Spinal Analgesic Interaction Between Non-Steroidal Anti-Inflammatory Drugs and *N*-Methyl-D-Aspartate Receptor Systems

A thesis presented in partial fulfilment
of the requirements for the degree of

Doctor of Philosophy in Veterinary Science

Institute of Veterinary, Animal and Biomedical Sciences
Massey University
Palmerston North, New Zealand

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2006

To Fernanda

Abstract

Activation of spinal *N*-methyl-D-aspartate (NMDA) receptors stimulates cyclooxygenase and nitric oxide pathways. Compounds that block the activity of these NMDA receptor systems reduce pain hypersensitivity. However, their usefulness is limited by the side effects they produce. One way of reducing side effects is by combining drugs that produce the same overt effect by different mechanisms, which hopefully increase the net effect. In these series of studies, drugs that interact with NMDA receptor systems and their combinations were screened *in vitro* to identify spinal antinociceptive synergistic combinations that could be assessed *in vivo*. Based on developmental changes in thresholds, conduction velocities and blocking actions of the local anaesthetic lignocaine in neonatal rat L4/L5 dorsal root potentials, it was decided to use spinal cord *in vitro* preparation from 5- to 7-day-old rat pups. In single drug studies, the NMDA receptor channel blocker ketamine (1-50 μM) and the non-steroidal anti-inflammatory drug (NSAID) ketoprofen (200-600 μM), but not the NSAID salicylate (1000 μM) and the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; 1-100 μM), reduced spinal NMDA receptor-mediated transmission. Ketamine also depressed non-NMDA receptor-mediated transmission. Using isobolographic and composite additive line analyses, fixed-ratio combinations of ketamine and ketoprofen, ketamine and L-NAME, and ketopofen and L-NAME synergistically depressed NMDA receptor-mediated transmission. The two former combinations had a subadditive effect on non-NMDA receptor-mediated transmission, and the latter had no significant effect. These studies identified that all combinations synergistically reduced both nociceptive transmission and potential side effects. In free-moving sheep implanted with indwelling cervical intrathecal catheters, 100 μl subdural administration of ketamine (25-400 μM) and ketoprofen (200-3200 μM) alone and in a fixed-ratio combination (873.95-3350.78 μM, 0.045:0.955) did not raise nociceptive thresholds as assessed by mechanical stimulation of one foreleg. Subdural administration of NMDA (2 mM) decreased mechanical nociceptive thresholds, and this was prevented by the highest concentrations of ketamine and ketoprofen alone and in combination. These findings demonstrated that NMDA receptor channel blockers and NSAIDs alone or in combination had no direct hypoalgesic effects when given onto the spinal cord of sheep, but they prevented NMDA-induced pain hypersensitivity. Simultaneous blockade of NMDA receptor systems could have important clinical implications.

Acknowledgements

I am extremely thankful to Paul Chambers and Craig Johnson for supervising this project. I appreciate all your academic and intelectual support, but mostly your friendship and generosity of time.

I could not have carried out this project without sponsorship of the National Autonomous University of Mexico, in particular the DGAPA scholarship I was granted and the support of the Faculty of Veterinary Medicine during my leave.

Special thanks to the Neuroscience Group in IVABS, in particular to Sheryl Mitchinson for her skilled assistance during sheep anaesthesia. Special thanks also go to Debbie Chesterfield, Judith Stanton and Louise Beazley from the Small Animal Production Unit for providing the rats for this project, and to Robin Whitson and Graeme King for looking after the sheep while housed in the Veterinary Teaching Hospital.

Thanks to Wendi Roe, Stuart Hunter, Mark Collett, and Alastair Johnstone for their expert advice in gross pathology and histopathology, to Nicolas Lopez-Villalobos for his advice on statistical analyses, to Aaron Hicks for processing the rat dorsal roots for histological examination, and to Allain Scott for her support with all postgraduate administrative requirements.

During this time in New Zealand I met extraordinary people who helped me in many different ways. Very special thanks to Alejandra, Victor, Natalia, Alfredo, Federico, Klelia, and the Whelan family. Also thanks to old friends, Kathy and Mike, Oscar and Sara, Sue and Godfrey, David and Diana, Patricia and Jose, and Sylvia.

I am grateful to my parents, Ignacio and Silvia, who have always been there for me and for being such great role models. Also to my family and friends in Mexico that kept me cheerful during this time.

Finally, I am most grateful to Fernanda. Thanks for all your help on every single part of this project, but mainly for being the wonderful person you are and sharing this journey with me. You mean all to me and I wish I could put in words how much I love you...

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Abbreviations

8-pCPT-cGMP	8-para-clorophenylthio cGMP, a membrane-permeable cGMP analogue
$[Ca^{2+}]_i$	intracellular calcium concentration
AA	arachidonic acid
$AACOCF_3$	arachydonyl trifluoromethylketone, a type-unspecific PLA_2 inhibitor
aCSF	artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxalone propionic acid
AUC	area under the curve
C5	fifth cervical vertebra
CaM	Ca^{2+}-calmodulin complex
cAMP	3',5'-cyclic adenosine monophosphate
CB	cannabinoid
cGMP	3',5'-cyclic guanosine monophosphate
CGRP	calcitonin gen-related peptide
CI	confidence intervals
CNS	central nervous system
COX	cyclo-oxygenases
$cPLA_2$	cytosolic Ca^{2+}-dependent PLA_2
CSF	cerebrospinal fluid
DAG	diacylglycerol
DRCAP	dorsal root compound action potential
DRG	dorsal root ganglion
DR-VRP	dorsal root evoked population ventral root potential
eNOS	endothelial NOS
epsp	excitatory postsynaptic potential
GABA	γ-aminobutyric acid
i.p.	intraperitoneal
i.t.	intrathecal
IC_{40}	inhibitory concentration at 40% depression of maximum possible effect
IC_{50}	inhibitory concentration at 50% depression of maximum effect, median inhibitory concentration
iNOS	inducible NOS
$InsP_3$	inositol-(1,4,5) triphosphate
$iPLA_2$	cytosolic Ca^{2+}-independent PLA_2

L-NAME	N^{ω}-nitro-L-arginine methyl ester
L-PGDS	lipocalin-type PGD synthase
MAFP	methyl arachydonyl fluorophosphanate, a type-unspecific PLA_2 inhibitor
MPE	maximum possible effect
mPGS1	microsomal PGE synthase 1
mPGS2	microsomal PGE synthase 2
MSR	monosynaptic compound action potential
N	Newtons
NF-κB	nuclear factor-κB
NK_1	$neurokinin_1$ receptor
NMDA	*N*-methyl-D-aspartate
nNOS	neuronal NOS
NO	nitric oxide
NOS	nitric oxide synthase
NSAIDs	non-steroidal anti-inflammatory drugs
ODQ	1*H*-[1,2,4]oxadiazolo[4,3-α]qinoxalin-1-one, a gyanylate cyclase blocker
PCOX-1a	partial COX-1a
PCOX-1b	PCOX-1a
PGDS	PGD synthases
PGES	PGE synthase
PGFS	PGF synthase
PGs	prostaglandins
PKA	protein kinase A
PKC	protein kinase C
PKG	cGMP-dependent protein kinases
PLA_2	phospholipase A_2
PS	phosphatidylserine
PSD-93	postsynaptic density-93
PSD-95	postsynaptic density-95
Rp-8-p-CPT-cGMPS	Rp-8-p[(4-Chlorophenyl)thiol]-cGMPS triethylamine, a selective PKG-Iα inhibitor
SP	substance P
$sPLA_2$	secretory PLA_2

1. Introduction and thesis outline

1.1 Introduction

The International Association for the Study of Pain has defined pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Lindblom *et al.*, 1986). The sensory component of pain requires pain signals to be transmitted from peripheral tissues through the spinal cord to higher centres of the brain and then back to the peripheral sites. This is not a passive, simple process using exclusive pathways. On the contrary, the circuitry within the spinal cord can modulate the relationship between the stimulus and the response to pain (Melzack and Wall, 1965). The interplay between both excitatory and inhibitory spinal neuronal systems determines the messages delivered to supraspinal levels, and this will depend on the particular circumstances. Additionally, there are descending inhibitory and excitatory controls subjected to alteration in accordance with the circumstances. This "plasticity" of spinal nociceptive systems to modulate the transmission can be induced over very short periods of time and a better understanding of this process may improve the way pain is managed.

The physiology and pathophysiology of sensory neurones in the dorsal horn of the spinal cord is complex, and an ever-growing number of neurotransmitters and neuromodulators and their receptors are known to be present. The neurotransmitters and neuromodulators are derived from afferent fibres, intrinsic neurones and neuroglia or descending fibres. Most neurotransmitters and neuromodulators and their receptors are concentrated in the substantia gelatinosa and other upper layers of the dorsal horn of the spinal cord, where nociceptive afferent messages from peripheral fibres are received and modulated. In general, high threshold, small diameter, thinly myelinated (Aδ-) and unmyelinated (C-) fibres terminate in laminae I-II, while lower threshold, large diameter, myelinated (Aβ-) fibres terminate in lamina III-V. Lamina V neurones also receive input from Aδ-fibres and from afferent fibres in laminae I-II via dendrites reaching into superficial layers (Doubeli *et al.*, 1999).

Amplification of low levels of C-fibre afferent stimulation by spinal mechanisms can result in marked and prolonged changes in the spinal cord, which is referred as central sensitisation (Dickenson, 1995; Woolf, 1996). Repeated constant C-fibre stimuli can induce the phenomenon of windup in which the response of deep dorsal horn neurones increases dramatically despite a steady input into the spinal cord. Windup needs certain minimum frequency of stimulation to produce its effects, but when produced, it can augment the responses of dorsal horn neurones by several times in amplitude and prolong the responses

even after cessation of the peripheral input (Dickenson, 1995; Woolf, 1996). It is therefore likely that the presence of windup-like events leads to the development of central sensitisation. 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 after apparently successful treatment of the lameness-causing lesion has been observed, respectively (Ley *et al.,* 1989; Ley *et al.,* 1995; Whay *et al.,* 1997; Whay *et al.,* 1998).

At the peripheral level most nociceptive signalling arises from activation of specialised nociceptors, which are innervated by C-fibres. The acute application of mechanical or thermal stimuli results in a good relation between the stimulus and the response since the stimulus directly activates nociceptors, which in turn activate simple spinal systems (Raja *et al.,* 1999). However, in the presence of tissue damage, these fibres respond to locally generated chemical stimuli and become sensitised. The large number of peripheral mediators that participate in this process (see Raja *et al.,* 1999) illustrates the complexity of transmission at the peripheral level leading to the development of allodynia (pain perception in response to normally non-painful stimuli) and hyperalgesia (increased pain perception to normally painful stimuli). The induction and maintenance of these excitatory events are enhanced in inflammatory states establishing peripheral hypersensitivity, which facilitates spinal excitation and plays an important role in the induction of central sensitisation. Under some circumstances, however, loss of inhibitory controls can also occur (Doubeli *et al.,* 1999).

There is considerable evidence for the involvement of excitatory amino acids glutamate and aspartate as well as a number of neurotransmitters and neuropeptides in the spinal modulation of nociceptive transmission (Dickenson, 1995; Doubeli *et al.,* 1999). Some of the transmitters implicated in nociception include noradrenaline, acetylcholine, serotonin, dopamine, adenosine and ATP, and some of the neuropeptides include opioid peptides, substance P, calcitonin gene-related peptide, somatostatin, vasoactive intestinal polypeptide and galanin (Figure 1.1).

1.1.1 The research problem

As the literature review (Chapter 2) will show, activation of the glutamate receptor *N*-methyl-D-aspartate (NMDA) stimulates arachidonic acid (AA) and nitric oxide (NO) pathways. These NMDA receptor systems play an important role in the spinal processing of nociceptive information, and compounds that block their activity reduce pain hypersensitivity. However, the usefulness of these drugs is often limited by the side effects they produce. One way of reducing side effects is by combining drugs that produce the same overt effect by different mechanisms, which hopefully increases the net effect.

Using appropriate statistical methods developed for the study of drug-drug interactions (Porreca

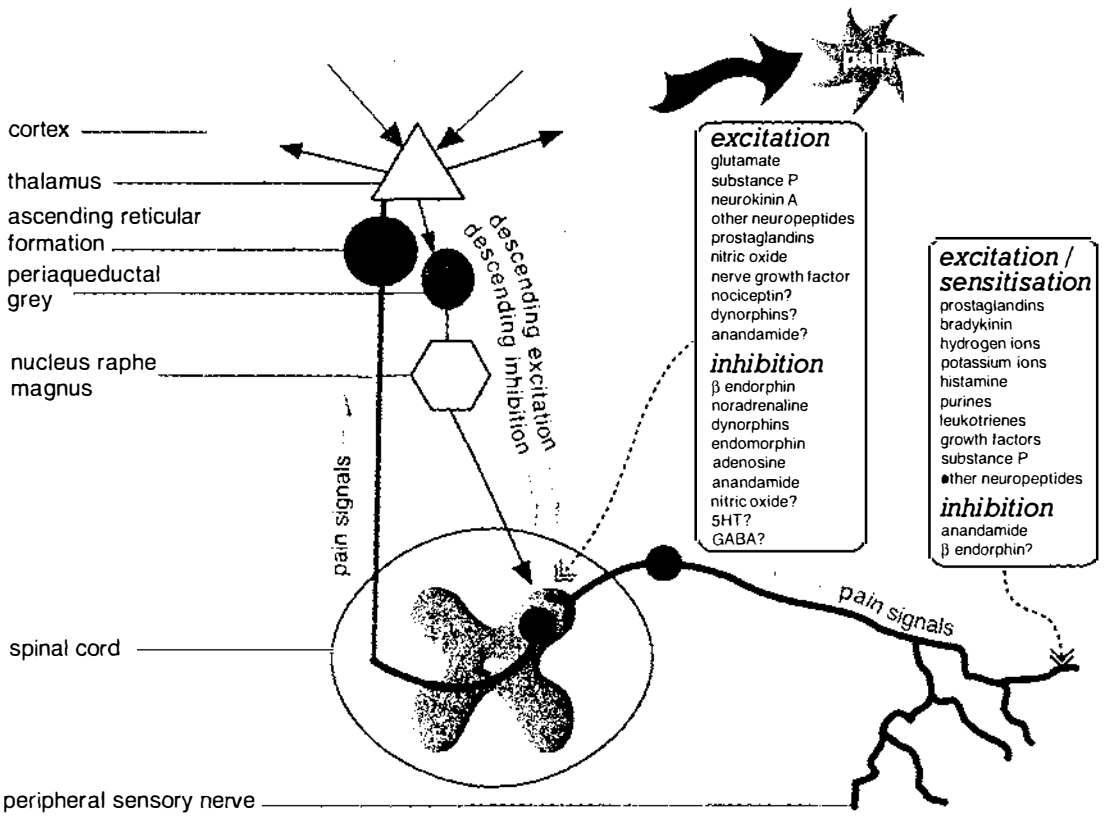

Figure 1.1 *Schematic representation of neurotransmission of pain signals from the periphery to higher centres of the brain and back to the spinal cord. Some peripheral and spinal neurotransmitters and neromodulators implicated in nociception are highlighted (Courtesy of Dr. Paul Chambers).*

et al., 1990; Tallarida *et al.,* 1997; Tallarida, 2000), the nature of the relationships between drugs known to interact with NMDA receptor systems was assessed in a series of studies reported in this thesis. The objective of these studies was to identify spinal antinociceptive synergistic combinations. To do this in a rational way, drugs and drug combinations were first screened *in vitro* using a neonatal rat spinal cord preparation. Further *in vivo* studies were carried out in sheep implanted with indwelling cervical intrathecal catheters following mechanical nociceptive stimulation of a foreleg.

All experimental protocols for reaserch involving animals in this thesis were approved by the Massey University Animal Ethics Committee (protocol numbers 02/103 and 04/118).

1.2 Thesis outline

The thesis is presented in 9 chapters. The literature on spinal NMDA receptor activation and further stimulation of AA and NO signalling pathways in the spinal processing of pain is reviewed in Chapter 2. This chapter also discusses the spinal effects of products of both cyclo-

oxygenase and NO synthase (NOS) enzymes on activity modulation of the other enzyme.

In vitro studies used neonatal rats since robust spinal cord preparations can be obtained from these animals. Firstly, it was determined the age of animals to be used based on developmental changes in thresholds, conduction velocities, and effects of the local anaesthetic lignocaine in electrically evoked potentials from sciatic nerve-L4/L5 dorsal root preparations from 0- to 12-day-old rats. From these findings, presented in Chapter 3, it was decided to use 5- to 7-day-old-rats in further studies.

Chapter 4 describes the effects of the NMDA receptor channel blocker ketamine, the non-steroidal anti-inflammatory drugs ketoprofen and salicylic acid, and the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) on non-NMDA and NMDA receptor-mediated transmission in *in vitro* spinal cord preparations. The spinal effects of fixed-ratio combinations of ketamine and ketoprofen, ketamine and L-NAME, and ketoprofen and L-NAME are presented in Chapters 5, 6, and 7, respectively.

In vivo studies, described in Chapter 8, used sheep with indwelling cervical intrathecal catheters and assessed threshold changes after mechanical nociceptive stimulation of a foreleg. The effects of intrathecal administration of ketamine and ketprofen alone and in a fixed-ratio combination were studied with and without previous subdural administration of NMDA. The thesis concludes in Chapter 9 with a general discussion of the results from both *in vivo* and *in vitro* studies, and what the implications may be for the treatment of pain. The limitations of the studies are addressed in this final chapter and suggestions are made for further research.

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2. Spinal processing of pain: a review on NMDA receptor-mediated activation of arachidonic acid and nitric oxide signalling pathways

2.1 Spinal NMDA receptors

The amino acid glutamate is the main excitatory neurotransmitter in the mammalian central nervous system and acts on two different classes of receptors: metabotropic and ionotropic glutamate receptors. The former receptors function by intracellular signalling through G-proteins and are subdivided into three groups (Group I-III). The latter class of receptors contain integral cation-specific ion channels and are subdivided into kainate, α-amino-3-hydroxy-5-methyl-4-isoxalone propionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA). There are at least seven subunits of NMDA receptors: NR1, NR2 (A, B, C, and D), and NR3 (A and B). The NR1 subunit exists in at least eight splice variants: 1a, 1b, 2a, 2b, 3a, 3,b, 4a, and 4b. The number of subunits a functional NMDA receptor is composed of is still a matter of debate, both tetrameric and pentameric structures have been proposed (Dingledine *et al.,* 1999). Although the specific functions for most subunits are to be determined, the diversity of subunits and splice variants, and the way they couple in the receptor itself could explain why NMDA receptors have been implicated in many forms of synaptic transmission, plasticity, and neurodegeneration.

It is recognised that spinal glutamate outflow is increased after Aβ- and C-fibre activation. During afferent terminal stimulation, intracellular calcium concentration ($[Ca^{2+}]_i$) increases, which allows depolarisation of cell membrane and release of stored glutamate. Activity of low threshold fibres, such as that required in acute painful stimulus, appears to activate normally only the AMPA receptor, which leads to a brief neuronal firing. The conditions needed for NMDA-receptor activation are much more complex and only achieved by repeated C-fibre activity, such as that gained after induction of peripheral inflammation which is accompanied by increased glutamate release in the dorsal horn of the spinal cord (Yang *et al.,* 1996; Vetter *et al.,* 2001) (Figure 2.1). Activation of NMDA receptors causes spinal cord neurones to become more responsive to all of its inputs, resulting in central sensitisation (Bennett, 2000). Apart from the release of glutamate and its binding to the receptor, activation of NMDA receptors can be regulated by other means.

2.1.1 Regulation of spinal NMDA receptors

Magnesium ions

At resting potential the channel is blocked by extracellular Mg^{2+} ions, but activation exceeding a defined threshold, usually through postsynaptic non-NMDA receptor activation, depolarises

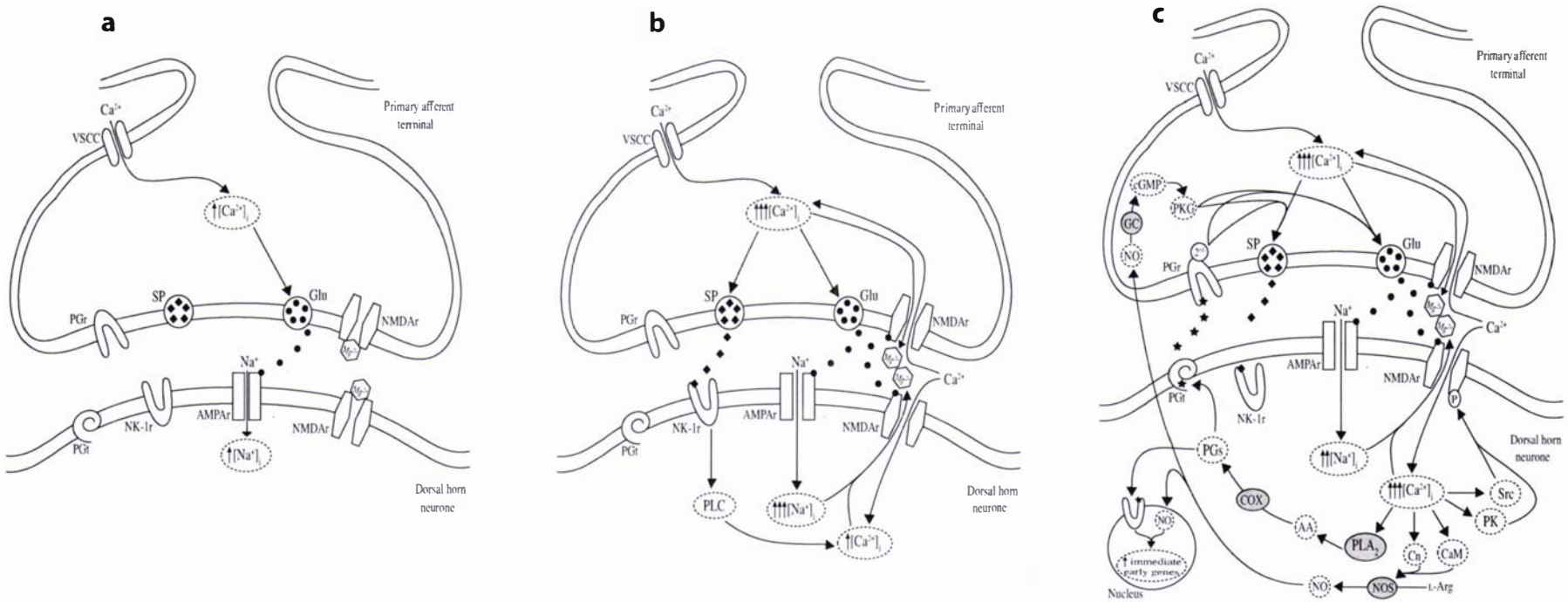

Figure 2.1 *Schematic representation of pain neurotransmission in the spinal cord. (a) Under normal conditions, afferent terminal stimulation (mainly that of Ad-fibres) increases intracellular calcium concentration ([Ca2+]i) allowing depolarisation of the cell membrane and release of stored glutamate (Glu). On dorsal horn neurones, Glu activates a-amino-3-hydroxy-5-methyl-4-isoxalone propionic acid receptors (AMPAr) leading to an increase in intracellular sodium concentration ([Na+]i) and a brief neuronal firing. (b) During repetitive stimulation of primary afferent C-fibres, a larger [Ca2+]i releases more Glu into the synaptic cleft producing a larger depolarisation of the dorsal horn neurone, which releases the magnesium (Mg2+) block from N-methyl-D-aspartate receptors (NMDAr). This increases [Ca2+]i even more in primary afferent terminals with the subsequent release of substance P (SP). Activation of neurokinin1 receptors (NK1r) by SP stimulates phospholipase C (PLC) to further increase [Ca2+]i from intracellular stores in dorsal horn neurones. (c) Although NK1r suffer internalisation, the increased [Ca2+]i in dorsal horn neurones activates protein kinases (PK) and Src-family of protein tyrosine kinases (Src), which increase the activity of NMDAr by phosphorylating them (P). The increased [Ca2+]i can also activate calcineurin (Cn) and bind to calmodulin to form the Ca2+-calmodulin complex (CaM). Both Cn and CaM activate nitric oxide synthase (NOS) to produce nitric oxide (NO) from the precursor L-arginine (L-Arg). NO then diffuses back to activate guanylate cyclases (GC) to increase the intracellular concentration of 3',5'-cyclic guanosine monophosphate (cGMP), which results in activation of cGMP-dependent protein kinases (PKG). PKG may increase the release of Glu and SP. In addition, the increased [Ca2+]i can activate PLA2to produce free arachidonic acid (AA), which is converted into prostaglandins (PGs) by cyclo-oxygenases (COX). A PG transporter (PGt) moves PGs from the intracellular space into the synaptic cleft. PGs then activate specific PG receptors (PGr) in primary afferent terminals, and second messengers (2nd) increase the release of Glu and SP. Both NO and PGs can also act in the nucleus of dorsal horn neurones and may play a role in gene transcription.*

the membrane removing the Mg^{2+} ion. This permits the opening of the channel and a massive depolarisation of the neurone results from the influx of Ca^{2+} and Na^{+} (Ghosh and Greenberg, 1995; Chaplan *et al.,* 1997). Hence, non-NMDA and 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 potentiates the activity of NMDA receptors (Yu and 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 protein tyrosine kinase (see below) associated with the channel (Yu and Salter, 1998). Nonetheless, removal of the Mg^{2+} block from the NMDA receptor channel is not a Na^{+}-mediated effect *per se*, but a voltage-sensitive one. Hence, Ca^{2+} influx through voltage-gated Ca^{2+} channels also potentiates NMDA-receptor functioning (Ikeda *et al.,* 2003).

Other divalent cations

In addition to the effects of Mg^{2+}, 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 (see below), and are not voltage-dependent to the same degree as those of Mg^{2+}, suggesting a different site of action (Reynolds and Miller, 1990; Dingledine *et al.,* 1999). Another cations, Sr^{2+}, Ca^{2+} and Ba^{2+}, may increase or decrease NMDA ligand binding, an effect that seems to be related to their concentration (Reynolds and Miller, 1990).

Glycine

The NMDA receptor is reciprocally coupled to a glycine receptor distinct from the classical receptor in the spinal cord because it is strychnine insensitive. Although glycine does not activate the channel itself, it potentiates NMDA responses and may be necessary for channel opening (Reynolds and Miller, 1990). Even glycine released from inhibitory interneurones facilitates the activation of spinal NMDA receptors (Ahmadi *et al.,* 2003). Blockade of glycine sites prevents channel opening (Viu *et al.,* 1998).

D-Serine

Similar to glycine, D-serine does not evoke NMDA-receptor responses by itself. This amino acid rather functions as an endogenous co-agonist of NMDA receptors since its depletion in the medium strongly diminishes NMDA-receptor activity (Mothet *et al.,* 2000). D-Serine has been regarded as an endogenous ligand for the glycine site of the NMDA receptor (Mothet *et al.,* 2000).

Phencyclidine

There is a phencyclidine binding site located close to the Mg^{2+} site within the channel,

and phencyclidine site ligands, such as ketamine and dizocilpine, reduce NMDA-receptor currents. Phencyclidine site ligands preferentially bind to NMDA receptors that are in a state consistent with channel opening, while ligands bind less well to inactive channels (Reynolds and Miller, 1990).

Polyamines

Polyamines such as spermidine and spermine act within the modulatory site on the NMDA receptor. They can induce a weak voltage-dependent inhibition, a glycine-dependent potentiation, and a voltage- and glycine-independent potentiation of NMDA receptor function (Dingledine *et al.,* 1999).

Protein phosphorylation

NMDA receptors are also regulated by protein phosphorylation at serine, threonine and tyrosine residues (Yu *et al.,* 1997). Activation of protein kinase C (PKC) potentiates the NMDA receptor function by phosphorylating serine and threonine residues (Yu *et al.,* 1997) on the NR1 subunit (Ghosh and Greenberg, 1995). Protein kinase A (PKA) also enhances NMDA receptor function (Dingledine *et al.,* 1999; Westphal *et al.,* 1999), and this is more evident when the NMDA receptor is composed of NR2A subunits. This receptor subunit is coupled to the associated protein yotiao, which anchors PKA and, therefore, an increase of the receptor currents in comparison to NMDA receptors not expressing yotiao is observed (Westphal *et al.,* 1999).

The Src-family of protein tyrosine kinases phosphorylates NMDA receptor subunits NR2A and NR2B (Yu *et al.,* 1997; Tezuka *et al.,* 1999), and the anchor proteins postsynaptic density-95 (PSD-95) (Tezuka *et al.,* 1999) and PSD-93 (Tao *et al.,* 2003) may play an important role in this process. Brain-derived neurotrophic factor, acting through tyrosine kinase B neurotrophin receptors, elicits NR1 and NR2B subunit phosphorylation-dependent enhancement of NMDA-channel functional activity (Levine *et al.,* 1998). Nerve growth factor, acting on tyrosine kinase A neurotrophin receptors, upregulates brain-derived neurotrophic factor which, apart from enhancing NMDA receptor phosphorylation, also induces changes in NR2A and NR2B receptor subunit expression (Thompson *et al.,* 1999). Calcium/calmodulin-dependent protein kinase II, when activated by Ca^{2+} influx through the NMDA receptor, phosphorylates NR1 and NR2B subunits increasing NMDA receptor-dependent Ca^{2+} influx (Leonard *et al.,* 1999).

Phosphorylation of the NMDA receptor sensitises the receptor so that its subsequent responsiveness to glutamate is enhanced. This allows subthreshold inputs to reach threshold levels, resulting in allodynia and hyperalgesia.

Protein desphosphorylation

Interestingly, increased $[Ca^{2+}]_i$ can also produce Ca^{2+}-dependent inactivation of NMDA receptors (Kyrozis *et al.,* 1996). Once in the neurone, Ca^{2+} activates calmodulin which then desphosphorylates NMDA receptors (Tong *et al.,* 1995). Yotiao proteins, in addition to anchor PKA to NR2A subunits, couple type I protein phosphatase to NMDA receptors, and this enzyme desphosphorylates NMDA receptors negatively regulating the receptors' currents (Westphal *et al.,* 1999).

Fluctuation of NMDA receptors between phosphorylated and desphosphorylated forms may depend on the rate of synaptic stimulation and the magnitude of the associated Ca^{2+} influx through them. Calmodulin may also act as a Ca^{2+} sensor contributing in breaking the recruitment of NMDA receptors during sensory synaptic input. 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.

The above sites and regulatory mechanisms are potential targets for reducing the activity of NMDA receptors and could be exploited for the pharmacological control of pain.

2.1.2 NMDA receptors and the spinal nociceptive process

NMDA receptor expression

Both mRNA and protein of NMDA receptors are constitutively expressed in spinal and dorsal root ganglion (DRG) neurones (Liu *et al.,* 1994; Tölle *et al.,* 1996; Parada *et al.,* 2003). The expression is high in DRG and the superficial layers of the dorsal horn of the spinal cord (Table 2.1), which are important areas for the transmission of nociceptive information. Interestingly, chronic monoarthritis did not affect the spinal staining of NMDA mRNA, but did increase the spinal binding of [^{3}H]PDBU, a molecule that binds and activates the diacylglycerol binding site on PKC (Tölle *et al.,* 1996). This suggests that arthritic pain may indirectly increase NMDA-receptor function by a PKC-mediated phosphorylation action.

NMDA receptors and pain

Direct activation of spinal NMDA receptors after intrathecal administration of NMDA produce nociceptive behaviours, allodynia, and hyperalgesia in different models of pain (Björkman *et al.,* 1996; Dolan and Nolan, 1999; Eguchi *et al.,* 1999; Kawamata and Omote, 1999; Fairbanks *et al.,* 2000; Tao and Johns, 2000; Minami *et al.,* 2001; Parada *et al.,* 2003; Muscoli *et al.,* 2004; Shimoyama *et al.,* 2005). The increased nociceptive response is believed to be due to the entry of Ca^{2+} into the neurone, which triggers different metabolic pathways activating a number of enzymes and ultimately immediate-early genes (Ghosh and Greenberg, 1995; Muscoli *et al.,* 2004). Retrograde second messengers resulting from this cascade cause

Table 2.1 *Expression and anatomical distribution of mRNAs encoding for the different NMDA receptor splice variants in the lumbar spinal cord of the rat.*[*]

Splice variant	Subtype	Dorsal horn	Lamina X	Ventral horn	Reference
NR1		√	√	√	(Tölle *et al.*, 1996)
		√			(Yung, 1998)
	a	√	√	√	(Tölle *et al.*, 1996)
	b	√ (few)	√	√ (few)	(Tölle *et al.*, 1996)
	1	√	nd	√ (few)	(Tölle *et al.*, 1996)
	2	√	√	√	(Tölle *et al.*, 1996)
	3	√ (few)	nd	nd	(Tölle *et al.*, 1996)
	4	√	√	√	(Tölle *et al.*, 1996)
NR2	A	nd	nd	nd	(Tölle *et al.*, 1996)
		nd			(Yung, 1998)
	B	nd	nd	nd	(Tölle *et al.*, 1996)
		√			(Yung, 1998)
	C	√ (few)	nd	nd	(Tölle *et al.*, 1996)
		nd			(Yung, 1998)
	D	√	√	√	(Tölle *et al.*, 1996)

[*] *Abbreviations:* √ = *present; nd = not detected.*

presynaptic augmentation of neurotransmitter and neuropeptide release, which can result in amplification and prolongation of the neural response, underlying many forms of central sensitisation. Candidates include the neuromediators arachidonic acid (AA), prostaglandins (PGs) and nitric oxide (NO) (Dumuis *et al.*, 1988; Herrero *et al.*, 1992; Dawson and Dawson, 1995; Chaplan *et al.*, 1997; Kawamata and Omote, 1999; Minami *et al.*, 1999).

Supporting evidence for the AA/PG/NO model comes from studies showing that non-steroidal anti-inflammatory drugs (NSAIDs, which inhibit PG synthesis) (Malmberg and Yaksh, 1992b; Björkman *et al.*, 1996; Dolan and Nolan, 1999; Parada *et al.*, 2003) and inhibitors of the enzyme NO synthase (NOS, which catalyses the formation of NO) (Dolan and Nolan, 1999; Kawamata and Omote, 1999; Fairbanks *et al.*, 2000) inhibited the increased nociceptive response induced after intrathecal administration of NMDA.

Directly or indirectly, NMDA receptors may contribute to the release of neuromodulators other than AA, PGs and NO, and substances can differ depending on the kind of stimulus. Some of these neuromodulators include glutamate, substance P (SP), γ-aminobutyric acid (GABA), opioid peptides, monoamines, and superoxide, just to mention a few.

NMDA receptor-mediated events are considered to be postsynaptic, but the existence of active presynaptic NMDA receptors (autoreceptors) in the dorsal horn of the spinal cord has been demonstrated (Liu *et al.*, 1994). Although presynaptic NMDA receptors seem to contribute to the release of glutamate (Robert *et al.*, 1998; Parada *et al.*, 2003) and SP (Liu *et al.*, 1997; Malcangio *et al.*, 1998), there is controversy on the nociceptive role of these receptors (Urban and Nagy, 1997).

NMDA receptor blockers and pain

Electrophysiological studies have shown that blockade of spinal NMDA receptors reduced

the excitability of dorsal horn neurones to electrical stimulation of the dorsal root at C-fibre strength (Ikeda *et al.,* 2003) or to mechanical stimulation of an inflamed joint (Neugebauer *et al.,* 1993). In accordance, there is strong evidence that intrathecal administration of NMDA receptor antagonists inhibited intrathecal NMDA-induced behaviours (Kawamata and Omote, 1999; Fairbanks *et al.,* 2000), as well as thermal (Fairbanks *et al.,* 2000; Tao and Johns, 2000; Yaksh *et al.,* 2001) and mechanical (Dolan and Nolan, 1999; Nishimura *et al.,* 2004) hypersensitivity. These antagonists also reduced hyperalgesia in animal models of inflammatory and neuropathic pain (Chaplan *et al.,* 1997), and produced postoperative analgesia in human beings (Koinig *et al.,* 2000; Ozyalcin *et al.,* 2004).

NMDA receptor deficiency and pain

Deletion of spinal NR1 receptor subunit by use of antisense oligonucleotides abolished nociceptive behaviours and thermal hyperalgesia observed after intrathecal injection of NMDA or intraplantar formalin in rats (Garry *et al.,* 2000; Shimoyama *et al.,* 2005). Similarly, gene disruption techniques in mice showed that NR1-1 receptor subunits were involved in allodynia induced by intrathecal administration of PGE_2, and NR1-4 receptor subunits in allodynia induced by intrathecal injection of $PGF_{2\alpha}$ (Minami *et al.,* 1999). Decreased spinal excitatory transmission along with antihyperalgesia in models of neuropathic and arthritic pain accompanied the reduced spinal expression of NR2A and NR2B subunits after disruption of the *psd-93* gene in mice (Tao *et al.,* 2003). Even knocking down of spinal *psd-93* (Zhang *et al.,* 2003) and *psd-95* (Tao *et al.,* 2000a), without affecting NMDA subunit expression, reduced thermal and mechanical hyperalgesia in rats.

However, not all NMDA receptor subunits seem to have an excitatory action. Knockout of the NR2A or NR2D subunit produced no difference in withdrawal thresholds as compared to wild-type mice in a postoperative pain model (Nishimura *et al.,* 2004). Compared to wild-type mice, mice lacking the NMDA receptor NR1-1A subunit gene had an enhanced nociceptive response to SP administered by the intrathecal route, which suggested an inhibitory role for the NR1-1A receptor subunit (Inoue *et al.,* 2000).

It seems that each NMDA receptor subunit plays a specific role in different types of pain. Although most NMDA receptor subunits are pronociceptive, some are antinociceptive; the net effect may depend on the subunit composition of the NMDA receptor. Blockade of spinal NMDA receptors may help to prevent or reduce the development of central sensitisation.

2.2 NMDA receptors and the arachidonic acid pathway

The increase in $[Ca^{2+}]_i$ after NMDA receptor stimulation activates phospholipases. Activation

of phospholipase A_2 (PLA_2) can make membrane phospholipids more susceptible to hydrolytic processes that are associated with an increasing level of free AA. Arachidonate, in turn, may be converted by cyclo-oxygenases (COX) to prostanoids. Supporting evidence for this model comes from: *(i)* increased AA release after administration of glutamate and NMDA to cultured cerebellar granule cells (Viu *et al.,* 1998) and astrocytes (Strokin *et al.,* 2003); and *(ii)* increased spinal release of PGs after spinal treatment with NMDA (Dirig and Yaksh, 1999) and after activation of high-threshold nociceptive afferent inputs (Ramwell *et al.,* 1966).

2.2.1 Phospholipases A_2 and the spinal nociceptive process

Arachidonic acid generation and functions

Arachidonate concentrations in the cytoplasm are low under physiological conditions, and formation of AA is generally considered to be the rate-limiting step in prostanoid synthesis. Arachidonic acid is found in the *sn*-2 position of membrane phospholipids, and PLA_2 hydrolyses the phospholipid *sn*-2 ester bond to generate free AA and lysophospholipids (Balsinde *et al.,* 2002). It is theorised that AA diffuses back to the primary afferent terminal to amplify pain signals, but this is unlikely to happen due to AA's physicochemical properties. AA certainly potentiates NMDA receptor currents by itself, probably by altering the receptor's lipid environment (Miller *et al.,* 1992), or by functionally converting K^+ channels from non-inactivating delayed rectifiers into rapidly inactivating A-type channels (Oliver *et al.,* 2004). Both increased release and decreased glial uptake of glutamate have also been proposed as possible excitatory mechanisms for AA (Dumuis *et al.,* 1988; Herrero *et al.,* 1992), but these effects seem to be mediated by products downstream of AA in the biochemical pathway (Bezzi *et al.,* 1998).

PLA_2 isozymes and functions

In mammals, 19 proteins possessing PLA_2 activity have been identified. Based on biological properties, the PLA_2 superfamily can be divided into three distinct classes: cytosolic Ca^{2+}-dependent PLA_2 ($cPLA_2$), cytosolic Ca^{2+}-independent PLA_2 ($iPLA_2$), and secretory PLA_2 ($sPLA_2$) (Balsinde *et al.,* 2002).

$cPLA_2$ are found in the cytosol of most cells and are translocated to nuclear/endoplasmic reticulum membranes in response to increased $[Ca^{2+}]_i$; their activity is enhanced if phosphorylated by mitogen-activated protein kinases (Balsinde *et al.,* 2002). These isozymes possess a preference for phospholipids containing AA and in fact, AA release from astrocytes has been linked to $cPLA_2$ activity (Strokin *et al.,* 2003). $iPLA_2$ are a group of related isozymes that require mM concentrations of Ca^{2+} for activation and are not selective for AA-containing phospholipids. These isozymes are released into the extracellular space and can induce phospholipid cleavage in adjacent cells. $sPLA_2$ are involved in AA remodelling rather

than AA liberation, incorporating and exposing AA and other fatty acids into membrane phospholipids (Balsinde *et al.,* 2002).

PLA_2 expression

Both PLA_2 mRNA and protein are constitutively expressed in the spinal cord, and they can be induced after lipopolysacharide, interleukin-1β and zymosan treatment (Svensson and Yaksh, 2002), but not after carrageenan injection (Lucas *et al.,* 2005).

PLA_2 inhibitors and pain

Corticosteroids, which among other actions inhibit PLA_2, have been used to study the role of these enzymes in the spinal processing of pain. Single intrathecal administration of methylprednisolone and triamcinolone did not affect flinching behaviour in rats submitted to a paw formalin test, and the former even induced mechanical allodynia (Abram *et al.,* 1994). Similarly, a single intravenous dose of dexamethasone did not increase mechanical nociceptive thresholds in normal and sensitised (due to footrot) sheep (Chambers *et al.,* 1995). These data suggest that corticosteroids have no central analgesic or antihyperalgesic actions.

Intrathecal administration of the type-unspecific PLA_2 inhibitors arachydonyl trifluoromethylketone ($AACOCF_3$) and methyl arachydonyl fluorophosphanate (MAFP) (Lucas *et al.,* 2005), and the preferential $cPLA_2$ inhibitor AX048 (Yaksh *et al.,* 2005) produced thermal antihyperalgesia in rats injected with carrageenan into one hind paw. Intrathecal injection of $AACOCF_3$ and AX048 also prevented both increased spinal release of PGE_2 and thermal hyperalgesia induced by intrathecal administration of NMDA or SP, respectively (Lucas *et al.,* 2005; Yaksh *et al.,* 2006). Although it could be argued that the spinal antihyperalgesic effects of $AACOCF_3$ and MAFP might be due to possible interaction with the cannabinoid (CB) system (Ates *et al.,* 2003), the spinal antihyperalgesic actions of AX048 were not blocked by intrathecal pre-treatment with the CB_1 receptor antagonist SR141716 (Yaksh *et al.,* 2006).

The presence and biological activity of PLA_2 suggest a role for this family of enzymes in the spinal processing on nociceptive information, but the lack of more selective inhibitors has left open the issue regarding the relative contribution of these enzymes. This is an area where further research is warranted.

2.2.2 Cyclo-oxygenases and the spinal nociceptive process

COX isozymes and functions

The membrane-bound enzymes COX exist in at least two isoforms: COX-1 and COX-2. Both COX isozymes, however, use the same endogenous substrate and form the same

products by the same catalytic reaction. This is be due to the highly conserved homology (>90%) of the COX and peroxidase enzyme regions between isozymes (Conn and Pin, 1997). Cyclooxygenation of AA produces PGG_2, which is reduced to PGH_2 by COX-mediated peroxidase activity. PGH_2 is converted to final active products via individual prostaglandin synthases (see below) (Appleton *et al.,* 1996).

The major difference in COX isoenzymes lies in their pathophysiological functions. COX-1 is constitutively expressed in most tissues at a different constant level, and it is believed to be responsible for the formation of PGs with physiological functions. COX-2 also produce PGs with physiological effects, but it is normally undetectable in most tissues and can be induced at high levels in a variety of cells by pro-inflammatory stimuli (Appleton *et al.,* 1996; Conn and Pin, 1997). The COX-2 mRNA contains consensus sequences for several nuclear transcription factors including nuclear factor-κB (NF-κB), providing potent mechanisms for induction. The gene also displays repeated motifs in the untranslated regions common to immediate early genes rendering the mRNA less stable, whereas during catalysis COX-2 undergoes auto-inactivation (Svensson and Yaksh, 2002). Such properties indicate an effective regulation of enzyme expression and activity.

The presence of additional COX enzymes was recently demonstrated in various tissues from both human beings and dogs. Canine neuronal tissues expressed three new identified mRNAs derived from the COX-1 gene. The enzyme products of these mRNAs were termed COX-3, partial COX-1a (PCOX-1a) and PCOX-1b (Chandrasekharan *et al.,* 2002). In transfected insect cells, canine COX-3 was selectively inhibited by various NSAIDs, including paracetamol, in comparison to COX-1 and COX-2; PCOX-1a had no COX activity (Chandrasekharan *et al.,* 2002). These data suggested that COX-3 could be involved in pain transmission. However, PGI_2 production in rat brain and cerebellum, which also expressed COX-3 mRNA, was not particularly sensitive to inhibition by paracetamol compared to other COX inhibitors (Warner *et al.,* 2004). It is possible that the dog COX-3 functions differently to that from the rat. For instance, although COX-3 protein (also termed COX-1b since it is a splice variant of COX-1) was constitutively expressed in the rat spinal cord, its amino acid sequence was completely different than the known COX and it lacked COX activity in transfected COS-7 cells (Snipes *et al.,* 2005). Various methodological issues in the original report describing the existence of COX-3 (Chandrasekharan *et al.,* 2002) have been recently highlighted and the conclusions questioned (Kis *et al.,* 2005).

COX expression

In the spinal cord, COX-1, COX-2 and COX-3 mRNAs and proteins are constitutively expressed, and their predominance may depend on the anatomical localisation, the age, and the species (Table 2.2). In the rat, COX-1 immunoreactivity was detected in small to medium

Table 2.2 *Constitutive expression of cyclo-oxygenase isozymes in the spinal cord.*[*]

Isozyme / Method	Species	Localisation: Dorsal horn	Lamina X	Ventral horn	DRG	Cell type	Reference
COX-1							
IHC	Mouse	√		√		Glial cells	(Maihofner *et al.*, 2000)
IHC	Rat	√		√		Neurones	(Zhu *et al.*, 2003)
IHC	Rat	√(few)	nd	nd	√	Neurones	(Willingale *et al.*, 1997)
ISH	Sheep	√	√	√		Neurones	(Dolan *et al.*, 2003)
RNA	Mouse	√ †		√ †			(Ballou *et al.*, 2000)
RT-PCR	Mouse	√ †		√ †			(Gühring *et al.*, 2000)
RT-PCR	Mouse	√ †		√ †			(Ballou *et al.*, 2000)
RT-PCR	Sheep	√ †		√ †			(Dolan *et al.*, 2000; Dolan *et al.*, 2003)
WB	Rat	√ †		√ †	√ †		(Yaksh *et al.*, 2001)
WB	Rat	√		√			(Seybold *et al.*, 2003)
WB	Rat	nd†		nd†			(Wen *et al.*, 2001)
WB	Rat	√ †		√ †			(Dirig and Yaksh, 1999)
WB	Sheep	√ †		√ †			(Dolan *et al.*, 2003)
COX-2							
IHC	Mouse	nd	√	√		Neurones	(Maihofner *et al.*, 2000)
IHC	Rat	√	√	√	nd	Neurones	(Willingale *et al.*, 1997)
ISH	Sheep	√		√		Neurones	(Dolan *et al.*, 2003)
RNA	Mouse	√ †		√ †			(Ballou *et al.*, 2000)
RT-PCR	Mouse	√ †		√ †			(Gühring *et al.*, 2000)
RT-PCR	Mouse	√ †		√ †			(Ballou *et al.*, 2000)
RT-PCR	Sheep	√ †		√ †			(Dolan *et al.*, 2000; Dolan *et al.*, 2003)
WB	Rat	√ †		√ †	√ †		(Yaksh *et al.*, 2001)
WB	Rat	√		√			(Seybold *et al.*, 2003)
WB	Rat	√ †		√ †			(Wen *et al.*, 2001)
WB	Rat	√ †		√ †			(Dirig and Yaksh, 1999)
WB	Sheep	√ †		√ †			(Dolan *et al.*, 2003)
COX-3							
WB	Rat	√ †		√ †			(Snipes *et al.*, 2005)

[*] *Abbreviations: √ = present; † = homogenate of whole spinal cord; COX-1 = cyclooxygenase-1; COX-2 = cyclooxygenase-2; IHC = immunohystochemistry; ISH = in situ hybridisation; nd = not detected; RNA = RNAsa protection assay; RT-PCR = reverse transcription-polymerase chain reaction; WB = western blotting.*

sized cell bodies in the DRG and the dorsal roots, and to a lesser extent in the dorsal horn of the spinal cord. COX-2 immunoreactivity was not present in DRG neurones, but it was observed in the superficial (laminae I-II) and deep (laminae V-VI) dorsal horn, around the central canal (lamina X), and in individual motor neurones in the ventral horn (Willingale *et al.*, 1997). Distribution of COX-1 and COX-2 in the dorsal horn was consistent with COX products being involved in the processing of nociceptive input, but COX metabolites may also be involved by stimulating afferent fibres.

