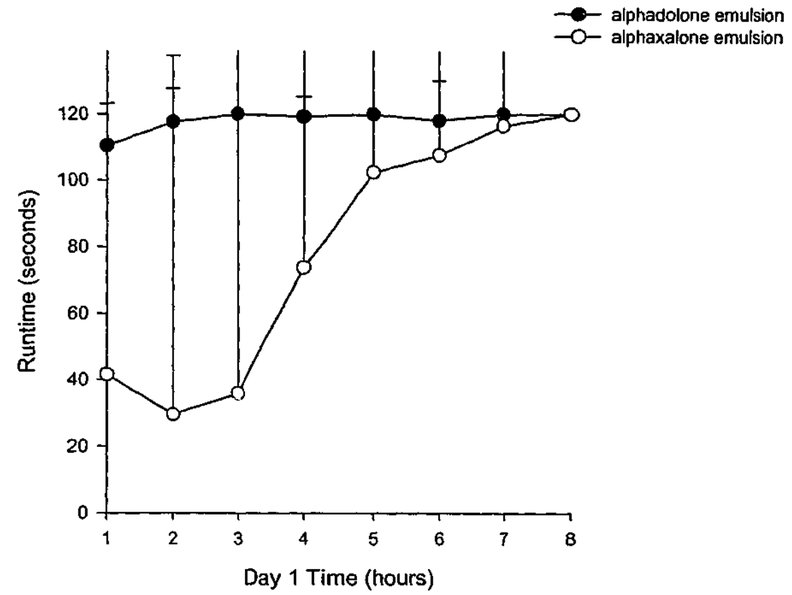


* denotes statistical significance compared to pre-tolerance
**denotes statistical significance compared to post-tolerance

Figure 7.3 The Effect of Co-administered Neurosteroid and Morphine Emulsions on Morphine Tolerance

Figure 7.4

This figure shows time response curves for the sedative effects of subcutaneous emulsions alphadolone (250mg/kg) and alphaxalone (80mg/kg) assessed by the rotarod test as described in Chapter 2, page 82. Run times were measured every hour for 8 hours after the subcutaneous injections were given on two consecutive days. Sedation was considered as a decrease in run time compared with the normal run time of 120 seconds. Alphadolone caused no sedative effects at any test point where as alphaxalone caused significant sedation for the first four hours on both days. Each point represents the mean of 8 rats ± SD.



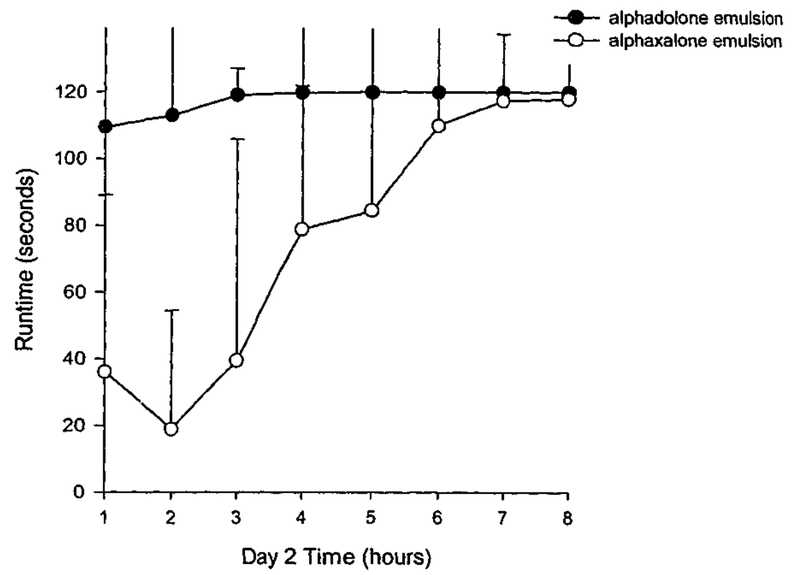
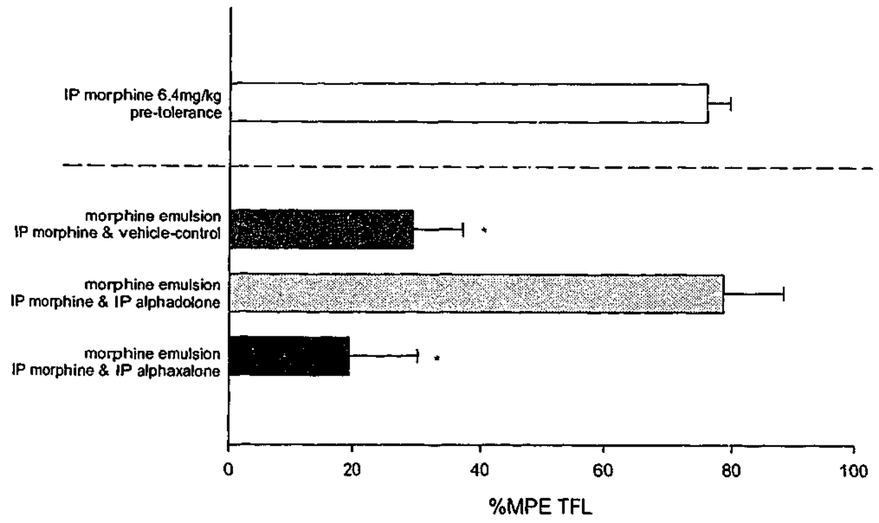


Figure 7.4 The Sedative Effects of Subcutaneous Alphadolone and Alphaxalone Emulsions

Figure 7.5

This graph shows the TFL response to the co-administration of either IP alphadolone or IP alphaxalone combined with IP morphine in morphine tolerant rats. Potent significant antinociceptive effects were caused by IP morphine (6.4mg/kg) given before tolerance induction (76.0% ± 3.6, n=39). After morphine tolerance was established, IP morphine 6.4mg/kg caused a significant smaller effect in TFL response (23.4% ± 7.6, n=11) i.e. morphine tolerance had occurred. Rats given a combination of IP alphadolone 10mg/kg with IP morphine 6.4mg/kg after tolerance induction, by contrast showed significant antinociceptive effects (78.6% ± 9.8, n=9) that were not significantly different from original, pre-tolerance TFL responses to morphine 6.4mg/kg. The combination of IP alphaxalone 10mg/kg and IP morphine 6.4mg/kg caused no such restoration of morphine antinociception (19.4% ± 10.4, n=10). Each histogram represents means ± SEM.



* denotes statistical significance compared to pre-tolerance

Figure 7.5 The Effects of Co-administered IP Neurosteroids and IP Morphine on Morphine Tolerant Rats

7.4 Discussion

The experiments reported in this chapter used an established model for the induction of morphine tolerance; the slow release morphine emulsion reliably produced tolerance to morphine in rats. The induction of tolerance was partially prevented when emulsions containing either alphadolone or alphaxalone were co-administered with the morphine emulsion. When tested for sedative effects, the alphaxalone emulsion caused sedation while the alphadolone emulsion caused none. In further experiments, the co-administration of IP alphadolone with IP morphine increased the reduced antinociceptive effect of morphine in tolerant rats. By contrast, the co-administration of IP alphaxalone with IP morphine had no effect on the reduced antinociceptive effects of morphine in tolerant rats.

