CENTRAL ANALGESIC EFFECTS

OF THE

NON-STEROIDAL ANTI-

INFLAMMATORY DRUGS

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ABSTRACT

In addition to their well-known peripheral analgesic effects, non-steroidal anti-inflammatory drugs also exert antinociceptive actions at the central level. However, it is not clear if these central effects are spinally or supraspinally mediated nor whether cyclooxygenase inhibition or the interaction of these drugs with neurones in a different way is responsible for these central analgesic actions. This project investigated the possible central analgesic mechanisms of action of these drugs. It involved the use of *in vitro* neonatal rat hemisected spinal cord preparations and sheep implanted with indwelling cervical intrathecal catheters.

The dorsal root of the neonatal rat hemisected spinal cord preparations was electrically stimulated in such a way to evoke excitatory postsynaptic potentials in the ipsilateral ventral root. All records from this *in vivo* technique were identified as artefacts and no further experiments were carried out.

Conscious, unrestrained, non-lame sheep chronically implanted with indwelling cervical intrathecal catheters were submitted to mechanical noxious stimulation of the left radius. They received cumulative intrathecal doses of ketoprofen, phenylbutazone, salicylic acid and tolfenamic acid as well as repetitive intrathecal administration of normal saline without significantly affecting mechanical thresholds. The same drugs and normal saline were also given intravenously and only ketoprofen and tolfenamic acid significantly raised the nociceptive thresholds.

The involvement of spinal opioidergic and α_2-adrenergic mechanisms in the hypoalgesia induced by the intravenous administration of ketoprofen in sheep was investigated. The prior intrathecal administration of naloxone and atipamezole at a dose that had no effect on nociceptive thresholds and reversed the analgesia mediated by intravenous fentanyl and xylazine, respectively, almost completely reversed the antinociceptive effects mediated by intravenous ketoprofen.

These studies confirm that non-steroidal anti-inflammatory drugs can produce hypoalgesia even when inflammation is absent and, although they did not have a direct effect on the spinal cord, their analgesic action appeared to be spinally mediated by opioidergic and adrenergic descending inhibitory systems.

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ABBREVIATIONS

$[Ca^{2+}]_i$	Intracellular calcium concentration.
5-HT	Serotonin.
AA	Arachidonic acid.
ACh	Acetylcholine.
ACSF	Artificial cerebral spinal fluid.
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxalone propionic acid.
ANOVA	Analysis of variance.
AUC	Area under the curve.
AUC30	Area under the threshold change *vs.* time curve values for 30 minutes.
AUC60	Area under the threshold change *vs.* time curve values for 60 minutes.
BDNF	Brain-derived neurotrophic factor.
C5	Cervical vertebra 5.
CaM	Calcium-calmodulin complex.
CaMKII	Calcium/calmodulin-dependent protein kinase II.
cAMP	3',5'-cyclic guanosine monophosphate.
CDI	Calcium-dependent inactivation.
cGMP	3',5'-cyclic guanosine monophosphate.
CGRP	Calcitonin gene-related peptide.
CNS	Central nervous system.
COX	Cyclooxygenases.
COX-1-li	COX-1-like immunoreactivity.
COX-2-li	COX-2-like immunoreactivity.
$cPGI_2$	Carbaprostacyclin.
CSF	Cerebral spinal fluid.
DHN	Dorsal horn neurones.
DLF	Dorsolateral funiculus.
DP	Prostaglandin D receptor.
DRCAP	Dorsal root compound action potential.
DRG	Dorsal root ganglion.

DR-VRP	Dorsal root-ventral root potential.
e.p.s.p.	Excitatory postsynaptic potential.
EEA	Excitatory amino acid.
eNOS	Eendothelial nitric oxide synthase.
EOX	Epoxygenases.
EP	Prostaglandin E_2 receptor.
GABA	γ-Aminobutyric acid.
HETE	Hydroxyeicosatetraenoic acids.
i.c.v.	Intracerebroventricular, intracerebroventricularly.
i.p.	Intraperitoneal, intraperitonaelly.
i.t.	Intrathecal, intrathecally.
i.v.	Intravenous, intravenously.
IL-1β	Interleukin-1β.
iNOS	Inducible nitric oxide synthase.
Ins(1,4,5)P3	Inositol-(1,4,5) triphosphate.
LOX	Lipoxygenases.
L-PGDS	Lipocalin-type prostaglandin D synthase.
LPS	Lipopolysacharide.
LTs	Leukotrienes.
mGluRs	Metabotropic glutamate receptors.
MSR	Monosynaptic compound action potential.
N	Newtons.
NFκB	Nuclear factor κB.
NMDA	N-methyl-D-aspartate.
nNOS	Neuronal nitric oxide synthase.
NO	Nitric oxide.
NOS	Nitric oxide synthase.
NRM	Nucleus raphe magnus.
NSAIDs	Non-steroidal anti-inflammatory drugs.
p.o.	*Per os*, oral administration.
PAG	Periaqueductal grey.
PGs	Prostaglandins.
PKA	Protein kinase A.
PKC	Protein kinase C.

PLA_2	Phospholipase A_2.
PLC	Phospholipase C.
PMNLs	Polymorphonuclear leukocytes.
PTK	Protein tyrosine kynase.
RVM	Rostral ventromedial medulla.
SP	Substance P.
TNF-α	Tumour necrosis factor-α.
trkA	Tyrosine kinase A.
trkB	Tyrosine kinase B
Tx	Thromboxane.

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Farm animal welfare has been an important issue during the last few years in governmental agencies and research institutions, and although several recommendations have been proposed to improve the wellbeing of these animals, only minor changes in the way of producing food from animal sources have been observed. Developed countries show the most concern for this topic but not many of them have adopted practices to achieve even the minimum standards of comfort for farm animals. These standards, derived from applied ethology studies, are grouped in a concept known as the "five-freedoms of animals" (Crook & Helder, 1994), which are:

1) freedom from hunger and thirst;
2) freedom from physical discomfort and pain;
3) freedom from injury and disease;
4) freedom from fear and distress; and,
5) freedom to conform to essential behaviour patterns.

The economic consequences of implementing animal welfare recommendations cannot be ignored. A major issue, therefore, is whether better animal welfare necessarily involves high cost or lower profit. Clearly, improvements to animal welfare incur additional costs, however, extra costs do not necessarily lead to lower profit, particularly when better welfare leads to increased performance, lower disease, or reduced mortality. Nevertheless, in these times of market globalisation and free trade, if the industry fails to compete on a commercial basis because of improvements in animal welfare, it will be more difficult to promote these changes (Logue *et al.* 1998). Moreover, when farmers have to cut costs, traditionally, one of the first areas to suffer is investment in animal health (Anonymous, 1999).

Lameness is one of the most serious welfare problems in farm animals. Not only is lameness detrimental to the well-being of animals it is, in addition,

detrimental to production. Economic losses arise from poor production because lame animals, in pastoral production systems, are unable to walk around to graze and, therefore, lose condition which negatively affects production parameters. Of course, lameness also affects animals raised in feedlot systems in a very similar way. Therefore, rapid and effective treatment is important.

The magnitude and diversity of the problem in British dairy herds was recently highlighted by a group from the University of Liverpool, who reported that lameness incidence was above 50% (Clarkson *et al.* 1996). In some instances, radical treatment and a long period for recovery is required but even so, lameness can be a recurring condition. One common feature of lameness is an increased sensitivity to normally painful stimuli. Cattle and sheep can continue in this state for up to 28 and 90 days, respectively, after an apparently successful treatment of the lameness causing lesion (Whay *et al.* 1997; Whay *et al.* 1998; Ley *et al.* 1989; Ley *et al.* 1995). Therefore, the use of analgesics at the time of treating the cause of lameness has been suggested (Whay *et al.* 1998). This practice, in addition to relieving pain and preventing or minimising some changes associated with pain (see below), might also help to restore the animal's normal feeding behaviour, and thus production, in a shorter period of time. Nevertheless, most of these animals do not receive analgesic drugs to ameliorate pain. Among other reasons, this is due to worries about drug residues in milk and meat, increase of cost production, and a lack of interest shown by pharmaceutical companies in this potential market.

Many analgesics exert their antinociceptive effect, at least, at the level of the spinal cord. Hence, by administering a small amount of drug by the intrathecal (i.t.) route it would be possible to achieve analgesia and reduce drug residues. In this regard, the i.t. injection of α_2-adrenoceptor (Waterman *et al.* 1988; Ley *et al.* 1989; Kyles *et al.* 1993), γ-aminobutyric acid (GABA) (Kyles *et al.* 1995) and, to a lesser extent, opioid receptor agonists (Ley *et al.* 1989) produced good analgesia in sheep. Other analgesic agents such as the non-steroidal anti-inflammatory drugs (NSAIDs) could represent a good option as well.

Acetylsalicylic acid, dipyrone, flunixin, ketoprofen, phenylbutazone, and tolfenamic acid are the NSAIDs licensed for use in cattle in New Zealand[1]. Although all of them are indicated for the relief of inflammation and pain associated with musculo-skeltal disorders, scarce data on their analgesic effects on cattle and other farm species are available.

This research attempted to evaluate the spinal analgesic effect of the NSAIDs. In order to do so rationally, it was firstly tried to determine *in vitro* if they have any effect at the spinal level and to further assess if they produce analgesia with minimum side effects. The *in vitro* neonatal rat hemisected spinal cord preparation and sheep with implanted indwelling i.t. cervical catheters submitted to mechanical nociceptive stimulation of the left radius, were used for these purposes.

An overview of the neurophysiological mechanisms involved in pain transmission as well as the possible modes of action by which NSAIDs elicit analgesia is presented in the following chapter.

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[1] The New Zealand Index of Veterinary Specialities Annual. (1999). MediMedia (NZ) Limited. Auckland, New Zealand.

2.1. PAIN

Although pain has been studied for centuries and great achievements in neurosciences have been made in the last two decades, the mechanisms involved in its transmission still are incompletely understood, and therefore a satisfactory definition of pain that encompasses all of its manifestations does not exist. In humans, pain is known to be a complex interaction between physical and emotional factors and is most commonly defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Merskey, 1986). Thus, pain is a uniquely individual experience consisting in part of purely physical sensation, which unpleasant aspects may be all in the mind.

The sensory component of pain, referred to as nociception, corresponds to the physiological responses associated with the stimulation of a subpopulation of sensory neurones, called nociceptors, and the subsequent signalling of the information through specialised nociceptive pathways. However, activity induced in nociceptors and the nociceptive pathways is not pain, which is always a psychological state (Merskey, 1986). At levels of stimulation sufficient enough to activate nociceptors, humans report an awareness of sensation but do not describe it as painful. This is referred to as the nociceptive threshold. Higher levels of stimulation are required to reach the pain threshold, which is the least experience of pain that a subject can recognise. Under controlled conditions, these two thresholds are quite consistent among different subjects. The greatest level of pain that a subject is prepared to tolerate is denominated as the pain tolerance level and is highly variable not only between subjects but even in the same subject at different times. The range of stimulus intensities between the pain threshold and the pain tolerance level is the pain sensitivity range. At the lower end of this range, the cognitive and emotional responses that characterise the stimulus as aversive are weak, and as the stimulus

intensity increases, these responses become stronger. Thus, it is the cognitive and emotional components of pain that contribute to the wide variability in the pain tolerance level (Danneman, 1997).

Pain perception, thereby, is a broad concept, even in humans. It is well accepted that all non-human animal species are capable of detecting and reacting to stimuli that may compromise their integrity but, do animals feel pain in the same way as we humans do? The only answer is that we do not know. However, many non-human mammal species possess similar nociceptive pathways which to activate require similar stimulus intensities to those observed in humans. Furthermore, human and non-human mammals exhibit the same behavioural and physiological responses to painful stimulation and, like humans, non-human mammals will try harder to escape more intense stimulation (Danneman, 1997). But, because we cannot measure the emotional component of pain in animals (see below), it is not possible to determine whether an animal perceives a noxious stimulus as unpleasant in the same way and extent as humans would. Despite our inability to measure all aspects of pain in animals, and even in humans, the assumption must nevertheless be made that non-human mammals have the same capacity to feel pain as humans do.

Generally, it is accepted that some kind of pain has a protective function within the organism (Iadarola & Caudle, 1997; Malmberg et al. 1997; Dray et al. 1994). This pain is referred to as acute or physiological pain and serves as a warning to prevent further injury. On the other hand, when severe tissue injury is experienced the development of chronic or pathological pain starts immediately with complex changes in the nociceptive pathways (e.g., cell phenotype, protein expression and neural structure alterations). These changes account for the expression of allodynia [pain perception in response to normally non-painful stimuli (Merskey, 1986)] and hyperalgesia [exaggerated pain sensations to normally painful stimuli (Merskey, 1986)] as constant features (Dray et al. 1994). It is important, therefore, to prevent and/or control this kind of pain.

Different groups of analgesic drugs are utilised in the treatment of pain. Opioid and α_2-adrenoceptor agonists are among the most used in veterinary clinical practice, followed by NSAIDs, benzodiazepines and local anaesthetics. Ketamine, an N-methyl-D-aspartate (NMDA) receptor antagonist, is indirectly used to produce analgesia when utilised as an anaesthetic agent. However, all of them produce diverse "levels" of analgesia and side effects, both attributed to their mechanism(s) of action. Therefore, alternative routes and timing of administration, as well as drug combination have been implemented to reduce the side effects of a given drug without affecting, or in the best case increasing, the analgesic effects. Thus, it has been reasoned that by administrating an analgesic drug before a noxious stimulus is experienced it would prevent, or at least diminish, the hypersensitisation of spinal cord neurones (see below), a concept known as pre-emptive analgesia which is useful in the pre-operative stage. Its effectiveness, of course, will depend on the drug's parmacokinetics/pharmacodynamics in the target species. Additionally, there have been attempts to combine analgesic drugs from different pharmacological groups to provide better pain relief with less side effects than if a greater dose of one single drug is used, this concept is referred to as balanced analgesia.

Regardless of all of these pharmacological manipulations, chronic pain is often very difficult to treat effectively (Iadarola & Caudle, 1997; Dray et al. 1994). Only a better understanding of how pain is generated and maintained will allow us to manage it in a more efficient way. Hence, an overview of basic neurophysiology of the sensory component of pain is next presented. The reader should note that this overview does not consider many of the complexities of the subject and those interested in a more complete review can refer to Wall and Melzack (1989).

2.1.1. The nociceptive pathways

The transmission of the sensory constituent of pain is due via the nociceptive pathways. First, the signal is perceived by the peripheral nerves and travels through them to the dorsal root ganglion (DRG) neurones. These neurones are

in communication with the spinal cord through the dorsal roots of the spinal cord, which then stimulate spinal cord dorsal horn neurones (DHN). These spinal neurones send further information through ascending pathways to different nuclei in the brain, where pain is actually "felt" and then appropriate signals are generated and transmitted back to the spinal cord through descending inhibitory systems. Modulation of pain transmission, therefore, is feasible in each one of these sites; however, stopping the signal before the brain gets the message, either by blocking the signal in the spinal cord or keeping it out of the cord in the first place is the best approach to control pain. Hence, the role that the spinal cord plays in the nociceptive pathway is of great importance because it "gates" the incoming information from the afferent fibres and the descending pathways, as well as the outgoing information towards the brain.

2.1.1.1. Peripheral afferent input

Under normal physiological conditions, peripheral nociceptors, mainly composed of high-threshold Aδ (thinly myelinated axons) and C (unmyelinated axons) nociceptive fibres, transmit protective pain signals from the periphery to the CNS; a concept known as acute pain. However, when tissue damage occurs (*e.g.,* chemical, mechanical or thermal stimuli) diverse substances (*e.g.,* histamine, catecholamines, eicosanoids, neuropeptides, cytokines, etc.) are released from both neuronal and non-neuronal tissues into the adjacent affected area. These substances facilitate and amplify pain signals, leading to the establishment of peripheral hyperalgesia (Dray *et al.* 1994; Lascelles, 1996). Such complex changes and interactions are referred to as chronic pain. The exact substance composition of the released mixture may directly influence nociceptors' transduction, transference and transmission of onset, intensity, duration and location of peripheral stimuli to the central nervous system (CNS). Table 2.1 summarises some of the most relevant substances that activate or enhance the sensitivity of primary afferent nociceptors to evoke pathological pain signals, as well as the receptor system which they interact with and the functions they mediate.

Table 2.1. Ligand-gated ion channels and ionotropic and metabotropic receptors expressed by nociceptive primary afferent neurones.

Receptor/Channel	Major subtypes	Ligand	Expression (cell size/type)	Notes	Function*
Ligand-gated ion channels					
Sodium TTXs	PN1, PN4, rB(I-III)	N/A*	All cells	NGF regulated	EMC
Sodium TTXr	SNS/PN3, SNS/NaN	N/A	Small	PKA and PKC substrate; NGF regulated	EMC
Calcium voltage-gated channels	T- (low threshold) L-, N- (high threshold)	N/A	T-, Small/medium L-, N-, Mainly small	Secondary calcium currents mediated by G-protein-coupled receptors; enhancement of high threshold current by PKC	SyMC
Ionotropic receptors					
Vanilloid	VR1	Heat, capsaicin	Small	Sensitised by heat; proton-gated; NGF-regulated	ST, IMR
Purine	P2X2, P2X3	ATP, ADP, adenosine	P2X3, IB4 positive cells	P2X2 modulated by protons; P2X3 BDNF regulated	IMR, SyMR, AR
Serotonin	$5-HT_3$	Serotonin	Small		IMR, ST
NMDA	NR1, NRgbs	Glutamate/aspartate	Small/large	Controls SP release	SyMR, AR
AMPA	GluR1-3	Glutamate	R1, Small; R2-3, Small/large		SyMR, AR
Kainate	GluR5	Glutamate	Small	Controls neurotransmitter release	SyMR, AR
Metabotropic receptors					
EP	EP_{1-4}	PGE_{1-4}	EP1, EP2, EP4, Unspecified. EP3, Mainly small	PGE2 sensitises some normally unresponsive intermediate size cells to bradikinin; PGE2-PKA/C mediated modulator of TTXr; Sensitises heat stimuli.	IMR, AR, SyMR?
IP	IP	PGI_2	Mainly small		
Histamine	H1	Histamine	Some small	Itch mediator	IMR
Serotonin	$5-HT_{1A}$, $5-HT_{2A}$, $5-HT_4$	Serotonin	$5-HT_{1A}$/$5-HT_4$, Small. $5-HT_{2A}$, Most	$5-HT_4$ increases TTXr small cell currents via PKA/C.	IMR
Bradykinin	B1, B2	Bradykinin	B1, Small/large; B2, Small	B1 sensitises small cells to heat via PKA. B2 increases the number of cells that respond to capsaicin and protons.	IMR
Cannabinoid	CB1-2	Anandamide	Unspecified	Inhibits peripheral activation	IMR(in)
Tachykinin	NK1	SP, NKA	Small		SyMR, AR
Opioid	μ, δ, κ	Enkephalins, dynorphins, β-endorphin	Small	Inflammatory cells release endogenous opioids. Inhibits peripheral activation.	SyMR, IMR(in)

*AR, autoreceptor; EMC, excitatory modulatory channel; IMR, inflammatory mediator receptor; IMR(in), IMR inhibitory; N/A, not applicable; ST, signal transducer; SyMC, synaptic modulator channel; SyMR, synaptic modulator receptor.

Modified from Woolf & Costigan, 1999.

In chronic pain states, the low-threshold Aβ fibres (highly myelinated axons), which normally transmit innocuous tactile stimuli, also acquire the capacity to increase neural excitability. This enhanced excitability is due, at least in part, to the phenotypic switch in a subpopulation of these fibres so that they, like C fibres, can also express the neuropeptide substance P (SP) (Neumann *et al.* 1996). Thus, Aδ, C and Aβ fibres contribute to the hyperexcitability observed in chronic pain states by enhancing synaptic transmission in sensory pathways, and Aβ fibres do it by switching their phenotype to one resembling pain fibres. This diversity of changes in the peripheral, as well as central (see below), nociceptive pathways may contribute to the apparent loss of the biological significance of pain signalling as an organic protective measure (Dray *et al.* 1994).

2.1.1.2. The spinal cord

The cell bodies of primary afferent nociceptors lay in the DRG; fibres enter the spinal cord via the dorsal roots and end in the dorsal horn of the spinal cord grey matter, which, based on its cytoarchitecture, is divided into 10 sections: laminae I-X (Figure 2.1). In general, Aδ fibres synapse in laminae I-II and IV, while C fibres do it in laminae I-II, and Aβ fibres in laminae III-V (Fitzgerald, 1989), where an every day increasing number of new discovered neuroactive agents take part in the pain-evoking process. From these, glutamate, an excitatory amino acid (EAA) that acts on at least four different class of receptors (see Section 2 of this Chapter for details): kainate, α-amino-3-hydroxy-5-methyl-4-isoxalone propionic acid (AMPA), NMDA, and metabotropic glutamate (mGluRs) receptors, plays a major role.

It is generally agreed, on the one hand, that non-NMDA receptor spinal cord mechanisms are involved in the transmission of acute nociceptive messages evoked by C and Aδ fibre activity. On the other hand, states of chronic pain, evoked by Aβ fibres too, may lead to NMDA receptor activation that, in turn, results in facilitation of second order neurones (Eisenach, 1997; Calcutt &

Chaplan, 1997; Sorkin, 1997; Liu *et al.* 1994; Dray *et al.* 1994). Therefore, it seems that the development and maintenance of central hyperexcitability, which is clinically manifested as allodynia and hyperalgesia (Kaneko & Hammond, 1997; Liu *et al.* 1997; Ilkjaer *et al.* 1998), requires the activation and continuous activation, respectively, of both non-NMDA and NMDA receptors (Neugebauer *et al.* 1993). Nevertheless, this assumption may be an oversimplification, since a wider variety of receptors and agents are present in the spinal cord and they may interact in a much more complex way to produce central sensitisation. In this regard, some of the same primary afferent nociceptors that release glutamate can also synthesise and co-release neuropeptides [*e.g.,* SP, neurokinin A, and calcitonin gene-related peptide (CGRP)] within the spinal cord, where their respective receptors are present and have been implicated in chronic pain states (Iversen, 1998; Cao *et al.* 1998; Zamponi *et al.* 1997).
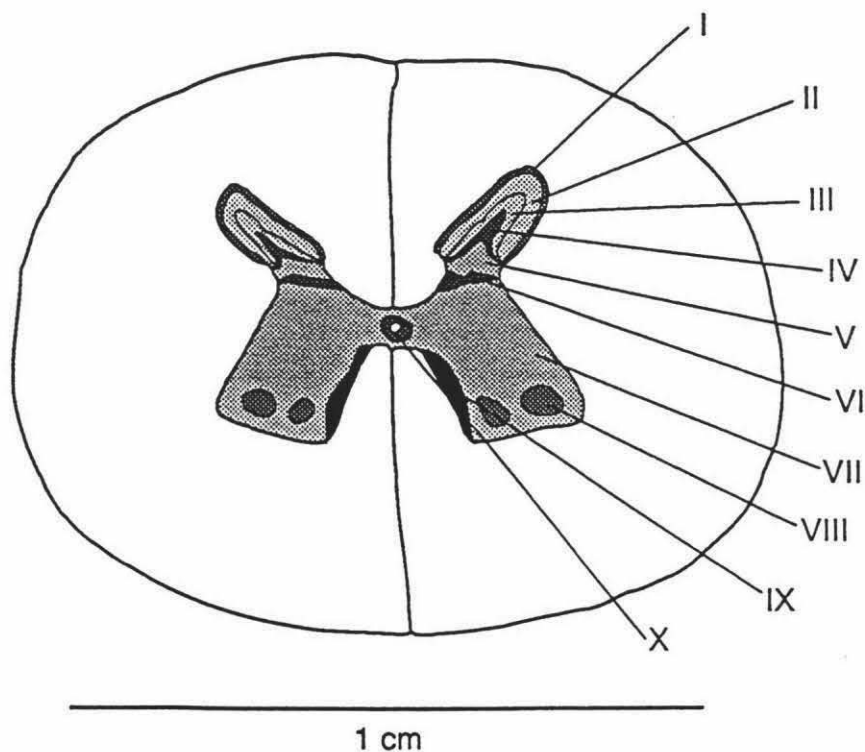
Figure 2.1. Transversal section of a sheep's spinal cord at the C5 vertebra showing the ten different laminae of the grey matter.

Central sensitisation, in a similar way to peripheral sensitisation, is also accompanied by prolonged alterations in the response properties, neurochemistry, genotype and phenotype of DHN (Kaneko & Hammond, 1997; Iadarola & Caudle, 1997; Liu *et al.* 1997; Liu *et al.* 1994; Lascelles, 1996). For example, both electrical activation of C and Aδ fibres and carrageenan injection into the hindpaw of rats enhanced spinal c-Fos protein expression [a nuclear protein encoded by the immediate-early gene *c-fos* which is widely used as an indirect marker of neurones involved in spinal nociceptive processes (Buritova & Besson, 1998)] in superficial (I-II) and deep (V-VI) laminae of the spinal cord dorsal horn (Buritova *et al.* 1996). These cellular changes, which take place immediately after peripheral afferent stimulation, may persist for long after the stimulus is terminated, blocked or interrupted. There is little information about how long these changes remain for, but in cattle and sheep suffering from lameness, a hyperexcited state for up to 28 and 90 days, respectively, after apparently successful treatment of the lameness causing lesion has been observed (Whay *et al.* 1997; Whay *et al.* 1998; Ley *et al.* 1989; Ley *et al.* 1995). Even less clear, but not less important, is the time course of all these changes. Undoubtedly, a better understanding of the timing of induction and the precise role of both excitatory and inhibitory elements in the nociceptive pathway might help to identify new therapeutic targets and establish better administration time protocols for analgesic drugs.

Thus, the spinal cord, and especially DHN, represents an important site for pain signal modulation. However, the physiology and, thereby, the pharmacology of hyperexcited spinal cord DHN differs markedly from that involved in the transmission of acute pain, resulting in pain being much more difficult to control and analgesic drugs less effective (Eisenach, 1993; Chambers, 1992; Chambers *et al.* 1995; Ley *et al.* 1991; Lascelles, 1996).

2.1.1.3. Ascending spinal pathways

Pain sensation requires nociceptive signals to be transmitted through ascending pathways from the spinal cord to appropriate nuclei in the brain. Five

somatosensory ascending pathways seem to be among the most important in most species; however, interspecies differences exist. In human beings and monkeys, for example, there is a similar arrangement of ascending spinal pathways, with most important tracts been ventrally located in the spinal cord white matter. In cats, on the other hand, important nociceptive ascending pathways are both dorsally and ventrally located, while in rats, long nociceptive tracts may not be as important as propriospinal pathways (Willis, 1983).

The spinothalamic, spinomesencephalic and spinoreticular tracts ascend through the ventrolateral white matter. Cells of origin of the spinothalamic tract are mainly situated in laminae I-VII and X of the spinal cord and their neurones project into several different nuclei of the thalamus (*e.g.,* medial and lateral parts of the ventral posterior lateral nucleus, central and medial part of the posterior complex, nucleus submedius, intralaminar complex). Most of the axons of this pathway, as well as those of the spinomesencephalic tract, cross to the contralateral side, but some travel ipsilaterally too (Willis, 1983; Willis, 1989).

The spinomesencephalic fibres originate from laminae I and V of the spinal cord and project their neurones to the midbrain reticular formation, the tectum and the periaqueductal grey (PAG). Fibres of the spinoreticular tract, on the other hand, arise from laminae V-VIII and project their neurones both to the lateral reticular nucleus and the medial reticular formation. There are also terminations in several medial reticular nuclei (*e.g.,* nucleus centralis medula oblongata, nucleus reticularis gigantocelularis, nucleus reticularis pontis caudalis and oralis, nucleus paragigantocelularis and lateralis) and in the nucleus subceruleus and the raphe nuclei (Willis, 1983; Willis, 1989).

The spinocervical tract and the second-order dorsal column pathway ascend through the dorsal aspect of the spinal cord white matter. The neurones of origin of both pathways are primarily located in laminae IV of the spinal cord. The spinocervical tract ascends ipsilaterally to the end of the lateral cervical nucleus which in turn projects to the ventral posterior lateral nucleus of the thalamus. The second-order dorsal column pathway projects ipsilaterally in the

dorsal funiculus and the dorsal part of the lateral funiculus, terminating into the dorsal column nuclei which in turn projects to the ventral posterior lateral nucleus of the thalamus (Willis, 1983; Willis, 1989).

2.1.1.4. The brain

The brain is the organ that decodifies the incoming information from the ascending spinal tracts and is where the signal is perceived as pain, but the precise brain structure that first perceives pain is still a matter of debate. Some areas within the brain that have been involved in the nociceptive process include the brain stem (e.g., median medullary reticular formation, midline medullary tegumentum), the thalamus (e.g., posterior lateral thalamus, medial and intralateral thalamus) (Casey & Morrow, 1989), and the cerebral cortex (e.g., parasylvian cortex) (Lenz et al. 1998). However, the higher the nociceptive pathway goes, the less is known about it

The great diversity of neuroactive agents is synthesised by different brain neurones that constitute specialised pathways, which run within the same brain and outside it. Most of these substances may play a major role in the nociceptive process and noradrenaline, serotonin (5-HT), dopamine, histamine, adenosine, acetylcholine (ACh), SP, CGRP, glutamate, glycine, GABA, and opioid peptides are among the best characterised, and for some of them the pathways have been described elsewhere (Rang et al. 1995).

2.1.1.5. Descending inhibitory systems

The descending inhibitory systems regulate the ongoing processing of nociceptive afferent input. Several different tracts have been identified and, like the ascending spinal pathways, species differences also exist. Some of the most important long descending tracts, which are found only in the lateral and ventral aspects of the spinal cord white matter, are the corticospinal pyramidal tract, coming from the sensorimotor cortex; the rubrospinal tract, originated from

the red nucleus in the midbrain; the reticulospinal tract, which fibres arise from the pontomedullary reticular formation; and the vestibulospinal fibres, which originate from the lateral vestibular nucleus of the medulla oblongata (Bowsher, 1975). Even though these pathways were anatomically identified several years ago, their physiopathological role is not well understood yet.

The most extensively studied regions implicated in pain control descending mechanisms are the PAG, the rostral ventromedial medulla (RVM), and the spinal cord. The PAG receives afferents from the frontal cortex, hypothalamus, amygdala, thalamus, nucleus cuneiformis and locus coeruleus, and projects to neurones in the RVM, which includes the nucleus raphe magnus (NRM), the reticular formation, and the nucleus reticularis gigantocellularis, magnocellularis and paragigantocellularis. RVM neurones in turn project via the dorsolateral funiculus (DLF) to control the transmission of nociceptive information by neurones in the superficial dorsal horn. In addition, these descending inhibitory fibres also synapse with neurones from ascending spinal tracts (Fields & Basbaum, 1989; Carstens, 1983). Thus, the PAG-RVM circuit control of nociceptive DHN is an important mechanism of pain modulation. In addition, the RVM contains both excitatory and inhibitory local circuit interneurones, which can activate either on-cells and facilitate pain transmission or off-cells and suppress nociceptive transmission (Tortorici *et al.* 1996).

Some of the most important descending modulatory pathways include opioidergic, monoaminergic, and GABAergic systems, which can be solely expressed or co-exist in some axons. The opioid-mediated analgesia system has well-established components in the PAG-RVM spinal projection, with DHN modulated by PAG activation containing all three opioid receptor classes (μ, δ, κ), as well as enkephalinergic interneurones and terminal fields (Budai & Fields, 1998).

Modulation of nociceptive transmission by bulbospinal pathways is largely attributed to the biogenic amines noradrenaline, 5-HT and dopamine, as well as to adenosine, which mainly exert inhibitory effects both in the brain and the spinal cord. Noradrenergic inputs are sent by the locus coerulus to the PAG,

which connects to noradrenergic pontomedullary cell groups in the RVM (Fields & Basbaum, 1989). Thus, activation of PAG neurones inhibited noxious responses of sacral DHN through a presynaptic α_2-adrenoceptor mechanism (Budai *et al.* 1998) and, interestingly, excitatory effects were also observed but these were mediated by α_1-adrecoceptors (Budai *et al.* 1998). Dopaminergic descending mechanisms seem to be also implicated in the nociceptive process and a pathway descending from diencephalic neurones and projecting into the spinal cord, where activation of dopamine D2 receptors produced analgesia, might play an important role (Barasi *et al.* 1987). Adenosine also mediates antinociception via the descending DLF; when the locus coeruleus was electrically stimulated a suppressing spiking response from parafascicular neurones was observed and this inhibitory effect was reversed by the i.t. administration of the adenosine receptor antagonists phentolamine and aminophylline (Zhao *et al.* 1999).

Serotoninergic, GABAergic and glycinergic mechanisms also play an important function within the descending inhibitory pathways. Serotoninergic and GABAergic fibres descending from the RVM, synapse with both spinothalamic projection neurones and local circuit neurones in the spinal cord dorsal horn (Xu *et al.* 1998), suggesting that both neurotransmitters may contribute to the supraspinal control of nociception. In fact, an interaction between these two neurotransmitters was observed in rat sacral dorsal neurones, where activation of 5-HT$_2$ receptors by 5-HT enhanced Cl$^-$ outflow indirectly by phosphorylate the GABA$_A$ receptor (Xu *et al.* 1998). Interestingly, GABA and glycine can be released by the same neurone and even by the very same vesicle (Nicoll & Malenka, 1998). Therefore, it is feasible that volleys in RVM-spinal tract could evoke the release of 5-HT, GABA and/or glycine from RVM terminals, and they may then act as co-transmitters to inhibit spinal and supraspinal nociceptive transmission through GABA$_A$ and/or glycine receptors, resulting in attenuation of pain.

Many other neuroactive agents (*e.g.*, cholecystokinin, prolactin, SP) are also produced in the brain and transported by diverse descending pathways to finally

act in the spinal cord; however, in most cases, their contribution to the modulation of sensory transmission is not completely understood. A more detailed overview of these agents surpasses the objectives of this work.

2.2. GLUTAMATE RECEPTORS

Although EAAs other than glutamate can also modulate synaptic transmission (Curtis *et al.* 1961), most excitatory synapses in the mammalian CNS use L-glutamate as their neurotransmitter (Nicholls, 1992; Woodburn & Woodruff, 1994; Sheng, 1997), which acts on two different classes of receptors: ionotropic and metabotropic glutamate receptors. The former receptors contain integral cation-specific ion channels and are subdivided into kainate, AMPA, and NMDA receptors, named on the basis of the selective agonist that has been discovered (Jin & Fredholm, 1997; Woodburn & Woodruff, 1994). The later receptors function by intracellular signalling through G-proteins and are subdivided into three groups (Group I-III) (Akam *et al.* 1997; Cartmell *et al.* 1997; Pinkney *et al.* 1997).

Recently, an ionotropic-heteromeric receptor molecule, XenU1/XenNR1G-receptor which combines non-NMDA and NMDA agonist sites on different subunits, was described. This receptor has fundamental attributes of the NMDA receptor (voltage-dependent Mg^{2+} block, glycine dependence; see below) into the channel opened by non-NMDA receptor agonists (Barnard, 1997). This unitary receptor exists also in mammals, but its function is still unknown.

Although all different glutamate receptors can be distributed within the same glutamatergic synapse and may share a similar structure for their agonist recognition domain (Conn & Pin, 1997), their functions are completely different (see below). This could be explained, at least in part, by the different receptor distribution within the same synapse and the differential regulation of by synaptic proteins that physically couple to them. Thus, these adaptor or anchor proteins (*e.g.*, GRIP, PSD-95 and Homer proteins coupled to AMPA, NMDA and kainate, and mGluRs, respectively) may partially regulate the downstream

signalling pathways for each receptor (Sheng, 1997; Dingledine *et al.* 1999). These pathways are discussed below.

Glutamate concentration in the synaptic cleft is mainly regulated by activation of presynaptic inhibitory receptors (Scanziani *et al.* 1997) and by re-uptake into glial cells (Billups & Attwell, 1996; Zerangue & Kavanaugh, 1996), which help to maintain glutamate concentration within a physiological level and, thereby, protect the CNS.

2.2.1. Ionotropic glutamate receptors

2.2.1.1. AMPA receptors

The AMPA receptors mediate most of the fast synaptic excitatory neurotransmission in the CNS and, to date, four subunits, GluR-1 through GluR-4, have been identified, which are found pre- and postsynaptically on neurones throughout all superficial laminae of the spinal dorsal horn (Sang *et al.* 1998). These receptors mainly conduct Na^+ currents (Sommer *et al.* 1990; Keinänen *et al.* 1990); however, and at least in a subpopulation of DHN, they also provide a gated route of Ca^{2+} influx that trigger changes in synaptic transmission (Gu *et al.* 1996). Interestingly, interactions of AMPA receptors with G-proteins, independent of Na^+ and Ca^{2+} influx, have been observed in rat cortical neurones (Wang *et al.* 1997).

Increased neuronal excitability by AMPA-receptor activation has been ascribed to different mechanisms. The presence of "flop" or "flip" modules into AMPA receptors is one example. The former module mediates the fast desensitising current component and the later regulates the steady-state current component, making the action of glutamate four to five times more effective when it interacts with the flip version and, hence, the synapse operates at an increased gain (Sommer *et al.* 1990). Another example is the mechanism is of interacting with protein kinases; the AMPA receptor contains phosphorylation sites for calcium/calmodulin-dependent protein kinase II (CaMKII), protein kinase C

(PKC) (Keinänen *et al.* 1990; Dingledine *et al.* 1999) and protein kinase A (PKA) (Dingledine *et al.* 1999). Recently, it was demonstrated that CaMKII, activated by Ca^{2+} influx through the NMDA receptor, enhanced the channel conductance of AMPA receptors (Leonard *et al.* 1999; Derkach *et al.* 1999). In a similar way, some AMPA receptors are attached to the kinase-anchoring protein AKAP-79 that, in turn, has linked calcineurin and PKA. When Ca^{2+} enters the cell calcineurin is dissociated and PKA activated. Then, PKA phosphorylates and enhances the AMPA channel activity (Dingledine *et al.* 1999). It also was demonstrated that AMPA receptors containing the GluR2 receptor subtype are physically associated with the protein tyrosine kynase (PTK) Lyn, which was activated, by an independent Ca^{2+} and Na^+ influx mechanism, following receptor activation. PTK Lyn, in turn, activated the mitogen-activated protein kinase pathway that increased the expression of brain-derived neurotrophic factor (BDNF) mRNA (Hayashi *et al.* 1999). Interestingly, activation of tyrosine kinase B (trkB) neurotrophin receptors by this trophic factor, markedly increased the translation and therefore protein expression of GluR1 and GluR2/3 AMPA receptor subunits in rat cultured neocortical neurones (Narisawa-Saito *et al.* 1999). In addition, BDNF enhances NMDA receptor activity (Levine *et al.* 1998) (see below). Therefore, activation of the mitogen-activated protein kinase pathway through AMPA receptor mechanisms may contribute to the development of neuronal hyperexcitability. In summary, phosphorylation of AMPA receptors by protein kinases may increase the receptor activity and calcineurin may decrease this action.

Apart from being activated by AMPA, the AMPA receptors can be activated by kainate and vice versa (Dingledine *et al.* 1999). Interestingly, receptor subunits from both AMPA and the kainate receptor can form heteromeric oligomers that mutually differ in electrophysiological properties and ionic selectivity and, although they selectively conduct monovalent cations, glutamate may trigger Ca^{2+}-dependent intracellular events (Woodburn & Woodruff, 1994; Varney *et al.* 1998), thus, contributing to the process of central sensitisation (Sang *et al.* 1998). In fact, co-activation of AMPA-kainate and mGluRs on astrocytes

stimulated these cells to release glutamate through a Ca^{2+}-dependent process mediated by prostaglandins (PGs) (Bezzi *et al.* 1998).

In keeping with the hypothesis that AMPA receptors take part in the process of central sensitisation, it was demonstrated that these receptors were involved in the development and/or maintenance of hyperalgesia and that administration of AMPA receptor antagonists was beneficial for its management (Zahn *et al.* 1997; Nishiyama *et al.* 1999; Sang *et al.* 1998).

2.2.1.2. Kainate receptors

Despite the fact that kainate receptors were identified almost 20 years ago and they are widely expressed in the CNS, the lack of selective ligands has greatly hindered progress in understanding the roles of these receptors. So far, there have been identified five different kainate receptor subunits: GluR5, GluR6, GluR7, KA1, and KA2 (Clarke *et al.* 1997; Chittajallu *et al.* 1999) as well as splice variants of GluR5 and GluR7 (Chittajallu *et al.* 1999).

The kainate receptors are expressed diffusely in the spinal cord dorsal horn, mostly in the superficial layers (Sang *et al.* 1998; Li *et al.* 1999) and to a lesser extent in synapses formed by low- rather than high-threshold nociceptive afferent fibres, where they are co-expressed with AMPA receptors (Li *et al.* 1999). Thus, kainate receptors might contribute to the transmission of somatosensory inputs between primary afferent fibres and dorsal DHN. In fact, the i.t. injection of kainate in mice activated afferent C fibres and increased the synthesis of nitric oxide (NO) and further mobilisation of SP, reflecting a mechanism similar to that underlying hyperalgesia (Larson & Kitto, 1995). However, this may not be a direct effect of kainate receptors but probably one mediated by NMDA receptors, which are much more accepted to be involved with the NO pathway (see below).

These glutamate receptors exist like homo- or heteromeric units which accounts for their physiological and pharmacological properties (Chittajallu *et al.* 1999).

Some of the kainate receptor-mediated effects include depolarisation, regulation of glutamate release and depression of excitatory and inhibitory synaptic transmission. In the hippocampus (Chittajallu *et al.* 1996) and the substantia gelatinosa of the spinal cord (Sequeira & Näsström, 1998), regulation of glutamate release and depression of excitatory synaptic transmission involved kainate receptors composed of, or containing, pre- and postsynaptic GluR6 subunits. This kainate inhibitory effect did not involve metabotropic glutamate, $GABA_A$, $GABA_B$, glycine and adenosine A1 receptor mechanisms (Chittajallu *et al.* 1996) and, although some hypothesis have been suggested (Mayer, 1997), the precise mechanism remains unknown. On the other hand, activation of hippocampal kainate receptors composed of, or containing, presynaptic GluR5 subunits depressed GABA-mediated inhibitory synaptic transmission (Clarke *et al.* 1997). Thus, different effects are attributed to kainate receptors; however, it is not largely known how these effects are mediated, nor where the kainate receptor is localised in the synapse. Furthermore, functional evidence suggests a metabotropic action of a population of kainate receptors (Chittajallu *et al.* 1999).

2.2.1.3. NMDA receptors

The NMDA receptor is, by far, the most studied glutamate receptor and has been implicated in many forms of synaptic plasticity, development and neurologic disorders. Six different receptor subunits have been identified: NR1, NR2A trough NR2D, and NR3A (Dingledine *et al.* 1999), but the specific functions for most of them are to be determined.