Induction of peripheral inflammatory hyperalgesia in rats increased the immunoreactive staining of spinal COX-2 mRNA and protein, but not that of COX-1, 2 days after injecting complete Freund's adjuvant into the hid paw (Seybold *et al.*, 2003). However, in a model of peripheral inflammatory hyperalgesia in which formalin was injected into the hind paw, increased spinal release of PGs during 60 min post-injection was accompanied by increased expression of COX-2 mRNA, but not protein, suggesting that this process is largely independent of the induction of spinal COX-2 (Tegeder *et al.*, 2001). Interestingly, Zhu *et al.* (2003) demonstrated that rats submitted to an incisional model of postoperative pain showed increased COX-1 immunoreactivity in the dorsal horn and the gracile nucleus of the spinal cord on postoperative days 2 to 5. Spinal COX-1 immunoreactivity also increased

in the superficial laminae (I-II), but decreased it in the deep dorsal horn (IV-VI), ventral horn, and white matter of rats after 2 weeks of peripheral nerve injury (Zhu and Eisenach, 2003). Concurrently, these data may suggest that both constitutive and inducible spinal COX isozymes play a role in the development and maintenance of spinal sensitisation, and that the expression of COX in the spinal cord is not static, but changes in a time- and laminar-dependent manner.

In addition to determining its biological importance, the spinal anatomical distribution and regulation of COX-3 remain to be characterised.

COX inhibitors and pain

Administration of COX inhibitors into the spinal canal induced antihyperalgesia as assessed using different algesiometric tests (Table 2.3). Support for the inhibition of spinal COX enzymes as a means of reducing this altered pain state comes from: *(i)* antihyperalgesic potency of NSAIDs injected intrathecally correlating with their rank order potencies as COX inhibitors (Malmberg and Yaksh, 1992a; Dirig *et al.,* 1998; Yaksh *et al.,* 2001); *(ii)* antihyperalgesia produced at the same time that inhibition of spinal PG occurred (Muth-Selbach *et al.,* 1999); and *(iii)* antihyperalgesia achieved after intrathecal injection of PG receptor antagonists (Malmberg *et al.,* 1994; Nakayama *et al.,* 2002).

Table 2.3 *Effects of spinal administration of cyclo-oxygenase inhibitors on different algesiometric tests.*

Reference	Species	Nociceptive test	COX inhibitor	Dose	Effects
Intrathecal					
(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.
(Seybold *et al.,* 2003)	Rats (spinalised)	C-fibre reflex recorded from the hamstring muscles after electrical stimulation of the sural nerve	S(+)-ibuprofen	1-100 nM	No effect on reflex response.
			R(-)-ibuprofen	100 nM	No effect on reflex response.
		Subcutaneous injection of Complete Freund's Adjuvant + C-fibre reflex recorded from the hamstring muscles after electrical stimulation of the sural nerve	S(+)-ibuprofen	10 and 100 nM	Dose-dependent reduction of the reflex response.
			R(-)-ibuprofen	100 nM	No effect on reflex response.
(Carlsson *et al.,* 1986)	Rats (intact)	Radiant thermal tail-flick test	Dipyrone	50-400 µg in 5 µL = 30-240 mM	Dose-dependent increase of tail withdrawal latencies.
	Rats (spinalised)	Radiant thermal tail-flick test	Dipyrone	400 µg in 5 µL = 240 mM	No effect on tail withdrawal latencies.
		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 lower dose but increased it with the highest dose.
		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 the reflex response.
(Yaksh *et al.,* 2001)	Rats	Intrathecal SP + radiant thermal stimuli of the hindpaws	S(+)-ibuprofen	8 – 80 nmol	Dose-dependent increase of paw withdrawal latencies.
			R(-)-ibuprofen	80 nmol	No effect on paw withdrawal latencies.
			SC-384	23 – 230 nmol	Dose-dependent increase of paw withdrawal latencies.
			SC-58125	5-50 nmol	Dose-dependent increase of paw withdrawal latencies.
			SC-236	Not specified	Dose-dependent increase of paw withdrawal latencies.
			SC-560	280 – 2800 nmol	No effect on paw withdrawal latencies.
		Intrathecal NMDA + radiant thermal stimuli of the hindpaws	SC-58125	50 nmol	Increase on paw withdrawal latencies.
			SC-560	Not specified	No effect on paw withdrawal latencies.

Table 2.3 *Effects of spinal administration of cyclo-oxygenase inhibitors on different algesiometric tests. (Cont.)*

Reference	Species	Nociceptive test	COX inhibitor	Dose	Effects
		Intraplantar carrageenan + radiant thermal stimuli of the hindpaws	Ibuprofen	80 nmol	Increase of paw withdrawal latencies.
			SC-58125	50 nmol	Increase of paw withdrawal latencies.
			SC-560	280 nmol	No effect on paw withdrawal latencies.
(Malmberg and Yaksh, 1992)[5]	Rats	Intrathecal NMDA + hot plate test	Acetylsalicylic acid	100 nmol	Increase of paw withdrawal latencies.
			Ketorolac	27 nmol	Increase of paw withdrawal latencies.
			S(+)-ibuprofen	27 nmol	Increase of paw withdrawal latencies.
			R(-)-ibuprofen	270 nmol	No effect on paw withdrawal latencies.
		Intrathecal AMPA + hot plate test	S(+)-ibuprofen	27 nmol	Increase of paw withdrawal latencies.
			R(-)-ibuprofen	270 nmol	No effect on paw withdrawal latencies.
		Intrathecal SP + hot plate test	S(+)-ibuprofen	27 nmol	Increase of paw withdrawal latencies.
			R(-)-ibuprofen	270 nmol	No effect on paw withdrawal latencies.
(Malmberg and Yaksh, 1992)[5]	Rats	Subcutaneous formalin into one hindpaw	Acetylsalicylic acid	27 nmol (18 – 41 nmol)*	Dose-dependent inhibition of phase 2; 70% inhibition with the highest dose.
			Indomethacin	1.9 nmol (1.2 - 4.0 nmol)*	Dose-dependent inhibition of phase 2; 80% inhibition with the highest dose.
			Flurbiprofen	2.1 nmol (1.0 - 4.3 nmol)*	Dose-dependent inhibition of phase 2; 70% inhibition with the highest dose.
			Ketorolac	5.2 nmol (3.2 - 8.3 nmol)*	Dose-dependent inhibition of phase 2; 80% inhibition with the highest dose.
			Zomepirac sodium	5.9 nmol (3.9 - 8.9 nmol)*	Dose-dependent inhibition of phase 2; 75% inhibition with the highest dose.
			S(+)-ibuprofen	15.7 nmol (6.7 – 36 nmol)*	Dose-dependent inhibition of phase 2; 60% inhibition with the highest dose.
			Racemic ibuprofen	18.9 nmol (9.3 – 38 nmol)*	Dose-dependent inhibition of phase 2; 55% inhibition with the highest dose.
			Acetaminophen	257 nmol (163 – 405 nmol)*	Dose-dependent inhibition of phase 2; 50% inhibition with the highest dose.
			R(-)-ibuprofen	>270 nmol	No effect on phase 2.
		Hot plate test	Acetylsalicylic acid	100 nmol	No effect on paw withdrawal latencies.
			Flurbiprofen	80 nmol	No effect on paw withdrawal latencies.
			Ketorolac	80 nmol	No effect on paw withdrawal latencies.
			Zomepirac sodium	80 nmol	No effect on paw withdrawal latencies.
			Indomethacin	30 nmol	No effect on paw withdrawal latencies.
(Malmberg and Yaksh, 1993)	Rats	Subcutaneous formalin into one hindpaw	Ketorolac	2.7 – 80 nmol	Dose-dependent inhibition of phase 1 and 2; 52% and 80% inhibition, respectively, with the highest dose.
(Pelissier *et al.*, 1996)	Rats	Paw pressure test	Acetaminophen	100 and 200 µg in 10 µL = 66 and 132 mM	About 600% and 800% increase in the area under the nociceptive threshold curve.
(Lorenzetti and Ferreira, 1996)	Rats	Intraplantar PGE_2 + paw pressure test	Dipyrone	2.5 – 80 µg in 20 µL = 0.375 – 12 mM	Dose-dependent reduction of escape behaviours.
(Akman *et al.*, 1996)	Mice	Radiant heat tail-flick test	Dipyrone	50 – 400 µg in 5 µL = 30 – 240 mM	No effect on tail withdrawal latencies.
		Hot plate test	Dipyrone	50 – 400 µg in 5 µL = 30 – 240 mM	No effect on 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 and Nozaki-Taguchi, 1996)	Rats	Subcutaneous formalin into one hindpaw	Indomethacin	0.03 – 30 µg in 10 µL = 8.3 µM-8.3 mM	Dose-dependent reduction in the number of flinches in both phase 1 and 2.
			NS-398	0.03 – 30 µg in 10 µL = 9.5 µM-9.5 mM	Dose-dependent reduction in the number of flinches in both phase 1 and 2.
		Hot plate test	Indomethacin	30 µg in 10 µL = 8.3 mM	No effect on paw withdrawal latencies.
			NS-398	30 µg in 10 µL = 9.5 mM	No effect on paw withdrawal latencies.
(Willingale *et al.*, 1997)[6]	Rats (anaesthetised)	Mechanical stimulation of the ankle joint	Meclofenamic acid	10 and 100 µM	No effect on dorsal horn neurone responses.
		Mechanical stimulation of the knee joint	Indomethacin	1 and 100 µM	No effect on dorsal horn neurone responses.
		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 lower dose. About 67% reduction of the reflex response with the higher dose.

Table 2.3 *Effects of spinal administration of cyclo-oxygenase inhibitors on different algesiometric tests. (Cont.)*

Reference	Species	Nociceptive test	COX inhibitor	Dose	Effects
(Dirig *et al.*, 1998)	Rats	Subcutaneous carragennan into one hindpaw + radiant thermal stimuli	S(+)-ibuprofen	27 and 80 nM	Dose-dependent increase of paw withdrawal latencies.
			R(-)-ibuprofen	80 nM	No effect on paw withdrawal latencies.
			SC58125	50 nM	Dose-dependent increase of paw withdrawal latencies.
(Zhu *et al.*, 2003)	Rats	Paw incision + von Frey filaments	Ketorolac	150 µg in 10 µL = 39.5 mM	Increase of paw withdrawal thresholds.
			SC560	100 µg in 10 µL	Increase of paw withdrawal thresholds.
			NS-398	50 µg in 10 µL = 15.8 mM	No effect on paw withdrawal thresholds.
(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	About 35% and 80% reduction of the reflex response.
			Indomethacin	200, 300 and 500 µg in 10 µL = 55, 83 and 139 mM	About 36%, 62% and 89% reduction of the reflex response.
			Ketoprofen	300 and 500 µg in 10 µl = 112 and 196 mM	About 23% and 40% reduction of the reflex response.
			Lysine clonixinate	300, 500 and 1000 µg in 10 µL = 73, 122 and 244 mM	About 30%, 48% and 63% reduction of the reflex response.
(Dolan and Nolan, 1999)	Sheep	Foreleg mechanical stimulation.	DFU	200 nmol in 200 µL	No effect on mechanical thresholds.
		Intrathecal NMDA + foreleg mechanical stimulation.	DFU	200 nmol in 200 µL	Increase of mechanical thresholds.
(Eisenach *et al.*, 2002)[e]	Humans	Thermal heat stimuli of the skin of the volar forearm and the lateral calf.	Ketorolac	0.25 – 2 mg	No effect on withdrawal thresholds.
Epidural					
(Lauretti *et al.*, 1998)	Humans	Cancer pain sufferers	Diclofenac	75 mg	Pain relief (VAS[a] = 0) for 48 h.
			Tenoxicam	20 mg	Pain relief (VAS = 2) for 22.5 h
(Aldrete, 2003)[e]	Humans	Postlaminectomy syndrome	Indomethacin	2 injections 2 weeks apart, 1 or 2 mg in 3 mL 0.5% bupivacaine	Reduced pain intensity as evaluated by the Pain Progress Score 2 weeks after each injection
(Masue *et al.*, 1999)	Rats	Intrathecal nitroglycerin + tail-flick test	S(+)-ibuprofen	52 µg (38-66 µg) /30 µL = 8.4 mM (6.1-10.7 mM)*	Dose-dependent increase of tail withdrawal latencies.
			Indomethacin	5 µg (2.8-7.3 µg) /30 µL = 460 µM (258-672 µM)*	Dose-dependent increase of tail-flick latencies.
			Diclofenac	6.3 µg (5.7-7.6 µg) /30 µL = 660 µM (597-796 µM)*	Dose-dependent increase of tail-flick latencies.
			R(-)-ibuprofen	>1000 µg/ 30µL = >161 mM*	No effect on tail-flick latencies.
		Subcutaneous formalin into one hindpaw	S(+)-ibuprofen	1000 µg/ 30µL = 161 mM	Inhibition of paw-lifting behaviour in the phase 2.
			R(-)-ibuprofen	1000 µg/ 30µL = 161 mM	No effect on paw-lifting behaviour.

*ID_{50} values and 95% confidence intervals; [a] administration volume = 10 µL; [e] administration volume = 40-50 µL; [e] administration volume not specified; [a] VAS = visual analogue scale.

COX-2 seems to be the major isozyme involved in analgesia since spinal administration of non-selective COX inhibitors and COX-2, but not COX-1, selective inhibitors reduced thermal hyperalgesia (Yamamoto and Nozaki-Taguchi, 1996; Smith *et al.*, 1998a; Yaksh *et al.*, 2001). Importantly, the spinal antihyperalgesic actions of COX-2 inhibitors can be shown under conditions and at times when enzyme induction has probably not occurred, stressing the importance of the constitutive COX-2 isozyme in spinal nociception. Recently, the importance of spinal COX-1 in analgesia has been highlighted by the intrathecal delivery of a non-selective COX inhibitor and a COX-1, but not a COX-2, selective inhibitor reducing both mechanical hyperalgesia in a model of incisional post-operative pain (Zhu *et al.*, 2003) and mechanical hyperalgesia and allodynia in a model of neuropathic pain (Hefferan *et al.*,

2003). Similarly, oral administration of a COX-1, but not a COX-2, selective inhibitor reduced flinching behaviour in rats injected formalin in a hind paw at the same time that inhibited spinal PG release (Tegeder *et al.,* 2001). It may be possible that selective inhibition of spinal COX-1 and COX-2 are necessary for induction of mechanical and thermal antihyperalgesia, respectively.

From information in Table 2.3, it is evident that COX inhibitors produced spinal analgesic effects in models of inflammatory pain and hyperalgesia, but lacked analgesic action in models of acute pain, suggesting that COX inhibitors produced analgesia at the spinal level only when an ongoing afferent barrage was established (central sensitisation). However, Pelissier *et al.* (1996) reported hypoalgesia after intrathecal injection of mM concentrations of paracetamol in rats with no inflammation submitted to a paw pressure test, suggesting an analgesic action in the absence of spinal facilitated states. Spinal administration of COX inhibitors, in concentrations that were one or two orders of magnitude smaller, did not increase mechanical nociceptive thresholds in animals with no inflammation (Willingale *et al.,* 1997; Dolan and Nolan, 1999; Lizarraga and Chambers, 2006). Most COX inhibitor-mediated spinal antihyperalgesic effects have been reported with drugs being administered in mM concentrations (Table 2.3). Since nM to μM drug concentrations are necessary to inhibit COX isozymes (Giuliano and Warner, 1999), it is possible that mechanisms other than, or complementary to, prostanoid synthesis inhibition may mediate the spinal antihyperalgesic action of COX inhibitors (see McCormack, 1994 for review).

COX isozyme deficiency and pain

COX-2 deficient, COX-1 homozygous, and wild-type mice showed similar latency times in a hot plate test, which agrees with the lack of analgesic action of NSAIDs in this test. Surprisingly, COX-1 heterozygous mice showed longer reaction times. In an abdominal stretching test in which acetic acid was intraperitoneally injected, fewer writhes were observed in COX-1 deficient mice and COX-2 heterozygous female mice, but not in COX-2 homozygous females or COX-2 deficient mice, as compared to wild-type mice. Although no increase in COX-2 mRNA was detected in the spinal cords of COX-1-null animals, a compensatory increase in COX-1 mRNA in the spinal cords of COX-2-null animals was noticed (Ballou *et al.,* 2000), which makes it difficult to interpret these results.

Intrathecal injection of an oligonucleotide antisense targeted to the *Ptgs-2* gene reduced spinal COX-2, but not COX-1, expression in rats and prevented thermal hyperalgesia induced by intrathecal NMDA injection, but not acute thermal nociception (Svensson and Yaksh, 2002).

In summary, a modulatory role for COX isozymes in the processing of pain is suggested

by the spinal distribution and expression of these enzymes. This is further supported by the antihyperalgesic action of NSAIDs injected intrathecally, although these drugs may also interfere with other systems involved in nociception.

2.2.3 Prostaglandins and the spinal nociceptive process

PG formation

Prostaglandins are formed by cyclooxygenation of AA and peroxidation of PGH_2. Individual PG synthases convert PGH_2 into prostanoids, including PGD_2, PGE_2, $PGF_{2\alpha}$, and PGI_2. Prostanoids are synthesised upon demand and are rapidly metabolised with biological half-lives of approximately 1 minute (Appleton *et al.*, 1996). In the normal rat spinal cord, PGD_2 and PGE_2 are the predominant PGs, followed by PGI_2 and $PGF_{2\alpha}$ (Willingale *et al.*, 1997).

PG synthase expression

Isozymes of specific PG synthases have been identified. PGF synthase (PGFS) exists in two variants: PGFS I (Suzuki-Yamamoto *et al.*, 2000) and PGFS II (Suzuki-Yamamoto *et al.*, 2003); whereas PGE synthase (PGES) subsists as microsomal (mPGS1 and mPGS2) and cytosolic glutathione transferases (GSTM2-2 and GSTM3-3, cPGES/p23) (Trebino *et al.*, 2003). There are three PGD synthases (PGDS), but lipocalin-type PGDS (L-PGDS) is believed to be responsible for the biosynthesis of PGD_2 in the central nervous system (Eguchi *et al.*, 1999). The mRNA and protein expression for some PG synthases in the spinal cord is summarised in Table 2.4.

Table 2.4 *Constitutive expression of prostaglandin and thromboxane synthases in the spinal cord.*

Synthase / Method	Species	Localisation			Cell type	Reference
		Dorsal horn	Lamina X	Ventral horn		
PGFS I						
IHC	Rat	√		√	Neurones and endothelial cells	(Suzuki-Yamamoto *et al.*, 2000)
PGFS II						
WB	Rat				Spinal cord†	(Suzuki-Yamamoto *et al.*, 2003)
IHC	Rat		√		Ependimal cells, tanacytes, endothelial cells	(Suzuki-Yamamoto *et al.*, 2003)
mPGES1						
NDB	Human				Spinal cord†	(Jakobsson *et al.*, 1999)
mPGES2						
NDB	Human				Spinal cord†	(Tanikawa *et al.*, 2002)
TxS						
IHC	Ovine	√	√	√	Neurones, astroglial and ependimal cells	(Husted *et al.*, 2003)
L-PGDS						
IHC	Mice	√	√	√	Leptomeninges and oligodendrocytes of the spinal cord	(Eguchi *et al.*, 1999)

** IHC = immunohistochemistry; L-PGDS = lipocalin-type prostaglandin D synthase; mPGES1 = microsomal prostaglandin E synthase 1; mPGES2 = microsomal prostaglandin E synthase 2;NDB = Northern blot dot, PGFS I = prostagalndin F synthase I; PGFS II = prostagalndin F synthase II; TxS = thromboxane synthase; WB = western blotting; † homogenate of whole spinal cord.*

PG synthase deficiency and pain

In a model of abdominal pain, mPGES1 knockout mice exhibited the same pain behaviour as NSAID-treated wild-type mice, whereas untreated mPGES$^{+/+}$ mice developed the full classic response. However, withdrawal latencies of mPGES-null and wild-type mice in a hot plate test were not different (Trebino *et al.,* 2003), which agrees with the lack of antinociceptive action of NSAIDs in this test.

L-PGDS seems to play a major spinal role in allodynia. After intrathecal injection of PGE_2, wild-type mice developed allodynia and hyperalgesia, but mice lacking L-PGDS developed hyperalgesia only. Similarly, intrathecal injection of the $GABA_A$ receptor antagonist bicuculline (increases neuronal excitability by decreasing Cl^- influx) produced allodynia in wild-type mice, but not in L-PGDS$^{-/-}$ mice (Eguchi *et al.,* 1999).

PG release

In the spinal cord, PGs can be released by activation of high-threshold nociceptive afferent inputs (Ramwell *et al.,* 1966), peripheral inflammatory stimuli (Yang *et al.,* 1996; Smith *et al.,* 1998a; Dirig and Yaksh, 1999; Muth-Selbach *et al.,* 1999; Tegeder *et al.,* 2001; Nakayama *et al.,* 2002) or direct spinal administration of neurotransmitters (Chaplan *et al.,* 1997; Dirig and Yaksh, 1999; Yaksh *et al.,* 2001). These events suggest certain level of repetitive stimulation of the primary afferent for the release of PGs and support the NMDA-receptor activation for their generation. Nonetheless, PG generation can also occur by co-activation of AMPA/kainate and mGluR receptors (Meller *et al.,* 1996; Bezzi *et al.,* 1998; Vane *et al.,* 1998), and PGs can even be released from neuronal and non-neuronal elements (Buritova *et al.,* 1996; Willingale *et al.,* 1997; Bezzi *et al.,* 1998). The release of PGs from their generating cells is mediated by the active carrier transporter multidrug resistance protein 4 (Reid *et al.,* 2003), but its function in the spinal modulation of pain remains to be elucidated.

PGs and pain

The intrathecal injection of PGs and PG analogues increase nociceptive responses. In mice, for instance, PGE_2 and $PGF_{2\alpha}$ produced allodynia by co-activating AMPA and NMDA receptors and AMPA and mGluR receptors, respectively (Minami *et al.,* 1994b). The constitutive presence of spinal PGD_2 was required for PGE_2, but not $PGF_{2\alpha}$, to evoke this altered pain sensation (Eguchi *et al.,* 1999). In rodents, PGE_2 given intrathecally produced allodynia and hyperalgesia (Taiwo and Levine, 1988; Minami *et al.,* 1995; Minami *et al.,* 2001; Parada *et al.,* 2003). The exact mechanisms by which PGs enhance nociception are not clear, but may include enhanced Ca^{2+}-dependent glutamate (Bezzi *et al.,* 1998), SP (Malcangio *et al.,* 1996; Southall and Vasko, 2001) and calcitonin gene-related peptide (CGRP) release (Andreeva and Rang, 1993; Southall and Vasko, 2001); noradrenaline release inhibition from the bulbospinal noradrenergic pathway (Taiwo and Levine, 1988); up-regulation of the neurokinin$_1$ receptor

(NK_1; binding site for SP) in DRG sensory neurones (Segond von Banchet *et al.,* 2003); direct depolarisation of wide dynamic range neurones in the deep dorsal horn of the spinal cord (Baba *et al.,* 2001); and reduction of the inhibitory tone of glycine onto neurones in the superficial laminae of the dorsal horn (Harvey *et al.,* 2004).

Although hypothesised that PGs may diffuse back to the primary afferent terminal to amplify pain signals, it is more likely that they use a specific carrier-mediated transport (PG transporters) (Kanai *et al.,* 1995) since they are charged anions at physiological pH and diffuse poorly across biological membranes. Thus, not just the release (Reid *et al.,* 2003), but also the up-take of prostanoids may be mediated by carrier transporters.

Interestingly, not all PGs increase pain sensation. In mice, for instance, intrathecal administration of PGD_2 blocked nociceptin-induced allodynia (Minami *et al.,* 1997), and PGE_2 played a physiological inhibitory role in the appearance of thermal hyperalgesia (Minami *et al.,* 2001).

In general, PGs are synthesised upon demand by specific PG synthases, and their release is increased in the spinal cord during development of central sensitisation. Intrathecal injection of some PGs induces allodynia and/or hyperalgesia. Preventing the formation of PGs may be useful for treating some types of pain.

2.2.4 Prostaglandin receptors and the spinal nociceptive process

PG receptors

Prostaglandins act as extracellular and intracellular messengers by activating specific G protein-coupled membrane receptors. Receptors for PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TxA_2 are denoted as DP, EP, FP, IP and TP receptors, respectively. For some of them, receptor subtypes and isoforms have been identified. After activation, the signal transduction pathway a given prostanoid receptor follows depends on the G protein species the receptor is coupled to. In general, there are changes in concentrations of the second messengers 3',5'-cyclic adenosine monophosphate (cAMP), inositol-(1,4,5) triphosphate ($InsP_3$) or Ca^{2+} (Table 2.5).

PG receptor expression

Both mRNA and protein for several prostanoid receptors are expressed in the spinal cord, especially in the superficial layers of the dorsal horn, and in DRG neurones (Table 2.6). In DRG from normal mice, mRNAs for EP_1, EP_3, EP_4 and IP receptors were expressed in about 30%, 50%, 20% and 40% of neurones, respectively. Co-expression of EP_1, EP_3 or EP_4 receptor mRNAs with IP receptor mRNA occurred in 25%, 41% and 24% of IP receptor mRNA-positive DRG neurones, respectively (Oida *et al.,* 1995). Further, mRNA for the SP precursor preprotachykinin A was present in 30% of total DRG neurones, and 70% of these

neurones co-expressed IP receptor mRNA and corresponded to C and Aδ fibres (Oida *et al.*, 1995). These data may suggest a nocimodulator role for IP receptors.

The four EP receptor subtypes were also expressed in nuclear membranes from porcine newborn brain cortex (Bhattacharya *et al.*, 1998), suggesting that they may play a role in gene transcription.

Table 2.5 *Classification and signal transduction pathways of prostanoid receptors.*[*]

Receptor			G protein	Second messenger and (effect)
Type	Subtype	Isoform		
DP			G_s	cAMP (↑)
EP	EP_1		Unidentified	Ca^{2+} (↑)
	EP_2		G_s	cAMP (↑)
	EP_3		G_i	cAMP (↓)
		EP_{3A}	G_s	cAMP (↑)
		EP_{3B}	G_s	cAMP (↑)
		EP_{3C}	G_i, G_s, G_q	cAMP (↓), cAMP (↑), $InsP_3$ (↑)
		EP_{3D}	G_i	cAMP (↑)
	EP_4		G_s	$InsP_3$ (↑)
FP			G_q, G_i	cAMP (↑), $InsP_3$ (↑)
IP			G_s, G_q	$InsP_3$ (↑), cAMP (↓)
TP		TP_α	G_q, G_i	$InsP_3$ (↑), cAMP (↑)
		TP_β		

[*] *Abbreviations: (↓) = decrease; (↑) = increase; Ca^{2+} = calcium; cAMP = 3',5'-cyclic adenosine monophosphate; $InsP_3$ = inositol-(1,4,5) triphosphate.*
Elaborated with information from (Coleman et al., 1994; Narumiya et al., 1999)

PG receptors and pain

Allodynia and hyperalgesia can develop after activation of PG receptors in sensory fibres (Bley *et al.*, 1998; Smith *et al.*, 1998b) by way of neurotransmitter and/or neuropeptide release from primary afferent fibres (Andreeva and Rang, 1993; Oida *et al.*, 1995; Buritova *et al.*, 1996; Malcangio *et al.*, 1996; Bezzi *et al.*, 1998;Southall and Vasko, 2001). This may be initiated in part by: *(i)* activation of adenylate cyclase, which increases cAMP levels and thus stimulates PKA; or *(ii)* activation of phospholipase C, leading to enhanced PKC activity and increased $InsP_3$ levels (Coleman *et al.*, 1994; Smith *et al.*, 1998b; Narumiya *et al.*, 1999). However, activation of some PG receptors has an inhibitory action —reducing cAMP production (Table 2.5). In fact, spinal activation of EP_{3A} receptors reduced the hypersensitivity of dorsal horn neurones in the spinal cord of rats suffering from peripheral inflammation, and decreased PGE_2-mediated increased responses in both spinal and DRG neurones (Bär *et al.*, 2004). Interestingly, spinal EP_1 receptors seemed to play an inhibitory role in the appearance of hyperalgesia induced by intrathecal injection of PGE_2 in both mice and rats (Minami *et al.*, 2001; Nakayama *et al.*, 2004).

The activation of spinal EP_1, EP_2 and EP_4 receptors was pharmacologically involved in the development of spinal hypersensitivity similar to that observed after knee inflammation in rats (Bär *et al.*, 2004). EP_1 receptors and EP_2 and EP_3 receptors (at high and low doses of PGE_2, respectively) were involved in PGE_2-evoked allodynia and hyperalgesia in mice, respectively (Minami *et al.*, 1994a). Accordingly, in mice intrathecal injection of the EP_1

Table 2.6 *Expression of prostanoid receptors in the spinal cord and dorsal root ganglia neurones.**

Receptor / Method	Species	Localisation			Cell type	Reference
		Dorsal horn	Ventral horn	DRG		
DP						
ISH		√	√		Neurones	(Wright *et al.*, 1999)
EP						
[^{3}H]PGE$_2$ AR	Rat	√		√ (few)	Neurones	(Bley *et al.*, 1998)
EP$_1$						
[^{3}H]iloprost AR	Rat	√		√ (few)	Neurones	(Bley *et al.*, 1998)
ISH	Mice			√ (30%)	Neurones	(Oida *et al.*, 1995)
ISH	Rat			√ (32%)	Neurones	(Nakayama *et al.*, 2004)
EP$_2$						
WB	Rat			√		(Southall and Vasko, 2001)
RT-PCR	Rat			√		(Southall and Vasko, 2001)
RT-PCR	Sheep	√ (few)	√ (few)			(Dolan *et al.*, 2000)
EP$_3$						
[^{3}H]iloprost AR	Rat	√		√ (few)	Neurones	(Bley *et al.*, 1998)
RT-PCR	Sheep	√	√			(Dolan *et al.*, 2000)
ISH	Mice			√ (50%)	Neurones	(Oida *et al.*, 1995)
EP$_{3C}$						
WB	Rat			√		(Southall and Vasko, 2001)
RT-PCR	Rat			√		(Southall and Vasko, 2001)
EP$_4$						
ISH	Mice			√ (20%)	Neurones	(Oida *et al.*, 1995)
WB	Rat			√		(Southall and Vasko, 2001)
RT-PCR	Rat			√		(Southall and Vasko, 2001)
IP						
ISH	Mice	√		√ (40%)	Neurones	(Oida *et al.*, 1995)
WB	Rat			√		(Southall and Vasko, 2001)
RT-PCR	Rat			√		(Southall and Vasko, 2001)
[^{3}H]iloprost AR	Rat	√		√ (few)	Neurones	(Bley *et al.*, 1998)

** Abbreviations: AR = autoradiography; DP = prostaglandin D receptor; EP = prostaglandin E receptor; IP = prostaglandin I receptor; ISH = in situ hybridisation; RT-PCR = reverse transcription-polymerase chain reaction; WB = western blotting.*

receptor antagonist ONO-NT-012 (also has TP receptor antagonist and EP$_3$ receptor agonist activities) reduced PGE$_2$-induced allodynia (Minami *et al.*, 1995) and that of the EP$_3$ receptor agonist ONO-AE-248 produced hyperalgesia (Minami *et al.*, 2001). Conversely, intrathecal administration of selective EP$_1$ receptor antagonists (SC-51089 and SC-51234A; Malmberg *et al.*, 1994) (ONO-8711; Nakayama *et al.*, 2002) produced antihyperalgesia in rats injected carrageenan or formalin into a hind paw. The discrepancy between antinociceptive effects (antiallodynia *vs.* antihyperalgesia) at the EP$_1$ receptor level could be due to differences in species (mice *vs.* rats) or experimental paradigms (intrathecal PGE$_2$ *vs.* inflammatory pain).

Activation of nuclear EP$_1$ receptors with 17-phenyltrinor PGE$_2$ increased the intranuclear concentration of Ca^{2+} and the transcription of the immediate-early gene *c-fos* (Bhattacharya *et al.*, 1998). These data support the hypothesis that prostanoids signalling from the cell membrane/cytoplasm to the nucleus may enhance spinal c-Fos protein expression (a nuclear protein encoded by *c-fos*, which, although not purely involved in nociception, is extensively used as an indirect marker of neurones involved in nociceptive processes) (Buritova *et al.*, 1996).

Blockade of spinal DP receptors with BW A868C (or lack of L-PGDS, see above) produced

allodynia (Minami *et al.,* 1996; Minami *et al.,* 1999). On the contrary, over-activation of DP receptors with BW 245C reversed nociceptin- and PGE_2-induced allodynia (Minami *et al.,* 1996; Minami *et al.,* 1997), suggesting complex interactions between opioid-like, EP and DP receptor systems.

The lack of more selective ligands has precluded the pharmacological exploration of possible physiological and pathophysiological functions of prostanoid receptor subtypes on the spinal processing of pain.

PG receptor deficiency

Deletion of both EP_{3C} and EP_4 receptors using antisense oligonucleotides reduced the PGE_2-evoked increased release of neuropepides from DRG neurones (Southall and Vasko, 2001). Mice lacking the EP_3 receptor ($EP_3^{-/-}$) developed allodynia after intrathecal injection of PGE_2, but mice lacking the EP_1 receptor ($EP_1^{-/-}$) did not (Minami *et al.,* 2001). $EP_3^{-/-}$ mice also showed hyperalgesia after high, but not low, doses of PGE_2 and did not develop hyperalgesia after intrathecal injection of the selective EP_3 receptor agonist ONO-AE-248 (Minami *et al.,* 2001). These data confirm the pharmacological findings (see above) that the EP_1 receptor and the EP_2 and EP_3 receptors (at high and low doses of PGE_2, respectively) are involved in PGE_2-induced allodynia and hyperalgesia, respectively. Interestingly, saline-treated $EP_1^{-/-}$ mice developed hyperalgesia and this was reversed by intrathecal PGE_2 (Minami *et al.,* 2001). In mice, pain induced by intraplantar injection of formalin was not ascribed to either EP_1 or EP_3 receptor subtype since wild-type, $EP_1^{-/-}$ and $EP_3^{-/-}$ animals developed similar behaviours after injection of the irritant (Segond von Banchet *et al.,* 2003). These data suggest that intrathecal PGE_2 and peripheral inflammation may induce spinal sensitisation by activating different EP receptors.

The comparable tail-flick and hot-plate latencies, but not writhing responses, between wild-type and IP receptor knockout mice did not support the role of IP receptors in spinal nociceptive transmission (Murata *et al.,* 1997) —the first two tests are sensitive to central acting drugs and the writhing test to both central and peripheral acting drugs.

Prostanoid receptors are present in DRG neurones and superficial layers of the spinal cord dorsal horn and, in general, their activation induces allodynia and/or hyperalgesia. They may do so, at least in part, by altering the concentrations of cAMP, $InsP_3$ or Ca^{2+}. Although, at present, there are few highly selective ligands for PG receptors, the active research in this field will certainly expand our knowledge on the role of these receptors under normal and increased nociceptive spinal transmission.

2.2.5 Protein kinases A and C and the spinal nociceptive process

Protein kinases A and C isoforms and functions

The mammalian PKA is a tetramer composed of a regulatory subunit dimer, which contains the cAMP binding sites, and a single catalytic subunit bound to each regulatory subunit. At least four regulatory (RIα, RIβ, RIIα, and RIIβ) and two catalytic (Cα and Cβ) subunits have been characterised. The subunit composition of a holoenzyme contributes to its activation properties by cAMP (Brandon *et al.,* 1997).

PKC represents a family of al least 12 serine/theorine kinases and they are divided in four groups: (*i*) conventional PKCs (α, βI, βII, and γ), which are Ca^{2+}-dependent and activated by phosphatidylserine (PS) and diacylglycerol (DAG); (*ii*) novel PKCs (δ, ε, η, and θ), which are Ca^{2+}-independent and regulated by PS and DAG; (*iii*) atypical PKCs (ζ and ι/λ), which are activated by PS and phosphatidylinositol-2,3,4-triphosphate; and (*iv*) recently described PKCs (μ and ν), which are activated by DAG and phospatidylinositol-4,5-diphosphate (Battaini, 2001).

Once activated, PKA and PKC may phosphorylate relevant substrates that control intracellular events permitting cell-to-cell communication.

Protein kinases A and C isoforms and expression

The α isoforms of the PKA subunits are ubiquitously expressed in neural and non-neural tissues, but the β isoforms are more restricted in the nervous system (Brandon *et al.,* 1997; Malmberg *et al.,* 2003). Immunocytochemistry studies have shown the presence of phosphorylated-PKA in the superficial laminae of the dorsal horn of the mice spinal cord, and immunoreactivity increased 7 days after injection of complete Freund's adjuvant into a hind paw (Yajima *et al.,* 2003).

Most PKC are widely distributed throughout the body, but the γ isoform is restricted mainly to the brain and the spinal cord (Battaini, 2001). Binding studies with [^{3}H]PDBU, a phorbol ester which binds to the DAG binding site on PKC, demonstrated that PKC was constitutively expressed in lamina I-II of the rat spinal cord (Tölle *et al.,* 1996). In mice and using immunocytochemistry techniques, the presence of phosphorylated-conventional PKCs has been demonstrated in superficial laminae of the dorsal horn of the spinal cord (Yajima *et al.,* 2003), which is consistent with PKC α, βI, and βII being distributed rather homogeneously in the DRG and all superficial layers of the dorsal horn of the spinal cord, and PKC γ only being found in interneurones of lamina II (Malmberg *et al.,* 1997).

An increased binding of [^{3}H]PDBU in lamina I-II of the rat spinal cord demonstrated up-regulation of PKC following 4 and 14 days of monoarthritis induced by injection of

complete Freund's adjuvant into the tibio-tarsal joint of one hind limb (Tölle *et al.,* 1996). Immunoreactivity of phosphorylated-conventional PKCs was also up-regulated in the superficial laminae of the spinal cord at 7 days after sciatic nerve ligation in mice (Yajima *et al.,* 2003). Acute (within 24 hours) spinal up-regulation of PKC γ protein also occurred after intrathecal administration of kainic acid in rats (Wen *et al.,* 2001).

Protein kinases A and C and pain

In mice, inflammatory thermal hyperalgesia was attenuated by chronic intrathecal treatment for 7 days with the selective PKA inhibitor KT-5720, but not by the selective PKC inhibitor RO-320432. The opposite was true in a model of neuropathic thermal hyperalgesia (Yajima *et al.,* 2003).

The possible mechanisms by which PKA increases nociception may include phosphorylation of NMDA receptors coupled to yotiao proteins resulting in enhancement of current flow through the receptor (Westphal *et al.,* 1999), phosphorylation of spinal glycine α_3 receptors resulting in decreased receptor's activity (Harvey *et al.,* 2004), up-regulation of NK_1 receptors in DRG neurones (Segond von Banchet *et al.,* 2003), and increased AA release from astrocytes (Strokin *et al.,* 2003). Hence, PKA may blunt the inhibition and facilitate the transmission of pain signals through the spinal cord.

Protein kinases A and C deficiency

As compared to wild-type mice, transgenic mice lacking the RIβ subunit of PKA exhibited less nociceptive responses to either intraplantar formalin and intrathecal PGE_2 injections, but neurophatic pain was not affected (Malmberg *et al.,* 2003). On the contrary, mice lacking the γ isoform of PKC were less prone to develop neuropathic pain than their naïve counterparts (Malmberg *et al.,* 1997).

Collectively, these data suggest that spinal activation of PKA and PKC may be involved in inflammatory and neuropathic pain, respectively. Targeting prostanoid receptor signal transduction pathways may serve to control some types of pain.

2.3 NMDA RECEPTORS AND THE NITRIC OXIDE PATHWAY

In addition to activating phospholipases, Ca^{2+} entering through NMDA receptors activates calcineurin and binds to calmodulin forming the Ca^{2+}-calmodulin complex (CaM). Calcineurin desphosphorylates the enzyme NOS, the catalytic activity of whose is usually reduced by PKC-induced phosphorylation, and the CaM complex further activates the enzyme (Dawson and Dawson, 1995). NOS catalyses the conversion of L-arginine to L-citrulline and NO. The

physical properties of NO [*e.g.*, small, diffusible, membrane permeable, reactive (Dawson and Dawson, 1995)] allow it to diffuse back to presynaptic terminals where it increases the release of neurotransmitters and/or neuropeptides, which augment the nociceptive input. Supporting evidence for this model comes from: *(i)* increased spinal release of NO after intrathecal perfusion of glutamate or NMDA (Kawamata and Omote, 1999; Rivot *et al.*, 1999); *(ii)* increased spinal release of citrulline, a marker of NO synthesis (Yang *et al.*, 1996), and NO metabolites nitrite/nitrate (Gühring *et al.*, 2000; Vetter *et al.*, 2001) in states of facilitated processing; *(iii)* development of allodynia and hyperalgesia after intrathecal administration of L-arginine (Eguchi *et al.*, 1999; Minami *et al.*, 2001) and NO donors (Minami *et al.*, 1995; Eguchi *et al.*, 1999; Tao and Johns, 2000; Minami *et al.*, 2001); and *(iv)* increased spinal release of glutamate, SP and CGRP after activation of NMDA receptors or NOS with the consequent release of NO (Aimar *et al.*, 1998; Kawamata and Omote, 1999).

2.3.1 Nitric oxide synthases and the spinal nociceptive process

NOS isozymes and functions

Three isoforms of NOS have been identified, two are constitutive enzymes: neuronal NOS (nNOS) and endothelial NOS (eNOS); and one is inducible (iNOS) (Dawson and Dawson, 1995; Appleton *et al.*, 1996). Apart from cell type, the NOS isoform synthesised is dependent on cell activation. Both nNOS and eNOS depend on CaM for catalytic activity, whereas iNOS is Ca^{2+}-independent and has calmodulin tightly bound to the enzyme. Calmodulin is considered a protein subunit of iNOS, which accounts for its resistance to Ca^{2+} activation (Dawson and Dawson, 1995; Appleton *et al.*, 1996). In contrast, induction of iNOS requires of protein tyrosine kinase activity (Kleinert *et al.*, 1998). Despite these differences, all NOS isoforms perform the same catalytic reaction: conversion of L-arginine to L-citrulline and NO.

NOS expression

Both protein and mRNA for all three NOS isozymes are present in the spinal cord of many mammal species, and are found predominantly in small- to medium-sized neurones of the superficial layers of the dorsal horn and around the central canal. The intermediolateral cell column from thoracic and sacral segments and DRG neurones are important sources of NOS also. Scattered expression of NOS in the ventral horn corresponds mainly to motoneurones (Table 2.7).

The distribution of NOS isozymes in dorsal horn and DRG neurones confers a nocimodulator role to these enzymes. In fact, up-regulation of nNOS (Maihofner *et al.*, 2000a; 2000b; Wu *et al.*, 2001) and iNOS (Maihofner *et al.*, 2000a; Wu *et al.*, 2001) protein expression in the superficial layers of the spinal cord occurred after peripheral inflammatory hyperalgesia.

Likewise, spinal nNOS protein expression increased after intrathecal kainate injection (Wen *et al.,* 2001), and nNOS protein (Qian *et al.,* 1996) and mRNA expression (Luo *et al.,* 1999) were up-regulated in rat DRG, but not in spinal cord, neurones after peripheral nerve injury.

Interestingly, intrathecal injection of SP(1-7), the major metabolite of SP, decreased nNOS mRNA expression and NOS activity in the rat spinal cord (Kovacs *et al.,* 2001), suggesting a possible feed-back mechanism.

Table 2.7 *Constitutive expression of nitric oxide synthase isozymes in the spinal cord.*[*]

Isozyme / Method	Species	Localisation					Cell type	Reference
		Dorsal horn	Lamina X	IML cell column	Ventral horn	DRG		
nNOS								
NADPH-d	Rat	√	√	√	√ (few)	√	N	(Valtschanoff *et al.,* 1992)
WB	Rat	√			√	√		(Qian *et al.,* 1996)
IHC	Rat	√	√	√	√	√	N	(Terenghi *et al.,* 1993)
IHC	Human	√	√	√	√	√	N	(Terenghi *et al.,* 1993)
NADPH-d	Sheep	√		√	√ (few)			(Xu *et al.,* 1996a)
IHC	Sheep	√	√		√ (few)		N	(Dolan *et al.,* 2000)
NADPH-d	Sheep	√			√ (few)		N	(Dolan *et al.,* 2000)
RT-PCR	Sheep	√[†]			√[†]			(Dolan *et al.,* 2000)
IHC	Cat	√	√	nd	√		N	(Pullen *et al.,* 1997)
IHC	Macaque	√	√	√ (few)	√		N	(Pullen *et al.,* 1997)
IHC	Human	√	√	√	√		N	(Pullen *et al.,* 1997)
WB	Rat	√[†]			√[†]			(Wu *et al.,* 2001)
RT-PCR	Rat	√[†]			√[†]	√		(Kovacs *et al.,* 2001)
IHC	Mouse	√	√				N	(Maihofner *et al.,* 2000)
WB	Rat	√[†]			√[†]			(Wen *et al.,* 2001)
IHC	Mouse	√					N	(Maihofner *et al.,* 2000)
NADPH-d	Cat	√	√	√		√	N	(Vizzard *et al.,* 1994)
NADPH-d	Mouse	√	√		nd		N	(Bruning, 1992)
IHC	Rat, mouse, cat, sqirrel monkey	√	√	√	√ (few)		N	(Dun *et al.,* 1994)
iNOS								
IHC	Sheep	√	√		√ (few)		N	(Dolan *et al.,* 2000)
RT-PCR	Sheep	√[†]			√[†]			(Dolan *et al.,* 2000)
IHC	Cat		√ (few)		nd		N	(Pullen *et al.,* 1997)
WB	Rat	√[†]			√[†]			(Wu *et al.,* 2001)
WB	Rat	nd			nd			(Wen *et al.,* 2001)
IHC	Mice	√					A	(Maihofner *et al.,* 2000)
eNOS								
IHC	Sheep	√	√		√ (few)		N	(Dolan *et al.,* 2000)
RT-PCR	Sheep	√[†]			√[†]			(Dolan *et al.,* 2000)
IHC	Cat	√	√		√ (few)		A	(Pullen *et al.,* 1997)

[*] *Abbreviations: √ = present; [†] = homogenate of whole spinal cord; eNOS = endothelial nitric oxide synthase; IHC = immunohystochemistry; iNOS = inducible nitric oxide synthase; NADPH-d = nicotinamide adenine dinucleotide phosphste-diaphorase staining; nd = not detected; nNOS = neuronal nitric oxide synthase; RT-PCR = reverse transcriptase-polymerase chain reaction; WB = western blotting; N = neurones; A = astrocytes.*

NOS inhibitors and pain

In rodents, electrophysiological data have showed that intrathecal infusion of NOS inhibitors reduced the increased responsiveness of dorsal horn neurones to noxious and innocuous stimulation of an inflamed peripheral tissue (Wu *et al.,* 2001), and genetic information indicated that blockade of spinal NOS reduced c-Fos labelling in laminae I-II of the spinal cord (Roche *et al.,* 1996). Behavioural evidence have demonstrated that selective or non-selective pharmacological inhibition of spinal NOS activity reduced hyperalgesia induced by intrathecal administration of glutamate or NMDA (Ferreira *et al.,* 1999; Kawamata and Omote, 1999; Fairbanks *et al.,* 2000; Park *et al.,* 2000), or by injection of irritants into

the paw (Roche *et al.,* 1996; Larson *et al.,* 2000; Park *et al.,* 2000; Wu *et al.,* 2001) or the intraperitoneal cavity (Larson *et al.,* 2000).

Interestingly, the analgesic effects of intrathecally injected NOS inhibitors were still observed well after the spinal activity of NOS had been re-established (Larson *et al.,* 2000), which suggests that transient changes in the spinal synthesis of NO may produce long-term changes in nociceptive processing, probably at the level of synthesis (up- or down-regulation) or activity (sensitisation or desensitisation) of proteins (Larson *et al.,* 2000). Hence, spinal NO seems to be an important modulator in the plasticity of nociceptive pathways.

Analgesia induced after intrathecal administration of non-selective inhibitors of NOS, such as N^{ω}-nitro-L-arginine methyl ester (L-NAME), cannot be solely attributed to inhibition of NOS in neurones. They also induce vasoconstriction by inhibiting eNOS, which may affect interpretation of results. The use of selective nNOS inhibitors, such as 7-nitroindazole, may overcome this pitfall, and they have been shown to reduce C-fibre evoked spinal responses in both normal and inflamed anaesthetised rats (Stanfa *et al.,* 1996).