7.4.1 Development of Morphine Tolerance

Other investigators have used the administration of a slow release morphine emulsion for the induction of morphine tolerance in rats. However the literature on the subject is often conflicting making it difficult to compare data from different laboratories. Different protocols have been used with varying opioid concentrations, formulations and dosing regimens. For example, Fredrickson and Smits used a single injection designed to release 300mg/kg morphine over a 24-hour period (Frederickson and Smits 1973), while Laska and Fennessy tested several different doses (75, 100 & 150mg/kg) (Laska and Fennessy 1976). Collier *et al.* used a slow release emulsion containing

morphine in the form of its base (Collier, Francis et al. 1972), while Warhurst *et al.* used the hydrochloride salt (Warhurst, Smith et al. 1984). Salem and Hope eventually published a validated protocol for the induction of morphine tolerance. These researchers demonstrated the administration of a slow release emulsion reliably produced tolerance that could be reproduced by other researchers (Salem and Hope 1998). The experiments reported in this chapter followed this protocol to induce morphine tolerance in rats. Tolerance to morphine was reliably produced in all control rats.

7.4.2 The Possible Overlap Between Mechanisms of Morphine Tolerance and Hyperalgesia

Recent experiments have reported similar neural mechanisms to be involved in morphine tolerance and chronic injury pain (Grisel, Watkins et al. 1996; Gutstein and Trujillo 1993; Laurido, Hernandez et al. 1996). These reports suggest hyperalgesia as a result of chronic injury and the development of morphine tolerance may be interrelated. Hyperalgesia associated with nerve injury involves neuronal plasticity that activates excitatory amino acid (EAA) receptors. This inturn causes subsequent intracellular cascades, including protein kinase C translocation and activation as well as nitric oxide production (Coderre, Katz et al. 1993). Similar EAA receptor-mediated cellular and intracellular mechanisms have now been implicated in the development of tolerance to the analgesic effects of morphine (Gilron, Biederman et al. 2003; Mao, Price et al. 1994). Furthermore, a common site for those drugs involved in both hyperalgesia and morphine tolerance is likely to be in the dorsal horn of the spinal cord (Mao, Price et al.

1994; Mayer, Mao et al. 1999). Thus although the mechanisms causing tolerance to opioids have essentially remained unexplained, the common link to other forms of neuronal plasticity may provide a scientific basis for improved pain management with opioid analgesics. This argument implies that the effect neurosteroids have on the development of morphine tolerance may be caused by the same mechanisms that underlie the effects of the compound on hyperalgesia associated with nerve injury and inflammation.

7.4.3 Neurosteroid Co-Administration with Morphine Emulsion Reduces Tolerance Development

Most published work suggests that the activation of NMDA receptors, increased concentrations of intracellular protein kinase C in neurones of the spinal cord and the production of nitric oxide all play important roles in the development of tolerance (Chevlen 2003; Trujillo 2002). Among the several possible mechanisms, the role of the GABAergic system has received considerable attention in recent years. Investigations have suggested morphine may produce some of its effects by interacting with GABAergic systems because chronic administration of morphine has been shown to modify central $GABA_A$ receptors (Ticku and Huffman 1980). Early work suggested the administration of GABA might increase the development of tolerance to morphine in mice, while blockade of $GABA_A$ receptors by bicuculline resulted in the reduction of tolerance (Ho, Loh et al. 1976). However this runs contrary to more recent findings, including those of this thesis, which suggest an inhibitory effect on the development of opioid tolerance by positive GABAergic modulation.

In support of the latter are the observations that parenteral administration of benzodiazepines like midazolam, diazepam and flunitrazepam have all been shown to delay the onset of opioid tolerance in rats by positively modulating $GABA_A$ receptors (Maldonado, Mico et al. 1991; Tejwani, Rattan et al. 1993). In addition, the administration of the benzodiazepine antagonist flumazenil was able to enhance naloxone-precipitated withdrawal symptoms in rats (Valverde, Mico et al. 1992). All of these studies suggest that benzodiazepine receptor agonists inhibit the development of both tolerance and physical dependence.

Like benzodiazepines, neurosteroids positively modulate $GABA_A$ receptors. Experiments by Reddy and Kulkarni showed that chronic administration of neurosteroids such as allopregnenolone, pregnenolone sulphate and progesterone effectively prevented the development of tolerance to the antinociceptive effect of morphine (Reddy and Kulkarni 1997). These experiments measured the antinociceptive effects of morphine using the tail-flick test in mice. The induction of tolerance was produced by the administration of morphine sulphate (10mg/kg) twice daily for 9 days. However, there are some concerns about this work when one considers practical application and clinical use; the test compounds used in those experiments did not produce analgesia on their own and they did cause sedation. Both these qualities render a compound less desirable for opioid tolerant patients. In addition, none of those compounds would be suitable for clinical use as they possess hormonal properties.

Previously, benzodiazepines such as midazolam, diazepam and flunitrazepam were shown to prevent opioid tolerance. Although benzodiazepines act at peripheral receptor

sites, it has been concluded that their inhibition of morphine tolerance involves $GABA_A$ receptors in the CNS (Tejwani, Rattan et al. 1993). Neurosteroids are known to modulate directly chloride ion channels that are distinct from those chloride ion channels associated with $GABA_A$ receptors. However, like benzodiazepines, their ability to attenuate the development of morphine tolerance might involve the $GABA_A$ receptor (Valeyev, Hackman et al. 1999).

The experiments described in this chapter tested two neurosteroids, alphadolone and alphaxalone that are both pregnanediones devoid of hormonal effects (Stock 1973). The rats treated with neurosteroid emulsions in combination with the slow release morphine emulsion responded to IP injections of morphine with a significant rise in tail flick compared with the tail flick of tolerant rats. One concludes from these results that neurosteroids partially prevent the development of tolerance. A possible argument against such conclusions is that these neurosteroids are analgesics (Lambert, Belelli et al. 2001); that the opioid tolerance remains and the results merely show the analgesic effect of the neurosteroids alone. However results shown in Chapter 3 (page 105) indicate that neither alphaxalone nor alphadolone cause any antinociceptive effects assessed by the TFL test.

One possible mechanism for neurosteroid prevention of opioid tolerance is a direct interaction of neurosteroids with opioid receptors. Perhaps neurosteroids protect opioid receptors from down regulation. Schwarz and Pohl investigated a wide variety of steroids for direct interactions with opioid receptors. Among all classes of steroids tested (oestrogens, androgens, glucocorticoids, mineralocorticoids, gestagens) only oestrogens competed with naloxone binding sites (Schwarz and Pohl 1994). Although

pregnanediones were not included in this study, studies have shown that alphaxalone and alphadolone both modulate $GABA_A$ receptors and do not directly interact with opioid receptors (Cottrell, Lambert et al. 1987; Nadeson and Goodchild 2000).

Neurosteroids such as pregnenolone sulphate and pregnenolone have also been shown to modulate calcium currents (ffrench-Mullen, Danks et al. 1994). Furthermore, Diaz and co-workers have shown that morphine tolerance is associated with an increase in calcium channel flux (Diaz, Ruiz et al. 1995). One might speculate that neurosteroids such as alphadolone and alphaxalone can modulate this morphine-induced increase in calcium channel currents to help prevent morphine tolerance.