2.2.1.3.1. Regulation of the NMDA receptor

The NMDA receptors, in contrast to most other ion channels, are blocked by Mg^{2+} in a voltage-dependent manner, and are allosterically regulated by glycine and polyamines (Dumuis *et al.* 1988; Jin & Fredholm, 1997; Ghosh & Greenberg, 1995; Reynolds & Miller, 1990; Woodburn & Woodruff, 1994; Gu *et*

al. 1996). The voltage dependence of the channel arises from the fact that at resting potential the channel is blocked by extracellular Mg^{2+} ions (Reynolds & Miller, 1990). Stimulation of primary afferents removes the Mg^{2+} block by activating postsynaptic non-NMDA receptors and activation exceeding a defined threshold depolarises the membrane removing the Mg^{2+} ion from the NMDA receptor, permitting the opening of the channel and the influx of Ca^{2+}, Na^+ and K^+ (Chaplan *et al.* 1997; Ghosh & Greenberg, 1995; Woodburn & Woodruff, 1994). Hence, the non-NMDA and the NMDA receptors might operate in succession (Neugebauer *et al.* 1993). In fact, Na^+ influx through either subtype of ionotropic glutamate receptor or voltage-gated Na^+ channels potentiated the activity of NMDA receptors (Yu & Salter, 1998). It seems that once Na^+ enters the postsynaptic neurone it functions as a signalling ion acting directly on the NMDA channel or on a closely associated regulatory site, which agrees with Na^+ sensitivity of NMDA receptors being set by a Src kinase associated with the channel (Yu & Salter, 1998).

The NMDA receptor is reciprocally coupled to the glycine site (distinct from the "classical" receptor in the spinal cord because is strychnine insensitive), and may also be coupled to a divalent-cation site outside the channel. Although glycine itself does not activate the channel, it potentiates NMDA responses and may be necessary for channel opening (Reynolds & Miller, 1990; Woodburn & Woodruff, 1994). In this regard, glycine site antagonists inhibited NMDA stimulated arachidonic acid (AA) release from rat cerebellar granule cells (Viu *et al.* 1998). Glycine effects are mimicked by D-serine and D-alanine (Reynolds & Miller, 1990). In addition to the effects of Mg^{2+} and glycine, Zn^{2+} and Cd^{2+} ions may be endogenous negative modulators of NMDA receptors because they affect the ability of NMDA ligands to operate the channel. Their actions are not competitive with respect to NMDA or glycine and are not voltage-dependent to the same degree as those of Mg^{2+}, suggesting a different site of action (Reynolds & Miller, 1990; Dingledine *et al.* 1999). Another cations, Sr^{2+}, Ca^{2+} and Ba^{2+}, can also increases or decrease NMDA ligand binding, an effect that seems to be related to their concentration (Reynolds & Miller, 1990).

Another binding site is the phencyclidine site, which is located close to the Mg^{2+} site within the channel and, although it is almost identical to the glutamate binding site in the NMDA receptor, phencyclidine site ligands do not alter the binding of agonists or antagonists at the NMDA site (Reynolds & Miller, 1990). Polyamines represent other compounds for the modulatory site on the NMDA receptor, it is possible that they produce Sr^{2+}-like effects with greater efficacy (Reynolds & Miller, 1990).

In addition, NMDA receptors are also regulated by protein phosphorylation at serine, threonine and tyrosine residues (Yu *et al.* 1997). Activation of PKC potentiates the NMDA receptor function in central neurones (Schoepp & Conn, 1993; Malmberg *et al.* 1997; Ghosh & Greenberg, 1995; Woodburn & Woodruff, 1994) by phosphorylating serine and threonine residues (Yu *et al.* 1997) on the NR1 subunit (Ghosh & Greenberg, 1995). The Src-family PTKs phosphorylate NMDA receptor subunits NR2A and NR2B (Yu *et al.* 1997; Tezuka *et al.* 1999) and the anchor protein PSD-95 may play an important role in this process (Tezuka *et al.* 1999). Furthermore, BDNF, acting trough the trkB receptor, elicits, at the NR1 and NR2B subunit level, phosphorylation-dependent enhancement of the NMDA channel functional activity (Levine *et al.* 1998). Interestingly, nerve grow factor treatment by acting in neurones expressing tyrosine kinase A (trkA) neurotrophin receptors, upregulates BDNF which, besides enhancing NMDA receptor phosphorilation, also induces changes in NR2A and NR2B receptor subunit expression (Thompson *et al.* 1999). CaMKII, when activated by Ca^{2+} influx through the NMDA receptor, phosphorylates the NR1 and NR2B subunits, thereby increasing Ca^{2+} influx too (Leonard *et al.* 1999). Phosphorylation of NMDA receptors by PKA also enhances the receptor function (Dingledine *et al.* 1999; Westphal *et al.* 1999) and this effect is even more evident when the NMDA receptor is composed of NR1A subunits. This receptor subunit is coupled to the associated protein yotiao which anchors PKA and type I protein phosphatase to NMDA receptors and PKA activation increases the receptor currents in comparison to NMDA receptors not expressing yotiao whereas type I protein phosphatase negatively regulates NMDA receptor currents (Westphal *et al.* 1999).

Interestingly, the development of NMDA receptor-dependent central sensitisation can be physiologically stopped by the increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$; e.g., Ca^{2+} entry through NMDA receptors, voltage-gated Ca^{2+} channels, Ca^{2+} permeable AMPA receptors, Ca^{2+} release from intracellular stores or Ca^{2+} entry induced by action potentials), a mechanism denominated Ca^{2+}-dependent inactivation (CDI) of NMDA receptors, which may act as a brake on the recruitment of NMDA receptors during sensory synaptic input to DHN, constituting a potent inhibitory control of NMDA currents (Kyrozis et al. 1996). In fact, L-, P/Q-, and R-type Ca^{2+} channels also suffer Ca^{2+}-mediated inactivation, and facilitation, by action of calmodulin (Ehlers & Augustine, 1999; Lee et al. 1999; Zühlke et al. 1999) which, also, desphosphorylates the NMDA receptor resulting in a negative feedback of NMDA receptor synaptic activation (Tong et al. 1995). Thus, NMDA receptors may fluctuate between phosphorylated and desphosphorylated forms, depending on the rate of synaptic stimulation and the magnitude of the associated Ca^{2+} influx through them, and calmodulin may act as a Ca^{2+} sensor in these and other channels, contributing, therefore, to modulate the cellular response. Nevertheless, activation of NMDA receptors responds almost immediately with stimulation of different metabolic pathways and the formation of retrograde messengers, which may account for the NMDA receptor-mediated excitability.

2.2.1.3.2. Effects of NMDA receptor activation

Activation of NMDA receptors permits the entry of Ca^{2+} into the cytoplasm, which triggers different metabolic pathways activating a number of enzymes and ultimately immediate-early genes (Figure 2.2) (Ghosh & Greenberg, 1995; Coderre et al. 1990; Woodburn & Woodruff, 1994). Retrograde second messengers resulting from this cascade have been posited to cause presynaptic amplification of neurotransmitter release; candidates include the neuromediators AA, PGs and NO, which may diffuse back to the primary afferent terminal (Chaplan et al. 1997; Dumuis et al. 1988; Nicholls, 1992; Schoepp & Conn, 1993; Dawson & Dawson, 1995; Woodburn & Woodruff,
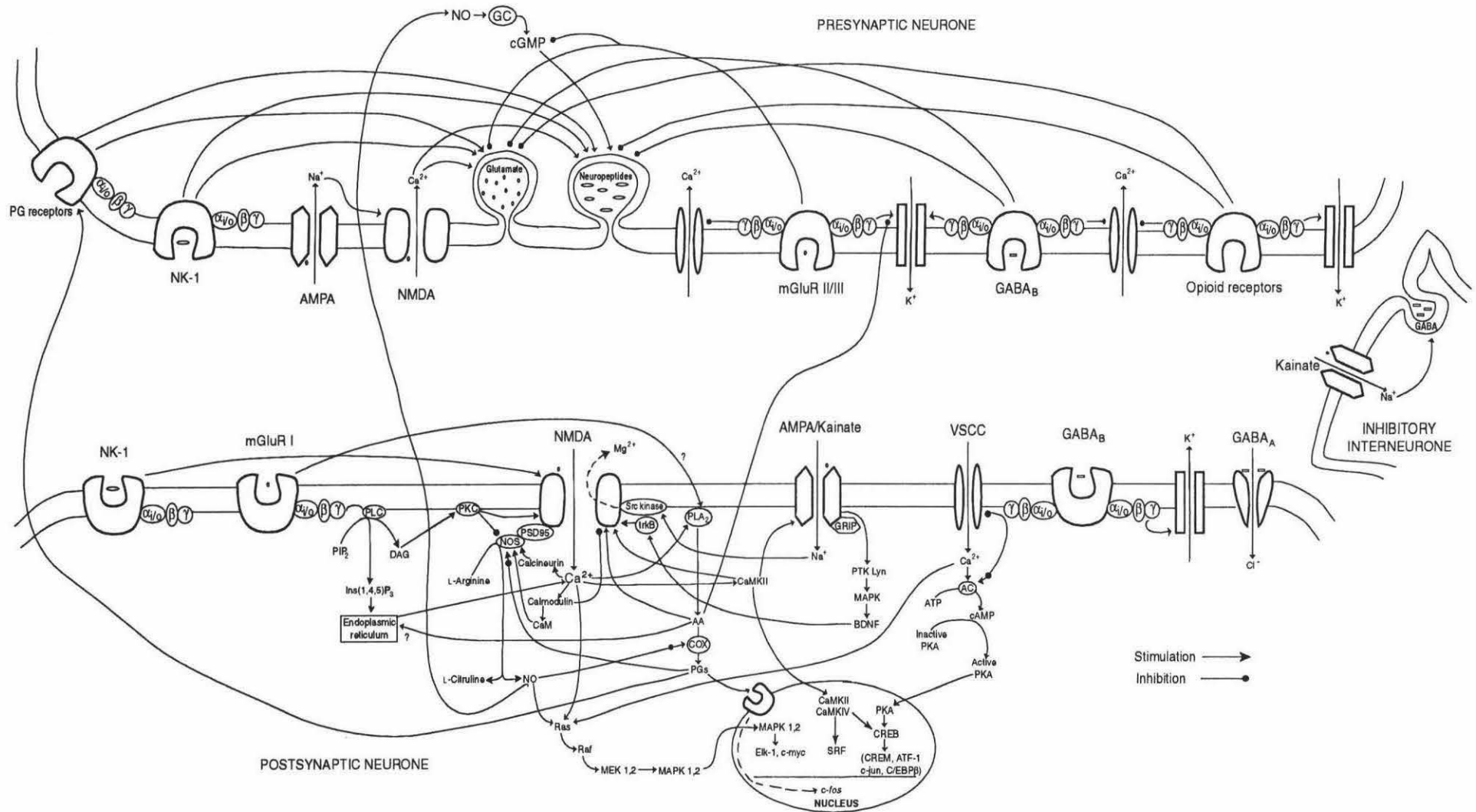
Figure 2.2. The role of glutamate receptors and their interaction with other receptor systems in the modulation of nociceptive processing. Refer to the text for details.

1994). This hypothesis could be true for NO because of its physical properties [*e.g.,* small, diffusible, membrane permeable, reactive (Dawson & Dawson, 1995)]; however, it may not apply for the PGs. Although these lipidic acids are often assumed to traverse membranes by simple diffusion, they are charged anions at physiological pH and diffuse poorly across model biological membranes. This limited simple diffusion appears to be augmented, at least in some circumstances, by a specific carrier-mediated transport (PG transporter) and AA is probably not transported to a significant extent by this transporter (Kanai *et al.* 1995). Nevertheless, both AA and PGs are involved in the process of pain signal amplification (see below).

2.2.1.3.2.1. NMDA receptors and the AA pathway

The resulting increase of $[Ca^{2+}]_i$, preceded by NMDA receptor activation, activates phospholipases. Activation of phospholipase A_2 (PLA_2) can increase the susceptibility of membrane phospholipids to hydrolytic processes that are associated with an increasing level of free AA, which, in turn, may be converted by cyclooxygenases (COX, also known as prostaglandin H synthase or prostaglandin endoperoxidase synthase) to prostanoids, including the PGs (Malmberg & Yaksh, 1992b; Buritova *et al.* 1996; Yaksh & Malmberg, 1993; Woodburn & Woodruff, 1994; Miller *et al.* 1992) (Figure 2.2). In agreement with this model, administration of glutamate and NMDA increased $[^3H]AA$ release from cerebellar granule cells (Viu *et al.* 1998), activation of high-threshold nociceptive afferent inputs, which activate NMDA receptors, released PGs within the spinal cord (Ramwell *et al.* 1966), and i.t. administration of NMDA evoked spinal increased release of PGE_2 and thromboxane (Tx) B_2 (Sorkin, 1997). Hence, a clear interaction between NMDA receptors and the AA pathway exists.

Arachadionic acid directly or indirectly potentiates NMDA currents, increases the release of glutamate from presynaptic terminals and impairs its glial reuptake (Dumuis *et al.* 1988; Nicholls, 1992). Amplification of NMDA receptor

currents by AA itself is probably due to its binding to a different site than the glutamate, glycine, Mg^{2+}, Zn^{2+} or polyamine binding sites, or by altering the receptor's lipid environment (Miller *et al.* 1992); however, the exact mechanism remains unknown. Glutamate increased release and decreased reuptake seem to be mediated by products downstream than AA in the biochemical pathway (Bezzi *et al.* 1998) but the effects and mechanisms of action of these AA metabolic products in the nociceptive process will be approached in further detail later in this work.

The generation of reactive oxygen species is the endpoint during the oxidative metabolism of AA and these free radicals contribute to originate neurocytotoxic states, by producing lipid peroxidation of cellular membranes and damage of proteins and DNA (Gunasekar *et al.* 1998). Their role in pain states is unknown.

2.2.1.3.2.2. NMDA receptors and the NO pathway

In addition to activation of phospholipases, Ca^{2+} entering though the NMDA receptor can also activate the enzyme nitric oxide synthase (NOS). Once in the cytoplasm, Ca^{2+} binds to calmodulin forming the Ca^{2+}-calmodulin complex (CaM), which activates NOS, which in turn, in the presence of O_2 and NADPH, is critical for the conversion of L-arginine to NO and L-citrulline (Dawson & Dawson, 1995; Machelska *et al.* 1997; Woodburn & Woodruff, 1994; Appleton *et al.* 1996; Knowles *et al.* 1989). This coupled NO production to NMDA receptor activation is thought to be mediated by a signalling pathway mediated by the PDZ-PDZ protein interaction domains between the NMDA receptor anchor protein PSD-95 and NOS (at least the neuronal isoform; see below) (Hillier *et al.* 1999; Sattler *et al.* 1999). NOS has to be in desphosphorylated form to be functionally active and when phosphorylated by PKC the enzyme catalytic activity is reduced (Dawson & Dawson, 1995; Snyder *et al.* 1998); desphosphorylation of NOS can be achieved by calcineurin (Snyder *et al.* 1998), which, in turn, is activated by Ca^{2+} entering through NMDA receptors and/or L-type Ca^{2+} channels (Genazzani *et al.* 1999). In addition to its

dephosphorylation action on NOS, calcineurin can also dephosphorylate the inositol-(1,4,5) triphosphate [Ins(1,4,5)P3] receptor and increase Ins(1,4,5)P3 receptor mRNA and protein levels in cultured cerebellar granule cells via an unidentified pathway (Genazzani *et al.* 1999), regulating, therefore, Ca^{2+} efflux from intracellular stores and contributing to the hyperexcited neuronal state.

Two isoforms of constitutively expressed NOS have been identified: 1) neuronal NOS (nNOS) which is found in a population of central and peripheral non-adrenergic, non-cholinergic neurones and is also present in skeletal muscle, pancreatic islets, endometrium, and respiratory and gastrointestinal epithelia; and 2) endothelial NOS (eNOS) present in endothelial cells. Macrophages synthesise an inducible isoform, iNOS (Dawson & Dawson, 1995; Appleton *et al.* 1996; Moncada *et al.* 1997; Bredt *et al.* 1990). Apart from the cell type, the NOS isoform synthesised is dependent on cell activation. Both nNOS and eNOS depend on CaM for catalytic activity, while iNOS is Ca^{2+}-independent and has calmodulin tightly bound to the enzyme and is considered a protein subunit of iNOS, which accounts for its resistance to Ca^{2+} activation (Dawson & Dawson, 1995; Appleton *et al.* 1996). In contrast, induction of iNOS requires of PTK activity (Kleinert *et al.* 1998). All isoforms require the cofactors flavin adenine nucleotide, flavin mononucleotide, nicotinamide adenine dinucleotide phosphate, and tetrahydrobioprotein (Appleton *et al.* 1996).

In the peripheral nervous system, NO is released by a widespread network of nerves which mediate some forms of neurogenic vasodilatation and regulate certain gastrointestinal, respiratory and genitourinary functions, presumably by direct action of NO (Moncada *et al.* 1997). In the CNS, NO synthesis can be mediated by either NOS isoform (Dawson & Dawson, 1995). Specifically in the spinal cord, the enzyme is found predominantly in the superficial layers of the dorsal horn and around the central canal (the intermediolateral cell column) (Machelska *et al.* 1997; Sakai *et al.* 1998), conferring a nocimodulator role to this gas in the CNS. In addition, NO modulates, among other functions in the CNS, the formation of memory and the coordination between neuronal activity and blood flow (Moncada *et al.* 1997). These physiological actions of NO are

mediated by activation of the soluble guanylate cyclase and consequently increase the concentration of 3',5'-cyclic guanosine monophosphate (cGMP) in target cells (Moncada *et al.* 1997; Knowles *et al.* 1989; Bredt *et al.* 1990).

Specifically during the nociceptive process, stimulation of NMDA receptors by L-glutamate permits Ca^{2+} influx in postsynaptic neurones to activate NOS, which in turn forms NO that diffuses to the presynaptic terminals and astrocytes where it activates guanylate cyclase to produce cGMP (Ohta *et al.* 1996). The precise effects of cGMP on the release of neuroactive agents are poorly understood but include a direct effect on ion channels, activation of protein kinases and stimulation or inhibition of phosphodiesterase activity (Fukuto & Chaudhuri, 1995). In support of this nociceptive action of NO, there are reports that NO donors enhance the release of SP and CGRP, causing hyperalgesia (Machelska *et al.* 1997; Sakurada *et al.* 1996; Masue *et al.* 1999), although an antinociceptive action, mediated at the NMDA receptor level, has also been demonstrated for these donors (Aizenman & Potthoff, 1999). S-nitrosylation of the NMDA receptor by NO or NO-derived compounds is believed to inactivate the receptor (Dawson & Dawson, 1995; Dingledine *et al.* 1999; Aizenman & Potthoff, 1999) and, therefore, may physiologically modulate glutamatergic neurotransmission. Another antinociceptive mechanisim for this gas could be by reacting with noradrenaline to form 6-nitro-noradrenaline in the spinal cord where produces analgesia presumably by inhibiting the amine re-uptake and metabolism (Chiari *et al.* 1999). A similar mechanism has been proposed for ACh antinociceptive action (Xu *et al.* 1996). These opposite pharmacological effects of NO, nociception and antinociception, have been attributed to the existence of a reduced and an oxidised form of this gas (Machelska *et al.* 1997); however, the circuitry of NO in the spinal pain processing remains to be determined.

All of the above support the neuromodulator role of NO on the nociceptive process acting as a retrograde messenger between neurones, as a modulator of postsynaptic function, and/or as a paracrine mediator in adjacent neurones or glia. Further support comes from the inhibitory effect of the nociceptive process by inhibiting the enzyme NOS with L-arginine analogues (Morgan *et al.* 1992;

Machelska *et al.* 1997; Moore *et al.* 1991; Moncada *et al.* 1997; Sakurada *et al.* 1996; Bulmer *et al.* 1998). However, the level within the CNS where NOS inhibitors exert their antinociceptive effects is still a matter of debate (Sakurada *et al.* 1996). Despite where in the CNS NOS inhibitors exert their action, it is possible that these agents have a dual antinociceptive action by inhibiting the synthesis of NO and by augmenting the levels of L-arginine. When the amino acid was administered in mice submitted to the carrageenan paw test, antinociception by an opioidergic mechanism independent of the NO pathway was observed (Kawabata *et al.* 1992). Interestingly, metabolism of arginine can occur by more than one pathway: by NOS to NO and citrulline, by arginase to ornithine and urea, and by arginine decarboxylase to agmatine. Ornithine and agmatine, in turn, can be converted into the polyamines putrescine, spermidine and spermine (Reis & Regunathan, 2000). Since agmatine and polyamines can modulate NMDA receptors (Appleton *et al.* 1996; Reis & Regunathan, 2000), they could also have contributed to L-arginine's antinociceptive action. Nonetheless, hyperalgesic actions have also been observed after epidural administration of L-arginine in rats (Masue *et al.* 1999).

Another pharmacological group that is also able to inhibit NOS are the anti-inflammatory steroids (also known as glucocorticoids), but they only affect the induction of iNOS and have no effect on the expression of either constitutive NOS (Salvemini *et al.* 1993; Salvemini *et al.* 1994). This inhibition, at least for dexamethasone, seems to involve enhanced lipocortin release (Appleton *et al.* 1996; Minghetti *et al.* 1999), a potent endogenous mediator of the anti-inflammatory actions of glucocorticoids. In fact, a lipocortin 1-derived N-terminus peptide (peptide Ac2-26) inhibited PGE_2 and NO production from lipopolysacharide (LPS)-stimulated microglial cells in a similar way to dexamethasone and these effects were largely due to down-regulation of COX-2 and iNOS (Minghetti *et al.* 1999).

2.2.1.3.2.3. Interactions between the AA and NO pathways

The products of both COX and NOS enzymes as well as having effects on various cellular systems, can also modulate the activity of the other enzyme. Thus, COX products can affect the activity of NOS and similarly NO can affect COX activity. These effects, however, are not clear, as both stimulatory and inhibitory actions have been ascribed. Discrepancies in the effect of PGs on NO synthesis may depend on the stimuli used as well as the source of the PG (Appleton *et al.* 1996). Thus, PGE_2 and $PGF_{2\alpha}$, but not PGD_2, stimulated NO release in rat spinal cord slices (Sakai *et al.* 1998), whereas PGE_2 and PGD_2 as well as their cyclopentenone derivates, PGA_2, 15-deoxy-$\Delta^{12,14}$-PGA_2, PGJ_2, and 15-deoxy-$\Delta^{12,14}$-PGJ_2, suppressed iNOS activity in a LPS-stimulated murine microglial cell line (Petrova *et al.* 1999). 15-Deoxy-$\Delta^{12,14}$-PGJ_2 also suppressed iNOS mRNA and protein accumulation, at least in part, by suppressing nuclear factor κB (NFκB) transcriptional activity via an independent pathway to the inhibition of nuclear translocation of NFκB or the nuclear activation of peroxisome proliferator-activated receptor γ (Petrova *et al.* 1999). In addition, eicosanoids, and more probably PGE_2, are strong candidates for determining which biochemical pathway (see above) arginine follows (Appleton *et al.* 1996).

On the other hand, stimulatory and inhibitory actions of NO on PG production may be explained by the relative concentrations of NO. Low levels of NO may stimulate PG formation and thus act as a pronociceptive agent, whereas high levels of NO may inhibit PG production, therefore acting as an antinociceptive agent (Machelska *et al.* 1997; Appleton *et al.* 1996). Interestingly, studies *in vitro* using mouse macrophage cells (Salvemini *et al.* 1993), and *ex vivo* utilising a rabbit hydronephrotic kidney model (Salvemini *et al.* 1994), have demonstrated a NO-mediated increase in PG production, supporting a target role of COX for NO. In human cultured astroglial cells incubated with interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α), the involvement of NO in the increase of PGE_2 has also been reported (Mollace *et al.* 1998). This effect was attributed to the activation of iNOS and subsequent release of NO which interacted with COX-2 to enhance the formation of PGE_2. However, reduced PG

production in human vascular smooth muscle cells was attributed to inhibition of COX-2 activity via activation of the NO-soluble guanylate cyclase pathway (Gitlin *et al.* 1999). Even more, lack of interaction between iNOS and COX-2 products in the plasma of LPS-treated rats was observed (Hamilton *et al.* 1998). Therefore, it may be possible that the interactions between these pathways could be tissue or species specific, or both.

The interaction between the two pathways has also been observed when L-arginine analogues are used because they are capable of interacting with other iron-containing enzymes, including COX, and, thereby, their effects may not be solely attributable to NOS inhibition (Appleton *et al.* 1996).

2.2.1.3.3. NMDA receptors and the release of other neuromediators

Directly or indirectly, the NMDA receptors may contribute to the evoked release of neuromediators other than AA, PGs and NO, and the released substance(s) can differ depending on the kind of stimulus. Some of the substances released after activation of NMDA receptors include glutamate, SP, GABA, opioid peptides and monoamines, just to mention some of them.

In general, direct NMDA receptor-mediated events are considered to be postsynaptic (Buritova *et al.* 1996). However, it was recently demonstrated the existence of active presynaptic NMDA receptors (autoreceptors) in the spinal cord dorsal horn (Liu *et al.* 1994) which seem to contribute to the release of glutamate (Conway *et al.* 1997; Robert *et al.* 1998) and SP (Liu *et al.* 1997; Malcangio *et al.* 1998). Interestingly, the neurokinin NK-1 receptor, to which SP primarily couples, can also be located either post- or preseynaptically (Urban & Nagy, 1997). Thus, both glutamate and SP, in addition to depolarising the postsynaptic terminal, can also act on presynaptic autoreceptors and may modulate neurotransmitter release. Nevertheless, controversy regarding the role of presynaptic NMDA receptors in nociception exists (Urban & Nagy, 1997).

Under certain conditions of afferent stimulation, GABA, the main inhibitory neurotransmitter in the mammalian CNS, is released in sufficient amounts to cause hyperpolarisation of DHN, thereby limiting the NMDA-mediated development of central sensitisation (Lorenzetti & Ferreira, 1996). In this regard, it is not surprising that GABAergic and glutamatergic synapses often exist in close apposition on DHN and so are stimulated to effectively modulate local membrane potentials (Kaneko & Hammond, 1997). GABA acts on three different receptor types: $GABA_A$, $GABA_B$ and $GABA_C$. The $GABA_A$ receptor, located postsynaptically, is a ligand-gated ion channel that, when activated by two molecules of GABA, allows an influx of Cl^- ions hyperpolarising the cell membrane and producing a cessation of the cell's electrical discharge. The $GABA_B$ receptor, which exists pre- and postsynapticallty, is linked to G-proteins and when activated by one molecule of GABA, the receptor then couples to associated G-proteins. One of these (G_i) inhibits adenylate cyclase, probably through its α-subunit, and opens a K^+ channel, probably trough its $\beta\gamma$ subunits. Another G-protein (G_o) retards the opening of the Ca^{2+} channel, probably through its $\beta\gamma$ subunit (Bowery & Brown, 1997), hyperpolarising the cell membrane, therefore, by a dual mechanism. The $GABA_C$ receptor is an ionotropic receptor, which mainly localises in mammalian retina cells (Bormann, 2000).

Stimulation of NMDA receptors also seems to be interrelated with the opioidergic and adrenergic pathways. Thus, noxious stimulation enhances the release of endogenous opioid peptides which, by acting on presynaptic receptors (Table 2.2), reduce the release of glutamate and SP from the primary afferent (Dhawan *et al.* 1996). Increased noradrenaline release after spinal NMDA receptor stimulation also has been observed (Nakai *et al.* 1999) and activation of α_2- subtype receptors mediates its antinociceptive effects. In this regard, α_2-adrenoceptor agonists depressed a NMDA receptor-mediated response of nociceptive neurones in the spinal cord (Faber *et al.* 1998; Zhang *et al.* 1998), an effect observed with synthetic opioids too (Faber *et al.* 1997a).

Enhanced release of dopamine (Jin & Fredholm, 1997), ACh (Jin & Fredholm, 1997) and adenosine (Jin & Fredholm, 1997; Conway *et al.* 1997) can also occur after NMDA receptor activation. Dopamine produces antinocicepcion at the spinal level by activation of dopamine D2 receptors (Barasi *et al.* 1987) and, interestingly, probably by blocking the NMDA receptor itself (Castro *et al.* 1999), whereas ACh does it by acting on muscarinic M2 receptors (Gomeza *et al.* 1999). Adenosine, in turn, inhibits NMDA-receptor mediated hyperalgesia and the concurrent spinal glutamate release by acting on adenosine A1 receptors (Kakinohana *et al.* 1998).

Table 2.2. Distribution and functions of opioid receptors.

Receptor	Distribution*	Functions
μ	Neocortex, thalamus, nucleus accumbens, hippocampus, amygdala, superficial layers of the spinal cord dorsal horn, periaqueductal gray, raphe nuclei, hypothalamus, perioptic area and globus pallidus. Widely distributed in the peripheral nervous system, particularly in myenteric neurones in the gut and the vas deferens.	μ-Opioid receptor agonists block the nociceptive responses to thermal, mechanical or chemical high intensity stimulation, indicating that μ receptors play an important role in nociception. Respiration, cardiovascular functions, intestinal transit, feeding, learning and memory, locomotor activity, thermoregulation, hormone secretion and immune functions, all of which, except hormone secretion, are most often depressed by μ receptor stimulation.
δ	More restricted than other opioid receptors within the CNS. Are present in olfactory bulb, neocortex, caudate putamen, nucleus accumbens, thalamus, hypothalamus, brainstem and spinal cord dorsal horn.	Role in analgesia, motor integration, gastrointestinal motility, olfaction, respiration, cognitive function, mood driven behaviour, etc. Activation of presynaptic receptors produce antinociception by inhibiting neuropeptide release from terminals of primary afferent fibres in the spinal cord dorsal horn. Involvement of supraspinal and peripheral δ receptors in analgesia is not excluded. Both peripheral and central δ receptors are involved in inhibition of gastrointestinal transit.
κ	Specific binding sites and species differences for tritiated arylacetamide derivatives and benzomorphans. In the rat, only low levels of tritiated arylacetamide compounds are found throughout the cerebral cortex and no area caudal to the forebrain is heavily labelled.	Regulation of nociception, diuresis, feeding, neuroendocrine secretions, and immune functions. κ-Opioid receptor agonists produce antinociception at the spinal level, and controversy about supraspinal sites of action exist. Apparently, both central and peripheral κ receptors mediate the anti-diarrhoeal properties of opioids.

Elaborated with information from Dhawan *et al.* 1996.

Increased IL-1β expression has also been observed after NMDA receptors activation and it seems that this cytokine releases some neurotransmitters (*e.g.,* noradrenaline, dopamine) or retrograde messengers (*e.g.,* AA, NO, platelet-activating factor), or both (Schneider *et al.* 1998). In this regard, cultured astroglial cells treated with IL-1b increased mRNA $P2Y_2$ expression (John *et al.* 1999) and activation of a P2Y receptor in cultured astrocytes resulted in increased COX-2 expression (Brambilla *et al.* 1999). Moreover, increased NO and subsequent PGE_2 formation in cultured astroglial cells incubated with IL-1β and TNF-α has been reported (Mollace *et al.* 1998), as well as enhanced SP release from spinal cord slices, presumably through the generation of endogenous prostanoids by the cytokine (Malcangio *et al.* 1996). Together, these data suggest the involvement of IL-1β in the nociceptive processing, an effect supported by an enhanced Aδ fibre-evoked response of rat spinal neurones after i.t. administration of the cytokine, and by inhibition of both Aδ and C fibre-evoked responses after i.t. application of the IL-1 converting enzyme inhibitor SDZ-224-015 (Reeve *et al.* 1998).

Inhibition of nerve growth factor secretion has also been observed in bladder smooth muscle cells treated with the NMDA receptor antagonist ketamine (Tuttle *et al.* 1999). If inhibition of this growth factor also occurs in nociceptive neurones, this mechanism may contribute to ketamine's analgesic effect by interfering with BDNF regulation and subsequent phosphorylation of the NMDA receptor (see above).

In summary, activation of NMDA receptors permits Ca^{2+} influx which in turns activates the AA and the NO pathways, resulting in the production of retrograde messengers that could act on presynaptic neurones, as well as non-neural structures or postsynaptic neurones. The effects of these molecules include the release of neurotransmitters or neuropeptides, or both, from the presynaptic site and inhibitory interneurones, as well as the formation and expression of new receptors. All these complex mechanisms serve to modulate pain transmission.

Pharmacological inhibition of NMDA receptors, hence, results in attenuation of nociceptive processing (Chaplan *et al.* 1997; Ilkjaer *et al.* 1998; Dupont *et al.* 1998). However, mechanisms other than or complementary to purely NMDA receptor antagonism may contribute to the analgesic effect of these receptor blockers. For example, the analgesic effect of intraperitoneal (i.p.) dizocilpine assessed by the hot plate test in rats was partially reversed by i.p. injection of the opioid receptor antagonist naloxone, and the dopamine D1 and D2 receptor antagonists SCH 23390 and sulpiride, respectively (Forman, 1999). Because co-administration of naloxone plus SCH 23390 or sulpiride did not completely abolish dizocilpine analgesia, contribution of serotoninergic, α-adrenergic, and muscarinic cholinergic mechanisms was proposed (Forman, 1999). In fact, enhanced [^{3}H]noradrenaline release evoked by electrical stimulation of rat spinal cord slices was observed with dizocilpine (Nakai *et al.* 1999), whereas increased dopamine and 5-HT release in the rat striatum was detected after i.p. and intra-striatum administration of the NMDA receptor antagonist D-CPPene (Potschka *et al.* 1999). Thus, it could be possible that NMDA receptor antagonists produce antinociception by partially increasing the release of natural occurring inhibitory mediators.

2.2.2. Metabotropic glutamate receptors

In the same synapses at which glutamate elicits fast excitatory responses, it can modulate fine-tune activity by activation of mGluRs which are coupled to effector systems through G-proteins. These G-protein-linked receptors are divided into three groups (Group I-III) based on their sequence homologies, the selectivity/potency of mGluR agonist and the signal transduction pathways to which each receptor preferentially couples. Group I receptors, which include the splice variants of mGluR1 and mGluR5, stimulate phosphoinositide hydrolysis by stimulating phospholipase C (PLC) to generate Ins(1,4,5)P3 and DAG; whereas Group II (mGluR2 and 3) and III (mGluR4, 6, 7 and 8) receptors evoke an inhibition of forskolin-stimulated 3',5'-cyclic guanosine monophosphate (cAMP) formation by negatively coupling to adenyl cyclase (Akam *et al.* 1997;

Cartmell *et al.* 1997; Pinkney *et al.* 1997; Woodburn & Woodruff, 1994; Wigmore & Lacey, 1998; Conn & Pin, 1997).

Because mGluRs are widely distributed, are present in different types, subtypes and splice variats, and are activated not only by glutamate but also by physiological extracellular Ca^{2+} concentrations (see below), it is not surprising that these receptors participate in a wide variety of neuronal functions. Moreover, within the nociceptive pathway, these receptors can produce both direct excitation and presynaptic modulation of neurotransmitter release.

2.2.2.1. Excitatory effects of mGluRs

The mGluRs can produce excitatory responses as powerful as those elicited by AMPA receptors (Batchelor & Garthwaite, 1997), but, of course, with very different kinetics. The group I mGluRs seem to be the most involved in this excitatory process. When activated, postsynaptic group I mGluRs increase phosphoinositide hydrolysis, forming Ins(1,4,5)P3 and DAG. On the one hand, Ins(1,4,5)P3 binds to specific receptors on the endoplasmic reticulum membrane and increases intracellular Ca^{2+} release (Schoepp & Conn, 1993; Ghosh & Greenberg, 1995; Malmberg *et al.* 1995) which, by binding to calmodulin, may activate CaMKII and then phosphorylates the NMDA receptor, releasing the Mg^{2+} block from it (Holehean *et al.* 1999) and contributing, thus, to the hyperexcited state. DAG, on the other hand, activates PKC, which may enhance presynaptic NMDA receptor function (Liu *et al.* 1994) with the subsequent increase of glutamate (Dumuis *et al.* 1988; Nicholls, 1992; Liu *et al.* 1997; Conn & Pin, 1997; Robert *et al.* 1998) or SP (Liu *et al.* 1997) release, or both.

In addition to the postsynaptic excitatory action, a presynaptic excitatory action for group I mGluRs has been suggested also. In this model, inhibition of K^+ influx is due via activation of a sensitised PKC by AA diffused back from the postsynaptic terminal (Dumuis *et al.* 1988; Nicholls, 1992) potentiating, thus,

action potentials and neurotransmitter release. Similarly, increased glutamate release through a Ca^{2+}-dependent process mediated by PGs was observed after co-activation of group I mGluRs and AMPA-kainate receptors in astrocytes (Bezzi *et al.* 1998). Furthermore, a decrease in dopamine levels after presynaptic stimulation of group III, and to a lesser extent group II, mGluRs was noticed (Hu *et al.* 1999) as well as those for GABA, but the specific mGluRs involved in this process are not mentioned (Conn & Pin, 1997). Thus, mGluRs are capable of enhancing the excitatory synaptic transmission by multiple mechanisms that may include potentiation of excitatory neurotransmitter and neuropeptide release, decreased glutamate reuptake, and/or suppression of inhibitory neurotransmitter release.

Recent studies from Kubo *et al.* (1998) have raised the possibility that mGluRs may also function as ionotropic receptors. They found that continuous stimulation of oocytes expressing mGluR1α by 2 mM Ca^{2+} solution, increased the concentration of Ins(1,4,5)P3 in chinese hamster ovary cells. Thus, extracellular physiological Ca^{2+} concentrations could represent another mechanism of mGluR-mediated excitatory transmission.

2.2.2.2. Inhibitory effects of mGluRs

Reduction of transmission by mGluRs is mediated typically by presynaptic autoreceptors that reduce glutamate release. Depression of glutamatergic transmission attributable to presynaptic group II and III mGluR activation has been observed at several synapses including those in the spinal cord (Wigmore & Lacey, 1998; Scanziani *et al.* 1997; Conn & Pin, 1997); however, the molecular mechanisms involved in this process are still to be determined. Reduction of voltage-dependent Ca^{2+} currents seems to be a possible mechanism. Recently, Takahashi *et al.* (1996) described a presynaptic inhibition of a high voltage-activated P/Q type Ca^{2+} conductance mediated by group II or III mGluR activation and postulated that these receptors can inhibit transmitter release by targeting ion channels.

Other likely mechanisms for inhibition of synaptic transmission could include the reduction of cGMP accumulation and the increased production of adenosine. The former mechanism was observed after group III mGluR activation in retinal cells (Conn & Pin, 1997); if this also happens in nociceptive neurones, the inhibition of this product of the NO pathway may represent an important mechanism in the modulation of synaptic neurotransmission, because it contributes to the release of neurotransmitters (Fukuto & Chaudhuri, 1995). On the other hand, increased adenosine production was ascribed to co-activation of group II mGluRs and β-adrenergic receptors which produced cAMP and subsequently adenosine (Conn & Pin, 1997) that, in turn, may inhibit spinal glutamate release and hyperalgesia by acting on presynaptic adenosine A1 receptors (Kakinohana *et al.* 1998).

In addition to the excitatory effects, group I mGluRs can also inhibit glutamate release. Although it was suggested that these postsynaptic receptors may mediate presynaptic inhibition by promoting the NMDA receptor-dependent release of an inhibitory retrograde messenger (Wigmore & Lacey, 1998), it is the presynaptic activation of these mGluRs that is the most involved in inhibitory effects. Recently, it was shown that presynaptic activation of group I mGluRs enhanced GABA-mediated synaptic inhibition via excitation of inhibitory interneurones in the rat frontal cortex (Chu & Hablitz, 1998). Increased dopamine release was also observed after stimulation of group I mGluRs from the rat striatum (Burton *et al.* 1999). Therefore, both excitatory and inhibitory effects could be expected with group I mGluR ligands and may depend on the mGluR splice variants present in the area of influence of the agents.

2.3. THE ARACHIDONIC ACID PATHWAY

This section intends to review the biochemical steps that lead to the formation of some of the most important autacoids in the AA pathway as well as their effects on the nociceptive process. The AA pathway (Figure 2.3) begins with the release of polyunsaturated fatty acids from the cell membrane's phospholipids which are converted to AA by action of different phospholipases, although PLA_2
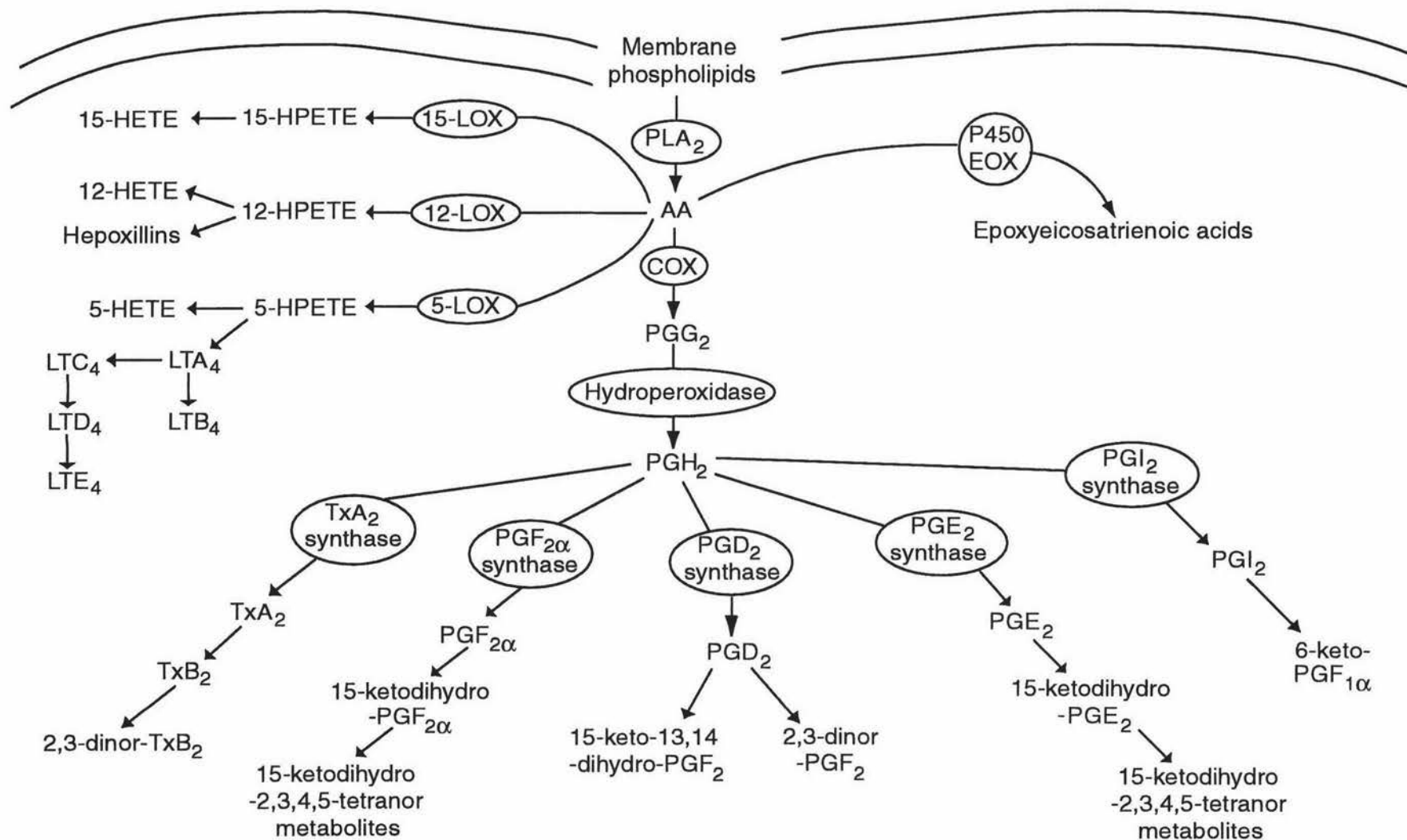
Figure 2.3. Major pathways of the arachidonic acid cascade.

seems to be the most important. AA is released into the cell and acts as a substrate for enzymes, lipoxygenases (LOX), COX and epoxygenases (EOX), which produce intermediate and final products. LOX are responsible for the production of hydroxyeicosatetraenoic acids (HETE), and specifically 5-LOX for that of leukotrienes (LTs); COX have a role on the synthesis of PGs and Txs; whereas EOX are involved in the formation of epoxyeicosatrienoic acids. The EOX biochemical pathway is not as well characterised as that for COX or LOX, and the effects of their products on nociception have hardly been studied.