It is also possible that NOS inhibitors have a dual antinociceptive action by inhibiting the synthesis of NO and by augmenting the levels of L-arginine, which has been shown to induce antinociception by an opioidergic mechanism independent of the NO pathway (Kawabata *et al.,* 1992). L-Arginine could follow alternative pathways to the NOS pathway: *(i)* by arginase to ornithine and urea, and *(ii)* by arginine decarboxylase to agmatine. Ornithine and agmatine, in turn, can be converted into the polyamines putrescine, spermidine and spermine (Reis and Regunathan, 2000). Agmatine and sometimes polyamines can negatively modulate NMDA receptors (Appleton *et al.,* 1996; Dingledine *et al.,* 1999; Reis and Regunathan, 2000), contributing so to reduce facilitated states. In fact, intrathecal administration of agmatine reduced inflammatory, chemical, and mechanical hyperalgesia in rodents (Fairbanks *et al.,* 2000).

NOS isozyme deficiency

As compared to wild-type mice, NMDA-stimulated glutamate release was reduced in nNOS knockout mice, and NMDA-stimulated GABA release was reduced in eNOS knockout mice (Kano *et al.,* 1998), suggesting a stimulatory and inhibitory role for nNOS and eNOS isoenzymes, respectively. Wild-type mice and nNOS knockout mice exhibited similar pain behaviours after intraplantar injection of formalin, but analgesia with intraperitoneal L-NAME occurred in wild-type mice only, which suggested that NO was involved in, but was not necessarily required for, the development of noxious stimulation-induced central sensitisation (Crosby *et al.,* 1995). In contrast, iNOS knockout mice showed reduced thermal hyperalgesia attributable to lack of spinal iNOS since intrathecal, but not intraperitoneal,

injection of the iNOS selective inhibitor L-NIL reduced thermal hyperalgesia in wild-type mice to a similar level to that observed in $iNOS^{-/-}$ mice. The role of nNOS and eNOS seemed to be minimal since intrathecal injection of the non-selective NOS inhibitor L-NAME into $iNOS^{-/-}$ mice had no effect on thermal hyperalgesia (Gühring *et al.*, 2000). These data highlight the importance of spinal iNOS in thermal hyperalgesia.

The above data evidence the prominent role of NOS isozymes in the development and/or maintenance of central sensitisation. Inhibiting the activity of these enzymes in the spinal cord could be an important target for treating some types of pain.

2.3.2 Nitric oxide and the spinal nociceptive process

NO formation and release

NO is formed by oxidation of L-arginine. NO is not stored, and, on the contrary, it is released and utilised immediately; it can travel about 50 μm before degradation (Gühring *et al.*, 2000). In the spinal cord, NO can be released by peripheral inflammatory stimuli (Gühring *et al.*, 2000), peripheral nerve damage (Luo *et al.*, 1999) or direct administration of glutamate and NMDA (Kawamata and Omote, 1999; Rivot *et al.*, 1999). These events suggest certain level of repetitive stimulation of the primary afferent for the release of NO, and support the NMDA-receptor activation theory for its generation.

NO and pain

Once formed, NO exerts a direct action on postsynaptic ion channel proteins or diffuses to the presynaptic terminals and astrocytes where it activates the enzymes guanylate cyclases to increase the concentration of 3',5'-cyclic guanosine monophosphate (cGMP) and the release of neuroactive substances (Fukuto and Chaudhuri, 1995; Kawamata and Omote, 1999; Gühring *et al.*, 2000).

It is clear that NO plays a neuromodulator role on the spinal nociceptive process, but its precise role is still a matter of debate. On the one hand, support for its nociceptive action at the spinal level comes from reports of intrathecal administration of NO donors promoting allodynia and hyperalgesia (Masue *et al.*, 1999; Tao and Johns, 2000; Sousa and Prado, 2001), probably by enhancing the release of glutamate, SP and CGRP (Aimar *et al.*, 1998; Kawamata and Omote, 1999; Masue *et al.*, 1999). Further support comes from the antinociceptive effect achieved by inhibiting the enzyme NOS with L-arginine analogues (Roche *et al.*, 1996; Ferreira *et al.*, 1999; Kawamata and Omote, 1999; Fairbanks *et al.*, 2000; Larson *et al.*, 2000; Park *et al.*, 2000; Wu *et al.*, 2001). On the other hand, spinal administration of NO donors (Sousa and Prado, 2001) and L-arginine (Masue *et al.*, 1999) can produce antinociception also. Possible antinociceptive mechanisim for NO could include s-nitrosylation and therefore inactivation of the NMDA receptor by NO or NO-derived

compounds (Dawson and Dawson, 1995; Aizenman and Potthoff, 1999; Dingledine *et al.*, 1999), and interaction with noradrenaline and acetylcholine to form 6-nitro-noradrenaline and 6-nitro-acetylcholine, respectively, which inhibit both the reuptake and metabolism of these inhibitory neurotransmitters (Xu *et al.*, 1996b; Chiari *et al.*, 2000).

The opposite nociceptive effects of NO have been pharmacologically and physiologically characterised. After increasing NO concentration with NO donors in the spinal cord of neuropathic rats, both antiallodynic and proallodynic effects were induced, and these effects were attributed to low and high NO concentrations, respectively (Sousa and Prado, 2001). This agrees with electrophysiological studies in new born rat spinal cord preparations in which low concentrations of NO gas-containing medium or NO donors inhibited electrically evoked nociceptive spinal reflexes, but higher concentrations evoked depolarisation of ventral roots (Kurihara and Yoshioka, 1996). However, other electrophysiological studies have demonstrated that NO-mediated excitation and inhibition occurred in different neurones. For instance, NO had an excitatory effect in the majority of rat spinal cord neurones tested in lamina X (93% of neurones), and predominantly inhibited neurones in lamina I-II (49% of neurones) while still excited some (28% of neurones) (Pehl and Schmid, 1997). Similarly, NO produced both mechanical excitation and inhibition of dural nociceptors in urethane-anaesthetised rats (Levy and Strassman, 2004). A difference between these last two studies was that neurones that were excited showed grater spontaneous activity than those that were inhibited in the former study (Pehl and Schmid, 1997), whereas a tendency towards the contrary was observed in the latter one (Levy and Strassman, 2004). Also in this latter study, mechanical activation thresholds of excited neurones were higher than those of inhibited neurones (Levy and Strassman, 2004). Hence, the opposite effects could depend on the level of excitability of the nociceptor (Levy and Strassman, 2004).

NO-mediated opposite effects have also been attributed to the existence of a reduced and an oxidised form of this gas (Machelska *et al.*, 1997). Another hypothesis is that NO may be a modulator of different types of spinal neurones, including excitatory neurones and inhibitory interneurones; NO-induced activation of excitatory neurones could result in hyperalgesia whereas NO-induced activation of inhibitory interneurones could result in hypoalgesia (Luo and Cizkova, 2000). Experimental conditions (*e.g.*, type of afferent fibres activated, intensity and duration of input) may also determine the outcome (Sousa and Prado, 2001). Hence, the circuitry of NO in the spinal processing of pain remains to be fully characterised.

2.3.3 Guanylate cyclases and the spinal nociceptive process

Guanylate cyclase isozymes and function

Guanylate cyclases exist in at leat five different isoforms: four heterodimers ($\alpha1\beta1$, $\alpha2\beta1$, $\alpha1\beta2$, $\alpha2\beta2$) and one homodimer ($v\beta2$) (Gibb *et al.*, 2003). In the rat spinal cord, NO

produced after subdural infusion of NMDA activated guanylate cyclases to increase cGMP concentrations (Kawamata and Omote, 1999).

Guanylate cyclase expression

In the dorsal horn of the rat spinal cord, guanylate cyclase α1 subunit, but not β1 subunit, was constitutively expressed and up-regulated 2 to 4 days after intraplantar injection of formalin (Tao and Johns, 2002).

cGMP and pain

The hyperalgesic actions of NO are mediated by spinal activation of guanylate cyclases as these effects were mimicked by membrane-permeable cGMP analogues (Ferreira *et al.,* 1999) and blocked by guanylate cyclase inhibitors (Ferreira *et al.,* 1999; Kawamata and Omote, 1999; Tao and Johns, 2002). Immunohistochemical studies demonstrated the constitutive presence of cGMP in the superficial laminae of the dorsal horn of the spinal cord, and up-regulation occurred after activation of the NMDA-NO pathway (Morris *et al.,* 1994). It is well accepted that the increased concentration of cGMP in the presynaptic terminal serves to release neuroactive agents. Although the exact mechanisms that take part in this spinal nociceptive process are poorly understood, these may include direct effect on ion channels, stimulation or inhibition of phosphodiesterase activity, and activation of protein kinases (Fukuto and Chaudhuri, 1995).

Interestingly, similar to NO, cGMP also can produce dual nociceptive effects. Pharmacological manipulation of spinal cGMP levels with 8-bromo-cGMP showed that low cGMP concentrations reduced, but high concentrations increased, the hyperalgesic response to injection of formalin into the hind paw of rats (Tegeder *et al.,* 2002), and spinal inhibition of gyanylate cyclase with 1*H*-[1,2,4]oxadiazolo[4,3-α]qinoxalin-1-one (ODQ) reduced both NO-mediated antiallodynic and proallodynic effects in neuropathic rats (Sousa and Prado, 2001)

Electrophysiological data have also demonstrated that both NO-induced spinal excitation and inhibition were mimicked by the cGMP analogue 8-bromo-cGMP (Kurihara and Yoshioka, 1996; Pehl and Schmid, 1997). However, only the excitatory affects were also mimicked by the cAMP analogue 8-bromo-cAMP (Kurihara and Yoshioka, 1996; Pehl and Schmid, 1997). In fact, the membrane-permeable cGMP analogue 8-para-clorophenylthio cGMP (8-pCPT-cGMP) inhibited, but not excited, dural nociceptors in anaesthetised rats, and the gyanylate cyclase blocker ODQ prevented NO-mediated inhibition (Levy and Strassman, 2004).

These data suggest that NO-mediated inhibition depends completely, and excitation partially, on cGMP mechanisms, and that NO-induced production of cGMP alone does not allow any

prediction about an excitatory or inhibitory effect on spinal neurones.

2.3.4 cGMP-dependent protein kinases and the spinal nociceptive process

cGMP-dependent protein kinase isoforms and function

Two isosymes of cGMP-dependent protein kinases (PKG) are recognised in mammals: cytosolic PKG-I and membrane-bound PKG-II. Further, PKG-I exists in two isoforms: Iα and Iβ (Tao *et al.,* 2000b). In the spinal cord, PKG serve as major effectors for NO and cGMP.

cGMP-dependent protein kinase expression

Immunohistochemical studies demonstrated that DRG neurones and axonal processes of laminae I and II of the spinal cord are rich sources of PKG-I, with small- and medium-sized neurones being intensely stained (Qian *et al.,* 1996). In neurones of the superficial laminae of the spinal cord, however, isoform Iα was the most abundant and isoform Iβ was scarcely expressed (Tao *et al.,* 2000b). DRG neurones expressing PKG-I also showed SP and CGRP immunoreactivity (Qian *et al.,* 1996). Up-regulation of spinal PKG-I, in particular that of isoform Iα, occurred after induction of peripheral inflammation (Tao and Johns, 2002; Tegeder *et al.,* 2002). Interestingly, in rats injected formalin into a hind paw, spinal PKG-I was further increased after intrathecal treatment with a high dose of cGMP analogue 8-bromo-cGPM, but low doses completely prevented any increase at all (Tegeder *et al.,* 2002).

cGMP-dependent protein kinases and pain

Facilitation of rat tail-flick latencies after intrathecal administration of NMDA or the NO donor NOC-12 was prevented by intrathecally injecting the selective PKG-Iα inhibitor Rp-8-p[(4-Chlorophenyl)thiol]-cGMPS triethylamine (Rp-8-p-CPT-cGMPS) (Tao and Johns, 2000). Intrathecal injection of the non-selective PKG inhibitor Rp-8-bromo-cGMPS and the PKG-Iα inhibitor Rp-8-p-CPT-cGMPS also reduced the hyperalgesia induced by injecting formalin into the hind paw of rats (Tao *et al.,* 2000b; Tegeder *et al.,* 2002). Together, these data suggest that PKG-Iα may be involved in the generation of spinal hyperalgesia, though the presynaptic targets for PKG-Iα that participate in the sensory processing remain uncertain.

Conversely, the PKG inhibitor KT5823 blocked both NO- and cGMP-mediated inhibition of electrically evoked nociceptive spinal reflexes, but had no effect on the excitatory response of NO (Kurihara and Yoshioka, 1996).

Further studies are necessary to better characterise the role of PKG in the spinal processing of nociceptive information and determine the effect of altering NO signal transduction

pathways to control some types of pain.

2.3.5 Poly(ADP-ribose) synthase and the spinal nociceptive process

NO can also produce postsynaptic effects by activating the nuclear enzyme poly(ADP-ribose) synthase. Interestingly, spinal inhibition of poly(ADP-ribose) synthase after intrathecal injection of benzamide into rats, reduced both mechanical and thermal neuropathic hyperalgesia as well as mechanical neuropathic allodynia in a similar way to that observed with the intrathecal administration of the NOS inhibitor L-NAME (Mao *et al.*, 1997). These data suggest that NO may facilitate the spinal nociceptive process not only by indirectly rising neurotransmitter/neuropeptide release from presynaptic terminals, but also by mechanisms within the same neurone from which is synthesised.

2.4 Spinal interactions between arachidonic acid and nitric oxide pathways

The products of both COX and NOS enzymes can modulate the activity of the other enzyme, but the interactions are complex as both stimulatory and inhibitory actions have been ascribed.

2.4.1 Effects of cyclo-oxygenase pathway modulation on nitric oxide synthesis

In rat spinal cord slices, PGE_2 and $PGF_{2\alpha}$ (but not PGD_2) further increased the release of NO induced by stimulation of the NMDA receptor-nNOS pathway (Sakai *et al.*, 1998). Accordingly, non-selective COX inhibitors reduced the increased release of NO and the increased protein expression of nNOS and iNOS induced by lipopolysacharide in rat cerebellar slices (DiGirolamo *et al.*, 2003). However, in lipopolysacharide-stimulated microglial cells, PGE_2 and PGD_2, and 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 and/or expression (Petrova *et al.*, 1999). Moreover, opposite effects on zymosan-induced iNOS protein expression in the rat spinal cord were observed after a single oral administration of the COX-2 selective inhibitor rofecoxib, with low doses increasing and high doses decreasing iNOS expression (Niederberger *et al.*, 2003). The decreased expression of iNOS was attributed to a simultaneous inhibition of NF-κB and activation of AP-1 transcriptor factors (iNOS transcription is stimulated by NF-κB, but inhibited by AP-1) (Niederberger *et al.*, 2003). Discrepancies may also depend on specific prostanoids, most likely PGE_2, determining what biochemical pathway L-arginine follows, or on the particular experimental conditions (Appleton *et al.*, 1996).

2.4.2 Effects of nitric oxide pathway modulation on prostaglandin synthesis

The NO donor sodium nitroprusside increased PGE_2 release from cultured astroglial cells in a similar way to that by incubation of cells with NMDA or cytokines, and incubation with the NOS inhibitor L-NAME prevented the increased release of PGE_2 (Mollace *et al.,* 1995; Mollace *et al.,* 1998), wich suggests that the NO pathway may enhance COX-2 activity. In the mouse spinal cord, NO, specially derived from iNOS, modulated the catalytic activity of COX-1 and/or COX-2 and increased PGE_2 concentration (Gühring *et al.,* 2000). In cultured rat DRG cells, NO mediated the release of SP by indirectly modulating (probably through NF-κB) the expression of COX-2 at the level of gene transcription (Morioka *et al.,* 2002). Behaviourally, inhibition of spinal NO synthesis with L-NAME diminished allodynia (Minami *et al.,* 1995) and hyperalgesia (Park *et al.,* 2000) induced by intrathecal administration of PGE_2 in mice and rats, respectively. The stimulatory and inhibitory actions of NO on PG production could be explained by the relative concentrations of NO. Low levels of NO may reduce PG formation, and thus act as an antinociceptive agent, whereas high levels of NO may raise PG production, therefore acting as a pronociceptive agent (Appleton *et al.,* 1996; Machelska *et al.,* 1997).

2.4.3 Nitric oxide pathway and spinal cyclo-oxygenase inhibition-mediated analgesia

Modulation of the NO pathway can affect the spinal analgesic effects of COX inhibitors. On the one hand, pharmacological blockade of the NO pathway reduced the spinal analgesic effect of COX inhibitors. For instance, paw treatment with the NOS inhibitor L-NMMA or the guanylate cyclase inhibitor methylene blue prevented the spinal antihyperalgesic action of the non-selective COX inhibitor dipyrone in rats injected PGE_2 into the paw (Lorenzetti and Ferreira, 1996). Similarly, the spinal antihyperalgesic effects of the COX-2 selective inhibitor lumeracoxib in rats injected formalin into a hind paw were prevented by the intrathecal administration of the NOS inhibitor L-NAME, the NO-sensitive soluble guanylyl cyclase inhibitor ODQ, and the ATP-sensitive K^+ channel inhibitor glibenclamide (Lozano-Cuenca *et al.,* 2005). Together, these data suggest that peripheral or spinal activation of the NO-cGMP pathway may be involved in the antihyperalgesia induced at the spinal cord level by some COX inhibitors.

On the other hand, stimulation of the NO pathway also reduced the spinal analgesic effects of COX inhibitors. The NO precursor L-arginine antagonised the analgesic responses of the COX inhibitors diclofenac and S(+)-ibuprofen, all given through the intraperitoneal route, in rats intrathecally injected NMDA (Björkman *et al.,* 1996). However, when combined in the same molecule, intravenous administration of NO donors linked to COX inhibitors (also known as nitro-NSAIDs) can produce synergistic analgesic effects mediated at the spinal level

(Romero-Sandoval *et al.*, 2002).

Modification of the NO pathway also had no effect on the spinal analgesic effects of some COX inhibitors. Pre-treatment with the NOS inhibitor L-NAME or the NO precursor L-arginine, either by the intrathecal or the intracerebroventricular routes, did not modify the analgesic effects of S(+)-ketoprofen given orally to arthritic rats (Díaz-Reval *et al.*, 2004). Also, the spinal/supraspinal analgesic effect of the COX-2 preferential inhibitor nimesulide in human beings persisted after administration of a hyperalgesic dose of the NO donor nitroglycerine, suggesting that COX-2 is necessary for NO-mediated hyperalgesia (Sandrini *et al.*, 2002). Clearly, the spinal nociceptive interaction between the NO pathway and COX inhibitors is complex and further studies are required to determine how, and under which experimental conditions, they affect each other.

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

2.5 References

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3. Developmental changes in threshold, conduction velocity and depressive action of lignocaine on dorsal root potentials from neonatal rats are associated with maturation of myelination

3.1 Abstract

Developmental changes in conduction velocity of neonatal rat primary afferent fibres have been suggested to be due to changes in myelination. However, data from those reports were interpreted with no indicators of statistical reliability, and no parallel assessment of myelination was undertaken. The blocking actions of local anaesthetics also seem to be developmentally regulated. The current work investigated all these ontogenic changes using *in vitro* sciatic nerve-dorsal root preparations from 0- to 12-day-old rats. The effect of age on physiological (threshold, conduction velocity, and myelination) and pharmacological [lignocaine (0.0625 to 2 mM) and capsaicin (2 μM)] properties of dorsal root potentials were assessed. As rats aged, stimulus intensities necessary to evoke A-fibre thresholds significantly decreased and A-fibre conduction velocities significantly increased. For C-fibres, thresholds significantly increased and conduction velocities significantly varied with age. The blocking potency of lignocaine varied with age; A-fibres from 4-day-old rats and younger were significantly more resistant than those from older rats, and C-fibres were blocked more uniformly amongst ages. Capsaicin significantly depressed C-fibres irrespective of age, and A-fibres were significantly reduced during the first postnatal week only. Myelination significantly increased as rats aged. A-fibre physiological parameters were significantly correlated with both other A-fibre physiological and pharmacological parameters, but C-fibre parameters were not. These data confimed previously reported physiological and pharmacological properties of afferent fibres, and demonstrated that, for A-fibres, these properties were correlated with increased myelination. Peripheral A-fibre transduction mechanisms seem to require time to acquire their full stimulus-response sensitivity, which coincides with development of myelination. In contrast, peripheral C-fibres seem to have mature transduction mechanisms from the first days of postnatal life.

3.2 Introduction

The physiological properties of primary afferents change rapidly during the postnatal developmental period. For example, Fitzgerald (1985; 1987) has described that conduction

velocity of afferent input volleys from L4 dorsal roots and single cutaneous afferent units from L4 dorsal root ganglions, evoked after electrical stimulation of the skin of the foot, increased with age in 0- to 15-day-old anaesthetised rats. From birth, afferent input volleys were divisible into two distinct waveforms, and conduction velocity and threshold of the early wave increased and decreased with age, respectively. In addition, from 6 days of age, afferent input volleys became more complex with the appearance of an intermediate wave (Fitzgerald, 1985). This was also observed for conduction velocities in single cutaneous afferent units, which showed a trimodal distribution (Fitzgerald, 1987). In *in vitro* studies, Fulton (1987) also described an age-dependent increase in conduction velocity in L4-L6 dorsal root ganglion neurones after electrical stimulation of the sciatic nerve from 0- to 14-day-old rats. In all these studies, changes in conduction velocity were attributed to maturation of myelination, and the three waveforms and patterns of distribution were considered to represent activity in thickly myelinated (immature Aβ), thinly myelinated (immature Aδ) and non-myelinated (C) fibres. However, none of these studies assessed myelination of primary afferents, making the above statement hypothetical. Further, their results were interpreted without indicators of statistical reliability, which render their data descriptive and inconclusive.

There are at least two possible causes for an increase in conduction velocity: increased myelination and increase in axon diameter. Sima (1974) found that axon diameter of myelinated fibres in rat L5 dorsal roots increased with age. However, myelinated fibres from 5- and 10-day-old rats had an unimodal pattern of distribution, and distribution became bimodal and trimodal in 20- and 40-day-old animals, respectively. (Sima, 1974). Although at a glance it may appear that conduction velocities and axon diameters of myelinated primary afferents follow a similar pattern of development, no correlation studies in neonate animals have been carried out to challenge this hypothesis. Furthermore, a poor correlation between conduction velocity and cell size in L4 dorsal root ganglion neurones was observed for Aα/β-fibres from young rats (35 to 55 days old) (Harper and Lawson, 1985).

Developmental changes have also been observed for the blocking actions of local anaesthetics. In carrageenan pre-treated rats, 3 day olds were more susceptible than 10 and 21 day olds to induction of mechanical analgesia and antihyperalgesia by epidural administration of bupivacaine (0.004-0.125%) (Howard *et al.,* 2001). Similarly, preoperative sciatic nerve block with 0.5% bupivacaine had a longer lasting effect on the development of mechanical allodynia in 14- than 28-day-old rats following paw incision (Ririe *et al.,* 2004). Although these data may suggest that nerve fibres from younger rats are more susceptible to the blocking action of local anaesthetics, there is evidence that sciatic nerve block with 0.5% bupivacaine just before wounding of one hindpaw, and repeated administrations at 6 and 24 h after surgery, did not prevent mechanical hypersensitivity in 0-day-old rats (de Lima *et al.,* 1999).

The present study sought to investigate how developmental changes in threshold, conduction velocity, and the effect of the local anaesthetic lignocaine on dorsal root potentials of neonatal rats correlate with the degree of myelination of primary afferent terminals. For this purpose, electrophysiological, pharmacological, and histological studies were carried out using an *in vitro* sciatic nerve-dorsal root preparation. Preliminary results have been presented in abstract form (Lizarraga *et al.*, 2005).

3.3 Material and methods

Electrophysiological and pharmacological studies

Dissection

After approval by the institutional Animal Ethics Committee, 0- to 12-day-old (with a maximum of 12 h error since dams were checked twice daily for delivery with the day of birth recorded as day 0) Sprague-Dawley rat pups of either sex (Small Animal Production Unit, Massey University, Palmerston North, New Zealand) were killed by cervical dislocation followed by decapitation. The abdomen ventral skin was removed and an incision made in the ventral abdominal wall and the thoracic cage. All abdomen and thoracic internal organs were removed and the vertebral canal was opened on the ventral side. Sciatic nerves together with L4 or L5 dorsal root ganglion (DRG) and respective dorsal and ventral roots were dissected free in aerated (O_2 95%/CO_2 5%) modified Tyrode's solution (118 mM NaCl, 24 mM $NaHCO_3$, 12 mM glucose, 3 mM KCl, 1.5 mM $CaCl_2$, 1.25 mM $MgSO_4 7H_2O$; pH 7.4). Sciatic nerve-spinal root preparations were placed in a petri dish containing modified Tyrode's solution. Preparations were desheathed and ventral roots removed.

Recordings

Preparations were mounted in a tissue bath with the sciatic nerve in contact with the stimulating electrodes and the dorsal root in contact with the recording electrodes. Electrical isolation of the Ag/$AgCl_2$ wire electrodes was achieved by using grease gaps made of Vaseline and liquid paraffin (5:4 v/v) (Figure 3.1a). Preparations were bathed in modified Tyrode's solution at room temperature (22-25°C).

Using an isolated stimulator (Model DS2, Digitimer Ltd., Welwyn Garden City, Hartfordshire, England), preparations were given single square-wave pulses of 500 μs duration to find their threshold voltage, defined as the minimum voltage necessary to evoke a clear response above the baseline level (A-fibre threshold). Stimuli at 2, 4, 8, 16, 24, 32, 48 and 64 times threshold, if necessary, were carried out 2 min apart to evoke a dorsal root compound action

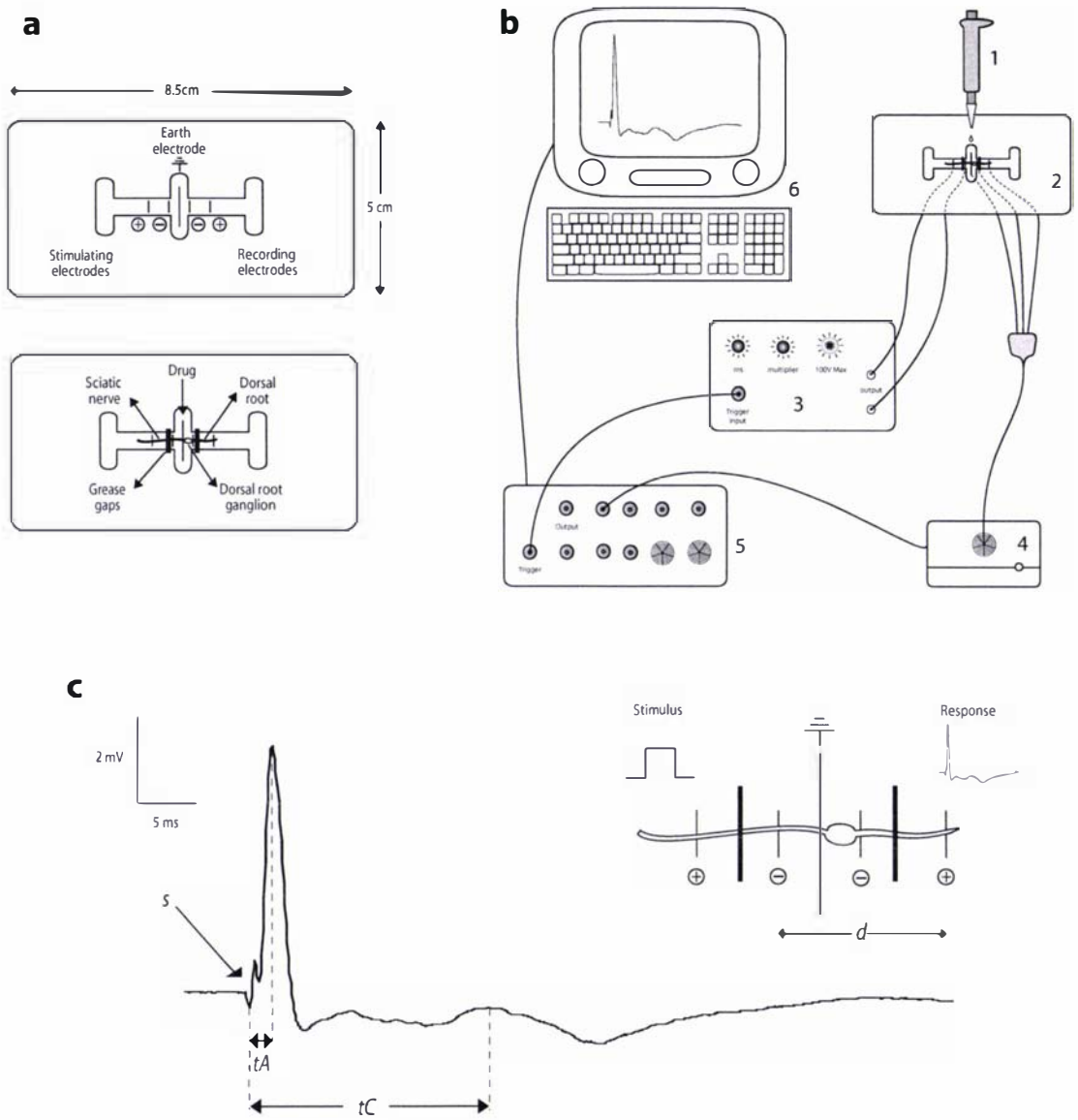

Figure 3.1 *Schematic representation of the experimental setting for the sciatic nerve-dorsal root preparation. (a) Tissue bath showing the arrangement of electrodes (top) and positioning of the preparation in it (bottom). (b) After treatment administration (1), the preparation (2) was stimulated (3) on the sciatic nerve to evoke dorsal root potentials, which were amplified (4), transferred to a PowerLab (5), and digitally stored (6). (c) Measurement of conduction velocities in DRCAPs. In the upper right diagram, the sciatic nerve to L4 or L5 dorsal root conduction distance (d) was constant at 15 mm. The illustration at the bottom is an example of a DRCAP. The times requiered by both A- and C-fibre waveforms to reach their peak amplitudes (tA and tC, respectively) were measured after apearance of the stimulus artefact (s). The conduction velocities for A- and C-fibre waveforms were calculated from d/tA and d/tC, respectively. In this example, from a 6-day-old rat preparation, tA and tC were 2.4 and 18.1 ms, yielding conduction velocities of 6.25 and 0.83 m/s, respectively.*

potential (DRCAP) containing a C-fibre waveform; this was considered to be the C-fibre threshold. All further experiments were carried out using voltage intensities at twice the C-fibre threshold. Extracellular potentials were amplified (BIO Amp, ML132, ADInstruments Pty Ltd, Castle Hill, Australia), transferred to a PowerLab 4/20 (ADInstruments), sampled

(10 kHz) and acquired by means of Scope software (Scope v3.5.8, MacLab® System© 1998, ADInstruments) for off-line analysis (Figure 3.1b).

Conduction velocity measurement

DRCAPs were recorded as described, and conduction times for A- and C-fibre waveforms were determined as the time elapsed between nerve stimulation and peak generation for the respective waveform (apex velocity). Conduction velocity was calculated directly from the conduction distance divided by the conduction time for each waveform (Figure 3.1c).

Drugs

Three control readings were recorded before lignocaine (Lopaine, Ethical Agents Ltd, Auckland, New Zealand), capsaicin (ICN Biomedicals Inc • K+K Labs, Plainview, NY, USA) or ethanol were applied to 5 mm of the preparation. Lignocaine (40 mM) and capasaicin (1 mM) were prepared as stock solution in double distilled water and 96% ethanol respectively, and were kept at 4°C. On experimental days, all treatments were diluted to the desired concentration in Tyrodes solution. Cumulative concentrations of lignocaine (0.0625, 0.125, 0.25, 0.5, 1, and 2 mM) were applied to all preparations. Similar lignocaine concentrations have been shown to decrease DRCAPs in preparations from 10- to 12-day-old rats (Nagy and Woolf, 1996). Some preparations also received 2 μM final concentration of capsaicin, which is known to depress the C-fibre waveform of DRCAPs from 3- to 6-day-old rats (Faber *et al.*, 1997). The final concentration of ethanol in the capsaicin treatment was 0.192%, and it was used as vehicle control in some preparations. All treatments were given in a volume of 400 μl.

The drugs were left in contact with the preparations for 2 min before another recording was taken. Drug exposure periods were selected based on data from preliminary experiments. Recovery of the preparations on wash out of the drugs (for about 50-60 min) was attempted for lignocaine only, and any results where DRCAP did not recover to at least 75% of pre-drug size were discarded. The effect of capsaicin and ethanol was studied in preparations that had previously received lignocaine, but only one of them was applied per preparation, and control readings were taken after recovery from lignocaine.

The absolute amplitude of both A- and C-fibre waveforms were used to assess the effect of each drug concentration on the DRCAP. Values were converted to percentage baseline values for individual preparations. For lignocaine, sigmoidal-concentration response curves were constructed and the concentrations necessary to reduce the amplitude of the A- and C-fibre waveforms by 50% (IC_{50}) were calculated using GraphPad Prism (Prism 4 for Macintosh, v4.0b, GraphPad Software Inc., San Diego, CA, USA).

Histological studies

Fixing and embedding procedures

As described above, L4 or L5 dorsal root-sciatic nerve tissue was harvested from 0-, 3-, 6-, 9-, and 12-day-old rats. The tissue was transferred into a petri dish containing Karnovsky fixative (2% formaldehyde, 3% glutaraldehyde in 0.1 M cacodylate buffer solution at pH 7.2). Using a razor blade, the dorsal root was transversely divided into approximately three thirds. The middle section was further subdivided into two halves and these were kept in vials with Karnovsky fixative for 24 h at 4°C. The old fixative was refreshed using a glass Pasteur pipette and after 3 h, the fixative was removed and the specimens triple rinsed (10-15 sec between rinses) with cacodylate buffer solution. Specimens were stained with reduced 1% osmium tetroxide solution in double distilled water for 1 h. Osmium solution was removed and specimens triple rinsed (3-5 min between rinses) with cacodylate buffer solution. Specimens were dehydrated using 25%, 50%, 70%, 90%, and 100% acetone, each for 10 min followed by 1 h in 100% acetone. Specimens were infiltrated with a 1:1 mixture of Spurr's resin and 100% acetone, and were left overnight in a rotary mixer. The diluted resin was removed and replaced with pure resin. Vials were left in the rotary mixer for 6 h. Specimens were moulded in pure resin, which was oven cured at 60°C for 48 h. Except for refrigeration and curing, all steps were carried out in a fume cupboard.

Blocks were cut to obtain 0.5 μm thick transverse sections. These were mounted on slides and stained with 1% toluidine blue in 0.1 M phosphate buffer solution at pH 7.2 on a hot plate set at 90°C for 30 sec.

Myelination

Under light microscopy, micrographs from all preparations were digitalised (Spot RT color camera, model 2.2.0, Diagnostic Instruments Inc, Sterling Heights, MI, USA) and stored for off line analysis. The area of myelin and myelinated axons, herein referred as myelination, and the total transverse area of individual dorsal root sections were automatically measured using commercial software (ImagoPro® Plus, v4.1.1.2 for Windows 95/NT/98, Media Cybernetics, L.P., Silver Spring, MD, USA). This was done by identifying objects by their colour. An object was considered to be any contiguous group of pixels that shared a specified range of intensity values. In nervous tissue, toluidine blue stains myelin dark blue colour and the rest of the tissue pale blue. This difference in optical density was used to select and highlight myelin sheaths (Figure 3.2). The total area of the micrograph was selected by also highlighting the pale blue colour. Percentage myelination values were obtained for individual micrographs. If more than one micrograph was taken for a given dorsal root, the average of individual micrograph percentage values for myelination was taken as the percentage myelination value for that particular preparation.

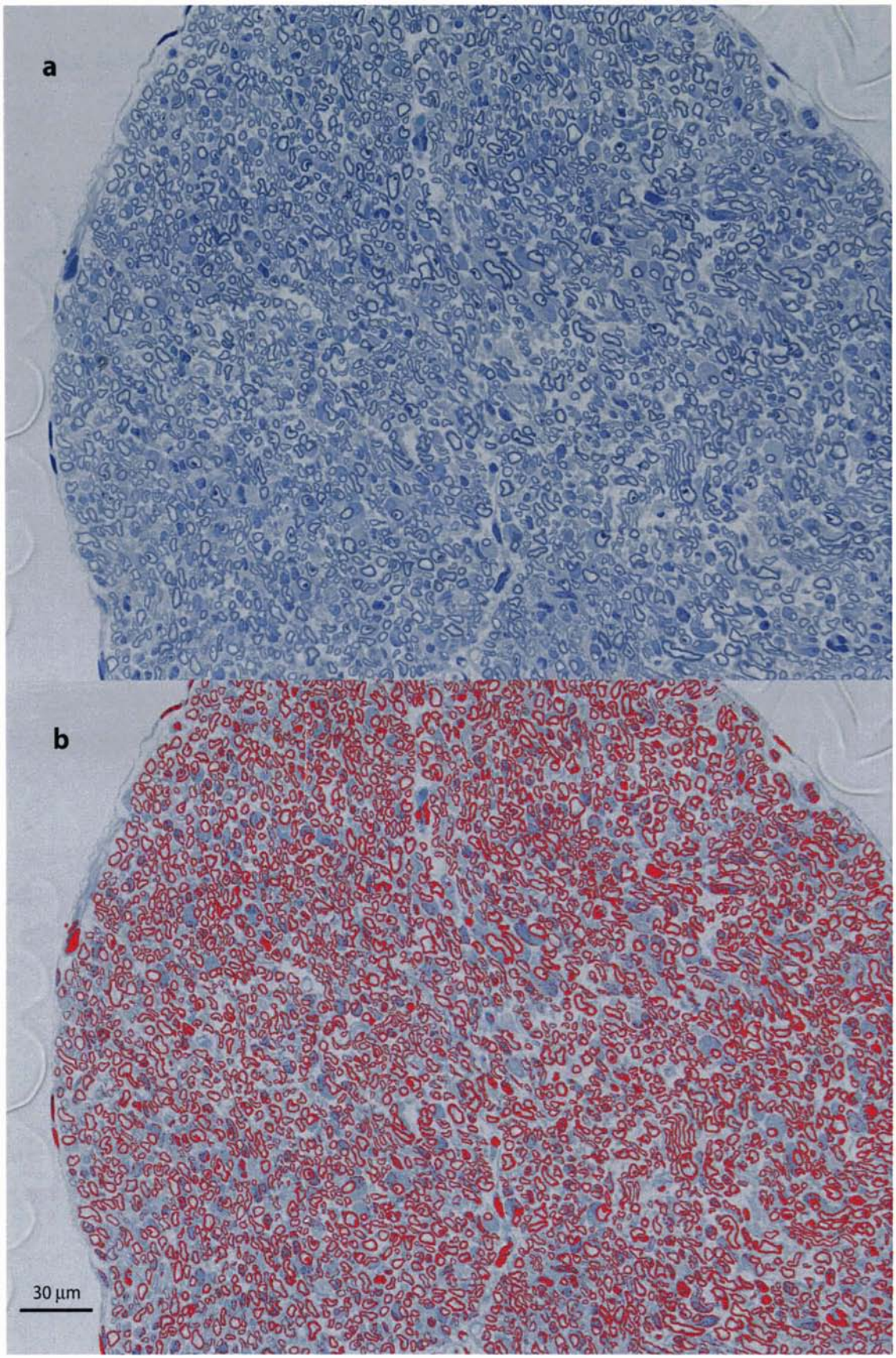

Figure 3.2 *High power images of transverse sections of L5 dorsal root from a 12-day-old rat. (a) Under toluidine blue staining, individual myelin sheaths resemble dark blue doughnuts. (b) Myelin was highlighted in red for automatic quantification as indicated in materials and methods. In this particular micrograph, myelination corresponded to 43.75% of the total tissue area.*

Statistical analysis

The effect of age on *i*) stimulus intensities required for evoking A- and C-fibre thresholds, *ii*) conduction velocities of A- and C-fibre waveforms, *iii*) inhibitory action of lignocaine (IC_{50} values) on A- and C-fibre waveforms, and *iv*) percentage area of myelination were assessed. The effect of capsaicin on A- and C-fibre waveforms was analysed with a linear model that considered the effect of age, time, and the interaction of these two. For ethanol, only the effect of time was considered. Correlation analysis between physiological (threshold, conduction velocity, and myelination) and both physiological and pharmacological (lignocaine, capsaicin) properties were carried out as appropriate for A- and C-fibre waveforms. For these purposes, the MIXED and CORR procedures of SAS (2002, SAS Institute Inc, Cary, NC, USA) were used. Least square means and standard errors were used for comparisons between ages and multiple comparisons between ages and time as appropriate. $P < 0.05$ was taken as the accepted level for statistical significance.

3.4 Results

Thresholds

The voltage threshold of the afferent input recorded from L4 or L5 dorsal root on electrical stimulation of the sciatic nerve significantly changed over the early postnatal period ($F(12, 44) = 15.64$, $P < 0.0001$). The highest level of stimulation to evoke a threshold was 2.25 ± 0.15 V in preparations from the youngest rats used in this study. As rat pups aged, preparations showed a trend to have lower thresholds until reaching the lowest value of 0.125 ± 0.19 V recorded from preparations of 12-day-old rats. However, preparations from 2-to-4-day-old rats showed a slight, but not statistically significant, increase in threshold values as compared to thresholds from 1-day-old rat preparations ($P \geq 0.2501$; Figure 3.3a).

A higher voltage than that required to evoke the first discernible response was necessary to evoke a DRCAP composed of C-fibres, and the age of the rat from which the preparation came from had a significant effect ($F(12, 44) = 2.04$, $P = 0.042$). Preparations from rats aged 0- to 2-days-old had significantly lower C-fibre thresholds (mean values between 5.6 and 6.75 V) than most preparations from older animals (mean values ≥ 8.6 V) ($P \leq 0.0479$). However, C-fibre thresholds for preparations from 3-day-old rats and older were not significantly different to one another ($P \geq 0.1024$) (Figure 3.3b).

Conduction velocities

From birth, the DRCAP was clearly divisible into two waves, A- and C-fibre waveforms.

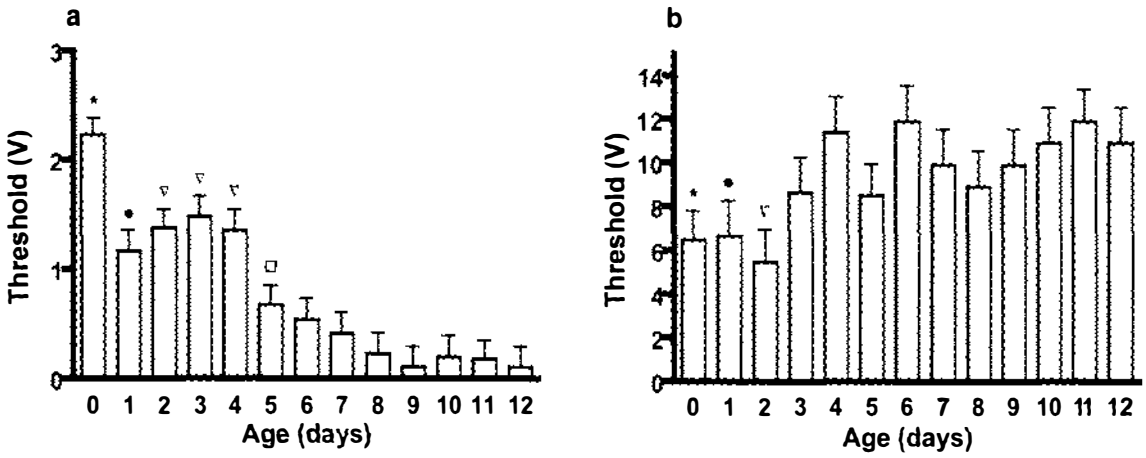

Figure 3.3 *Developmental changes in L4 or L5 dorsal root A- (a) and C-fibre thresholds (b) after electrical stimulation of the sciatic nerve of 0- to 12-day-old rats. In panel (a), ★ significantly different to ages 1-12 days ($P \leq 0.0037$); ● significantly different to ages 6-12 days ($P \leq 0.0244$); ∇ significantly different to ages 5-12 days ($P \leq 0.011$); □ significantly different to ages 9, 11, and 12 days ($P \leq 0.0429$). In panel (b), ★ significantly different to ages 4, 6, and 10-12 days ($P \leq 0.0394$); ● significantly different to ages 4, 6 and 11 days ($P \leq 0.043$); ∇ significantly different to ages 4, 6, 7, and 9-12 days ($P \leq 0.0479$). Data are least square means ± standard error, n = 4-6 preparations per age group.*

From day 6, the DRCAP became more complex with the presence of a third wave, which was not investigated further (Figure 3.4a). Besides having different thresholds, A- and C-fibre waveforms travelled at different speeds and this was significantly affected by the age of the rat from which the preparations were harvested ($F(12, 44) = 46.55$, $P < 0.0001$ for A-fibre waveforms, and $F(12, 44) = 3.42$, $P = 0.0014$ for C-fibre waveforms). In preparations from 0-day-old rats, the faster A-fibre waveform travelled at 1.138 ± 0.388 m/s and the slower C-fibre waveform at 0.607 ± 0.042 m/s. As rats aged, A-fibre waveforms showed a trend to increase in velocity reaching 9.769 ± 0.475 m/s in preparations from 12-day-old rats. The C-fibre waveforms did not change much as rats aged; mean conduction velocities were between 0.593 and 0.861 m/s for preparations from all studied ages. Statistical differences were found, however, when conduction velocities of C-fibre waveforms from different age preparations were compared ($P \leq 0.0476$) (Figure 3.5).