Neuroactive steroids regulate neuronal function through their effects on both transmitter-gated ion channels and steroid receptor-regulated gene expression (Baulieu, Robel et al. 1999). Neurosteroids may have potent anaesthetic and anticonvulsant effects occurring within a few minutes of administration by interacting directly with a surface membrane receptor-complex to cause a rapid change in central nervous system excitability (Puia, Santi et al. 1990; Purdy, Moore, Jr. et al. 1992). However, neurosteroids may also bind to the intracellular progestin receptor after metabolic conversion and therefore start gene transcription (Rupprecht, Reul et al. 1993). This long-term regulatory effect caused by steroids may result in morphological changes. Such changes may protect receptor down regulation or enhance receptor concentrations, and hence reduce the development of tolerance to opioids. Since neither alphadolone nor alphaxalone possess any hormonal effects, it is unlikely that the mechanism described is involved in the reduction of the development of morphine tolerance.

7.4.4 Sedative Properties of Neurosteroid Emulsions

Experiments described in Chapter 3 (page 101 & 103) showed dose dependent sedative effects for the neurosteroids, alphadolone and alphaxalone. It has been reported that alphaxalone is two times more potent as an anaesthetic compared with alphadolone (Stock 1973). This is why the alphaxalone emulsion was given at a much lower dose compared with alphadolone. Even so, the alphaxalone emulsion still caused significant sedation while the alphadolone emulsion caused none. The sedative effects of alphaxalone were gone after 8 hours. Thus residual sedative effects did not affect TFL testing performed on the following day. In spite of the structural similarities of these two neurosteroids, their pharmacological properties are distinct. This further supports the conclusions from work described in Chapter 3, where the different activities of alphaxalone and alphadolone are highlighted.

Chronic administration of opioids, particularly at high doses, results in the development of tolerance to their analgesic activity. This is most often seen in patients seeking relief for pain of moderate to severe intensity, for example cancer (Bhargava 1994). Unfortunately, in order to prevent opioid tolerance in these patients, it would not be practical to co-administer a neurosteroid that is associated with sedation. Such effects can decrease quality of life for chronically ill patients or cause serious adverse effects like respiratory depression. In preventing morphine tolerance in long-term pain patients, the work in this chapter demonstrated a much wider therapeutic index for co-administration of alphadolone with morphine. Alphaxalone might cause a similar effect on opioid tolerance but only at the expense of causing some sedation of the subject.

7.4.5 Effects of Neurosteroids on Established Morphine Tolerance

Experiments reported in this chapter showed co-administration of alphadolone with morphine led to normal morphine antinociceptive effects in rats with established tolerance. Morphine tolerant rats were tested with IP morphine 6.4mg/kg given with IP alphadolone 10mg/kg. These rats showed a TFL response that was no different to the TFL response of rats before tolerance induction. By contrast, IP alphaxalone (10mg/kg) co-administered with IP morphine 6.4mg/kg did not produce any significant increase in TFL responses in morphine tolerant rats compared with rats receiving only IP morphine at the same dose.

It is most likely the increased antinociceptive effect produced in morphine tolerant rats was caused by an interaction between alphadolone and the reduced, but remaining, antinociceptive effect of morphine. Past experiments have shown a dose of 10mg/kg of alphadolone potentiates morphine TFL effects (Winter, Nadeson et al. 2003). This work showed that only a small dose of morphine was required when co-administered with alphadolone, to cause an increased antinociceptive effect. In the experiments reported in this chapter almost all the antinociceptive effects of morphine were abolished due to the effects of tolerance. After tolerance production, the small but significant effect remaining for morphine antinociception may have been enough for potentiation by alphadolone. Previous experiments showed co-administration of neurosteroids progesterone, allopregnenolone and pregnenolone sulphate prevented morphine tolerance. However, none of these compounds were able to reverse established morphine tolerance (Reddy and Kulkarni 1997). This might be because those

compounds were not associated with any analgesic effects and they therefore, like alphaxalone, which also has no analgesic activities, were not capable of potentiating the remaining effects of morphine in established tolerance.

It is well known that after chronic treatment with morphine, receptor down regulation is observed in the central nervous system (CNS) (Bhargava 1994). Investigators have concluded that since morphine targets μ-opioid receptors, it is this receptor that is responsible for the development of morphine tolerance (DeLander, Portoghese et al. 1984). Those alterations of opioid receptors might contribute to tolerance but may not necessarily affect the activity of other compounds interacting with morphine. For example, even though the antinociceptive effects of morphine are diminished due to tolerance, the co-administration of alphadolone with morphine might produce antinociceptive effects by activating other receptors that were not down regulated in tolerance development. This is supported by work described in Chapter 4 (page 123) that demonstrated an increase in morphine antinociception caused by alphadolone co-administration, was mediated by spinal cord $GABA_A$ receptors. The experiments reported in this chapter showed co-administration of alphadolone with morphine produced potent antinociception in morphine tolerant rats. This effect might also involve $GABA_A$ receptors.

Once tolerance to the analgesic effect of the opioid is observed, and in order to avoid unnecessary further development of tolerance, simultaneous administration of other analgesics is often advocated in clinical practice. In the perioperative period, the simultaneous administration of low-dose ketamine (Duncan and Spiller 2002), co-administration of an α_2 agonist (clonidine, dexmedetomidine), and the administration of

a selective cyclooxygenase-2 (COX-2) inhibitor (refecoxib) (Freye and Latasch 2003), are all multimodal strategies employed in situations of opioid tolerance. In chronic pain therapy opioid rotation of a more potent ligand such as methadone, fentanyl or oxycodone has been suggested (Inturrisi 2002). With regard to tolerance development in the intensive care unit, co-administration of an α_2 agonist, and daily intermittent cessation of benzodiazepine administration are advocated (Freye and Latasch 2003). None of these outlined strategies actually reverse the down regulation of opioid receptors; they are merely able to function effectively in a state of opioid tolerance.

7.4.6 Summary

The development of tolerance to morphine limits its use for long-term pain control (Portenoy 1987). Morphine-mediated analgesic effects are decreased when tolerance develops. Therefore the morphine dose requirement must be increased to obtain the same therapeutic effect. A combination of drugs that can maintain the analgesic effect of morphine and/or reduce the degree of tolerance may help patients suffering from chronic pain.

The experiments described in this chapter suggest alphaxalone and alphadolone can both partially prevent the development of morphine tolerance in rats. This indicates the potential for other neurosteroids to be assessed for a role in morphine tolerance. During the induction of tolerance, alphadolone produced no sedative effects. In contrast, alphaxalone produced significant sedation when co-administered with the slow release morphine emulsion. Therefore alphadolone offers a more beneficial role for co-administration with opioid treatment in patients suffering from chronic pain.

These studies also showed that in established tolerance, co-administration of alphadolone and morphine produced potent antinociceptive effects. This is most likely caused by alphadolone increasing the remaining antinociceptive effects of morphine. Alphaxalone did not increase the antinociceptive effects of morphine in tolerant rats. This further demonstrates the different pharmacological properties of two similar neurosteroid structures. These results support a role for the addition of alphadolone to opioids in the clinical setting of tolerance. The concept of multimodal analgesia, consisting of the simultaneous use of analgesics with a different mode of action, may in fact counteract tolerance development.