2.3.1. Leukotrienes

The LTs are metabolites derived from lipooxiganation of AA. They are potent smooth muscle contracting agents (Freedman *et al.* 1993) involved in inflammatory and painful reactions; however, these effects seem to be indirectly mediated by producing microvascular changes on the blood flow and increasing the vascular permeability (Bisgaard *et al.* 1982; Bisgaard, 1987; Ford-Hutchinson, 1985). Their contribution to the pain process, therefore, comes from sensitisation of cutaneous nociceptors rather than producing a direct effect on them (Madison *et al.* 1992) and this is expressed as a delay in the onset of hyperalgesia after their experimental administration (Taiwo *et al.* 1987). This sensitising effect of LTs seems to be specific for different agents; for example, LTB_4 sensitised nociceptive fibres to become hyperalgesic to the action of PGE_2 (Martin *et al.* 1987) but not PGE_1, which was affected by LTD_4 (Rackham & Ford-Hutchinson, 1983). Furthermore, the induction of hyperalgesia by LTB_4, a potent chemotactic factor for polymorphonuclear leukocytes (PMNLs) (Ford-Hutchinson, 1985), might also depend on the generation by cutaneous PMNLs of mediators derived from the 15-lipoxygenation of AA, which modulate primary afferent functions and contribute to inflammatory hyperalgesia (Levine *et al.* 1986). All these studies illustrate LT-mediated nociceptive effects at the peripheral level and, because both centrally administered LTC_4 and LTD_4 had no effect on thermal pain sensation in mice (Brus *et al.* 1986), it seems that the effects of these agents on nociception are restricted to this level.

Together, the above data suggest that LTs may indirectly modulate pain responses by sensitising peripheral nociceptors to the action of PGs or by increasing the recruitment of vascular agents that may enhance pain transmission, or both. Inhibition of the LOX pathway, therefore, was postulated as an advantageous adjunct to the effect of the NSAIDs (Bisgaard & Kristensen, 1985). Interestingly, the 5-LOX/COX dual inhibitor SK&F 105809 reduced hyperalgesia as assessed by the phenyl-benzoquinone-induced writhing test in mice (Griswold *et al.* 1991); however, the antinociceptive importance of inhibiting LT production remains uncertain. In the same nociceptive test, the 5-LOX inhibitors BW A4C and BW A797C had no analgesic effect (Higgs *et al.* 1988) but the COX inhibitors indomethacin, naproxen, and ibuprofen did produce analgesia (Griswold *et al.* 1991). More research is necessary to establish the analgesic importance of inhibiting the LOX pathway.

2.3.2. Prostaglandins

The PGs are polyunsaturated fatty acid derivatives, which basic structural unit is a 20-carbon unsaturated carboxylic acid with a characteristic cyclopentane ring structure. The term was first used to describe the presence of these substances in semen and the belief that they came from the prostate; nevertheless, they appear to be present in almost all tissues. Their synthesis is due by oxygenation of AA by COX, which produces PGG_2, unstable peroxide that is converted to PGH_2. PGH_2, in turn, is converted via distinct synthases to PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, or TxA_2, generally only one prostanoid is formed in abundance and this effect is cell specific. None of these agents are stored within cells but rather are synthesised in demand, and once formed, all are rapidly metabolised with biological half-lives of approximately 1 minute (Appleton *et al.* 1996). Some of the most important metabolites of these prostanoids are shown in Figure 2.3.

Besides catalysing the formation of PGG_2, COX has a peroxidase activity, reducing the 15-hydroperoxyl group of PGG_2 to PGH_2. These two reactions occur on the same enzyme but the active sites differ and COX inhibition by NSAIDs has no effect on peroxidase activity (Appleton *et al.* 1996).

Interestingly, PGs, to some extent, can modulate COX activity (Appleton *et al.* 1996; Kurumbail *et al.* 1996). Different COX isozymes have been identified and an overview of their pathophysiological functions as well as their expression and regulation is next presented. For those interested in this particular subject various recent reviews have been published elsewhere (Smith & Dewitt, 1996; Vane *et al.* 1998; Cryer & Dubois, 1998; Mitchell & Warner, 1999; Dubois *et al.* 1998).

2.3.2.1. Cyclooxygenases

2.3.2.1.1. Cycoolxygenase isoforms and functions

For many years it was thought that COX was a single enzyme present constitutively in most tissues and that inhibition of this enzyme led to decreased production of pro-inflammatory as well as physiological PGs. However, now it is known the existence of at least two COX isozymes: COX-1 and COX-2. Both isoforms use the same endogenous substrate, AA, and form the same products by the same catalytic reaction (Langenbach *et al.* 1995; Morham *et al.* 1995); this is due to the highly conserved homology (>90%) of the COX and peroxydase enzyme regions between the two isozymes (Cryer & Dubois, 1998). Their major difference lies in their pathophysiological functions. On the one hand, COX-1 is constitutively expressed in most tissues at a different constant level, and is believed to be responsible for the formation of PGs with physiological functions. COX-2, on the other hand, is normally undetectable in most tissues, but it can be induced at high levels in a variety of cells by pro-inflammatory stimuli (Geisslinger & Schaible, 1996; Yamamoto & Nozaki-Taguchi, 1996; Appleton *et al.* 1996; Seibert *et al.* 1994; Kurumbail *et al.* 1996; Vane *et al.* 1998; Langenbach *et al.* 1995; Morham *et al.* 1995; Mahler *et al.* 1996). This activation of COX-2 by pro-inflammatory stimuli lead to the suggestion that the NSAIDs' therapeutic effects are mediated via the inhibition of COX-2 whereas their side effects are due to inhibition of COX-1 (Geisslinger & Schaible, 1996; Seibert *et al.* 1994; Langenbach *et al.* 1995), an hypothesis that has created a lot of controversy (see below). Furthermore, important

structural peculiarities in the regulation of both COX isozymes exist (Table 2.3). Of great importance is the presence of a glucocorticoid regulatory region on the COX-2 gene which accounts for cytokine or endotoxin induction of COX-2 being inhibited *in vitro* and *in vivo* by glucocorticoids such as dexamethasone, whereas COX-1 is unaffected (Appleton *et al.* 1996; Seibert *et al.* 1994; Salvemini *et al.* 1993; Salvemini *et al.* 1994; Vane *et al.* 1998; Langenbach *et al.* 1995; Morham *et al.* 1995). Together, all these differences suggest that the two isozymes can act independently.

Table 2.3. Comparison between COX-1 and COX-2.

Properties	COX-1	COX-2
Proposed role	Housekeeping	Inflammatory response
Amino acid homology within the same species (human, mouse, avian)	60%	60%
Intracellular localisation	Endoplasmic reticulum membrane (mainly), nuclear envelope (some)	Nuclear envelope (mainly), endoplasmic reticulum membrane (some)
Regulation	Constitutive	Inducible
Range of expression	2 to 4 fold	10 to 80 fold
Tissue expression	Platelets, endothelial cells, stomach, kidney, smooth muscle, most tissues	Most tissues, especially inflammatory cells. Requires stimulation by: grow factors, cytokines & hormones

Elaborated with data from Appleton *et al.* 1996; Cryer & Dubois, 1998.

Although most of our knowledge on the functions of both COX isozymes comes from studies of pharmacological inhibition with NSAIDs and COX-2 selective inhibitors, the development of *Ptgs 1* and *Ptgs 2* gene knockout mice (Langenbach *et al.* 1995; Morham *et al.* 1995; Mahler *et al.* 1996) showed interesting findings (Table 2.4) about these two isozymes. For example, COX-1, to some degree, contributes to inflammation, parturition and luteolysis, and its absence alone is not sufficient to cause gastric ulceration. On the other hand, COX-2 is not essential for an inflammatory response and may be involved in cell differentiation, mitogenesis and specific reproductive functions. Therefore, it is highly likely that both COX isozymes have physiological and pathophysiological functions and that inhibition of COX-2 is not devoid of side

effects. Thus, COX-1, besides its well-known role in gastroprotective functions, plays a part in the inflammatory process also (Smith *et al.* 1998; Wallace, 1999; Wallace *et al.* 1999). On the other hand, up-regulation of COX-2 has a homeostatic role in the inflamed tissue surrounding gastrointestinal ulcers and PGs produced via this isozyme may be responsible for the beneficial effects on ulcer healing (Wallace, 1999; Wallace & Cirino, 1994; Shigeta *et al.* 1998). Therefore, when COX-2 selective inhibitors were used in rodents suffering from induced gastric or colonic ulceration there was a significant reduction in mucosal PG synthesis and a marked increase in the severity of the organ damage, delaying the healing (Wallace, 1999; Shigeta *et al.* 1998). Other possible side effects could occur in the kidneys, lungs or the CNS, where COX-2 is constitutively expressed (Vane *et al.* 1998; Ermert *et al.* 1998; Cryer & Dubois, 1998; Phillips & Pearce, 1999), and data from COX-2 knockout mice (Morham *et al.* 1995) indicates that reproductive functions might be affected as well. Recently, it was highlighted that COX-2 selective inhibitors reduced systemic and renal synthesis of PGI_2 in humans (Catella-Lawson *et al.* 1999; Cullen *et al.* 1998), suggesting that more than the predicted side effects could be expected with the use of these compounds. A comprehensive review of possible adverse effects by COX-2 inhibition in these and other body organs and systems (*e.g.*, pancreas, cardiovascular system) has been published elsewhere (Mitchell & Warner, 1999).

2.3.2.1.2. Cycoolxygenases and the nociceptive process

As stated above, in the CNS both COX isozymes are constitutively expressed and their prevalence may depend on the anatomical localisation, the age, and the species. In the rat spinal cord COX-2 is the predominant isoform and it is believed that it's here where the nociceptive process is most influenced by this isozyme (Willingale *et al.* 1997; Seibert *et al.* 1994; Vane *et al.* 1998). Thus, increased immunoreactive staining of COX-2 mRNA and protein, but not for COX-1, in the rat spinal cord resulted from inflammatory hyperalgesia to the paw (Vane *et al.* 1998), and i.t. administration of the selective COX-2 inhibitor NS-398 produced analgesia in the rat paw formalin test (Yamamoto & Nozaki-Taguchi, 1996). Further support for the relevance of COX-2 on the spinal

nociceptive process comes from anatomical studies of COX-1- and COX-2-like immunoreactivity (COX-1-li and COX-2-li, respectively) in the spinal cord of normal rats (Willingale *et al.* 1997). On the one hand, COX-1-li was detected in small to medium sized cell bodies in the DRG and the dorsal roots, and to a lesser extent in the spinal cord dorsal horn. On the other hand, although COX-2-li was not present in DRG neurones, it was observed in the superficial dorsal horn (laminae I and II), around the central canal (lamina X), in some neurones in the deep dorsal horn layers (medial aspect of laminae V and VI) and in individual motor neurones in the ventral horn. Therefore, distribution of COX-2 in the dorsal horn is consistent with COX products being involved in the processing of nociceptive input, but COX-1 metabolites may also be involved in stimulating afferent fibres.

Table 2.4. Functions of COX-1 and COX-2 determined by *Ptgs 1* and *Ptgs 2* gene depletion in mice, respectively.

	Ptgs 1 gene knockout mice	*Ptgs 2* gene knockout mice
Gastric ulceration	Absence of COX-1 alone was not sufficient to cause gastric ulceration. Its absence decreased rather than increase the incidence of indomethacin-induced gastric ulceration. PGE_2 levels were about 1% of wild type mice.	No gross or microscopic lesions of the stomach.
Platelet aggregation	Reduced.	Not determined.
Inflammation response	Decreased response to AA*, but not to TPA*.	No decreased response to AA, TPA or carrageenan. Normal response to bacterial invasion of the peritoneum.
Kidneys	No deleterious effects.	Development of nephropathy that becomes more severe and complex with the age.
Fertility	Matings between *Ptgs 1* deficient females and mutant males resulted in reduced fertility and prolonged gestation length (1-2 d). Matings between heterozygous and wild type females and mutant males increased fertility and number of live pups in comparison to matings between mutant mice.	Mutant males were fertile. Homozygous mutant females wee infertile due to ovulatory failure.

* AA, arachidonic acid; TPA, tetradecanoyl phorbol acetate.
Elaborated with information from Langenbach *et al.* 1995; Morham *et al.* 1995; Mahler *et al.* 1996.

This last statement agrees with the study from Dirig *et al.* (1997) who, based on the blockade of paw carrageenan-evoked hyperalgesia by spinal and systemic pre-administration of, as well as systemic, but not spinal, post-administration of S(+)-ibuprofen or SC58125 (a COX-2 selective inhibitor) in rats, concluded that at least spinal COX-2, if not both spinal COX-1 and COX-2, are essential for the initiation of hyperalgesia, whereas non-spinal sources of COX products are important for their maintenance. Using the same pain model but different drugs (SC-560 and celecoxib; COX-1 and COX-2 selective inhibitors, respectively) and drug administration protocols, Smith *et al.* (1998) concluded something similar. They proposed that, in addition to the role of peripherally produced PGs, there is a critical, centrally mediated component of inflammatory pain transmission mediated in part by COX-2.

The unexplained analgesic effect for some NSAIDs by COX-1 or COX-2 inhibition (see below) has lead to the proposal of a third COX isoform present in the CNS (Appleton *et al.* 1996; Vane, 1994), but it has not been demonstrated yet. However, a possible variant of COX-2 or a third COX that is co-regulated with COX-2, and that may play an important role in apoptosis, has been recently reported (Simmons *et al.* 1999).

2.3.2.1.3. Cycoolxygenase inhibition by NSAIDs

It has been postulated (Appleton *et al.* 1996) that the effect of a NSAID will depend on both the relative amount of COX-1 and COX-2 present in a tissue and on its COX-1:COX-2 inhibitory ratio. Moreover, a classification for NSAIDs according to their COX-1:COX-2 inhibitory ratio was proposed by Frölich (1997) who argued that its use would permit a useful prediction of the major effects and side-effects of these drugs, a statement that has been debated (Griswold *et al.* 1997). One of the major conflicts in establishing such a classification is the direct comparison of results obtained from different assay systems or conditions in different laboratories which can produce widely disparate results for the COX1:COX-2 inhibition ratio and even change the precise ranking of a drug from model to model (Griswold *et al.* 1997; Cryer & Dubois, 1998).

Numerous methodological details, including the source (endogenous or exogenous) and concentration of substrate (AA), the use of intact cells or purified enzymes, the protein concentration in the incubation mixture and the time of incubation, are contributing factors to the great range of reported values for most NSAIDs (Frölich, 1997; Ricketts *et al.* 1998). Therefore, the relative ranking of compounds in a single assay system has more meaning than comparisons between several assay systems (Ricketts *et al.* 1998). However, even comparing results from different laboratories that use the same assay system, small differences in small parameters (*e.g.*, pH, buffer solutions) can cause considerable differences in COX kinetics (Cryer & Dubois, 1998). In addition, important species differences may exist among COX structures and they may account for the different inhibition of COX isozymes by NSAIDs; hence, it is recommended to use enzymes derived from the target species when attempting to predict efficacy and toleration (Ricketts *et al.* 1998; Cryer & Dubois, 1998).

In vitro assays, furthermore, do not entirely parallel the *in vivo* effect of a given drug. For example, the *in vitro* highly selective COX-2 inhibitor NS-398 showed only a slight difference between its oral (p.o.; *per os*) dose to selectively inhibit COX-2 (less than 6 mg/kg) and that for dual COX inhibition (10 mg/kg) in the ulcerated gastric mucosa of rats (Shigeta *et al.* 1998). Moreover, it was recently documented that COX-2 selective inhibitors significantly reduced inflammation only when given at doses that inhibited COX-1 too (Wallace, 1999; Wallace *et al.* 1999). It was proposed, in fact, that highly selective COX-2 inhibitors might not be more therapeutically effective than the existing NSAIDs (Wallace & Cirino, 1994).

2.3.2.2. Prostaglandins and the nociceptive process

Within the nociceptive pathway, PGs are very well known for their sensitising action on peripheral nociceptors (Ferreira, 1972; Appleton *et al.* 1996); however, their function(s) in the CNS is still debated and far from being well understood. They can be released from the spinal cord by activation of high-

threshold nociceptive afferent inputs (Ramwell *et al.* 1966), by direct administration of neurotransmitters (Chaplan *et al.* 1997; Malmberg & Yaksh, 1993; Dirig & Yaksh, 1999) or by peripheral inflammatory stimuli (Smith *et al.* 1998; Muth-Selbach *et al.* 1999). This suggests that a certain level of repetitive stimulation of the primary afferent is required for their release (Buritova *et al.* 1996), which agrees with the NMDA receptor-mediated generation of PGs (see above). Nonetheless, their generation also can be stimulated by co-activation of AMPA/kainate and mGluRs (Bezzi *et al.* 1998; Conn & Pin, 1997) which activation does not necessarily require repetitive stimulation. The origin of spinal prostanoids is not well established either, and release into the extracellular space may result not only from neuronal but also glial structures (Buritova *et al.* 1996; Willingale *et al.* 1997; Bezzi *et al.* 1998; Phillips & Pearce, 1999).

At the spinal level, these polyunsaturated fatty acids augment the synapting processing of pain information (Minami *et al.* 1997; Yaksh & Malmberg, 1993; Willingale *et al.* 1997; Minami *et al.* 1994b) by enhancing Ca^{2+}-dependent glutamate (Bezzi *et al.* 1998), SP (Malcangio *et al.* 1996) and CGRP (Andreeva & Rang, 1993). Furthermore, it has been suggested that spinal PGs may exert a presynaptic inhibition on noradrenaline release from the bulbospinal noradrenergic pathway (Taiwo & Levine, 1988). Thus, the PG-mediated neurotransmitter/neuropeptide release and noradrenaline release inhibition could be mechanisms for amplification of pain signals, and therefore induce a hyperalgesic state. In this regard, i.t. administration of PGs increase nociceptive responses in rodents (Minami *et al.* 1994a; Minami *et al.* 1994b; Minami *et al.* 1996; Eguchi *et al.* 1999); however, not all the PGs are pain-evoking, and some of them diminish pain sensitivity (Eisenach, 1993; Minami *et al.* 1997; Matsumura *et al.* 1993; Eguchi *et al.* 1999; Minami *et al.* 1996).

In addition to their presynaptic actions, these lipidic acids also may influence postsynaptic terminals. For example, it was shown that activation of the AA pathway is required for NMDA receptor-dependent induction of c-Fos protein in the spinal cord (Buritova *et al.* 1996), suggesting a signalling role for products of this pathway from the cell membrane/cytoplasm to the nucleus, where prostanoid receptors (see below) are present (Bhattacharya *et al.* 1998). In this

regard, $PGF_{2\alpha}$ is necessary, but not sufficient, for NMDA receptor-induced expression of c-Fos protein (Kaufmann *et al.* 1997).

Recently, PGD_2 and PGE_2 were identified as the predominant PGs in the normal rat spinal cord, while 6-keto-$PGF_{1\alpha}$, the stable metabolite of PGI_2, and $PGF_{2\alpha}$ were detected in minor concentration (Willingale *et al.* 1997). Most prostanoids, to some extent, have been investigated for their effects on the pain signalling process, but special interest has been put on the four PGs mentioned above. PGE_2, the major studied prostanoid, partially because is released from the spinal cord in response to noxious stimuli (Dirig & Yaksh, 1999) and because it can also release neurotransmitters (Bezzi *et al.* 1998) and neuropeptides (Yamamoto & Nozaki-Taguchi, 1996; Yaksh & Malmberg, 1993), has been adopted as the major PG involved in hyperalgesia. Nevertheless, when a comparison was made between the effect of PGI_2, PGE_2, and some analogues on sensory neurones, PGI_2 analogues were equally or more effective than those of PGE_2 as hyperalgesic or sensitising agents (Bley *et al.* 1998; Smith *et al.* 1998). However, this was not true for the paintbrush test in mice where the PGI_2 analogue cicaprost produced a smaller allodynic effect than various PGE_2 analogues (Minami *et al.* 1994b). Together, these data suggest that allodynia and hyperalgesia may be mediated by action of different PGs.

Interestingly, the spinal glutamatergic system has been implicated in both PGE_2- and $PGF_{2\alpha}$-evoked allodynia but subtypes of glutamate receptors involved may differ between them. Allodynia, assessed by the paintbrush test, was evoked by i.t. injection of PGE_2 or $PGF_{2\alpha}$ in conscious mice. The NMDA receptor antagonists MK-801 and D-AP-5 blocked PGE_2- but not $PGF_{2\alpha}$-induced allodynia; the non-NMDA receptor antagonists GAMS and CNQX inhibited both PGE_2- and $PGF_{2\alpha}$-induced allodynia; and L-AP-3 and L-AP-4, mGluR antagonists, reduced $PGF_{2\alpha}$- but not PGE_2-induced allodynia (Minami *et al.* 1994a). Therefore, it may be feasible that PGE_2-iduced allodynia is mediated by co-activation of AMPA and NMDA receptors, whereas $PGF_{2\alpha}$-induced allodynia is due to co-activation of AMPA and mGluRs. In addition, the NO pathway may be involved in the PGE_2-induced allodynia because PGE_2

stimulated NO release from the spinal cord by activation of NMDA receptors; therefore, NO may be implicated in allodynic responses induced by PGE_2 (Sakai *et al.* 1998). Even more, recent studies on lipocalin-type PGD synthase (L-PGDS; responsible enzyme for the conversion of PGH_2 to PGD_2 in the CNS) knockout mice (Eguchi *et al.* 1999) showed a possible spinal interaction between PGD_2 and PGE_2 through the $GABA_A$ receptor on the PGE_2-evoked allodynia. This conclusion was driven from the fact that i.t. injection of PGE_2 evoked allodynia in wild-type mice but not in L-PGDS deficient mice and i.t. co-administration of PGD_2 or BW 245C, a PGD receptor (DP) agonist, plus PGE_2 or bicuculline ($GABA_A$ antagonist) evoked allodynia in L-PGDS$^{-/-}$ mice similarly to that observed in wild-type mice by PGE_2 and bicuculline alone. Furthermore, the DP antagonist BW A868C reversed the PGD_2 plus PGE_2- or bicuculline-induced allodynia in L-PGDS$^{-/-}$ mice, as well as that evoked by PGE_2 or bicuculline in wild-type mice. This interaction between PGD_2 and PGE_2 at the $GABA_A$ receptor level seems to be PG-specific because the i.t. injection of $PGF_{2\alpha}$ induced allodynia in both wild-type mice and L-PGDS deficient mice (Eguchi *et al.* 1999), suggesting that its allodynic effect does not depend on PGD_2.

Contrary to the contribution of PGD_2 to evoke allodynic responses, there are reports suggesting that this PG, by acting on DP receptors, might play an inhibitory role in the appearance of this pathologic process. For instance the i.t. injection of PGD_2 in mice blocked both nociceptin- and PGE_2-induced allodynia (Minami *et al.* 1997; Minami *et al.* 1996) whereas in rat spinal cord slices, PGD_2 inhibited the PGE_2-evoked NO release (Sakai *et al.* 1998).

Thus, our current knowledge on the role of spinal PGs in nociception is largely scattered and the interaction of these lipidic acids with diverse nociceptive systems seems to be quite complicated and is still poorly understood.

2.3.2.3. Prostanoid receptors

Although our knowledge about the PG-mediated mechanisms of nociception is scarce, some incursions into understanding the role of prostanoid receptors in this area have been made. This section aims to overview the current knowledge about these receptors and their involvement within the nociceptive pathway. For those interested in extra information about prostanoid receptors the review from Coleman *et al.* (1994) is recommended.

As mentioned above, prostanoids act as extracellular and intracellular messengers. After being synthesised, they are released immediately acting, on cell membrane receptors to elicit their actions (Oida *et al.* 1995; Vane *et al.* 1998) (Table 2.5). It is known that prostanoid receptor-mediated sensitisation of sensory nerve fibres is a key contributor to the generation of hyperalgesia (Bley *et al.* 1998; Smith *et al.* 1998), probably by facilitating neurotransmitter/neuropeptide release from the primary afferent (Geppetti *et al.* 1991; Malcangio *et al.* 1996; Buritova *et al.* 1996; Yaksh & Malmberg, 1993; Andreeva & Rang, 1993; Bezzi *et al.* 1998). Indications for an intracellular function of prostanoids have been strengthened by the demonstration that PGD_2, PGJ_2, and PGI_2 can activate the nuclear peroxisome proliferator-activated receptors and, thus, alter transcription factors (Vane *et al.* 1998; Bhattacharya *et al.* 1998). Interestingly, increased c-Fos protein expression through some AA pathway elements signalling from the cell membrane/cytoplasm to the nucleus, where the four different PGE_2 receptor subtypes (EP_1-EP_4) were recently demonstrated (Bhattacharya *et al.* 1998), has been suggested (Kaufmann *et al.* 1997; Buritova *et al.* 1996). In fact, activation of the EP_1 nuclear receptor with the EP_1 agonist 17-phenyltrinor PGE_2 increased $[Ca^{2+}]_i$ concentration and *c-fos* transcription (Bhattacharya *et al.* 1998). However, to date the knowledge about these intracellular receptors is small.

The diversity of actions of PGE_2 is ascribed to PGE receptor subtypes EP_1, EP_2, EP_3 and EP_4 coupled to different signal transduction pathways. While the EP_1

Table 2.5. Properties and distribution of prostaglandin receptors.

Receptor / Subtype	Distribution	Biological Functions	Transduction System	Observations / Notes
DP	Platelets; vascular, gastrointestinal, uterine, and airway smooth muscles. Nervous tissue including CNS.	Inhibitory responses. Excitatory and inhibitory events.	↑ cAMP via G_s.	Distribution highly species specific. Rich population in human platelets, and few in lab animals if any; in uterine smooth muscle appear to be confined to humans. In afferent sensory nerves they can induce hyperalgesia
EP		Contraction and relaxation of smooth muscle; immunoregulation; inhibition and enhancement of neurotransmitter release and non acid (water) secretion; inhibition of lipolysis, gastric acid secretion, inflammatory mediator release, immunoglobulin expression; etc.		
EP$_1$	Smooth muscle of trachea, GIT, uterus and bladder.		↑ Intracellular Ca^{2+} via an IP_3-independent mechanism.	Although are more sparse in non-rodent species, they do exist in superior species including humans.
EP$_2$	Smooth muscle. Epithelial cells. Inflammatory cells (mast cells and macrophages). Sensory afferent nerves.	Relaxation. Non-acid secretion. Inhibition of mediator release. Activation.	↑ cAMP via G_s.	
EP$_3$	GI, uterine and vascular smooth muscle. Autonomic nerves. Adipocytes. Gastric mucosal cells. Renal medulla.	Contraction. Inhibition of neurotransmitter release. Inhibition of lipolysis. Inhibition of acid secretion. Inhibition of water reabsorption.	↓ cAMP via G_i. ↑ PI turnover via G_q.	Development of EP$_3$ receptor agonist as gastric antisecretory agents. In nervous tissue the mRNA is expressed only in neurones and highly expressed in dorsal root ganglion and brain regions such as hippocampus, preoptic area, hypothalamus, maxillary body, locus coerulus, and raphe nuclei.
EP$_4$	Piglet saphenous vein; rabbit jugular and saphenous veins; rat trachea; hamster uterus.		↑ cAMP via G_s?	

Table 2.5. Properties and distribution of prostaglandin receptors. (Cont.)

Receptor / Subtype	Distribution	Biological Functions	Transduction System	Observations / Notes
FP	Corpus luteum. Rodents myometrium. Cat and dog iris sphincter and airway smooth muscle.	Luteolysis. Contraction. Contraction. Contraction.	$\uparrow$ PI turnover via G_q.	$PGF_{2\alpha}$ and analogues serve to synchronise the oestrus cycles of farm animals. They are also effective in lowering intraocular pressure in various species, including human, and are used to treat glaucoma. In dogs and cats FP agonists are lethal.
IP	Platelets, arterial smooth muscles. Sensory afferent nerves.	Inhibition of arterial tone and platelet aggregation. Excitation.	$\uparrow$ cAMP via G_s. $\uparrow$ PI turnover.	There is no convincing evidence that IP receptors can be subclassified; the only differences that exist probably result from species variants rather than from true subtypes.
TP	Vascular and airway smooth muscles and platelets. Myofibroblasts. Mesangial cells in the kidney. Epithelium of GIT.	Excitation. Wound healing and scar formation. Increase in filtration rate. Increase in secretion.	$\uparrow$ PI turnover via G_q.	They appear to regulate the closure of umbilical vessels at birth, and thymocyte differentiation and development. There is controversy regarding different subtypes.

cAMP = cyclic AMP; PI = phosphatidylinositol; G_s = stimulatory G-protein; G_i = inhibitory G-protein; G_q = pertussis toxin-insensitive G-protein.
Elaborated with information from Coleman *et al.* 1994.

receptor is coupled to Ca^{2+} mobilisation, EP_2 and EP_4 receptors stimulate adenylate cyclase and the EP_3 receptor inhibits this enzyme (Coleman *et al.* 1994). Using different approaches (*e.g.,* radio-labelled markers, hybridisation studies and pharmacological screening), EP receptors have been identified in diverse areas of the CNS. For example, binding sites for [^{3}H]iloprost, an EP_1 selective agonist, were observed in DRG, the spinal cord dorsal horn, nodose ganglia, and the nucleus tractus solitarius of rats (Bley *et al.* 1998), and EP_1, EP_3 and EP_4 receptor mRNAs were expressed in about 30%, 50% and 20%, respectively, of mice DRG neurones (Oida *et al.* 1995). These findings match with the spinal EP receptor system being involved in pain transmission on high and low threshold afferents in interaction with neurotransmitters and neuropeptides. In this regard, allodynia induced by i.t. administered PGE_2 in mice was attributed to stimulation of the EP_1 receptor and PGE_2-evoked hyperalgesia through EP_2 and EP_3 receptors (at high and low doses of PGE_2, respectively) (Minami *et al.* 1994b). Furthermore, activation of the EP_1 receptor by PGE_2 enhanced NO release, via the NMDA receptor-nNOS pathway, from rat spinal cord slices (Sakai *et al.* 1998), suggesting that PGE_2 may stimulate nNOS and thus release NO following NMDA receptor activation via EP_1 receptors and, thus, evoke allodynic responses. However, EP_1-mediated allodynia may need the presence of PGD_2 because L-PGDS deficient mice did not show PGE_2-induced allodynia whereas wild type mice did (Eguchi *et al.* 1999).

The presence of DP receptor mRNA transcripts has been demonstrated, by *in situ* hybridisation reactions, in the periphery of the rat brain (leptomeninges and choroid plexus) and in both dorsal and ventral horns of the lumbar, but not cervical or thoracic, spinal cord (Wright *et al.* 1999). Contrary to the need of PGD_2 for the development of PGE_2-induced allodynia (Eguchi *et al.* 1999) is the inhibition of the allodynic response by PGD_2, which was reversed by the DP receptor antagonist BW A868C (Minami *et al.* 1996). Therefore, it seems that activation of DP receptors may either participate in the development of or reversal of PGE_2-induced allodynia. Even more, the solely blockage of spinal DP receptors with BW A868C in mice produced allodynia (Minami *et al.* 1996).

Together, these results suggest important roles for EP and DP receptor systems in nociception as well as complex interactions between them and other nociceptive systems.

Prostacyclin receptors may also play a crucial role in nociception and, although they were very little labelled in the mouse spinal cord, 40% of DRG neurones expressed IP receptor mRNA. Both small- and large-sized cell bodies, but not glial structures, were labelled. Furthermore, expression of preprotachykinin A (a SP precursor) mRNA was noted in 30% of total DRG neurones and 70% of these neurones co-expressed IP receptor mRNA, which corresponded to C and $A\delta$ fibres (Oida et $al.$ 1995). Hence, it is likely that IP receptors contribute to nociception by facilitating SP release from the afferent terminals. However, other sensory mechanisms may also be modulated by these prostanoid receptors because half of the IP receptor mRNA-positive DRG neurones did not express preprotachykinin A mRNA, and 25%, 41% and 34% of the IP receptor mRNA-positive DRG neurones co-expressed EP_1, EP_3 and EP_4 receptor mRNAs, respectively (Oida et $al.$ 1995). Whatever the mechanism by which IP receptors modulated nociception, it was biochemically [cAMP and $Ins(1,4,5)P3$ accumulation in cultured sensory neurones] and electrophysiologically (isolated vagus nerve preparation) demonstrated that responses to IP receptor agonists [cicaprost and carbaprostacyclin ($cPGI_2$)] were greater than those of other endogenous prostanoids (PGE_1, PGE_2, PGD_2, TxB_2, $PGF_{2\alpha}$) (Smith et $al.$ 1998). Interestingly, in the vagus nerve preparation, sustained applications of either $cPGI_2$ or PGI_2 caused receptor desensitisation. Furthermore, pre-treatment with $cPGI_2$ and PGI_2 attenuated the response to a subsequent application of PGE_2, possibly by depolarising actions of these three agents through action at a single receptor, because high K^+-evoked nerve depolarisation after PGI_2 or $cPGI_2$ pre-treatment still occurred. However, the possibility that PGI_2 and $cPGI_2$ had caused heterologous receptor desensitisation was not excluded (Smith et $al.$ 1998). Together, these results suggest that IP receptor activation may modulate the release of neuropeptides from afferent terminals by a process that involve the adenylate cyclase and PLC

pathways, and that some interaction or cross-talk between IP and EP receptors may also exist.

Supporting evidence to the role of prostacyclin receptors in nociception comes from the reduced response of IP receptor knockout mice to the acetic acid-induced writhing test to a similar level as that observed in indomethacin-treated wild-type mice (Murata *et al.* 1997). However, latency in the tail-flick and hot-plate tests was comparable between wild-type mice and mice lacking the prostacyclin receptor, suggesting that IP receptors might not be involved in nociceptive transmission at the spinal and supraspinal levels (Murata *et al.* 1997).

2.4. CENTRAL ANALGESIC EFFECTS OF NONSTEROIDAL ANTI-INFLAMMATORY DRUGS

The NSAIDs have been commercially available for over one century, but their use in veterinary clinical practice became more popular from the 50's with the introduction of phenylbutazone. However, the mechanism of action of these drugs remained unknown until 1971, when Vane (1971) demonstrated that they work by inhibiting prostanoid production. Actually, in spite of their structural diversity, it is accepted that all NSAIDs inhibit COX to a greater or lesser extent and has been assumed that this action causes analgesia by preventing the sensitisation of peripheral nerve endings by PGs, particularly PGE_2 and PGI_2 (Ferreira, 1972). In fact, neutralisation of PGE_2 with a monoclonal antibody (2B5) prevented and reduced inflammatory hyperalgesia in rats as effectively as indomethacin or ketorolac (Portanova *et al.* 1996; Zhang *et al.* 1997). Thus, since antibodies do not cross the blood-brain barrier, it seems that peripherally derived PGs are essential for establishing and sustaining the hyperalgesic state.

The above information implies that the NSAIDs' analgesic effects are peripherally mediated and that their mechanism of action is by inhibiting PG synthesis. However, several reports (Bustamante *et al.* 1997; Malmberg &

Yaksh, 1992a; Malmberg & Yaksh, 1992b; Malmberg & Yaksh, 1993; Chambers *et al.* 1995; Pelissier *et al.* 1996; Brune *et al.* 1992; Björkman *et al.* 1996; Willingale *et al.* 1997; Piletta *et al.* 1991; Devoghel, 1983) attribute a central analgesic effect to these agents, even when inflammation is absent (Bustamante *et al.* 1997; Chambers *et al.* 1995; Pelissier *et al.* 1996; Piletta *et al.* 1991). In fact, dissociation between the analgesic and anti-inflammatory effects of NSAIDs has been suggested (McCormack, 1994a). Nevertheless, to date, it is not fully understood if the central analgesic effects are achieved by inhibition of prostanoid production, or if NSAIDs interact with nociceptor neurones in other ways.

2.4.1 Possible central antinociceptive mechanisms of action for NSAIDs

For a drug to produce analgesia at the central level this has to be present in the neural tissue in high enough concentration to modulate the nociceptive process. Thus, the drug can be administered directly in the cerebral spinal fluid (CSF) or onto/into the CNS or, if it was systemically administered, it will have to cross the blood-brain barrier. This barrier regulates the CNS environment's homeostasis and the diffusion of compounds across the plasma membranes of the endothelial cells of this barrier depends on the physicochemical properties of these compounds, such as lipid solubility, molecular weight, electrical charge and extent of ionisation (Vries *et al.* 1997). In this regard, some NSAIDs and their metabolites have the capacity to cross this barrier (Muth-Selbach *et al.* 1999; Edwards *et al.* 1999).

The central analgesic mechanisms of action for the NSAIDs range from the classic COX inhibition to disruption of G-protein signalling and interaction with cell and nucleus membrane receptors. These mechanisms, and others, have been reviewed elsewhere (Yaksh & Malmberg, 1993; McCormack, 1994a; McCormack, 1994b).

2.4.1.1. NSAIDs and prostanoid synthesis inhibition

Spinal prostanoid synthesis inhibition has been postulated as NSAIDs' major analgesic mechanism of action. Support to this hypothesis comes from the antinociception observed after i.t. administration of various NSAIDs in rats submitted to the paw formalin test and, according to the authors, the analgesic potency of these drugs matched with COX inhibition values (Malmberg & Yaksh, 1992b). Furthermore, the sole inhibition of PGE_2 seems to be sufficient to mediate their spinal antinociceptive effects since i.t. injection of the EP receptor antagonists SC-5108 and SC-51234A produced analgesia in rats submitted to the same nociceptive test (Malmberg et al. 1994). Nevertheless, oral dipyrone administered to patients suffering from neurological diseases did not decrease PGE_2 levels in the CSF as compared to that in patients suffering from neurological diseases not receiving the NSAID; however, reduction of TxB_2 was observed (Levy et al. 1998).

In addition to those three reports, there is the suggestion that paracetamol's analgesic effects are due to selective PG synthesis inhibition in the CNS (Flower & Vane, 1972), which is supported by the PGE_2 spinal inhibition, but lack of effect on PGE_2 $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ urinary excretion levels, after i.p. administration of paracetamol in rats at the same time that produced antinociception in the formalin test (Muth-Selbach et al. 1999). However, failure to demonstrate tissue specific differences in COX sensitivity to paracetamol and other NSAIDs is also reported (McCormack, 1994a). Even more, a recent study demonstrated that inhibition of central COX might not be involved in the antinociceptive effects of paracetamol (Pelissier et al. 1996), suggesting that NSAIDs' analgesic effects are mediated by additional mechanisms to central prostanoid synthesis inhibition.

To try to clarify if NSAIDs-mediated analgesia is due to inhibition of prostanoid production or not, anti-inflammatory steroids have been used for comparison. Because anti-inflammatory steroids indirectly inhibit PLA_2 and, therefore, prostanoid synthesis in an earlier step within the AA pathway (Figure 2.3), it could be reasoned that these drugs might produce similar analgesia to that

observed with NSAIDs. By comparing the antinociceptive effect of i.t. injected NSAIDs (Malmberg & Yaksh, 1992a; Malmberg & Yaksh, 1992b; Malmberg & Yaksh, 1993) and anti-inflammatory steroids (Abram *et al.* 1992) in the formalin test in rats, it is clear that a single injection of a NSAID reduced the nociceptive behaviour observed in the second phase of this test, whereas chronic administration of the anti-inflammatory steroid triamcinolone was required to produce similar analgesia to that of the NSAIDs. Even more, the single i.t. injection of another anti-inflammatory steroid, methylprednisolone, produced allodynia (Abram *et al.* 1992). Although the analgesic effect of both NSAIDs and anti-inflammatory steroids was quite different, the authors attributed it in both cases to changes on spinal prostanoid levels. However, Chambers *et al.* (1993; 1995) demonstrated no increase on mechanicical nociceptive thresholds of sheep when the anti-inflammatory steroid dexamethasone was intravenously (i.v.) administered, whilst flunixin or dipyrone, both given i.v. too, did increase them. Therefore, comparison of the analgesic action of NSAIDs and anti-inflammatory steroids suggests that the former compounds are highly likely to have central antinociceptive mechanisms of action other than or additional to prostanoid synthesis inhibition.

Interestingly, an almost complete dissociation between analgesic efficacy and potency to inhibit COX has been evidenced for some NSAIDs (McCormack, 1994a; McCormack, 1994b). For instance, the central analgesic effects of R(-)-flurbiprofen [does not inhibit PG synthesis and does not undergo significant chiral inversion to S(+)-enantiomer in rats and humans] and S(+)-flurbiprofen (inhibits PG synthesis) were almost equal in various algesiometric models in rats (Brune *et al.* 1992; Geisslinger & Schaible, 1996; Neugebauer *et al.* 1995a) suggesting an additional mode of action other than prostanoid synthesis inhibition for the antinociceptive effects of some NSAIDs.

2.4.1.2. NSAIDs and glutamate receptors

Because NSAIDs blocked the hyperalgesia elicited by i.t. injection of NMDA, AMPA or SP (Malmberg & Yaksh, 1992a; Yaksh & Malmberg, 1993; Buritova *et*

al. 1996; Björkman *et al.* 1996; Kakinohana *et al.* 1997; Sorkin, 1997; Dolan & Nolan, 1999), the spinal antinociceptive mechanism of these drugs may involve glutamatergic synapses, where the AA pathway plays an important role and the possible actions of the NSAIDs on this cascade were already discussed. The NO pathway is another important biochemical pathway modulated by glutamate receptors and because its interaction with the AA pathway was previously discussed in this work, here will only be exposed the potential analgesic mechanisms of NSAIDs related to this signal transduction pathway.

Interaction of NSAIDs with the NO pathway could also represent a possible antinociceptive mode of action for these drugs; however, this interaction is far too complex since stimulation and inhibition of NO production seems to regulate NSAID-induced analgesia. Thus, diclofenac and S(+)- but not R(-)-ibuprofen, inhibited the spinal NMDA-induced behavioural response in rats and pre-treatment with L-arginine, but not D-arginine, antagonised their analgesic effect (Björkman *et al.* 1996). Whereas i.t. administered dipyrone attenuated the rat paw PGE_2-induced hyperalgesia, and antinociception was abolished by pre-treating the paws with L-NMMA (a NOS inhibitor) or methylene blue (an inhibitor of soluble guanylate cyclase) (Lorenzetti & Ferreira, 1996).

Reduced NO production by NSAIDs has been observed in both *in vivo* and *in vitro* studies. Ibuprofen reduced NO production, as measured by alveolar NO flow rate and urinary excretion of nitrite and nitrate, in both normal and endotoxin challenged human volunteers by probably diminishing NOS activity (Vandivier *et al.* 1999). In fact, *in vitro* inhibition of both nNOS activity and iNOS induction has been demonstrated for ketoprofen, but not paracetamol (Schubert *et al.* 1999). Interestingly, modulation of NO transport and delivery has been postulated as possible mechanisms of action for indometacin and indoprofen. These drugs, but not acetylsalicylic acid and diclofenac, reduced the NO donor-increased production of cGMP in human whole platelets by an independent inhibitory mechanism on the COX pathway neither by interference with nitrite production (Failli *et al.* 1998). Nevertheless, the relevance of these *in vitro* findings in the nociceptive process, or any other biological process, is unknown

because inhibition was only observed with very high drug concentrations, within the mM range.