Effects of lignocaine

Lignocaine depressed, in a concentration-dependent manner, both A- and C-fibre waveforms with IC_{50} values being significantly affected by age ($F(12, 42) = 6.29$, $P < 0.0001$ for A-fibre waveforms, and $F(12, 42) = 3.11$, $P = 0.0032$ for C-fibre waveforms; Figures 3.4b and 3.6). For depression of A-fibre waveforms by 50%, preparations from rats 4-day-old and younger required significantly higher concentrations of lignocaine than those from older rats ($P \leq 0.0487$). Interestingly, preparations from 4-day-old rats required significantly higher concentrations than preparations from any other age group ($P \leq 0.0356$). Except for

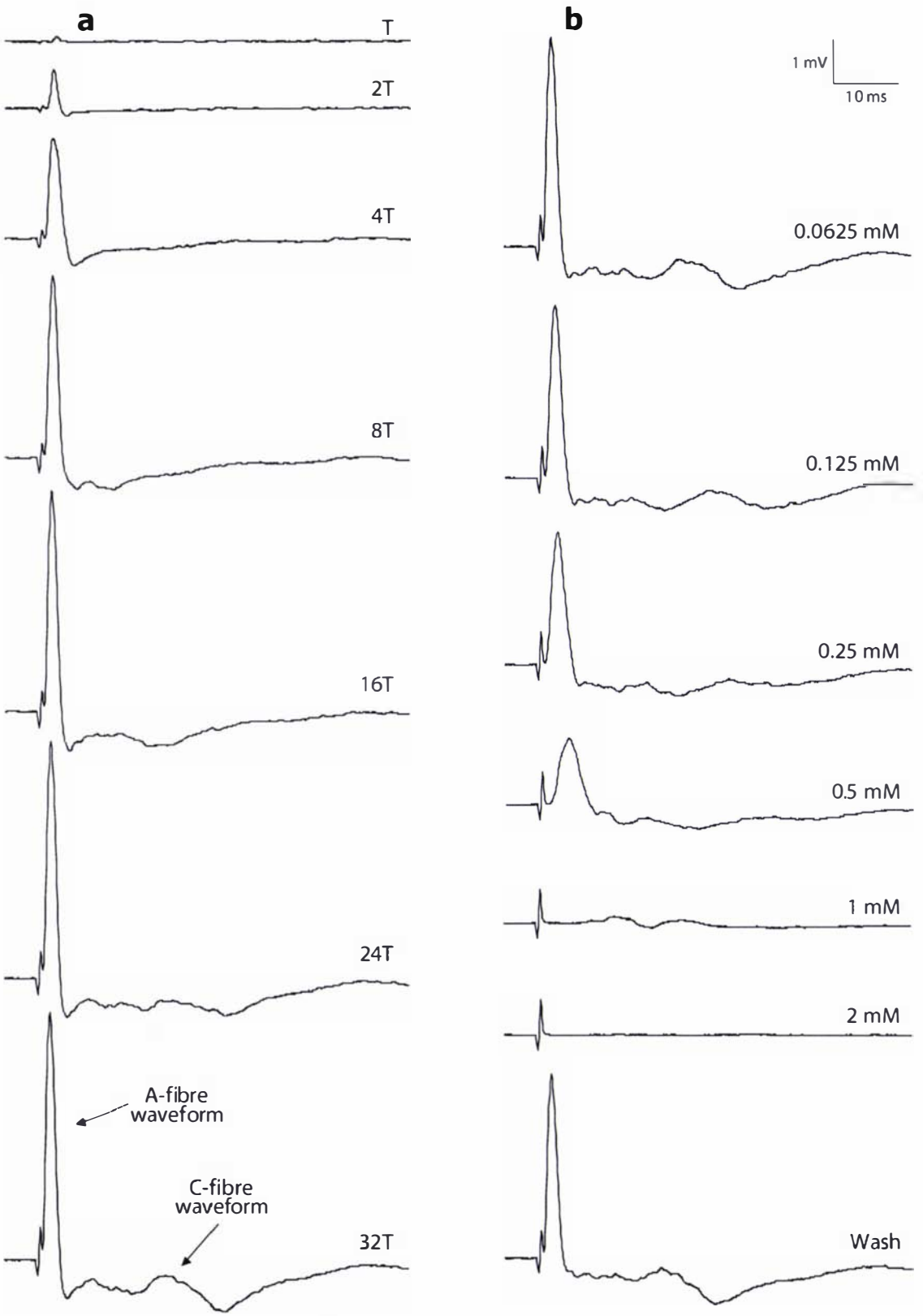

Figure 3.4 *Action potential recordings from a L4/L5 dorsal root-sciatic nerve preparation from a 6-day-old rat. (a) Recordings in this column show the effect of increasing intensities of stimulation. In this case, only at stimulation intensities at or above 16 times threshold (T) is a C-fibre waveform apparent. (b) Recordings in this column show the depressive action of cumulative concentrations of lignocaine (0.0625 - 2 mM) on the DRCAP. Recovery is shown in the lowest trace 60 min following return to lignocaine-free medium.*

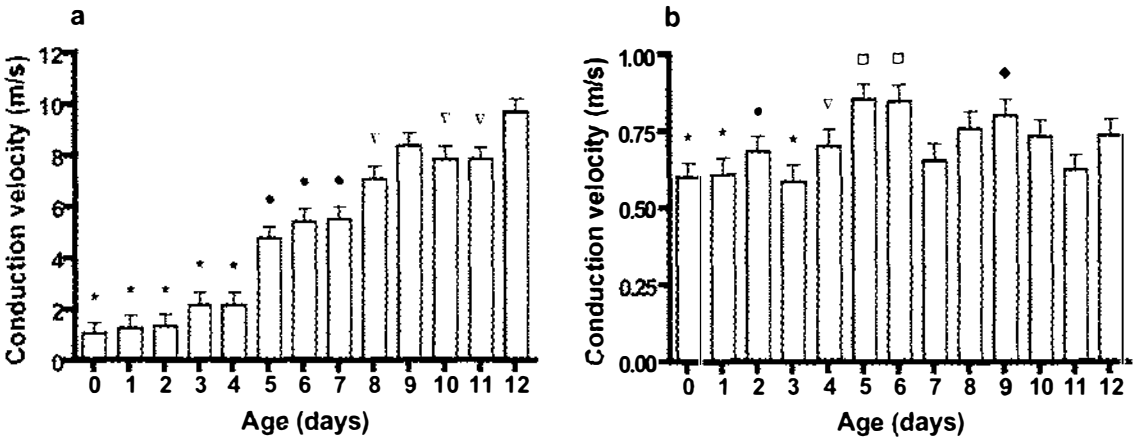

Figure 3.5 *Developmental changes in conduction velocity of A- (a) and C-fibre waveforms (b) from L4 or L5 DRCAPs of 0- to 12-day-old rats. In panel (a), ★ significantly different to ages 5-12 days ($P \leq 0.0002$); ● significantly different to ages 8-12 days ($P \leq 0.0224$); ∇ significantly different to age 12 days ($P \leq 0.0093$). In panel (b), ★ significantly different to ages 5, 6, 8, and 9 days ($P \leq 0.0476$); ● significantly different to ages 5 and 6 days ($P \leq 0.0248$); ∇ significantly different to age 5 days ($P = 0.0328$); □ significantly different ages 7 and 11 days ($P \leq 0.0128$); ◆ significantly different to age 11 days ($P = 0.0165$). Data are least square means ± standard error, n = 4-6 preparations per age group.*

preparations from 1- and 10-day-old rats, mean lignocaine IC_{50} values for C-fibre waveforms were between 0.105 and 0.192 mM. Preparations from 1-day old rats were significantly more sensitive to the depressive actions of lignocaine than those from 2-, 4-, 9-, 10-, and 12-day-old rats ($P \leq 0.0457$). In contrast, preparations from 10-day-olds were significantly more resistant than those from any other age group ($P \leq 0.0439$).

Characterised by IC_{50} values, C-fibre waveforms were significantly more susceptible than A-fibre ones to the depressant actions of lignocaine in preparations from 4-day-old rats and younger ($P \leq 0.0212$).

Effects of capsaicin and ethanol

Figure 3.7 shows an example of the effects of capasaicin and ethanol on DRCAPs. The age of the pup from which the preparation came was not significant on the effects of capsaicin on A-fibre waveforms ($F(12, 30) = 0.78$, $P = 0.6662$), but the time and the interaction age x time were both significant ($F(3, 90) = 15.4$, $P < 0.0001$ and $F(36, 90) = 1.76$, $P = 0.0169$, respectively). Control readings were not significantly different to each other for each age group ($P = 0.2526$), but some of them were significantly different to post-capsaicin treatment readings in preparations from 0- to 6-day-old animals only ($P \leq 0.0285$; Figure 3.8a).

The effect of age in the action of capsaicin on C-fibre waveforms was not significant ($F(12, 30) = 1.93$, $P = 0.0714$), but that of time ($F(3, 90) = 908.79$, $P < 0.0001$) and the interaction between them (age by time interaction, $F(36, 90) = 2.28$, $P = 0.0009$) were both significant.

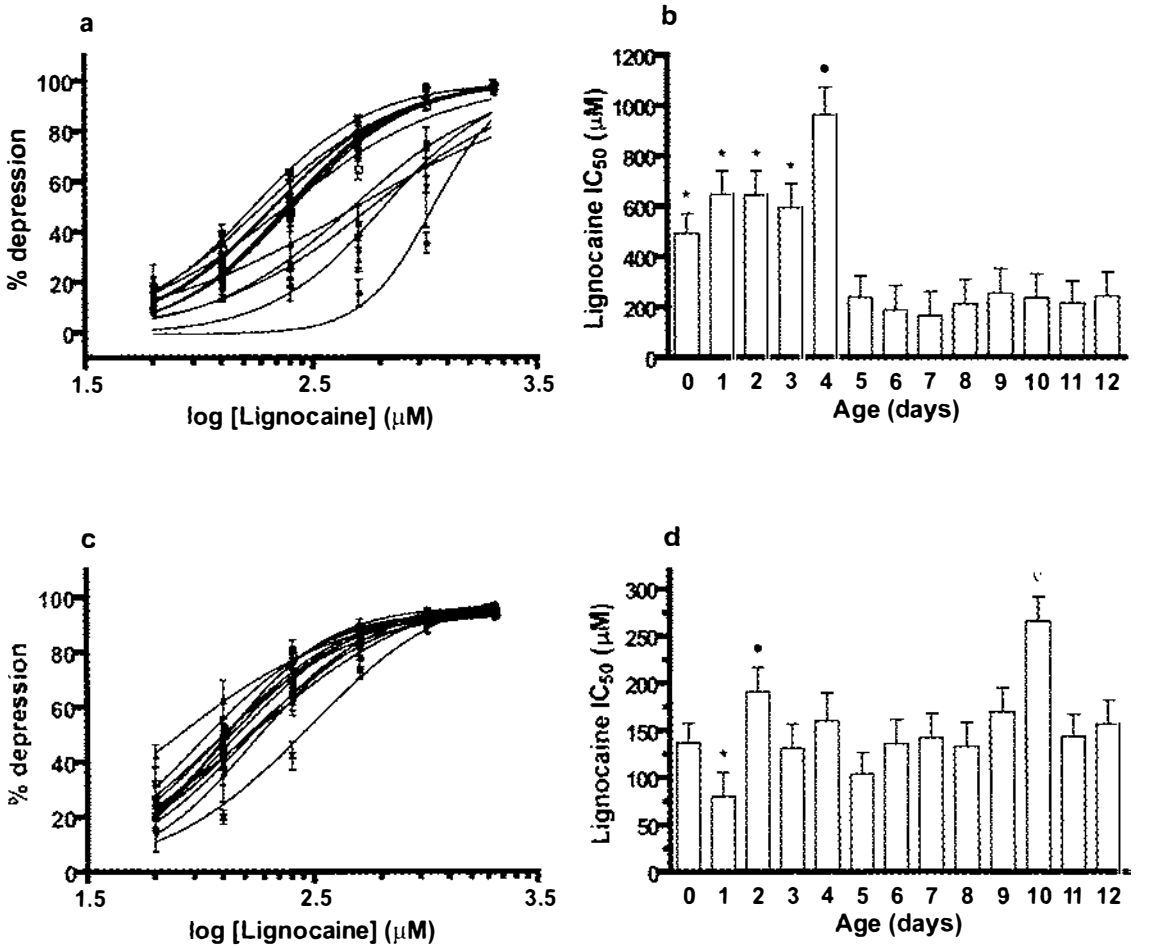

Figure 3.6 *Developmental changes in the depressive action of lignocaine on A- and C-fibre waveforms from L4 or L5 DRCAPs of 0- to 12-day-old rats. (a and c) Concentration-effect plots showing the depressant action of lignocaine on A- (a) and C-fibre waveforms (c). The keys for age (in days) in these graphs are: ■ 0, ▲ 1, ▼ 2, ◆ 3, ● 4, □ 5, △ 6, ▽ 7, ◇ 8, ○ 9, x 10, + 11, and * 12. (b and d) Lignocaine concentrations necessary to inhibit the amplitude of A- (b) and C-fibres (d) by 50% (IC50). In panel (b), ★ significantly different to ages 4-12 days ($P \leq 0.0487$); ● significantly different to ages 5-12 days ($P < 0.0001$). In panel (d), ★ significantly different to ages 2, 4, 9, and 12 days ($P \leq 0.0457$); ● significantly different to age 5 days ($P = 0.0152$); ▽ significantly different to all other ages ($P \leq 0.0439$). Data are least square means ± standard error, n = 3-6 preparations per age group.*

Within the same age group, control readings were not significantly different to each other ($P \geq 0.137$), but they were always significantly larger than those obtained after capsaicin treatment ($P < 0.0001$; Figure 3.8b).

Since very few preparations were tested with ethanol, the effect of age could not be reliable assessed and preparations were pooled across age. Ethanol had no effect on the amplitude of A- ($F(3, 9) = 1.85$, $P = 0.1619$) and C-fibre waveforms ($F(3, 9) = 1.67$, $P = 0.1974$) as compared to baseline levels (Figures 3.8c and 3.8d).

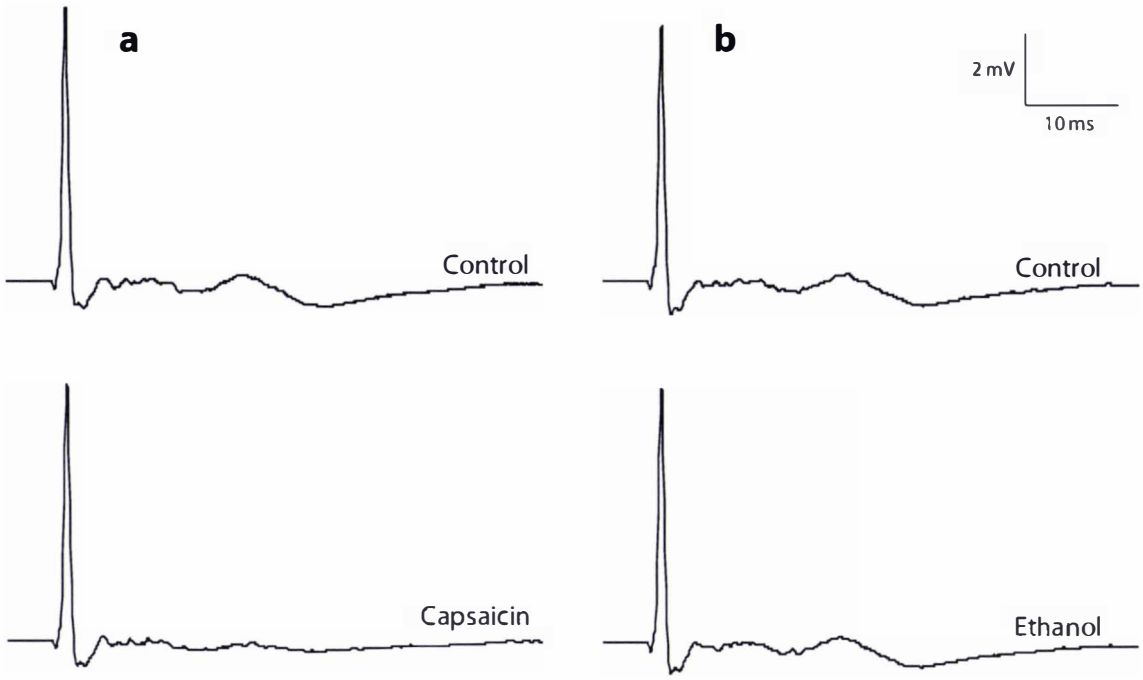

Figure 3.7 *DRCAPs form 11-day-old rat preparations. (a) Recordings in this column show the selective depressive action of capsaicin (2 μM) on the C-fibre waveform. (b) Recordings in this column show the lack of effect of ethanol (0.192%).*

Myelination

In L4 or L5 dorsal root transverse sections, the proportional area of myelin and myelinated axons varied significantly with age ($F(4, 14) = 12.80$, $P < 0.0001$). Dorsal roots from younger rats had lower percentage of myelination than those from older animals; values ranged from 19.28 ± 3.56% in 0-day olds to 50.64 ± 3.56% in 12-day olds. Myelination was significantly lower in 0- and 3-day-old rats than in 6-, 9-, and 12-day-old animals ($P \leq 0.0247$). Significantly lower values were also found in sections from 6-day-old rats than those from 12-day olds ($P = 0.0471$). There was no significant difference between sections from 9- and 12-day-old rats ($P = 0.1048$) (Figure 3.9).

Correlations

As seen in Table 3.1, all studied A-fibre waveform physiological parameters had a significant correlation with both other A-fibre physiological and pharmacological properties. In contrast, C-fibre waveform parameters were non-significantly correlated.

3.5 Discussion

Thresholds and conduction velocities of A- and C-fibres changed with age in electrically evoked DRCAPs from *in vitro* sciatic nerve-dorsal root preparations from 0- to 12-day-

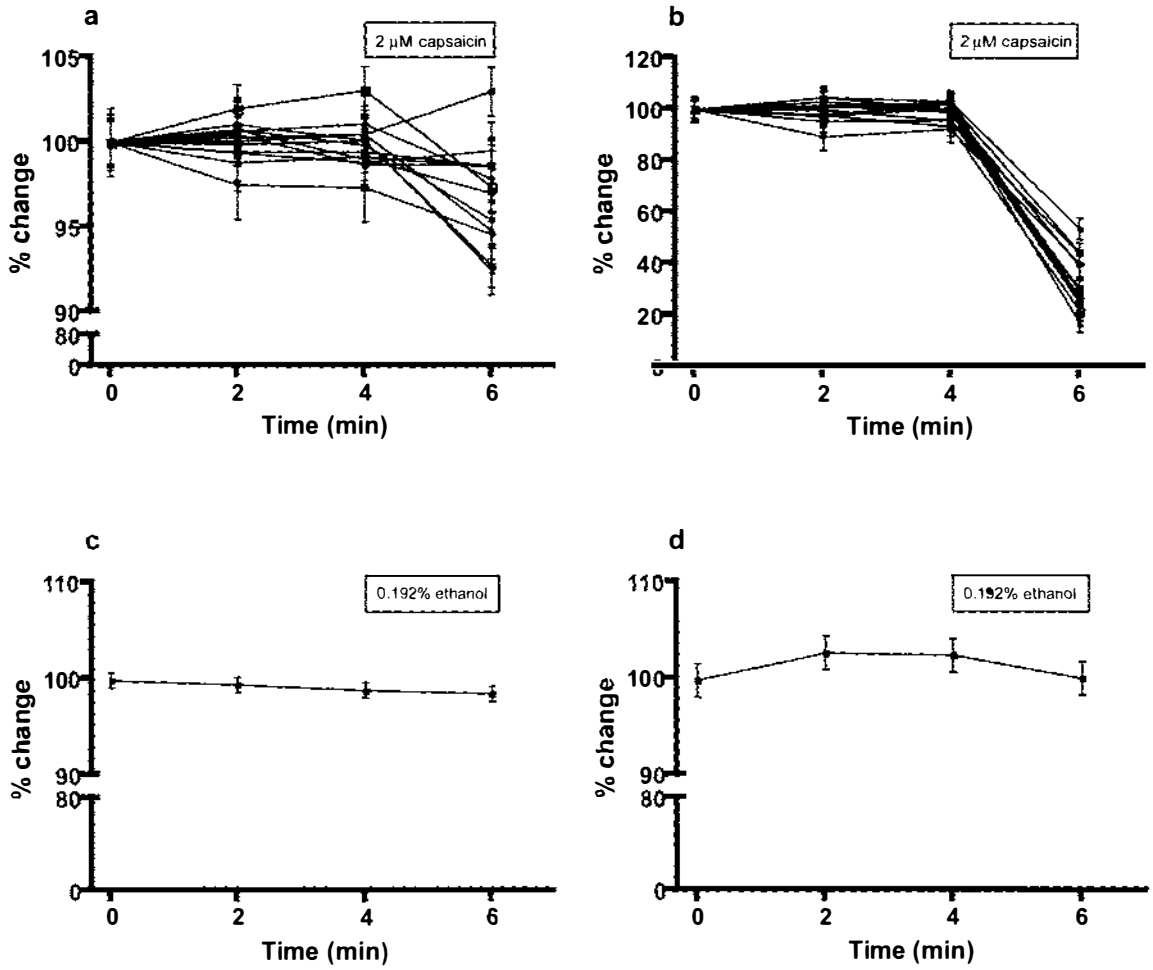

Figure 3.8 *Effect of capsaicin and ethanol on A- and C-fibre waveforms from L4 or L5 DRCAPs of 0- to 12-day-old rats. (a and b) Capsaicin (2 μM) reduced A-fibre waveforms (a) in ages 0-3, 5, and 6 days, and C-fibre waveforms (b) in all ages. A-fibre values at time 6 min were significantly different to those at times 0, 2, and 4 min for ages 0 days ($P < 0.0001$), 1 day ($P < 0.0001$), 2 days ($P \leq 0.0277$), and 6 days ($P \leq 0.0102$); to times 2 and 4 min for age 5 days ($P \leq 0.0087$); and to time 0 min for age 3 days ($P = 0.0285$). C-fibre values at time 6 min were significantly different to those at times 0, 2, and 4 min for ages 0-12 days ($P < 0.0001$). The keys for age (in days) are: ■ 0, ▲ 1, ▼ 2, ◆ 3, ● 4, □ 5, △ 6, ▽ 7, ◇ 8, ○ 9, x 10, + 11, and ⋆ 12. Data are least square means ± standard error, n = 3-5 preparations per age group. (c and d) Ethanol (0.192%) had no effect on A- (c) and C-fibre waveforms (d). Data are least square means ± standard error, n = 10.*

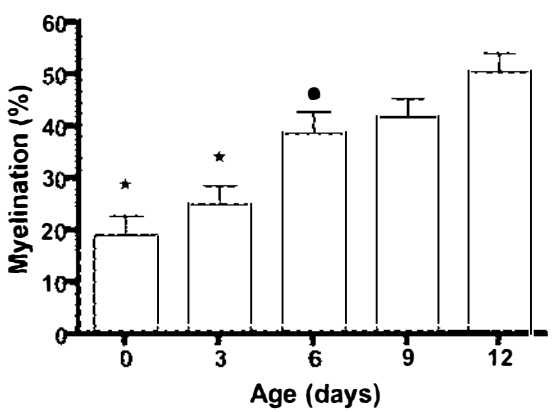

Figure 3.9 *Developmental changes in myelination of L4 or L5 dorsal roots in 0- to 12-day-old rats. ★ Significantly different to ages 6, 9, and 12 days ($P \leq 0.0247$); ● significantly different to age 12 days ($P = 0.0471$). Data are least square means ± standard error, n = 3-4 sections per age group.*

Table 3.1 *Correlation and (P) values for threshold (t) and conduction velocity (cv) of, and effect of lignocaine (lingo) and capsaicin (caps) on A- (A) and C-fibre waveforms (C), and for myelination (myelin).*

	t A	t C	cv A	cv C	ligno A	lingo C	caps A	caps C
t A				-0.81814 (<0.0001)		0.54294 (<0.0001)		-0.44765 (0.0026)
t C					-0.02458 (0.8560)		0.08596 (0.5326)	0.23278 (0.1331)
cv A						-0.59283 (<0.0001)		0.52539 (0.0003)
cv C							0.16375 (0.2322)	0.08442 (0.5904)
myelin	-0.81220 (<0.0001)		0.90266 (<0.0001)		-0.49322 (0.0319)		0.63049 (0.0088)	

old rats. The depressant action of lignocaine on A-fibres also had an age-dependent effect. These ontogenic changes on A-fibres were associated with an increased percentage area of myelination in primary afferent terminals.

Thresholds

From birth, and as previously described (Fitzgerald, 1985), DRCAPs were clearly divisible into two waveforms: A- and C-fibre waveforms. Direct comparison between both waveforms was possible since the same stimulus duration (500 μs) was always used. The former waveform consistently required lower levels of stimulation than the latter one, but both showed developmental changes on their thresholds. As pup rats aged, the intensity of stimulation to evoke A-fibre thresholds showed a decreasing trend and that of C-fibre thresholds had a tendency to slightly increase (Figure 3.3). This widened the about 3-fold threshold difference between A- and C-fibre thresholds in preparations from 0-day-old rats to almost 90 times threshold in preparations from 12-day-old animals. This data may suggest that, within the first 12 postnatal days, A-fibre waveforms become more sensitive and C-fibre waveforms slightly more resistant to electrical activation in rats.

Although the experiments reported here were not designed to assess the biological significance of age-related changes on A- and C-fibre thresholds, these changes could have potential protective functions. Various studies have shown that mechanical nociceptive thresholds increase as a function of age (Howard *et al.,* 2001; Ririe *et al.,* 2003; Ririe *et al.,* 2004) and reach similar levels to those of young adult rats (110 days old) by approximately 40-70 days of age (Ririe *et al.,* 2003). A developmental increase in mechanical thresholds has also been observed in human beings (Fitzgerald, 1991). Interestingly, mechanical nociceptive information, which is mainly transmitted by Aδ- and C-fibres in adult rats (Woolf, 1983), is transmitted by immature Aβ-fibres in neonatal rats (Fitzgerald and Gibson, 1984). Although central mechanisms may also be involved in response to mechanical stimulation, these data are

consistent with findings from this study in which the gap between A-and C-fibre thresholds widened as rats aged.

Age-related differences in thermal withdrawal thresholds have also been reported. Hu *et al.* (1997) found that 1- and 5-day-old rats had shorter paw withdrawal latencies than 9- to 70-day-old animals in a modified hot plate test, and Conway *et al.* (1998) showed that 5- and 10-day-old rats exhibited shorter withdrawal latencies than older animals (15- to 125-day-old) when their tails were exposed to radiant or conductive heat. This is in agreement with the tendency of C-fibre thresholds to slightly increase during the early postnatal days. However, Ririe *et al.* (2003) observed that withdrawal latencies to radiant thermal nociceptive stimulation of the paw decreased as a function of age in 14- to 110-day-old rats. The lack of consistency on whether thermal thresholds increase or decrease with development may be due to differences in the intensity of stimuli and maturational changes in radiant absorption properties of the stimulated area (Hu *et al.,* 1997; Conway *et al.,* 1998). Despite this, these studies found that at about 28 days postnatal, thermal thresholds were not different to those from young adult rats (70-125 days old) (Conway *et al.,* 1998; Hu *et al.,* 1997; Ririe *et al.,* 2003).

In a model of acute postoperative pain, mechanical and thermal thresholds decreased in a similar manner in 14-, 28-, and 110-day-old rats. The duration of mechanical allodynia was shorter in younger that older animals (2, 5, and 8.5 days, respectively), but that of thermal hyperalgesia was similar through all ages (7, 7, and 10 days, respectively) (Ririe *et al.,* 2003). The authors suggested that the more rapid decrease in mechanical allodynia in very young animals may indicate a biological difference in A-fibres between young and older animals, and the similar duration in thermal hyperalgesia may suggest that C-fibres are functionally the same along the studied age range (Ririe *et al.,* 2003). Although the age of rats from this study and the current one does not match, a similar changing pattern in thresholds can be identified, with A-fibre thresholds changing constantly and C-fibre thresholds being more stable.

Conduction velocity

Conduction velocities of A-fibre waveforms significantly and consistently increased within the first 12 postnatal days in rats. Significant age-related changes in conduction velocities of C-fibre waveforms were also found, with a trend to increase velocity within the first 6 postnatal days. These developmental changes were similar to those previously described from recordings of L4 dorsal roots and dorsal root ganglion neurones after electrical stimulation of the skin of the hind limb of 0- to 14-day-old anaesthetised rats (Fitzgerald, 1985; Fitzgerald, 1987). In addition to confirm these descriptive observations (no statistical analyses were used), results from the current study demonstrate, utilising reliable statistical resources, age-

related changes on conduction velocities of afferent fibres in the early postnatal period.

However, Nagy and Woolf (1996) calculated conduction velocities for A- and C-fibre waveforms from DRCAPs in 10- to 12-day-old rats to be much slower: 3.43 and 0.29 m/s, respectively. From studies with adult rat sciatic nerve preparations, it is known that conduction velocity of Aα/β-fibres varies 1.2 m/s with every Celcius degree change within the range 20 to 40°C (Birren and Wall, 1956). Although not known if this is also true for neonatal peripheral nerves, but even assuming it is, it would be difficult to determine if temperature could have contributed to these different results. Considering that the normal core temperature of newborn rats when not under the mother is 27 to 29°C (Brockmeyer and Kendig, 1995), conduction velocities in the current study may be slightly underestimated since were obtained at room temperatures between 22 and 25°C. Conduction velocities reported by Fitzgerald may be overestimated since they were obtained with rat pups on a heated pad set at 38°C (Fitzgerald, 1985; Fitzgerald, 1987), and those from Nagy and Wolf may be uncertain as no room temperature was specified (Nagy and Woolf, 1996). However, this would not be consistent with calculated conduction velocities in this study being so similar to those reported from Fitzgerald (1985; 1987). The way conduction velocities were computed could also have had a minor impact. In Aα/β-fibres from adult rat sciatic nerve preparations, apex velocity was 10 to 13% slower than inflexion velocity, which is considered as the first evidence of rise in the wave (Birren and Wall, 1956). Apex conduction velocities were calculated in the current study, but the methodology for computing conduction velocities was not specified in the other studies. Further research is necessary to clarify these differences in conduction velocity.

Conduction velocities of both A- and C-fibre waveforms were slower and similar to Aβ- and C-fibres in adult rat sciatic nerves (33.9 and 1.18 m/s, respectively; Huang *et al.,* 1997). Interestingly, the developmental trend of C-fibre waveforms to increase in velocity was also observed in C-fibres from the rat cervical sympathetic trunk. Mean C-fibre conduction velocity in 300-day-old rats was 2.02 m/sec and about half this value in 10- to 20-day-olds; by 50 days of age, values lied within the adult range (Hopkins and Lambert, 1973). Factors responsible for increasing conduction velocity are different for myelinated and unmyelinated fibres. For the former, the increase with age may be related to changes in fibre diameter, number of myelin lamellae, and membrane capacitance. Changes which may account for the latter are increase in fibre diameter and less likely a decrease in axoplasmic resistance or membrane capacitance (Hopkins and Lambert, 1973). Hence, A- and C-fibre waveforms have been proposed to be produced by activation of immature myelinated Aβ-fibres and non-myelinated C-fibres, respectively (Fitzgerald, 1985; Fitzgerald, 1987; Fitzgerald, 1988; Nagy and Woolf, 1996).

Capsaicin and ethanol

Capsaicin, a crystal alkaloid that is the main pungent ingredient in hot chilli peppers, selectively destroys primary afferent nociceptors expressing the TRVP1 receptor (Caterina *et al.,* 1997). Electrophysiologically, this is expressed as a relatively selective conduction block in afferent C-fibres. In saphenous nerve compound action potentials from anesthetised adult rats, capsaicin (33 μM to 33 mM) depressed, in a concentration-dependent manner, the amplitude of C-fibres by 95%. The amplitude of Aδ-fibres suffered a smaller reduction (25% depression) and that of Aα/β-fibres was depressed by 30%, but only by the highest concentration (Baranowski *et al.,* 1986). One to eight days after subcutaneous administration of capsaicin (50 mg/kg) to neonatal rats (0 or 2 days old), C-fibre waveforms were prevented from appearing in L4 dorsal root ganglion neurones after electrical stimulation of the skin of the hind limb of decerebrated animals (Fitzgerald, 1988) and in L4 dorsal roots after electrical activation of the sciatic nerve in *in vitro* preparations (Akagi *et al.,* 1985). Similarly, a low capsaicin concentration (2 μM) depressed *in vitro* the amplitude of C-, but not A-, fibre waveforms in DRCAPs from 3- to 6-day-old rats (Faber *et al.,* 1997). This is in line with the consistent, non-age-dependent depressive action of capsaicin (2 μM) on C-fibre waveforms in this study (46 to 83% depression). However, it was also observed here that A-fibre waveforms were slightly, but significantly, depressed (up to 7.5%) by capsaicin during the first week of age only. These data, together with conduction velocity results, confirm that A- and C-fibre waveforms are indeed the result of activation of immature Aβ- and C-fibres, respectively (Fitzgerald, 1985; Fitzgerald, 1987; Fitzgerald, 1988).

Since ethanol was used in the capsaicin stock solution and it is known to block compound action potentials from adult rat sciatic nerves *in vitro* (Staiman and Seeman, 1974), some preparations were tested with this drug. Contrary to capsaicin, 0.192% ethanol produced no effect on A- or C-fibre waveforms. This is equivalent to 34 mM concentration of ethanol, which is much lower than the 1.2 M concentration necessary to depress compound action potentials from adult rat sciatic nerve preparations (Staiman and Seeman, 1974), and the calculated 0.762 M and 1.07 M concentrations to depress by 50% A- and C-fibre waveforms in DRCAPs from 10- to 12-day-old rats (Chambers and Lizarraga, unpublished data). These data demonstrated that the depressive effects of capsaicin in the current study were not due to the action of ethanol.

Lignocaine

Local anaesthetics block nerve impulse conduction by inhibiting voltage-gated Na^+ channels, which exist in at least three different states: resting, open, and inactivated. Local anaesthetics block and bind to open and inactivated Na^+ channels more effectively than to resting ones. This confers local anaesthetics a more effective action at blocking nerves with more rapid firing rates (*i.e.* action potentials passing along the nerve; use-dependence or phasic inhibition) than

those with low firing rate (tonic inhibition) (Butterworth and Strichartz, 1990). In the current study, marked developmental changes in the depressant actions of lignocaine were observed. A-fibre waveforms in preparations from 5-day-old rats and older were more susceptible to the depressant action of lignocaine than those from younger animals. An increased access of lignocaine through the perineurium in preparations from younger rats seems unlikely since a similar age-dependent trend was not observed on C-fibre waveforms. In fact, these latter waveforms showed no particular trend, though significant differences were found between some ages; preparations from 1-day-old rats were the most susceptible (IC_{50} = 81.17 μM) and those from 10-day-olds the most resistant (IC_{50} = 276.53 μM) to lignocaine's depressant actions. This is the first time that such age-related, depressant actions have been reported for lignocaine. Differences cannot be attributed to a longer length of nerve being exposed to the local anaesthetic in preparations from younger rats (Berde, 2004). The length of nerve bathed in lignocaine was a constant 5 mm for all preparations, which was as long as anatomically possible.

In preparations from 4-day-old rats and younger, A-fibre waveforms were also more resistant than those of C-fibre to the depressant actions of lignocaine. Within this age range, mean lignocaine IC_{50} values for A-fibre waveforms were between 499.73 and 968.13 μM, and those for C-fibre waveforms between 81.17 and 192.33 μM. In contrast, adult mammal myelinated peripheral nerve fibres have been reported to be more susceptible to the depressant actions of lignocaine than unmyelinated ones. In rabbit sciatic and vagus nerve preparations, lignocaine IC_{50} values for Aα-, Aβ-, and C-fibres were 0.085, 0.335, and 0.724 mM, respectively (Gissen *et al.,* 1980). Similar IC_{50} values were computed for single Aβ- (0.41 mM) and C-fibre (0.80 mM) units from rat sciatic nerves *in situ* (Huang *et al.,* 1997). All these studies assessed lignocaine tonic inhibition, which suggests that differences may result from developmental changes.

Interestingly, neonates have been regarded as showing shorter block duration and requiring larger weight-scaled doses of local anaesthetics for epidural or intrathecal anaesthesia as compared to adult human beings (Berde, 2004). Considering that Aβ-fibres may play an important role in the transmission of pain in neonates (Fitzgerald and Gibson, 1984), these data concur with the *in vitro* lignocaine results reported here. These results may also help to explain the lack of mechanical antihyperalgesia of 0.5% bupivacaine administered as a percutaneous sciatic nerve block before hindpaw incision in 0- (de Lima *et al.,* 1999), but not in 14-day-old rats (Ririe *et al.,* 2004). However, this last report also found that bupivacaine had a longer lasting antihyperalgesic action in 14-day-old rats than in 28 day olds (72 *vs.* 2 h, respectively) (Ririe *et al.,* 2004), which is somehow congruent with mechanical analgesia and antihyperalgesia being observed at lower epidural bupivacaine concentrations in 3- than in 10- and 21-day-old rats injected with carrageenan into one hindpaw (lower effective

concentrations: 0.004, 0.03, and 0.0625%, respectively) (Howard *et al.,* 2001). It is obvious that nerve fibre block by local anaesthetics is developmentally regulated, but further studies are required to assess whether fibres from newborns are more or less susceptible than those from adults.

In addition to length of nerve exposed to the drug, rate of firing, and nerve fibre size, the concentration of local anaesthetic necessary to block impulse conduction can be affected by degree of myelination, distance between nodes of Ranvier in myelinated fibres, and density and gating behaviour of Na^+ channels (Gissen *et al.,* 1980; Huang *et al.,* 1997; Alvares and Fitzgerald, 1999; Howard *et al.,* 2001). Developmental changes in these anatomical and functional factors may have contributed to the observed differences. However, exactly how these factors may affect lignocaine's depressive actions is not known and requires further investigation.

Myelination

The axon diameter of peripheral myelinated fibres has been shown to increase during the postnatal period (Sima, 1974). This has lead to the proposal that age-related changes in conduction velocity of A-fibre waveforms increase with myelination (Fitzgerald, 1985). However, until now, no parallel studies had been carried out to correlate these features. In the current study, myelination, considered as percentage area of myelin and myelinated axons, consistently increased with age in L4/L5 dorsal roots from 0- to 12-day-old rats (Figure 3.9). Further, there was found a good positive correlation between myelination and conduction velocities of A-fibre waveforms. These data give convincing statistical support to the statement that conduction velocity of A-fibre waveforms increase with myelination in neonates (Fitzgerald, 1985; Fitzgerald, 1987; Fulton, 1987).

Although not possible to determine from results in this study if axon diameter of myelinated fibres or myelin itself was responsible for the increase in conduction velocity in A-fibre waveforms, some insight can be gained by comparing them with those from other studies. Sima (1974) found that axon diameter of myelinated fibres in L5 dorsal roots increased with age in 5- to 180-day-old rats. However, peak axon diameter did not change much during the first two postnatal weeks, and fibre thickness growth was fast during the third and fourth postnatal weeks (Sima, 1974). These data may suggest that myelin could have been more important than axon diameter for increasing conduction velocity of A-fibre waveforms in this study. This is also supported by a loose positive correlation between soma transverse-sectional area and conduction velocity of sciatic nerve $A\alpha/\beta$ fibres in 35- to 55-day-old rats (Harper and Lawson, 1985). However, further studies are necessary to determine the individual importance of these two factors on A-fibre waveform conduction velocities in neonates.

In conclusion, the results reported here support with statistical reliability that developmental increases in conduction velocities of A-fibre waveforms from electrically evoked DRCAPs in *in vitro* sciatic nerve-dorsal root preparations from 0- to 12-day-old rats depend on myelination. Age-dependent changes of A-fibre waveforms were also found on threshold and response to lignocaine and capsaicin. These data indicate that the underlying peripheral A-fibre transduction mechanisms require some time to acquire their full stimulus-response sensitivity, which coincides with development of myelination. In contrast, C-fibre waveforms had few developmental changes indicating that peripheral C-fibres have mature transduction mechanisms from the first days of postnatal life. Further *in vivo* studies are required to investigate the ontogenic changes described here. If confirmed, they could have important clinical implications for the control of pain in neonates.

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4. Depression of NMDA receptor-mediated segmental transmission by ketamine and ketoprofen, but not L-NAME, on the *in vitro* neonatal rat spinal cord preparation

4.1 Abstract

Activation of spinal *N*-methyl-D-aspartate (NMDA) receptors, arachidonic acid, and nitric oxide pathways are important in pain transmission. This study assessed the effects of the NMDA receptor channel blocker ketamine, the cyclo-oxygenase inhibitors ketoprofen and salicylic acid, and the nitric oxide synthase inhibitor L-NAME on nociceptive transmission using an *in vitro* neonatal rat spinal cord preparation. Supramaximal electrical stimulation of the dorsal root evoked A-fibre- and C-fibre-mediated high intensity excitatory postsynaptic potentials (epsp) in the ipsilateral ventral root. Low intensity stimulation evoked A-fibre-mediated monosynaptic compound action potentials (MSR) superimposed on low intensity epsps. Both low and high intensity epsps contain NMDA receptor-mediated components. Only ketamine and ketoprofen depressed synaptic responses. Ketamine depressed all three spinal reflexes with IC_{50} values (with 95% CI) of 10.80 (5.97 to 19.54) μM for the MSR, 8.29 (4.53 to 14.17) μM for the low intensity epsp, and 5.35 (3.05 to 9.40) μM for the high intensity epsp. Naloxone (1 μM) did not reverse the depressant actions of ketamine. Ketoprofen depressed the low intensity epsp and the high intensity epsp only; IC_{50} values (with 95% CI) were 354.5 (217.5 to 576.8) μM and 302.7 (174.0 to 526.7) μM, respectively. Reflexes recovered after drug washout. These data demonstrate that ketamine and ketoprofen, but not L-NAME, depressed NMDA-mediated nociceptive transmission on the *in vitro* neonatal rat spinal cord preparation.

4.2 Introduction

The processing of nociceptive information in the spinal cord is subject to modulation by a number of local receptor systems, in particular that of spinal *N*-methyl-D-aspartate (NMDA) receptors and the signalling pathways they interact with. Among them, nitric oxide (NO) and arachidonic acid (AA) pathways have been highlighted (for reviews see Luo and Cizkova, 2000; Svensson and Yaksh, 2002). In keeping with this hypothesis, NMDA receptor antagonists, nitric oxide synthase (NOS) inhibitors, and cyclo-oxygenase (COX) inhibitors produce analgesia in a wide range of nociceptive tests when given by the intrathecal (i.t.) route (Malmberg and Yaksh, 1992; Chaplan *et al.*, 1997; Dolan and Nolan, 1999; Larson *et al.*, 2000; Park *et al.*, 2000). Under most circumstances, the site of analgesic action is thought

to be the spinal cord close to the injection site. However, absorption of the drug by blood vessels, changes in the local circulatory pattern, and spreading of the drug to supraspinal sites can contribute to their analgesic effect (Thompson *et al.*, 1995; Payne *et al.*, 1996). One way of studying a true spinal antinociceptive effect for analgesic drugs is using an *in vitro* spinal cord preparation.

The neonatal rat spinal cord preparation is a robust *in vitro* preparation, which allows stable recordings for several hours (Otsuka and Konishi, 1974). The reflex evoked in the ventral root after supramaximal stimulation of the dorsal root [the high intensity excitatory postsynaptic potential (epsp)] is considered to be due to activation of C-fibre primary afferents and therefore to reflect nociceptive activity (Akagi *et al.*, 1985; Faber *et al.*, 1997). Stimulation of the dorsal root at low intensities can evoke two A-fibre responses: the monosynaptic compound action potential (MSR) and the low intensity epsp. All three spinal reflexes have glutamate receptor components. The MSR is dependent on non-NMDA receptors (Long *et al.*, 1990), whereas the low intensity epsp and the high intensity epsp are known to possess NMDA receptor components. The two latter reflexes are depressed by NMDA receptor antagonists (Thompson *et al.*, 1995; Brockmeyer and Kendig, 1995; Faber *et al.*, 1997), and application of NMDA produces similar depolarisations to those evoked after supramaximal electrical stimulation (Thompson *et al.*, 1995). They are also suppressed by many analgesics including opioids and α_2-adrenoceptor agonists, and drug inhibition of such reflexes correlates well with analgesic activity *in vivo* (Faber *et al.*, 1997).

The aim of this study was to assess the role of the NMDA receptor channel blocker ketamine, the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME), and the non-steroidal anti-inflammatory drugs (NSAIDs) ketoprofen and salicylic acid on spinal nociceptive transmission in a hemisected rat spinal cord *in vitro* preparation. NSAIDs inhibit COX activity (Warner *et al.*, 1999) and they have not been assessed previously on the *in vitro* spinal cord preparation. This model was used to determine the spinal antinociceptive effects of these compounds and gain additional information on their spinal mechanisms of action.

4.3 Material and methods

Spinal cord preparation

The study protocol was approved by the institutional animal care committee. Minor modifications were made to the method described by Otsuka and Konishi (1974). Briefly, 5– to 7-day old, unsexed Sprague-Dawley rats were killed by cervical dislocation and decapitation. All thoracic and abdominal 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.

The spinal cord was dissected out of the dorsal part of the vertebral column together with attached L4 or L5 dorsal and ventral roots and was placed in a Petri dish containing artificial cerebrospinal fluid (aCSF) of the following composition (mM): NaCl, 118; $NaHCO_3$, 24; glucose, 12; $CaCl_2$, 1.5; KCl, 3; and $MgSO_4 7H_2O$, 1.25. The aCSF was gassed with 95% O_2 and 5% CO_2, and was kept at room temperature.

All nerve roots other than L4 or L5 were removed along with the dura mater covering the dorsal side of the spinal cord. The spinal cord was hemisected in the sagittal midline by separating the left and right sides of the cord at the cranial end with scissors and gently tearing the two halves apart. The ventral and dorsal roots were separated at the dorsal root ganglion, leaving the dorsal root intact with the dorsal root ganglion. The hemisected spinal cord was placed on a chamber with the dorsal root and attached dorsal root ganglion in contact with a stimulating Ag–AgCl wire electrode. The corresponding ventral root was in contact with a recording electrode of similar construction. The hemisected spinal cord was superfused with aCSF at a rate of 2 ml/min, which was dripped off the chamber and collected as waste (Figure 4.1).

The preparations were allowed to equilibrate for at least 60 min before any recordings were made. To determine the performance of the experimental setting, aCSF alone was infused and recordings taken every 5 min until reflex responses started to decline in magnitude. At least three baseline readings were recorded, 5 min apart, before any drug was infused. Ketamine (1-50 μM), L-NAME (1-100 μM), ketoprofen (200-600 μM), salicylic acid (1000 μM), and anandamide (0.1-1 μM) were applied to preparations in cumulative concentrations by adding them to the superfusate. Responses were recorded every 5 min until the last concentration was infused and then once more after a 90 min-washout period with aCSF. If no significant effect was produced with the drug being tested, a washout period of 30 min only was set. Each preparation was infused one drug only. Preliminary experiments were carried out to determine the time each drug needed to reach equilibrium. The concentration ranges for ketamine and L-NAME were selected from previous studies using a neonatal rat spinal cord preparation (Brockmeyer and Kendig, 1995; Thompson *et al.*, 1995; Kurihara and Yoshioka, 1996). Those for ketoprofen and salicylic acid were selected on the basis of COX inhibition, with the former but not the latter drug inhibiting COX activity *in vitro* (Warner *et al.*, 1999). The choice of concentrations of anandamide was based on inhibition of miniature excitatory postsynaptic currents in substantia gelatinosa neurones in spinal cord slices from 15- to 25-day old rats (Morisset and Urban, 2001). The contribution of opioidergic mechanisms to ketamine depressive effects, was assessed by adding naloxone (1 μM) to a further 50 μM ketamine-containing perfusate at the end of each ketamine trial. Naloxone infused in such a way reversed the depressive effects of morphine on spinal nociceptive reflexes (Faber *et al.*, 1997).

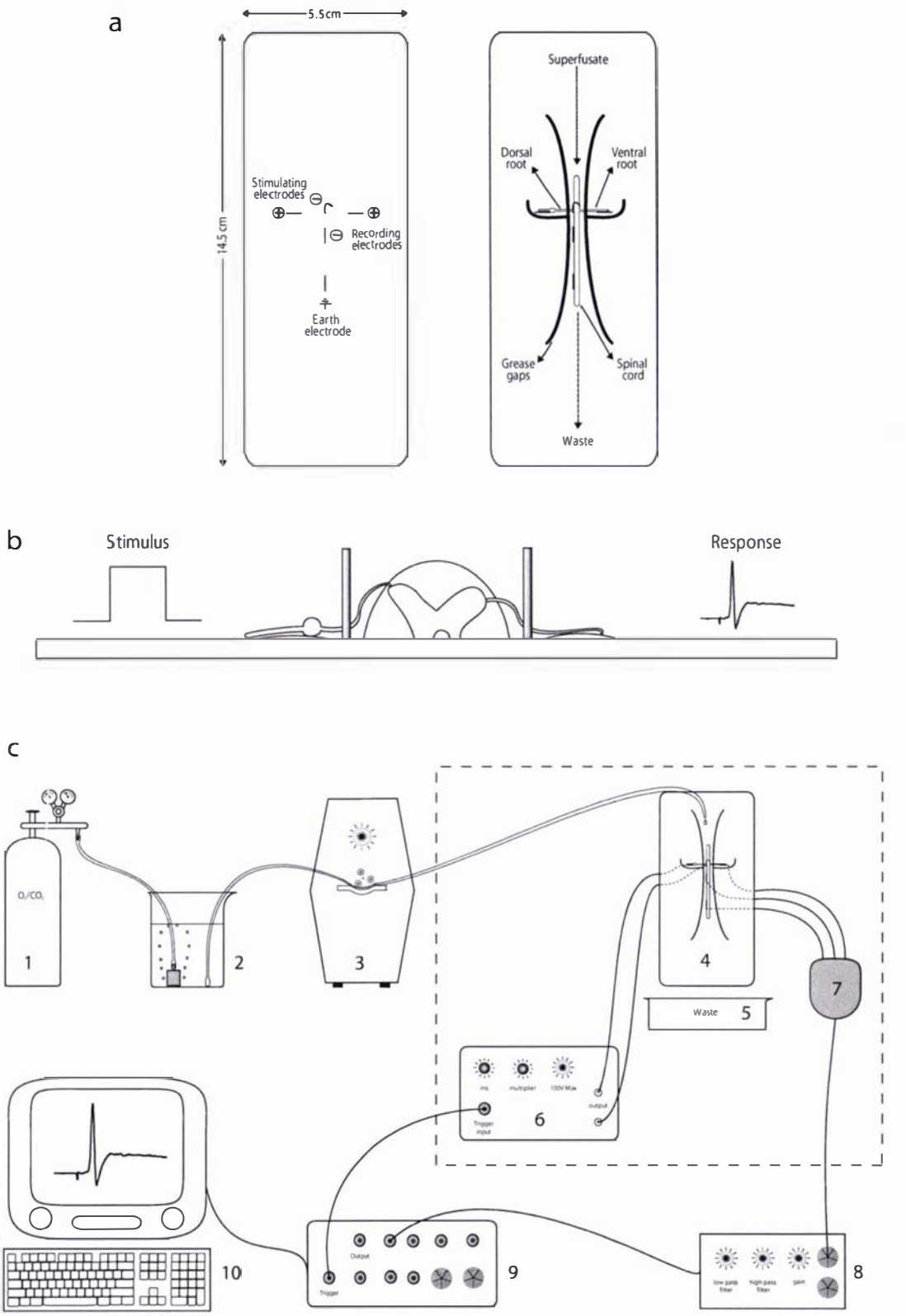

Figure 4.1 *Schematic representation of the experimental setting for the in vitro neonatal rat spinal cord preparation. (a) Recording chamber showing the arrangement of electrodes (left) and positioning of the hemicord on it (right). (b) Transverse section of the recording chamber with the hemisected spinal cord on it. (c) A mixture of O_2 95%/CO_2 5% (1) was delivered to the superfusate (2), which was pumped at 2 ml/min (3) onto the spinal cord preparation (4) and then collected as waste (5). The dorsal root was stimulated (6) to evoke ventral root potentials, which were pre-amplified (7), amplified (8), transferred to a PowerLab (9), and digitally stored (10). The dashed line represents a Farady cage.*

Recording techniques

Stimulation of the dorsal root evoked a population ventral root potential (DR-VRP) in the corresponding ipsilateral ventral root. Low intensity stimulation activated the A-fibre-mediated components of the DR-VRP. An initial MSR was superimposed on the low intensity epsp, which lasted up to two seconds (Faber *et al.,* 1997). High intensity stimulation evoked an additional polysynaptic C-fibre-mediated response, which lasted up to tens of seconds, the high intensity epsp (Akagi *et al.,* 1985; Faber *et al.,* 1997).