Chapter 8 Conclusions

Although there are many analgesics available for human use, opioids are still the most common treatments for severe pain states. Unfortunately they and many other analgesics are associated with adverse side effects that sacrifice patient comfort. This thesis investigated the novel neurosteroid alphadolone as an effective analgesic therapy for pain management. The goals for a new analgesic are to provide pain relief of an improved quality if used alone, or when used in combination with other established compounds to improve the overall analgesic profile. Such use of combinations of drugs, acting via different mechanisms to provide more effective pain relief, is called multimodal analgesia. Low doses of individual drugs when used in combination can achieve the same level of pain relief gained with single analgesics used at high doses but with decreased side effects. Multimodal analgesia is based on the concept of an integrated nociceptive system where neurotransmitters work in complex ways together, rather than alone, in the modulation of analgesia. Thus, in addition to investigating the antinociceptive effects of neurosteroids given alone, this thesis investigated the extent of the concomitant side effects. Sedation was used as a surrogate that suggested the occurrence of CNS mediated side effects. In all experiments only doses that were below a drug's sedative threshold were used. This minimised the masking of potential analgesia by sedation, and therefore allowed the demonstration of the analgesic/sedative profile of alphadolone and alphadolone/opioid combinations in a variety of animal pain models.

Pain is a complex sensation involving both psychological and physiological components. The psychological aspects of pain are difficult to classify because it is a very personal and individual experience (Horn and Munafo 1997). Therefore the majority of research has focused on experiments aimed at understanding the physiology

and mechanistic nature of pain. In order to gain better knowledge of these physiological mechanisms both human and animal models are used for pain studies. Not surprisingly, clinical experiments provide the most insight into human pain. However they do hold a number of limitations. Pre-clinical work using animal models of human pain states, such as those used throughout this thesis, is valuable because it allows manipulation of experimental variables not possible in humans. There has been a vast increase in animal models specifically for the screening of new drugs. It is widely accepted that drugs shown to be effective in such studies are worthy of further investigation including testing in human clinical trials.

Because pain is a multifaceted experience, its study can be broad and confusing. There are a wide variety of methods that exist for producing nociception in animals. Noxious heat, mechanical, electrical and chemical stimulation procedures can be used in models of both acute and chronic pain states (Vyklicky 1980). A range of these have been used in the studies presented in this thesis.

Various researchers have outlined many specific prerequisites for the study of pain (Beecher 1959; Dubner 1983; Wolff 1977). However these outlines are often conflicting and contain general assumptions about research that cannot be held to individual experiments. The assorted pain models and tests for sedation used throughout this thesis helped provide a general overview for the properties of alphadolone as an analgesic. Of course there are many other models that could be employed for further characterisation. However those utilised supplied ample preliminary data suggesting alphadolone's analgesic actions.

In the 1950's, much research led to the recognition of the importance of GABA as an inhibitory transmitter (Rang, Dale et al. 1996). Today the enhancement of neuronal inhibition by GABA is one of the most powerful therapeutic strategies for the treatment of CNS diseases. Pharmacological studies have demonstrated the existence of different subclasses of GABA receptors with a wide variety of binding sites that can modulate GABA activity. This suggests a plethora of therapeutic opportunities. Because GABAergic transmission has some level of control for all CNS processes, $GABA_A$ receptors have become targets for many drugs in clinical use, e.g. treatments for anxiety disorders, sleep disturbances, muscle spasms and seizures (Rudolph, Crestani et al. 2001).

In persistent nociception, transformations within the CNS cause a dysfunctional state of increased neuronal excitability. This plasticity involves alterations in GABAergic transmission. These events, or their underlying mechanisms, have not been elucidated entirely. However there is consensus that an overall loss of inhibition occurs. In the published literature attention has been directed to a reduction in GABA concentrations or GABAergic interneurons (Castro-Lopes, Tavares et al. 1993), as well as altered binding of GABA to $GABA_A$ receptors (Castro-Lopes, Malcangio et al. 1995). Since positive modulation of $GABA_A$ receptors increases GABA activity, one would expect compounds with these properties could ameliorate this form of nociception. The neurosteroid alphadolone has been shown to positively modulate $GABA_A$ receptors (Nadeson and Goodchild 2000) and therefore could have a role in the treatment of persistent nociception.

Persistent pains of seemingly different origins have a commonality in mechanisms. Peripheral *tissue injury* causes hyperexcitability resulting from neuronal plasticity, persistent pain of *neuropathic* origin also results in excessive neuronal firing caused by altered processing. Both of these phenomena are most commonly associated with, and identified by, the development of hyperalgesia. Intracellular processes associated with hyperalgesia are also common to the mechanisms that cause tolerance to opioids. As GABA is the prevailing inhibitory neurotransmitter, it has some level of control in all nociceptive processes. As demonstrated by the experiments described in this thesis, the positive $GABA_A$ receptor modulator, alphadolone, was active in all these models, although the magnitude of its effectiveness varied.

Since Selye first discovered fast acting steroids, various neurosteroids have been investigated for activities within the central nervous system (CNS). There have been considerable efforts to delineate their physiological roles and mechanisms of action. Neurosteroid compounds have been shown to selectively modulate $GABA_A$ receptors, glycine chloride channels, voltage gated calcium channels and to potentiate NMDA receptor responses (Gambhir, Mediratta et al. 2002). However, most neurosteroids exhibit neuromodulatory roles that are caused by their GABAergic functions. Numerous endogenous steroids with diverse biological specificities do exist. Interestingly, endogenous neurosteroids are thought to be partially responsible for gender-dependent differences in the responsiveness to nociceptive stimuli (Liu and Gintzler 2000). This topic has long been the subject of speculation and investigation. Such neurosteroids may have an influence on nociception by modulating GABA transmission, similar to the actions of alphadolone. Furthermore, some experimental studies have shown there are gender differences in opioid analgesia that range from

mice to humans (Craft 2003). Since several investigators have shown different sensitivities for different genders with respect to antinociceptive effect, the experiments in this thesis were all conducted in male rats to prevent any interference or variation due to fluctuations of hormones in the female menstrual cycle. Some steroids have antinociceptive properties along with their hormonal activities. However, prospective analgesics for human use should be without hormonal effects. Although it has been proved that alphadolone is devoid of hormonal effects, the influence of different genders and hormonal levels should be taken into consideration in any nociceptive testing.

Some endogenously occurring neuroactive steroids have been reported to inhibit and potentiate the binding of other exogenously administered $GABA_A$ receptor modulators (Gee 1988; Majewska, Harrison et al. 1986). Furthermore, such endogenous neurosteroids have also been reported to reach concentrations that are capable of potentiating the actions of GABA (Paul and Purdy 1992; Purdy, Morrow et al. 1991). Studies have revealed that neurosteroids with varying efficacies as modulators of the $GABA_A$ receptor complex can cause direct agonist, partial agonist and antagonist activity. Such factors may have significant implications for the development of pharmacological agents with therapeutic value. Therefore it is necessary to determine the physiological roles of endogenous neuroactive steroids and their possible influence on alphadolone's activity.