Another possible mode of analgesic action for the NSAIDs is interference on glutamate and/or other EAAs metabolism, which would rebound on reduced activation of glutamate receptors. According to McCormack (1994a; 1994b) some NSAIDs might compete with palmitic acid for the long-chain acyl-CoA synthase and, thus, inhibit β-oxidation of palmitic acid. This would lead to the lack of palmitoyl-CoA for regulation of the enzyme glutaminase and, therefore, produce analgesia through reducing levels of the EAA glutamate. On the other hand, increased levels of the endogenous broad-spectrum EEA antagonist kyanurenate may contribute also to the central antinociceptice effects of NSAIDs. Kyanurenate concentrations were increased in the rat spinal cord and diencephalon after systemic diclofenac administration (McCormack, 1994a) and it is believed that this EEA antagonist attenuates NMDA receptor-mediated activity by modulating the glycine site on this receptor (McCormack, 1994a; Edwards et al. 1999). Although kyanurenate is synthesised in the CNS by action of the enzyme indolamine 2,3-dioxygenase, its increased presence in the CNS after NSAID treatment might be due to rapid uptake of its biosynthetic precursor kynunerine, which is produced by the action of the enzyme tryptophan 2,3-dioxygenase in the liver (McCormack, 1994a; Edwards et al. 1999).

2.4.1.3. NSAIDs and descending inhibitory mechanisms

Supraspinal mechanisms where NSAIDs may exert descending inhibitory influences on the spinal transmission of nociceptive inputs have also been proposed. These effects are probably mediated by activation of on- and off-cells of the RVM, which receive an input from the PAG and project to the spinal dorsal horn, facilitating and depressing the spinal nociceptive transmission, respectively (Tortorici et al. 1996). Thus, PAG-micoinjection of NSAIDs may depress on-cell activity, facilitate off-cell activity and, therefore, depress excitation of spinal sensory neurones (Vanegas et al. 1997; Tortorici et al. 1996). In this regard, descending opioidergic (Chambers et al. 1993; Chambers

et al. 1995; Akman *et al.* 1996), noradrenergic (Chambers *et al.* 1992; Chambers, 1995), serotoninergic (Pelissier *et al.* 1996; Björkman *et al.* 1996) and muscarinic cholinergic (Stets & Slivko, 1989) inhibitory mechanisms have been implicated in NSAIDs-mediated analgesia.

Nevertheless, indications against a supraspinal antinociceptive action for the NSAIDs come from the lack of analgesia in the rat paw PGE_2-induced hyperalgesia test (Lorenzetti & Ferreira, 1996) and the failure to depress a C fibre reflex in anaesthetised rats (Bustamante *et al.* 1997) by intracerebroventricular (i.c.v.) administered NSAIDs.

2.4.1.4. NSAIDs and other receptor systems and signal transduction pathways

A wide variety of receptors and biochemical pathways, other than glutamate receptors and AA and NO pathways, have been related to NSAIDs-mediated analgesia. For example, evidence for the interference of NSAIDs on G-protein-dependent functions has been reviewed (McCormack, 1994a; McCormack, 1994b), where intercalation of NSAIDs into the plasma membrane with resultant changes in viscosity and alteration of α-subunit configuration and the PLC/Ins(1,4,5)P3 cascade are proposed as possible analgesic mechanisms for some of these drugs.

The possibility that NSAIDs exert their analgesic action, at least in part, by an inhibitory action via vanilloid receptors has been raised due to the inhibition of capsaicin-induced current in rat DRG neurones (Buritova & Besson, 1998) and attenuation of capsaicin-induced pain and allodynia in human volunteers (Szallasi & Blumberg, 1999) by NSAIDs. Similarly, inhibition of the cAMP second messenger system, which is involved in the modulation of voltage- and ligand-gated ion channels in sensory neurones (Bley *et al.* 1998), by a mechanism unrelated to COX inhibition was suggested for indomethacin-mediated analgesia (Yamamoto & Nozaki-Taguchi, 1996). However, those both inhibitory actions, vanilloid receptors and cAMP cascade, could still be mediated

through direct PG synthesis inhibition, because some of these lipidic acids stimulate the cAMP/PKA cascade and phosphorylation of the capsaicin receptor by PKA increases its conductance or open probability (Bley *et al.* 1998; Kress & Zeilhofer, 1999).

Blockade of L- and T-type Ca^{2+} currents by the COX-2 selective inhibitor nimesulide was observed in cultured human myometrial myocytes (Knock & Aaronson, 1999); if this is also true in neuronal tissue, this mechanism might reduce neurone depolarisation and represent a potential analgesic mode of action for NSAIDs. On the other hand, neurone hyperpolarisation through an outward K^+ conductance has been ascribed to the antinociceptive action of some NSAIDs (Geisslinger & Schaible, 1996). Together, these results suggest that NSAIDs could produce analgesia by interacting, directly or indirectly, with ion channels. Interestingly, direct opposite effects of mefenamic acid on different types of β human recombinant $GABA_A$ receptor subunits have recently been identified. Interaction of the NSAID with $\beta_{2/3}$ subunits produced excitatory responses and with β_1 either zero or inhibitory responses (Halliwell *et al.* 1998). Thus, it seems that NSAIDs could produce analgesia by direct activation of $GABA_A$ receptors; however, this could also be linked to some side effects (*e.g.*, convulsions and coma) observed with these drugs.

2.4.1.5. NSAIDs and interference with nuclear related events

The NSAIDs have been shown to down- or up-regulate the level of diverse transcription factors, enzymes and even receptors that could be involved in the nociceptive process. Interference of these processes, which take place at the nuclear level, may represent very important mechanisms of analgesia, because long-lasting neuronal responses may depend on them; however, they are poorly understood. Thus, as mentioned above, NSAIDs are able to decrease c-Fos protein expression in spinal cord neurones (Buritova *et al.* 1996; Buritova & Besson, 1998) and this transcription factor, although its function within the nociceptive process is not known, is used as an indirect marker of neurones

involved in spinal nociceptive processes (Buritova & Besson, 1998). Another transcription factor inhibited by acetylsalicylic acid and salicylic acid is NFκB (Xu *et al.* 1999; Cronstein *et al.* 1999). These two salicylates, but not indomethacin, also inhibit the transcriptor factor Erk, which activation was reduced in neutrophils, but only at the mM range concentration of the drugs, by a COX-independent inhibitory mechanism upstream of Erk (Pillinger *et al.* 1998). If this effect by salicylates also occurs in neurones, nNOS could be involved in this process. Inhibition or depletion of nNOS, and therefore NO diminished production, in mice primary cortical neurones prevented activation of the Ras pathway and thereby the transcriptor factor Erk by an independent action in cGMP increase (Yun *et al.* 1998). Thus, acetylsalicylic acid and salicylic acid could inhibit nNOS and this could be a possible analgesic mode of action for these drugs; in fact inhibition of nNOS has been demonstrated for high-dose ketoprofen (Schubert *et al.* 1999).

Further to the information mentioned above about acetylsalicylic acid and salicylic acid, these salicylates, but not indomethacin nor the COX-2 selective inhibitor NS398, reduced *in vitro* and *in vivo* both COX-2 mRNA and protein expression (Xu *et al.* 1999). Regarding receptor levels, in human mononuclear cells, acetylsalicylic acid increased both *ex vivo* and *in vitro* secretion of soluble IL-1 type II receptor, which is believed not to transduce signals (Daun *et al.* 1999). Because IL-1β is present in the CNS (Schneider *et al.* 1998), enhances SP release from the spinal cord (Malcangio *et al.* 1996) and NO and PGE$_2$ from astroglial cells (Mollace *et al.* 1998), and is involved in the processing of noxious inputs in the spinal cord dorsal horn (Reeve *et al.* 1998), it follows that inactivation of the cytokine by coupling to the soluble IL-1 type II receptor may contribute to the analgesic effects of acetylsalicylic acid, only if the same increment in receptor level happens in neuronal tissue.

Thus, interference with diverse biochemical functions in glutamatergic synapses and non-neuronal structures, interaction with natural occurring inhibitory systems as well as modulation of other receptors, transduction signal pathways and even nuclear events related to nociceptive signals represent a potential explanation for the central analgesic effects of NSAIDs. However, the current

knowledge on their actions at the molecular level is incomplete and, therefore, far to be well understood.

2.5. INDUCTION AND ASSESSMENT OF PAIN

As mentioned before, pain consists of both sensory and emotional components and the physiological changes associated with the former component can be measured, but the emotional component is not so easy quantified, even in humans, who can verbally describe their experience. In non-verbal beings, however, it is not possible to know their feelings and therefore not possible to measure pain directly on them (Danneman, 1997). Of course, this does not mean that animals experience no emotional response to the sensation, it is highly likely that they do, but we cannot evaluate it. Nevertheless, because mammal animals have similar nociceptive pathways, alike pain thresholds, and exhibit the same motor behaviours and physiological responses to noxious stimuli as humans (Danneman, 1997), as well as respond in a similar way to analgesic drugs, it is possible to infer that they have the same capacity to feel pain as we humans do. Hence, it is assumed that stimuli are noxious and strong enough to give rise to the perception of pain in animals if stimuli are either detected as pain by humans, at least approach tissue damage proportions, and produce escape behaviour in animals (Kitchell & Erickson, 1983).

Most nociceptive tests, in fact, measure a behavioural response as an indicator that the stimulus was perceived as painful, and this would correspond to the pain threshold (see Section 1 of this Chapter). The next sections overview some of these tests, but for those wishing a more profound description on these and other assays, the works by Danneman (1997) and Dubner (1989), and references therein are recommended.

2.5.1. Animal models of acute pain

The pain involved in these tests is generally brief, mild and escapable. These tests measure simple reflex responses or more complex unlearned or learned behaviours. Considered as simple reflex tests are the tail flick test and the jaw-opening reflex. The former test is based on withdrawal of the animal's tail in response to cutaneous thermal stimulation, either by focussing a beam of intense light to the tail or dipping it into hot water (Vanegas et al. 1997; Tortorici et al. 1996; Gray et al. 1999; Gomeza et al. 1999; Murata et al. 1997). The later model measures the reflex response (e.g., licking, chewing, head jerk, dropping of the lower jaw) to electrical stimulation of the tooth pulp, passed trough electrodes implanted in the dentin of the incisor or canine tooth. Electromyographic activity of the digastricus muscle of the lower jaw and cortical evoked potentials are additionally recorded to monitor the response (Danneman, 1997). In the tail flick test an increase or decrease in nociception is indicated by a shorter or longer latency to observe the response, whereas in the jaw-opening reflex by a higher or smaller level of stimulation. Both assays have safety cut-off points, maximum amount of time for the tail flick test and maximum level of stimulation for the jaw-opening reflex are set.

Models that involve more complex unlearned behaviours, which reflect supraspinal processing and, presumably, pain perception, include the hot plate and the paw pressure tests. In the hot plate test, mice or rats are placed on a heated (50-56°C) metal surface and the latency for the animal to show the first avoidance response (e.g., jumping, licking of one hind paw, or rapidly stamping the paws) is measured (Moore et al. 1991; Minami et al. 1994b; Minami et al. 1996; Nishiyama et al. 1999; Gomeza et al. 1999; al-Swayeh et al. 2000). In the paw pressure test one of the animal's hind paw is placed on a plinth and a piston pressures, in a linear increased constant rate, onto its dorsal aspect until the animal vocalises or tries to escape, pressure is recorded at this point (Pelissier et al. 1996; Machelska et al. 1997; Kawabata et al. 1992). A similar mechanical nociceptive test has been developed for larger species (Nolan et al. 1987a; Chambers et al. 1990; Chambers, 1992) In these tests, safety cut-off

points, maximum amount of time and pressure, respectively, in the absence of a response are pre-determined to avoid major tissue damage.

In some experiments, animals are trained to change their behaviour to indicate recognition of or reluctance to tolerate noxious stimulation. For example, animals are thought to perform a task to terminate or prevent a noxious stimulus, which correspond to learned escape or learned avoidance paradigms, respectively. Both these tests measure the length of time in which the animal detects the noxious stimulus. More sophisticated paradigms, conflict paradigms, require an animal to choose what level of nociception it will tolerate in order to receive a reward, and at some stage of stimulation it will refuse to work for the reward. Discrimination paradigms are highly complex and animals, generally non-human primates trained during months, discriminate between different stimuli, at least one of them is within the noxious range. These last two paradigms measure the strength of the response as an indicator of the degree of aversiveness to the stimulus (Danneman, 1997; Dubner, 1989).

2.5.2. Animal models of chronic pain

Contrary to acute pain models, chronic pain tests cause inescapable pain that may last up to several weeks and is always accompanied with obvious tissue damage. This tissue injury is generally directed to specific areas within the organism, giving us the opportunity to investigate chronic pain associated with different conditions. Thus, inflammatory, arthritic, visceral and neurophatic models of chronic pain have been developed. Models of inflammatory pain and hyperalgesia, such as the formalin and the carrageenan tests, are widely used. In the former, formalin is injected in the hind paw of rats or mice and two behavioural phases are observed. The first one, denominated phase 1, is thought to correspond to acute pain, stars immediately after the formalin injection and lasts about 5 minutes. Phase 2, which is believed to represent chronic pain, begins after 10 minutes, peaks at around 25 to 35 minutes and diminishes at about 45 to 60 minutes (Malmberg & Yaksh, 1992b; Coderre et al. 1990). The carrageenan test involves the s.c. administration of carrageenan into

the hind paw, developing inflammation whit localised hyperthermia and oedema in less than 3 hours (Portanova *et al.* 1996; Zhang *et al.* 1997; Traub, 1996; Ochi *et al.* 1999). Alternatively, injection of capsaicin in the hind paw has also been used (Sakurada *et al.* 1996). Different scoring systems and methodologies are used to evaluate nociception in these tests.

Arthritis is a common entity that always is accompanied by chronic pain, and for which different experimental models exist. Systemic administration of Freund's complete adjuvant in rats produces, after 10 to 21 days, polyarthritis accompanied of inflammation, hyperalgesia, and destruction of bone and cartilage of the affected joints. The animals show spontaneous behaviours suggesting pain (*e.g.,* licking and biting the affected limbs, decrease ambulatory and exploratory behaviour, vocalisation when an affected joint is pressured) but they also become systematically ill, with ulcerative skin lesions, hepatopathy and lymphadenopathy (Danneman, 1997). With such effects it is difficult to determine which changes are due to pain itself and which are a result of illness. Therefore, alternative models where irritants (*e.g.,* kaolin and capsaicin) (Neugebauer *et al.* 1993; Neugebauer *et al.* 1995a; Neugebauer *et al.* 1995b) or microorganisms (*e.g., Mycobacterium tuberculosis*) (Ochi *et al.* 1999) are injected into a joint to induce arthritis of that particular joint, with no signs of systemic disease, have been designed.

The writhing test is an experimental model of visceral, chronic pain, where an irritant substance (*e.g.,* acetic acid or phenylbenzoquinone) is i.p. administered to mice or rats and the number of abdominal constrictions (abdomen flattened, back depressed and hind limbs extended) are counted to evaluate nociception (Gray *et al.* 1998; Gray *et al.* 1999; Murata *et al.* 1997).

Large number of experimental models of neurophatic pain, one of the most troublesome and poorly understood forms of pain, have been developed. Among them, gentle ligation of the sciatic nerve (Liu *et al.* 1994) or lumbar dorsal roots (Chaplan *et al.* 1997; Liu *et al.* 1994) of rats are some of the most commonly used. These two tests produce partial peripheral denervation because of demylination of large fibres and destruction of some small

unmyelinated axons, which is shown as allodynia, hyperalgesia and spontaneous pain. Furthermore, nerve injury models associated with systemic disease as the streptozotocin-induced painful diabetic neuropathy in rats (Calcutt & Chaplan, 1997), are also of great value.

It is worth mentioning that analgesic drugs can modify nociceptive tests' behavioural responses but their antinociceptive efficacy varies widely with the experimental model employed. This has been partially explained by specific mechanisms activated in the spinal cord (Yamamoto & Nozaki-Taguchi, 1996). Therefore, results obtained with one model should not be directly compared with different experimental models.

2.6. AIMS OF THE PROJECT

The aims of this research were to evaluate the spinal effect of NSAIDs as well as their interaction with opioidergic and α_2-adrenergic mechanisms with the hope to elucidate the central analgesic mode of action of these drugs. For these purposes, a neonatal rat spinal cord preparation and sheep with indwelling cervical i.t. catheters submitted to mechanical nociceptive stimulation of the left radius were utilised.

Chapter 3
EFFECT OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS ON NMDA-RECEPTOR SYNAPTIC TRANSMISSION IN THE *in vitro* RAT SPINAL CORD PREPARATION

3.1. INTRODUCTION

It has been shown that NSAIDs produce analgesia in rats (Bustamante *et al.* 1997; Malmberg & Yaksh, 1992a; Malmberg & Yaksh, 1992b; Malmberg & Yaksh, 1993; Pelissier *et al.* 1996), mice (Akman *et al.* 1996) and humans (Devoghel, 1983; Lauretti *et al.* 1988) after spinal administration of these drugs. However, is still debated if these effects are mediated by spinal COX inhibition or if NSAIDs interact with nociceptors in a different way, or both. The present experiments were designed to investigate possible mechanisms of action by which NSAIDs might reduce nociceptive spinal transmission. The *in vitro* neonatal rat hemisected spinal cord preparation was used for this purpose.

In this preparation, supramaximal electrical stimulation of a lumbar dorsal root evokes a reflex in the ipsilateral ventral root (the high intensity excitatory postsynaptic potential). This reflex is due to the activation of C fibres and therefore is considered to reflect a nociceptive reflex (Faber *et al.* 1997a; Akagi *et al.* 1985). It can be depressed by opioids (Faber *et al.* 1997a) and α_2-adrenoceptor agonists (Faber *et al.* 1997a; Faber *et al.* 1997b; Faber *et al.* 1998). In addition, stimulating the dorsal root at low intensities can evoke an A fibre-mediated response (the low intensity excitatory postsynaptic potential), which is also sensitive to these drugs (Faber *et al.* 1997a; Faber *et al.* 1998).

Both the high and low intensity excitatory postsynaptic potentials have NMDA receptor-mediated components which must be mediated by the enhanced glutamate release after electrical stimulation of A fibres (Suzuki *et al.* 1998) and the enhanced glutamate and SP and CGRP release after C fibre stimulation (Akagi *et al.* 1985; Andreeva & Rang, 1993; Malcangio *et al.* 1998; Suzuki *et al.* 1998). NMDA receptor-mediated synaptic excitation seems to be depressed by

centrally acting analgesic (Faber *et al.* 1997a). Therefore, if NSAIDs produce analgesia at the spinal level, they should depress the NMDA receptor-mediated reflexes of the *in vitro* neonatal rat hemisected spinal cord preparation.

3.2. MATERIALS AND METHODS

3.2.1. Spinal cord preparation

Minor modifications were made to the method described by Otsuka and Konishi (1974), which allows a stable recording and good reflex responses for at least several hours. After approval from the Massey University Animal Ethics Committee, neonatal Sprague-Dawley rats [3–9 days old, unsexed, 9–16 g; preparations from older pups cannot be maintained *in vitro* (Wong *et al.* 1998)] (Small Animal Production Unit, Massey University, Palmerston North, New Zealand) were cervically dislocated and decapitated. The abdomen ventral skin was removed and an incision was performed on the thoracic cage and the ventral abdomen wall, all thoracic and abdomen internal organs were removed and the vertebral canal was opened on the ventral side. The dura mater covering the ventral side of the spinal cord was removed and the spinal cord was carefully dissected out of the dorsal part of the vertebral column together with attached L4 or L5 dorsal and ventral roots. The spinal cord was placed in a petri dish containing artificial cerebral spinal fluid (ACSF) containing the following (mM): NaCl 118, NaHCO$_3$ 24, C$_6$H$_{12}$O$_6$ 12, CaCl$_2$ 1.5, KCl 3 and MgSO$_4$7H$_2$O 1.25 (all reagents purchased from BDH Laboratory Supplies, Poole, England). The ACSF was gassed with 95% O$_2$ and 5% CO$_2$, (Carbogen 5; BOC Gases, New Zealand) maintaining the solution at pH 7.4, and was kept at a temperature of 27 ± 1°C, normal temperature for pups of this age when they are not under the mother (Wong *et al.* 1998).

The roots, but not from L4 or L5, were removed along with the dura mater covering the dorsal side of the spinal cord. The spinal cord was hemisected sagittally by making a small incision at the centre of the cord at the cranial end with a scalpel and gently tearing the two halves apart. The ventral and dorsal

roots were separated at the DRG, leaving the dorsal root intact with the DRG. Then, the hemisected spinal cord was placed in a chamber (made up from Perspex glass; Figure 3.1) with L4 or L5 dorsal root and attached DRG in contact with the stimulating silver – silver chloride wire electrode, which was bent into the shape of a hook in order to hold the spinal cord in place on the preparation chamber. The corresponding ventral root was in contact with the recording silver wire electrode. Electrical isolation of the electrodes was made by using grease gaps made of vaseline and liquid paraffin (5:4 v/v).
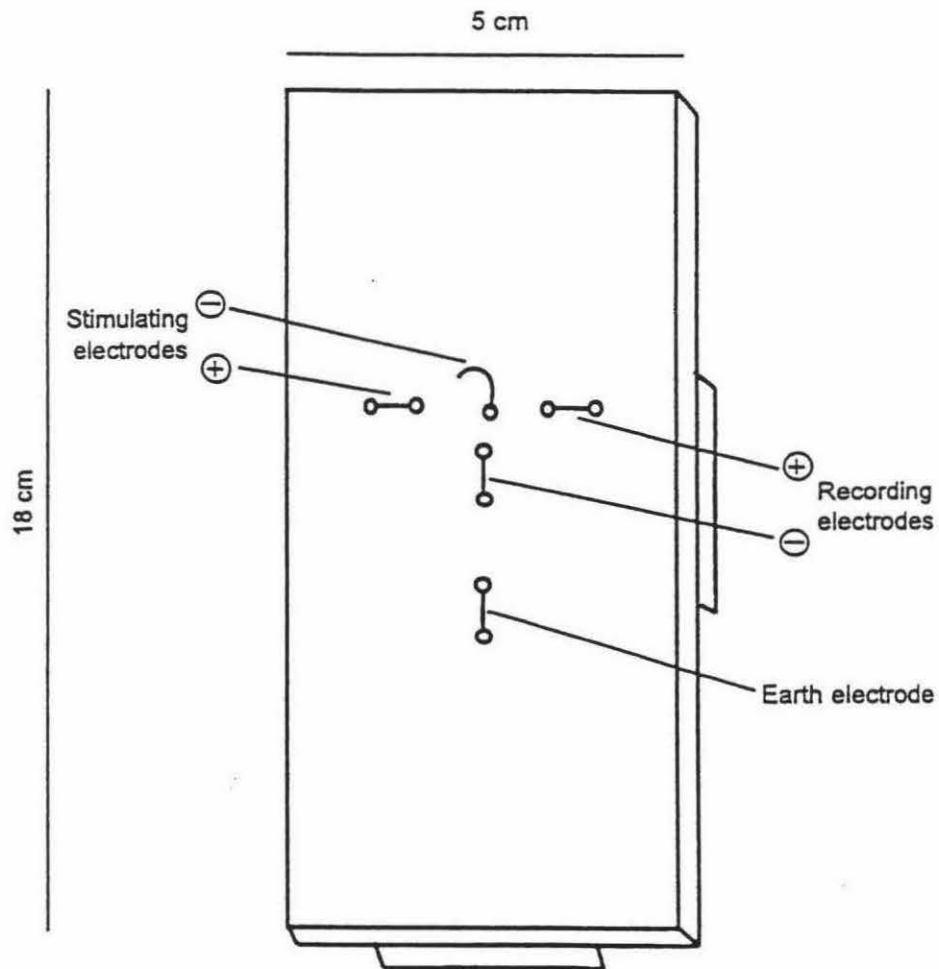
Figure 3.1. The preparation chamber. The hemicord was placed to the right of the negative stimulating electrode with the dorsal root in contact to the positive stimulating electrode. The ventral root was in contact with the positive recording electrode.

Once in the chamber, the spinal cord preparation was superfused with ACSF as it is shown in Figure 3.2. The ACSF, contained in a glass reservoir and gassed with 95% O_2 / 5% CO_2, was pumped (H.R. Flow Inducer, model MHRE 22, Watson-Marlow Ltd., Cornwall, England) at a rate of 2 mL/minute into polyethylene tubes (2 mm diameter; SMA Flow Rated Pump Tubes, model 116-0549P12, Technicon Instruments Corporation, Tarrytown, N.Y., U.S.A.) which passed by a heated water jacket ensuring a temperature of 27 ± 1°C. The chamber was placed with an inclination of about 30° to maintain constant flow of ACSF over the hemisected spinal cord, which ensures a stable baseline of recordings. The ACSF was dripped off the chamber and collected as waste. The preparations were allowed to equilibrate for at least 60 minutes before recordings were performed and they were only accepted if two subsequent control measurements, 15 minutes apart, differed in amplitude by less than 10%. Drugs were applied to the preparation in known concentrations by adding them to the superfusate. The distance between the superfusion reservoir and the chamber was 75 cm, approximately. The polyethylene tubes were cleaned for each experiment by flushing them through with HCl (37% diluted by a factor of two in distilled water; BDH Laboratory Supplies, Poole, England) and rinsing with distilled water.
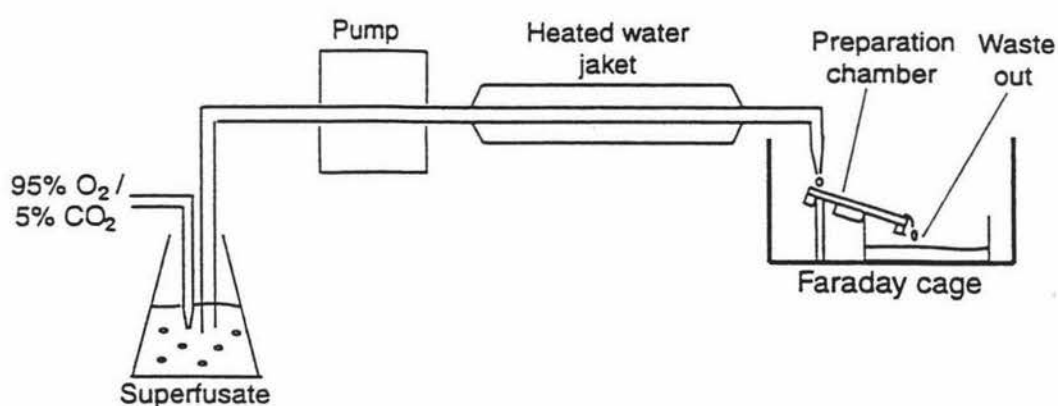
Figure 3.2. The superfusion system. The ACSF was continuously gassed with 95% O_2 / 5% CO_2 and pumped through a heated water jacket. The ACSF was dripped onto the preparation chamber and then it was collected as waste.

Following the technique described above, and to assess the dorsal root compound action potential (DRCAP), the sciatic nerve was also isolated together with the hemisected spinal cord to be used for the experiment. In these experiments the nerve laid across the preparation chamber with the peripheral end of the sciatic nerve in contact with the stimulating electrode and the dorsal root in contact with the recording electrode. Both electrodes were isolated with grease gaps as described above.

3.2.2. Recording techniques

All trials were carried out in a Faraday cage with the front covered by aluminium foil in order to isolate the preparation from electrical interference and to produce a stable environment. The dorsal root was electrically stimulated (isolated stimulator JP, IVABS Workshop, Massey University, Palmerston North, New Zealand) to evoke a population ventral root potential (DR-VRP) in the corresponding ipsilateral ventral root. There are three longer time sweeps. Low intensity stimulation activates the A primary afferent fibre-mediated components of the DR-VRP. An initial monosynaptic compound action potential (MSR) (Otsuka & Konishi, 1974) superimposes on a population excitatory postsynaptic potential (e.p.s.p.) (the low intensity e.p.s.p.), which last up to two seconds. High intensity stimulation evokes a polysynaptic presumed C fibre-mediated response, which has duration of up to forty seconds, the high intensity e.p.s.p. (Akagi *et al*. 1985). Additionally, a long duration response similar to that elicited by a single supramaximal pulse was evoked following a train of five stimuli (the train e.p.s.p.) at low intensity stimulation (Faber *et al*. 1997a).

The A fibre-mediated MSR and the low intensity e.p.s.p. were evoked by a single impulse 0.5 ms in duration at three times the threshold (the threshold being the intensity at which a discernible response first appears in the ventral root). From previous experiences (Faber *et al*. 1997a), it is known that approximately one volt (v) is required to evoke a threshold. The MSR was measured over 120 ms at a sampling rate of 10 kHz and the low intensity e.p.s.p. was measured over 1200 ms at 1kHz. The high intensity e.p.s.p. was

evoked by a single pulse 0.5 ms in duration at 16 times the threshold, this response was measured over 60 seconds at 20 Hz. The train e.p.s.p. was evoked with a train of 5 stimulus (20 Hz) at 2 times the threshold and was measured over 25 seconds at 40 Hz. In order to elucidate which primary afferent fibres (A or C) are activated following stimulation of the dorsal root at different intensities, the DRCAP was evoked in the central end of the sciatic nerve following a single pulse of 0.5 ms duration and was measured over 60 ms at 10 kHz (Table 3.1).

Table 3.1. Electrical stimuli applied to the spinal cord preparation and data acquisition parameters.

	Electrical stimulation		Data acquisition parameters	
*Stimulus**	*Duration*	*Intensity*	*Measuring time*	*Sampling rate*
DRCAP	0.5 ms	1-16 v	60 ms	10 kHz
MSR	0.5 ms	3 thresholds	120 ms	10 kHz
Low intensity e.p.s.p.	0.5 ms	3 thresholds	1200 ms	1 kHz
High intensity e.p.s.p.	0.5 ms	16 thresholds	60 s	20 Hz
Train e.p.s.p.	5 stimuli of 20 Hz	2 thresholds	25 s	40 Hz

* DRCAP = dorsal root action potential; MSR = monosynaptic compound action potential; e.p.s.p. = excitatory postsynaptic potential.

3.2.3. Drugs

Bupivacaine HCl (Marcain), Astra Pharmaceutics Pty Ltd, N. Ryde, NSW, Australia.

Ketamine HCl (Ketamine Injection), Parnell Laboratories NZ Limited, East Tamaki, New Zealand.

Lignocaine HCl (Lopaine), Ethical Agents Ltd, Auckland, New Zealand.

Mepivacaine HCl (Mepivacaine), Nature Vet Pty Limited, Agnes Banks, NSW, Australia.

Morphine sulphate (Morphine Sulphate Injection), David Bull Laboratories, Victoria, Australia).

Xylazine HCl (Phoenix Xylazine 2% Injection), Phoenix Pharm Distributors Ltd, Auckland, New Zealand.

3.2.4. Data analysis

The actions of drugs on each of the population responses were assessed by measuring changes in the peak amplitude of the MSR and in the A and C waves of the DRCAP, as well as changes in the areas under the curve (AUC) of the low intensity e.p.s.p., high intensity e.p.s.p. and train e.p.s.p. Responses were amplified, monitored and analysed using commercially available software (Scope v3.5.7, MacLab®System © 1998 ADInstruments Pty Ltd, Castle Hill, Australia).

3.3. RESULTS

No physiological responses were recorded from the hemisected spinal cord. All records were identified as artefacts. Figure 3.3 shows the responses obtained after electrical stimulation of the dorsal root and the sciatic nerve, both attached to the spinal cord. These responses were not depressed by any of the drugs used in this study, even when very high concentrations were administered (data not shown). In addition, similar responses were recorded the next day after leaving the spinal cord overnight on the chamber and without being infused with ACSF. Similar records were obtained by putting a piece of cloth in the same position the spinal cord was placed on the chamber. Moreover, these same artefacts were recorded even in the absence of this cloth.

In another set of experiments, isolated sciatic nerves from mice were electrically stimulated to evaluate the performance of the equipment. They were laid on the same chamber used for the spinal cord, with the peripheral end in contact with the stimulating electrode and the proximal end in contact with the recording one. Not all the preparations displayed consistent responses. However, in some of them, nerve action potentials were recorded and conduction blockade was observed with the local anaesthetic lignocaine when used at 1 mM concentration (Figure 3.4).

In vitro spinal cord preparation
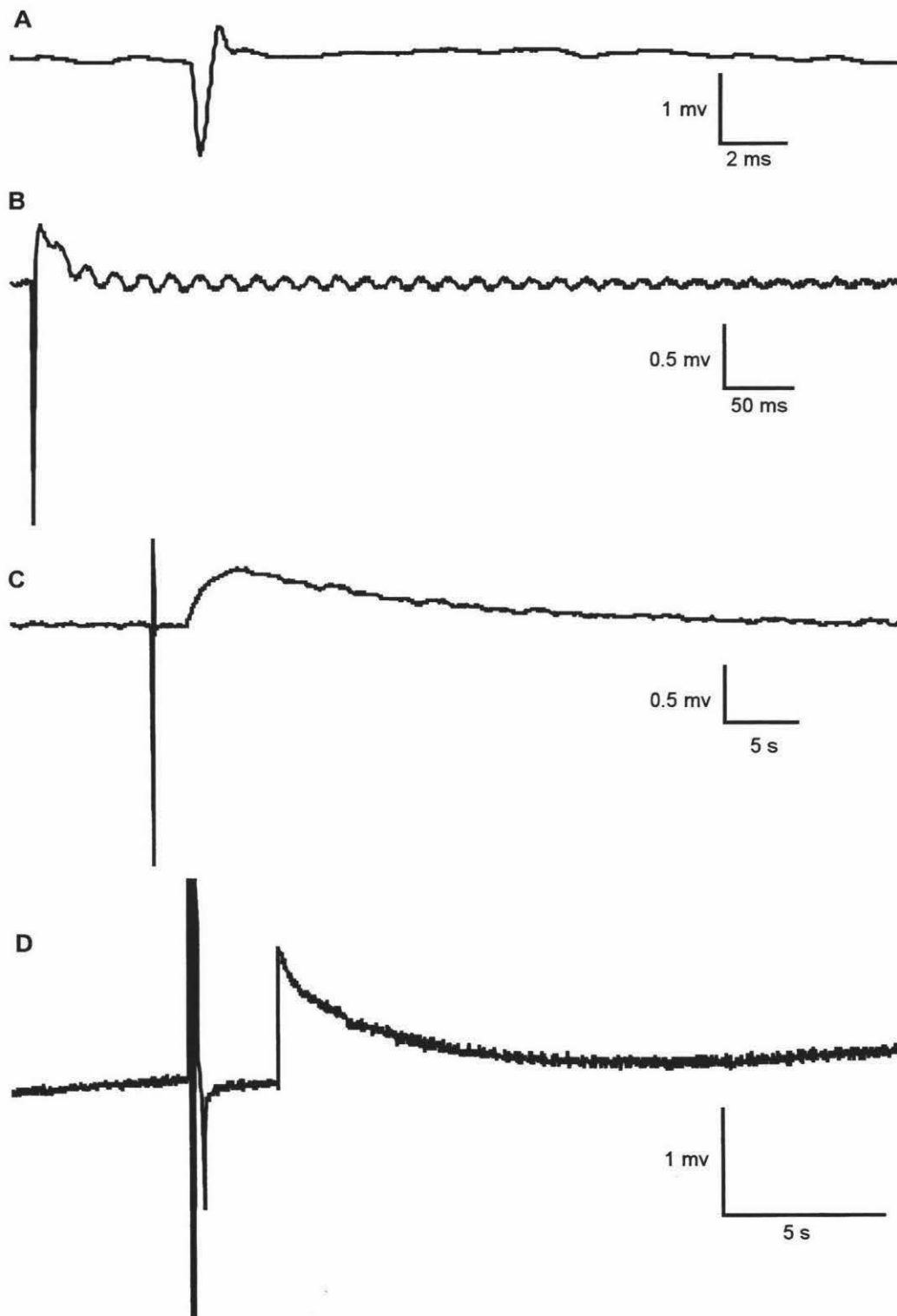
Figure 3.3. Artefacts obtained after electrical stimulation to evoke the DRCAP (A), the low intensity e.p.s.p. (B), the high intensity e.p.s.p. (C) and the train e.p.s.p (D) in the neonatal rat hemisected spinal cord preparation.
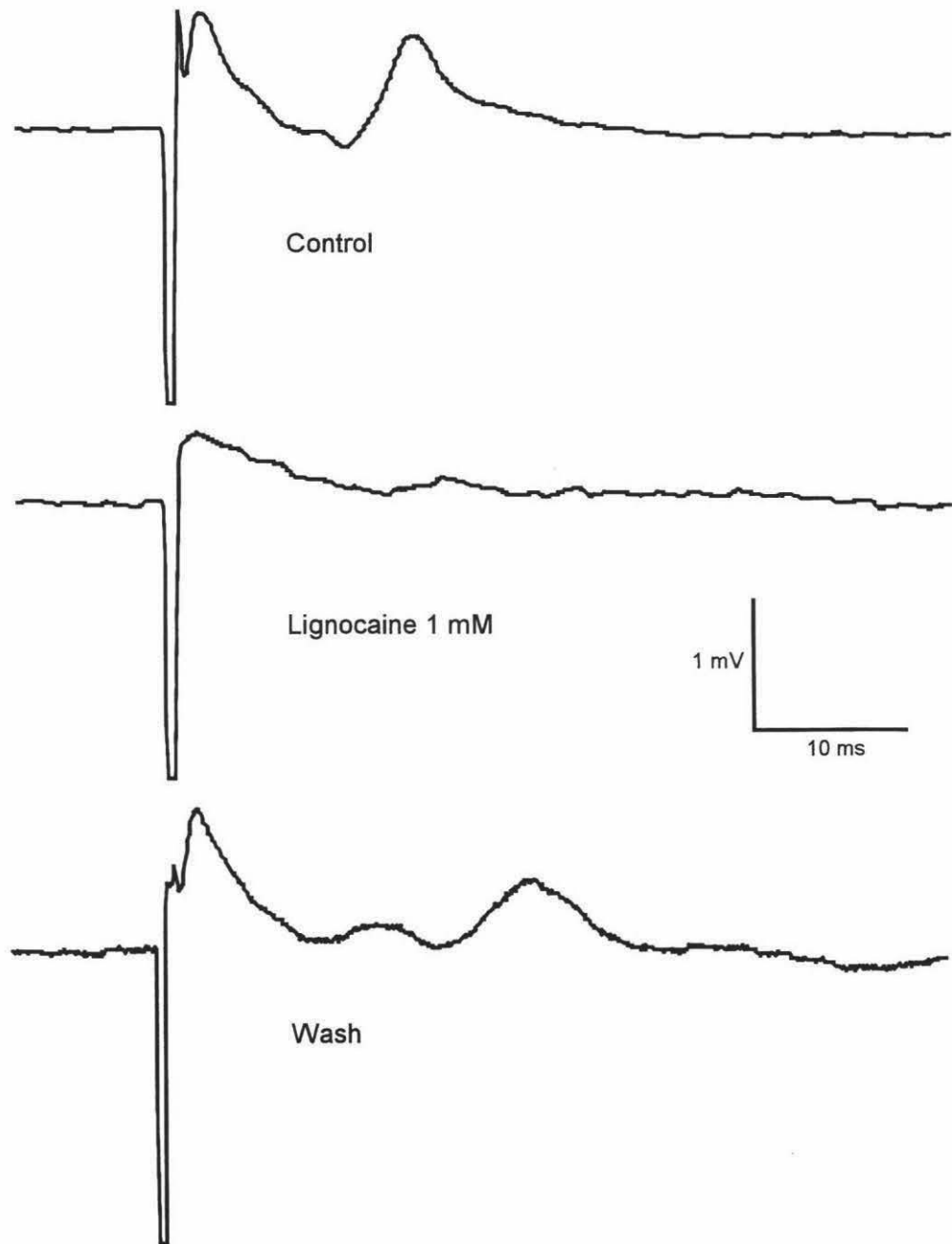
Figure 3.4. Effect of lignocaine (1 mM) on nerve action potentials from mice sciatic nerve. The threshold of this nerve was 0.5 v (not showed) and a 6 v stimulus was used to evoke both A and C fibre compuond action potentials (A), which amplitude was blocked and reduced, respectively, by lignocaine (B). Recovery is shown 25 minutes following return to lignocaine-free medium (C).

3.4. DISCUSSION

The hemisected neonatal rat spinal cord preparation is a robust *in vitro* preparation that permits the study of the spinal actions of centrally acting analgesics (Faber *et al.* 1997a; Faber *et al.* 1998; Brockmeyer & Kendig, 1995). However, in this trial, proper setting up of this technique was not possible and all records obtained from this preparation were identified as artefacts. Several lines conveyed us to this conclusion. Firstly, none of the analgesic and anaesthetic drugs used in this study depressed these "responses". The opioid agonist morphine, known to depress both low and high intensity e.p.s.p. with EC_{50} values of 99 nM and 79 nM, respectively (Faber *et al.* 1997a), produced no change in the records even when it was administered at concentrations of 1–10 μM. Similarly the α_2-adrenoceptor agonist xylazine, which inhibits both spinal reflexes with EC_{50} values of 760 nM and 910 nM, respectively (Faber *et al.* 1998), did not depress the obtained records after infusing the drug at 1–500 μM concentration. The NMDA receptor antagonist ketamie, which has concentration-dependent (1–50 μM) depressant actions on both spinal reflex components (Brockmeyer & Kendig, 1995), did not modify the records either when a 150 μM concentration was used. The local anaesthetic lignocaine also depresses the high intensity e.p.s.p. with an EC_{50} values of 0.15 μM (J.P. Chambers, unpublished observations) but here, when used at 0.5–1 μM, it did not alter the recorded responses. The same results were observed with the more potent local anaesthetics mepivacaine (0.2 μM) and bupivacaine (0.2 μM).

Secondly, spinal cord preparations left overnight on the preparation chamber without being infused with ACSF showed similar responses to those not depressed by the analgesic and anaesthetic drugs. Although this preparation is capable of stable responses for several hours (Brockmeyer & Kendig, 1995), it is difficult to believe that this fragile nervous tissue could survive for almost 24 hours and even more so if no oxygen was supplemented for more than 10 hours.

Thirdly, the same artefacts were recorded by replacing the spinal cord with pieces of cloth that were laid on the chamber in the same way as was the spinal cord. Moreover, the same records were detected even in the absence of this cloth.

There could be several reasons why no physiological responses were obtained from this preparation: misdissection of the spinal cord, misplacement of the spinal cord on the preparation chamber, contamination of the pumping system or material in contact with the spinal cord with toxic substances, and faulty equipment. It was necessary to practice dissecting the spinal cord with attached lumbar nerves in order to perform it quickly and without inferring too much damage to the cord. However, once the dissection technique was mastered, it usually took a period of 40 to 60 minutes to obtain an almost intact preparation and have it all set up on the chamber. Furthermore, the spinal cord was always immersed in ACSF during the dissection procedure. Thus, it seems unlikely that the dissection itself had any influence on the results. In fact, with practice, each dissection became easier than previous ones.