The MSR and the low intensity epsp were evoked by a single impulse 0.5 ms in duration at 3 times the threshold (the threshold being the intensity at which a discernible response first appeared in the ventral root). Approximately 0.5-1 V was required to evoke a threshold. The high intensity epsp was evoked by a single pulse 0.5 ms in duration at 32 times the threshold. In sciatic nerve-dorsal root preparations from age-matched rats, voltage stimulation at 3 times the threshold activated A-fibre afferents only, and at 32 times the threshold C-fibre afferents were also activated (Lizarraga *et al.,* 2005; Chapter 3).

Responses were pre-amplified (Pre-amplifier PA62, Medelec Ltd., Woking, Surrey, England) and a.c. amplified (AA6 Mk III, Medelec Ltd., Woking, Surrey, England) using a Medelec MS6 system (Medelec Ltd., Woking, Surrey, England). The signal was transferred to a PowerLab 4/20 (ADInstruments Pty Ltd, Castle Hill, Australia) and then digitally stored using commercially available software (Scope v3.6.8, MacLab System© 1998 ADInstruments Pty Ltd, Castle Hill, Australia).

Drugs

Ketamine hydrochloride and naloxone hydrochloride were dissolved in distilled water, ketoprofen and salicylic acid in distilled water and NaOH (no more than 1.12 mM, final concentration), anandamide in 96% ethanol, and L-NAME in aCSF. Stock solutions of all drugs were kept in refrigeration, except for L-NAME, which was prepared freshly as required. All drugs were obtained from Sigma (St. Louis, MO, USA).

Data analysis

The actions of drugs were assessed by measuring percentage changes in the peak amplitude of the MSR, and in the areas under the curve of the low intensity epsp and the high intensity epsp (Faber *et al.,* 1997). Scope software was used for this purpose. Data were expressed as mean ± s.e.m. Preliminary experiments on which spinal reflexes were used to determine time taken to reach equilibrium for each drug were analysed with repeated measures ANOVA followed by Tukey's *post-hoc* tests (GraphPad Prism v4.0b for Macintosh, GraphPad Software Inc., San Diego, CA, USA). Equilibrium was defined as three consecutive readings being not significantly different to each other after administration of the tested drug. When any

drug produced no obvious effect, it was infused for at least 30 min. Data from cumulative concentration experiments were fitted to a sigmoidal curve (GraphPad Prism). The concentrations required to produce 50% depression of the maximal effect of the drug (IC_{50} values) with 95% confidence intervals (CI) were computed and compared using the extra sum-of-squares F test. $P<0.05$ was considered significant.

4.4 Results

After at least a 60 min equilibration period, infusion of aCSF only permitted stable recordings of all three measured spinal reflexes for over 5 h (Figure 4.2).

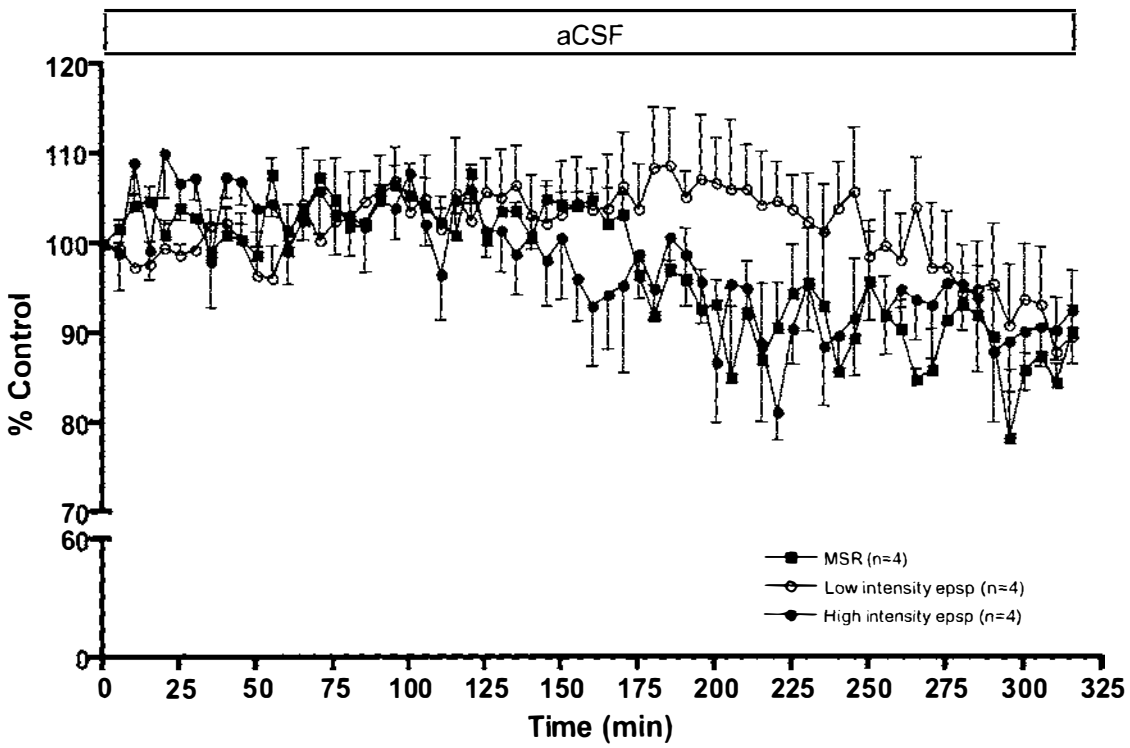

Figure 4.2 *Time course showing the effect of artificial cerebrospinal fluid (aCSF) on the different components of the synaptic response (mean ± s.e.m.).*

Effects of ketamine

Ketamine reached equilibrium within 30 min of application (Figure 4.3), and all three spinal reflexes were depressed in a concentration-dependent manner (Figure 4.4a). The mean IC_{50} values (with 95% CI) were 10.80 (5.97 to 19.54) μM for the MSR (n=4), 8.29 (4.53 to 15.17) μM for the low intensity epsp (n=5), and 5.35 (3.05 to 9.40) μM for the high intensity epsp (n=4). There were no significant differences between the IC_{50} values for depression of the three reflexes (P=0.2534, F test). However, the maximum depressant action of ketamine on the MSR was about 20% higher than those on the low and high intensity epsps (Figure 4.4b).

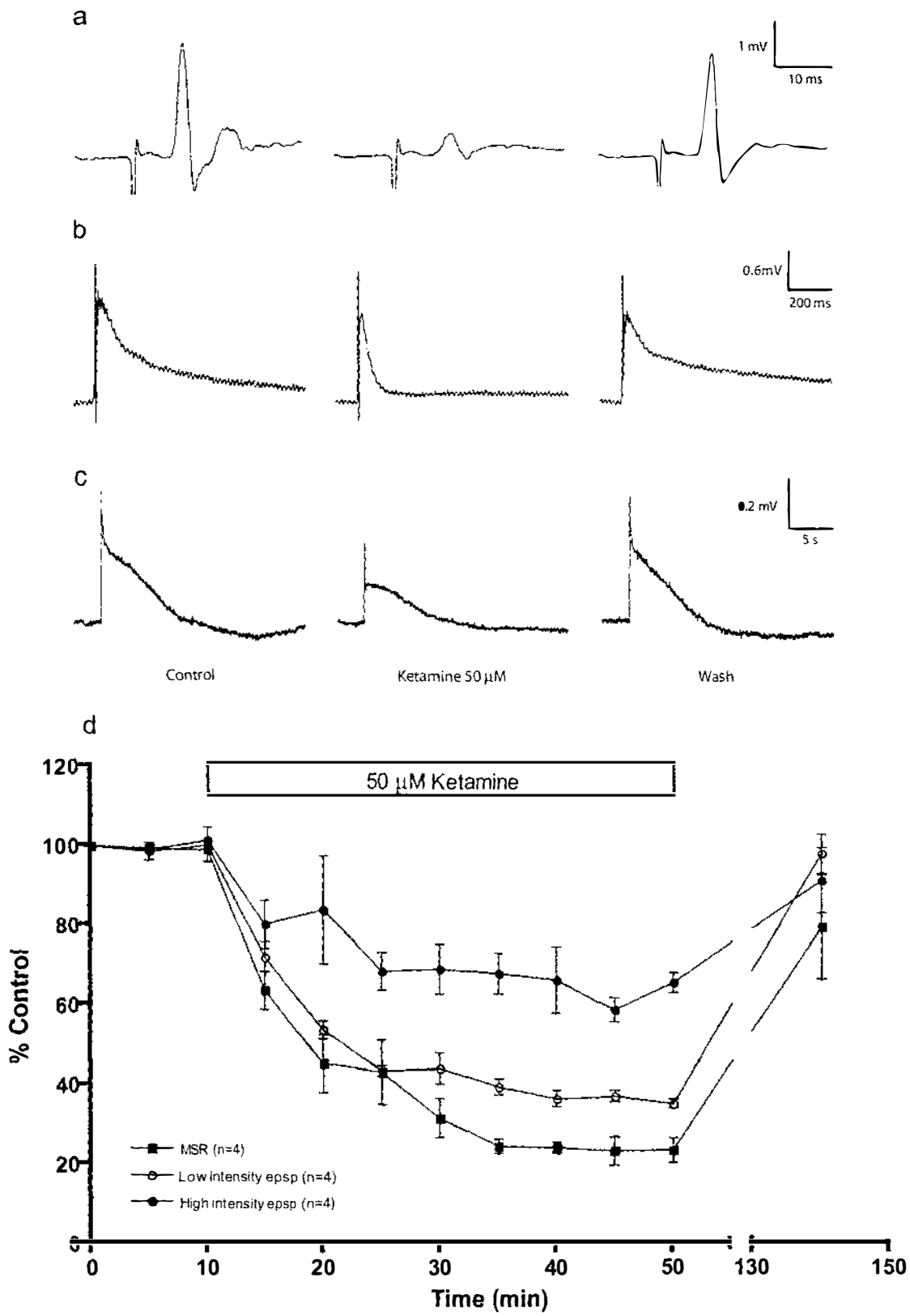

Figure 4.3 *(a-c) Effects of ketamine (50 µM) on the synaptic responses. Ketamine depressed the MSR (a), the low intensity epsp (b), and the high intensity epsp (c). Recovery is shown in the right panels after 90 min of ketamine-free medium. (d) Time course showing the depressant actions of ketamine on the different components of the synaptic response (mean ± s.e.m). At the time that reached equilibrium (30 min after infusion), 50 µM ketamine depressed the MSR by 76.03 ± 1.39 %, the low intensity epsp by 63.70 ± 1.99 %, and the high intensity epsp by 43.9 ± 8.35 %.*

The depressant actions of ketamine were not reversed by naloxone (1 μM), but partial recovery was achieved after drug washout.

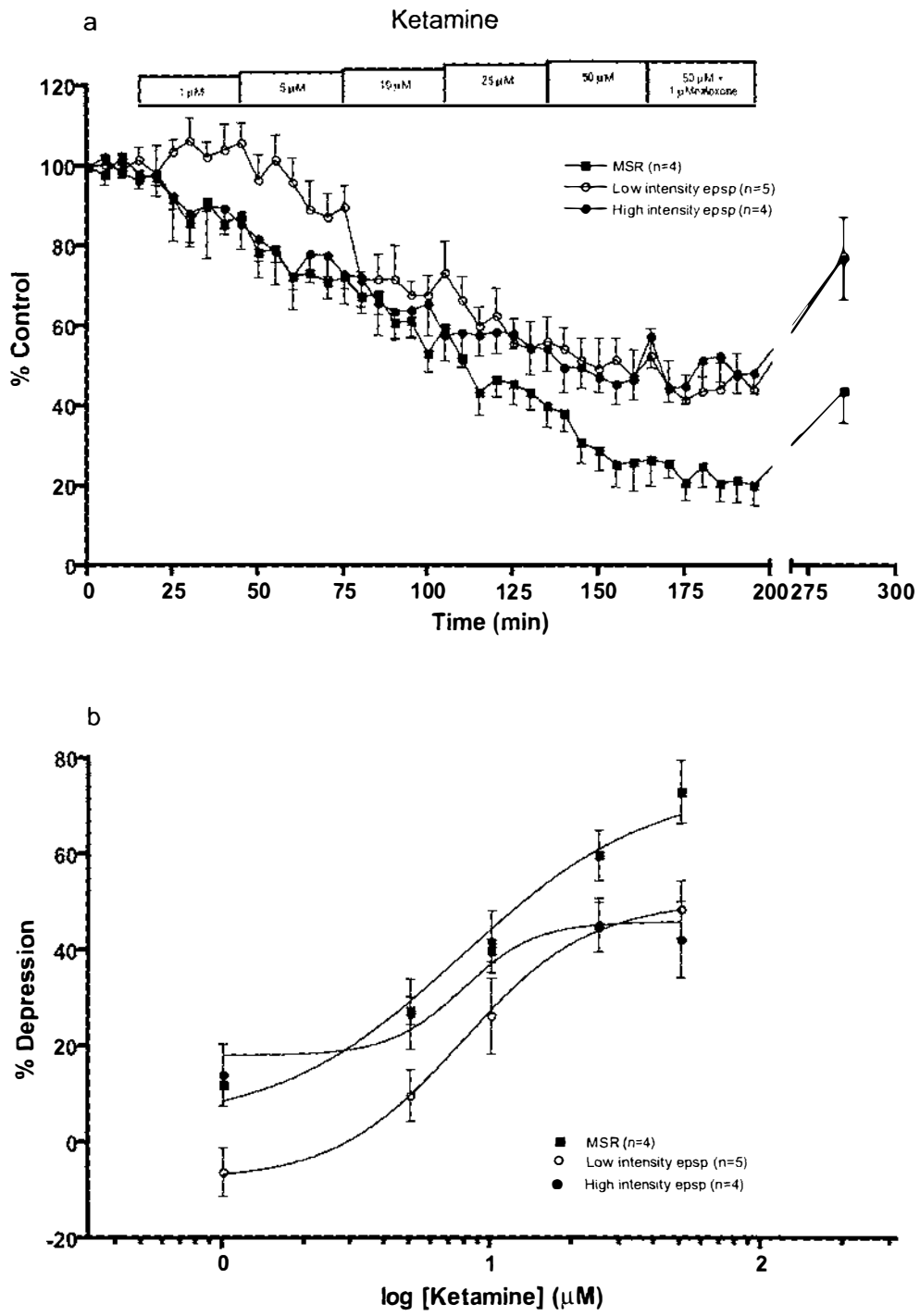

Figure 4.4 *(a) Time course showing the depressant actions of ketamine (1-50* μM*) and the lack of activity of naloxone (1* μM*) to reverse ketamine's depressant effects (mean ± s.e.m). (b) Concentration-effect plot showing the effect of ketamine.* IC_{50} *values (with 95% CI) were 10.8 (5.97 to 19.54)* μM *for the MSR, 8.29 (4.53 to 15.17)* μM *for the low intensity epsp, and 5.35 (3.05 to 9.40)* μM *for the high intensity epsp.*

Effects of L-NAME

After applying increasing concentrations for 45 min for each concentration, L-NAME (1-100 μM) had no significant effect on any of the spinal reflexes (n=4, Figure 4.5a). Construction of dose-response curves for the three spinal reflexes yielded slopes not significantly different to zero ($P \geq 0.348$, F test; Figure 4.5b).

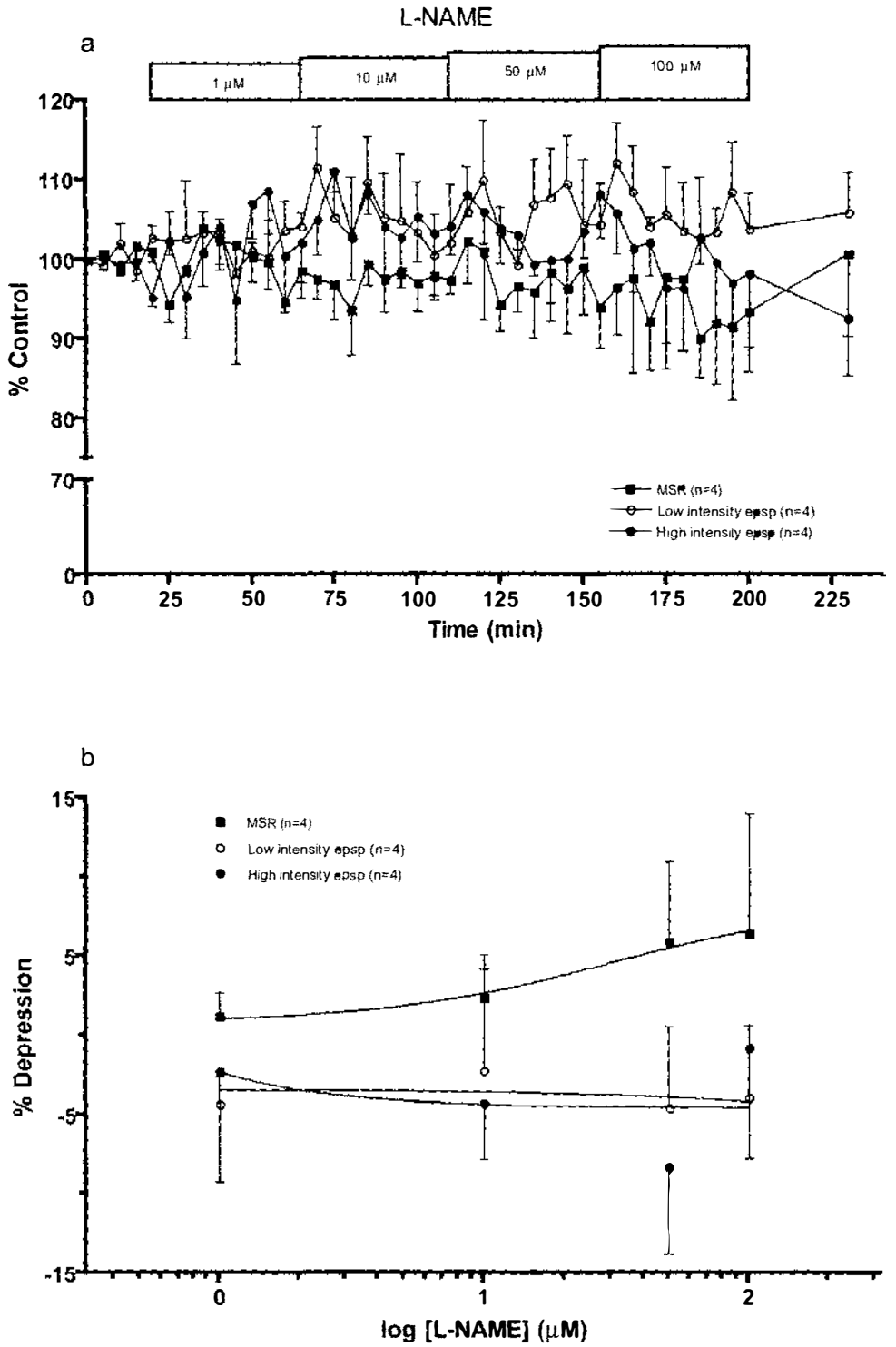

Figure 4.5 *(a) Time course showing the lack of depressive effect of L-NAME (1-100* μM*) on the different components of the synaptic response (mean ± s.e.m). (b) Concentration-effect plot showing the lack of effect of L-NAME.*

Effects of NSAIDs

Application of salicylic acid (1000 μM) for 60 min produced no significant effect on any of the three spinal reflexes (n=4, Figure 4.6). In contrast, ketoprofen, which reached steady state after 35 min (Figure 4.7), depressed both the low intensity epsp and the high intensity epsp, but not the MSR (Figure 4.8a). The IC_{50} values (with 95% CI) were 354.5 (217.5 to 576.8) μM for the low intensity epsp (n=5), and 302.7 (174.0 to 526.7) μM for the high intensity epsp (n=4). As seen in Figure 4.8b, both the low and the high intensity epsps were depressed progressively by increasing concentrations of ketoprofen, and a maximum depressant effect was not attained at the highest concentration tested (600 μM). These IC_{50} values for ketoprofen are therefore an underestimate. There were no significant differences between the IC_{50} values for depression of the low intensity epsp and the high intensity epsp (P=0.8021, F test).

Recovery of both low and high intensity epsps was observed after a 90 min washout period, but the amplitude of the MSR was reduced.

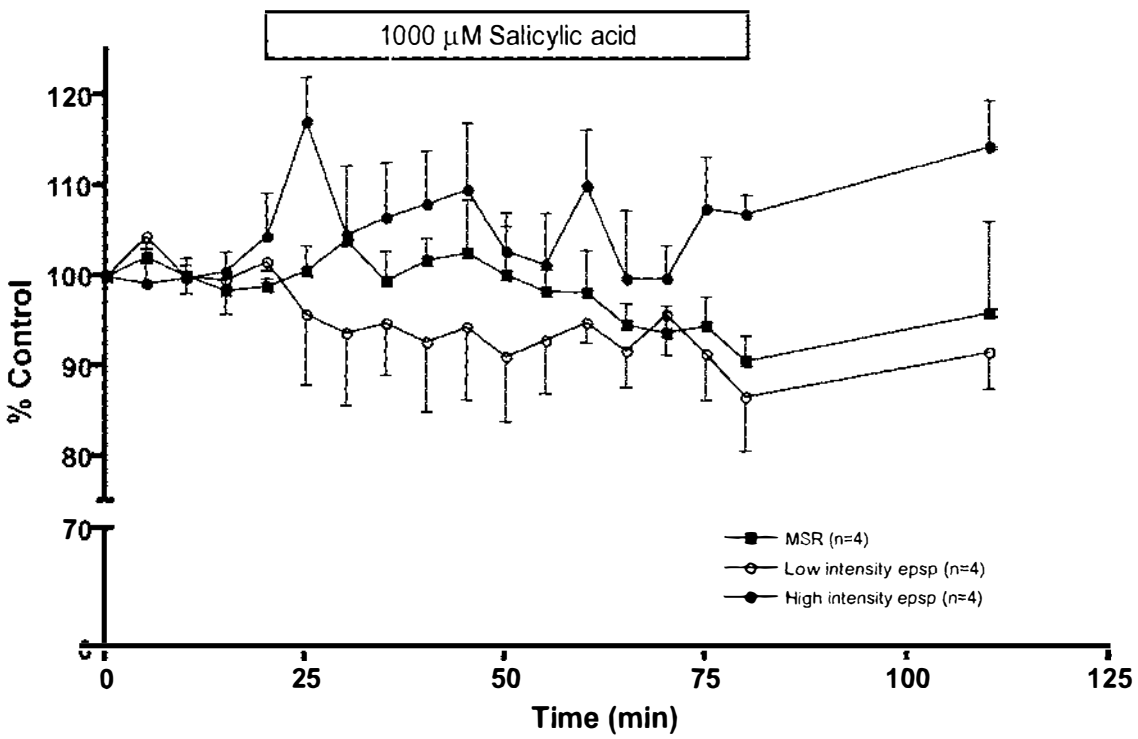

Figure 4.6 *Time course showing the lack of depressive effect of salicylic acid (1000* μM*) on the different components of the synaptic response (mean ± s.e.m).*

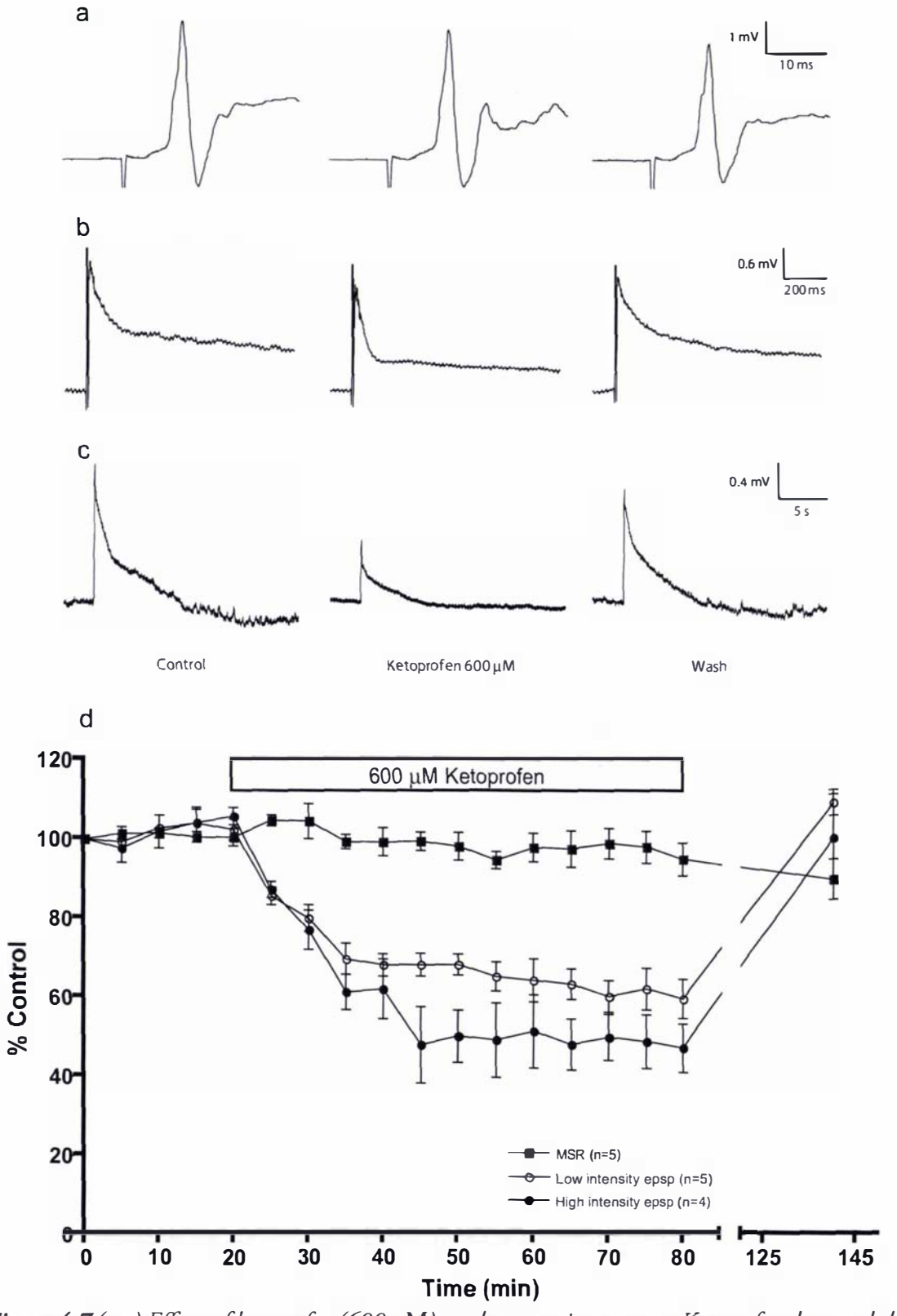

Figure 4.7 *(a-c) Effects of ketoprofen (600 µM) on the synaptic responses. Ketoprofen depressed the low intensity epsp (b) and the high intensity epsp (c), but had no effect on the MSR (a). Recovery is shown in the right panels after 60 min of ketoprofen-free medium. (d) Time course showing the actions of ketoprofen on the different components of the synaptic response (mean ± s.e.m). At the time that reached equilibrium (35 min after infusion), 600 µM ketoprofen depressed the low intensity epsp by 35.00 ± 3.69 % and the high intensity epsp by 51.08 ± 9.38 %, but had a minor depressive effect on the MSR (5.54 ± 2.17 %).*

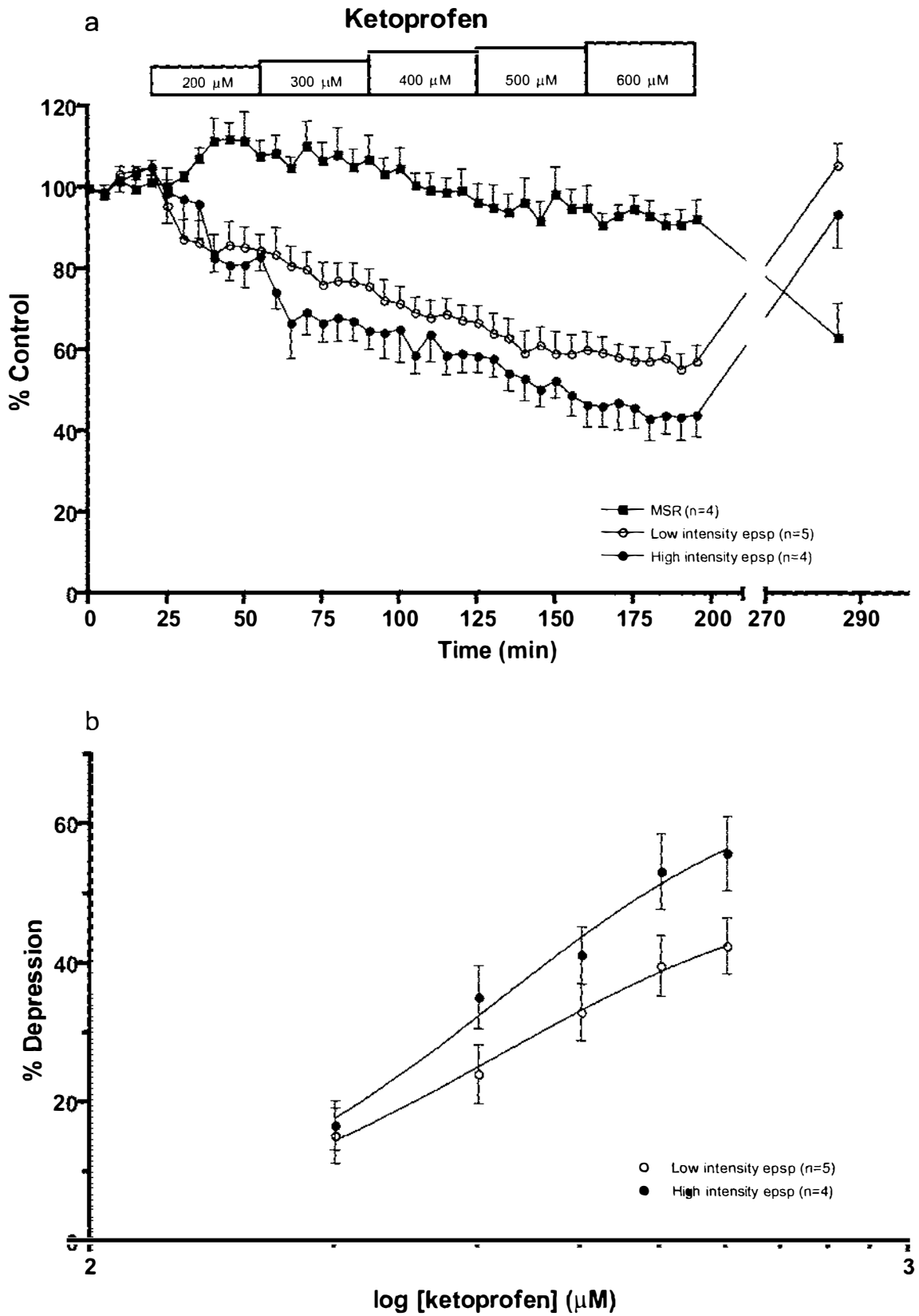

Figure 4.8 *(a) Time course showing the depressant actions of ketoprofen (200-600* μM*) on the synaptic responses (mean ± s.e.m). (b) Concentration-effect plot showing the effect of ketoprofen.* IC_{50} *values (with 95% CI) were 354.2 (217.5 to 576.8)* μM *for the low intensity epsp, and 302.7 (174.0 to 526.7)* μM *for the high intensity epsp.*

Effects of anandamide

Although only tested on two preparations per reflex, perfusion of increasing concentrations of anandamide (0.1-1 μM), for 30 min for each concentration, produced no apparent effect on the low and high intensity epsps. For the MSR, one preparation was slightly depressed and the other increased (Figure 4.9). No dose-response curves were plotted.

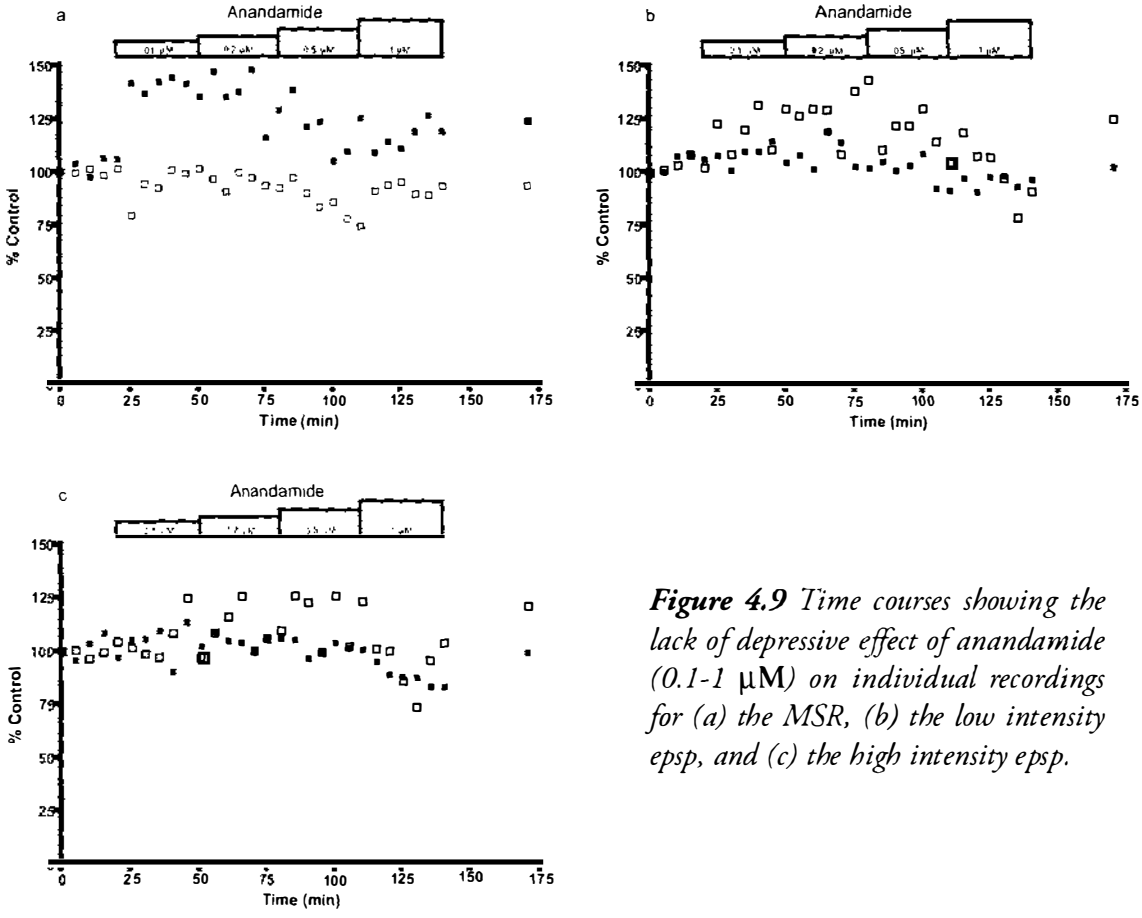

Figure 4.9 *Time courses showing the lack of depressive effect of anandamide (0.1-1 μM) on individual recordings for (a) the MSR, (b) the low intensity epsp, and (c) the high intensity epsp.*

4.5 Discussion

These data demonstrate that the NMDA receptor channel blocker ketamine and the NSAID ketoprofen, but not the NOS inhibitor L-NAME, depressed NMDA-receptor mediated transmission on an *in vitro* neonatal rat spinal cord preparation. This is the first time that an NSAID has been shown to depress spinal nociceptive transmission in this preparation.

As shown with the infusion of aCSF only, several hours of stable recordings were possible from the spinal cord preparation. Long lasting spinal reflexes in this preparation have been regarded as a consequence of C-fibre primary afferent activation (Akagi *et al.,* 1985; Faber *et al.,* 1997). However, a brief train of low A-fibre afferent impulses can evoke a synaptic

response similar in duration to that observed after a single supramaximal stimulus (Faber *et al.,* 1997). Also, several studies using this preparation have not employed stimulus intensities low enough to activate A-fibre primary afferents only (Akagi *et al.,* 1985; Brockmeyer and Kendig, 1995; Kurihara and Yoshioka, 1996). Therefore, all responses may be a combination of A- and C-fibre primary afferent responses. In this study, the voltage threshold of each preparation was tuned and stimulus intensities were applied so that only A-fibre primary afferents were activated to evoke the MSR and the low intensity epsp, whereas C-fibre primary afferents also were activated to evoke the high intensity epsp (see Materials and Methods). The MSR is reported to be non-NMDA dependent (Long *et al.,* 1990), whereas the low intensity epsp and the high intensity epsp are NMDA dependent (Brockmeyer and Kendig, 1995; Thompson *et al.,* 1995; Faber *et al.,* 1997) with the latter also having a neuropeptide component (Akagi *et al.,* 1985).

Effects of ketamine

At the spinal level, ketamine produces no analgesia in models of acute pain (Kawamata *et al.,* 2000; Bulutcu *et al.,* 2002), but reduces allodynia and hyperalgesia in models of inflammatory pain (Chaplan *et al.,* 1997; Kawamata *et al.,* 2000; Bulutcu *et al.,* 2002). This is congruent with ketamine's analgesic action being mediated by blocking the NMDA channel, inhibiting glutamate activation of the channel in a non-competitive manner (Liu *et al.,* 2001).

In an *in vitro* neonatal rat hemisected spinal cord preparation, ketamine 1-50 μM reversibly depressed (~60% maximum depression) the high intensity epsp in a concentration-dependent manner, whereas the MSR was unaffected at these concentrations. It was suggested that ketamine acted as a non-competitive antagonist at NMDA receptors (Brockmeyer and Kendig, 1995). Using the same range of concentrations, a concentration-dependent depressive action (~45% maximum depression) of ketamine on the high intensity epsp was found, but the concentration-effect plot showed a plateau of resistance (Figure 4.4b), which may reflect the significant neuropeptide component of this reflex (Akagi *et al.,* 1985). The concentration-effect plot for the low intensity epsp showed no clear tendency towards a plateau. There was, however, no significant difference on IC_{50} values for depression of the low and the high intensity epsps, suggesting a non-competitive antagonistic effect of ketamine at NMDA receptors. If competitive antagonism had occurred, the high intensity epsp, which presumably causes a maximal release of synaptic glutamate, would have been more resistant than the low intensity epsp, as was demonstrated with the competitive NMDA receptor antagonist CGP40116 (Faber *et al.,* 1997).

Contrary to Brockmeyer and Kendig (1995), a concentration-dependent depressant action of ketamine on the MSR was recorded here (Figure 4.4). This discrepancy could be accounted for by the conditions of stimulation to evoke the MSR. Whereas they evoked this reflex using

supramaximal stimuli (7-40 V) and therefore activating A- and C-fibre primary afferents, stimulus intensities necessary to activate A-fibres only were used in the present study. This paradoxical action also has been observed with the NMDA receptor antagonist AP5, which depressed the MSR when stimulus intensities of less than 2 threshold were applied, but not when supramaximal stimuli of 16 threshold were used (Evans, 1995). These data suggest that the MSR may have an NMDA receptor component, which can be inhibited by NMDA receptor antagonists when the reflex is evoked at low levels of stimulation, or that some NMDA receptor antagonists possess local anaesthetic-like effects. The former hypothesis seems unlikely since the NMDA receptor antagonist CGP40116 did not affect the MSR when evoked after activation of A-fibre primary afferents only (Faber *et al.,* 1997). The latter hypothesis has been demonstrated in the dog spinal cord, where amplitudes of intraspinal evoked potentials were reduced and latencies increased after intravenous (2 and 10 mg/kg) and i.t. (1 mg/kg in 0.5 ml) ketamine administration (Iida *et al.,* 1997). Further support comes from a transient (less than 30 min duration) motor disfunction after each i.t. injection of ketamine (5 mg in 0.5 ml) administered once a day for 14 days to rabbits (Borgbjerg *et al.,* 1994), and from increased tail-flick latencies after i.t. injection of large concentrations of ketamine (280 to 310 mM) in rats (Ahuja, 1983). It is possible that blockade of spinal action potential conduction may be partially responsible for the analgesia induced by i.t. administration of ketamine (Iida *et al.,* 1997). Interestingly, interaction of ketamine with Na^+ channels has been reported (Hirota and Lambert, 1996).

The opioidergic system also has been implicated in the analgesic effects of ketamine. Intraperitoneal (i.p.) injection of naloxone prevented the analgesic effect of i.p. or i.t. ketamine in rats (Smith *et al.,* 1980; Ahuja, 1983). Receptor binding studies have shown that ketamine displaced ^{3}H-naloxone from binding sites on rat brain membranes (IC_{50} = 14.8 μM) (Smith *et al.,* 1980) and ^{3}H-dihydromorphine from binding sites in rat brain homogenates (IC_{50} = 23 μM) (Finck and Ngai, 1982). In mice, ketamine displaced ^{3}H-etorphine from binding sites in the striatum, hippocampus, brain stem and thalamic region (Finck and Ngai, 1982). The interaction with opioid receptors is enantioselective with S(+)-ketamine been about three times more effective than R(-)-ketamine (Smith *et al.,* 1980; Finck and Ngai, 1982). These data suggest that the analgesic effect of ketamine is mediated by central opioidergic mechanisms and that S(+)-ketamine is primarily responsible for this action. In support of this theory is the greater analgesia produced by S(+)-ketamine in wild-type mice compared to μ-opioid receptor knockout mice, and it seemed that analgesia depended on spinal μ-opioid receptors and partially on supraspinal μ-opioid pathways (Sarton *et al.,* 2001). In the current study, however, infusion of naloxone at a concentration sufficient to antagonise the depressant actions of morphine on both the low intensity epsp and the high intensity epsp in an *in vitro* neonatal rat spinal cord preparation (Faber *et al.,* 1997), produced no effect on the depressant action of ketamine on any of the three reflexes studied here (Figure 4.4a).

Though not conclusive, these data suggest that ketamine did not interact with spinal opioid receptors to depress segmental transmission in the cord. A more robust approach to assess the involvement of opioid receptors would have been to compare the effect of ketamine in the absence and presence of naloxone.

After washout of the ketamine for 90 min, recovery of all three reflexes was only partial. There are at least two possible explanations for this: *i)* the preparations were dying, and/or *ii)* ketamine produced a direct toxic effect on the spinal cord. The former explanation seems unlikely since spinal reflexes were very stable for at least 3 h after infusion of aCSF only. It was afterwards that reflexes started to decline consistently but slightly, so that recordings above 80% of the control were usually achieved for over 5 h. All experiments with ketamine lasted less than 5 h, including the washout period (Figure 4.4a).

There is evidence that preservatives cholobutanol and benzethonuim chloride, but not preservative-free ketamine, produce neurotoxic and vosotoxic effects in the spinal cord (Malinovsky *et al.,* 1993; Karpinski *et al.,* 1997; Stotz *et al.,* 1999). However, spinal cord vacuolation has also been observed after i.t. injection of large doses of preservative-free ketamine (1600 μg/kg i.t.) in rats, and 3 out of 33 rats died shortly after injection (Ahuja, 1983). Preservative-free ketamine was used in this study and a toxic effect of the drug seems unlikely. Moreover, at least 80% recovery in all three reflexes was attained in preliminary experiments in which 50 μM ketamine was infused for 40 min (Figure 4.3), and recovery was also achieved after infusion of a single concentration of ketamine (1-50 μM) for 30 min (Brockmeyer and Kendig, 1995). However, it cannot be ruled out that chronic administration of ketamine could produce spinal toxicity.

Effects of L-NAME

The role of NO in spinal nociception is not completely understood. There is agreement that NOS inhibitors produce analgesia in several models of inflammatory and neuropathic pain. This has been attributed to a spinal action (Dolan and Nolan, 1999; Larson *et al.,* 2000; Park *et al.,* 2000). However, assessment of NOS inhibitors on *in vitro* neonatal rat spinal cord preparations has yielded inconsistent results. On the one hand, both the NOS inhibitor L-NAME (30-1000 nM) and the NO scavenger carboxy-PTIO (100-200 μM) increased the area under the curve of the high intensity epsp, suggesting that NO may exert an inhibitory influence in the cord (Kurihara and Yoshioka, 1996). On the other hand, the NOS inhibitors 7-nitroindazole (30 μM) and L-NAME (100 μM), and the NO donor sodium nitroprusside (50 μM) had no effect on the high intensity epsp, suggesting lack of participation of NO in spinal nociception (Thompson *et al.,* 1995). Results with L-NAME (1-100 μM) in the current study agree with the latter observations. The discrepancy between studies could not be accounted for by differences in the concentration of L-NAME since a range of concentrations

that cover the highest concentrations administered in the other two studies was used here. The discrepancy, however, could be attributed to possible postnatal changes in NO-mediated spinal responses. Increase of the high intensity epsp response was observed in 0- to 2-day-old rats (Kurihara and Yoshioka, 1996), whereas lack of effect was seen in older animals: 5- to 7-day-old rats in the current study and 10- to 12-day-old animals in the other study (Thompson *et al.,* 1995).

Lack of effect of L-NAME (1-100 μM) on both A-fibre-mediated reflexes was also found here, which agrees with the unresponsiveness of the MSR to both NO gas-containing medium and NO donors (Kurihara and Yoshioka, 1996). However, in this last study, the MSR was evoked at stimulus intensities that may have recruited C-fibre primary afferents.

The possibility that L-NAME blocks nociceptive spinal transmission in hyperalgesic animals but not in naïve ones has already been challenged (Thompson *et al.,* 1995). NOS inhibitors and a NO donor did not affect the high intensity epsp or the NMDA-induced ventral root depolarisations in *in vitro* neonatal rat spinal cord preparations from either animal. In age matched rats, L-NAME (30 mg/kg i.p.) significantly increased hindpaw withdrawal latencies from a radiant heat stimulus in both naïve and hyperalgesic rats. These data suggest that antinociceptive effects of NOS inhibitors do not result from a direct action on spinal neurones, a hypothesis supported by the current findings. Although these studies did not demonstrate differences in righting reflexes between naïve and hyperalgesic rats, they did not assess blood pressure in these animals. It is possible that reduced peripheral blood flow after systemic administration of L-NAME may have contributed to the analgesic effect. Instead, the authors proposed a supraspinal site of action and local regulation of spinal blood flow as possible explanations for the analgesic effect of NOS inhibitors (Thompson *et al.,* 1995).

Effects of NSAIDs

In situ electrophysiological experiments have demonstrated a central antinociceptive effect after intravenous administration of different NSAIDs, including ketoprofen, in anaesthetised rats, but this route of administration does not rule out the possibility that these drugs acted on supraspinal sites (Willingale *et al.,* 1997; Herrero *et al.,* 1997). In fact, an indirect spinal analgesic action of intravenous ketoprofen in sheep mediated by descending opioidergic and α_2-adrenergic mechanisms has been identified (Lizarraga and Chambers, 2006). Using the same electrophysiological methodology but subdural drug administration, Willingale *et al.* (1997) proposed that indomethacin (100 μM, 50 μl) had an antinociceptive spinal effect. However, it can be argued that spread of the drug to supraspinal sites or even systemic absorption after administration of a rather large volume (50 μl) could have contributed to the reported depressive effects. It is also possible that the anaesthetic (sodium thiobutabarbitone) could have interacted with indomethacin in this study. Since *in vitro* spinal cord preparations

were obtained after cervical dislocation and decapitation, here any supraspinal components were obviated and the confounding effects of other dugs avoided. Inhibition of both the low and the high intensity epsps in a concentration-dependent, reversible manner, without affecting the MSR as seen in this study (Figure 4.8), demonstrates for the first time a true spinal antinociceptive effect for ketoprofen. This is the first time that this has been demonstrated for any NSAID.

Antihyperalgesia after i.t. delivery of NSAIDs is well documented in experimental animals (Malmberg and Yaksh, 1992; Dolan and Nolan, 1999; Park *et al.,* 2000) and there is anecdotal evidence of their efficacy against cancer pain (Devoghel, 1983). The most accepted mechanism of spinal antinociceptive action for NSAIDs is inhibition of COX enzymes and therefore reduction of prostaglandin production (Svensson and Yaksh, 2002). Prostaglandins and all the machinery necessary for their synthesis are present in the spinal cord (Willingale *et al.,* 1997), where their release can be increased by activation of high-threshold nociceptive afferent inputs (Ramwell *et al.,* 1966). Ketoprofen (200-600 μM) was infused at concentrations high enough to inhibit the synthesis of prostaglandins (reported IC_{50} 0.047 μM and 2.9 μM for COX-1 and COX-2, respectively) (Warner *et al.,* 1999), but the calculated IC_{50} values for both the low and the high intensity epsps were above 300 μM. It is possible, therefore, that mechanisms other than, or complentary to, prostaglandin synthesis inhibition may have mediated the depressive effects of ketoprofen in the spinal cord. Several other spinal analgesic mechanisims of action for NSAIDs have been proposed (McCormack, 1994).