Past experiments demonstrated antinociceptive effects of alphadolone were mediated by $GABA_A$ receptors in the spinal cord (Nadeson and Goodchild 2000). Alterations of spinal cord responsiveness to incoming nociceptive information may be via a direct

interaction of a drug with spinal cord receptors or by the activation of descending projections from higher centres. Adverse side effects like sedation are most commonly associated with high drug dosing that affects the brain. For this reason, many drugs that could act at brain and spinal cord sites because of their unselective nature are given via epidural or intrathecal administration methods. The benefit of such direct administration is to reduce adverse effects by limiting drug dosing and action in the brain. In non-spinal administration, antinociceptive drugs are dispersed throughout the body before they reach their target site, which is most commonly within the CNS. Using this form of delivery, high concentrations are often required for effective results. By contrast, spinal administration uses lower doses of drugs because they are directly administered to their site of analgesic action. Lower doses help minimise adverse dose dependent side effects, but with the added dangers of spinal administration; neurotoxicity as well as other adverse responses due to rostral spread of the spinal drug. For all experiments in this thesis alphadolone was injected via the intraperitoneal route of administration. Even though it was administered via a non-spinal route, alphadolone showed potent antinociceptive effects while demonstrating a low sedative profile. This suggests even after non-spinal administration, alphadolone selectively interacts with spinal rather than supraspinal receptors. The unique selective actions of alphadolone on spinal cord receptors suggest clinical potential, where a drug can target nociceptive systems within the spinal cord without being administered spinally. It would have been interesting to test intrathecally-administered alphadolone in the nociceptive test models used throughout this thesis. This would help resolve whether the effect of alphadolone is localised within the spinal cord. However, the nature of the alphadolone vehicle made it unsuitable for intrathecal testing.

Currently, opioids are the most widely used analgesics. However a major concern for their use in clinical analgesia is their associated adverse side effects, which may prevent dose escalation and thus limit the amount of analgesia achievable. In such cases where higher opioid doses are administered, patients' safety or quality of life may be compromised. Limitations of opioids have led to the concept of combination therapy in the hope of attaining a favourable balance of analgesia and side effects with a reduction in the dose. The work described in this thesis investigated the interaction of the opioid and $GABA_A$ nociceptive systems. Opioids were chosen as adjuncts for combination experiments, firstly because they are clinically available, and secondly their actions are associated with adverse side effects like sedation. Previous research has already described an interaction between these two systems using drugs known to act at $GABA_A$ receptors like midazolam and muscimol. Combinations of drugs acting at opioid and GABA systems have been shown to produce clinically useful analgesia across a range of human pain conditions. These combination therapies sometimes use parenteral application to activate the GABA antinociceptive system, however many cause potent sedation. This thesis demonstrated interactions between alphadolone and the opioid antinociceptive system mediated by spinal cord $GABA_A$ receptors. The increased antinociceptive effect was not paralleled by an increase in sedative effects. Since the antinociceptive effects of alphadolone are restricted to spinal cord receptors, the interaction between opioids and alphadolone might also be confined to spinal cord receptors, thus explaining the lack of increased sedative effects.

The interaction between opioids and alphadolone was further confirmed in established opioid tolerance, where small but significant remaining antinociceptive effects of morphine in tolerant rats were enhanced by alphadolone to produce maximum

antinociception. In addition, when co-administered with morphine ahead of tolerance development, alphadolone was also able to partially prevent the development of tolerance. Hence alphadolone may be beneficial to patients receiving chronic opioid treatment and also for those requiring pain therapies but are already tolerant to opioids.

The experiments in this thesis did not investigate the development of tolerance to alphadolone for the prospect of *its* long-term delivery. There has been evidence to suggest $GABA_A$ receptor subunit plasticity occurs in various pathological situations and in response to long-term exposure of the receptor to compounds targeting $GABA_A$ receptors such as ethanol (Devaud, Smith et al. 1995; Fenelon and Herbison 1996). This thesis studied pathological situations of neuropathic pain and inflammation. However the effect of alphadolone was only evaluated in their short-term management. Further analysis is required for the long-term effects of neurosteroid administration, i.e. for the *duration* of a pathological situation.

GABA is ubiquitous throughout the central nervous system, so it is no surprise that different $GABA_A$ receptors exist with such diverse functions. The different subunits that make up the structure of native $GABA_A$ receptors help generate numerous combinations for $GABA_A$ receptor subtypes. Henceforth their exploitation may lead to new and effective analgesic therapies. Neurosteroids have only recently been discovered for clinical use as anticonvulsants and anaesthetics but further detailed analysis will undoubtedly extend their profile. The clinical anaesthetic Althesin® used in the 1980's was comprised of two neurosteroids, alphaxalone and alphadolone. Alphaxalone was the main anaesthetic component, while alphadolone, also an anaesthetic but with half the potency, was present to increase the solubility of the

mixture. The investigations of this thesis demonstrated these two compounds with almost identical molecular structures have pharmacological actions that are quite distinct. Alphaxalone produced potent sedative effects without antinociception, probably by targeting supraspinal $GABA_A$ receptors. Conversely, alphadolone produced potent antinociception in the absence of sedation, by targeting spinal cord $GABA_A$ receptors. This supports the idea that different $GABA_A$ receptors exist throughout the CNS, and when activated they can cause varied effects. The different actions described for alphadolone and alphaxalone may help delineate receptor specificity respectively important in nociception versus sedation. The selective actions of alphadolone on spinal cord receptors, even when administered parenterally, may suggest a specific subtype of $GABA_A$ receptor or even a subunit is important for its precise action. It would be advantageous to study the receptors involved in alphadolone antinociception to help broaden the knowledge of spinal cord receptor subtypes and to selectively target those receptors important in pain modulation as opposed to other regions where the same drug may produce unwanted side effects.

The study of a variety of neurosteroid analogues would be beneficial for expanding the knowledge acquired from the experiments reported in this thesis. Small chemical variations may produce active compounds for various clinical applications. The step-wise assay used throughout this thesis, including several nociceptive and sedative tests, would be a practical way to analyse analogues with analgesic potential. A detailed study of the structure-activity relationship for neurosteroids would help provide necessary information to anticipate which neurosteroids might be valuable for designated physiological functions.

Alphadolone has an exciting antinociceptive profile in the absence of sedation, both when used alone and in combination with opioids, in a variety of acute and chronic pain models. Other neurosteroids have yet to show such potent and diverse antinociceptive properties. In fact no other investigations have found a neurosteroid devoid of hormonal, behavioural and/or sedative effects, to show *any* analgesic capacity. Indeed alphadolone's potential for clinical use appears more promising than many analgesics used today.

Appendix A

Table A.1 TFL Response for Control Animals

	DAY 1			DAY 2		
Animal Number	TFL at 5-minute Intervals			TFL at 5-minute Intervals		
	0	*5*	*10*	*0*	*5*	*10*
76	3.6	3.4	3.4	3.2	2.6	3.9
77	2.8	3.1	2.8	2.5	2.0	3.5
78	3.5	3.7	3.1	3.1	2.6	2.3
79	2.6	2.3	3.0	1.8	2.5	2.5
82	3.7	4.3	3.8	2.3	2.1	2.6
83	2.6	2.7	2.8	3.4	2.7	3.2
103	2.9	2.9	3.0	3.9	3.3	4.0
104	2.5	2.7	2.6	2.2	2.1	2.4
105	2.5	2.3	2.3	3.7	4.3	3.8
106	2.5	2.6	2.1	3.2	3.2	3.2
259	1.2	1.3	1.2	1.3	1.3	1.4
260	1.6	2.0	2.0	1.8	2.2	2.0
261	1.2	1.7	1.5	1.6	1.5	1.5
262	2.2	2.5	2.4	2.8	2.7	2.9

Animals tested at three consecutive 5-minute intervals (time -10, -5 and 0 minutes); intensity of 55. Testing was completed on consecutive days.

No individual animal demonstrated any significant difference for any 5-minute interval reading (ANOVA). No animals demonstrated any significant difference between readings on Day 1 compared with Day 2 (*t*-test).