The lumbar dorsal root was placed in contact with the stimulating electrode whereas the ipsilateral ventral root was in contact with the recording electrode. Differentiation between dorsal and ventral roots is not difficult. Under the microscope, the dorsal root looks like a continuation of the DRG. The ventral root, on the other hand, looks like an "independent" structure from the DRG. To confirm this, black ink was injected in the ventral aspect of the cranial spinal cord of some preparations, allowing us to identify the ventral aspect of the spinal cord. This process validated the differentiation as stated above. Electrical isolation of the electrodes was achieved by utilising grease gaps that prevented the ACSF from flowing freely through the entire chamber. Hence, inappropriate setting up of the spinal cord on the chamber does not seem to be a likely cause for the recording of artefacts. Furthermore, on several different occasions placing of the spinal cord on the chamber was carried out by more than one person and the results were the same, artefacts.

In order to reduce the possibility of having contaminants in contact with the spinal tissue, the material where the neonatal rat and the spinal cord were dissected as well as the flasks and the tubing system in which the ACSF was contained and pumped through were properly cleaned with HCl and then thoroughly rinsed with distilled water before each experiment. Moreover, they were replaced by new material at some stage and still no physiological responses were recorded. Thus, the chance of having a nasty substance in contact with the spinal tissue, which may have affected its viability, seems unlikely. Pieces of absorbent cloth were used to keep the spinal tissue in contact with the electrodes, and different medical materials were tried (*e.g.* masks, hats, shoe covers, etc.) without producing different results. The preparation chamber was the only material in contact with the spinal cord that was not replaced. It was made from previously used Perspex glass and although it was washed, the possibility that a toxic substance had remained on it cannot be excluded.

The possibility that the ACSF could have adversely affected the spinal cord preparation was considered but it was ruled out by checking its pH and continuously monitoring its temperature. In addition, it was observed that solutions with very similar composition to the one used here have been successfully used on this *in vitro* preparation (Faber *et al.* 1997a; Faber *et al.* 1998; Andreeva & Rang, 1993; Wong *et al.* 1998; Brockmeyer & Kendig, 1995; Suzuki *et al.* 1998).

Suspicion of a fault in the system came from interference signals being picked up at the time of recording. It was recognised that the stimulator was sending signals by itself and had to be returned to the workshop for repair. Some interference was still detected, but this time was not from the stimulator. An selftest was run for the MacLab and no fault was detected, leading us to consider a probable fault in the bioamplifier which was replaced by a new one. Using the new bioamplifier reduced interference and by isolating the chamber with aluminium foil and connecting an earth cable to the cage, interference was reduced even more. To corroborate that the equipment was working properly, mice sciatic nerve was used instead of the spinal cord. Although no consistent

responses were obtained from all these preparations, physiological responses consistent with A and C fibre conduction velocities (Gissen *et al.* 1980) were recorded and abolished by lignocaine at a similar reported concentration needed to depress by 50% C wave amplitudes (Gissen *et al.* 1980) (Figure 3.4). This may demonstrate that the technical difficulties were apparently solved and that the equipment was working properly. Nonetheless, still no physiological responses were obtained from the rat spinal cord preparation.

Although the neonatal rat hemisected spinal cord preparation is a very well established technique (Faber *et al.* 1997a), we failed to record physiological responses from it. No obvious reason for only recording artefacts was perceived. Several possible faults were looked at and no major mistakes were detected, it even was demonstrated that the same material and equipment used for the spinal cord produced physiological responses when tested with mice sciatic nerves. However, it is important to mention that not all sciatic nerves displayed the same responses and some of then did not respond at all. Whether the dissection technique of both sciatic nerve and spinal cord affected the preparations' viability can be debated; however, the presence of a toxic substance in the chamber could be a more likely explanation since both tissues showed no response after electrical stimulation. Fabrication of another chamber with brand new Perspex glass would answer this issue.

4.1. INTRODUCTION

The NSAIDs are widely used for their analgesic, anti-inflammatory and sometimes antipyretic and antithrombotic effects. In spite of their structural diversity, it is accepted that all NSAIDs inhibit COX to a greater or lesser extent and it has been assumed that this action causes analgesia by preventing the sensitisation of peripheral nerve endings by PGs, particularly PGE_2 and PGI_2 (Ferreira, 1972). However, for some NSAIDs there is only a poor correlation between their ability to inhibit COX and their analgesic efficacy (McCormack, 1994a; McCormack, 1994b), implying that mechanisms other than or in addition to COX inhibition may be involved in the analgesic action of these drugs.

In addition to their peripheral effects, it has been demonstrated that NSAIDs exert central analgesic effects on the spinal cord (Bustamante *et al.* 1997; Malmberg & Yaksh, 1992a; Malmberg & Yaksh, 1992b; Malmberg & Yaksh, 1993; Pelissier *et al.* 1996) and on structures in the midbrain (Carlsson *et al.* 1986; Carlsson & Jurna, 1987; Vanegas *et al.* 1997; Tortorici *et al.* 1996). This may mean that some if not all the analgesic actions of these drugs are centrally mediated. Nevertheless, to date, it is not fully understood if the central analgesic effects are achieved by inhibition of prostanoid production, or if NSAIDs interact with nociceptive neurones in other ways.

This study was undertaken to determine both the site and the mechanisms of action on/by which NSAIDs produce analgesia. The technique of mechanical noxious stimulation of a sheep's leg has demonstrated to be sensitive to the analgesia produced by NSAIDs in this species (Chambers *et al.* 1993; Chambers *et al.* 1995; Welsh & Nolan, 1994) and the i.t. cervical catheterisation of sheep has been useful to determine the spinal actions of different analgesic drugs (Waterman *et al.* 1988; Ley *et al.* 1989; Kyles *et al.* 1993) but not the

NSAIDs. Therefore, ketoprofen, phenylbutazone, salicylic acid and tolfenamic acid were i.t. and i.v. injected to non-lame, healthy sheep implanted with indwelling cervical catheters (Kyles *et al.* 1992) and submitted to mechanical noxious stimulation of one forelimb. It was reasoned that if NSAIDs-mediated analgesia is due to spinal COX inhibition, increase in nociceptive thresholds should be observed after injecting these drugs into the subarachnoid space at concentrations high enough to inhibit the activity of these enzymes (Laneuville *et al.* 1994; Ricketts *et al.* 1998; Giuliano & Warner, 1998). In a similar way, it was assumed that if NSAIDs induce analgesia by activating descending inhibitory mechanisms then the i.t. injection of the opioid receptor antagonist naloxone (Emmerson *et al.* 1994) and the α_2-adrenoceptor antagonist atipamezole (Schwartz & Clark, 1998) should decrease the analgesic actions of i.v. administered NSAIDs.

4.2. MATERIALS AND METHODS

4.2.1. Animals and intratecal catheterisation

Sixteen adult Romney-cross, non-lame female sheep (age range 2 – 4 years old, weight range 40 – 70 kg) were used in this study that was carried out according to a protocol approved by the Massey University Animal Ethics Committee. The animals were housed indoors and red clover hay and water were provided *ad libitum* throughout the investigation. The sheep had chronic indwelling cervical i.t. catheters (Kyles *et al.* 1992). The ewes were anaesthetised, thiopentone (15 mg/kg i.v.; Bomathal, Bomac Laboratories Ltd, Auckland, New Zealand) followed by halothane (Fluothane, ICI New Zealand Ltd, Auckland, New Zealand), and placed in left lateral recumbency with the head held horizontally and flexed at an angle of about 90° to the neck. Then, after aseptic skin preparation of the dorsal neck area, a 2 to 3 cm skin incision on the midline at the atlanto-occipital joint level was performed and a 16G Tuohy needle (Portex Epidural Minipack System 1, SIMS Portex Ltd, Hythe, Kent, U.K.) inserted in the subarachnoid space. Once CSF was flowing freely through the needle, a 16G nylon catheter was passed through it until about 25

cm were inserted. In all cases a good flow of CSF was obtained through the catheter. A luer lock connector affixed to a 0.22 μm filter was attached to the catheter and 1 mL (the volume of the system) of contrast media (Iodixanol; Visipaque 320 mg I/mL, Nycomed Ireland Ltd, Cork, Ireland) was administered. Next, a radiograph was taken to determine the position of the catheter tip and according to this the catheter was moved to locate the tip in the region of the cervical vertebra 5 (C5) which was confirmed radiographically (Figure 4.1). In this position, the innervation of the brachial plexus is susceptible to any administered agent via the catheter (Kyles *et al.* 1992). The luer fitting cap and the filter were disconnected from the catheter and the guide needle removed. The catheter was tunnelled s.c. to emerge in the lower neck region and the excess length of the catheter cut. The catheter was sutured firmly in the place where it emerged from the subcutaneous tissue. The skin incision was closed with 2-0 monofilament nylon suture using a continuous suture pattern. Soon after, the luer lock connector and the filter, this last with an affixed butterfly made from waterproof strapping (Sleek™; Smith & Nephew Pty. Ltd., Clayton, Australia), were reattached to the catheter and both secured to the skin with non-absorbable suture. A non-adherent absorbent dressing (Melolin™; Smith & Nephew Pty. Ltd., Clayton, Australia) covered with elastic adhesive (Elastoplast™; Smith & Nephew Pty. Ltd., Clayton, Australia) was stitched to the skin to minimise damage to the catheter. Following surgery, the animals were allowed to recover for at least two weeks before any drugs were given. Catheters were maintained by periodic resuturing of the dressings, the luer lock connectors and the butterflies, and were flushed daily with 1 mL sterile normal saline when no experiment was carried out.

Because several placed catheters were lost and others migrated rostrally, it was decided that in the future they should be anchored. Hence, before passing the catheter subcutaneously a knot made with the catheter itself was tried (Figure 4.2). However, the knot became so tight that it blocked the catheter and therefore this technique was abandoned and replaced by advancing the catheter in a loop manner through a Yates draining tube approximate 1 cm long consisting of four catheter lumens joined together, each of 3 mm diameter (Flexi-Drain; Obex Medical Ltd., Auckland, New Zealand) (Figure 4.2). This
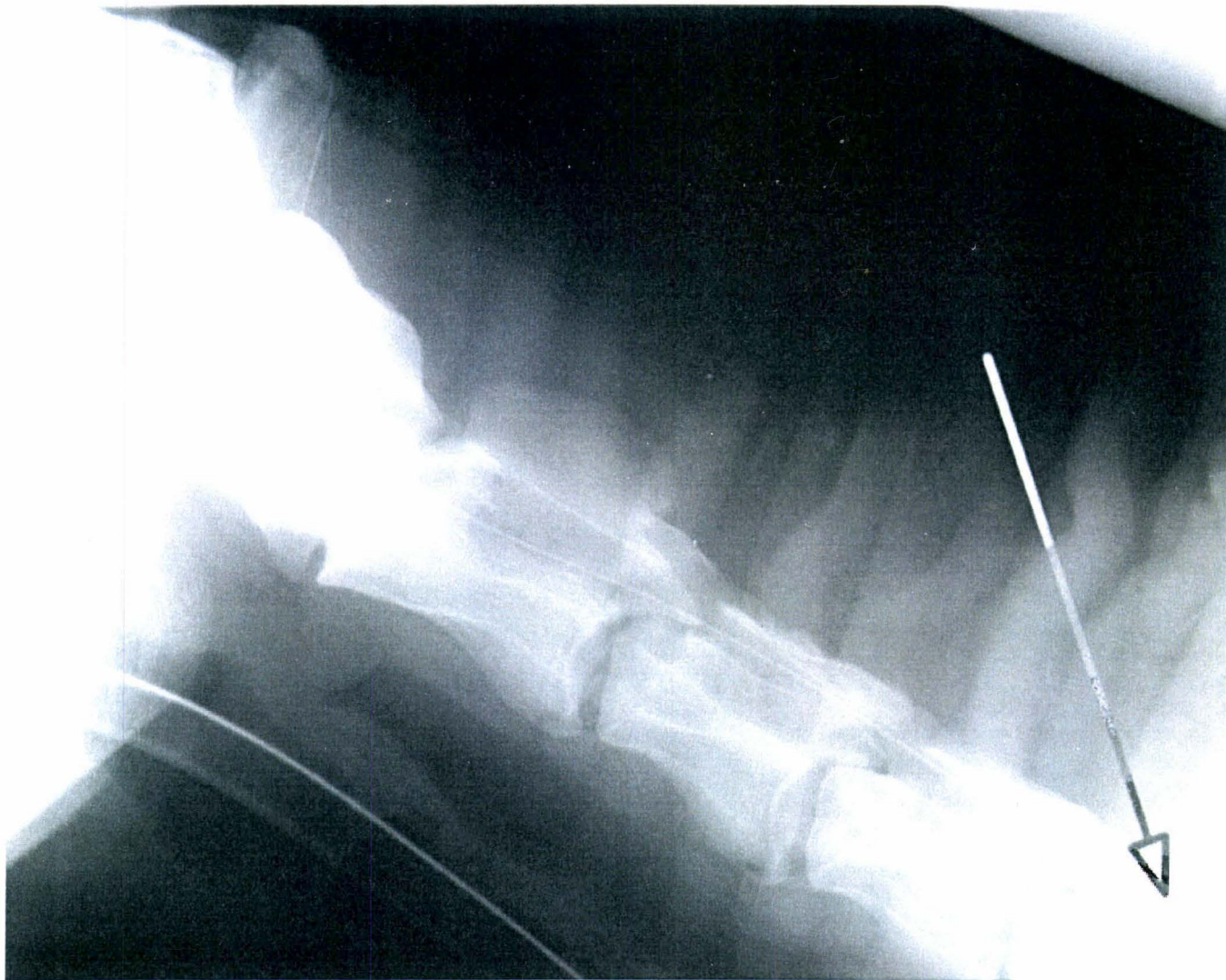
Figure 4.1. Radiograph showing the correct placement of an intrathecal catheter in sheep, with the catheter tip (arrow) at the C5 level.
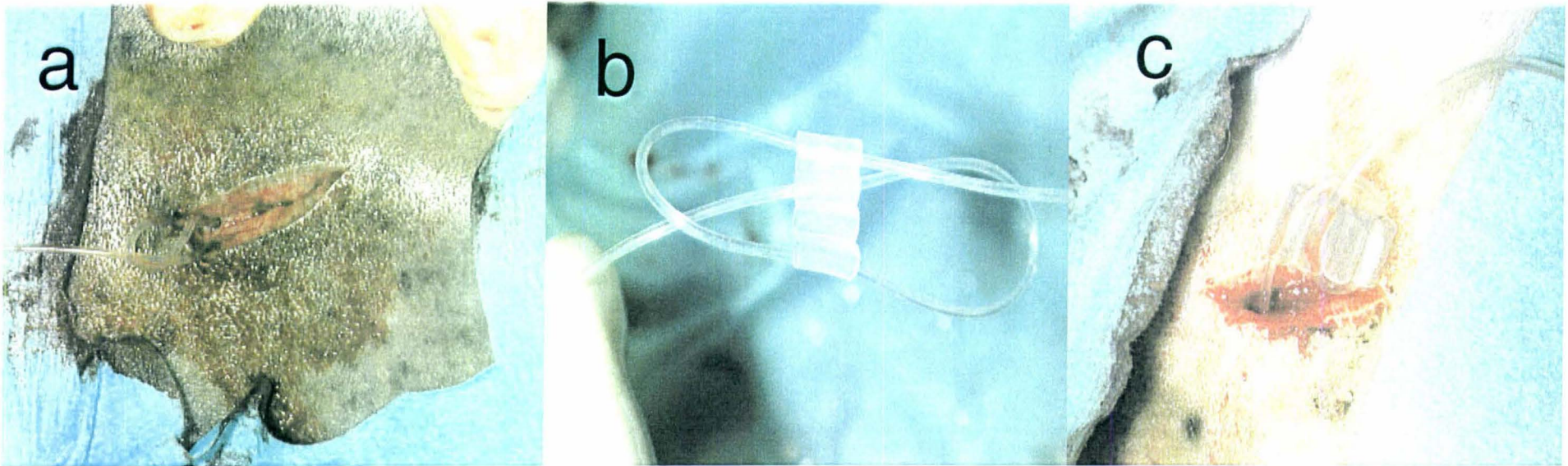
Figure 4.2. A knot made with the intrathecal catheter itself (a) and a silicon tubular drain (a) were used to anchor the intrathecal catheter. In both cases the anchoring element, knot or drain tube, was kept subcutaneously (a and c).

proved to be more efficacious in keeping in place a functional i.t. catheter. The knot or the draining tube was placed subcutaneously at the level of the skin incision creating a physical barrier between the catheter inside the sheep's body and the external part of the catheter.

Demonstration of functional i.t. catheters was made by injecting xylazine (10 and 50 µg/ 100 µL; equivalent to 388 and 1940 µM, respectively) through them (see below), which produces good analgesia in sheep (Waterman *et al.* 1988; Kyles *et al.* 1993). Furthermore, throughout the investigation, catheters were tested periodically with xylazine (10 µg) and thresholds were measured for 10 to 15 minutes after drug administration to confirm proper placement only. If no increase in nociceptive thresholds was observed, catheters were replaced.

4.2.2. Mechanical nociceptive testing

A special, light (18 g) device (Figure 4.3) was made from a loss of resistance syringe cut at the 5 mL mark (2.9 cm long) and had a 2 mm diameter blunt-ended nail attached to the plunger. The apparatus was screwed to a polyethylene band stapled to a Velcro tape, which permitted the device to hold to the sheep's limb. The loss of resistant device was connected to a polyethylene tube of 2 mm internal diameter and 120 cm long which was attached to a three-way stopcock that, in turn, was coupled through a 5 mm diameter, 80 cm long silicon rubber tube to a bourdon tube dial gauge (Teltherm, Independent Vacuum Services Ltd, Auckland, New Zealand) with an operating range of 0 to 160 kPa. The system was closed by using a 60 mL plastic syringe which was used to apply pressure into the system and push the blunted pin against the sheep's leg. The force applied by the pin to the sheep's leg was gradually increased by increasing the pressure in the system with the syringe. When the pain threshold was reached, the sheep lifted its leg; the pressure in the system was immediately reduced to atmospheric by opening the three-way tap. The required pressure to elicit this response was read on the dial gauge's face and recorded. If the sheep did not lift its leg at a pressure
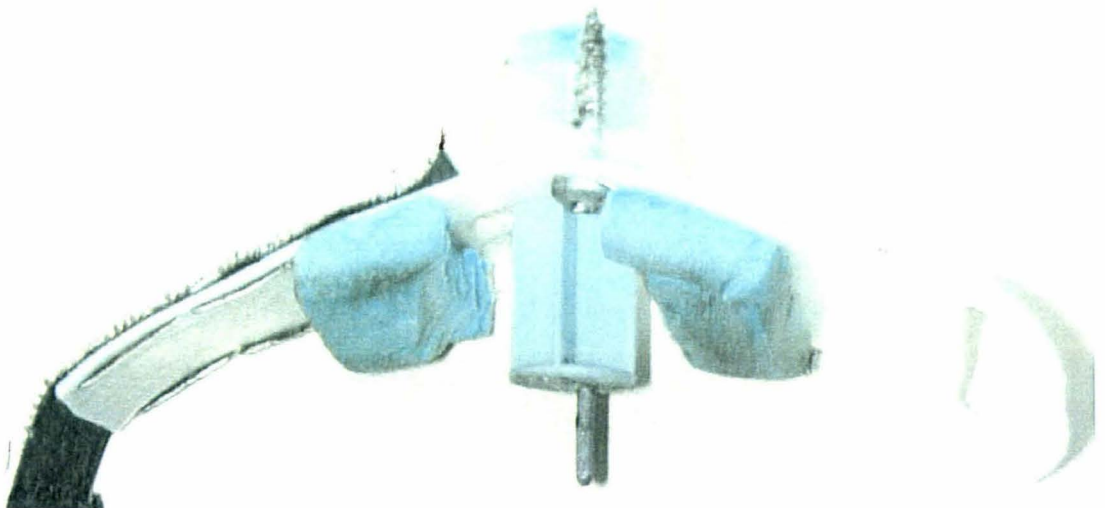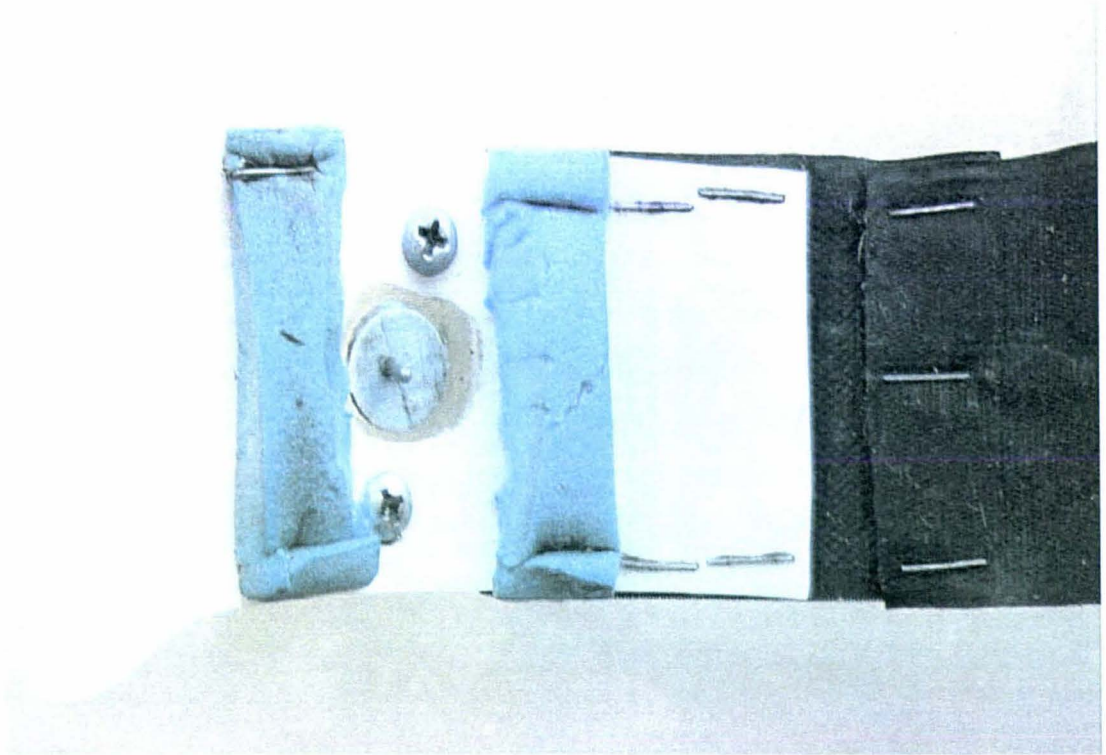
Figure 4.3. Mechanical nociceptive device. The 2 mm diameter blunted pin attached to the plunger was pushed against the sheep's leg.

equivalent to 20 Newtons (N), the stimulus was terminated to prevent tissue damage and this value recorded.

The device was calibrated using an electronic kitchen scale (Propert, Model 1000, Auckland, New Zealand). Pressure was applied to push down the blunted pin against the scale's plate and both weight (g) and the corresponding pressure (kPa) were recorded. This procedure was repeated ten times. For calibration purposes 100 g was taken as equivalent as 1 N, thus N were plotted against the pressure media value required to elicit that force (Figure 4.4). The data on this graph was analysed for linearity and the obtained equation used to transform the readings taken in pressure into a force value.
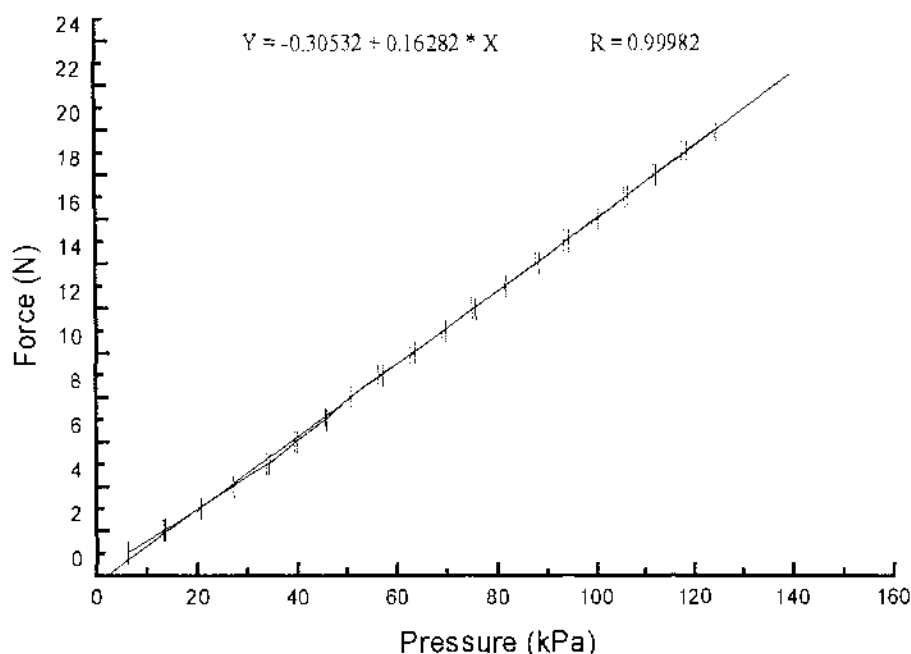
Figure 4.4. Mechanical device's calibration curve. The *X* axis shows the pressure applied to elicits the corresponding force at 1 Newton intervals.

During the experiments, the sheep were placed individually in steel, mobile crates (86 cm high x 112 cm long x 53 cm wide), which were constructed in such a way to allow the sheep to see each other easily, have room to move slightly without turning round and give easy access to their lower legs. The sheep were content to stand in these crates for long periods (up to 7 hours) and

always were kept in pairs since individual sheep become distressed when isolated from the flock. A portable radio was used to provide background noise to prevent the sheep from being distracted by events outside. The skin on the cranial aspect of the left antebrachium was clipped and the device, to which sheep rapidly became accustomed, was attached to the lower end of the radius. The sheep were given a 15 to 20 minute period to acclimatise to the new environment, then at least four baseline readings were taken. After administration of the drug being tested, readings were recorded every 5 minutes for the first hour, every 10 minutes for the second hour and every 30 minutes for up to 6 hours, or until values returned to pre-administration baseline levels.

4.2.3. Drug admninistration protocol

4.2.3.1. Intrathecal administration of NSAIDs

The drugs used for these experiments were ketoprofen (MW 254.3), phenylbutazone (MW 308.4), salicylic acid (MW 138.1), tolfenamic acid (MW 261.7), and xylazine HCl (MW 256.8). All purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

The NSAIDs were dissolved in saline and NaOH (not more than 2.4 mM final concentration of NaOH for the highest drug concentration; pH between 7.0 and 7.8 for all different drugs and concentrations). Xylazine was dissolved in saline only (pH 6.0 and 6.5). Once dissolved, drugs were filtered (26 mm, 0.2 μm syringe filter; Sartorius, Minisart CE, Goettingen, Germany) and then injected through a 0.2 μm filter (4 mm filter unit, Phenomenex, Torrance, California, U.S.A.) attached to the luer lock connector affixed to the catheter. All drugs were administered i.t. in a 100 μL volume followed by a wash-in injection of 300 μL (the volume of the system) of saline. The volume of 100 μL was adopted because previous studies (Waterman *et al.* 1988; Kyles *et al.* 1993) have shown that the drug distribution is limited to about 3 or 4 vertebral segments either side of the catheter tip when administered in such a volume.

Demonstration of a functional i.t. catheter was made by injecting xylazine (10 and 50 μg/ 100 μL) through it, which is known to produce good analgesia in sheep (Waterman et al. 1988; Kyles et al. 1993). Therefore, xylazine was used as the positive control. Once confirmed that the catheter was working properly, cumulative doses (0.375 to 200 μM) of NSAIDs and repetitive doses of saline solution (10 doses; negative control), were i.t. administered. The sheep received all agents but xylazine in a randomised manner with at least one week interval between successive treatments on each sheep to avoid drug residue interference. The operator was blind to the drug treatment except xylazine in all trials.

The mechanical nociceptive testing was carried out as described above and, during the time frame of the trial, the behavioural effects produced by the i.t. injection of any drug were subjectively assessed. Animals were observed for signs of increased or decreased movement, head droop, ataxia or recumbency, agitation, vocalisation, chewing movements, changes in respiratory patterns and salivation.

4.2.3.2. Intravenous aministration of NSAIDs

The drugs used for these experiments were ketoprofen (3 mg/kg i.v.; Ketofen 10%, Rhône Mérieux, Lyon, France), phenylbutazone (8 mg/kg i.v.; Bute IV, Virbac Laboratories (NZ) Ltd, Auckland, New Zealand), salicylic acid sodium salt (10 mg/kg i.v.; Sigma Chemical Co., St. Louis, MO, U.S.A.), and tolfenamic acid (2 mg/kg i.v.; Tolfedine CS, Vétoquinol Veterinary Pharmaceuticals, Lure Cedex, France).

All drugs were injected into the left jugular vein in a 3 mL dose volume, which was achieved by adding 0.9% saline solution as necessary. Salicylic acid was dissolved in 3 mL 0.9% saline solution and then passed through a sterile 0.2 μm filter (Sartorius, Minisart CE, Goettingen, Germany). Normal saline solution, 3 mL, was used as the control drug. Both, the NSIADs and saline solution were administered in a randomised manner with at least one week interval between

treatment, to which the operator was blind also. The nociceptive testing was performed as described above and, during the time frame of the trial, the behavioural effects produced by the i.v. injection of any drug were subjectively assessed as mentioned before for the i.t. drug injection.

4.2.3.3. Involvement of opioidergic and α-adrenergic systems in the NSAIDs-induced analgesia

In order to investigate the interaction between NSAIDs and spinal adrenergic and opioidergic systems another set of experiments were carried out. The drugs used in these trials were atipamezole HCl (MW 248.75, 100 μL, 4.03 mM; Ciba-Geigy (New Zealand) Ltd, Auckland, New Zealand), fentanyl citrate (10 μg/kg i.v.; Fentanyl Citrate Injection U.S.P., David Bull Laboratories, Melbourne, Australia), ketoprofen (3 mg/kg i.v.; Ketofen 10%, Rhône Mérieux, Lyon, France), naloxone HCl (MW 363.8; 100 μL, 5.49 mM; Sigma Chemical Co., St. Louis, MO, U.S.A.), yohimbine HCl (MW 390.9; 100 μL, 5.11 mM; Himbine, Virbac Laboratories (NZ) Ltd, Auckland, New Zealand), and xylazine HCl (20 μ g/kg i.v.; Phoenix Xylazine 2% injection, Phoenix Pharm Distributors Ltd, Auckland, New Zealand).

Naloxone (pH 5.9) and atipamezole (pH 5.5; when pH was adjusted to 7 drug precipitation occurred) were dissolved in normal saline solution, whereas yohimbine was first evaporated and then reconstituted in double distilled water (pH 5.1; when pH was adjusted to 7 drug precipitation occurred). These three drugs were filtered and i.t. injected in a 100 μL dose volume as explained above. Ketoprofen was i.v. administered in a 3 mL dose volume as mentioned before.

Firstly, it was necessary to demonstrate that the i.t. injection of naloxone blocked the analgesic effect of an i.v. administered opioid receptor agonist. Thus, the analgesic effect of i.v. fentanyl (10 μg/kg) alone and in combination with i.t. naloxone (100 μL, 5.49 mM) was assessed. Similarly, the effect of i.t.

atipamezole and and yohimbine on the analgesia elicited by an i.v. administered α2-adrenoceptor agonist was evaluated. The analgesic effect of xylazine (20 μ g/kg i.v.) alone and in combination with i.t. atipamezole (100 μL, 4.03 mM) and yohimbine (100 μL, 5.11 mM) was studied. In addition, 100 μL i.t. saline solution was used as control drug in combination with i.v. fentanyl and xylazine. The i.t. antagonist administration took place 10 minutes before the i.v. agonist injection. The effect of i.t. naloxone, atipamezole and yohimbine alone on nociceptive thresholds was also studied.

Once it was established an i.t. dose of naloxone and atipamezole that had no effect on nociceptive thresholds and blocked the analgesic effect of i.v. fentanyl and xylazine, respectively, their effect on the analgesia produced by i.v. NSAIDs was evaluated. Intrathecally, naloxone (100 μL, 5.49 mM) and atipamezole (100 μL, 4.03 mM) were separately administered before the i.v. injection of ketoprofen (3 mg/kg). Saline solution, 100 μL i.t., was used again as control drug but now in combination with i.v. ketoprofen. The i.v. drug administration was made 10 minutes after the i.t. drug injection.

Drugs and drug combinations were administered in a randomised manner and the operator was blind to the treatment the sheep were given. Again, an interval of at least one week between treatments was allowed to avoid drug residue interference. The nociceptive testing was performed as described above, but when drug combinations were studied, responses were only taken after administration of the analgesic drug. Behavioural effects produced by any drug or drug combination were subjectively assessed as mentioned before.

4.2.4 Statistical analysis

All the results are expressed as the mean ± s.e.m. An analysis of variance (ANOVA), which assessed the variation produced by each sheep, the drug treatment and time, was used to determine variations in threshold over time. The peak effects of each treatment were compared with pre-drug administration values using a Student's t test. The areas under the threshold change vs. time

curve values for 30 and 60 (AUC30 and AUC60) minutes after i.t and i.v. drug administration, respectively, for individual sheep (with the baseline subtracted) were calculated and compared using a Student's t test. Differences with $P < 0.05$ were considered significant.

4.3. RESULTS

4.3.1. Intrathecal catheterisation of sheep

The results of the i.t. catheterisation of sheep are summarised in Table 4.1. In the sixteen sheep, thirty-one different catheterisations were performed and proper placing of functional catheters was achieved in 54.84% of the attempts. The first three attempts were unsuccessful and insertion of the needle into the subarachnoid space was not possible. By practising on dead sheep it was noticed that the arachnoid membrane seems to be tightly attached to the spinal cord in this species (Figure 4.5). Thereafter, the subarachnoid space was not reached in one more sheep only on those which no previous catheteriation attempts had been performed. Three more catheterisation attempts did not reach the sbarachnoid space, in two of these sheep a catheterisation attempt had taken place before and in the remaining one three attempts had previously been performed.

Only one case required removal of the catheter and it was because of pronociceptive problems in the sheep's front limbs, which also dramatically increased the basal nociceptive thresholds of this animal (from 3.5-4 N to 10-12 N). A few days after removal of the catheter the sheep looked completely normal with no neurological problems, and after 10 days the nociceptive thresholds returned to previous basal levels, which persisted until the end of the trials and even after two successful catheterisations.

Two animals in which the catheter's distal tip migrated rostrally with the tip lying at the level of C3 were reanaesthetised and the catheter was successfully pushed back in at the C5 level on one of them. For the other, a 16G i.v. Teflon

Table 4.1. Summary of the intrathecal catheterisation in the sheep.

Sheep	Attempt	Radiography	Xylazine	Outcome	Comments
1	1	Readjust catheter tip position	Yes	30 days	Knot made with the catheter itself. Postoperative wound infection which reached the spinal canal and produced ataxia. Euthanasia.
2	1	Readjust catheter tip position	Yes	147 days	Knot made with the catheter itself was too tight and next day silicon draining tube was used for anchoring instead.
	2	Catheter tip at C5	Yes	29/2/00	I.v. catheter over the i.t. one was intended but excessive scar tissue prevented for advancing it further in. A Tuohy needle was inserted and silicon draining tube used for anchoring.
3	1	Catheter tip at C5	Yes	150 days	I.t. catheter anchored with silicon draining tube. Rostrally migration with distal tip at C3 level.
	2	Readjust catheter tip position	Yes	22/3/00	I.v. catheter passed over the i.t. one. I.t. catheter anchored with silicon draining tube.
4	1	Readjust catheter tip position	Yes	107 days	I.t. catheter anchored with silicon draining tube.
	2	Unsuccessful surgery	-		Old i.t. catheter unintentionally cut during dissection to pass an i.v. catheter over it. The inner end was not found. Euthanasia.
5	1	Readjust catheter tip position	Not tested	Removed	Pronociceptive problems in both forelimbs. Catheter was pulled out and sheep recovered by itself.
	2	Catheter tip at C5	Yes	56 days	I.t. catheter anchored with silicon draining tube. Rostrally migration with distal tip at C3 level.
	3	Catheter tip at C5	Yes	22/3/00	I.v. catheter over the i.t. one was intended but excessive scar tissue prevented for advancing it further in. A Tuohy needle was inserted and silicon draining tube used for anchoring.
6	1	Readjust catheter tip position	Yes	76 days	I.t. catheter anchored with silicon draining tube. Rostrally migration with distal tip at C3 level.
	2	Readjust catheter tip position	Yes	21/3/00	I.v. catheter passed over the i.t. one. I.t. catheter anchored with silicon draining tube.
7	1	Unsuccessful surgery	-	-	
12	1	Unsuccessful surgery	-	-	
	2	Unsuccessful surgery	-	-	
93	1	Unsuccessful surgery	-	-	
	2	Readjust catheter tip position	Yes	55 days	Pulled out.
	3	Readjust catheter tip position	Yes	60 days	I.t. catheter anchored with silicon draining tube. Pulled out.
	4	Unsuccessful surgery	-	-	
95	1	Readjust catheter tip position	Not tested	21 days	Pulled out.

Table 4.1. Summary of the intrathecal catheterisation in the sheep. (Cont.)

Sheep	Attempt	Radiography	Xylazine	Outcome	Comments
102	1	Readjust catheter tip position	Not tested	6 days	Pulled out.
103	1	Catheter tip at C5	Not tested	14 days	Pulled out.
116	1	Readjust catheter tip position	Yes	17 days	Dead because of lung and liver damage.
385	1	Unsuccessful surgery	-	-	
	2	Unsuccessful surgery	-	-	
404	1	Readjust catheter tip position	No	31 days	Rostrally migration with distal tip at C3 level.
	2	Readjust catheter tip position	Yes	25 days	Catheter from attempt 1 was pushed back in.
	3	Readjust catheter tip position	Yes	75 days	I.v. catheter passed over the i.t. one. I.t. catheter anchored with silicon draining tube. Catheter leakage.
	4	Catheter tip at C5	Yes	45 days	I.v. catheter passed over the i.t. one. I.t. catheter anchored with silicon draining tube. Catheter was removed because of signs of ataxia.
1572	1	Readjust catheter tip position	No	21 days	Rostrally migration with distal tip at C3 level.
	2	Readjust catheter tip position	Yes	33 days	Catheter from attempt 1 was unsuccessfully tried to be pushed back in. Instead, i.v. catheter passed over the i.t. one. Dead because of lung and liver damage.

Figure 4.5. Sheep's head medial section with the spinal cord exposed until the caudal aspect of C2. The Tuohy needle was superimposed on the atlanto-occipital joint to indicate the position where the needle had to be inserted through for the implantationof intrathecal catheters.

catheter (Quick-cath, Travenol Laboratories Inc, Dearfield, IL, U.S.A.) was necessary to reposition a new i.t. catheter. The i.v. catheter was passed over the old i.t. catheter until CSF flowed. This i.t. catheter was replaced with a new one which was freely moved until it reached the C5 level. This procedure was successfully carried out four more times. On two more occasions, however, it was not possible to perform this procedure and the catheter was pulled out to insert a Tuohy needle instead and to place a new catheter. One other time, while dissecting around the old i.t. catheter to pass over the i.v. one, the i.t. catheter was unintentionally cut and the distal part never found. This sheep was killed.

On other fourteen occasions the Tuohy needle was inserted into the subarachoind space and a catheter placed in. Nine of these catheterisations displayed a functional catheter as confirmed by i.t. injection of xylazine, whereas two of them did not respond to the i.t. injection of xylazine. The catheter tip had migrated to the C3 level. The other three catheters pulled out even before xylazine was tried.

In all the cases in which the catheter tip lay at the C5 level, the i.t. injection of 100 µL xylazine produced a marked elevation in the nociceptive thresholds with no obvious supraspinal behaviours such as sedation. This confirmed that the catheters were functional and that the effect of the volume dose was restricted to the spinal area. Two doses of xylazine, 388 and 1940 µM (10 and 50 µg/100 µL, respectively), were used in these experiments and their effect on the nociceptive thresholds seemed to be dose dependent (Figure 4.6). The 388 µM xylazine dose increased the nociceptive thresholds above 5 N from 5 to 60 minutes after drug administration with a peak effect above 8 N at 20 minutes. Thresholds returned to baseline levels after 70 minutes. When the 1940 µM dose was injected, the nociceptive thresholds raised after the 5 minute reading and lasted above the baseline level for up to 3 hours, the time frame of the experiment. From 20 to 90 minutes after xylazine administration the thresholds were above 15 N and at 40 minutes reached almost 19 N. No behavioural changes were observed with any dose of xylazine used in these trials.
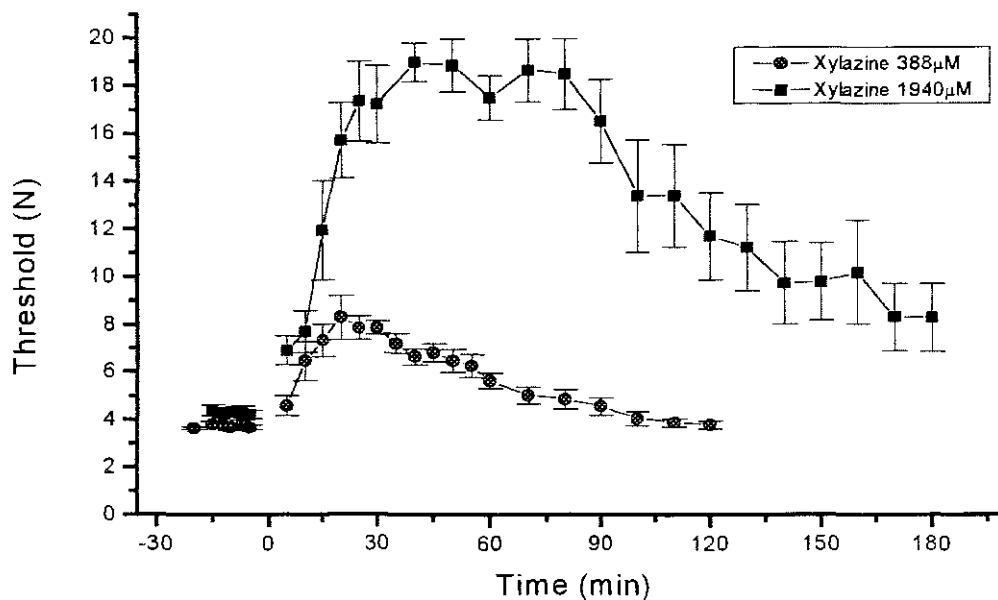
Figure 4.6. Effect of 100 μL xylazine (388 and 1940 μM i.t.) on nociceptive thresholds of healthy sheep over two or three hours after administration (time 0). The data is the mean ± s.e.m. for n = 11 cathterisations on 7 sheep for the 388 μM dose and n = 6 cathterisations on 6 sheep for the 1940 μM dose.