Recent studies have shown that spinal analgesic actions for some NSAIDs are mediated by endocannabinoid mechanisms (Gühring *et al.,* 2002; Ates *et al.,* 2003). In an attempt to investigate the role of the endocannabinoid system on ketoprofen-induced antinociception, anandamide was perfused onto hemisected spinal cord preparations. The reasoning behind this was that anandamide might depress nociceptive transmission as has been shown in spinal cord neurones from juvenile rats (Morisset and Urban, 2001). In a second set of experiments, the depressive action of anandamide would be challenged together with the cannabinoid$_1$ receptor antagonist AM251, which is known to prevent the antihyperalgesic action of anandamide in rats submitted to a paw formalin test (Gühring *et al.,* 2002). Once the effects of AM251 alone would have been assessed, it would be infused together with ketoprofen to determine a possible cannabinoidmimetic effect of the NSAID. In the few preparations tested, however, anandamide had no obvious depressive effects on any of the spinal reflexes. It is unlikely that the concentrations used in this study were too low to depress spinal synaptic transmission since electrophysiological studies using spinal cord slices from 15- to 25-day old rats have shown that anandamide (0.5-1 μM) inhibited miniature excitatory postsynaptic currents in lamina II neurones (Morisset and Urban, 2001). The lack of effect may be due to adsorption of the drug by polyurethane. Karlsson *et al.* (2004) demonstrated that anandamide

(200 µl, nominal assay concentration 10 µM) was adsorbed by about 50% to plastic cell culture wells (Karlsson *et al.*, 2004). Anandamide solution was stocked in 1 ml polyurethane vials and was pumped through 60 cm long polyurethane tubes. Although these studies were abandoned due to time constrains, further studies should consider non-plastic storage and infusion systems.

In contrast to ketoprofen, salicylic acid did not depress any of the spinal reflexes. Salicylic acid was infused at a lower concentration than the reported IC_{50} for inhibition of COX-1 and COX-2 (4956 µM and 34440 µM, respectively) (Warner *et al.*, 1999), which precluded us from drawing any conclusions regarding the role of COX enzymes in nociceptive transmission on the neonatal rat spinal cord. Infusion of higher concentrations of salicylic acid would have been a better strategy to assess their role.

In conclusion, the studies reported here have shown that ketamine produced concentration-dependent inhibitory effects on spinal nociceptive and non-nociceptive transmission possibly mediated by a non-competitive antagonism at the NMDA receptor and by a local anaesthetic effect. In contrast, L-NAME produced no effect on all three synaptic reflexes supporting the hypothesis that spinal NO may not play a direct role on nociceptive transmission in the *in vitro* neonatal spinal cord preparation (Thompson *et al.*, 1995). They also have demonstrate for the first time that the NSAID ketoprofen produced a direct depressive effect on spinal nociceptive transmission, though the mechanism of this effect remains to be elucidated.

4.6 References

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5. Synergistic depression of nociceptive segmental reflexes by ketamine and ketoprofen on the *in vitro* neonatal rat spinal cord preparation

5.1 Abstract

Spinal *N*-methyl-D-aspartate (NMDA) receptors play a major role in nociceptive processing and prostaglandins (PGs) are a key contributor to such an effect. Despite possible synergistic analgesic effects, drug synergism studies have not been carried out between drugs that inhibit these two systems. Using isobolographic and composite additive line analyses, the depression of synaptic reflexes from an *in vitro* neonatal rat spinal cord preparation by the NMDA receptor channel blocker ketamine together with the PG synthesis inhibitor ketoprofen were assessed. Two A-fibre-mediated reflexes, the monosynaptic reflex (MSR) and the low intensity excitatory postsynaptic potential (epsp), and one C-fibre-mediated reflex, the high intensity epsp, were evoked electrically. Infusion of ketamine alone decreased all three reflexes, whereas that of ketoprofen alone decreased both the low intensity epsp and the high intensity epsp only. Co-infusion of fixed ratios of IC_{40} fractions of ketamine and ketoprofen were synergistic for depression of both the low intensity epsp and the high intensity epsp. In contrast, the combined depressive effect of ketamine and ketoprofen on the MSR was sub-additive. These results suggest that co-administration of ketamine and ketoprofen resulted in a synergistic depression of nociceptive spinal transmission, with potentially decreased motor side effects. This interaction could carry important clinical implications.

5.2 Introduction

Although many methods and drugs are available for pain therapy, the search for more effective ways of relieving pain continues. As a vast number or receptor systems and pathways are involved in the nociceptive processing of information (Dickenson, 1995), intuitively it would be more effective to influence them simultaneously. Spinal administration of drug combinations that act at different nociceptive receptor systems may be an adequate way to overcome some of the limitations of conventional therapies. Analgesic combinations should, however, satisfy two important pharmacodynamic criteria: the drugs in the combination should display additive or synergistic antinociception and fewer side effects (Yaksh and Malmberg, 1994).

Currently, subdural administration of opioid agonists is a common procedure to control some types of pain, but the side effects and limitations of spinally administered opioid analgesics (*e.g.*

delayed respiratory depression, pruritus) (Channey, 1995) confirm the need for alternatives. It is widely accepted that spinal *N*-methyl-D-aspartate (NMDA) receptors play a key role in nociception (Bennett, 2000). Intrathecal (i.t.) administration of NMDA receptor antagonists reduces spinal hyper-excitability (Neugebauer *et al.,* 1993) and induces antinociception in a number of animal pain models (Malmberg and Yaksh, 1992; Dolan and Nolan, 1999; Yaksh *et al.,* 2001). Analgesia has also been reported following i.t. administration of the NMDA receptor channel blocker ketamine in human beings (Benrath *et al.,* 2005). Although relatively safe, i.t. administration of ketamine can produce unwanted side-effects such as motor block and acute psychotic alterations (Stotz *et al.,* 1999; Hawksworth and Serpell, 1998; Lauretti *et al.,* 2001). These side effects may arise from the fact that NMDA receptors are widespread in the central nervous system and are involved in many physiological processes (Dingledine *et al.,* 1999). This may limit the use of i.t. ketamine as monotheraphy for the treatment of pain.

Activation of spinal NMDA receptors can trigger the arachidonic acid (AA) / cyclooxygenase (COX) / prostaglandin (PG) signalling pathways and induce allodynia and hyperalgesia (Svensson and Yaksh, 2002). The i.t. administration of non-steroidal anti-inflammatory drugs (NSAIDs, which inhibit COX enzymes) reduce the hyper-excitability of spinal dorsal horn neurnones (Willingale *et al.,* 1997) and inhibit the hyperalgesic response after i.t. NMDA administration (Malmberg and Yaksh, 1992; Dolan and Nolan, 1999; Yaksh *et al.,* 2001). Case reports also suggest that i.t. injection of NSAIDs produce analgesia in humans (Devoghel, 1983). The i.t. route of administration for these drugs has recently entered clinical trials (Eisenach *et al.,* 2002).

Since NMDA receptor antagonists and NSAIDs are likely to have overlapping influences in the intracellular events that subserve spinal nociceptive transmission, a spinal antinociceptive interaction between these two classes of compounds remains a strong possibility. In fact, the analgesic effects of several NSAIDs were increased by a non-analgesic dose of the NMDA receptor antagonist dextromethorphan in a rat model of arthritic pain (Price *et al.,* 1996). However, double-blinded, placebo-controlled, randomised trials showed that pain relief associated with dextromethorphan and NSAID treatment was not superior to that obtained with an equal dose of dextromethorphan or the NSAID alone in patients scheduled for surgical termination of pregnancy (Ilkjær *et al.,* 2000) or elective laparoscopic cholecystectomy (Yeh *et al.,* 2004). These studies did not use a fixed-ratio combination design and no isobolar analysis could be carried out. This precluded the characterisation of the drug interaction rendering the findings suggestive, but inconclusive.

The current study examined the interaction between ketamine and the NSAID ketoprofen in nociceptive segmental reflexes using an *in vitro* neonatal rat spinal cord preparation. A fixed-

ratio combination design was used to create an additive composite line, which was compared to the actual combination line having the same proportions. It was hypothesised that both drugs would act synergistically to depress spinal nociceptive transmission. Preliminary results have been published in abstract form (Lizarraga *et al.*, 2004).

5.3 Material and methods

Spinal cord preparation

Experiments were carried out as previously described (Faber *et al.*, 1997). Briefly, spinal cords with attached dorsal and ventral roots were harvested from unsexed, 5- to 7-day old Sprague-Dawley rats. The spinal cords were hemisected sagittally and placed in a chamber with L4 or L5 dorsal root in contact with the stimulating electrode and the corresponding ventral root in contact with the recording electrode. The hemicords were perfused (2 ml/min) with artificial cerebrospinal fluid (aCSF) of the following composition (mM): NaCl, 118; $NaHCO_3$, 24; glucose, 12; $CaCl_2$, 1.5; KCl, 3; and $MgSO_4 7H_2O$, 1.25. The aCSF was gassed with 95% O_2 and 5% CO_2, and was kept at room temperature.

Recording techniques

Electrical stimulation of the dorsal root with single 0.5 ms square-wave pulses at 3 times threshold (threshold being the intensity at which a discernible response first appeared in the ventral root) produced two A-fibre-mediated synaptic responses: the monosynaptic compound action potential (MSR) superimposed on a population excitatory postsynaptic potential (epsp), the low intensity epsp, which lasts up to two seconds (Faber *et al.*, 1997). Stimulation at 32 times threshold evoked an additional C-fibre-mediated response, the high intensity epsp, which lasts up to tens of seconds (Akagi *et al.*, 1985; Faber *et al.*, 1997). Responses were amplified (AA6 Mk III, Medelec MS6 system, Medelec Ltd., Woking, Surrey, England), transferred to a PowerLab 4/20 (ADInstruments Pty Ltd, Castle Hill, Australia), and digitally stored for off-line analysis (Scope v3.6.8, MacLab® System© 1998 ADInstruments Pty Ltd, Castle Hill, Australia).

Responses were recorded every 5 min and after obtaining at least three baseline readings, cumulative concentrations of ketamine (1-50 μM) and ketoprofen (200-600 μM) were applied to the preparations by adding them to the superfusate. Each ketamine concentration was infused for 30 min and those of ketoprofen for 35 min, which were the times required for each drug to reach equilibrium (Chapter 4). The concentration range for ketamine was selected from previous studies using a neonatal rat spinal cord preparation (Brockmeyer and Kendig, 1995), and that for ketoprofen as being one order of magnitude less than that required for spinal inhibition of a C-fibre-evoked reflex in adult rats (Bustamante *et al.*, 1997).

The composite additive line

For calculation of concentration responses, the peak amplitude of the MSR and the area under the curve of both the low intensity epsp and the high intensity epsp were converted to percentage maximal possible effect (%MPE) according to the formula: %MPE = 100 - [(post-drug effect/baseline effect) x 100]. Linear regression analysis of the log concentration-effect data (4-5 preparations per reflex, at least 4 concentrations) was performed using GraphPad Prism (v4.0b for Macintosh, GraphPad Software Inc., San Diego, CA, USA). After assessing the regression lines for parallelism (F test, GraphPad Prism), an effect level that each drug alone was capable of achieving (concentration to achieve 40% MPE; IC_{40}) with 95% confidence intervals (CI) were determined, and the individual concentration-response data were used to construct the composite additive line (Tallarida *et al.*, 1997). This used a fixed ratio combination in which the constituents were infused in amounts that kept the proportions of each constant and were tested experimentally. The fixed ratio combination was arbitrarily determined by taking 0.5-fractions of the IC_{40} of each compound, and then adding them together.

For interactions involving the MSR, the method described by Porreca *et al.* (1990), in which one drug lacks efficacy, was conducted. In this case, 0.5-fractions of the IC_{40} of the active drug were combined with the inactive drug in a fixed ratio combination, which proportion was arbitrarily chosen.

Synergism was defined as the effect of a drug combination being higher and statistically different (IC_{40} significantly less) than the theoretical calculated equieffect of a drug combination with the same proportions. When the drug combination gave an experimental IC_{40} not statistically different from the theoretical calculated IC_{40}, the combination was deemed to have an additive effect. Additivity meant that each constituent contributed to the effect in accord with its own potency, and the less potent drug was merely acting as a diluted form of the other (Tallarida, 2000).

Drugs

Ketamine was dissolved in distilled water and ketoprofen in distilled water and NaOH (no more than 672 μM, final concentration). Stock solutions of both drugs were kept at 4°C. Drugs were obtained from Sigma (St. Louis, MO, USA).

Statistics

Results were presented as IC_{40} values with 95% CI. IC_{40} values for the composite additive line and the actual combination line were compared using the Student's t-test. To assess whether synergism extended to other concentration levels, the composite additive line and the actual combination line were compared using the F-distribution test (Tallarida, 2000). A *P* value

less than 0.05 was regarded as significant.

5.4 Results

Single drug studies

Cumulative increases in ketamine concentrations decreased all three types of reflexes, whereas keoprofen decreased both the low intensity epsp and the high intensity epsp, with very minor efficacy on the MSR (maximum depression of 7.42±4.61%, slope significantly different to zero, $P = 0.0097$, F test). IC_{40} values with 95% CI for each drug for each reflex response are shown in Table 5.1.

Table 5.1 *IC_{40} values with 95% confidence intervals (CI) for the depressive effect of ketamine (1-50 μM) and ketoprofen (200-600 μM) on the three evoked spinal segmental responses.*

Segmental response	IC_{40} values and 95% CI (μM)	
	Ketamine	Ketoprofen
MSR	7.75 (5.94 to 10.00)	N/E*
Low intensity epsp	24.85 (16.78 to 42.80)	528.64 (452.85 to 683.19)
High intensity epsp	18.63 (8.62 to 79.87)	368.55 (326.72 to 414.85)

* N/E = no effect.

Ketamine plus ketoprofen

The concentration-response lines obtained with ketamine and ketoprofen alone were not parallel for both the low intensity epsp and the high intensity epsp ($P > 0.05$, F test), suggesting that the relative potency of both drugs was not constant. Considering this, individual concentration-response data were used to construct the composite additive lines (Figures 5.1a and 5.2a). These lines provide a view of the total log concentration-effect relation over the entire range of concentrations used. The effect level 40% MPE is illustrated as a horizontal dashed line that intersects to give the log IC_{40} concentrations of the additive lines. The IC_{40} values with 95% CI were acquired from linear regression analysis and are shown in Table 5.2.

Co-infusion of fixed ratios of 0.5 fractions of the IC_{40} of ketamine and ketoprofen resulted in a synergistic depressive effect at the level of 40% reduction of the control response on both the low intensity epsp ($P < 0.01$; Figure 5.1a) and the high intensity epsp ($P < 0.05$; Figure 5.2a) The theorethical and experimental IC_{40} values with 95% CI are given in Table

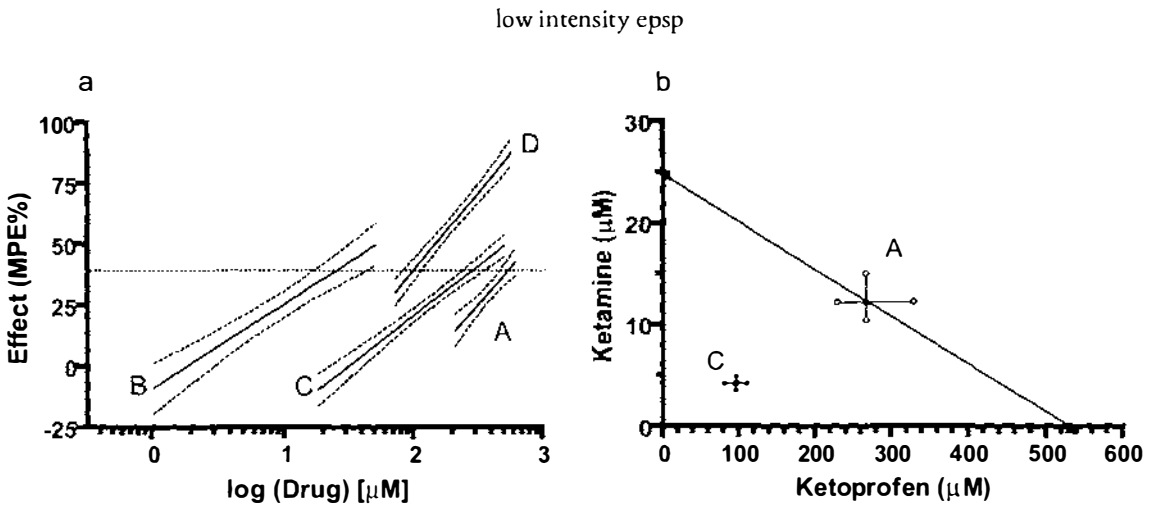

Figure 5.1 *(a) Percent maximal possible effect (MPE%) of ketoprofen alone (A), ketamine alone (B), ketoprofen and ketamine in a theoretical additive fixed ratio proportion (0.955 : 0.045) in which the quantities of the constituents are in proportion to their respective IC_{40} values (C), and the actual combination of ketoprofen and ketamine having the same proportion (D) for the low intensity epsp. The horizontal dashed line represents the effect level 40% MPE. (b) Isobologram for low intensity epsp at the effect level 40% MPE for the combinations ketoprofen and ketamine in a fixed ratio proportion as indicated above. The solid line is the line of additivity and contains point A representing the calculated additivity quantities for this proportional combination. Point C is the combination point determined experimentally with this same proportional mix. Coordinates of point A are [(264.32 (223.72 - 327.80)* μM, *12.43 (10.52 - 15.41)* μM*] and those of point C [(93.23 (77.48 - 108.27)* μM, *4.38 (3.64 - 5.09)* μM*].*

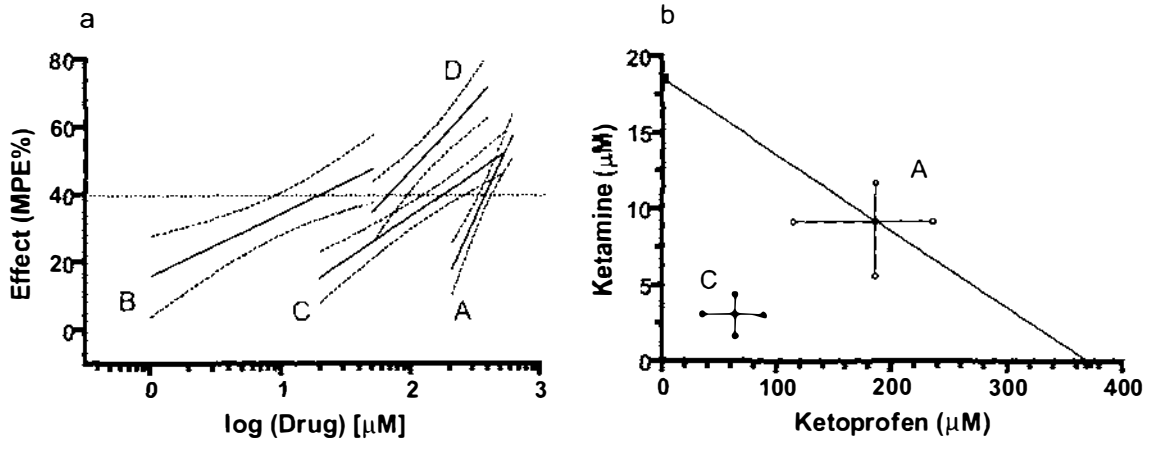

Figure 5.2 *(a) Percent maximal possible effect (MPE%) of ketoprofen alone (A), ketamine alone (B), ketoprofen and ketamine in a theoretical additive fixed ratio proportion (0.952 : 0.048) in which the quantities of the constituents are in proportion to their respective IC_{40} values (C), and the actual combination of ketoprofen and ketamine having the same proportion (D) for the high intensity epsp. The horizontal dashed line represents the effect level 40% MPE. (b) Isobologram for the high epsp at the effect level 40% MPE for the combinations ketoprofen and ketamine in a fixed ratio proportion as indicated above. The solid line is the line of additivity and contains point A representing the calculated additivity quantities for this proportional combination. Point C is the combination point determined experimentally with this same proportional mix. Coordinates of point A are [184.28 (113.10 - 234.88)* μM, *9.31 (5.72 - 11.86)* μM*] and those of point C [61.61 (33.13 - 86.96)* μM, *3.11 (1.68 - 4.40)* μM*].*

5.2, and are also displayed in the isobolograms of Figure 5.1b and 2b. To test if synergism extended to other concentration levels, the composite additive lines and their respective actual combination lines were compared. For the low intensity epsp, both lines were significantly different ($P < 0.01$, F-distribution test), and as shown in Figure 5.1a, synergism was observed over the entire concentration range studied. For the high intensity epsp, the composite additive line and its respective actual combination line were also significantly different ($P < 0.01$, F-distribution test), but synergism might be dependant on the total concentration on this fixed proportion combination, and might not be observed at lesser effect levels than 40% reduction of the control response (Figure 5.2a).

Table 5.2 *Theoretical and experimental IC_{40} values with 95% confidence intervals (CI) for combinations of ketoprofen and ketamine in a fixed drug proportion on the three evoked spinal segmental responses.*[*]

Segmental response	IC_{40} values and 95% CI (μM)	
	Theoretical	Experimental
MSR	172.24 (131.97 to 222.18)	5407.67 (1114.01 to 3.82 e+20)[‡]
Low intensity epsp	276.75 (234.23 to 343.21)	97.62 (81.12 to 113.63)[‡]
High intensity epsp	193.6 (118.82 to 246.75)	64.72 (34.81 to 91.36)[†]

[*] *Ketoprofen : ketamine proportions were 0.955 : 0.045 for the MSR and the low intensity epsp, and 0.958 : 0.042 for the high intensity epsp.*
[†] *$P < 0.05$ and [‡] $P < 0.01$ between theoretical and experimental values.*

For the MSR, on which ketoprofen had very minor efficacy when infused alone, the same ketoprofen : ketamine proportion used for the low intensity epsp (0.955 : 0.045) was chosen arbitrarily to calculate the additive IC_{40} value with its corresponding 95% CI (Table 5.2). Administration of ketoprofen and ketamine in amounts that kept the proportions of each constant was not sufficient to achieve a 40% reduction of the control response (mean ± s.e.m. maximum reduction of 25.92 ± 8.25%). The estimated mean IC_{40} value and 95% CI of the actual mix were significantly higher than those of the corresponding additive mixture with these proportions, which suggests a sub-additive effect (Figure 5.3a, Table 5.2). In this case the theoretical line of additivity in the isobologram was horizontal (Figure 5.3b). Although no composite additive line was constructed, comparison of the actual combination line with the concentration-response line of ketamine alone showed that both lines were significantly different ($P < 0.01$, F-distribution test). Figure 5.3a suggests that sub-additivity extended over the entire concentration range studied.

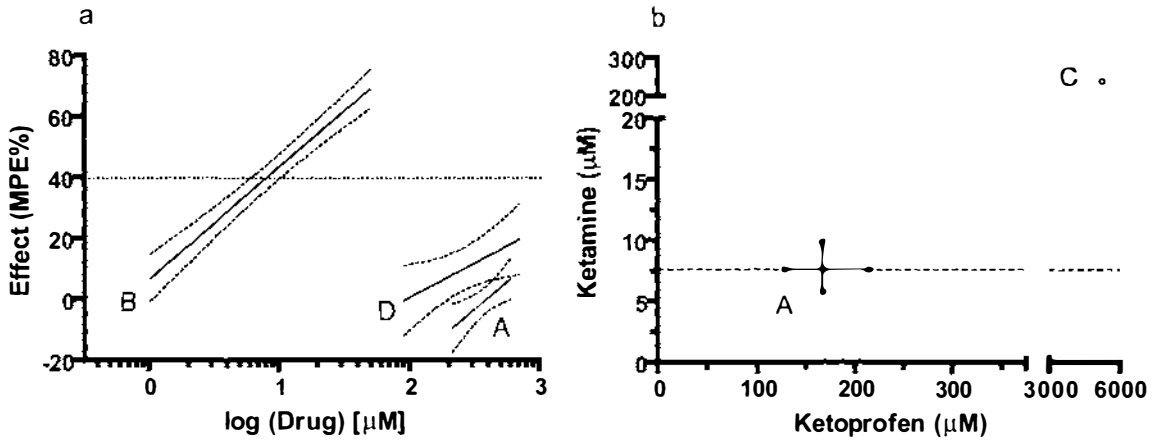

Figure 5.3 *(a) Percent maximal possible effect (MPE%) of ketoprofen alone (A), ketamine alone (B), and the actual combination of ketoprofen and ketamine in a fixed ratio proportion (0.955 : 0.045) in which the quantities of ketamine are in 0.5 fractions to its respective* IC_{40} *value (D) for the MSR. The horizontal dashed line represents the effect level 40% MPE. (b) Isobologram for MSR at the effect level 40% MPE for the combinations ketoprofen and ketamine in a fixed ratio proportion as indicated above. The dashed line is the line of additivity and contains point A representing the calculated additivity quantities for this proportional combination. Point C is the combination point determined experimentally with this same proportional mix. Coordinates of point A are [(164.49 (126.03 - 212.18)* μM, *7.75 (9.94 - 10.00)* μM*] and those of point C (5164.32* μM, *243.35* μM*; CI were too wide and hence not included).*

5.5 Discussion

The present study examined the nature of the interaction between ketamine and ketoprofen on spinal nociceptive reflexes. The results demonstrated a synergistic antinociceptive interaction, which may allow lower doses of drugs, with reduced potential side effects, to be used.

The fact that both ketamine and ketoprofen alone reduced the low intensity epsp and the high intensity epsp, which have NMDA components (Brockmeyer and Kendig, 1995; Faber *et al.,* 1997), may indicate that they have an effect on NMDA receptor-mediated transmission. This was expected for ketamine since it is a non-competitive NMDA receptor channel blocker and, similar to other NMDA receptor antagonists (Faber *et al.,* 1997), has been shown to reduce the high intensity epsp (Brockmeyer and Kendig, 1995). The depressive effects of ketoprofen alone were also expected since NSAIDs are capable of preventing NMDA-induced nociception (Malmberg and Yaksh, 1992; Dolan and Nolan, 1999; Yaksh *et al.,* 2001).

Few studies have addressed the analgesic interaction between NMDA receptor antagonists and NSAIDs. Until now, the most detailed study was that in which the analgesic effects of several NSAIDs, including ibuprofen, naproxen, piroxicam, etodolac, diclofenac and ketorolac, were increased by a non-analgesic dose of the putative NMDA receptor antagonist

dextromethorphan in a rat model of arthritic pain (Price *et al.,* 1996). The authors concluded that the interaction was synergistic despite not using isobolographic analysis, justifying that such analysis was not carried out due to the lack of analgesic efficacy of dextromethorphan alone. However, isobolographic analysis in which one of the two drugs of interest lacks efficacy alone can be performed (Porreca *et al.,* 1990), as was done here for the MSR. This highlights the lack of isobolographic analysis in the literature and does not rule out the possibility of additive, but not detectable, effects between these compounds. Another drawback of the dextromethorphan study was the uncertainty of the location of the suggested synergistic effect (*i.e.* peripheral, central or both), since drugs were administered systemically (i.e. orally and intraperitoneally). Dextromethorphan undergoes extensive first-pass metabolism (Wills and Martin, 1988) and the parent drug binds with high affinity to σ_1 receptors in comparison to the phencyclidine binding site in the NMDA receptor (K_i = 419 nM vs 3500 nM, respectively), whereas its metabolite dextrorphan binds with similar affinity to both receptors (K_i = 559 nM vs 460 nM, respectively) (Newman *et al.,* 1996). Hence, the effects of dextromethophan or even dextrorphan cannot be solely attributed to blockade of the NMDA receptor ion pore.

The interactions between ketamine and ketoprofen described in this study occurred at the level of the spinal cord. Using statistical methods that were developed for the analysis of drug-drug interactions (Tallarida *et al.,* 1997), current data demonstrated that this interaction was not additive, but rather synergistic for the depression of both the low intensity epsp and the high intensity epsp. By decreasing the concentrations of ketamine and ketoprofen, an equal depressive effect (40% MPE) was obtained than with higher concentrations of each drug alone. In fact, only about one third of the calculated concentrations were necessary to achieve 40% MPE in the actual experiments. The computed 95% CI for the IC_{40} values of the composite additive line and the experimental line did not overlap suggesting synergism, which was corroborated statistically (Figures 5.1b and 5.2b, Table 5.2). Construction of composite additive lines allowed for comparison of drug combinations over the range of effects. In both cases, the synergistic depressive effect of the combinations seemed to extend to other concentration levels. For the low intensity epsp, synergism was observed over the entire concentration range studied (Figure 5.1a), whereas for the high intensity epsp synergism may be dependant on the total concentration on its fixed proportion combination, and may not be observed at lesser effect levels than 40% reduction of the control response (Figure 5.2a). Thus, synergism depended on the fixed ratio combination and the total concentration in the combination.

These spinal antinociceptive synergistic interactions between ketamine and ketoprofen could have important clinical implications. This is especially so if the side effects of these drugs do not overlap or do not facilitate each other. Interestingly, the depressive actions of ketamine

alone on the MSR were significantly reduced when combined with ketoprofen (Figure 5.3b, Table 5.2). The MSR is dependent on AMPA and kainate receptor activation (Long *et al.*, 1990), and depression of this reflex may be interpreted as a local anaesthetic effect. In fact, ketamine is known to produce local anaesthetic effects, manifested as motor impairment when administered by the i.t. route (Iida *et al.*, 1997). Thus, the sub-additive effect of the combination ketamine plus ketoprofen could imply a potential to produce a reduction in side effects compared to ketamine alone.

Whether a spinal synergistic antinociceptive interaction with reduced potential side effects between ketamine and ketoprofen also occurs *in vivo* needs to be addressed, as well as the mechanism(s) by which these interactions take place. An interaction at the level of the Ca^{2+} activation of phospholipase A_2 (PLA_2) and subsequent activation of the AA/COX pathway has been proposed for the NMDA receptor antagonists and NSAIDs (Price *et al.*, 1996). However, other or additional mechanisms may have contributed to the depressant actions between ketamine and ketoprofen since the NSAID was infused at much higher concentrations than those needed to block COX activity (Warner *et al.*, 1999).

Using visual analogue scales and post-operative opioid consumption, the analgesic interaction of dextromethorphan and NSAIDs has been studied in human beings. Contrary to results in the current study, combination of an ineffective oral dose of dextromethorphan (120 mg) with an analgesic oral dose of the NSAID ibuprofen (400 mg) produced no additive or synergistic analgesic effects in patients scheduled for surgical termination of pregnancy (Ilkjær *et al.*, 2000). Similarly, preincisional administration of an analgesic dose of dextromethorphan (120 mg, intramuscularly) and the NSAID tenoxicam (40 mg, intravenously) produced no additive or synergistic analgesia in patients scheduled for elective laparoscopic cholecystectomy (Yeh *et al.*, 2004). However, these single dose studies are inappropriate to determine synergism (Yaksh and Malmberg, 1994). Also contrary to results presented here, there is anecdotal evidence, presumably obtained using isobolographical analysis, of additive analgesic effects after i.t. delivery of an unspecified NMDA receptor antagonist together with an unspecified COX inhibitor in a paw formalin test in rats (Yaksh and Malmberg, 1994).

Synergism depends not only on the drugs and the effect measured, but also on the fixed ratio combination and the total dose in the combination, which may explain the different results between the literature and the present study. It would be interesting to establish if the antinociceptive synergism between ketamine and ketoprofen is specific for these drugs or if it extends to the NMDA receptor antagonists and the NSAIDs as a drug class interaction. It was recently demonstrated that the analgesic interaction between opioids and NSAIDs in a radiant heat tail-flick test in mice was dependent upon both the opioid and the NSAID. The NSAIDs naproxen and ibuprofen, but not acetylsalicylic acid or ketorolac, potentiated

the analgesic effect of the opiate hydrocodone, but ibuprofen did not potentiate fentanyl or morphine analgesia (Zelcer *et al.,* 2005). Although studies in arthritic rats suggest that the antinociceptive effect of the NSAIDs as drug class is increased by the putative NMDA receptor antagonist dextromethorphan (Price *et al.,* 1996), further studies will be necessary to determine if differences between the drugs within each class also occur between NMDA receptor antagonists and NSAIDs.

In conclusion, the current study provides support for a synergistic interaction between ketamine and ketoprofen to depress spinal nociceptive transmission *in vitro*. Results also provide support for a sub-additive interaction between these two compounds to depress the MSR, which may cause less motor impairment than with ketamine alone. Drug combinations are widely used clinically and, if such a combination profile between ketamine and ketoprofen also occurs *in vivo*, current findings raise the possibility of ultimate therapeutic exploitation of increased analgesia with fewer side effects.

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6. Ketamine and L-NAME synergistically depress NMDA receptor-mediated transmission on the *in vitro* neonatal rat spinal cord preparation

6.1 Abstract

Activation of spinal *N*-methyl-D-aspartate (NMDA) receptors triggers the nitric oxide (NO) pathway, which has been implicated in the nociceptive processing of information in both adults and neonates. Despite this, few studies have investigated the nature of the interaction between NMDA receptor antagonists and nitric oxide synthase (NOS) inhibitors. Using statistical methods designed for the study of drug-drug interactions, the current study assessed the interaction between the NMDA receptor channel blocker ketamine and the NOS inhibitor L-NAME on the *in vitro* neonatal rat spinal cord preparation. Two A-fibre-mediated reflexes, the monosynaptic response (MSR) and the low intensity excitatory postsynaptic potential (epsp), and one C-fibre-evoked reflex, the high intensity epsp, were evoked. All three reflexes have glutamate receptor-mediated components; the MSR contains non-NMDA receptor-mediated components, and the last two reflexes contain NMDA receptor-mediated components. L-NAME produced no effect on its own on any of the spinal reflexes, but synergistically increased the depressive effect of ketamine on both NMDA receptor-mediated responses. The combination was sub-additive for the depression of the MSR. All interactions seemed to depend on the total concentration in the fixed proportional combinations. The combination of ketamine and L-NAME synergistically reduced nociceptive transmission in the *in vitro* neonatal rat spinal cord and at the same time decreased the potential local anaesthetic actions of ketamine alone. This drug combination could have important clinical implications in neonates. Further studies are necessary to assess the analgesic efficacy of this drug combination and its mechanisms of action.

6.2 Introduction

Activation of *N*-methyl-D-aspartate (NMDA) receptors and subsequently the nitric oxide (NO) pathway have been shown to play an important role in spinal nociceptive processing. For example, in adult rats, intrathecal (i.t.) administration of NMDA has been reported to induce pain-related behaviours and at the same time increase the concentration of the stable products of NO, nitrite and nitrate in cerebrospinal fluid (Kawamata and Omote, 1999). I.t. administration of drugs which antagonise NMDA receptors or inhibit nitric oxide synthases (NOS) and soluble guanylate cyclases blocked NMDA-induced nociception (Kawamata and Omote, 1999; Fairbanks *et al.,* 2000). In addition, *in vitro* experiments have shown that

NMDA treatment increased the release of 3',5'-cyclic guanosine monophosphate (cGMP) from the spinal cord of adult rats (Kawamata and Omote, 1999), and this increase was blocked by NOS inhibitors (Kawamata and Omote, 1999). These data may indicate that activation of spinal NMDA receptors induce pain at least by stimulating NOS to produce NO, which in turn may activate soluble guanylate cyclases to increase cGMP formation.

Similarly, in spinal cord preparations from neonatal rats, infusion of NMDA, as well substance P and calcitonin gene-related peptide, induces a long lasting depolarisation in the ventral root, which is similar to that evoked by electrical stimulation of the dorsal root at C-fibre strength (Woodley and Kendig, 1991; Thompson *et al.,* 1995). This electrically evoked response, hereafter referred to as the high intensity excitatory postsynaptic potential (epsp), is markedly depressed by opioid and α_2-adrenoceptor agonists (Faber *et al.,* 1997). In addition, NMDA and neurokinin-1 receptor antagonists depress the high intensity epsp (Akagi *et al.,* 1985; Woodley and Kendig, 1991). These data suggest that the high intensity epsp represents a C-fibre-evoked, NMDA receptor- and neuropeptide receptor-mediated nociceptive response.

Although the spinal NMDA receptor-NO pathway has been studied in neonatal rats, the results are inconsistent. On the one hand, infusion of low concentrations of NO and NO donors to spinal cord preparations from 0- to 2-day-old rats depressed the high intensity epsp, and higher concentrations evoked depolarisations of the ventral root (Kurihara and Yoshioka, 1996). In addition, infusion of the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) and the NO scavenger carboxy-PTIO potentiated the high intensity epsp, and that of the membrane permeable analogue of cGMP 8-bromo-cGMP induced both inhibition of the high intensity epsp and depolarisation of ventral roots (Kurihara and Yoshioka, 1996). These data may suggest that NO is produced endogenously by the spinal cord of newborn animals and exerts inhibitory actions at relatively low concentrations, but NO may also exert a facilitatory influence at higher concentrations; both actions seem to be mediated, at least partially, by cGMP (Kurihara and Yoshioka, 1996). On the other hand, infusion of L-NAME to spinal preparations from 10- to 12-day-old rats produced no effect on the high intensity epsp (Thompson *et al.,* 1995), but administration of NMDA to spinal cord slices from 12- to 16-day-old rats increased the release of cGMP and the staining of nicotinamide adenine dinucleotide phosphate diaphorase (a marker of NOS activity); both NMDA-induced effects were blocked by a NOS inhibitor (Morris *et al.,* 1994).

Together, data from neonatal rats may indicate that although activation of spinal NMDA receptors is related to nociception, its linked NO pathway induces different responses throughout postnatal development. This may be attributed to ontogenic changes in the distribution of NOS in the dorsal horn of the spinal cord (Soyguder *et al.,* 1994; Vizzard *et al.,* 1994).

Despite the association between NMDA receptors and the NO pathway in spinal nociceptive processing, there are very few data on the type of interaction between drugs that affect these two systems. The objective of this study was to determine the nature of the interaction between the NMDA receptor channel blocker ketamine and the NOS inhibitor L-NAME at the spinal cord level. It was reasoned that, because NMDA receptors are linked to the synthesis of NO (see above), L-NAME would enhance the antinociceptive action of ketamine. For this purpose, an *in vitro* neonatal rat hemisected spinal cord preparation was used, which may also help to broad our knowledge on the physiology and pharmacology of spinal nociceptive transmission, especially during the postnatal period.

6.3 Material and methods

Neonatal (5- to 7-day-old) Sprague-Dawley rat pups were cervically dislocated and decapitated. The spinal cord, with attached L4 or L5 dorsal and ventral roots, was rapidly removed and hemisected. The hemicords were placed in a chamber with the dorsal root in contact with the stimulating electrode and the ipsilateral ventral root in contact with the recording electrode. The hemicords were superfused (2 ml/min) with artificial cerebrospinal fluid (aCSF), which was kept at room temperature. The aCSF consisted of (in mM) NaCl 118, $NaHCO_3$ 24, glucose 12, $CaCl_2$ 1.5, KCl 3 and $MgSO_4 7H_2O$ 1.25, and was equilibrated with 95% O_2 and 5% CO_2.

Preparations were allowed to equilibrate for at least 60 min and were electrically stimulated thereafter with single square-wave pulses, 0.5 ms duration at 5 min intervals. Low intensity voltage stimulation at 3x threshold was used to evoke A-fibre-mediated responses, the monosynaptic compound action potential (MSR) and the low intensity epsp. Both responses have glutamate receptor-mediated components, with the former being mediated by kainite and AMPA receptors and the latter by NMDA receptors (Long *et al.,* 1990; Woodley and Kendig, 1991; Faber *et al.,* 1997). The high intensity epsp was evoked by activation of C-fibres at 32x threshold. Responses were amplified (AA6 Mk III, Medelec MS6 system, Medelec Ltd., Woking, Surrey, England), transferred to a PowerLab 4/20 (ADInstruments Pty Ltd, Castle Hill, Australia), and digitally stored for off-line analysis (Scope v3.6.8, MacLab® System© 1998 ADInstruments Pty Ltd, Castle Hill, Australia).

Five baseline readings were recorded before any treatment was infused. Cumulative concentrations of ketamine (1-50 μM) and L-NAME (1-100 μM) were applied to the hemicords in the superfusate. Each ketamine concentration was infused for 30 min, which was the necessary time for the drug to reach equilibrium (Chapter 4). L-NAME concentrations were infused for 45 min each. Drug concentrations were selected from previous studies

using neonatal rat spinal cord preparations (Brockmeyer and Kendig, 1995; Thompson *et al.,* 1995; Kurihara and Yoshioka, 1996). A final recording was taken 90 min after washout when the drug depressed the synaptic response, or 30 min after washout when the drug had no apparent effect.

Ketamine was made up as stock solution in distilled water and kept at 4°C, and L-NAME was prepared freshly in aCSF each experimental day. Drugs were purchased from Sigma (St. Lous, MO, USA).

The effect of treatment was assessed by converting the amplitude of the MSR and the area under the curve of both the low intensity epsp and the high intensity epsp into percentage maximum possible effect (MPE%) values using the formula: %MPE = 100 - [(post-drug effect/baseline effect) x 100]. Linear regression analysis was performed on log concentration-effect data (4-5 preparations per reflex, at least 4 concentrations) using GraphPad Prism (v4.0b for Macintosh, GraphPad Software Inc., San Diego, CA, USA). The inhibitory concentration necessary to achieve 40% MPE (IC_{40}) together with 95% confidence intervals (CI) were computed for each drug alone. A theoretical additive concentration that would produced this effect level was calculated for the drug combination with the active : inactive drugs given in a 0.8805 : 0.1195 fixed proportion, and CI were resolved according to the individual drugs in the combination. This fixed ratio combination, in which the constituents were administered in amounts that kept the proportions of each constant, was tested experimentally. The interaction of ketamine and L-NAME was assessed for synergism considering that one of the drugs lacked efficacy (Porreca *et al.,* 1990).

Data were presented as IC_{40} values with 95% CI. IC_{40} values for the theoretical additive combination and the actual drug combination tested experimentally were compared using the Student's t-test. Synergism was defined as the effect of an actual drug combination being higher and statistically different than the theoretical calculated equieffect of a drug combination with the same proportions (Tallarida, 2000). To assess whether synergism extended to other concentration levels, the actual drug combination line and the concentration-effect line of the efficacious dug given alone were compared using the F-distribution test (Tallarida, 2000). Differences were taken to be significant when $P < 0.05$.

6.4 Results

Single dug studies

Ketamine depressed all three spinal reflexes, whereas L-NAME had no effect on any of them. Ketamine's IC_{40} values were 7.75 μM (5.94 to 10.00 μM) for the MSR, 24.85 μM (16.78

to 42.80 μM) for the low intensity epsp, and 18.63 μM (8.62 to 79.87 μM) for the high intensity epsp.

Combinations

Theoretical additive and actual combination IC_{40} values for each one of the segmental responses are presented in Table 6.1. The IC_{40} for the actual combination was significantly higher than the theoretically calculated value for the MSR ($P < 0.01$, t test), which indicates a sub-additive interaction for the combination, and this is expressed isobolographically in Figure 6.1b. Comparison of the actual combination line with that of ketamine alone demonstrated that both lines were significantly different ($P < 0.01$, F test), which indicates that sub-additivity may extend to other effect levels. As seen in Figure 6.1a, sub-additivity may only occur at levels of 40% inhibition of the control response and lower.

Table 6.1 *Theoretical and experimental IC_{40} values with 95% confidence intervals (CI) for combinations of ketamine and L-NAME in a fixed drug proportion on the three evoked spinal segmental responses.*[•]

Segmental response	IC_{40} values and 95% CI (μM)	
	Theoretical	Experimental
MSR	8.80 (6.74 to 11.36)	16.43 (13.12 to 21.63)[‡]
Low intensity epsp	28.23 (19.06 to 48.60)	13.06 (10.37 to 15.71)[†]
High intensity epsp	21.16 (9.79 to 90.71)	9.14 (5.71 to 12.39)

[•] *Ketamine: L-NAME proportions were 0.8805 : 0.1195 for all three segmental responses.*
[†] *$P < 0.05$ and [‡] $P < 0.01$ between theoretical and experimental values.*

Contrary to the MSR, IC_{40} values for the low intensity epsp were significantly higher for the theoretical additive combination than the actual combination ($P < 0.05$, t test), demonstrating a synergistic interaction between ketamine and L-NAME (Figure 6.2b). Synergism may also occur at higher, but not lower, effect levels than 40% inhibition of the control response, as the actual combination line was significantly different to that of ketamine alone ($P < 0.05$, F test; Figure 6.2a)

For the high intensity epsp, the combination of ketamine and L-NAME was additive at the IC_{40} level since both theoretical additive and actual combination values were not significantly different ($P > 0.05$, t test; Figure 6.3b). However, the actual combination line was significantly different to that of ketamine alone ($P < 0.05$, F test), indicating that an interaction, other than an additive one, occurred between ketamine and L-NAME. Figure 6.3a shows that synergism may only occur at higher effect levels than 40% inhibition of the control response.

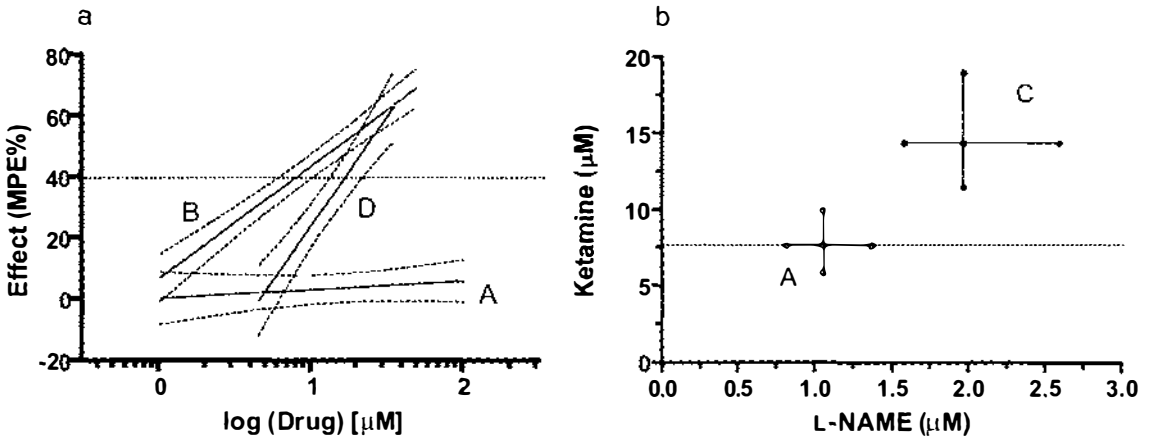

Figure 6.1 *(a) Percent maximal possible effect (MPE%) of L-NAME alone (A), ketamine alone (B) and the actual combination of ketamine and L-NAME in a fixed ratio proportion (0.8805 : 0.1195) in which the quantities of ketamine are in 0.5 fractions to its respective IC_{40} value (D) on the MSR. The horizontal dashed line represents the effect level 40% MPE. (b) Isobologram for the MSR at the effect level 40% MPE for the combinations ketamine and L-NAME in a fixed ratio proportion as indicated above. The dashed line is the line of additivity and contains point A representing the calculated additivity quantities for this proportional combination. Point C is the combination point determined experimentally with this same proportional mix. Coordinates of point A are [7.75 (5.94 - 10.00)* μM, *1.05 (0.81 - 1.36)* μM*] and those of point C [14.46 (11.55 - 19.05)* μM, *1.96 (1.57 - 2.59)* μM*].*

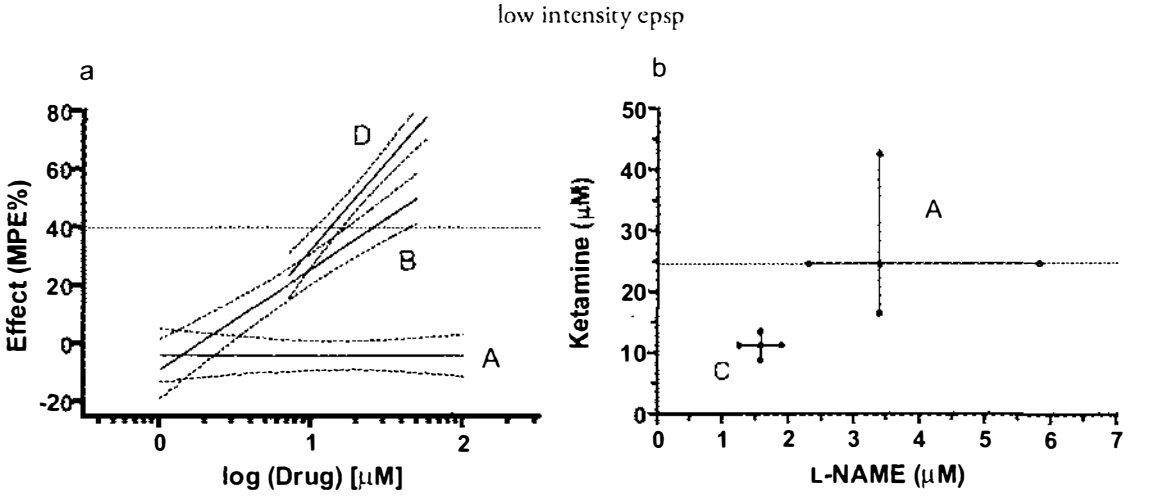

Figure 6.2 *(a) Percent maximal possible effect (MPE%) of L-NAME alone (A), ketamine alone (B) and the actual combination of ketamine and L-NAME in a fixed ratio proportion (0.8805 : 0.1195) in which the quantities of ketamine are in 0.5 fractions to its respective IC_{40} value (D) on the low intensity epsp. The horizontal dashed line represents the effect level 40% MPE. (b) Isobologram for the low intensity epsp at the effect level 40% MPE for the combinations ketamine and L-NAME in a fixed ratio proportion as indicated above. The dashed line is the line of additivity and contains point A representing the calculated additivity quantities for this proportional combination. Point C is the combination point determined experimentally with this same proportional mix. Coordinates of point A are [24.85 (16.78 - 42.79)* μM, *3.37 (2.28 - 5.81)* μM*] and those of point C [11.5 (9.13 - 13.83)* μM, *1.56 (1.24 - 1.88)* μM*].*

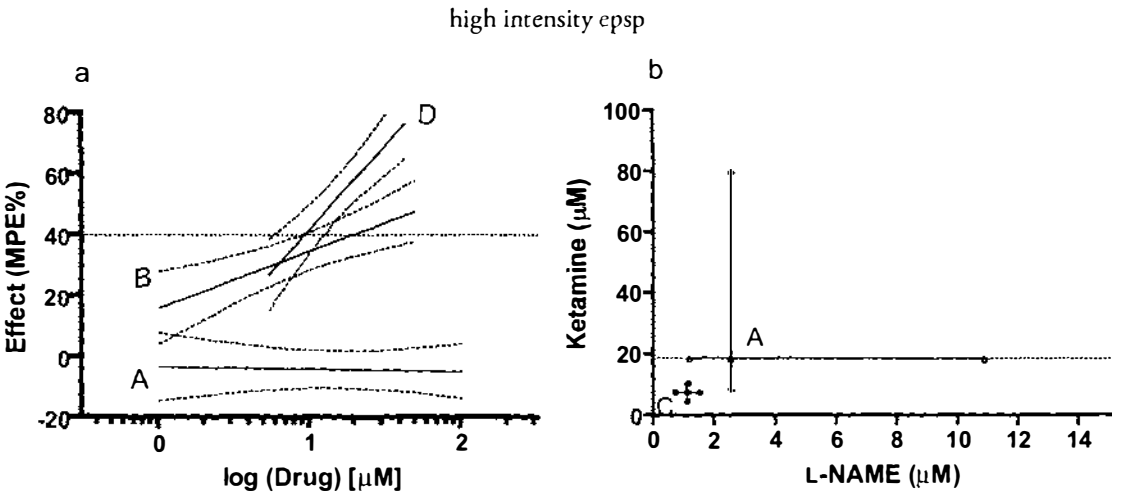

Figure 6.3 *(a) Percent maximal possible effect (MPE%) of L-NAME alone (A), ketamine alone (B) and the actual combination of ketamine and L-NAME in a fixed ratio proportion (0.8805 : 0.1195) in which the quantities of ketamine are in 0.5 fractions to its respective* IC_{40} *value (D) on the high intensity epsp. The horizontal dashed line represents the effect level 40% MPE. (b) Isobologram for the high intensity epsp at the effect level 40% MPE for the combinations ketamine and L-NAME in a fixed ratio proportion as indicated above. The dashed line is the line of additivity and contains point A representing the calculated additivity quantities for this proportional combination. Point C is the combination point determined experimentally with this same proportional mix. Coordinates of point A are [18.63 (8.62 - 79.87)* μM, *2.53 (1.17 - 10.84)* μM*] and those of point C [8.05 (5.03 - 10.91)* μM, *1.09 (0.68 - 1.48)* μM*].*

6.5 Discussion

The present study has demonstrated that the NMDA receptor channel blocker ketamine and the NOS inhibitor L-NAME acted synergistically to depress both the low intensity epsp and the high intensity epsp in the spinal cord of neonatal rats, at the same time that they induced a sub-additive depressive effect on the MSR. These results may indicate that simultaneously blocking NMDA receptors and inhibiting NO production in the spinal cord could be a new strategy to reduce nociceptive transmission, at least in neonates.