Appendix B

Figure B.1 Time Response of IP Opioids on TFL

(A) Fentanyl

Testing time is plotted against change in TFL response (seconds), both before and after fentanyl administration. Three stable baseline measurements were taken at 5-minute intervals (-10, -5, 0 minutes), after which fentanyl was administered. Fentanyl caused an increase from baseline values within 10 minutes of administration. This effect remained elevated for the following 15 minutes. Each point represents the mean for 18 animals ± SD.

(B) Morphine

Testing time is plotted against change in TFL response (seconds), both before and after morphine administration. Three stable baseline measurements were taken at 5-minute intervals (-10, -5, 0 minutes), after which morphine was administered. Morphine caused an increase from baseline values within 5 minutes of administration. This effect remained elevated for the following 15 minutes. Each point represents the mean for 13 animals ± SD.

(C) Oxycodone

Testing time is plotted against change in TFL response (seconds), both before and after oxycodone administration. Three stable baseline measurements were taken at 5-minute intervals (-10, -5, 0 minutes), after which oxycodone was administered. Oxycodone caused an increase from baseline values within 5 minutes of administration. This effect remained elevated for the following 15 minutes. Each point represents the mean for 12 animals ± SD.

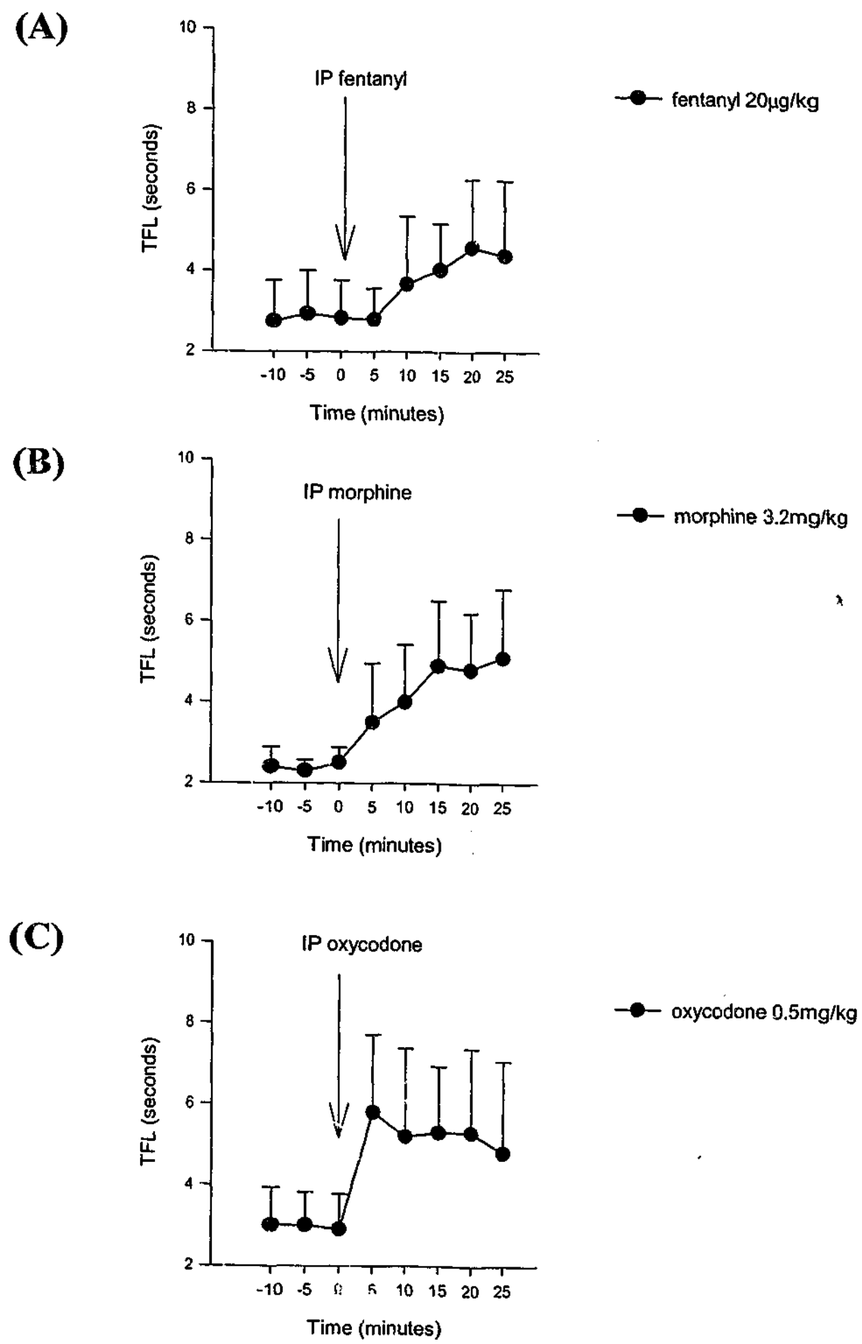


Figure B.1 Time Response Curves for IP Opioids Assessed by TFL

Figure B.2 Time Response of IP Opioids on ECT

(A) Fentanyl

Testing time is plotted against change in ECT response (mA), both before and after fentanyl administration. Three stable baseline measurements were taken at 5-minute intervals (-10, -5, 0 minutes), after which fentanyl was administered. Fentanyl caused an increase from baseline values that peaked within 5 minutes of administration. This effect remained elevated for the following 15 minutes. Each point represents the mean for 4 animals ± SD.

(B) Morphine

Testing time is plotted against change in ECT response (mA), both before and after morphine administration. Three stable baseline measurements were taken at 5-minute intervals (-10, -5, 0 minutes), after which morphine was administered. Morphine caused an increase from baseline values that peaked within 5 minutes of administration. This effect remained elevated for the following 15 minutes. Each point represents the mean for 4 animals ± SD.

(C) Oxycodone

Testing time is plotted against change in ECT response (mA), both before and after oxycodone administration. Three stable baseline measurements were taken at 5-minute intervals (-10, -5, 0 minutes), after which oxycodone was administered. Oxycodone caused an increase from baseline values that peaked within 5 minutes of administration. This effect remained elevated for the following 15 minutes. Each point represents the mean for 12 animals ± SD.