Complications after the placement of the i.t. catheter were rarely seen. All animals, but the one that developed pronociceptive problems, appeared completely normal within a few hours of surgery and remained so until the end of the trials. Only two sheep developed signs of ataxia. In one of them antibiotic treatment was instituted at the same time that the i.t. catheter was removed. This sheep clinically recovered showing normal thresholds after two weeks, which persisted for three more, at which time the sheep was moved to a teaching unit. The other sheep developed postoperative wound infection that appeared to respond to antibiotic therapy; however, a few days later, after the therapy had been suspended, severe ataxia was noticed. This sheep was euthanased and at histopatological examination of the cervical spinal cord there was observed marked diffuse oedema of the leptomeninges and necrosis and cavitation of dorsal horns at the level of C1 and C2.

Two more sheep died during these experiments. Both of them showed extensive adhesions between the lungs, diaphragm and thoracic wall as well as between the liver and the diaphragm. Large (4 – 7 cm) abscesses were present in both organs. Fibropurulent pneumonia and septicaemia were the diagnosis. Histopatological examination of the cervical spinal cord showed in both cases extensive accumulation of neutrophils in the dura matter along with neutrophilic infiltration into both the grey and white matter of the cord, supporting a diagnosis of suppurative spinal meningitis (Figure 4.7).

In these three sheep on which postmortem examination was performed, the position of the catheter tip was visually assessed and in all cases it lay at the C5 level.
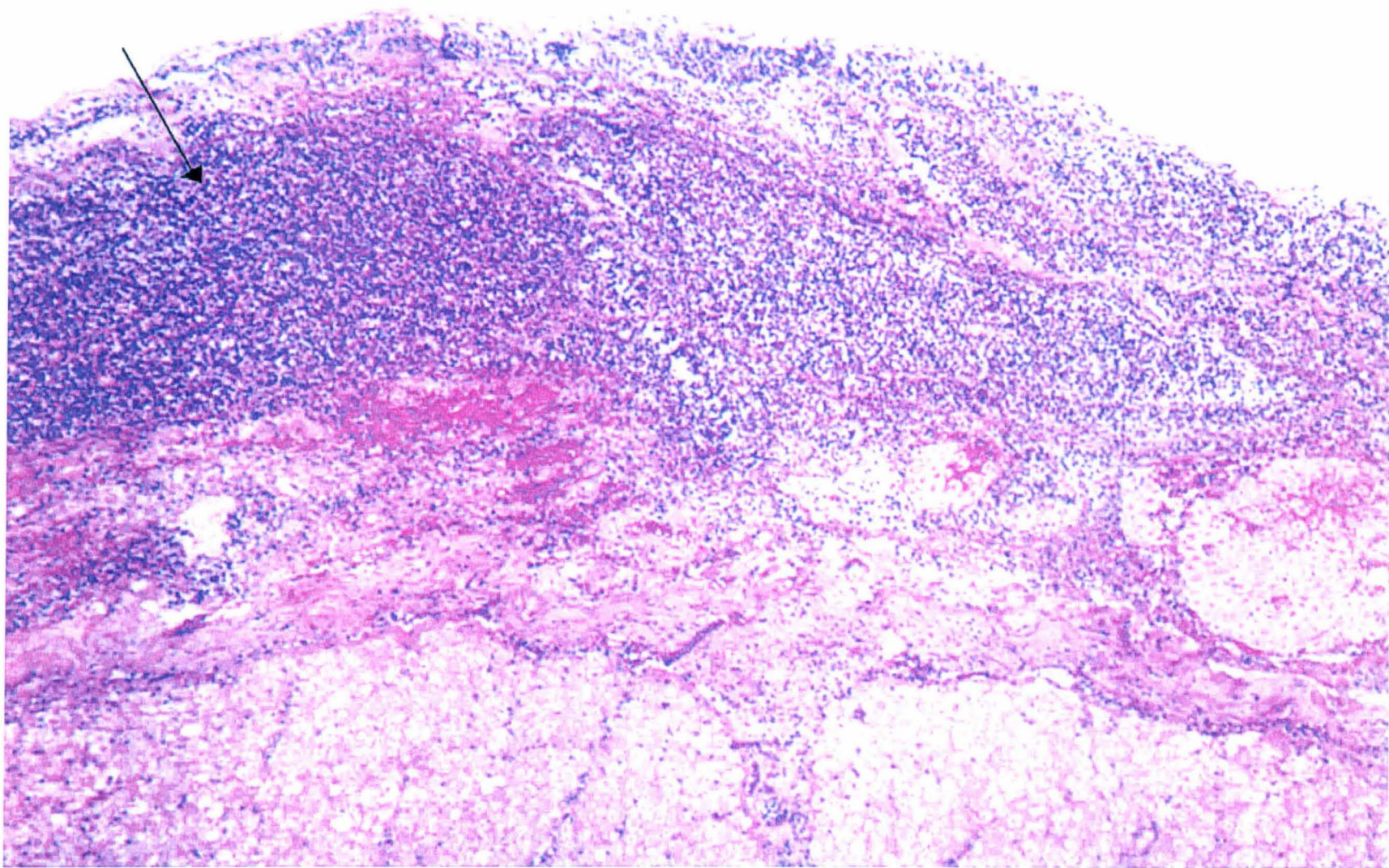
Figure 4.7. Spinal cord histopatology showng neutrophil accumulation in the dura mater (arrow) of sheep no. 116. Magnification 4x.

4.3.2. Intrathecal administration of NSAIDs

The nociceptive thresholds were not significantly raised after the repetitive i.t. administration of saline and from a baseline level of 3.86 ± 0.05 N, the highest threshold value of 4.32 ± 0.14 N was recorded 10 minutes after the second injection of saline ($P = 0.2989$, t-test; Figure 4.8). Similarly, no evident peak effect was observed after the cumulative i.t. injection of NSAIDs at different concentrations. Thus, from a baseline level of 4.27 ± 0.13, 3.76 ± 0.05, 3.84 ± 0.04, and 3.77 ± 0.08 N, the highest threshold value of 5.34 ± 1.34, 4.09 ± 0.13, 4.32 ± 0.14, and 5.88 ± 2.12 N was recorded at 10, 15, 20, and 5 minutes after the administration of 50 µM ketoprofen (Figure 4.9), 12.5 µM phenylbutazone (Figure 4.10), 1.5 µM salicylic acid (Figure 4.11), and 1.5 µM tolfenamic acid (Figure 4.12), respectively ($P \geq 0.3692$, t-test).

Comparison of the AUC30 revealed no significant difference when i.t. normal saline was administered repetitively up to 10 times ($P \geq 0.3556$, t-test) (Figure 4.8). Similarly, the AUC30 for the different doses of i.t. ketoprofen (0.8 – 200 µM), phenylbutazone (0.375 – 200 µM) and salicylic acid (0.375 – 200 µM) showed no significant difference between them ($P \geq 0.2954$; 0.1621 and 0.1022, respectively, t-test) (Figure 4.9 – 4.11). Most AUC30 for i.t. tolfenamic acid doses (0.375 – 200 µM) revealed no significant difference in between ($P \geq 0.0657$, t-test). However, when the AUC30 for the 0.375 µM dose was compared to that of 1.5 and 200 µM a small but significant difference was found ($P = 0.0160$ and 0.0367, respectively, t-test) (Figure 4.12).

When the AUC30 for i.t. ketoprofen, phenylbutazone, salicylic acid and tolfenamic acid doses were compared with their respective saline control injection, no significant difference between treatments was found ($P \geq 0.2956$, 0.3713, 0.3304 and 0.0923, respectively, t-test).

Apart from transitory mild excitement after the i.t. administration of tolfenamic acid in some sheep, no behavioural changes were observed with any i.t. injected NSAID under the conditions here described.
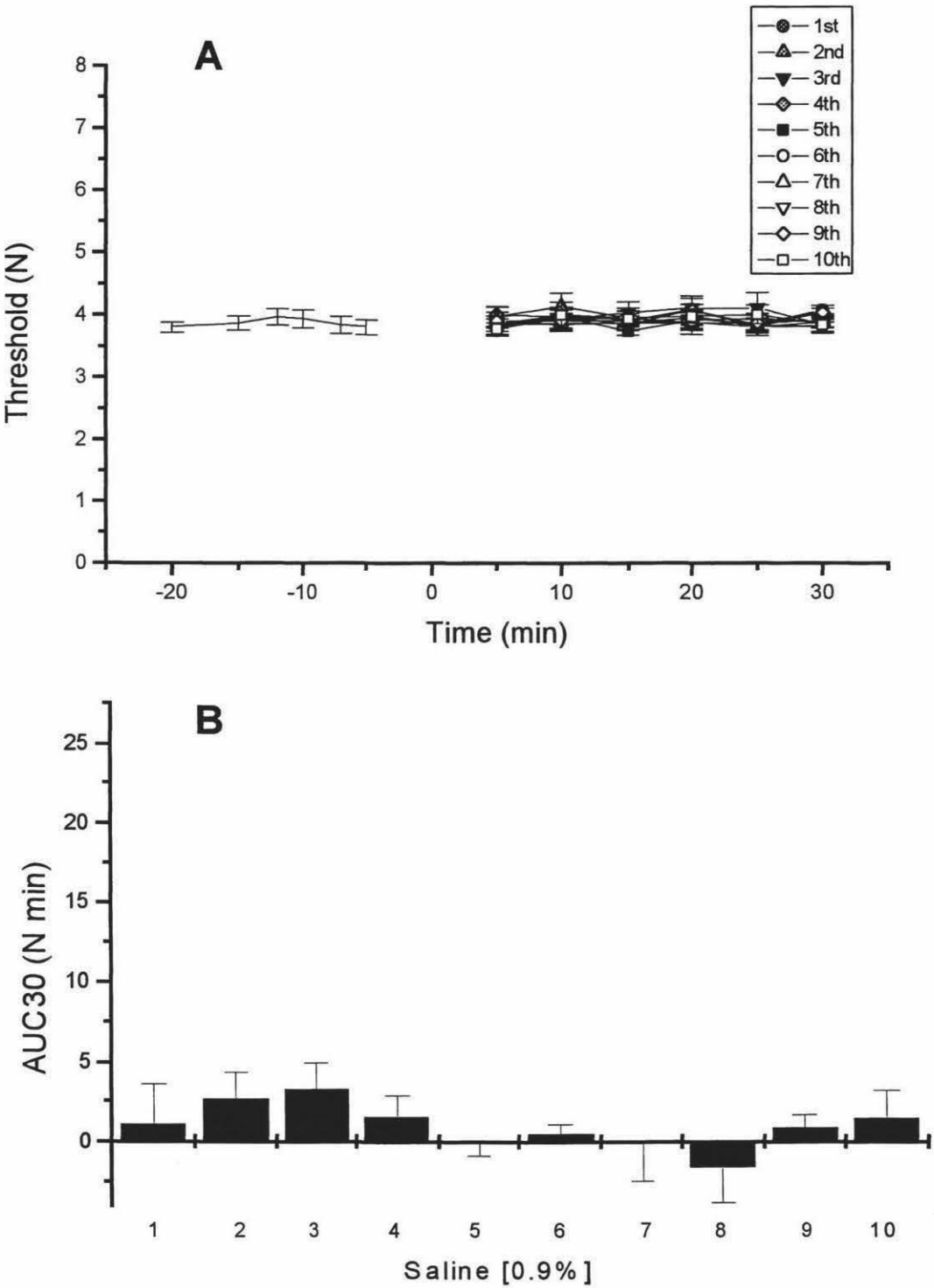
Figure 4.8. Effect of intrathecal repetitive administration of 100 μL saline solution (0.9%) on nociceptive thresholds of healthy sheep over 30 minutes after administration (time 0) (A) and their respective AUC30 (B). The data is the mean ± s.e.m. for n = 5 sheep.
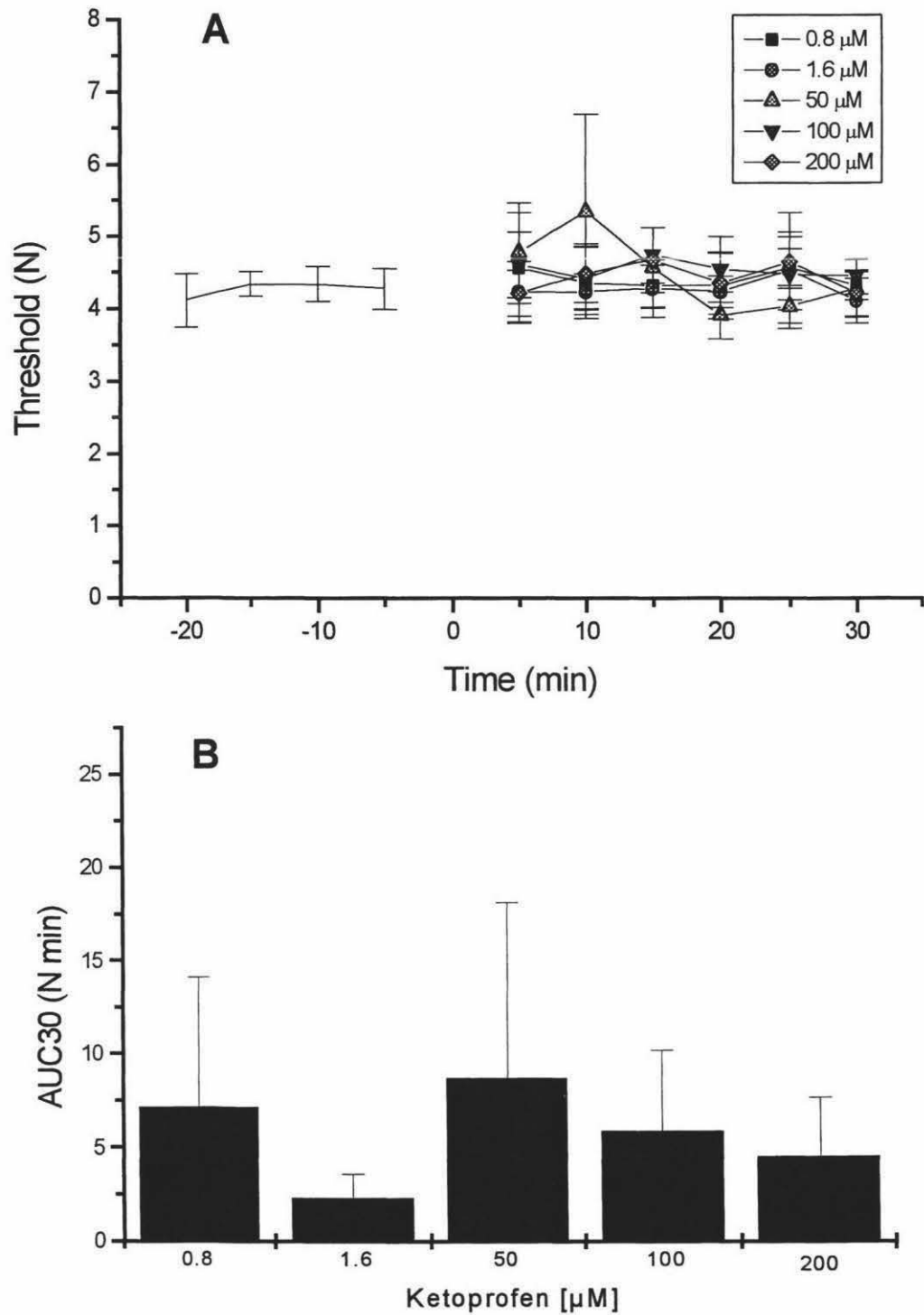
Figure 4.9. Effect of intrathecal cumulative doses of 100 μL ketoprofen (0.8 – 200 μM) on nociceptive thresholds of healthy sheep over 30 minutes after administration (time 0 (A) and their respective AUC30 (B). The data is the mean ± s.e.m. for *n* = 4-5 sheep.
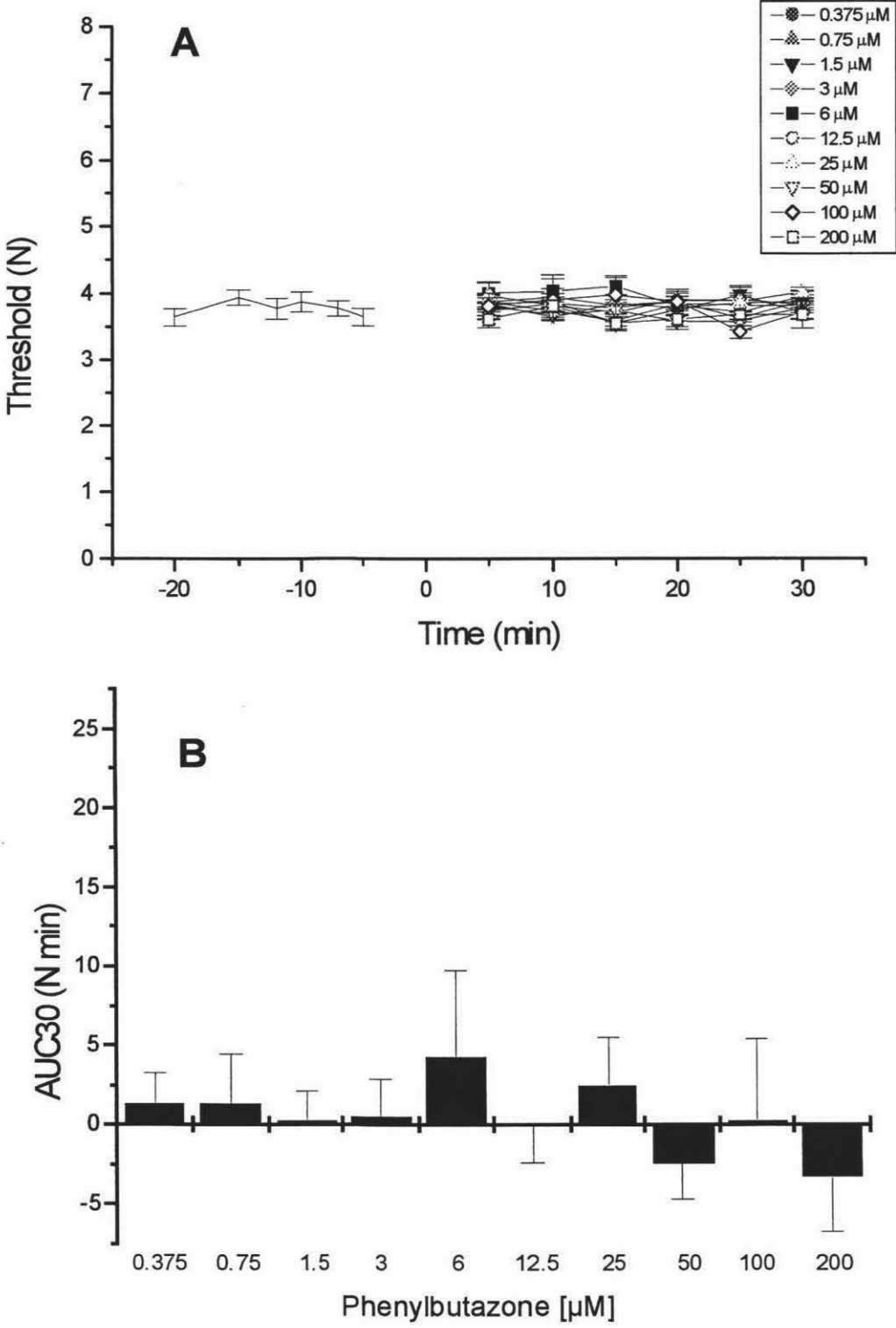
Figure 4.10. Effect of intrathecal cumulative doses of 100 µL phenylbutazone (0.375 –
200 µM) on nociceptive thresholds of healthy sheep over 30 minutes after administration
(time 0) (A) and their respective AUC30 (B). The data is the mean ± s.e.m. for *n* = 5 sheep
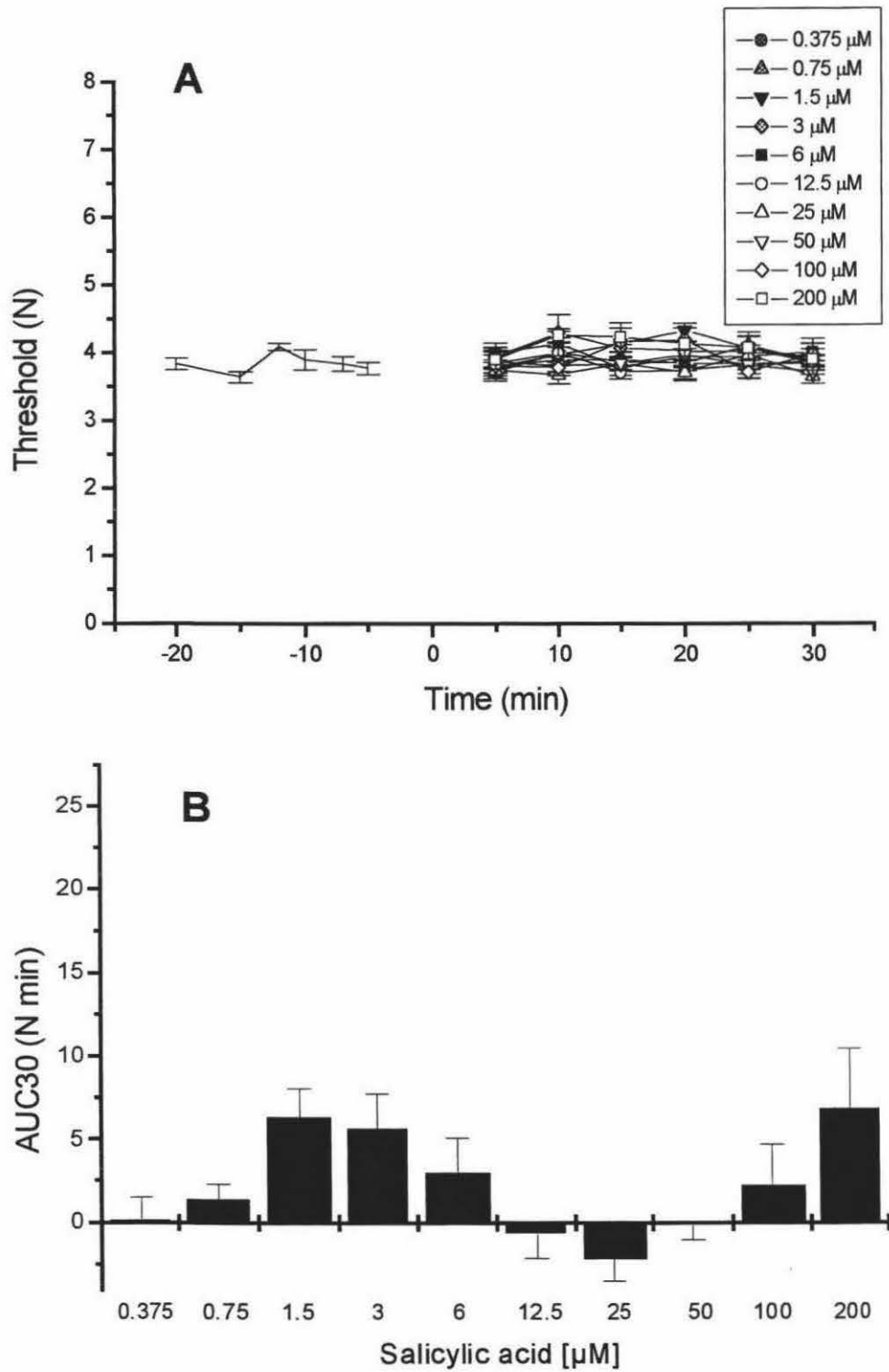
Figure 4.11. Effect of intrathecal cumulative doses of 100 μL salicylic acid (0.375 — 200 μM) on nociceptive thresholds of healthy sheep over 30 minutes afte administration (time 0) (A) and their respective AUC30 (B). The data is the mean s.e.m. for *n* = 5 sheep.
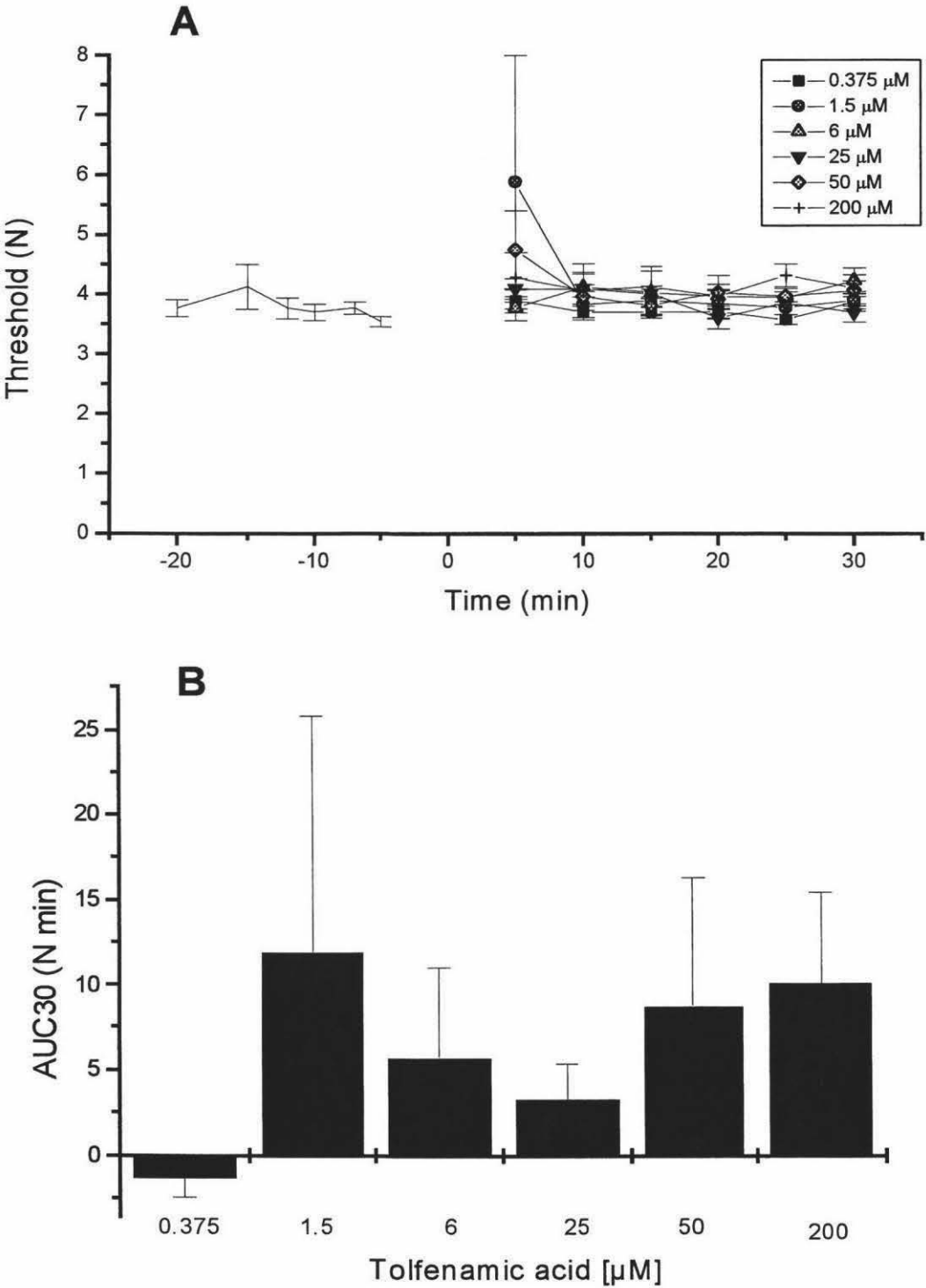
Figure 4.12. Effect of intrathecal cumulative doses of 100 µL tolfenamic acid (0.375
200 µM) on nociceptive thresholds of healthy sheep over 30 minutes after administratio
(time 0) (A) and their respective AUC30 (B). The data is the mean ± s.e.m. for n = 4-
sheep.

4.3.3. Intravenous administration of NSAIDs

From the four NSAIDs utilised plus saline, ketoprofen and tolfenamic acid were the only drugs that increased the nociceptive thresholds under the conditions of these experiments. The nociceptive thresholds were significantly raised after ketoprofen (3 mg/kg i.v.) injection from 3.76 ± 0.04 N to a maximum of 6.59 ± 0.12 N at 25 minutes post-drug administration ($P = 0.0001$, t-test). The thresholds returned to baseline levels by 180 minutes after drug injection (Figure 4.13). Ketoprofen antinociceptive behaviour was followed for up to 360 minutes in two sheep and a second, smaller peak (5.55 ± 0.16 N), not significantly different from pre-drug administration levels ($P = 0.0575$, t-test), was observed at 240 minutes.

Tolfenamic acid (2 mg/kg i.v) had a similar behaviour to that of ketoprofen, although only one peak was observed. The nociceptive thresholds were significantly raised from 3.78 ± 0.04 N up to 6.83 ± 0.66 N at 30 minutes after tolfenamic acid administration ($P < 0.006$, t-test), and returned to baseline levels by 210 minutes (Figure 4.13).

Saline (3 mL i.v.), salicylic acid (10 mg/kg i.v.) and phenylbutazone (8 mg/kg i.v.) showed no obvious peak effect on nociceptive thresholds (from 3.74 ± 0.05, 3.69 ± 0.28 and 3.74 ± 0.05 N to 4.28 ± 0.32, 4.54 ± 0.57 and 4.67 ± 1.06 N at 15, 10, and 25 minutes, respectively, $P > 0.12$, t-test).

Comparison of the AUC60 revealed no significant difference between saline (12.38 ± 2.77 N min), salicylic acid (21.87 ± 9.35 N min), and phenylbutazone (30.65 ± 16.89 N min) ($P \geq 0.2787$, t-test). However when ketoprofen and tolfenamic acid's AUC60 (111.31 ± 5.42 N min and 120.38 ± 14.58 N min, respectively) were compared with that of saline, or even any of the other two NSAIDs, a significant difference was found ($P = 0.0001$, t-test). No significant difference was observed between ketoprofen and tolfenamic acid's AUC60 ($P = 0.5519$, t-test) (Figure 4.14).
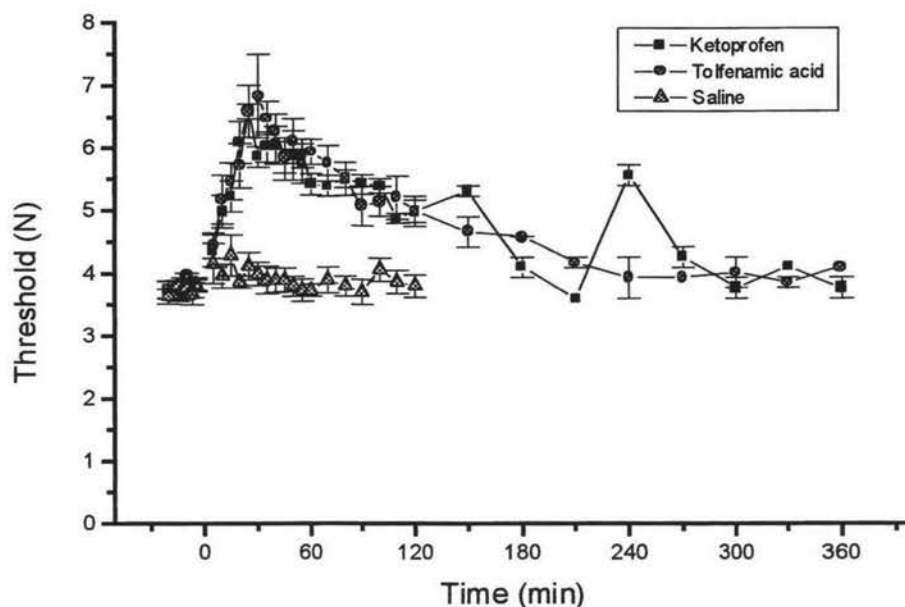
Figure 4.13. Effect of ketoprofen (3 mg/kg i.v.) and tolfenamic acid (2 mg/kg i.v.) on nociceptive thresholds of healthy sheep over six hours after administration (time 0) compared with saline (3 mL i.v.). The data is the mean ± s.e.m. for n = 5-6 sheep from –20 to 120 minutes and n = 2 sheep from 150 to 360 minutes.

When the effect of treatment on nociceptive thresholds was compared over the time, a significant difference was found between ketoprofen and tolfenamic acid in comparison to saline from 10 to 120 minutes after drug injection ($P \le 0.0235$, ANOVA) (Figure 4.15). At 5 minutes post-drug administration saline was not significantly different to ketoprofen and tolfenamic acid ($P \ge 0.4888$, ANOVA). Ketoprofen and tolfenamic acid treatments produced very similar effects on nociceptive thresholds and only at 30 minutes, when tolfenamic acid's peak effect was observed, was a significant difference between treatments found ($P = 0.014$, ANOVA).

Comparison of the effect of phenylbutazone and salicylic acid against that of saline on nociceptive thresholds over the time showed no significant difference at any time ($P > 0.05$, ANOVA).

No behavioural changes with any i.v. administered NSAID were noticed during the frame time of each experiment.
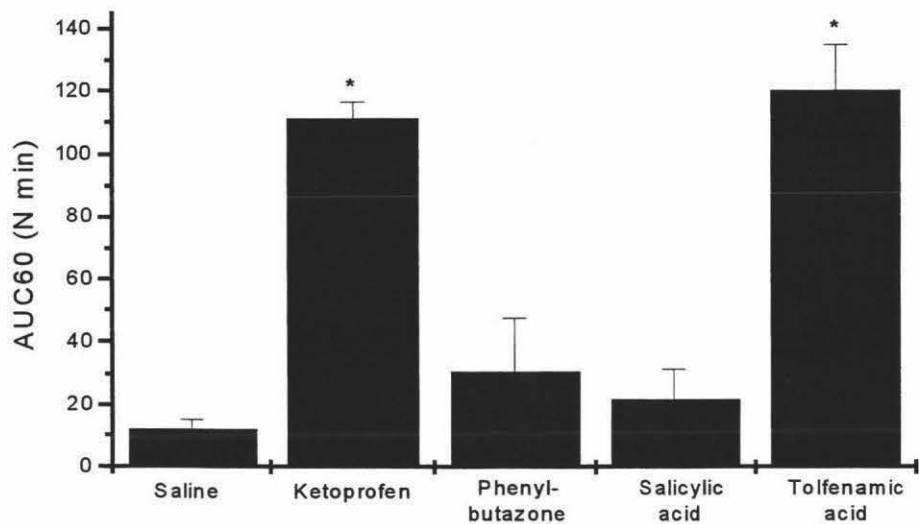
Figure 4.14. AUC60 for i.v. injected NSAIDs. The data is the mean ± s.e.m. for n = 5-6 sheep. * Represents a significant (P = 0.0001, t-test) difference between treatment and saline.
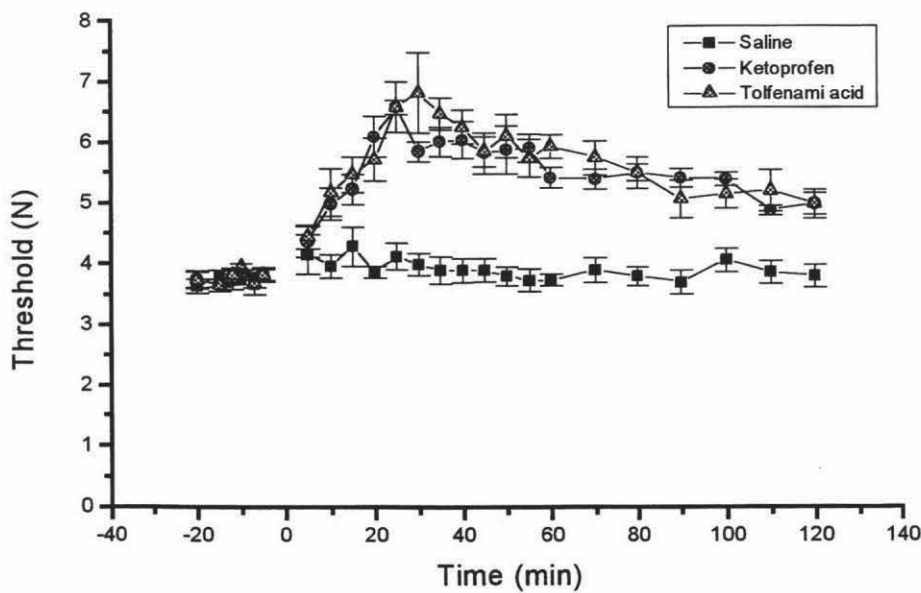
Figure 4.15. Effect of ketoprofen (3 mg/kg i.v.) and tolfenamic acid (2 mg/kg i.v.) on nociceptive thresholds of healthy sheep over two hours after administration (time 0) compared with saline (3 mL i.v.). The data is the mean ± s.e.m. for n = 5-6 sheep.

4.3.4. Involvement of opiodergic and α_2-adrenergic systems in NSAID-induced analgesia

In order to establish any possible interaction at the spinal level between the opioidergic and α_2-adrenergic systems with the analgesia produced by the i.v. administration of NSAIDs, it was necessary to find a high enough i.t. dose for opioid and α_2-adrenergic drug receptor antagonists to block the analgesia produced by i.v. fentanyl and xylazine, respectively, without affecting baseline nociceptive thresholds when these receptor antagonists were i.t. injected alone.

4.3.4.1. Blockade of spinal opioid receptors

The i.v. administration of fentanyl (10 µg/kg) alone produced a marked increase in the nociceptive thresholds, reaching its peak over 12 N at 5 minutes after drug administration and returning to baseline levels around 45 minutes (Figure 4.16). The AUC60 for fentanyl i.v. alone (207.37 ± 32.68 N min) was significantly different from that of 3 mL i.v. 0.9% saline solution (12.38 ± 2.77 N min) (P = 0.0001, t-test) (Figure 4.16).

Once it was demonstrated that i.v. fentanyl raised the nociceptive thresholds in sheep, a 100 µL dose i.t. naloxone (5.49 mM) was challenged to block the fentanyl-induced analgesia. At this dose, naloxone significantly reduced the i.v. fentanyl AUC60 by 89.95%, producing an AUC60 of only 20.84 ± 13.9 N min for the combination i.t naloxone / i.v. fentanyl (P = 0.0001, t-test) (Figure 4.16). Furthermore, i.t. naloxone alone had no effect on the nociceptive thresholds when these were read 10 minutes post-drug administration and then for up to 60 minutes (Figure 4.16), yielding an AUC60 of 1.44 ± 4.50 N min (Figure 4.16).

To be sure that the reduced analgesic effect of fentanyl in the combination i.t. naloxone / i.v. fentanyl was due to an antagonist effect on opioid receptors in the spinal cord and not just to a volume effect, i.t. 0.9% saline solution (100 µL) followed by i.v. fentanyl was injected. Saline failed to block the fentanyl-mediated analgesia (Figure 4.16) and an AUC60 equivalent to 87.82% for that

of i.v. fentanyl alone was obtained (182.12 ± 25.82 vs. 207.37 ± 32.68 N min; P = 0.3639, t-test) (Figure 4.16).

Immediately after the i.v. administration of fentanyl alone or in combination with i.t. saline, sheep were excited and chewed compulsively and in some cases some ataxia, recumbency and salivation were noticed. These behavioural changes were transitory, lasting around 30 to 40 minutes, but by the end of the experiment all sheep looked calm and normal. When naloxone was i.t. pre-administered, these fentanyl-mediated behavioural changes were less marked and only milder excitation and moderate chewing behaviour were observed in some sheep. Naloxone i.t. alone and saline i.v. did not produce any behavioural change in the sheep.

4.3.4.2. Blockade of spinal α_2-adrenoceptors

Xylazine (20 µg/kg i.v.) alone raised the nociceptive thresholds from 5 minutes after drug administration, reached its peak between 10 to 15 minutes with more than 9 N and gradually returned to baseline levels at about 45 minutes (Figure 4.17). The AUC60 was 149.31 ± 21.95 N min, which was significantly different from i.v. saline solution alone (12.38 ± 2.77 N min) (P = 0.0001, t-test) (Figure 4.17).

After it had been established that xylazine produced antinociception in the sheep, yohimbine (100 µL, 5.11 mM) was i.t. preinjected to prevent this hypoalgesic effect. Surprisingly, yohimbine increased, instead of decreasing, the nociceptive thresholds in two sheep on which the drug combination was employed. Nociceptive thresholds were elevated over 13 N after 10 minutes post-agonist drug administration and returned to baseline levels after 240 minutes (Figure 4.18). In one sheep on which yohimbine alone was injected, no significant effect on nociceptive thresholds was observed over 210 minutes. No further experiments were done with yohimbine.
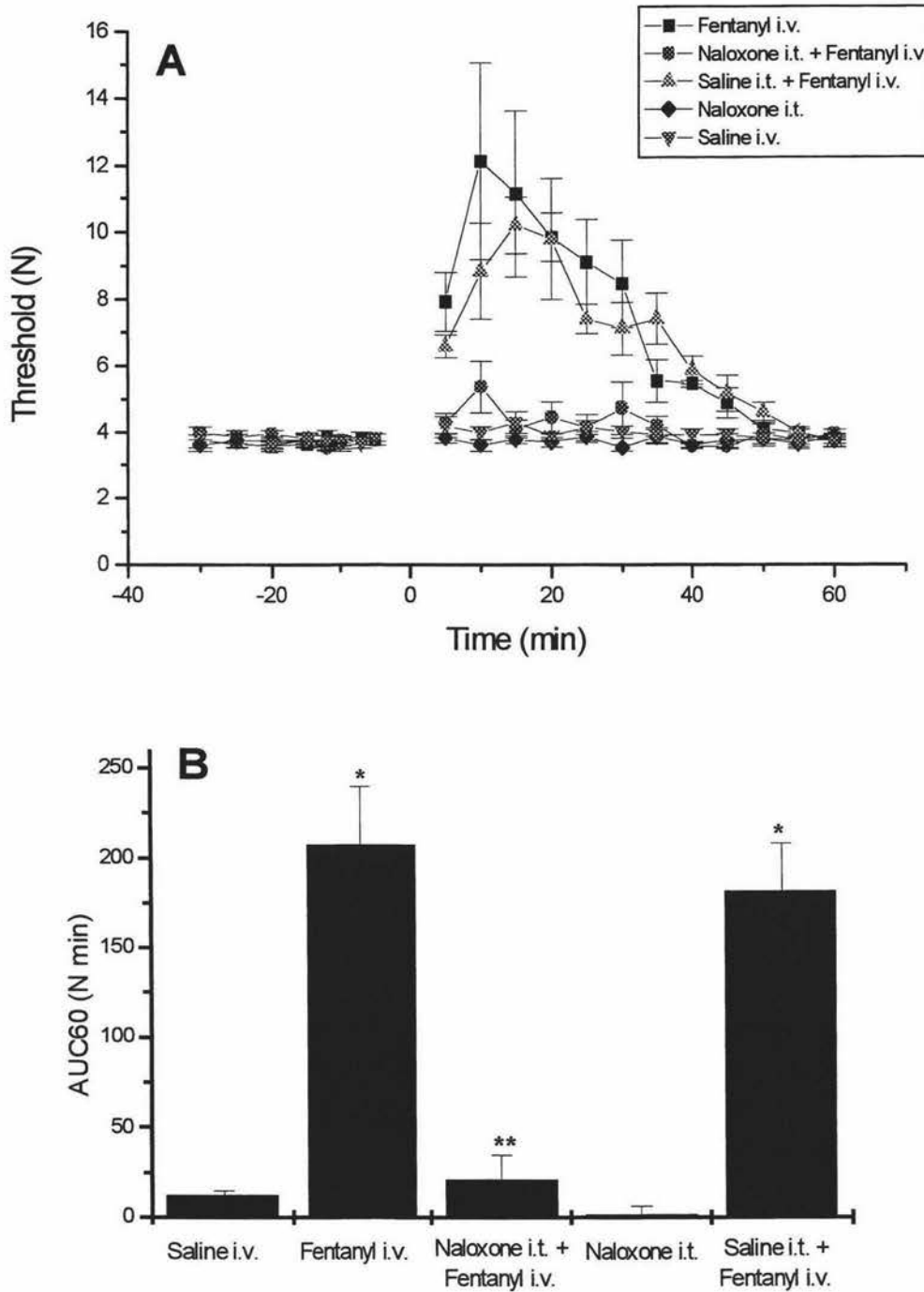
Figure 4.16. Effect of fentanyl (10 µg/kg i.v.) and naloxone (100 µL, 5.49 mM i.t.) alone and the combinations i.t. naloxone + i.v. fentanyl and i.t. saline (100 µL) + i.v. fentanyl on nociceptive thresholds of healthy sheep over one hour after administration (time –10 and 0 for i.t. and i.v. treatments, respectively) compared with saline (3 mL i.v.) (A) and their respective AUC60 (B). The data is the mean ± s.e.m. for n = 4 sheep, except saline i.v. where n = 5. * Represents a significant (P = 0.0001, t-test) difference between treatment and saline and ** between treatment and fentanyl i.v. alone.