A synergistic interaction between ketamine and L-NAME has been described previously, but tadpoles were used as experimental animals and anaesthesia, rather than analgesia, was taken as the end response (Tonner *et al.*, 1997). The authors found that a non-anaesthetic concentration of L-NAME (1 mM) decreased by 63% the concentration of ketamine required to induce anaesthesia in 50% of individuals (59.7 μM vs. 22 μM) (Tonner *et al.*, 1997). In the current study, despite L-NAME alone producing no effect on spinal transmission, it reduced by more than 50% the IC_{40} values for depression of both NMDA receptor-mediated responses when infused together with ketamine, but at this effect level the interaction was synergistic for the low intensity epsp only. The large 95% CI for the calculated theoretical value may

have precluded the actual drug combination from reaching statistical significance for the high intensity epsp. However, synergism may occur for both responses at effect levels above than 40% inhibition of the control response, suggesting that synergism may be dependant on the total concentration on their fixed proportion combinations. This is the first time that spinal antinociceptive synergism has been reported between an NMDA receptor antagonist and a NOS inhibitor.

The mechanisms by which the synergistic interactions between ketamine and L-NAME took place remain to be elucidated. However, since synergism was only observed on both the low intensity epsp and the high intensity epsp, spinal antinociceptive synergism with this drug combination may only occur when NMDA receptors are activated. This may suggest an interaction between the NMDA receptor-NO pathway. Ketamine is an NMDA receptor channel blocker and it would be expected to indirectly decrease NOS activity by reducing the entry of extracellular Ca^{2+} into the cell. Rivot *et al.* (1999) showed that ketamine (100 mg/kg, intraperitoneal) decreased NO concentrations in the spinal cord of spinalised adult rats and decreased the glutamate- and NMDA-induced increase of NO concentrations in the cord. Similarly, Gonzalez *et al.* (1999) demonstrated that ketamine (30-100 μM) blocked the NMDA-induced, NOS-dependent increased production of cGMP in cultured cerebral cortical neurones from foetal rats, and Miyawaki *et al.* (1997) reported that ketamine (10-100 μM) prevented the increase in cGMP concentrations induced by NMDA in cerebellar slices from 6- to 8-day-old rats. However, Tobin *et al.* (1994) found that ketamine (10-1000 μM) did not affect the activity of NOS in cultured cerebellar tissue from adult rats, and Wu *et al.* (2000) even showed that systemic administration (25-100 mg/kg, intraperitoneal) and local perfusion (50 and 100 μM) of ketamine increased nitrite and nitrate concentrations in the hippocampus and striatum of adult rats. Assessment of the biochemical activity of NOS during ketamine perfusion onto the *in vitro* spinal cord preparation may help to clarify this issue.

Whatever the net effect of ketamine on spinal NO production may have been, ketamine depressed nociceptive transmission in the neonatal rat spinal cord most likely by non-competitively blocking the NMDA receptor channel (Brockmeyer and Kendig, 1995; Lizarraga *et al.*, 2004; Chapter 4). Although stimulation of NOS with the subsequent production of NO triggered by activation of NMDA receptors plays an important role in the spinal nociceptive processing of information (see Introduction), there are other receptor systems that can also activate the NO pathway. For instance, activation of glutamate receptors other than NMDA receptors can also increase cGMP concentrations in spinal cord slices from neonate rats (Morris *et al.*, 1994) and in cultured cerebellar cortical neurones from foetal rats (Gonzales *et al.*, 1995). In both cases, NOS inhibitors, including L-NAME, blocked the increase in cGMP concentrations (Morris *et al.*, 1994; Gonzales *et al.*, 1995). Therefore,

blockade of NMDA receptors and reduction of NO production may be mechanisms by which ketamine and L-NAME synergistically reduced NMDA receptor-mediated transmission in the *in vitro* neonatal rat spinal cord.

In addition to elucidating the mechanisms of action, it remains to be determined if antinociceptive synergism between ketamine and L-NAME also *i*) occurs in live, neonate animals; *ii*) takes place in mature, adult animals; and *iii*) extends to other NMDA receptor antagonists and NOS inhibitors. If synergism is demonstrated in any of these situations, the combination described in this study could have important clinical implications for the control of pain transmission at the spinal cord level.

Contrary to the present study, there are reports suggesting an antagonistic action of L-NAME on the effects of ketamine in the central nervous system (CNS). Wu *et al.* (2000) observed that intraperitoneal administration of L-NAME (100 mg/kg) prevented the intraperitoneal ketamine (100 mg/kg)-induced increase of nitrite and nitrate concentrations in the hippocampus of adult rats. Mueller and Hunt (1998) demonstrated that chronic subcutaneous administration of L-NAME (50 mg/kg) prevented the anaesthetic effect of ketamine (75 mg/kg) given intramuscularly to adult rats. Bulutcu *et al.* (2002) reported that intraperitoneal (10 mg/kg), but not i.t. (30 µg in 5 µl), administration of a non-antinociceptive dose of L-NAME prevented the antinociceptive action of intraperitoneal (1-10 mg/kg) and i.t. (10-60 µg in 5 µl) administration of ketamine in models of inflammatory pain in adult mice. Lauretti *et al.* (2001) even found that transdermal application of the nitric oxide generator nitroglycerin increased the analgesic effect of epidurally administered S(+)-ketamine in patients undergoing orthopaedic knee surgery. These data suggest that ketamine may activate the NO pathway to exert its effects in the CNS, which contrast with the synergistic effect between ketamine and L-NAME described here. Although the differences between the above studies and the present one could be attributed to developmental changes in the CNS, in particular those of spinal NMDA receptors (Kalb *et al.,* 1992) and NOS (Vizzard *et al.,* 1994; Soyguder *et al.,* 1994), it is more likely that these differences may be due to changes in the absorption and distribution of ketamine induced by NO modulating drugs. For instance, systemic administration of L-NAME, in addition to preventing the anaesthetic effect of systemically administered ketamine, reduced by 75% and 36% the plasma and cerebellar tissue concentration of this drug, suggesting an L-NAME-mediated reduction in blood flow to both the site of administration of ketamine and to its site(s) of action in the CNS (Mueller and Hunt, 1998).

The effect of the combination ketamine and L-NAME on the MSR was the opposite of those seen on the NMDA receptor-mediated responses. Surprisingly, the combination produced a sub-additive effect; addition of L-NAME to ketamine reduced the depressive effect of the

latter drug alone on the MSR. Sub-additivity, in this case, may have depended on the total concentration in the fixed proportion combination, and may not be evident at effect levels higher than 40% inhibition of the control response.

The MSR is dependent on AMPA and kainate receptors (Woodley and Kendig, 1991; Long *et al.*, 1990), and inhibition of this reflex represents a local anaesthetic effect (Chapter 4). Ketamine indeed produced local anaesthetic effects in dogs, which can be manifested as motor impairment, when administered through the i.t. route (Iida *et al.*, 1997). Although the mechanisms involved in this sub-additive effect are not known, it may appear that depression of the MSR by ketamine is dependent on NO. Again, assessment of ketamine on NOS biochemical activity in the *in vitro* rat spinal cord may help to explain this. Nonetheless, the sub-additive effect of the combination of ketamine and L-NAME could potentially produce less motor impairment than ketamine alone, rendering this drug combination as a possible important analgesic combination with reduced side effects.

Despite not modifying electrically evoked spinal transmission, L-NAME interacted synergistically with ketamine *in vitro* to depress NMDA receptor-mediated transmission in the neonatal rat spinal cord preparation. In addition, the combination was sub-additive for the depression of the MSR response. These data imply that, at the level of the spinal cord in neonates, the combination of ketamine and L-NAME produced antinociception in a synergistic way at the same time that reduced some possible side effects of the former drug. Further studies are required to determine the mechanisms by which these interactions occur and to assess the *in vivo* efficacy of this drug combination.

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7. Synergistic depression of C-fibre-mediated synaptic transmission by ketoprofen and L-NAME on the *in vitro* neonatal rat spinal cord preparation

7.1 Abstract

Both non-steroidal anti-inflammatory drugs (NSAIDs) and nitric oxide synthase (NOS) inhibitors induce antinociception at the spinal cord level, but the spinal antinociceptive interaction of both classes of compounds has not been investigated. This study assessed the interaction between the NSAID ketoprofen and the NOS inhibitor L-NAME in reflex responses to electrical stimulation in an *in vitro* spinal cord preparation. Low intensity stimulation was sufficient for activation of only A-fibres to evoke a monosynaptic response (MSR) superimposed on a low intensity excitatory postsynaptic potential (epsp). Supramaximal intensity also activated C-fibres to evoke a high intensity epsp. Cumulative increases in ketoprofen concentrations decreased both the low intensity epsp [IC_{40} and (95% CI) in μM = 528.64 (452.85 to 683.19)] and the high intensity epsp [368.55 (326.72 to 414.85)] without affecting the MSR. L-NAME had no significant effect on any of the spinal reflexes. Compared to the theoretical additive value at the level of 40% reduction of the control response, the combination of ketoprofen and L-NAME (0.8805:0.1195) was synergistic for the high intensity epsp [418.57 (371.06 to 471.15) *vs.* 185.86 (135.64 to 235.38)], but was not significantly different for the low intensity epsp [600.37 (514.31 to 775.91) *vs.* 503.25 (403.59 to 646.54)] and the MSR (no significant effect). Despite producing no effect on spinal transmission, L-NAME acted synergistically with ketoprofen at the level of the spinal cord to depress C-fibre-mediated responses. The mechanisms by which these drugs produce this effect and the analgesic evaluation of this combination *in vivo* require further study.

7.2 Introduction

The circuitry of nociceptive systems within the spinal cord modulates the relationship between the stimulus and the response to pain, which makes this tissue a potential target for the pharmacological control and management of pain. Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for the control of pain. They induce analgesia after intrathecal (i.t.) injection, either in animal models of pain (Malmberg and Yaksh, 1992; Yaksh *et al.*, 2001; Zhu *et al.*, 2003) or in human beings suffering from cancer pain (Devoghel, 1983). The analgesic effect is manly attributed to the inhibition of cyclo-oxygenase (COX) enzymes, which are constitutively present in the spinal cord in the form of at least two different isozymes: COX-1

and COX-2 (Svensson and Yaksh, 2002). Both isozymes have been implicated in the spinal transmission of nociceptive information (Yaksh *et al.,* 2001; Zhu *et al.,* 2003).

Interestingly, alternative or complementary mechanisms to COX inhibition have been suggested for the spinal analgesic action of NSAIDs (McCormack, 1994).

Subdural administration of nitric oxide synthase (NOS) inhibitors also reduce hyperalgesia in various models of pain (Kawamata and Omote, 1999; Gühring *et al.,* 2000). Analgesia, probably partially mediated at the level of the dorsal horn of the spinal cord, has also been demonstrated after systemic administration of a NOS inhibitor in patients with chronic tension-type headache (Ashina *et al.,* 1999). Inhibitors of the enzymes NOS decrease the formation of NO, which has also been associated with the transmission of pain at the spinal cord level (Kawamata and Omote, 1999).

Some studies have evaluated a possible interaction between the NO pathway and analgesia induced by NSAIDs both at the peripheral and spinal cord levels (Morgan *et al.,* 1992; Björkman *et al.,* 1996; Lorenzetti and Ferreira, 1996; Sandrini *et al.,* 2002; Díaz-Reval *et al.,* 2004; Dudhgaonkar *et al.,* 2004; Lozano-Cuenca *et al.,* 2005). However, no isobolographic studies investigating the spinal antinociceptive effect of the interaction between NSAIDs and NOS inhibitors have been reported. Since neurotransmission of pain information through the spinal cord to higher centres of the brain is not a simple process and several neurotransmitters, neuropeptides and neuromodulators regulate this transmission of nociceptive information (Dickenson, 1995), it is of interest to characterise the interaction of both classes of compounds.

This study assessed the effect of the NSAID ketoprofen in combination with the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) on electrophysiological segmental transmission in the *in vitro* neonatal rat spinal cord preparation. Preliminary data have been published in abstract form (Lizarraga *et al.,* 2005).

7.3 Material and methods

As previously described (Faber *et al.,* 1997), the spinal cords with attached L4 or L5 dorsal and ventral roots from Sprague-Dawley rats (unsexed, 5–7 day old) were isolated, hemisected sagitally, and placed in a recording chamber. The dorsal root was in contact with the stimulating electrode and the corresponding ventral root with the recording electrode. The hemicords were perfused (2 ml/min) with artificial cerebrospinal fluid (aCSF) of the following composition (mM): NaCl 118, $NaHCO_3$ 24, glucose 12, $CaCl_2$ 1.5, KCl 3 and

$MgSO_4 7H_2O$ 1.25. The aCSF was gassed with 95% O_2 and 5% CO_2, and was kept at room temperature. Preparations were allowed to stabilise for 60 min before the start of recordings.

The dorsal root was electrically stimulated (single square-wave pulse of 500 μs duration) to evoke three segmental reflexes. Two of them, the monosynaptic compound action potential (MSR) and the low intensity excitatory postsynaptic potential (epsp), were primary afferent A-fibre-mediated and evoked by low intensity voltage stimulation at 3 times threshold. Threshold was defined as the intensity at which a discernible response first appeared in the ventral root. The other reflex, the high intensity epsp, was evoked by activation of primary afferent C-fibres at supramaximal intensity of 32 times threshold. Responses were amplified (AA6 Mk III, Medelec MS6 system, Medelec Ltd., Woking, Surrey, England), transferred to a PowerLab 4/20 (ADInstruments Pty Ltd, Castle Hill, Australia), and digitally stored for off-line analysis (Scope v3.6.8, MacLab® System© 1998 ADInstruments Pty Ltd, Castle Hill, Australia).

Segmental reflexes were recorded every 5 min. After obtaining five consecutive baseline readings, cumulative concentrations of ketoprofen (200-600 μM) and L-NAME (1-100 μM) were applied to the hemicords by adding them to the superfusate. Each ketoprofen concentration was infused for 35 min and those of L-NAME for 45 min. The lower concentration for ketoprofen was chosen as being one order of magnitude less than that necessary to inhibit a C-fibre response in adult rats (Bustamante *et al.,* 1997). The concentration-range for L-NAME was based on previous studies with neonatal rat spinal cord preparations (Thompson *et al.*, 1995; Kurihara and Yoshioka, 1996) .

The peak amplitude of the MSR and both the area under the curve of the low intensity epsp and the high intensity epsp were converted to percentage maximal possible effect values (%MPE) according to the formula: %MPE = 100 - [(post-drug effect/baseline effect) x 100]. All log concentration-effect data (4-5 preparations per reflex, at least 4 concentrations) were analysed using linear regression methods (GraphPad Prism, v4.0b for Macintosh, GraphPad Software Inc., San Diego, CA, USA).

The interaction of ketoprofen and L-NAME was evaluated for synergism utilising the procedure described by Porreca *et al.* (1990), in which one of the drugs lacks efficacy. The drug concentration necessary to achieve 40% MPE (IC_{40}), with the active : inactive drugs being given in a 0.8805 : 0.1195 proportion, was calculated. Confidence intervals (CI) were resolved according to the individual drugs in the combination. This fixed ratio combination, in which the constituents were administered in amounts that kept the proportions of each constant, was tested experimentally. Synergism has been defined as the effect of an actual drug combination being higher and statistically different than the theoretical calculated equieffect

of a drug combination with the same proportions. Additivity means that each constituent contributes to the effect in accord with its own potency and the less potent drug is acting merely as a diluted form of the other (Tallarida, 2000).

Results were presented as IC_{40} values with 95% CI. IC_{40} values for the actual combination line and the concentration-effect line of the efficacious dug given alone were compared using the Student's *t*-test. To assess whether synergism extended to other concentration levels, the actual combination line and the concentration-effect line of the efficacious dug given alone were compared using the F-distribution test (Tallarida, 2000). Significance was $P < 0.05$.

7.4 Results

Single drug studies

Cumulative increases in keoprofen concentrations decreased both the low intensity epsp and the high intensity epsp, with very minor efficacy on the MSR (mean + s.e.m. maximum depression of 7.42 ± 4.61%; slope significantly different to zero, $P = 0.0097$, F test). L-NAME had no significant effect on any of the spinal reflexes, and the concentration-response lines yielded slopes not significantly different to zero ($P \geq 0.3479$, F test). IC_{40} values with 95% CI for each drug for each reflex response are shown in Table 7.1.

Table 7.1 *IC_{40} values with 95% confidence intervals (CI) for the depressive effect of ketoprofen (200-600 μM) and L-NAME (1-100 μM) on the three evoked spinal segmental responses.*

Segmental response	IC_{40} values and 95% CI (μM)	
	Ketoprofen	L-NAME
MSR	N/E*	N/E*
Low intensity epsp	528.64 (452.85 to 683.19)	N/E*
High intensity epsp	368.55 (326.72 to 414.85)	N/E*

* *N/E = no effect.*

Combination studies

As L-NAME produced no effect when infused alone, an arbitrary fixed drug proportion of ketoprofen and L-NAME (0.8805 : 1195) was used to calculate the additive IC_{40} values with their corresponding 95% CI for the three spinal reflexes (Table 7.2). Co-infusion of ketoprofen and L-NAME, in amounts that kept the proportions of each constant, produced a synergistic depressive effect on the C-fibre-mediated high intensity epsps at the level of 40% reduction of the control response (Figure 7.1b). Synergism, however, may have not extended

to other effect levels since the actual combination line was not significantly different to the concentration-effect line of ketoprofen alone ($P > 0.05$, F-distribution test; Figure 7.1a).

Table 7.2 *Theoretical and experimental IC_{40} values with 95% confidence intervals (CI) for combinations of ketoprofen and L-NAME in a fixed drug proportion on the three evoked spinal segmental responses.*[•]

Segmental response	IC_{40} values and 95% CI (μM)	
	Theoretical	Experimental
MSR	N/E[¨]	N/E[¨]
Low intensity epsp	600.37 (514.31 to 775.91)	503.25 (403.59 to 646.54)
High intensity epsp	418.57 (371.06 to 471.15)	185.86 (135.64 to 235.38)[†]

[•] *Ketoprofen : L-NAME proportions were 0.8805 : 0.1195 for all three evoked segmental responses.*

[¨] *N/E = no effect.*

[†] *$P < 0.05$ between theoretical and experimental values.*

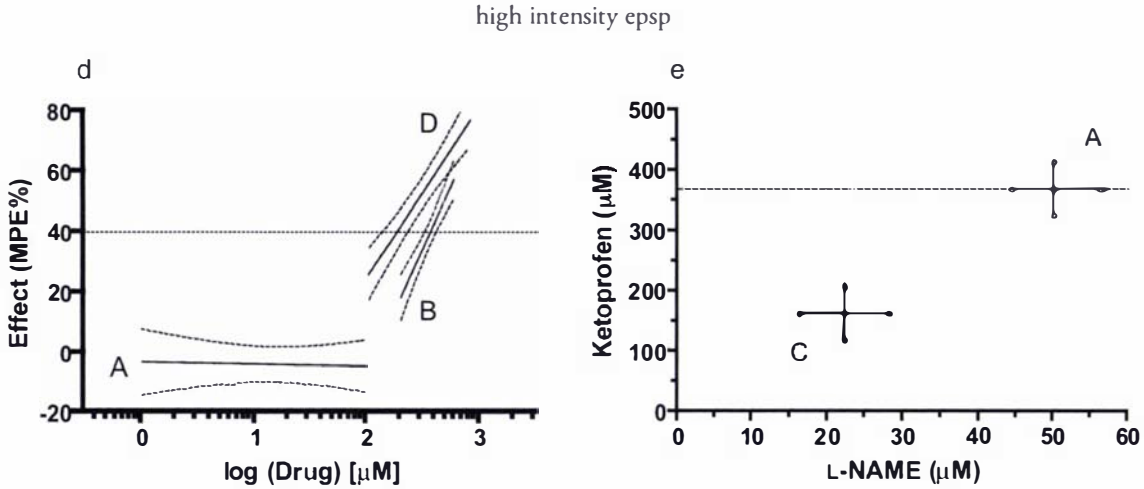

Figure 7.1 *(a) Percent maximal possible effect (MPE%) of L-NAME alone (A), ketoprofen alone (B) and the actual combination of ketoprofen and L-NAME in a fixed ratio proportion (0.8805 : 0.1195) in which the quantities ketoprofen are in 0.5 fractions to its respective IC_{40} value (D) on the high intensity epsp. The horizontal dashed line represents the effect level 40% MPE. (b) Isobologram for the high intensity epsp at the effect level 40% MPE for the combinations ketoprofen and L-NAME in a fixed ratio proportion as indicated above. The dashed line is the line of additivity and contains point A representing the calculated additivity quantities for this proportional combination. Point C is the combination point determined experimentally with this same proportional mix. Coordinates of point A are [368.55 (326.72 - 414.85)* μM, *50.02 (44.34 - 56.30)* μM*] and those of point C [163.65 (119.43 - 207.25)* μM, *22.21 (16.21 - 28.13)* μM*].*

A-fibre-evoked reflexes, on the other hand, showed an additive response to the co-infusion of ketoprofen and L-NAME (Table 7.2). The actual combination line for the low intensity epsp was almost identical to the concentration-effect line of ketoprofen alone ($P > 0.05$, F-distribution test; Figure 7.2a). Isobolographically, Figure 7.2b illustrates this at an effect level

of the 40% reduction of the control response. Similar to the administration of either dug alone, co-infusion of ketoprofen and L-NAME in equal quantities to those used on the high intensity epsp, had only a minor effect on the MSR; mean ± s.e.m. maximum depression of 11 ± 1.20% of the control response (Figure 7.3).

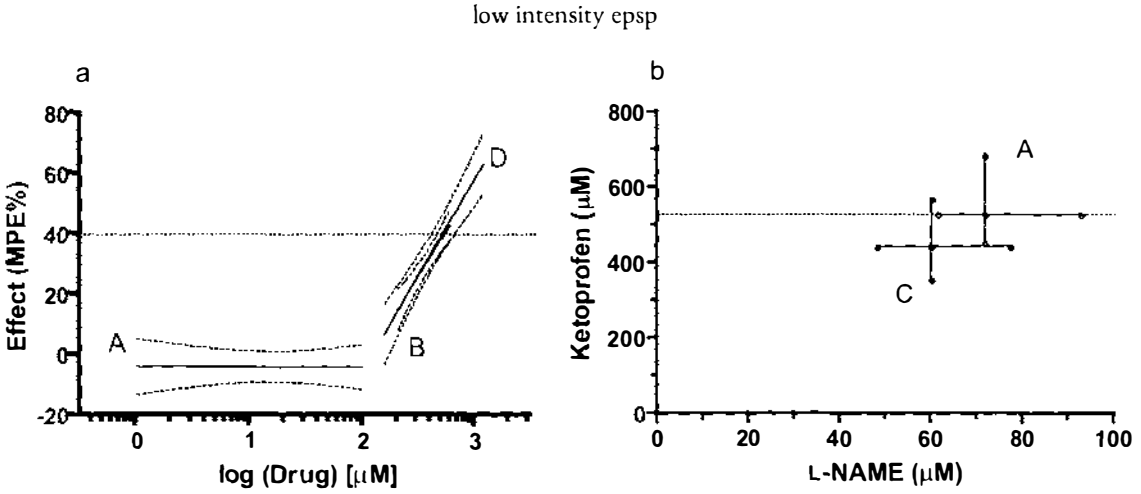

Figure 7.2 *(a) Percent maximal possible effect (MPE%) of L-NAME alone (A), ketoprofen alone (B) and the actual combination of ketoprofen and L-NAME in a fixed ratio proportion (0.8805 : 0.1195) in which the quantities ketoprofen are in 0.5 fractions to its respective* IC_{40} *value (D) on the low intensity epsp. The horizontal dashed line represents the effect level 40% MPE. (b) Isobologram for the low intensity epsp at the effect level 40% MPE for the combinations ketoprofen and L-NAME in a fixed ratio proportion as indicated above. The dashed line is the line of additivity and contains point A representing the calculated additivity quantities for this proportional combination. Point C is the combination point determined experimentally with this same proportional mix. Coordinates of point A are [528.64 (452.85 - 683.19)* μM, *71.75 (61.46 - 92.72)* μM*] and those of point C [443.11 (355.36 - 569.28)* μM, *60.14 (48.23 - 77.26)* μM*].*

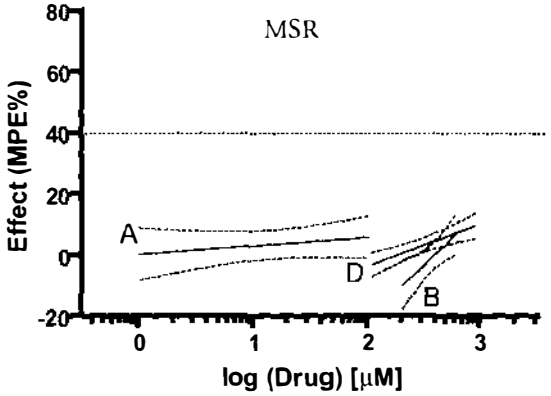

Figure 7.3 *Percent maximal possible effect (MPE%) of L-NAME alone (A), ketoprofen alone (B) and the actual combination of ketoprofen and L-NAME in a fixed ratio proportion (0.8805 : 0.1195) in which the quantities of the constituents were the same as those used on the high intensity epsp. The horizontal dashed line represents the effect level 40% MPE.*

7.5 Discussion

This is the first report to isobolographically assess spinal interaction between an NSAID and a NOS inhibitor. The present data demonstrated that, despite producing no effect when infused alone, the NOS inhibitor L-NAME synergistically depressed C-fibre-mediated transmission at the level of the spinal cord when co-infused with the NSAID ketoprofen. Synergism, however, was only observed after co-activation of afferent C- and A-fibres since the depressant action of ketoprofen on A-fibre-mediated spinal reflexes was not modified when combined with L-NAME.

Various studies have investigated a possible interaction at the spinal cord level between the NO pathway and the analgesic effects induced by NSAIDs, but there is lack of consistency among them. NSAID-mediated analgesia was reduced (Björkman *et al.,* 1996; Lorenzetti and Ferreira, 1996; Lozano-Cuenca *et al.,* 2005) or unaffected (Sandrini *et al.,* 2002; Díaz-Reval *et al.,* 2004) either by activating or blocking the NO pathway. Even inclusion of an NO moiety into the structure of NSAIDs, including S(+)-ketoprofen, produced enhanced analgesia in comparison to the NSAIDs alone (Romero-Sandoval *et al.,* 2002; Gaitan *et al.,* 2004). Discrepancies could be due to differences in experimental procedures, including the drugs, routes of administration, and pain models used.

Using dose-effect experimental designs in models of peripheral inflammatory pain (injection of formalin into the paw) and visceral pain (intraperitoneal administration of acetic acid), Morgan *et al.* (1992) suggested that synergism occurred in adult mice after intraperitoneal co-administration of L-NAME and the NSAIDs flurbiprofen and indomethacin. Although they provided evidence that subanalgesic doses of both drugs produced analgesia when given together, the authors failed to carry out isobolographic analysis rendering their data as suggestive, but not conclusive. Recently, Dudhgaonkar *et al.* (2004) utilised isobolographic analysis to show that the analgesic interaction between the NSAID rofecoxib (selective for COX-2) and the selective inhibitor of the inducible isoform of NOS aminoguanidine was synergistic after oral administration in adult rats submitted to a peripheral inflammatory model of pain (intraplantar formalin). Our data with ketoprofen and L-NAME in the immature spinal cord, which was also obtained using isobolographic analysis, corroborate that the nature of the antinociceptive interaction between NSAIDs and NOS inhibitors is synergistic, and provide evidence that such an interaction can take place in the spinal cord.

However, the synergistic depressive action between ketoprofen and L-NAME in the *in vitro* neonatal spinal cord contrasts with the lack of effect of i.t. administered L-NAME on the analgesia induced by S(+)-ketoprofen given orally to arthritic adult rats (Díaz-Reval *et al.,* 2004), and with the need of a functional spinal NO pathway for the NSAID lumeracoxib to

induce antihyperalgesia after being injected i.t. to adult rats submitted to a paw formalin test (Lozano-Cuenca *et al.,* 2005). It may be possible that *in vitro* and *in vivo* data do not correlate well or that experimental procedures (i.e., drugs, nociceptive models) affect the outcome of the interaction. Further investigation is necessary to clarify these issues.

The mechanism of the synergistic interaction between ketoprofen and L-NAME is clearly of interest, but cannot be established by the present data. However, the fact that synergism was detected only on the C-fibre-mediated reflex and that the drugs were applied in known concentrations in the superfusate allow us to formulate some hypotheses. The low intensity epsp has NMDA receptor components and the high intensity epsp also has neuropeptide components (Woodley and Kendig, 1991; Faber *et al.,* 1997). Since ketoprofen depressed both reflexes, antinociception may have been mediated through inhibition of the NMDA, neurokinin$_1$ (NK_1) and calcitonin gene-related peptide (CGRP) receptor systems, as has been shown with other NSAIDs (Malmberg and Yaksh, 1992). However, for the synergistic interaction between ketoprofen and L-NAME, a further inhibition of neuropeptide receptor systems may be possible. Reduction of glutamate, substance P (SP, the endogenous agonist of NK_1 receptors) and CGRP release by NSAIDs and NOS inhibitors has been demonstrated (Kawamata and Omote, 1999; Yaksh *et al.,* 2001) and this could also be the case for ketoprofen and L -NAME. The mechanisms for these effects could include inhibition of prostaglandin production by ketoprofen [prostaglandins increase the release of glutamate, SP and CGRP (Andreeva and Rang, 1993; Malcangio *et al.,* 1996; Bezzi *et al.,* 1998; Southall and Vasko, 2001)] and of NO synthesis by L-NAME [NO increase the release of glutamate and SP (Aimar *et al.,* 1998; Kawamata and Omote, 1999)]. It may be possible that they act on different effector systems in presynaptic neurones or that they interact with each other's pathways ("cross-talk") (Sakai *et al.,* 1998; Gühring *et al.,* 2000).

However, mechanisms other than, or complementary to, COX inhibition have to be considered since *in vitro* inhibition of COX-1 and COX-2 activity by 50% required only 0.047 μM and 2.9 μM of ketoprofen, respectively (Warner *et al.,* 1999), and the IC_{40} values for ketoprofen alone were 368 μM for the high intensity epsp and 568 μM for the low intensity epsp. These values are in fact more similar to those needed by ketoprofen (650 μM) to inhibit the activity of fatty acid amidohydrolase by 50% in rat brains (Fowler *et al.,* 1997). This enzyme is responsible for the breakdown of the endocannabinoid anandamide, which produced antihyperalgesia when injected i.t. to rats submitted to a model of peripheral inflammatory pain (Richardson *et al.,* 1998). In fact, a spinal endocannabinoid-dependent analgesic effect has been demonstrated for the NSAIDs flurbiprofen and indomethacin (Gühring *et al.,* 2002; Ates *et al.,* 2003).

In conclusion, using isobolographical analysis we have demonstrated that the NSAID

ketoprofen and the NOS inhibitor L-NAME synergistically depressed a C-fibre-mediated reflex from an *in vitro* spinal cord preparation. The mechanisms by which these drugs produce this effect and the analgesic evaluation of this combination *in vivo* require further study.

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8. Reduction of NMDA-induced mechanical hypersensitivity by intrathecal administration of ketoprofen and ketamine in sheep

8.1 Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) and *N*-methyl-D-aspartate (NMDA) receptor antagonists produce analgesia when given by the intrathecal (i.t.) route in various animal species, but their effect in farmed animals has hardly been studied. The present work assessed the spinal analgesic effects of the NSAID ketoprofen (200-3200 μM; 100 μl) and the NMDA receptor channel blocker ketamine (25-400 μM; 100 μl) given alone and in combination (837.95-3350.78 μM; 100 μl; 0.955:0.045 proportion) on mechanical nociceptive thresholds in sheep implanted with indwelling cervical i.t. catheters with the tip at C5. The sheep also received NMDA (2 mM; 100 μl) preceded by the highest concentration of ketoprofen and ketamine alone or in combination. Saline solution (0.9%; 100 μl) and xylazine (1.95 mM; 100 μl) were used as negative and positive controls, respectively. All treatments were given by the i.t. catheter and flushed through with saline. Nociceptive thresholds were measured for up to 3 h post-treatment and the area under the nociceptive threshold *vs.* time curve values (AUC) used to estimate the total effect of treatment. Compared to saline, xylazine significantly increased AUC values for 60 min and 180 min post-treatment. Ketoprofen and ketamine alone or in combination produced no significant effect on AUC values for 60 min and 180 min post-treatment. NMDA alone decreased the AUC value for 30 min post-treatment, and this hypersensitivity was prevented by ketoprofen and ketamine alone or in combination. In sheep, i.t. administration of ketoprofen and ketamine alone or in combination produced no hypoalgesia; however, they prevented the hypersensitivity induced by NMDA. The antihypersensitive effects of ketoprofen and ketamine could have important clinical implications in cases associated with persistent pain in farm animals.

8.2 Introduction

Analgesia in food animals has been a neglected subject until relatively recently. The need for adequate analgesia in sheep and cattle, particularly those suffering from many types of lameness, is now acknowledged (Whay *et al.,* 1998; Dobromylskyj *et al.,* 2000). In some cases, the pain induced by lameness can prevent the animals from moving around efficiently, and therefore to eat and have an adequate energy intake, which is likely to be reflected in lower production. Thus, pain detriments both the welfare of the animal and the income of the farmer.

Despite this, farm animals hardly ever receive analgesics. Some of the reasons may include lack of information on pain in farm animals, lack of approved analgesic drugs for the target species, the cost of analgesics, and fear of residues in meat and milk. One way of inducing analgesia with minimum risk of residues is by injecting low doses of drugs that act synergistically directly on their site of analgesic action. The spinal cord is one such place where the processing of nociceptive information is modulated and where many analgesics exert their action. In particular, the spinal *N*-methyl-D-aspartate (NMDA) / cyclooxygenase (COX) pathway seems to mediate changes in the sensitivity found in different pain states (Yaksh *et al.,* 1999). Accordingly, intrathecal (i.t.) administration of NMDA antagonists and non-steroidal anti-inflammatory drugs (NSAIDs, which inhibit COX enzymes) reduce pain hypersensitivity (Malmberg and Yaksh, 1992; Dirig *et al.,* 1998; Dolan and Nolan, 1999; Kawamata *et al.,* 2000; Park *et al.,* 2000), and the combination of both classes of drugs synergistically reduces nociceptive transmission at the spinal cord level in rats *in vitro* (Lizarraga *et al.,* 2004).

This study assessed the spinal analgesic effects of the NSAID ketoprofen and the NMDA channel blocker ketamine given alone and in combination, in sheep implanted with indwelling cervical i.t. catheters and submitted to mechanical stimulation of a foreleg.

8.3 Material and methods

Animals

Twenty-five adult (3-5 years old) Perendale ewes weighing (mean ± SD) 51.24 ± 10.26 kg were utilised in these studies. The ewes were accustomed to living indoors and to eat forage. The sheep were feed baylage and lucern chaff and were offered water *ad libitum.* On arrival, the sheep were clinically examined, and only apparently healthy sheep with no footrot were included in the experiments. The sheep were drenched with ivermectin (200 µg/kg *per os*; Ivomec® Liquid, Merial New Zealand Ltd., Manakau City, New Zealand) one week prior to any manipulation.

Intrathecal catheterisation

Catheterisation of the cervical subarachnoid space was carried out as previously described (Kyles *et al.,* 1992). Briefly, the ewes were anaesthetised with a mixture of ketamine (10 mg/kg; Ketamine Injection, Parnell Laboratories New Zealand Ltd., East Tamaki, New Zealand) and diazepam (0.5 mg/kg; Pamlin Injection, Parnell Laboratories New Zealand Ltd., East Tamaki, New Zealand) given into the right cephalic vein. Immediately after induction, the sheep were endotracheally intubated and maintained under anaesthesia with halothane vaporised in oxygen. The sheep were positioned in left lateral recumbency on a surgical table

and were instrumented to continuously monitor respiratory rate, heart rate, arterial blood pressure, oxygen saturation, end tidal halothane, and end tidal CO_2, which were recorded every 5 min throughout anaesthesia. The sheep were allowed to breath spontaneously.

Once on the table, the fleece from the neck region was clipped and the skin scrubbed with diluted chlorexhidine (Chemiscrub, Multichem Laboratories Ltd., Auckland, New Zealand) and sprayed with ethanol (Andrew Methylated Spirits, Andrew Brands Ltd., North Shore City, New Zealand). Prophylactic administration of cephazolin sodium (1 g cephazolin; Cephazolin sodium for injection, Mayne Pharma Pty. Ltd., Wellington, New Zealand) was given intravenously about 20 min before skin incision. The neck was kept flexed to facilitate Tuohy needle insertion, and the nose was kept elevated so it was level with the neck.

Under aseptic conditions, a 2-3 cm skin incision was made at the midline level of the atlanto-occipital joint. The surrounding subcutaneous tissue was dissected and a 16G Tuohy needle was inserted through the midline, approximately halfway between the occipital protuberance and the level of the wings of the atlas and directed cranially towards the tip of the nose until cerebrospinal fluid (CSF) flowed back (~3 cm deep). An 18G nylon epidural catheter (Portex Epidural Minipack System 1, Portex Ltd., Hythe, Kent, UK), previously filled with contrast media (Iohexol; Omnipaque 300, Amersham Health Pty. Ltd., North Ryde, NSW, Australia), was then passed through the needle until its tip reached the fifth cervical vertebra (C5). After catheter tip positioning was confirmed radiographically (Figure 8.1), contrast media was checked to flow back through the external catheter end, and then the needle was removed and the neck left free.

A 1 x 1 cm Yates drain tube was used to anchor the catheter at its exit from the neck muscles before tunnelling the catheter subcutaneously for about 5 cm caudally; the catheter excess was removed. The skin was sutured with 3-0 nylon (Nylene®, Dynek Pty. Ltd., Hendon, Australia) leaving the catheter and the anchoring device underneath. The remaining contrast media and some CSF were left to flow back through the catheter. A luer lock connector was attached to the distal end of the catheter and to a flat filter (0.2 μm) previously filled with sterile 0.9% saline solution. A butterfly made of strapping tape was put around the filter and it was stitched to the skin. The luer lock connector was also secured to the skin. A cotton dressing (Melolin®, Smith and Nephew Medical Ltd., Hull, England), covered on the top with strapping tape for stiffness, was stitched to the skin to cover the filter and the skin incision (Figure 8.2).

The catheters were flushed every second day with saline solution (0.9%, 500 μl), and the sheep were allowed to recover for 14 days before any experiments were conducted. Sheep that had locomotor deficits after surgery and/or reacted to flushing the catheter were excluded from the experiment.

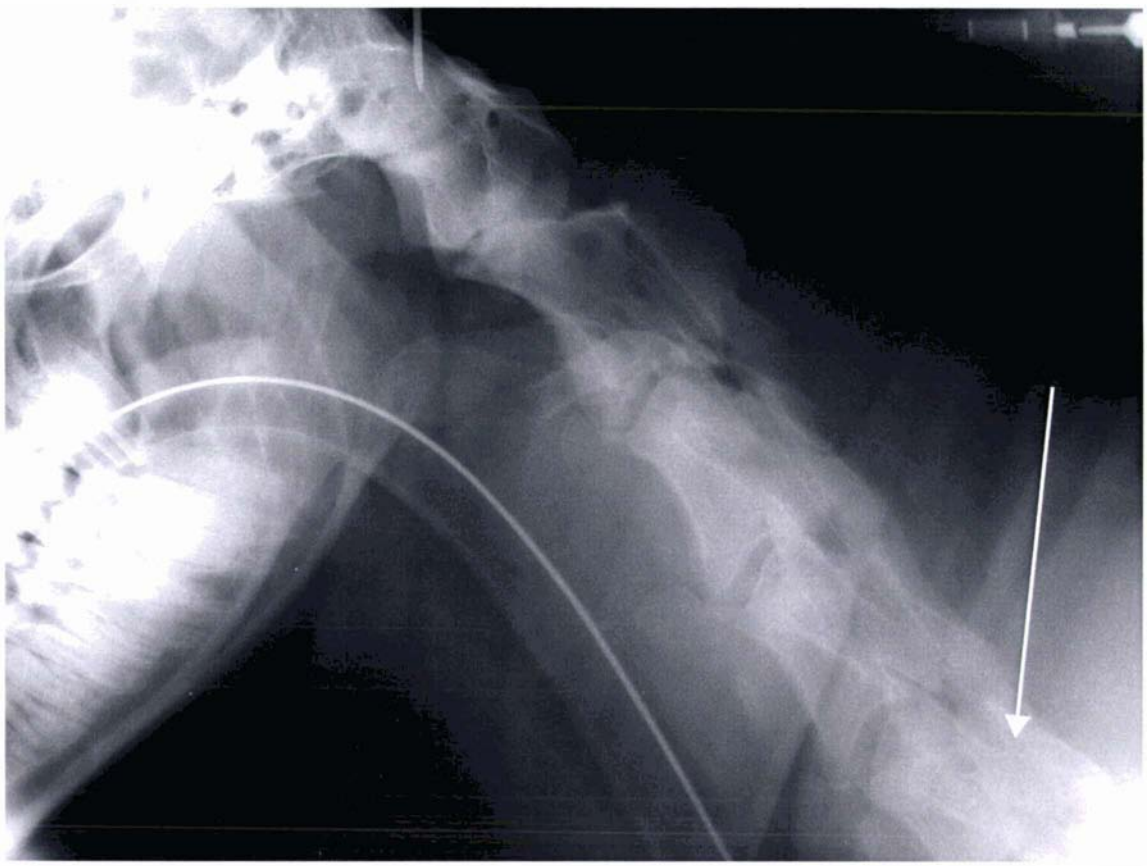

Figure 8.1 *Radiograph showing the correct placement of an intrathecal catheter at the level of the fifth cervical vertebra in sheep.*

Mechanical nociceptive testing

A light weight nociceptive device was made from a loss of resistance syringe cut at the 5 ml mark and screwed to a polyurethane piece; the latter had a Velcro strap stapled which was used to secure the device to the sheep's leg (Figure 8.3). The syringe plunger contained a 2 mm diameter, blunt-ended pin. The loss of resistance syringe was connected to a 2 mm internal diameter, 120 cm long polyethylene tube attached to a three-way stopcock connecting, through a 5 mm internal diameter, 50 cm long silicon rubber tube, to a bourdon tube dial gauge (operating range 0-160 kPa; Teltherm, Independent Vacuum Services Ltd., Auckland, New Zealand) and, through a 2 mm internal diameter, 120 cm long polyethylene tube, to a 60 ml syringe. Pressure was gradually applied into the system with the syringe, and this displaced the blunted pin towards the sheep's leg. When the sheep clearly lifted its limb the pressure was read on the dial gauge face and immediately reduced to atmospheric to terminate the stimulus. The pressure was recorded and this was considered to be the nociceptive threshold. If the sheep did not lift its leg, a cut off pressure equivalent to 20 Newtons (N) force was applied to the leg to prevent tissue damage and this value recorded.