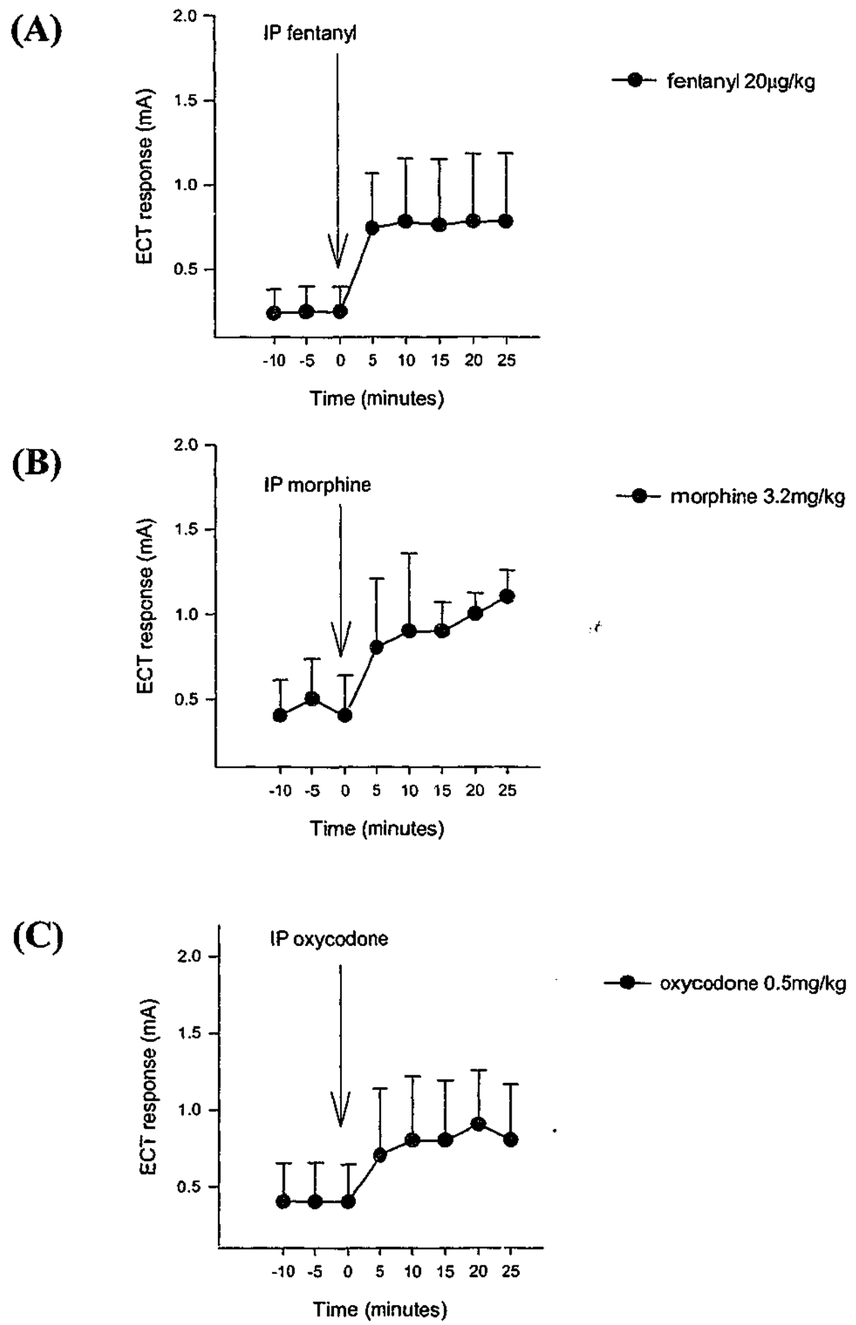


Figure B.2 Time Response Curves for IP Opioids Assessed by ECT

Figure B.3 Time Response of IP Opioids on PP

(A) Fentanyl

Testing time is plotted against change in PP response (g), both before and after fentanyl administration. Three stable baseline measurements were taken at 5-minute intervals (-10, -5, 0 minutes), after which fentanyl was administered. Fentanyl caused an increase from baseline values within 5 minutes of administration. Each point represents the mean for 5 animals ± SD.

(B) Morphine

Testing time is plotted against change in PP response (g), both before and after morphine administration. Three stable baseline measurements were taken at 5-minute intervals (-10, -5, 0 minutes), after which morphine was administered. Morphine caused an increase from baseline values within 5 minutes of administration. Each point represents the mean for 11 animals ± SD.

(C) Oxycodone

Testing time is plotted against change in PP response (g), both before and after oxycodone administration. Three stable baseline measurements were taken at 5-minute intervals (-10, -5, 0 minutes), after which oxycodone was administered. Oxycodone caused an increase from baseline values within 5 minutes of administration. Each point represents the mean for 7 animals ± SD.

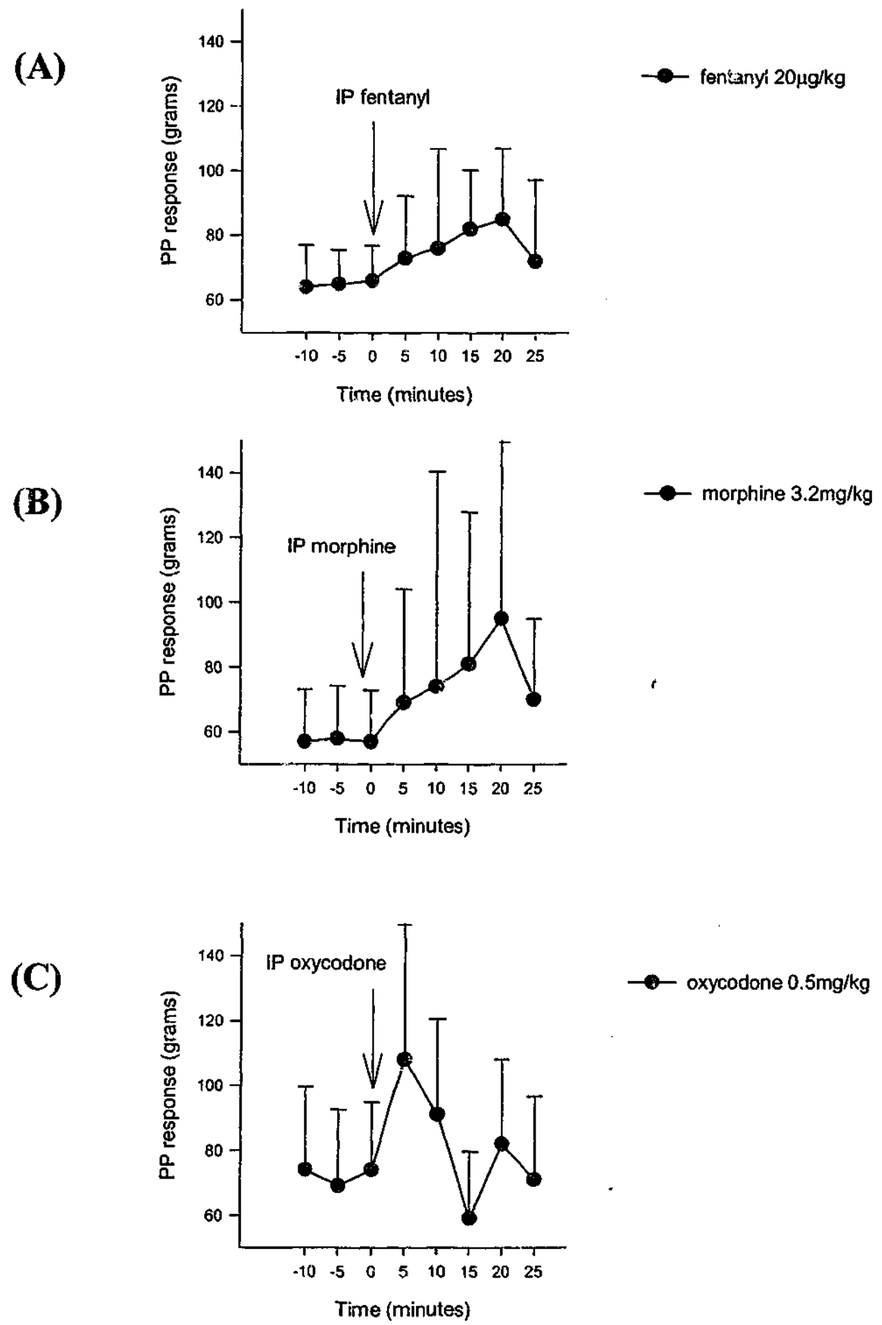


Figure B.3 Time Response Curves for IP Opioids Assessed by PP

Appendix C

Table C.1 The Neck ECT Response (r) for Intrathecal Bicuculline After Co-Administration of Alphadolone and Opioids

alphadolone 10mg/kg & drug dose	**neck ECT** *pre IP drug*	*post IT drug*	*alphadolone 0.1mg/kg & drug dose*	**neck ECT** *pre IP drug*	*post IT drug*
fentanyl 5µg/kg	0.16 (0.02)	0.32 (0.08)	fentanyl 5µg/kg	0.21 (0.04)	0.37 (0.06)
morphine 1.6mg/kg	0.14 (0.01)	0.27 (0.02)	morphine 1.6mg/kg	0.27 (0.04)	0.49 (0.05)
oxycodone 0.25mg/kg	0.15 (0.03)	0.25 (0.05)	oxycodone 0.25mg/kg	0.19 (0.04)	0.29 (0.03)

Mean (± SEM)
pre IP drug signifies the average of 3 readings before IP drug combination
post IT drug signifies the average of 3 readings after IT bicuculline administration

All neck ECT values were significantly increased after the co-administration of alphadolone and opioids ($p \leq 0.05$, *t*-test). No neck response was reversed by IT bicuculline at the doses recorded.