Figure 4.17. Effect of xylazine (20 µg/kg i.v.) and atipamezole (100 µL, 4.03 mM i.t.) alone and the combinations i.t. atipamezole + i.v. xylazine and i.t. saline (100 µL) + i.v. xylazine on nociceptive thresholds of healthy sheep over one hour after administration (time –10 and 0 for i.t. and i.v. treatments, respectively) compared with saline (3 mL i.v.) (A) and their respective AUC60 (B). The data is the mean ± s.e.m. for n = 4 sheep, except saline i.v. where n = 5. * Represents a significant (P = 0.0001, t-test) difference catween treatment and saline and ** between treatment and xylazine i.v. alone.

Figure 4.18. Effect of xylazine (20 µg/kg i.v.; mean ± s.e.m. *n* = 4) and yohimbine (100 µL, 5.11 mM i.t.; *n* = 1) alone and the combinations i.t. yohimbine + i.v. xylazine (mean ± s.e.m. *n* = 2 from –30 to 120 minutes and *n* = 1 from 130 to 330 minutes) and i.t. saline (100 µL) + i.v. xylazine (mean ± s.e.m. *n* = 4) on nociceptive thresholds of healthy sheep for up to 5.5 hours after administration (time –10 and 0 fot i.t. and i.v. treatments, respectively) compared with saline (3 mL i.v.; mean ± s.e.m. *n* = 5).

Atipamezole was i.t. injected to prevent i.v. xylazine-mediated analgesia. The selected dose of 100 µL, 4.03 mM inhibited by 88.13% the AUC60 of i.v. xylazine alone, generating an AUC60 of 17.72 ± 13.57 N min for the coadministration i.t. atipamezole / i.v. xylazine (*P* = 0.0001, *t*-test) (Figure 4.17). Atipamezole alone had no significant effect on the nociceptive thresholds when these were read 10 minutes post-drug administration and then for up to 60 minutes (Figure 4.17), producing an AUC60 of -10.19 ± 9.31 N min (*P* = 0.3121, *t*-test; when compared to i.v. saline alone) (Figure 4.17).

To demonstrate that atipamezole inhibition of xylazine-mediated antinociception was not due to a mere volume effect, the analgesic effect of i.t. saline solution (0.9%, 100 µL) followed by i.v. xylazine was evaluated. Saline failed to block the increase in nociceptive thresholds induced by the i.v. injection of xylazine. In fact, very similar results in comparison to those from i.v. xylazine alone were obtained (Figure 4.17). The AUC60 for this drug combination (141.85 ± 24.92 N min) was not found significantly different from that of i.v xylazine alone (*P* = 0.7479, *t*-test) (Figure 4.17).

One to two minutes after xylazine being administered transit short lasting (1-2 minutes) hyperventilation was observed, soon after light sedation for 15-20 minutes was perceived as well. Yohimbine and atipamezole produced no obvious behavioural effects and had no moderating influence on xylazine-induced behavioural changes.

4.3.4.3. Influence of spinal opioid and α_2-adrenergic receptor blockade on i.v. ketoprofen-induced analgesia

The i.t. injection of naloxone and atipamezole, at doses that blocked the analgesic action of fentanyl and xylazine i.v., respectively, and had no effect on baseline thresholds (100 µL, 5.49 mM and 100 µL, 4.03 mM, respectively, Figures 4.16 and 4.17), 10 min before i.v. ketoprofen administration (3 mg/kg) reduced the AUC60 by 85.64% and 91.78%, respectively, when compared to that of ketoprofen alone (15.98 ± 13.66 and 9.15 ± 8.08 vs. 111.31 ± 5.42 N min; P = 0.0001, t-test) (Figure 4.19). Intrathecal preadministration of saline solution had no significant effect on the i.v. ketoprofen AUC60 by reducing it by 2.46% (108.57 ± 9.82 vs. 111.31 ± 5.42 N min; P = 0.8297, t-test) (Figure 4.19).

Comparison of the AUC60 for i.v. ketoprofen alone and i.t. saline plus i.v. ketoprofen against that for i.v. saline alone (111.31 ± 5.42 and 108.57 ± 9.82 vs. 12.38 ± 2.77 N min, respectively) revealed a significant difference between treatments (P = 0.0001, t-test) (Figure 4.19). However, no significant difference was found between the AUC60 for saline i.v. alone and the combinations i.t. naloxone and atipamezole plus i.v. ketoprofen (12.38 ± 2.77 vs. 15.98 ± 13.66 and 9.15 ± 8.08 N min; P = 0.7881 and 0.8091, respectively, t-test) (Figure 4.19).
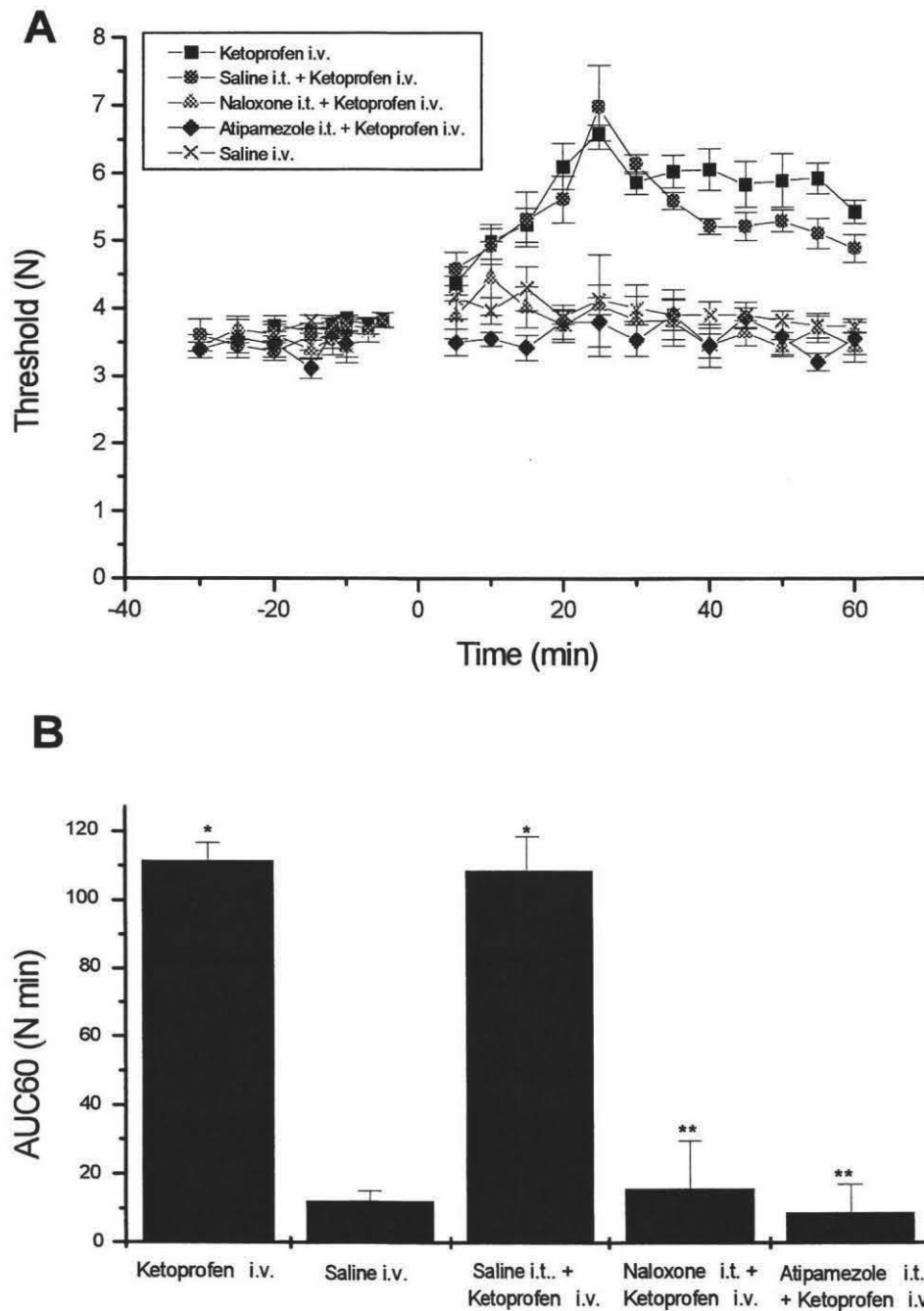
Figure 4.19. Effect of ketoprofen (3 mg/kg i.v.) alone and the combinations i.t. saline (100 μL) + i.v. ketoprofen, i.t. naloxone (100 μL, 5.49 mM) + i.v. ketoprofenand and i.t. atipamezole (100 μL, 4.03 mM) + i.v. ketoprofen on nociceptive thresholds of healthy sheep over one hour after administration (time −10 and 0 for i.t. and i.v. treatments, respectively) compared with saline (3 mL i.v.) (A) and their respective AUC60 (B). The data is the mean ± s.e.m. for n = 6 sheep, except saline i.v. where n = 5. * Represents a significant (P = 0.0001, t-test) difference between treatment and saline and ** between treatment and ketoprofen i.v. alone.

4.4. DISCUSSION

4.4.1. Intrathecal catheterisation in sheep

4.4.1.1. Catheter implantation technique

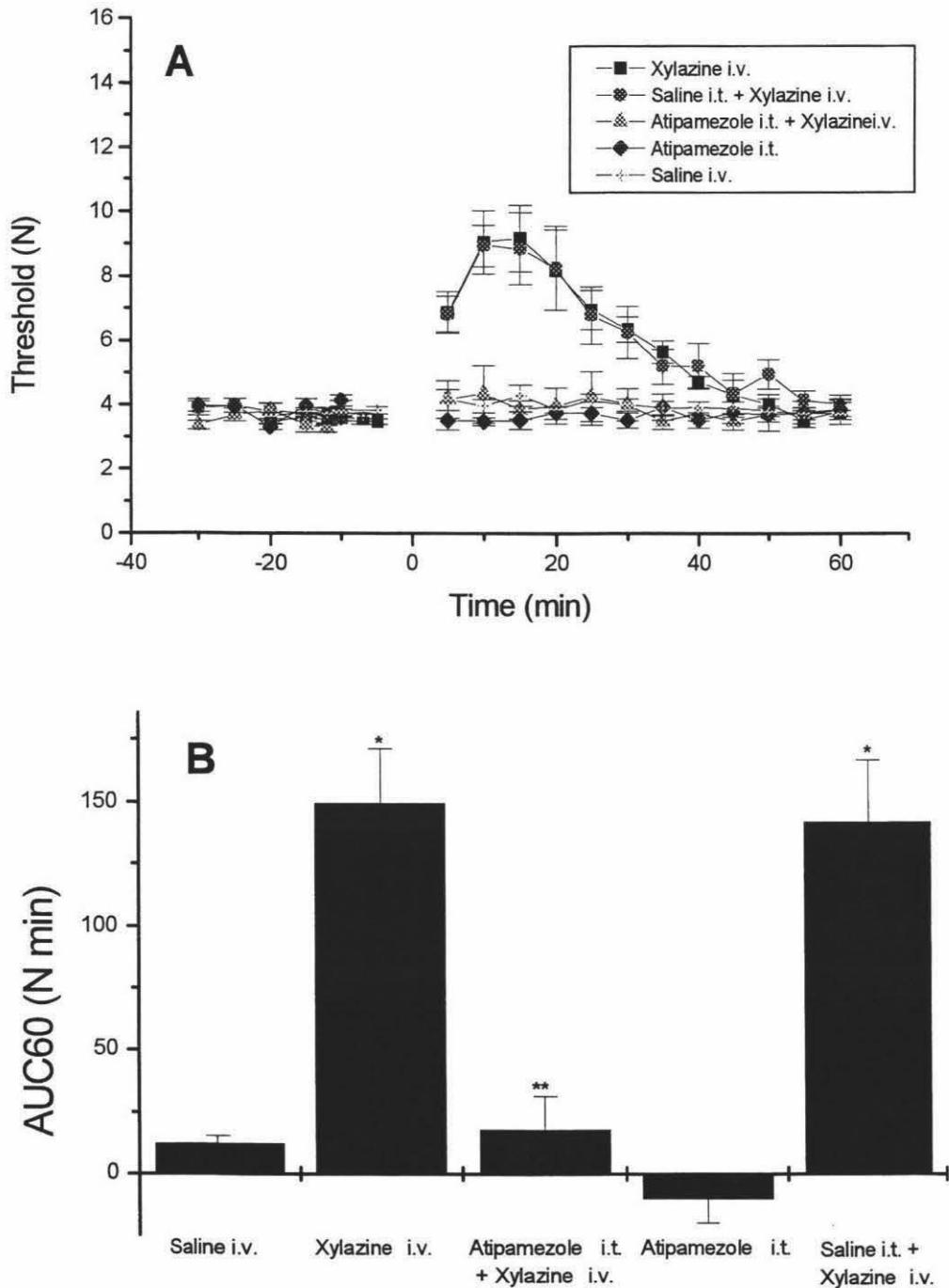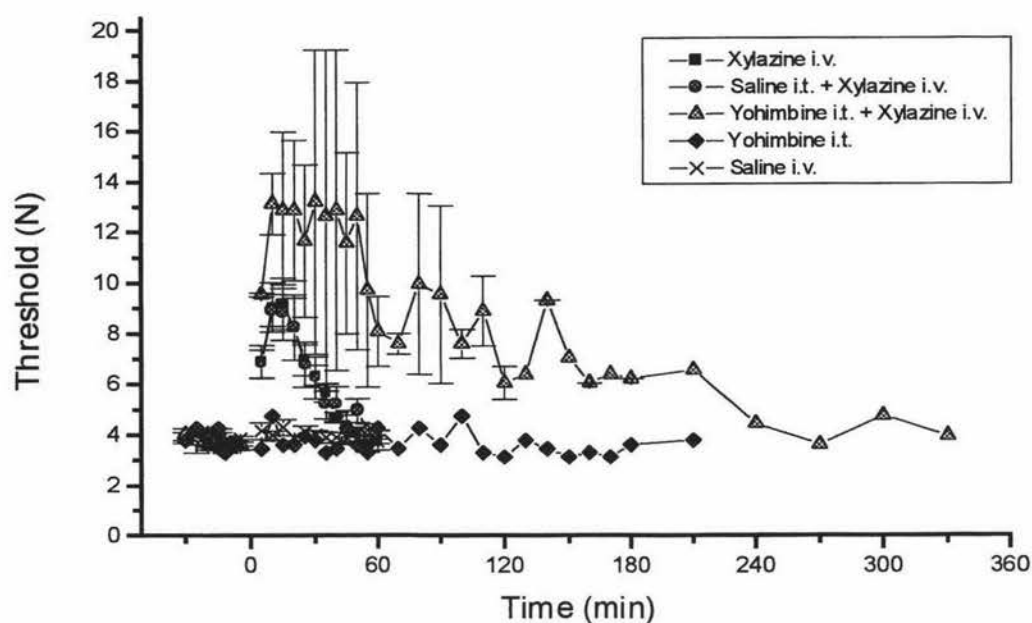The catheterisation of the spinal subarachnoid space is a well-established experimental technique in various animal species, such as rats (Malmberg & Yaksh, 1992b), cats (Chua *et al.* 1998), sheep (Kyles *et al.* 1992) and is used clinically in humans (Follett & Naumann, 2000) for anaesthesia or analgesia. This allows the investigation of spinal mechanisms of drug action as well as providing a method of treating intractable pain. The catheterisation procedure in sheep is technically difficult and review of the sheep's cervical anatomy and practice on dead sheep were necessary. It was noticed that roughly a distance of about 3 to 4 cm separates the brachiocephalic muscles from the spinal canal at the level of the atlanto-occipital joint and that the arachnoid membrane seems to be tightly attached to the spinal cord in mature Romney-cross sheep. With a good knowledge of the anatomy and taking care to keep the needle exactly in the midline, the i.t. catheterisation technique was relatively straightforward and quick. The 54.84% overall successful rate for correctly placing of functional i.t. catheters in this study was very similar to the 58.82% reported by a research group familiar with this technique (Kyles *et al.* 1992). An important issue for the correct needle placement into the subarachnoid space is the insertion of the needle through the middle line in a parallel position to the sheep's neck. Otherwise it is too easy to miss the small area through which the needle has to penetrate the meninges on the approximate 1 cm diameter sheep's cervical spinal cord (Figure 4.5).

4.4.1.2. Catheter maintenance

The catheters were daily flushed with 1 mL sterile 0.9% saline solution to prevent blockage, compared to once a week with 0.35 mL injected by Kyles *et al.* (1992) in their sheep. These same authors suggested that scar tissue

formation in blocked catheters depends on the length of time the catheter has been in position and the number of injections made through it. We did not find fibrous plugs in catheters that were removed even after being in place and in regular use for up to 150 days. This procedure neither affected the nociceptive thresholds of sheep, since similar baseline levels were obtained every experiment. Therefore, it seems that daily flush of i.t. catheters in sheep is a harmless, adequate procedure that helps to maintain catheters in good operating order.

4.4.1.3. Catheter replacing and anchoring

Some catheters needed replacing when the tip migrated rostrally, when there was leakage as result of damage to the catheter, or when the catheter pulled out completely. One catheter that migrated rostrally was successfully pushed back in; however, it migrated again 25 days after (sheep no. 404). This procedure was unsuccessfully tried on another rostrally migrated catheter (sheep no. 1572). In both occasions, while trying to push the catheter back in a lot of resistance was noticed and the catheter bent at different points which could have damaged its integrity, making the effort not worthwhile. This procedure was not repeated. An alternative technique was used where an i.v. catheter was passed over the old i.t. catheter until CSF flowed and this i.t. catheter was pulled out and replaced with a new one which was freely moved through the i.v. catheter until it reached the C5 level. This was very effective for replacing five catheters (sheep no. 3, 6, 404 twice, 1572). However, with both procedures, pushing the i.t. catheter back in and passing the i.v. catheter over i.t. one, there is always a higher risk of contamination than when a completely new catheter is to be placed because the existing catheter can never be completely sterilised. Meticulous antiseptic preparation of the area as well as the catheter resulted in clinical manifestations of spinal infection in one animal only, which had four different catheterisations (sheep 404). Nevertheless, there is a chance that microorganism contamination could have occurred even in the absence of neurological clinical signs. In humans, for example, bacterial contamination was found in 18% of the needles used for spinal-epidural

anaesthetic procedures even after aseptic technique skin preparation (Raedler *et al.* 1999); hence, strict hygienic measures are recommended when a foreign object is to be introduced into the spinal canal.

The technique of passing an i.v. catheter over the i.t. one was unsuccessfully performed three times. One attempt ended with the i.t. catheter being unintentionally cut while dissection of the surrounding tissue was taking place. The inner end of the catheter was not found and the ewe was killed (sheep no. 4). Therefore, extremely careful dissection around the i.t. catheter is necessary when performing this technique and failure to do so could lead to the worst scenario such as is described here. The two other attempts failed because excessive scar tissue formation around the i.t. catheter prevented the i.v. one passing over it (sheep no. 2, 5). One of these sheep had had a catheter for 147 days and the other one for 56 days on its second catheterisation; hence, it seems reasonable to suggest that the chances of performing a successful catheterisation using this procedure are inversely related to the period of time the i.t. catheter has been placed in and to the number of previous catheterisations. In these cases, the best thing to do, perhaps, is to completely pull the catheter out and try to insert a Tuohy needle; however, the spinal needle placement is more difficult under these circumstances (Kyles *et al.* 1992). These difficulties were experienced in sheep no. 93 on which, after three catheterisation attempts and being catheterised for over 100 days, it was not possible to properly place a Tuohy needle.

Needle and catheter placement were successfully carried out on 15 different occasions (sheep no. 1, 2, 3, 4, 5 twice, 6, 93 twice, 95, 102, 103,116, 404, 1572). However, three of these catheters (sheep no. 95, 102, 103) pulled out completely before even trying xylazine and two more (sheep no. 404, 1572) migrated rostrally and no analgesia was observed when xylazine was administered through them. Thus, to keep catheters in place became a major problem and even when into the subarachnoid space, if the tip was not at the C5 level no analgesic drug effect was perceived with the algesiometric test used here.

A possible reason for this migration could be attributed to the catheter design itself (*e.g.* wall thickness, fabrication material), which is known to have an impact upon complication-free "survival" of the catheter (Follett & Naumann, 2000). Actually, the manufacturer changed the material of the catheter and this has resulted in chronic kinking and occlusions in different parts of the world[1]. We continued working with the same type of catheters because we had them in stock and we did not realise, until later, that the new material could be causing this problem. A number of different anchoring techniques for the catheters were used, a knot made with the catheter itself was tried twice (sheep no. 1, 2) but blockage of the catheter was noticed. To anchor the catheter, a Yates silicon draining tube (Figure 4.2) was used instead twelve different times (sheep no. 2 twice, 3 twice, 4, 5 twice, 6 twice, 93, 404 twice). This technique was more effective in keeping catheters in the right position for longer period of time than by only suturing the catheter in place where it emerged from the subcutaneous tissue. The occurrence of migration is associated with failure to anchor the catheter, and in humans, a migration incidence of more than 45% for non-anchored catheters was reported (Follett & Naumann, 2000).

4.4.1.4. Assessment of catheter functionality

Catheter insertion in a length of about 25 to 28 cm was estimated as necessary to reach the C5 level in dead sheep. In most experimental animals, this length was enough to position the catheter tip around C4 and C5, as it was assessed radiographically. If catheter tip was not placed at the C5 level, it was repositioned until it got in the right place. In all cases, radiographic confirmation of catheter placement, catheter tip position at the C5 level as well as assessment of the integrity of the catheter was used to validate the catheterisation technique (Figure 4.1). This radiographic procedure has been accurately used by others to assess the functional integrity of the catheters (Kyles *et al.* 1992). In our study, catheter functionality was evaluated by administering the α_2-adrenoceptor agonist xylazine through them and assessing its effect on mechanical nociceptive thresholds.

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[1] J.P.Chambers 2000, E-mail 27 March.

Xylazine is known to produce good analgesia in sheep when administered through the i.t. route in a 100 μL volume and this effect has been solely attributed to spinal mechanisms because the bulk of radioactivity from [^{3}H]-clonidine (Waterman et al. 1988) and [^{3}H]-idazoxan (Kyles et al. 1993) injected in such a volume was distributed within two to three vertebral segments to either side of the catheter tip and because no detectable behavioural changes such as sedation were perceived after the i.t. injection of xylazine (Waterman et al. 1988; Kyles et al. 1993). The two doses of xylazine used in this study (10 and 50 μg/ 100 μL; equivalent to 388 and 1940 μM, respectively) produced dose dependent analgesia when the catheter tip was located at the C5 level. Similar results were reported by Waterman et al. (1988). The major difference between the two studies was observed with the 50 μg dose. Waterman and co-workers observed that mechanical nociceptive thresholds returned to baseline level after 120 min of xylazine being administered (Waterman et al. 1988), whereas this study recorded hypoalgesia for up to 180 min, the time frame of the experiment, with thresholds not returning to baseline levels within that time. This longer lasting xylazine-mediated analgesia seems unlikely to be due to a combined spinal-supraspinal action of the drug in our experiments since no sedation was evident. This effect was not investigated further. Nevertheless, this probably reflects different levels, subtypes and distribution of α_2-adrenoceptors in the spinal cord of these two distinct sheep populations (Clun-cross and Romney-cross). The antinociceptive effects of i.v. xylazine (50 μg/kg), for instance, varied in different breeds of sheep (Ley et al. 1990) but no conclusive information in this respect is available when xylazine is i.t. injected. Other possible explanations might include differences in the operation technique of the mechanical nociceptive device in both trials and the recovery time between insertion of the i.t. catheter and xylazine testing. Waterman et al. (1988) allowed at least one-week intervals whereas we allowed at least two before the α_2-adenoceptor agonist was administered.

While in use, catheters were periodically tested to verify that they were working properly. Regular injection of xylazine through the catheter is recommended for this purpose (Kyles et al. 1992) and here a 10 μg dose proved to be very useful.

When no antinociception was achieved after i.t. xylazine injection, catheter replacing was carried out as explained above.

4.4.1.5. Complications

Apart from catheter migration, complications with the catheterisation technique were rarely seen. Only two sheep (1, 404) developed clinical signs of spinal meningitis including ataxia of both rear and front limbs. In one of them (1) postoperative wound infection was detected and although response to antibiotic therapy was perceived, microorganisim migration into the spinal canal must have occurred because severe ataxia was noticed some days after treatment discontinuation. The sheep was destroyed and at postmortem examination the severe pathological changes in the cervical spinal cord matched the diagnosis of meningitis and neurodegeneration. The other sheep (404) had had four catheterisations and over 175 days of catheter placement before any clinical sign was noticed. This ewe was treated similarly and, in addition, the i.t. catheter was removed. Clinical improvement was observed and this animal was returned to a teaching unit. Infection has also been recognised as the most common complication after i.t. cateterisation in humans, counting for 30% of all complications with meningitis representing 4% (Follett & Naumann, 2000). Thus, although spinal meningitis was observed in 12.5% of the sheep (2 out of 16), when the number of catheterisations, the time that catheters were kept in place and the number of experiments run are taken into account, it seems that the technique of i.t. catheterisation in sheep offers a relatively safe way to evaluate the spinal antinociceptive effects of analgesic drugs. However, complications have to be expected when working with i.t. catheters and it seems that, at least in humans, complication rates do not necessarily decrease as experience is gained in implantation techniques (Follett & Naumann, 2000); an issue perceived during this trial.

Two other sheep (116, 1572) that came from a different farm than the others died of pneumonia and septicaemia with the cervical spinal cord showing histological changes consistent with suppurative spinal meningitis. These

changes in the CNS could be attributed to the septicaemia itself. It seems that a greater chance of infection exists when a surgical procedure is performed in an animal with septicaemia. Therefore, it could be argued that these changes were due more to the animal's condition than to the implantation technique. However, as mentioned before, the risk of contamination when introducing a foreign object into the spinal canal is always present (Raedler *et al.* 1999); hence, although less unlikely, spinal infection via the i.t. catheter cannot be ruled out.

On only one occasion (sheep no. 5) postoperative neurological problems, consistent with pronociceptive deficits in both front limbs, were observed. This was a minor misfortune when compared to the reported laceration and hemorrhage of the spinal cord or the insertion of a catheter into the central canal of the spinal cord while practising this technique (Kyles *et al.* 1992). Nociception in this sheep was altered and by simply removing the catheter the ewe went back to normal within a few days suggesting that the catheter must have been compressing some nerves.

4.4.2. Mechanical nociceptive testing

The mechanical nociceptive apparatus proved to be a reliable, inexpensive way to assess the analgesic action of drugs in sheep. The device was made mainly from medical waste material that was available all the time, making replacing of the parts not a problem. The polyethylene tube connecting the device to the three-way valve and the rubber end of the plunger were the parts that needed replacement. The sheep sometimes chewed or stepped on the tubes causing leaks in the system and the normal usage of the device wore off the lubricant on the rubber end of the plunger. Hence, continuous greasing with liquid paraffin, as well as periodic replacement, was required.

At the beginning of the trial, the device was calibrated before each experiment; however, this practice was abandoned since very similar responses were obtained each time, and once a month calibrations were carried out instead with no different results. The only difference observed during the entire experiment,

was some variation in the pressure needed to release the plunger which means that a bigger force was required for the blunted pin to make contact with the scale's plate. This was corrected by simply greasing or replacing the plunger. Occasionally, the device was attached to the operator's wrist and thresholds were measured to verify that it was working properly. No problems were detected at any time.

The sheep became rapidly accustomed to carrying the device on their legs as well as to the testing itself that sometimes was carried out for up to 7 hours. During the experiments, the sheep were placed individually in mobile crates and were always kept in pairs to minimise stress. Once in the crate, a 15 to 20 minute settle down period was allowed for the sheep to avoid, as much as possible, the effect of stress-induced analgesia (Fields & Basbaum, 1989). The continuous use of a portable radio, always on the same radio station, throughout the experiments masked outside noises. The very first time each sheep was submitted to the mechanical nociceptive test a period of about 20 minutes was necessary to train them not to lift their legs when the blunted pin was first felt on their skin (less than 1 N). After this, six, and in some cases four, baseline readings were recorded before any drug was administered with the intention that each sheep acted as its own control. This provided a more faithful way to evaluate the effect of the treatment. This procedure was carried out for each experiment.

The mechanical nociceptive device was simple to use and produced reproducible responses as long as it was utilised by the same operator. When different operators tried the device the same stimulus was difficult to obtain since the rate at which the pressure was applied into the system varied with the rate at which the operator pushed the syringe's plunger. In his more sophisticated mechanical nociceptive apparatus, Chambers (1992) solved this problem by utilising a flow pneumatic system powered by compressed oxygen which allowed entrance into the device to be controlled by valves producing always the same stimulus regardless of the operator. Here, to minimise this variation only one operator, blinded to the treatment the sheep were given to avoid bias, was in charge of taking readings throughout the entire trial. This

allowed baseline thresholds to remain constant from test to test. It has been shown that sheep, in contrast to horses, show no sign of learning to respond before the mechanical stimulus become painful (Chambers, 1992). For instance, the control thresholds stayed constant for at least 5 hours and others have reported this for longer periods of time (Chambers, 1992; Chambers *et al.* 1995). Furthermore, the nociceptive test has been shown to discriminate between sedation and analgesia. When sheep were systemically administered the phenothiazine derivative acepromazine, sedation but no change on mechanical nociceptive thresholds were observed (Nolan *et al.* 1987a; Chambers, 1992).

Ambient air temperature has been shown to influence mechanical nociceptive thresholds of sheep. When the temperature was below 8°C thresholds were raised and even reached about 20 N at 0°C. Between 10 and 25°C there was not significant variation on nociceptive thresholds (Chambers, 1992). Hence, a thermometer was put in the experimental room and it was decided that experiments would be carried out only if the temperature was higher than 10°C. All experiments were done between September and April and the lowest registered ambient temperature was 12°C and the highest 24°C; therefore, this environmental factor should have not influenced the results of this study.

4.4.3. Effect of NSAIDs on sheep's nociceptive mechanical thresholds

The main finding of this study is that the i.t. injection of ketoprofen, tolfenamic acid, phenylbutazone and salicylic acid produced no change on mechanical nociceptive thresholds of non-lame healthy sheep whereas a rise in pain thresholds was detected after the i.v. injection of ketoprofen and tolfenamic acid though not after phenylbutazone and salicylic acid. Furthermore, the hypoalgesic effect of i.v. ketoprofen was reversed by naloxone and atipamezole both injected i.t.

The analgesic effects of NSAIDs have been demonstrated in various species, including human beings. These effects are mainly attributed to peripheral tissue

COX inhibition which, therefore, prevents sensitisation of peripheral nerve endings by PGs, particularly PGE_2 and PGI_2 (Ferreira, 1972). This is further supported by the fact that neutralisation of PGE_2 with the monoclonal antibody 2B5 prevented and reduced inflammatory hyperalgesia in rats as effectively as indomethacin (Portanova *et al.* 1996) or ketorolac (Zhang *et al.* 1997) did. Thus, since antibodies do not cross the blood-brain barrier, it seems that peripherally derived PGs are essential for establishing and sustaining hyperalgesia. Nevertheless, extensive evidence for a central analgesic site of action for the NSAIDs also exists (Bustamante *et al.* 1997; Malmberg & Yaksh, 1992a; Malmberg & Yaksh, 1992b; Malmberg & Yaksh, 1993; Chambers *et al.* 1995; Pelissier *et al.* 1996; Brune *et al.* 1992; Björkman *et al.* 1996; Willingale *et al.* 1997; Piletta *et al.* 1991; Devoghel, 1983). It is not clear, however, if these central effects are mediated by prostanoid synthesis inhibition, or if these drugs interact with neurones in different ways.

4.4.3.1. Intrathecal injection of NSAIDs

4.4.3.1.1. Spinal COX inhibition as analgesic mechanism of action of NSAIDs?

The PGs and all the necessary machinery for their synthesis are present in the spinal cord (Willingale *et al.* 1997). They can be released from the cord by activation of high-threshold nociceptive afferent inputs (Ramwell *et al.* 1966), by direct administration of neurotransmitters (Chaplan *et al.* 1997; Malmberg & Yaksh, 1993; Dirig & Yaksh, 1999) or by peripheral inflammatory stimuli (Smith *et al.* 1998; Dirig & Yaksh, 1999; Muth-Selbach *et al.* 1999), suggesting that a certain level of repetitive stimulation of the primary afferent is required for their release. This agrees with the involvement of the NMDA receptor system in the generation of PGs, but their synthesis also can be stimulated by co-activation of AMPA/kainate and mGluRs (Bezzi *et al.* 1998; Conn & Pin, 1997), an activation which does not necessarily require repetitive stimulation. The origin of spinal prostanoids is not well established either, and release into the extracellular space may not only be from neuronal but also glial structures (Buritova *et al.* 1996; Willingale *et al.* 1997; Bezzi *et al.* 1998; Phillips & Pearce, 1999).

Little is known about the functions of the PGs in the spinal cord. In rodents, the i.t. administration of PGs increase nociceptive responses (Minami *et al.* 1994a; Minami *et al.* 1994b; Minami *et al.* 1996; Eguchi *et al.* 1999) but not all the PGs are pain-evoking and some diminish pain sensitivity (Minami *et al.* 1996; Minami *et al.* 1997; Matsumura *et al.* 1993; Eguchi *et al.* 1999). The mechanisms by which PGs mediate hyperalgesia at the spinal level are unknown, but it seems likely that they augment the synaptic processing of pain information by enhancing Ca^{2+}-mediated glutamate (Bezzi *et al.* 1998), SP (Malcangio *et al.* 1996) and CGRP (Andreeva & Rang, 1993) release through activation of prostanoid receptors on high and low threshold afferent terminals (Bley *et al.* 1998; Oida *et al.* 1995; Wright *et al.* 1999) and further modulation of cAMP and/or Ins(1,4,5)$P3$ transduction cascades. Additionally, it has been suggested that spinal PGs may exert a presynaptic inhibition on noradrenaline release from the bulbospinal noradrenergic pathway (Taiwo & Levine, 1988). Thus, PG-mediated neurotransmitter/neuropeptide release and noradrenaline release inhibition could be mechanisms for amplification of pain signals. Spinal prostanoid synthesis inhibition, therefore, could represent a mode of action by which NSAIDs exert antinociceptive effects.

The administration of NSAIDs into the spinal canal produced analgesia as assessed by different algesiometric tests (Table 4.2). The possibility that these effects had been mediated by inhibiting spinal COX enzymes is supported by the antinociceptive potency of various i.t. injected NSAIDs matching with COX inhibition values (Malmberg & Yaksh, 1992b). The possibility is also supported by the fact that paracetamol produced analgesia at the same time that inhibited spinal PGE_2 release (Muth-Selbach *et al.* 1999) and the EP receptor antagonists SC-5108 and SC-51234A generated antinociception after being i.t. injected (Malmberg *et al.* 1994). The spinal COX isozyme responsible for the formation of these "pain-evoking" PGs is also a matter of debate. It is believed that the nociceptive process in the cord is more influenced by COX-2 rather than COX-1, mainly because COX-2 is the predominant isoform in this organ (Willingale *et al.* 1997; Seibert *et al.* 1994; Vane *et al.* 1998) and, contrary to COX-1, it is up-regulated by inflammatory hyperalgesia (Vane *et al.* 1998) which

Table 4.2. Evaluation of the analgesic effect of intrathecally and epidurally administered non-steroidal anti-inflammatory drugs in different species.

Reference	Species	Nociceptive test	NSAID	Dose	Effect
Intrathecal					
Bustamante *et al.* 1997	Rats (anaesthetised)	C fibre reflex recorded from the ipsilateral biceps femoris muscle after electrical stimulation within the area of the sural nerve	Acetylsalicylic acid	100 and 500 μg in 10 μL = 55 and 277 mM	Reduction of the area under the curve by 35 and 80%
			Indomethacin	200, 300 and 500 μg in 10 μL = 55, 83 and 139 mM	Reduction of the area under the curve by 36, 62 and 89%
			Ketoprofen	300 and 500 μg in 10 μL = 112 and 196 mM	Reduction of the area under the curve by 23 and 40%
			Lysine clonixinate	300, 500 and 1000 μg in 10 μL = 73, 122 and 244 mM	Reduction of the area under the curve by 30, 48 and 63%
Malmberg & Yaksh, 1992a^	Rats	Intrathecal NMDA + hot plate test	Acetylsalicylic acid	100 nM	Reversal of thermal hyperalgesia in about 75-90%
			Ketorolac tromethamine	27 nM	Reversal of thermal hyperalgesia in about 85-95%
			S(+)-ibuprofen	27 nM	Reversal of thermal hyperalgesia in about 90-95%
			R(-)-ibuprofen	270 nM	Failure to reverse thermal hyperalgesia
		Intrathecal AMPA + hot plate test	S(+)-ibuprofen	27 nM	Reversal of thermal hyperalgesia in about 85-95%
			R(-)-ibuprofen	270 nM	Failure to reverse thermal hyperalgesia
		Intrathecal substance P + hot plate test	S(+)-ibuprofen	27 nM	Reversal of thermal hyperalgesia in about 110-130%
			R(-)-ibuprofen	270 nM	Failure to reverse thermal hyperalgesia
Malmberg & Yaksh, 1992b^	Rats	Subcutaneous formalin injection into one hindpaw	Acetylsalicylic acid	27 nM (18-41 nM)*	About 70% inhibition of the phase 2 with the two highest doses
			Indomethacin	1.9 nM (1.2-4.0 nM)*	About 80% inhibition of the phase 2 with the two highest doses

Table 4.2. Evaluation of the analgesic effect of intrathecally and epidurally administered non-steroidal anti-inflammatory drugs in different species. (Cont.)

Reference	Species	Nociceptive test	NSAID	Dose	Effect
Malmberg & Yaksh, 1992b^	Rats	Subcutaneous formalin injection into one hindpaw	Flurbiprofen	2.1 nM (1.0-4.3 nM)*	About 70% inhibition of the phase 2 with the two highest doses
			Ketorolac tromethamine	5.2 nM (3.2-8.3 nM)*	About 80% inhibition of the phase 2 with the two highest doses
			Zomepirac sodium	5.9 nM (3.9-8.9 nM)*	About 75% inhibition of the phase 2 with the two highest doses
			S(+)-ibuprofen	15.7 nM (6.7-36 nM)*	About 60% inhibition of the phase 2 with the two highest doses
			Racemic ibuprofen	18.9 nM (9.3-38 nM)*	About 55% inhibition of the phase 2 with the two highest doses
			Acetaminophen	257 nM (163-405 nM)*	About 50% inhibition of the phase 2 with the two highest doses
			R(-)-ibuprofen	>270 nM	Failure to inhibit the phase 2
		Hot plate test (52.5°C)	Acetylsalicylic acid	100 nM	No significant effect on response latency
			Flurbiprofen	80 nM	No significant effect on response latency
			Ketorolac tromethamine	80 nM	No significant effect on response latency
			Zomepirac sodium	80 nM	No significant effect on response latency
			Indomethacin	30 nM	No significant effect on response latency
Malmberg & Yaksh, 1993	Rats	Subcutaneous formalin injection into one hindpaw	Ketorolac tromethamine	2.7-80 nM	Dose-dependent inhibition on phase 1 and 2. Maximal inhibition of 52% and 80% for each phase at the highest dose.
Pelissier, 1996	Rats	Paw pressure test	Acetaminophen	100 and 200 μg in 10 μL = 66 and 132 mM	Increased the area under the nociceptive threshold cure by 6 and 8 times when compared to saline.
Carlsson et al. 1986	Intact rats	Radian heat tail-flick test	Dipyrone	50-400 μg in 5 μL =30-240 mM	Dose-dependent increase of tail withdrawal latencies.

Table 4.2. Evaluation of the analgesic effect of intrathecally and epidurally administered non-steroidal anti-inflammatory drugs in different species. (Cont.)

Reference	Species	Nociceptive test	NSAID	Dose	Effect
Carlsson *et al.* 1986	Spinal rats	Radian heat tail-flick test	Dipyrone	400 μg in 5 μL = 240 mM	Failure to modify tail withdrawal latencies.
	Spinal rats	C fibre reflex recorded from the ipsilateral tibialis anterior muscle after electrical stimulation of the sural nerve	Dipyrone	100 and 400 μg in 5 μL = 60 and 240 mM	No change on flexor reflex activity with the 100 μg dose but increased it with the 400 μg dose.
	Spinal rats	Ascending axon activity in response to C fibre stimulation of the sural nerve	Dipyrone	100 and 200 μg in 5 μL = 60 and 120 mM	About 150% increase in C fibre evoked activity.
Lorenzetti & Ferreira 1996	Rats	Paw pressure test + paw PGE$_2$ injection	Dipyrone	2.5-80 μg in 20 μL = 0.375-12 mM	Dose-dependent reduction in escape behaviours.
Akman *et al.* 1996	Mice	Radian heat tail-flick test	Dipyrone	50-400 μg in 5 μL = 30-240 mM	Failure to modify tail withdrawal latencies.
		Hot plate test (55°C)	Dipyrone	50-400 μg in 5 μL = 30-240 mM	Failure to modify paw withdrawal latencies.
		Abdominal constriction test	Dipyrone	100 and 200 μg in 5 μL = 60 and 120 mM	About 50 and 65% reduction in the number of stretches.
Yamamoto & Nozaki-Taguchi 1996	Rats	Subcutaneous formalin injection into one hindpaw	Indomethacin	0.03-30 μg in 10 μL = 8.3 μM-8.3 mM	Decreased number of flinches in both phase 1 and 2 in a dose-dependent manner.
			NS-398	0.03-30 μg in 10 μL = 9.5 μM-9.5 mM	Decreased number of flinches in both phase 1 and 2 in a dose-dependent manner.
		Hot plate test (52.5°C)	Indomethacin	30 μg in 10 μL = 8.3 mM	Failure to modify paw withdrawal latencies.
			NS-398	30 μg in 10 μL = 9.5 mM	Failure to modify paw withdrawal latencies.
Willingale *et al.* 1997[&]	Rats (anaesthetised)	Mechanical noxious stimulation of the ankle joint	Meclofenamic acid	10 and 100 μM	Failure to modify dorsal horn neurone responses
		Mechanical noxious stimulation of the knee joint	Indomethacin	1 and 100 μM	Failure to modify dorsal horn neurone responses

Table 4.2. Evaluation of the analgesic effect of intrathecally and epidurally administered non-steroidal anti-inflammatory drugs in different species. (Cont.)