Appendix D

Figure D.1 Opioid Dose Response Relationships Assessed by ECT and TFL

(A) Fentanyl

This graph shows the effect of increasing doses of fentanyl assessed by ECT and TFL.

Each point represents the mean for 5-18 animals ± SEM.

(B) Morphine

This graph shows the effect of increasing doses of fentanyl assessed by ECT and TFL.

Each point represents the mean for 5-13 animals ± SEM.

(C) Oxycodone

This graph shows the effect of increasing doses of fentanyl assessed by ECT and TFL.

Each point represents the mean for 5-12 animals ± SEM.

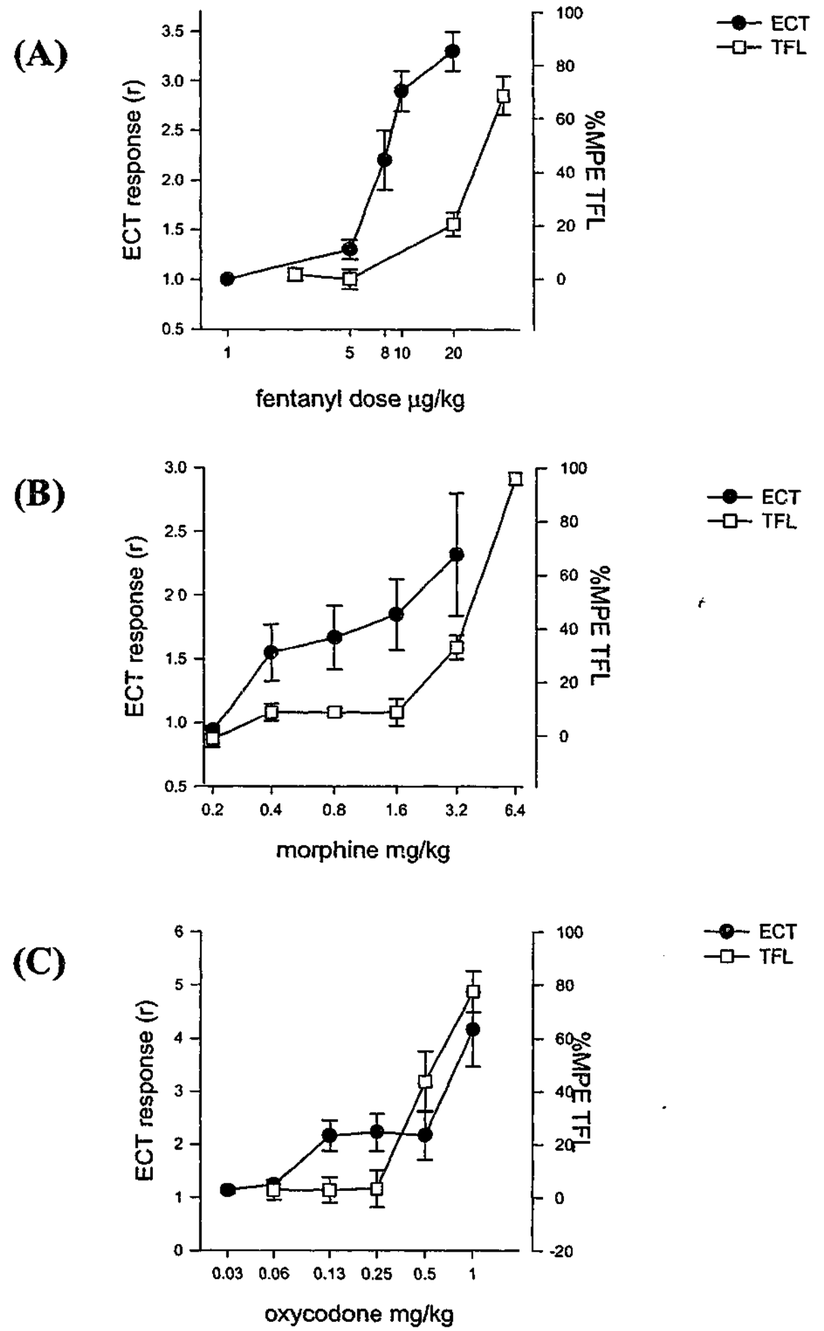


Figure D.1 Dose Response Relationships for IP Opioids Assessed by ECT and TFL

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ADDENDUM

p 2 sentence 4 insert bracket:
"neurosteroids...potential use in pain [management] has only recently been shown"

p 31 sentence 7 should read:
"Although *small* numbers..."

p 41 para 2 should read:
Tail ECT testing used a pair of exposed wire electrical stimulating electrodes coated in electrode gel (Pate Reux, Hewlet Packard). They were each placed on the tail 5cm and 7cm from the base of the tail *held in a constant position with waterproof adhesive tape.* Neck ECT testing used two needle electrodes placed in the skin at the base of the neck 1cm apart *held in a fixed position with scissor clamps.* A single half-second train of one millisecond pulses with a frequency of 50 Hz was delivered individually, in turn, to both sets of electrodes by a constant current electrical stimulator.

p 94 para 2 after sentence 3 add in:
Cyclodextrins (CD) are crystalline, water soluble, cyclic, non-reducing, oligosaccharides built up from six, seven, or eight glucopyranose units. Poorly soluble drugs can be encapsulated when bound to cyclodextrins and then released in water to make an aqueous solution. HPβCD has the best balance of enhanced aqueous solubility and can form a stable complexe with a wide range of drugs. Furthermore it has the most extensive collection of safety data with no adverse reactions reported.

p 161 para 2 should read:
The measurement of neck ECT values was used to determine any rostral spread of the intrathecally administered drug. Any experiments that showed an animal to have decreased neck ECT values after IT bicuculline were *required to be* excluded from collated data. Neck ECT data from all rats are tabulated in Appendix C (page 271) and show that the thresholds after IT bicuculline did not change. Therefore all these results could be included for interpretation.

p 170 para 2 sentence 1 should read:
"The series...uses the *chemical mediator* carrageenan..." .

p 170 para 3 sentence 2 insert bracket:
"so [that] rats' response..."

p 246 para 2 sentence 2 comment:
The rats treated with neurosteroid emulsions in combination with the slow release morphine emulsion responded to IP injections of morphine with a significant rise in tail flick. This result was compared with the diminished tail flick response to IP morphine for rats treated with *only* morphine emulsion.