Reference	Species	Nociceptive test	NSAID	Dose	Effect
Willingale *et al.* 1997[&]	Rats (anaesthetised)	C fibre reflex recorded from the ipsilateral biceps femoris muscle after electrical stimulation of the sural nerve	Indomethacin	10 and 100 μM	No change with the low dose. About 67% reduction in the magnitude of C fibre-evoked reflex with the high dose.
Sorkin 1997	Rats (anaesthetised)	Intrathecal NMDA injection	Ketorolac	30 μg in 10 μL = 7.9 mM	Blockage of PGE_2 and TxB_2 increased release by 94% and 119% of basal levels, respectively.
Dirig *et al.* 1997	Rats	Radiant thermal stimulus + subcutaneous carragennan injection into one hindpaw	S(+)-ibuprofen	Not specified	Prevented decrease of paw withdrawal latencies in a dose-dependent manner.
			R(-)-ibuprofen	Not specified	Failed to prevent decrease of paw withdrawal latencies
			SC58125	Not specified	Prevented decrease of paw withdrawal latencies in a dose-dependent manner.
Devoghel 1983	Humans	Cancer pain sufferers	Lysine-acetylsalicylate	60 mg in 1 mL = 33 mg acetylsalicylic acid in 1 mL = 5.55 M	Quick (within 15 minutes) and log-lasting (1-22 days) pain relief.
Dolan & Nolan 1999[@]	Sheep	Mechanical nociceptive thresholds	DFU	200 nM	No modifying effect.
		Mechanical nociceptive thresholds + intrathecal NMDA	DFU	200 nM	Attenuated reduction on pain thresholds from 57% to 17% only.
Epidural					
Masue *et al.* 1999	Rats	Tail-flick test + intrathecal nitroglycerin	S(+)-ibuprofen	52 μg (38-66 μg) /30 μL = 8.4 mM (6.1-10.7 mM)*	Dose-dependent prevention on shortening of tail-flick latencies.
			Indomethacin	5 μg (2.8-7.3 μg) /30 μL = 460 μM (258-672 μM)*	Dose-dependent prevention on shortening of tail-flick latencies.

Table 4.2. Evaluation of the analgesic effect of intrathecally and epidurally administered non-steroidal anti-inflammatory drugs in different species. (Cont.)

Reference	Species	Nociceptive test	NSAID	Dose	Effect
Masue *et al*. 1999	Rats	Tail-flick test + intrathecal nitroglycerin	Diclofenac	6.3 μg (5.7-7.6 μg) /30 μL = 660 μM (597-796 μM)*	Dose-dependent prevention on shortening of tail-flick latencies.
			R(-)-ibuprofen	>1000 μg/ 30μL = >161 mM*	Failure to prevent shortening of tail-flick latencies.
		Subcutaneous formalin injection into one hindpaw	S(+)-ibuprofen	1000 μg/ 30μL = 161 mM	Suppressed the paw-lifting behaviour in the phase 2.
			R(-)-ibuprofen	1000 μg/ 30μL = 161 mM	Failure to modify pawl-lifting behaviour.
Lauretti *et al*. 1998	Humans	Cancer pain sufferers	Diclofenac	75mg	Complete pain relief (VAS[#] = 0) for 48 h.
			Tenoxicam	20mg	Improvement in pain relief (VAS = 2) for 22.5 h

*ID_{50} values and 95% confidence intervals.
^ Administration volume was 10 μL.
& Administration volume was 40-50 μL.
@ Administration volume not specified.
# VAS = visual analogue scale.

is reversed by the i.t. injection of selective COX-2 inhibitors (Yamamoto & Nozaki-Taguchi, 1996). Nevertheless, COX-1 products may also be involved in the processing of nociceptive input by stimulating afferent fibres (Willingale et al. 1997). In fact, the probable role of spinal COX-1 in the initiation of inflammatory hyperalgesia has recently been highlighted (Dirig et al. 1997; Smith et al. 1998).

As presented above, documentation exists for a feasible role of spinal PGs in the nociceptive process and synthesis inhibition of these lipidic acids by NSAIDs. Nonetheless, we did not record analgesia with the i.t. injection of ketoprofen, salicylic acid, phenylbutazone and tolfenamic acid, which were administered in concentrations high enough to inhibit COX activity in in vitro (Laneuville et al. 1994; Ricketts et al. 1998) and ex vivo (Giuliano & Warner, 1998) studies. These differences may be accounted for by algesiometric test and dosage differences. Most papers describing the central analgesic effects of NSAIDs assessed nociception by using models of inflammatory pain and hyperalgesia (Malmberg & Yaksh, 1992a; Malmberg & Yaksh, 1992b; Malmberg & Yaksh, 1993; Lorenzetti & Ferreira, 1996; Akman et al. 1996; Yamamoto & Nozaki-Taguchi, 1996; Sorkin, 1997; Dirig et al. 1997; Dolan & Nolan, 1999; Masue et al. 1999) but failure to produce analgesia was observed when models of acute pain were utilised (Malmberg & Yaksh, 1992b; Akman et al. 1996; Yamamoto & Nozaki-Taguchi, 1996; Willingale et al. 1997; Dolan & Nolan, 1999). It seems, therefore, that NSAIDs produce analgesia at the spinal level only when an ongoing afferent barrage is established (peripheral and central sensitisation). However, a report in which rats were submitted to a paw pressure test without induction of inflammation, shows i.t. paracetamol had an antinociceptive effect (Pelissier et al. 1996), which suggests that these drugs are able to produce analgesia in the absence of spinal facilitated states. This effect was only evident by injecting the drug in very high concentrations (mM range) and when NSAIDs were administered at smaller concentrations, in one or two orders of magnitude, they failed to increase mechanichal nociceptive thresholds (Willingale et al. 1997; Dolan & Nolan, 1999), as was observed here. In fact, most NSAID-mediated central analgesic effects have only been reported with the drugs administered in mM concentrations, the only exception are those

papers from Yaksh's laboratory which reported analgesia in the rat paw formalin test by utilising drug concentrations within the nM range (Table 4.2). Since COX inhibition by NSAIDs only requires these drugs to be present in the medium in nM to μM range concentrations (Laneuville *et al.* 1994; Ricketts *et al.* 1998; Giuliano & Warner, 1998), it seems reasonable to suggest that mechanisms other than or complementary to prostanoid synthesis inhibition might mediate NSAIDs-induced central antinociception. Interestingly, detailed reviews of the literature have emphasised an almost complete dissociation between the analgesic efficacy of these agents and their COX inhibition potency (McCormack, 1994a; McCormack, 1994b).

Nevertheless, it is important not to overvalue the usefulness of assays systems utilised to characterise the effects of NSAIDs on COX inhibition. It is known that *in vitro* systems do not always correlate with *in vitro* situations (Shigeta *et al.* 1998) and that COX inhibition seems to be specific for different species (Ricketts *et al.* 1998; Cryer & Dubois, 1998) as well as for different organs (Flower & Vane, 1972). Thus, given that data describing NSAIDs-mediated COX inhibition in sheep's spinal neurones have, to our knowledge, not been published, there is a slight possibility that the concentrations utilised in this study were not high enough to inhibit PG production in the sheep's spinal cord. Moreover, a possible lack or reduced interaction of the NSAIDs with spinal neurones in sheep cannot be excluded either. This has been observed with opioid agonists in sheep. These drugs produce good analgesia when injected i.v. (Waterman *et al.* 1990; Waterman *et al.* 1991) but their effects are minimal when administered i.t. (Ley *et al.* 1989), even when the specific receptors for them are present in the spinal cord (Brandt & Livingston, 1990). Intrathecal naloxone, however, substantially blocks the analgesic effects produced by i.v. opioid agonists (Waterman *et al.* 1991); hence, it seems that i.t. opioid agonists are unable to penetrate into the spinal neurones in sufficient concentration to exert an effect. From results in this study, therefore, it is not possible to rule out a possible central analgesic effect for NSAIDs mediated by COX inhibition.

4.4.3.1.2. Protocol and safety of NSAID injected i.t.

Although the distribution of i.t. injected NSAIDs was not studied, it was assumed that the volume administered would be confined within the cervical region since previous studies have demonstrated it for other drugs injected in the same volume (Waterman et al. 1988; Kyles et al. 1993). However, because NSAIDs were administered in cumulative doses, it is highly likely that these drugs had spread away to supraspinal centres. Thus, apart from the first injection dose, the next doses may have had a supraspinal component as well as the spinal component only for the dose assessed at the time. A similar procedure has been utilised to evaluate noradrenergic drugs in cats (Chua et al. 1998). Since no analgesia was recorded with i.t. NSAIDs, this procedure had a minor impact, if any, on our results. Furthermore, repetitive doses of saline, injected in the same way that NSAIDs were, did not affect mechanical nociceptive thresholds of sheep over the time (Figure 4.8), validating this drug injection protocol.

At concentrations used in this study, the NSAIDs were found to be highly insoluble in normal saline and once dissolved the pH of the solutions was around 2. Therefore, NaOH was used to dissolve them as well as adjust pH. No control solution with NaOH was intended since dilution of this substance in saline at equivalent concentrations to those used with the NSAIDs produced alkaline solutions with pH between 11 and 12, which were considered inappropriate for injection into the subarachnoid space. Other workers have used dimethyl sulfoxide (Yamamoto & Nozaki-Taguchi, 1996) cyclodextrin (Malmberg & Yaksh, 1992b) and ethanol (Malmberg & Yaksh, 1992b) to dissolve NSAIDs for i.t. injection and although they did not find these agents affected motor function, it raises a question about the extent to which these known antinociceptive agents may have affected or contributed to the reported analgesic effect of those NSAIDs. The possibility exists that the interaction between NaOH and the NSAIDs could also have modified the pharmacological properties of the NSAIDs; however, it was not studied here and clarification on this point is required.

No modification in sheep's general behaviour was observed after the i.t injection of any NSAIDs used in this study. This is in agreement with studies on rats, in which administration of different NSAIDs into the subarachnoid space did not produce behavioural changes or motor dysfunction (Malmberg & Yaksh, 1992b; Malmberg & Yaksh, 1993; Yamamoto & Nozaki-Taguchi, 1996). However, transit allodynia with squeaking and agitation has been observed in rats after 1 and 3 hours of i.t. indomethacin (28 nM) and ketorolac (27 nM) administration, respectively (Malmberg & Yaksh, 1992b). Alterations in cardiovascular and respiratory parameters were not observed during or after the i.t. injection of NSAIDs in anaesthetised rats (Bustamante et al. 1997; Sorkin, 1997) and, apart from light congestion and oedema, no histopatological lesions were observed with i.t. dipyrone or saline in mice (Akman et al. 1996). Moreover, no sensory, motor and neurovegetative side effects were observed in humans who received i.t. (Devoghel, 1983) or epidural (Lauretti et al. 1998) injections of NSAIDs. Thus, although the spinal administration of these analgesic agents seems to be devoid of major side effects, preclinical scrutiny of especially designed preparations should be carried out before any recommendations are made for these drugs to be administered through this route.

4.4.3.2. Intravenous injection of NSAIDs.

In contrast to the effects of i.t. injection of NSAIDs, i.v. administration of ketoprofen and tolfenamic acid, but not phenylbutazone and salicylic acid, raised pain thresholds in healthy sheep, which confirms that NSAIDs produce hypoalgesia in the absence of inflammation (Bustamante et al. 1997; Chambers et al. 1993; Chambers et al. 1995; Pelissier et al. 1996; Piletta et al. 1991; Ochi et al. 1999) and gives further support for the dissociation between the analgesic and anti-inflammatory effects of these drugs (McCormack, 1994a). As reported by others (Chambers et al. 1992; Chambers, 1995; Welsh & Nolan, 1994), no behavioural changes were observed in sheep after the i.v. administration of any NSAIDs. Therefore, their analgesic effects should be solely attributed to interaction with the pain pathway and not to modification of motor function.

4.4.3.2.1. Lack of analgesia with salicylic acid and phenylbutazone

Previous reports have demonstrated an antinociceptive action of i.v. NSAIDs (flunixin, dipyrone and carprofen) in sheep submitted to mechanical nociceptive stimulation (Chambers *et al.* 1993; Chambers *et al.* 1995; Welsh & Nolan, 1994) but the present study is the first to report failure of systemically administered NSAIDs, namely salicylic acid and phenylbutazone, to increase pain thresholds in this species.

Clinically, acetylsalicylic acid is almost devoid of anti-inflammatory effects and is considered as weak analgesic. This NSAID decreased serum TxB_2 values in sheep after i.v. administration at a rate of 10 mg/kg (Nolan *et al.* 1990); however, since acetylsalicylic acid is rapidly metabolised to salicylic acid (Higgs *et al.* 1987), this effect must be attributed to salicylic acid rather than the prodrug acetylsalicylic acid. In the present study, i.v. injection of salicylic acid (10 mg/kg) did not increase mechanical pain thresholds of healthy sheep. Thus, if salicylic acid produced in our sheep a similar effect on prostanoid synthesis inhibition to that described for acetylsalicylic acid (Nolan *et al.* 1990), it is possible to suggest that peripheral COX inhibition is not an analgesic mechanism of action for NSAIDs. Determination of plasma TxB_2 or PGE_2 would have been useful to clarify this point. Furthermore, the possibility that the lack of analgesia observed with salicylic acid had been due to a dose effect rather than an action of the drug itself cannot be ruled out, a dose-response trial would answer this.

Phenylbutazone, a pyrazolone derivative widely used in the equine clinic more for its anti-inflammatory effects than for its analgesic properties, produced no change on nociceptive thresholds of healthy sheep either, even when a quite large dose was used. This contrasts with the reported analgesic action of this drug in sheep submitted to unilateral stifle surgery (Dowd *et al.* 1998); however, this effect was achieved by administering the NSAID over three days and we only used a single dose. In addition, inflammation must have been present after surgery and an anti-inflammatory action by phenylbutazone may have contributed to the analgesic effect of this drug. Interestingly, dipyrone, another pyrazolone derivative with similar structure and molecular weight to

phenylbutazone and known for having good analgesic but poor anti-inflammatory action, raised the mechanical nociceptive thresholds in healthy sheep (Chambers *et al.* 1995). However, this analgesic effect was observed after injecting dipyrone in a 3-fold molar equivalent dose of phenylbutazone. The potential toxic effects of phenylbutazone may restrict its evaluation at bigger doses, which also may argue for different mechanisms of action between these two drugs. Thus, it seems that the analgesic effects of NSAIDs, even when classified within the same group, differ for each individual agent. Although the ability to inhibit COX enzymes for these two drugs seems to vary markedly with phenylbutazone and dipyrone being potent and weak COX inhibitors, respectively, it was shown that flunixin (potent COX inhibitor) and dipyrone had similar effects on healthy sheep's nociceptive thresholds and on TxB2 plasma levels (Chambers *et al.* 1995). It is not clear therefore, whether or not COX inhibition accounts for the analgesic action of NSAIDs.

4.4.3.2.2. Supraspinal analgesic action of NSAIDs

Given that no peripheral inflammation was present in our sheep, the antinociceptive effects observed with i.v. ketoprofen and tolfenamic acid cannot be explained by inhibition of COX enzymes in the periphery unless there is tonic production of COX. Therefore, this raises the possibility of an analgesic effect mediated at the brain level. After systemic administration, some NSAIDs cross the blood-brain barrier and can be found in the CSF (Zecca *et al.* 1991; Muth-Selbach *et al.* 1999) and central nervous tissue (Edwards *et al.* 1999). Prostanoid synthesis inhibition in the CNS has been observed after NSAIDs have been systemically administered both in laboratory animals (Muth-Selbach *et al.* 1999) and human beings (Levy *et al.* 1998), which supports the possibility of a central analgesic mechanism of action for NSAIDs by COX inhibition. Although the permeability of NSAIDs into the CSF and the inhibition of prostanoids at this level were not studied, the results obtained from the i.t. administration of NSAIDs do not reinforce this theory. Hence, alternative modes of action were investigated.

Opiodergic (Chambers, 1992; Chambers *et al.* 1993; Chambers *et al.* 1995; Akman *et al.* 1996; Tortorici *et al.* 1996; Ochi *et al.* 1999) and adrenergic (Chambers, 1992; Chambers *et al.* 1995) links have been implicated with the analgesic effects of some NSAIDs. To evaluate the significance of these two systems on the hypoalgesia produced by ketoprofen, drug antagonists at these receptor systems were injected i.t. The opioid receptor antagonist naloxone and the α_2-adrenoceptor antagonists yohimbine and atipamezole were used for these purposes.

4.4.3.2.1.1. Blockage of spinal opioid and α_2-adrenergic receptors

Firstly, it was confirmed that the selective μ-opioid receptor agonist fentanyl (Emmerson *et al.* 1994) and the α_2-adrenoceptor agonist xylazine (Faber *et al.* 1998) produced analgesia in sheep when given i.v., as it has been reported by others (Nolan *et al.* 1987b; Ley *et al.* 1991; Waterman *et al.* 1990; Waterman *et al.* 1991; Kyles *et al.* 1993; Welsh & Nolan, 1994). Secondly, it was necessary to find an i.t. dose for the opioid receptor and the α_2-adrenoceptor antagonists that prevented the rise of mechanical thresholds by fentanyl and xylazine, respectively, and that, when administered alone, had no effect on nociceptive thresholds.

Naloxone has been administered i.t. to laboratory animals in a broad range of doses, such as 0.055 mM (100 ng/ 5 µL) in mice (Akman *et al.* 1996), 2.75 mM (10 µg/ 10 µL) in rats (Pelissier *et al.* 1996), and 27.45 mM (1000 µg/ 100 µL) and 68.62 mM (2500 µg/ 100 µL) in sheep (Waterman *et al.* 1991; Kyles *et al.* 1990). Although some of these doses blocked the analgesic effects of different opioid receptor agonists without having a significant effect on control readings, Kyles *et al.* (1990) observed that the "low" naloxone dose used in their study (27.45 mM) increased mechanical thresholds. The onset of analgesia occurred around 50 minutes post-injection and persisted for 60 more minutes with the peak analgesic effect (325% increase from pretest values) seen at about 90 minutes after administration. Contrary to this is the lack of effect of low and high doses of systemically administered naloxone (0.4 and 10 mg, respectively) on

mechanical nociceptive thresholds of humans (Kaiser *et al.* 1997). Even more, excitatory effects on rat DHN (Budai & Fields, 1998) and increased secondary hyperalgesia in humans (Ilkjaer *et al.* 1998) have also been reported for naloxone. It seems, therefore, that paradoxical effects can be expected with this antagonist. Here, we found that a 5.49 mM dose of naloxone (200 µg/ 100µL) had no effect on mechanical nociceptive thresholds of sheep for at least 70 minutes. Since analgesia was observed in sheep about 50 minutes after naloxone administration (Kyles *et al.* 1990), it is unlikely that the dose chosen in our study produces hypoalgesia. However, it would be interesting to test this i.t. naloxone dose for longer periods to confirm that it is void of altering nociceptive thresholds in sheep. This was not done here because the paper by Kyles and co-workers (1990) was not available to us at the time of running the experiments.

When the same 5.49 mM i.t. naloxone dose was injected before administering i.v. fentanyl, it decreased the antinociceptive activity of the µ-opioid receptor agonist by almost 90%. In sheep too, Waterman *et al.* (1991) observed a 63.4% reduction in the analgesic effect of i.v. fentanyl by i.t. naloxone. Since both tests utilised a mechanical nociceptive device to assess antinociception, differences cannot be explained by this means. However, differences in drug doses, time of administration and the way the AUC was calculated could account for these slight differences. While we injected a smaller dose of naloxone (5.49 mM) 10 minutes before fentanyl at a rate of 10 µg/kg i.v. and estimated the AUC for the 60 minutes following fentanyl injection, Waterman *et al.* used a higher dose of the antagonist (68.62 mM) and injected it 30 minutes before a 11.3 µg/kg i.v. fentanyl dose, evaluating the AUC for 40 minutes only.

Naloxone inhibition of fentanyl-induced analgesia should be attributed to the blockage of spinal µ-opioid receptors since fentanyl binds to them with high affinity (Emmerson *et al.* 1994). Although naloxone was injected in mM concentration and this concentration was surely lower by the time the antagonist diffused in to where the receptors are, the possibility of an interaction with κ- and probably δ-opioid receptors cannot be excluded. This is due to naloxone's weak selectivity for µ-opioid receptors which is only 3 times greater

than that for κ-opioid receptors and 79 times greater than that for the δ- subtype (Emmerson *et al.* 1994).

Alpha$_2$-adrenoceptor antagonists have also been administered i.t. to laboratory animals. For example, yohimbine was used at a 76 nM (300 pg/ 10 μL) (Malmberg & Yaksh, 1993) and 7.66 mM concentration (30 μg/ 10 μL) in rats (Grabow *et al.* 1999) and at 2.6 mM (102 μg/ 100 μL) in cats (Chua *et al.* 1998), whereas idazoxan at a 4.15 mM concentration (100 μg/ 100 μL) and RX811059 at a 3.84 mM concentration (33 μg/ 100 μL) were used in sheep (Waterman *et al.* 1991; Kyles *et al.* 1993).

The results obtained with i.t. yohimbine were quite surprising, but the number of sheep used in the trials was too small to draw any firm conclusions. The selected 5.11 mM dose of yohimbine (200 μg/ 100 μL) administered i.t., dramatically increased the magnitude and duration of analgesia by i.v. xylazine (Figure 4.18). Since the yohimbine utilised here was one available for clinical use and it comes dissolved in a substance "X" (not mentioned here because is commercially sensitive information provided to us by the manufacturer in the strictest of confidence) that is known to produce analgesia when spinally injected on its own, these effects could be attributed to this substance. However, a direct effect of substance "X" on nociceptive thresholds seems unlikely since no significant change was observed when yohimbine was i.t. injected alone. This enhanced analgesic effect cannot be attributed to yohimbine blocking α_2-adrenergic receptors either, since the α_2-adrenoceptor antagonist atipamezole did not produce the same effects (this study). It is known that high concentrations of α_2-adrenoceptor agonists produce a local anaesthetic action independent of their effect on α_2-adrenoceptors (Chambers, 1993; Faber *et al.* 1998) and this has also been observed for some α_2-adrenoceptor antagonists (Chambers, 1993). Most α_2-adrenoceptor agonists are small molecules (MW 220-250) with a similar structure to lignocaine; however, yohimbine is quite large (MW 390) and different in structure to the local anaesthetic, so a local anaesthetic effect seems also unlikely. Molecular structure modification of yohimbine during the extraction technique utilised to separate the α_2-adrenoceptor agonist from substance "X" may also account for

this activity. Thus, by using the pure salt, yohimbine should be thoroughly evaluated to clarify the increased analgesic effect of i.v. xylazine described here.

Atipamezole was utilised instead of yohimbine in all other experiments that required blockage of α_2-adrenergic receptors. Although a local anaesthetic action has been described for atipamezole (Chambers, 1993), the i.t. injection of this antagonist in sheep at a 4.03 mM concentration (100 µg/ 100 µL) was void of effect on mechanical nociceptive thresholds for at least 70 minutes and, contrary to yohimbine, prevented the i.v. xylazine-induced increase in nociceptive thresholds by almost 90%. Also in sheep, the administration of the α_2-adrenoceptor antagonists idazoxan (4.15 mM) and RX811059 (3.84 mM) into the subarachnoid space reduced in about 60-65% the analgesic effect of i.v. xylazine (Waterman *et al.* 1991; Kyles *et al.* 1993). By comparing these reports with our experiments, it was found that all the antagonists were administered in very similar doses but the dose and time of administration for xylazine and the way in which the AUC was calculated were distinct. In our experiments, 10 minutes after atipamezole injection, all sheep received the same xylazine dose (20 µg/kg) and the AUC was evaluated for the 60 minutes after its administration. When idazoxan and RX811059 were administered, the xylazine dose was individualised for each sheep (12.5 to 41.6 µg/kg) and injected 30 minutes after the i.t. administration of the antagonist and the AUC was appraised for 40 minutes. Therefore, differences on methodological and analytical practices could explain these differences. Breed differences could also account for these analgesic variations (Ley *et al.* 1990). In this study, Romney-cross sheep were used whereas Swaledale, Swaledale cross Leicester and Clun Forest sheep were represented in the other study (Kyles *et al.* 1993). However, these breed differences might have been minimised by individualising the dose of xylazine.

The source of atipamezole was a commercial clinical product and, therefore, it came dissolved together with another ingredient, "Y" (not mentioned here for reasons already exposed). The sole administration of "Y" and in combination with xylazine was not performed as a measure of control and it can be argued

that the blockage or the bigger blockage in comparison to idazoxan or RX811059 (Waterman *et al.* 1991; Kyles *et al.* 1993) of xylazine-mediated analgesia may be attributed to "Y". Nonetheless, "Y" is a non-active preservative widely used in eye drops (Parfitt, 1999) and, hence, it is unlikely that the antagonistic effects observed with the administration of atipamezole would have been mediated by "Y".

It is possible that atipamezole, apart from acting on α_2-adrenergic receptors may have also blocked non-adrenoceptor imidazoline receptors (Sjöholm *et al.* 1996), which are localised in the substantia gelatinosa of the spinal cord and other sensory processing centres (Ruggiero *et al.* 1998). Binding to these sites by idazoxan is also documented (Mallard *et al.* 1991; Ruggiero *et al.* 1998) but RX811059 seems to have a low affinity for them (Mallard *et al.* 1991). Kyles *et al.* (1993) proposed that the reduction of i.v. xylazine-induced analgesia in sheep by i.t. idazoxan was due to the blockage of α_2-adrenoceptors but not non-adrenoceptor imidazoline receptors, since similar reduction was observed with the i.t. injection of RX811059. It would be interesting to compare the effect of atipamezole and RX811059 under the conditions here studied and, thus determine the impact of imidazoline receptor blockage on the attenuation of xylazine-mediated analgesia by atipamezole. However, it is reasonable to anticipate that a similar reduction by both antagonists would be observed because the spinal depressant actions of α_2-adrenoceptor agonists do not involve imidazoline receptors (Kyles *et al.* 1993; Faber *et al.* 1998). It seems, therefore, that the differences in the reduction of xylazine-induced antinociception by atipamezole (this study) and idazoxan and RX811059 (Kyles *et al.* 1993) are due to the methodological approaches discussed above.

Precipitation out of solution occurred when the antagonists' pH was adjusted to 7; therefore, they were administered with a slightly acid pH. Because no control experiments with an acid solution injected into the subarachnoid space were carried out, it is possible to argue that the pH of the solutions containing the anatagonists used in this study could have had an effect on mechanical nociceptive thresholds of sheep. However, it has been observed that the i.t. injection of a citric acid solution with pH as low as 4.4 produced no evidence of

discomfort and did not affect mechanical nocicetive thresholds in sheep (Kyles et al. 1995). Hence, it seems unlikely that the effects produced by naloxone, yohimbine and atipamezole were due to a merely pH effect.

The behavioural changes observed with the systemic administration of fentanyl and xylazine were similar to those reported previously (Waterman et al. 1990; Kyles et al. 1993; Welsh & Nolan, 1994). The sole i.t. injection of the three antagonists used here produced no behavioural changes and had very little moderating influence on the behavioural changes induced by their respective agonist, indicating that most of these effects were mediated at the supraspinal level.

4.4.3.2.1.2. Activation of descending inhibitory mechanisms as analgesic mode of action of NSAIDs

Interestingly, the i.t. administration of naloxone and atipamezole at concentrations sufficient to prevent the antinociceptive action of i.v. fentanyl and xylazine, respectively, were also capable of affecting the analgesic action observed with i.v. ketoprofen, which confirms that NSAIDs-induced analgesia is mediated by the opioidergic (Chambers, 1992; Chambers et al. 1993; Chambers et al. 1995; Akman et al. 1996; Tortorici et al. 1996; Ochi et al. 1999) and adrenergic (Chambers, 1992; Chambers et al. 1995) systems. These findings also support a supraspinal mechanism of action by which NSAIDs may indirectly cause descending inhibitory influences on the spinal transmission of nociceptive inputs.

The PAG seems to be the main brain structure in which NSAIDs exert their analgesic action. It was found that microinjection of dipyrone into the PAG of rats depressed peripheral C fibre-evoked activity of spinal ascending axons (Carlsson et al. 1986) and peripheral noxious pressure-evoked responses of spinal DNH (Vanegas et al. 1997), as well as increased heat-evoked tail-flick latencies (Carlsson et al. 1986; Tortorici et al. 1996). The increase in tail-flick latencies produced by i.p dipyrone was blocked by PAG-microinjected procaine

(Carlsson & Jurna, 1987), and an increased neural discharge rate in the PAG and depressed neural activity in the substantia nigra was recorded after i.v. dipyrone and aminophenazone administration (Carlsson *et al.* 1986; Carlsson & Jurna, 1987). Similarly, microinjection of dipyrone into the PAG depressed and facilitated on- and off-cell activity in RVM neurones, respectively (Tortorici *et al.* 1996). Thus, it seems that NSAIDs may increase the firing of "inhibitory" neurones in the PAG and RVM and decrease the discharge rate from "excitatory" neurones in the substantia nigra and RVM. This facilitates the release of endogenous inhibitory substances, which, by acting on spinal cord DHN and neurones from ascending spinal tracts, control the transmission of nociceptive information. If this is also true for ketoprofen, the increase of sheep's mechanical nociceptive thresholds detected after i.v. administration of the NSAID may be attributed to stimulation of descending inhibitory systems that involve the release of endogenous opioid peptides and noradrenaline since prevention of antinociception was observed with naloxone and atipamezole.

In agreement with the above model, naloxone i.v. or injected into the PAG of rats partially reverted the depression and facilitation of on- and off-cell activity in RVM neurones, respectively, as well as the retardation of tail-flick latencies achieved by PAG-microinjection of dipyrone (Tortorici *et al.* 1996). This same opioid receptor antagonist inhibited the analgesic effect of dipyrone injected i.c.v. to mice submitted to an abdominal constriction test (Akman *et al.* 1996). Naloxone-induced reversal of NSAID-mediated analgesia was also observed in sheep (Chambers *et al.* 1993; Chambers *et al.* 1995) and rats (Ochi *et al.* 1999) submitted to mechanical nociceptive stimulation. Together, these reports suggest that NSAIDs-induced analgesia may be partially mediated by an enhanced release of endogenous opioid peptides. In this regard, dipyrone and diclofenac increased β-endorphin plasma levels in humans (Tortorici *et al.* 1996). However, this was not true for S(+)- and racemic ibuprofen which reduced β-endorphin plasma levels and at the same time produced analgesia in patients submitted to the oral surgery model of acute pain (Dionne & McCullagh, 1998). Quantification of endogenous opioid peptides was not attempted here, but it would have been interesting to compare CSF and plasma levels for the different opioid peptides before and after administration of

ketoprofen in sheep to have a more complete picture of the analgesic mechanism of action of this drug.

From results of the current study, it is not possible to identify the class of spinal opioid receptors on which the supposedly released opioid peptides were acting to produce analgesia after injection of ketoprofen, since we used the weak-selective μ-opioid receptor antagonist naloxone (Emmerson et al. 1994) at a very high concentration. However, it has recently been demonstrated that dorsal horn μ-opioid receptors play an important role in the PAG-elicited descending modulation of nociceptive transmission (Budai & Fields, 1998). Intrathecal administration of selective antagonists for the three opioid receptor classes [e.g. CTOP, naltrindole and nor-binaltorphine for μ, δ and κ opioid receptors, respectively (Alexander & Peters, 1988)] would help to elucidate the significance of these receptors in ketoprofen-mediated analgesia in sheep.

Support for the involvement of adrenergic mechanisms in NSAIDs-mediated analgesia come from the blockage of i.v. dipyrone- and flunixin-induced increase of mechanical nociceptive thresholds in sheep by the α_2-adrenoceptor antagonist atipamezole injected i.v. as well (Chambers, 1992; Chambers et al. 1995). Interestingly, NSAIDs-mediated effects other than analgesia seem to also involve adrenergic mechanisms. Gastrointestinal motor disturbances after lysine-acetylsalicylic acid treatment in sheep were abolished by injecting i.v. the α-adrenoceptor antagonists phentolamine and tolazoline (Hondre & Buéno, 1984). Thus, it seems that some therapeutic and side effects of the NSAIDs could be attributed to an increase in noradrenaline release. Determination of this neurotransmitter in the sheep's CSF was not carried out either in this experiment; however, its measurement before and after the administration of ketoprofen may serve to give a better understanding of the mode of analgesic action of this NSAID.

The α_2-adrenoceptor subtype (α_{2A}, α_{2B}, and α_{2C}) involved in the analgesic action here described was not investigated; however, the α_{2A}-adrenoceptor subtype is a major candidate since it has been shown to mediate antinociception (Millan et al. 1994). In addition, the possibility that ketoprofen

had induced an increased release of an endogenous substance that by acting on imidazoline receptors had produced analgesia cannot be excluded since atipamezole, the α_2-adrenoceptor antagonist used in this study, has some affinity for these receptors (Sjöholm *et al.* 1996) and their activation at the spinal level has been implicated with antinociceptive responses (Diaz *et al.* 1997). It would be interesting to test this hypothesis in sheep by injecting i.t. an imidazoline receptor antagonist [*e.g.* BU-224 (Diaz *et al.* 1997)] followed by i.v. ketoprofen.

The mechanisms by which NSAIDs increase the discharge rate of neurones in the PAG (Carlsson *et al.* 1986; Carlsson & Jurna, 1987) and, thus, supposedly increase the release of endogenous antinociceptive substances are unknown. However, it has recently been suggested that μ-opioid receptor activation in GABAergic presynaptic terminals of the PAG reduces the probability of neurotransmitter release. It uses a mechanism that involves a voltage-dependent K^+ conductance via a $PLA_2/AA/12LOX$ pathway (Vaughan *et al.* 1997), with inhibition of COX-1, more than that of COX-2, accounting more for this action (Vaughan, 1998). Thus, by inhibiting COX enzymes in GABAergic presynaptic terminals in the PAG, NSAIDs may make AA more available for conversion to 12-LOX products and these may contribute to hyperpolarise these neurones and reduce GABA release, rendering neurones in the synapse more susceptible to firing and releasing endogenous antinociceptive agents. In fact, microinjection of the $GABA_A$ receptor antagonist bicuculine into the PAG inhibited cutaneous noxious heat-induced responses in spinal cord DHN by releasing endogenous substances that activated μ-opioid (Budai & Fields, 1998) and α_2-adrenergic (Budai *et al.* 1998) receptors on these spinal neurones.

The binding of ketoprofen to opioid and α_2-adrenergic receptors was not studied. However, the possibility that this drug had directly interacted with these receptors to produce analgesia seems unlikely. Receptor binding studies have shown no affinity of paracetamol for adrenergic (α_1, α_2 and β), opioid (μ, δ and κ), muscarinic, adenosine (A1 and A2), dopamine (D1 and D2), 5-HT (5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2C} and 5-HT_3) histamine (H1), benzodiazepine, $GABA_A$, NMDA and NK_1 receptors (Pelissier *et al.* 1996). Flunixin did bind to α_2-

adrenergic receptors with low affinity at concentrations that are unlikely to be found clinically (Chambers, 1992).

Despite all above evidence implicating opioidergic and adrenergic mechanisms in the antinociceptive action of NSAIDs, some reports do not support this theory. For instance, the antinociception observed in the acetic acid abdominal constriction test in mice following s.c. acetylsalicylic acid injection was not reversed by the opioid receptor antagonists naloxone and naltridone nor the enkephalin catabolism inhibitor acetorphan (Gray et al. 1998; Gray et al. 1999) or by the α_2-adrenoceptor antagonist RX821002 (Gray et al. 1999), all administered s.c. Because some inflammation may have occurred in this assay, peripheral COX inhibition could be argued as the mechanism of analgesic action in this case. However, in a rat paw pressure test without induction of inflammation, i.v. paracetamol-induced analgesia was not inhibited by i.t. naloxone or i.v yohimbine (Pelissier et al. 1996), which may suggest that not all NSAIDs share the same analgesic mechanisms of action or that these mechanisms may be influenced by the employed nociceptive test, or both.

The possibility that ketoprofen had activated other descending inhibitory mechanism to produce analgesia in sheep cannot be excluded since serotoninergic and cholinergic pathways have also been implicated in NSAIDs-induced analgesia. On the one hand, the i.t. injection of the 5-HT$_3$ receptor antagonist tropisetron inhibited the antinociceptive action of paracetamol administered systemically and i.t. in rats submitted to a paw pressure test (Pelissier et al. 1996) and the analgesic effect of diclofenac in rats was also attributed to enhancement of descending 5-HT neuronal activity (Björkman et al. 1996). On the other hand, the muscarinic cholinergic blocking agent atropine reversed the analgesic effect of a pyrazolone derivative NSAID as it was assessed in mice by a hot-plate test (Stets & Slivko, 1989). It would be interesting to investigate if these two descending inhibitory mechanisms take part in the antinociceptive action of ketoprofen, even though serotonergic mechanisms may only be relevant in rats, since large inter-species differences exist in central distribution, pharmacology and allosteric modulation of 5-HT$_3$ receptors (Fletcher & Barnes, 1998). Other substances that are released by

descending inhibitory mechanisms to modulate nociceptive transmission in the spinal cord include dopamine (Barasi et al. 1987), adenosine (Zhao et al. 1999), GABA and glycine (Xu et al. 1998; Nicoll & Malenka, 1998). However, whether these agents contribute to the analgesic action of NSAIDs remains to be elucidated.

However, there are some reports not supporting an antinociceptive supraspinal site of action for the NSAIDs. For instance, failure to modify rat behavioural responses to a paw PGE_2-induced hyperalgesia test (Lorenzetti & Ferreira, 1996) and to depress a C fibre-mediated reflex in anaesthetised rats (Bustamante et al. 1997) was observed after i.c.v. administration of NSAIDs. This may indicate that NSAIDs have no supraspinal analgesic effect on these nociceptive models or that not enough drug diffusion into the PAG, or other specific structure where these drugs exert their supraspinal analgesic action (Tortorici et al. 1996), was achieved. Although some drug may have reached supraspinal structures after the i.t. cumulative administration of NSAIDs in the current study (see above), no analgesia was recorded. However, it is difficult to know the amount of drug that spread into brain structures; therefore, a set of experiments in which known concentrations of ketoprofen are injected through the i.c.v. route in sheep would serve to further confirm a supraspinal site of action for this drug.

Interestingly, it was recently postulated that the endocannabinoid anandamine, known to produce analgesia by activation of cannabinoid CB1 receptors, could be synthesised from AA through the action of the enzyme anandamine aminohydrolase (Piomelli et al. 2000). To test if an interaction between ketoprofen-induced analgesia and the endocannabinoid system exists in sheep, a selective cannabinoid CB1 receptor antagonist [e.g. SR141716A (Piomelli et al. 2000)] could be injected i.t. before administering the NSAID. Some other mechanisms by which NSAIDs could produce analgesia include competition with palmitic acid for the enzyme long-chain acyl-CoA synthase and final reduction of glutamate levels (McCormack, 1994a; McCormack, 1994b); increase levels of the EAA antagonist kyanurenate (McCormack, 1994b; Edwards et al. 1999); alteration of G protein dependent functions (McCormack,

1994a; McCormack, 1994b); interactions with the NO pathway (Lorenzetti & Ferreira, 1996; Björkman *et al.* 1996; Schubert *et al.* 1999) as well as vanilloid (Buritova & Besson, 1998; Szallasi & Blumberg, 1999) and $GABA_A$ receptors (Martin *et al.* 1987); blockage of L- and T-type Ca^{2+} channels (Knock & Aaronson, 1999); inhibition of the transcription factors *c-fos* (Akagi *et al.* 1985; Buritova & Besson, 1998), NFκB (Xu *et al.* 1999) and Erk (Pillinger *et al.* 1998); inhibition of COX-2 mRNA and protein expression (Xu *et al.* 1999); and increment if IL-1 type II receptor (Daun *et al.* 1999). Nonetheless, the clinical implication of these molecular systems in antinociception is yet to be elucidated.

In conclusion, our results showed that although the NSAIDs did not have a direct effect on the spinal cord, their analgesic action appeared to be spinally mediated, probably by activating inhibitory descending opioidergic and adrenergic mechanisms. This raises the possibility of exploring drug combinations of NSAIDs and other agents which act on these spinal neurones with the hope of finding better ways to control pain.

Chapter 5
GENERAL CONCLUSIONS

Both *in vitro* and *in vivo* experiments were designed to evaluate the central analgesic effects of NSAIDs. For unknown reasons, it was not possible to properly set up the neonatal rat hemisected spinal cord preparation and therefore, experiments using this technique were not carried out.

The i.t. cervical catheterisation of sheep permitted the evaluation of NSAIDs at the spinal level. Catheter implantation is technically difficult in this species and although practice was gained over the time, pitfalls were still encountered. Maintaining the catheters in place for longer periods seems to represent the major problem with this procedure. In an attempt to overcome this aspect the Yates draining tube anchoring technique described here proved to be efficacious in keeping functional catheters for longer periods; however, any modification to improve the catheters' functional life-time will be always welcome. It seems, therefore, that if a well-designed catheter is implanted carefully and anchored properly, it will be likely to perform adequately over the long term. This technique conveys some risks of iatrogenias (*e.g.* spinal infection, neurological problems), but these seem to be minimal overall.

The NSAIDs used in this study were devoid of effect on mechanical nociceptive thresholds of sheep when given i.t. However, i.v. ketoprofen and tolfenamic acid, but not phenylbutazone nor salicylic acid, raised the thresholds. From these results it could be inferred that the nociceptive test employed here is adequate to evaluate the analgesic actions of NSAIDs, since ketoprofen and tolfenamic acid seem to be clinically more potent analgesics than phenylbutazone or salicylic acid. Discrepancies in the analgesic action of NSAIDs have also been reported in humans, with paracetamol producing analgesia but not acetylsalicylic acid (Piletta *et al.* 1991). Hence, our findings confirm that different NSAIDs have different analgesic potency. They also support the antinociceptive action of these drugs in the absence of inflammation

(Chambers *et al.* 1993; Chambers *et al.* 1995; Pelissier *et al.* 1996; Ochi *et al.* 1999).

The antinociceptive action of i.v. ketoprofen was inhibited by preinjecting naloxone and atipamezole into the cervical subarachniod space. Therefore, activation of supraspinal centres to enhance the release of endogenous opioid peptides and noradrenaline that, in turn, act on spinal sensory neurones to produce hypoalgesia seems to be the central analgesic mode of action of the NSAIDs. However, the contribution of COX inhibition in the analgesic action of these drugs cannot be ruled out.

The indirect spinal analgesic action of the NSAIDs raises the possibility of exploring more efficient forms of control by combining analgesic drugs which site of action is also on spinal sensory neurones but which receptor coupling system is different (*e.g.,* NMDA antagonists). In addition, if the activation of supraspinal centres is not due to COX inhibition, it would be possible to create drugs from NSAIDs that retain their analgesic action without producing gastric and renal side effects attributed to enzyme inhibition in these organs.

Finally, it would be interesting to test the clinical useful of NSAIDs in farm animals suffering from painful lame conditions to evaluate their impact on pain relief as well as on production parameters. A study like this will give us valuable information about the practical use of these often misused and underused analgesic drugs.

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