The nociceptive device was calibrated using an electronic scale (PG5002-S Delta Range®, Mettler-Toledo (Schweiz) AG, Greifensee, Switzerland). Pressure was applied to push down

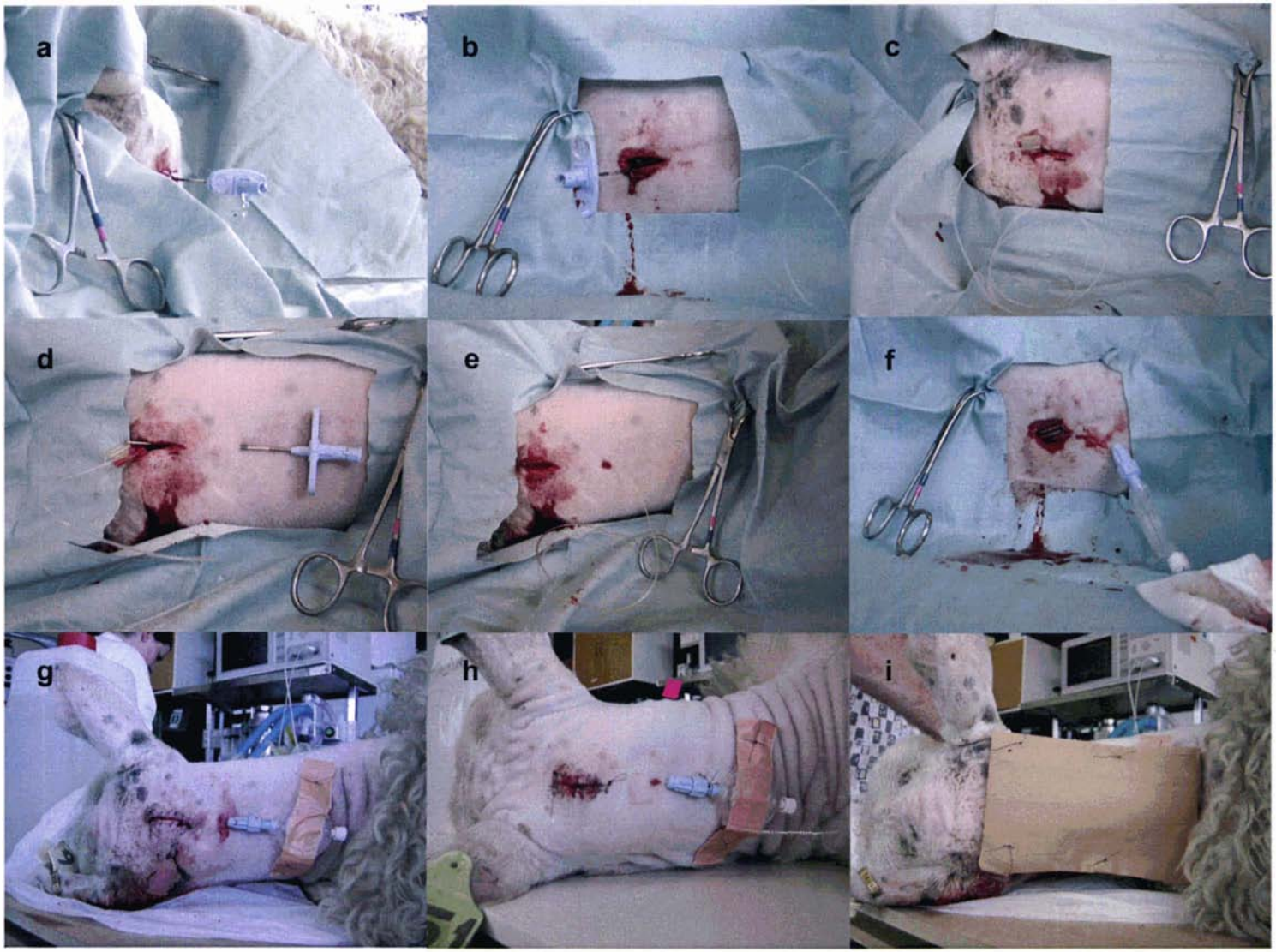

Figure 8.2 *Intrathecal catheterisation of sheep. (a) A 2-3 cm skin incision at the level of the atlanto-occpital joint was performed and through which a 16G Touhy needle was inserted into the subarachnoid space. (b) A 18G nylon catheter was advanced until its tip reached the fifth cervical vertebra. (c-e) The catheter was anchored with a piece of Yates drain tube and tunnelled subcutaneously. (f) The excess catheter was removed and a Luer lock connected the catheter with a 2 mm filter. (g-h) The skin incision was closed and a butterfly put on the filter; both the butterfly and the Luer lock were secured to the skin. (i) A dressing was stitched to the skin to cover the skin incision and the filter.*

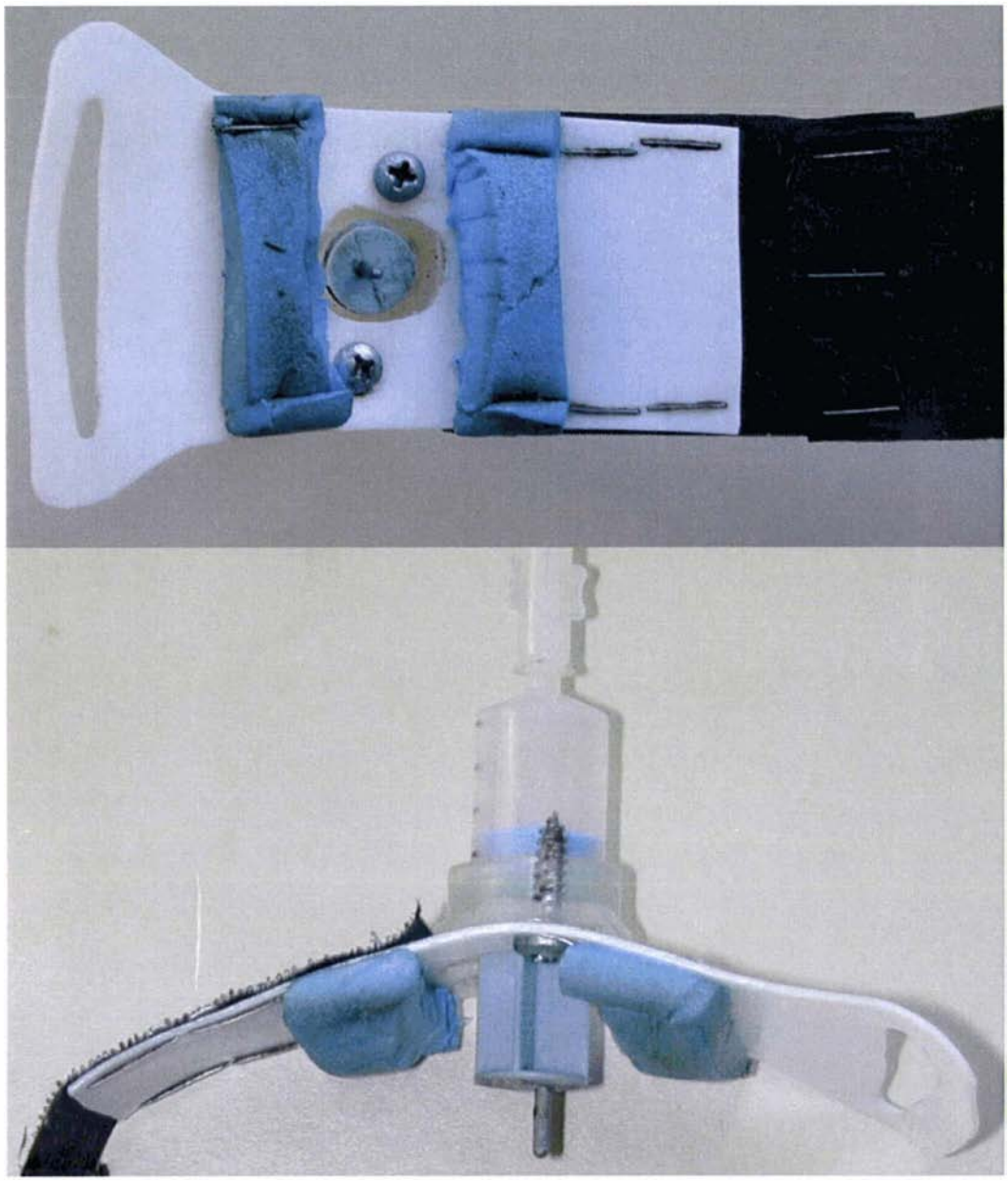

Figure 8.3 *Mechanical nociceptive device used to stimulate a foreleg in sheep. The device was constructed from a loss of resistance syringe with a blunted end 2 mm diameter pin. The syringe was screwed to a polyethylene piece stapled to a Velcro strap.*

the blunted pin against the scale's plate and both pressure (kPa) and the corresponding weight (g) were recorded; this procedure was repeated ten times. For calibration purposes 100 g was taken as equivalent as 1 N. Force was plotted against pressure and these data were analysed for linearity (GraphPad Prism v4.0b for Macintosh, GraphPad Software Inc., San Diego, CA, USA). The obtained equation was used to transform the readings taken in pressure into force values (Figure 8.4).

On experimental days, the sheep were put individually in mobile, steel crates (115 cm long x 50 cm wide x 90 cm high), which allowed the sheep to see each other, stand comfortably for up to 4 h, and permitted easy access to the sheep's legs and head. The sheep were taken

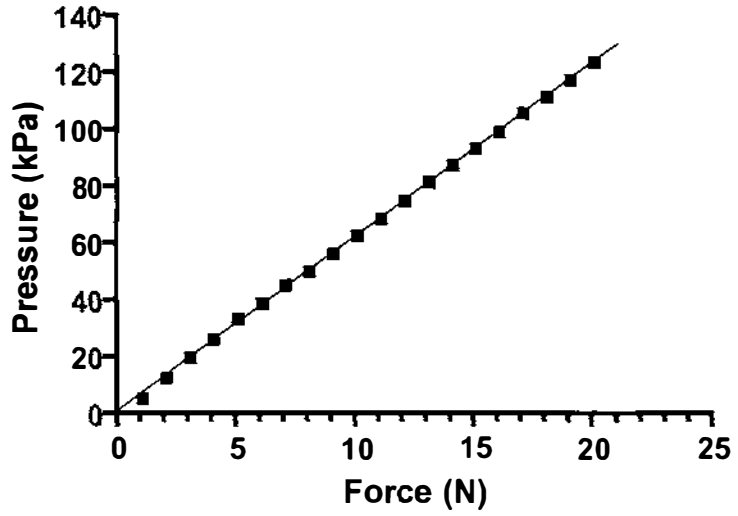

Figure 8.4 *Mechanical device calibration curve. The Y axis shows the pressure (kPa) applied to elicit the corresponding force at 1 Newton (N) intervals. y = -0.3093 + 0.1629 * x; r = 0.9988.*

to the laboratory located in a quiet room within the Massey University Large Animal Teaching Hospital. Ambient temperature in the laboratory was monitored throughout each experiment, and was between 11°C and 24°C. Temperatures below 8°C have been shown to raise nociceptive thresholds in sheep (Chambers, 1992). Animals were always kept in pairs to diminish the stress of being isolated from the flock and possible stress induced analgesia. The nociceptive device was attached to the lateral aspect of the dorsal end of the right metacarpal bone and, after a 20 min acclimation period, nociceptive testing started. Six basal consecutive readings of similar intensity were recorded at 2 min intervals before any treatment was given. After injection of the drug(s) being tested, readings were recorded every 5 min for the first hour, every 10 min for the second hour, and every 30 min for the third hour, inclusive. To minimise the possibility of drug residue interference, a minimum of 7 days was elapsed between treatments on each sheep. The operator was blinded to the drug concentrations the sheep were given, but not to the treatment they received and the xylazine injection at the end of each experiment in which no increase in nociceptive thresholds was detected with the tested drug.

Experimental design

Xylazine treatment was given first to assess the functionality of the i.t. catheters. In sheep that received more than one treatment, apart from xylazine, these were assigned as in Table 8.1. This decision was based on the lack of behavioural, motor or neurological side effects in sheep given i.t. ketoprofen at the lowest concentration used in this study (Lizarraga and Chambers, 2006), and the spinal neurotoxic potential of NMDA receptor antagonists administered i.t. to sheep (Hassenbusch *et al.,* 1999). Although no specific information on i.t. ketamine exists for sheep, data from human beings suggest that this drug may induce neurotoxic and vasotoxic effects at the spinal cord level after i.t. administration (Karpinski *et al.,* 1997; Stotz

et al., 1999; Vranken *et al.,* 2005). The ketoprofen and ketamine experiments were originally designed to construct concentration-response curves and assess the combination of both drugs for synergism. Hence, a combination of ketoprofen : ketamine was tested next.

Then, it was assessed that the concentration of NMDA used in this study enhanced pain sensitivity in sheep, as previously reported (Dolan and Nolan, 1999), to later evaluate the effects of ketoprofen and ketamine alone and in combination on the hypersensitivity induced by i.t. NMDA.

Table 8.1 *Distribution of sheep used in the experiments.*

Treatment	Sheep no.													*n*
	1	2	3	4	5	6	7	8	9	14	15	537	599	
Xylazine*	√	√	√	√	√	√	√	√	√	√	√	√	√	13
Ketoprofen	√	√	√	√	√							√		6
Ketamine		√		√		√	√		√			√		6
Ketoprofen : ketamine		√		√				√				√		4
NMDA						√	√	√	√	√	√	√		7
NMDA + treatment**						√	√	√	√		√			5

* *Only sheep used for statistical analysis were included.*
** *Refers to ketoprofen, ketamine or a combination of both.*

Treatment 1. Intrathecal xylazine

Before any other treatment was given, sheep were randomly assigned to receive xylazine (1.95 mM, equivalent to 50 µg) or saline (0.9%) in a crossover design. Xylazine in such concentration has been shown to produce mechanical hypoalgesia in sheep (Waterman *et al.,* 1988), and is the most potent known analgesic drug when given i.t. to sheep (Lizarraga & Chambers, unpublished). Therefore, the effect of xylazine on each individual sheep was considered as the maximal possible analgesic effect for that particular animal and to which all other treatments were compared.

Treatment 2. Intrathecal ketoprofen

Six sheep received ketoprofen (200, 400, 800, 1600, and 3200 µM; equivalent to 5.1 to 81.4 µg) and saline (0.9%) in a crossover, Latin square design. The lowest concentration of ketoprofen used in this study was selected on the basis that it has been used in sheep before, although with no analgesic efficacy (Lizarraga and Chambers, 2006), and that it decreased nociceptive transmission in the *in vitro* neonatal rat spinal cord preparation (Lizarraga *et al.,* 2004; Chapter 4).

Treatment 3. Intrathecal ketamine

Six sheep received ketamine (25, 50, 100, 200, and 400 µM; equivalent to 0.7 to 11 µg)

and saline (0.9%) in a crossover, Latin square design. At recovery from anaesthesia with ketamine, similar drug concentrations in the CSF of sheep have been reported (Waterman and Livingston, 1978). The two lower concentrations of ketamine used in this study have also been shown to depress nociceptive transmission in the *in vitro* neonatal rat spinal cord preparation (Lizarraga *et al.,* 2004; Chapter 4).

Treatment 4. Intrathecal ketoprofen and ketamine

Four sheep were given a combination of ketoprofen and ketamine (837.695, 1675.39, and 3350.78 μM) in a 0.955:0.045 proportion and saline (0.9%). This proportion was synergistic at decreasing nociceptive transmission in the *in vitro* neonatal rat spinal cord preparation (Lizarraga *et al.,* 2004; Chapter 4).

Treatment 5. Intrathecal NMDA

Seven sheep were randomly injected NMDA (2 mM, equivalent to 29.42 μg) and saline (0.9%) in a crossover design. This concentration of NMDA was chosen from a previous study demonstrating that it produced mechanical hypersensitivity in sheep (Dolan and Nolan, 1999).

Treatment 6. Intrathecal NMDA followed by ketamine, ketoprofen, and ketamine plus ketoprofen

Five sheep were allocated to receive the following treatments in a crossover, Latin square design:

1) Saline (0.9%) followed 10 min later by saline (0.9%).
2) Saline (0.9%) followed 10 min later by NMDA (2 mM).
3) Ketoprofen (3200 μM) followed 10 min later by NMDA (2 mM).
4) Ketamine (400 μM) followed 10 min later by NMDA (2 mM).
5) Ketoprofen (3200 μM) plus ketamine (150.78 μM) followed 10 min later by NMDA (2 mM).

Drug concentrations for these experiments were chosen as for treatments 2 to 5 in the current study.

All treatments were administered through the i.t. catheter and were given in a 100 μl volume. Drugs injected in such a volume mainly distribute 2 to 3 vertebral segments at either side of the catheter tip (Waterman *et al.,* 1988; Kyles *et al.,* 1993). Drugs were flushed-in with 350 μl of saline solution (0.9%), which was the volume of the system. If no apparent increase in nociceptive thresholds was noticed by the end of each experiment, xylazine (1.95 mM) was injected followed by 350 μl of saline solution (0.9%) and nociceptive thresholds recorded

for 20 min. This was done to confirm that data were always collected using functional catheters. All treatments were sterile filtered (4 mm filter unit, 0.2 µm, Phenomenex® (NZ) Ltd., Auckland, New Zealand) to minimise the risk of bacterial contamination. Nociceptive thresholds were measured as above for 3 h for treatments 1 to 4, and for 1 h for treatments 5 and 6.

Behavioural effects

The behavioural effects produced by any treatment were subjectively assessed during mechanical nociceptive testing. 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.

Drugs

Stock solutions of xylazine (1.95 mM), ketoprofen (10 mM), ketamine (1 mM) and NMDA (2 mM) were kept at 4°C. All drugs were diluted in saline solution (0.9%) as required, and ketoprofen was also diluted in NaOH (maximum concentration given: 3.58 mM). All drugs were obtained from Sigma (St. Louis, MO, USA).

Statistical analysis

Unless indicated, data were expressed as mean ± s.e.m. The areas under the threshold *vs.* time curve values for 30, 60, and 180 min (AUC_{30}, AUC_{60} and AUC_{180}, respectively) for individual sheep (with the mean baseline subtracted) were calculated as pertinent using the trapezoidal method. This gives an estimate of the total effect of the drug in a single figure, although a graph of threshold plotted against time gives useful information on peak effects and duration of action, and these graphs were included for visual reference. Data were analysed for normality using the D'Agostino-Pearson omnibus K2 normality test. Student *t* test and one-way ANOVA followed by Tukey's *post-hoc* tests were used as appropriate to compare AUC values. The percentage maximal possible analgesic effect for treatments 2 to 4 in each sheep was calculated using AUC_{60} and AUC_{180} values, and then contrasting them with corresponding individual AUC_{60} and AUC_{180} values with xylazine, respectively. Data from the resulting concentration-effect plots were subjected to linear regression analysis. GraphPad Prism was used for these purposes. Differences with $P < 0.05$ were considered significant.

8.4 Results

Intrathecal catheterisation

Table 8.2 summarises the results of i.t. catheterisation in sheep. In two sheep (11 and 13)

no CSF was obtained through the Tuohy needle, in the remaining 23 sheep CSF flowed back through the needle and a catheter was implanted at the C5 level. The mean time of anaesthesia (± SD) was 90.37 (± 22.05) min.

Table 8.2 *Summary of cervical intrathecal catheterisation and effects of xylazine (1.95* μM*) injected though the intrathecal catheter on nociceptive mechanical thresholds in healthy sheep.*

Sheep no.	Effect of xylazine	Persistence (days) (dates)	Comments
1	↑	64 (17/01/05 – 25/03/05)	Sheep pulled out the catheter
2	↑	166 (17/01/05 – 2/07/05)	Sheep pulled out the catheter
3	↑	72 (18/01/05 – 31/03/05)	Sheep pulled out the catheter
4	↑	154 (17/01/05 – 20/06/05)	Sheep pulled out the catheter
5	↑	72 (14/02/05 – 27/04/05)	Sheep pulled out the catheter
6	↑	130 (21/03/05 – 29/07/05)	Catheter was removed
7	↑	130 (21/03/05 – 29/07/05)	Catheter was removed
8	↑	127 (24/03/05 – 29/07/05)	Catheter was removed
9	↑	127 (24/03/05 – 29/07/05)	Catheter was removed
10	N/T	9 (24/03/05 – 2/04/05)	Killed / meningitis
11	N/T	0 (22/04/05)	Unsuccessful catheterisation. Quadraparesis after surgery. Killed (23/04/05)
12	N/T	16 (22/04/05 – 10/05/05)	Ataxia after surgery. Reacted to flushing the catheter. Catheter removed
13	N/T	0 (22/04/05)	Unsuccessful catheterisation
14	↑	71 (19/05/05 – 29/07/05)	Catheter was removed
15	↑	71 (19/05/05 – 29/07/05)	Catheter was removed
46	N/T	16 (22/11/04 – 8/12/04)	Killed / meningitis
275	↑*	20 (23/11/04 – 13/12/04)	Dead / meningitis
537	↑	204 (24/11/04 – 16/06/05)	Sheep pulled out the catheter
597	N/T	20 (21/12/04 – 10/01/05)	Ataxia after surgery. Reacted to flushing the catheter / catheter was removed. Killed (11/01/05)
599	↑	35 (21/12/04 – 25/01/05)	Sheep pulled out the catheter. Unsuccessful re-catheterisation on 10/02/05
603	N/T	20 (21/12/04 – 10/01/05)	Ataxia after surgery. Reacted to flushing the catheter / catheter was removed
687	N/C	38 (23/11/04 – 31/12/04)	Sheep pulled out the catheter
851	N/C	51 (24/11/04 – 14/01/05)	Catheter was removed
1233	N/T	5 (21/03/05 – 26/03/05)	Ataxia after surgery. Reacted to flushing the catheter / catheter was removed
2003	N/C	22 (22/11/04 – 14/12/04)	Ataxia after surgery. Catheter was removed

↑ = *increased nociceptive thresholds; N/T = not tested; N/C = no change; * = not used for statistical analysis.*

Four sheep (12, 597, 603, 1233) exhibited locomotor disorders after catheter implantation, which progressively improved and recovered after about 4-6 days. However, these four sheep also exhibited signs of discomfort every time the catheter was flushed. The signs lasted up to 5 min, and duration and intensity of the signs seemed to be proportional to the injected volume. The signs included tonic contraction of the fore limbs, neck extension, lateral recumbency and abrupt moving of all limbs, attempts of scratching the neck with a hind limb, and rubbing of the head and neck against other sheep. These sheep were excluded from the trial. Histopathological examination of the spinal cord from the first sheep displaying these behaviours (597) showed that the catheter was not implanted into the subarachnoid

space, but rather into the ventrolateral spinal cord white matter (Figure 8.5). The catheters were removed from the other three sheep, and the animals seemed to be normal afterwards.

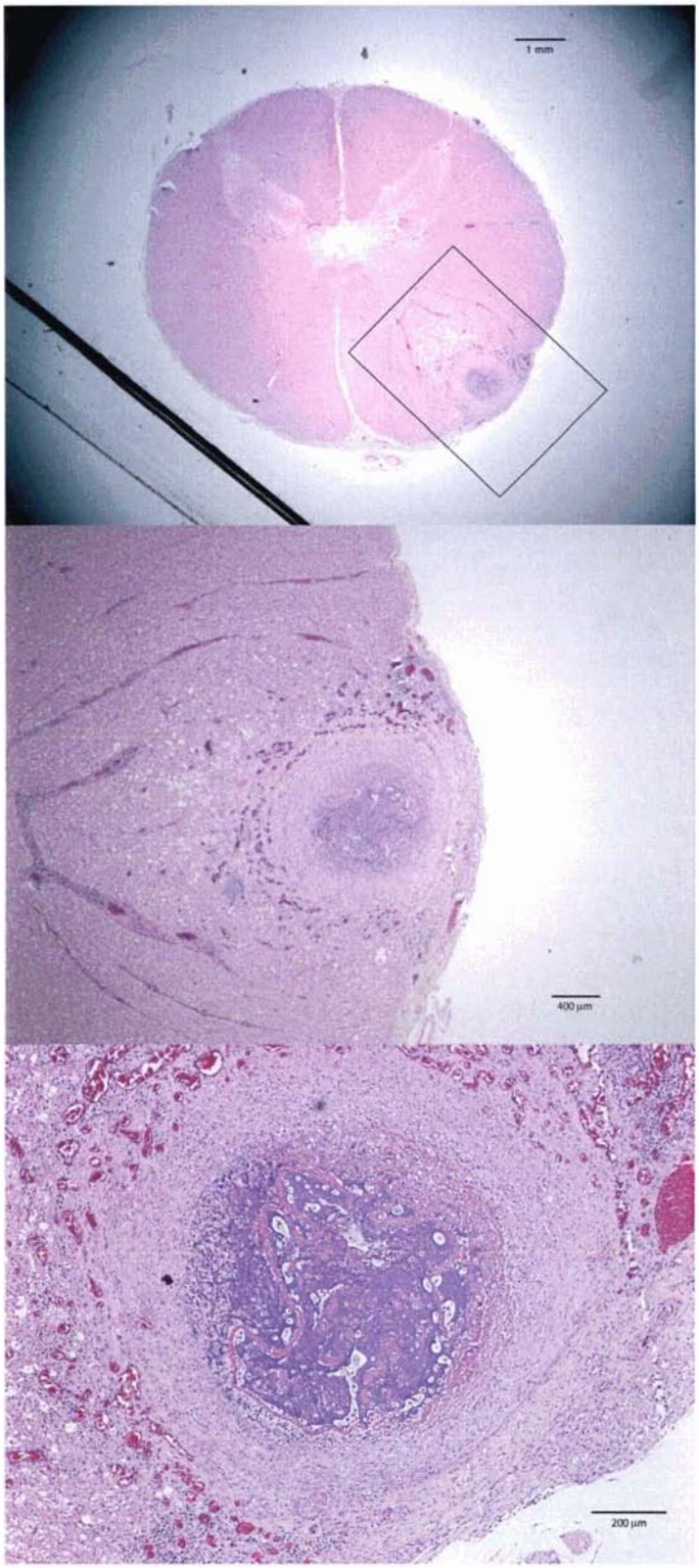

Figure 8.5 *Transverse sections of the cervical spinal cord of sheep 597 showing histopatological changes associated with penetration of the catheter into the spinal parenchyma. A 850 µm diameter, circular mass of granular amorphous debris admixed with moderate numbers of entrapped neutrophils, located in the ventrolateral white matter, and adjacent to the ventral horn. This mass is surrounded by a 400 µm thick fibrous capsule, the lateral boundary of which extends to the meningeal surface. The adjacent white matter is markedly spongiotic and irregularly replaced by sheets of macrophages, most of which contain large amounts of lacy to granular, light eosinophilic cytoplasm (Gitter cells), and proliferating, small blood vessels, and scattered glial cells. Aggregates of lymphocytes and plasma cells are located predominantly perivascularly, and swollen, eosinophilic axons are scattered throughout. Elsewhere, the central white matter is irregularly spongiotic, with dilation of myelin sheaths and infiltration with Gitter cells. There is irregular, mild neuronal degeneration (chromatolysis) in the adjacent ventral horn, with bilateral rarefaction and oedema of the grey matter in the dorsal horns.*

Two sheep (10, 46) were killed due to meningitis. Histopathological examination of the brain and the cervical spinal cord showed changes consistent with severe, acute, fibronosuppurative meningoencephalitis (Figure 8.6). It was not possible to identify the infectious microorganism(s) due to sample contamination.

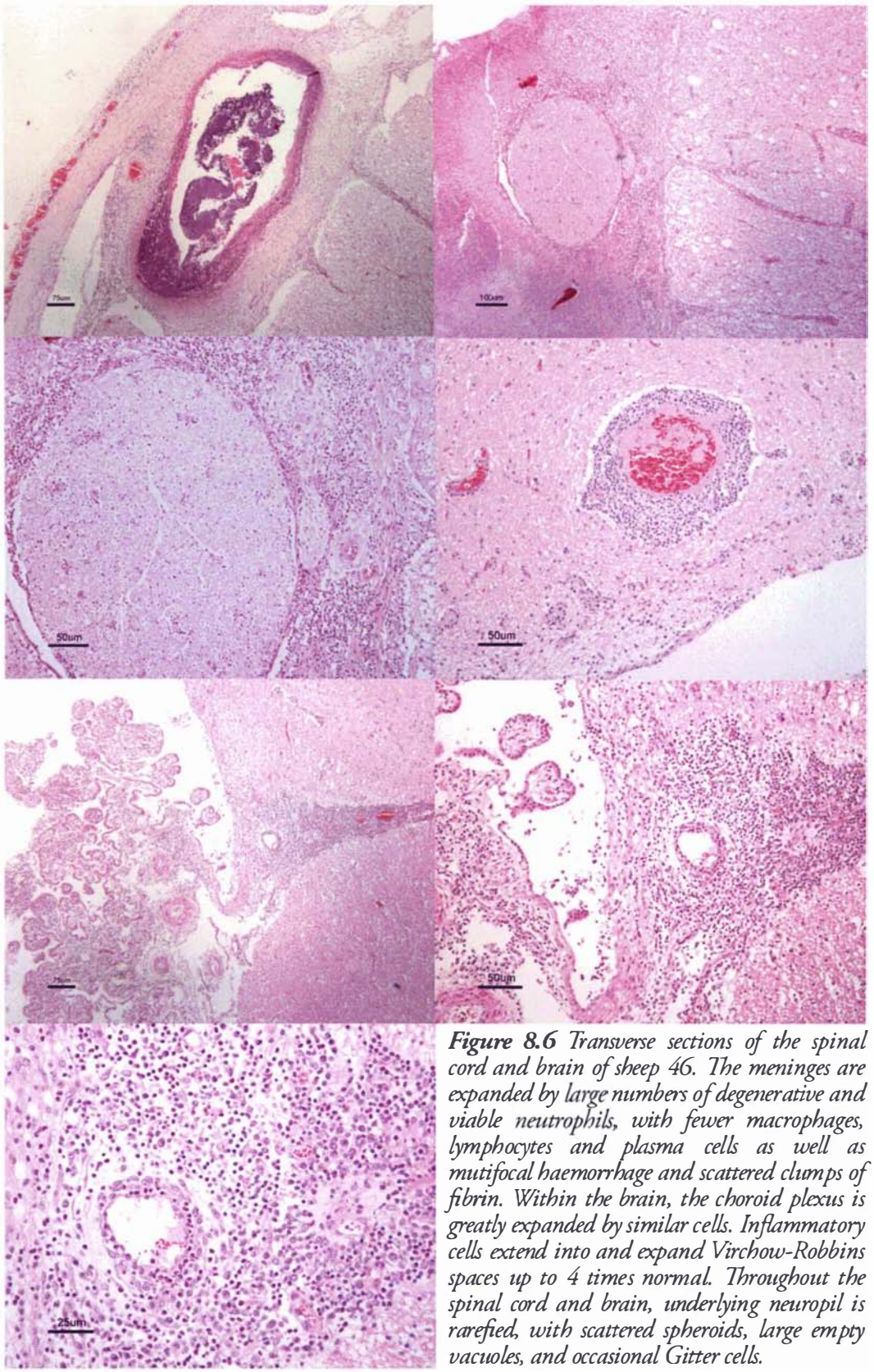

Figure 8.6 *Transverse sections of the spinal cord and brain of sheep 46. The meninges are expanded by large numbers of degenerative and viable neutrophils, with fewer macrophages, lymphocytes and plasma cells as well as mutifocal haemorrhage and scattered clumps of fibrin. Within the brain, the choroid plexus is greatly expanded by similar cells. Inflammatory cells extend into and expand Virchow-Robbins spaces up to 4 times normal. Throughout the spinal cord and brain, underlying neuropil is rarefied, with scattered spheroids, large empty vacuoles, and occasional Gitter cells.*

The mean persistence (± SD) for functional catheters (those through which xylazine induced hypoalgesia, n = 13) in this study was 109.5 (± 49.12) days.

Intrathecal xylazine

Xylazine was tested in 17 sheep. Three sheep (687, 851, 2003) showed no increase on nociceptive thresholds after injection of xylazine and were excluded from the trial as the catheter tip was assumed to be in the wrong place. In the remaining 14 sheep, xylazine increased nociceptive thresholds, but data from sheep 275 was excluded from the analysis since she died 4 days after xylazine injection. Histological changes were consistent with severe, acute, fibronosuppurative meningoencephalitis.

Xylazine produced mechanical hypoalgesia in sheep as its AUC values were significantly larger than those for saline (AUC_{60}: 629.0 ± 45.01 *vs.* 16.57 ± 3.67, $P < 0.0001$; AUC_{180}: 1510 ± 168.7 *vs.* 45.19 ± 13.48, $P < 0.0001$). However, there was marked variation in AUC values among individual sheep (Figure 8.7)

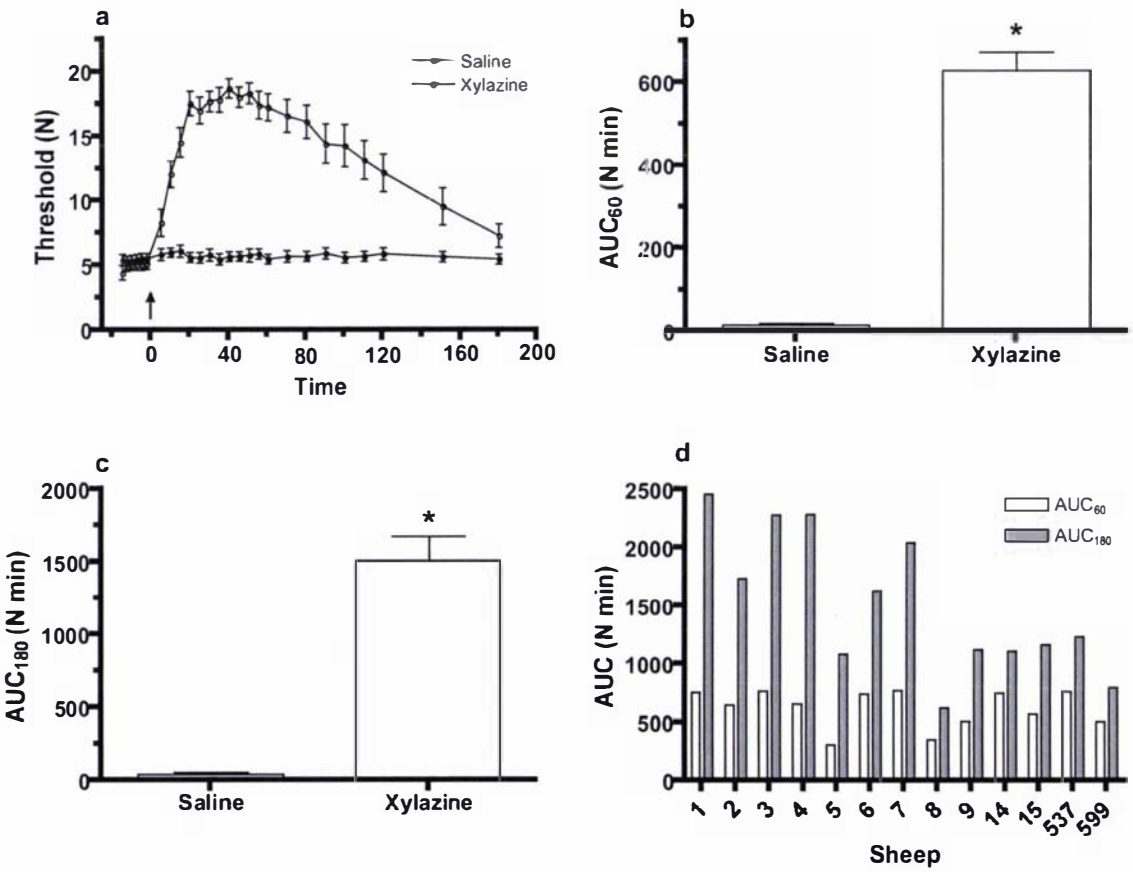

Figure 8.7 *(a) Time course showing the effect of intrathecal administration of xylazine (1.95 μM, 100 μl) and saline (0.9%, 100 μl) on mechanical nociceptive thresholds in sheep. The arrow represents the time treatments were given. (b-d) Xylazine induced hypoalgesia as assessed by the area under the curve (AUC) for 60 min (b) and 180 min post-treatment (c), but there was marked variation in individual AUC values (d). * Represents significant difference between xylazine and saline (P < 0.0001). Data are the mean ± s.e.m. of 13 sheep.*

Intrathecal ketoprofen

At the concentrations tested, ketoprofen did not significantly modify the AUC_{60} and AUC_{180} as compared to saline (Figure 8.8). Plotting of AUC_{60} and AUC_{180} values for ketoprofen concentrations against to their corresponding values for xylazine for individual sheep, yielded slopes not significantly different to zero ($P \geq 0.1134$, Figure 8.8).

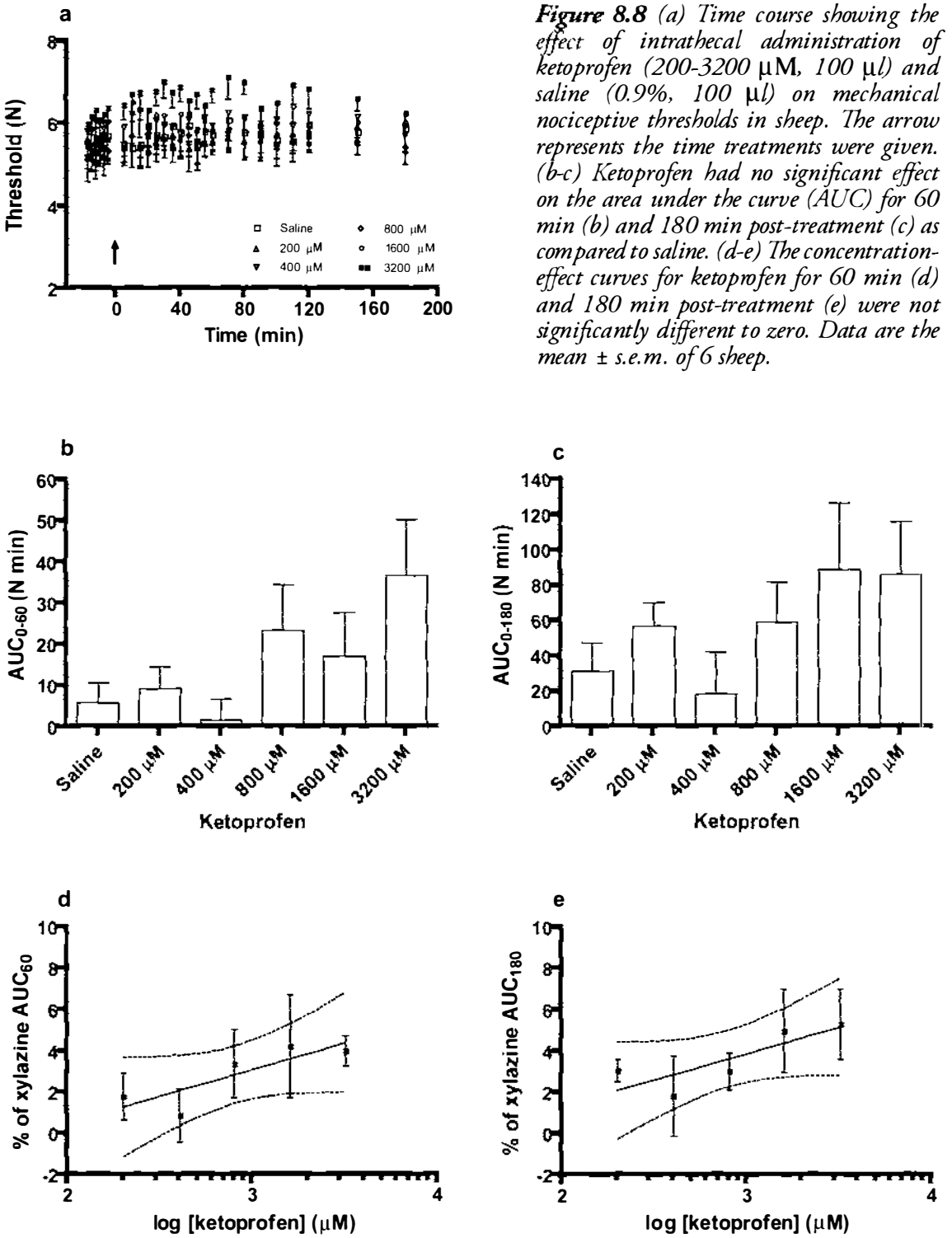

Figure 8.8 *(a) Time course showing the effect of intrathecal administration of ketoprofen (200-3200 μM, 100 μl) and saline (0.9%, 100 μl) on mechanical nociceptive thresholds in sheep. The arrow represents the time treatments were given. (b-c) Ketoprofen had no significant effect on the area under the curve (AUC) for 60 min (b) and 180 min post-treatment (c) as compared to saline. (d-e) The concentration-effect curves for ketoprofen for 60 min (d) and 180 min post-treatment (e) were not significantly different to zero. Data are the mean ± s.e.m. of 6 sheep.*

Intrathecal ketamine

Similar to ketoprofen alone, ketamine did not significantly change the AUC_{60} and AUC_{180} as compared to saline (Figure 8.9). The maximal possible analgesic effect of ketamine concentrations for AUC_{60} and AUC_{180} values gave slopes not significantly different to zero ($P \geq 0.6835$, Figure 8.9).

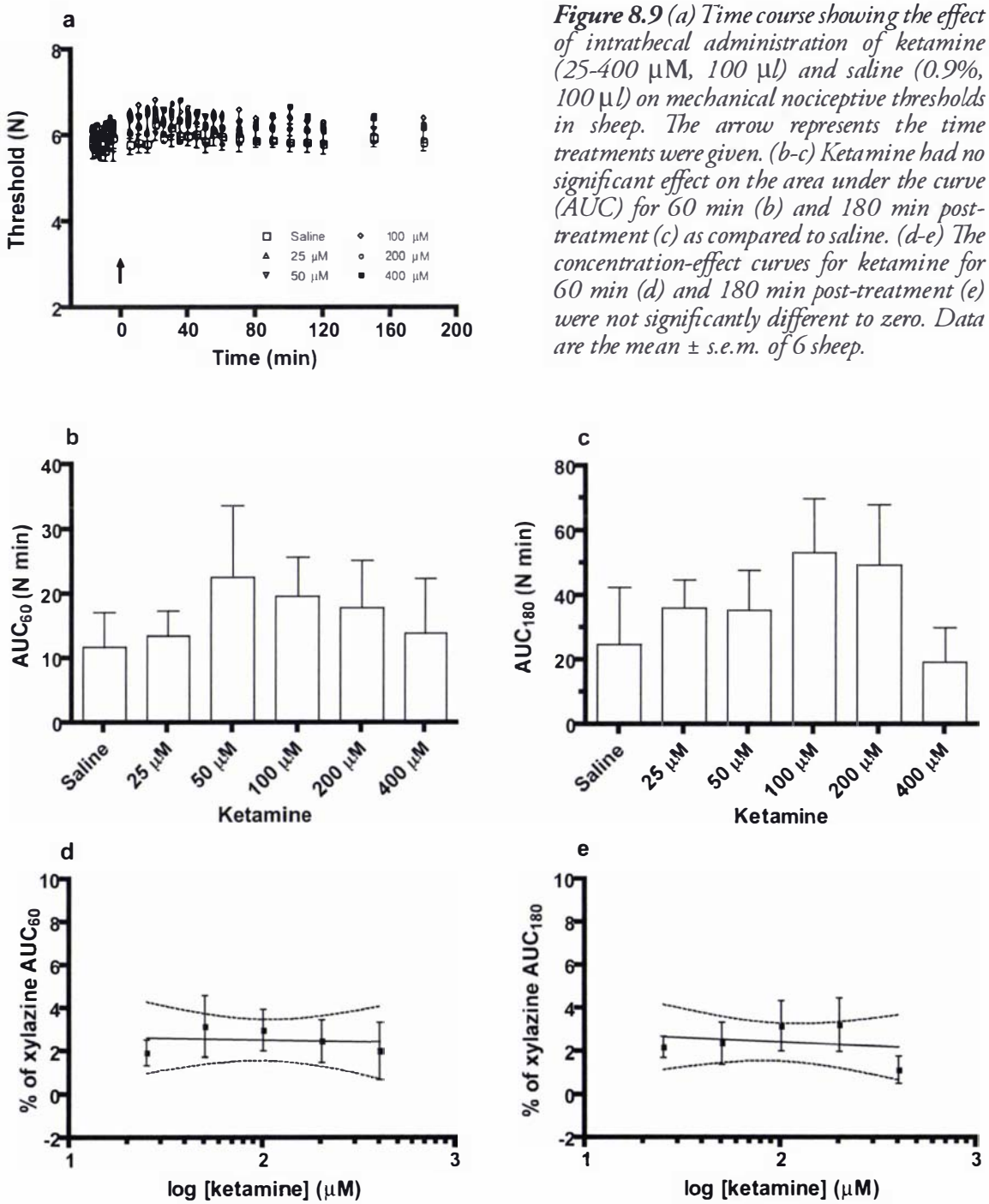

Figure 8.9 *(a) Time course showing the effect of intrathecal administration of ketamine (25-400 μM, 100 μl) and saline (0.9%, 100 μl) on mechanical nociceptive thresholds in sheep. The arrow represents the time treatments were given. (b-c) Ketamine had no significant effect on the area under the curve (AUC) for 60 min (b) and 180 min post-treatment (c) as compared to saline. (d-e) The concentration-effect curves for ketamine for 60 min (d) and 180 min post-treatment (e) were not significantly different to zero. Data are the mean ± s.e.m. of 6 sheep.*

Intrathecal ketoprofen and ketamine

Analysis of variance on AUC_{60} values for the combination ketoprofen and ketamine showed a significant difference between treatments ($P = 0.0144$). The highest ketoprofen and ketamine concentration gave AUC_{60} values significantly lower than those of lower concentrations ($P < 0.05$), but no significant differences were found between ketoprofen and ketamine concentrations and saline ($P > 0.05$). There were no significant differences for AUC_{180} values ($P = 0.1569$). The slopes for the maximal possible analgesic effect for both AUC_{60} and AUC_{180} values were not significantly different to zero ($P \geq 0.0683$, Figure 8.10).

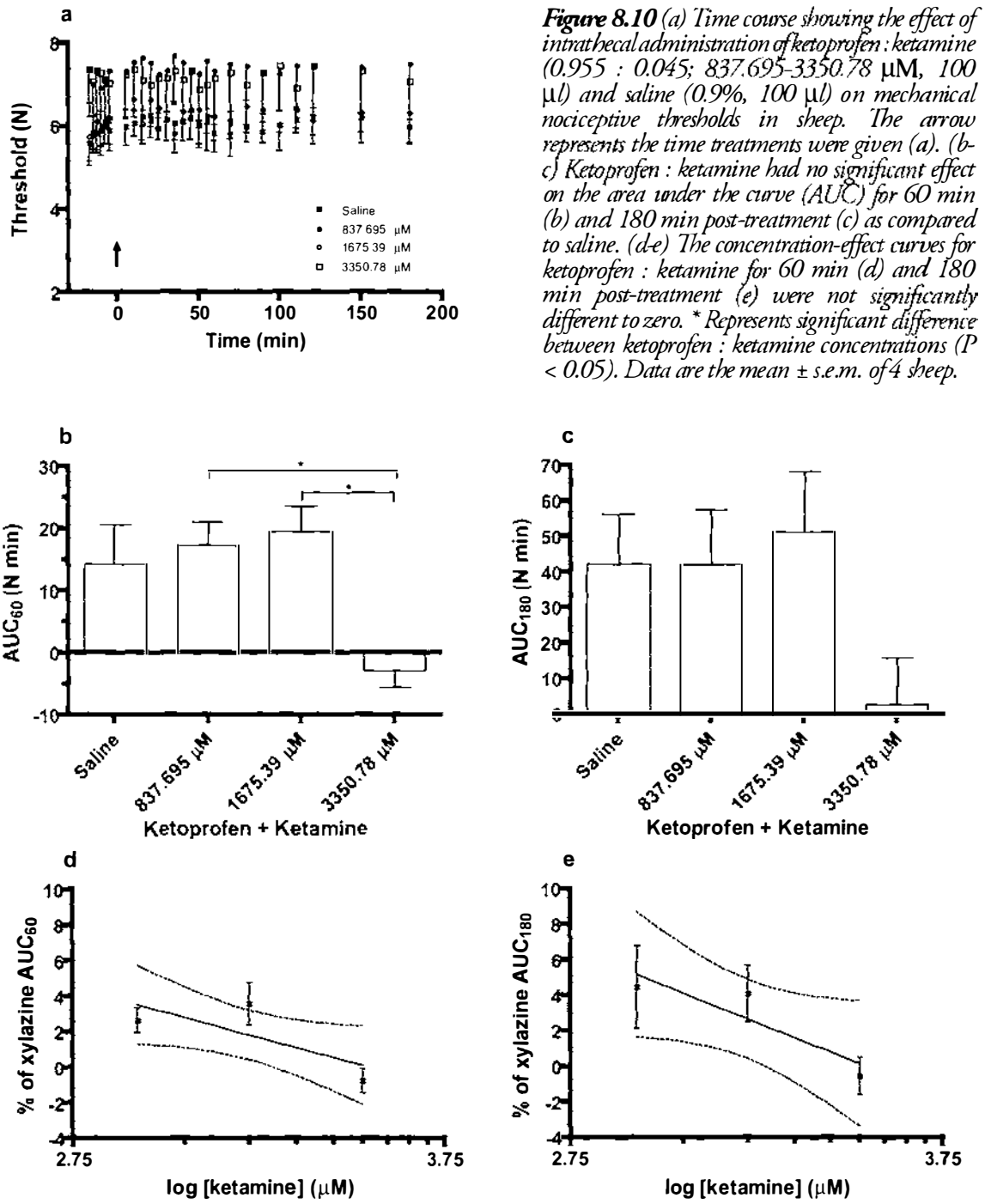

Figure 8.10 *(a) Time course showing the effect of intrathecal administration of ketoprofen : ketamine (0.955 : 0.045; 837.695-3350.78* µM, *100* µl*) and saline (0.9%, 100* µl*) on mechanical nociceptive thresholds in sheep. The arrow represents the time treatments were given (a). (b-c) Ketoprofen : ketamine had no significant effect on the area under the curve (AUC) for 60 min (b) and 180 min post-treatment (c) as compared to saline. (d-e) The concentration-effect curves for ketoprofen : ketamine for 60 min (d) and 180 min post-treatment (e) were not significantly different to zero. * Represents significant difference between ketoprofen : ketamine concentrations (P < 0.05). Data are the mean ± s.e.m. of 4 sheep.*

Intrathecal NMDA

Figure 8.11 shows the depressive action of NMDA on mechanical nociceptive thresholds, which returned to basal levels by 30 min post-administration. In comparison to saline values, AUC_{30} values for NMDA were significantly lower (7.64 ± 1.94 *vs.* -22.41 ± 2.90, $P < 0.0001$).

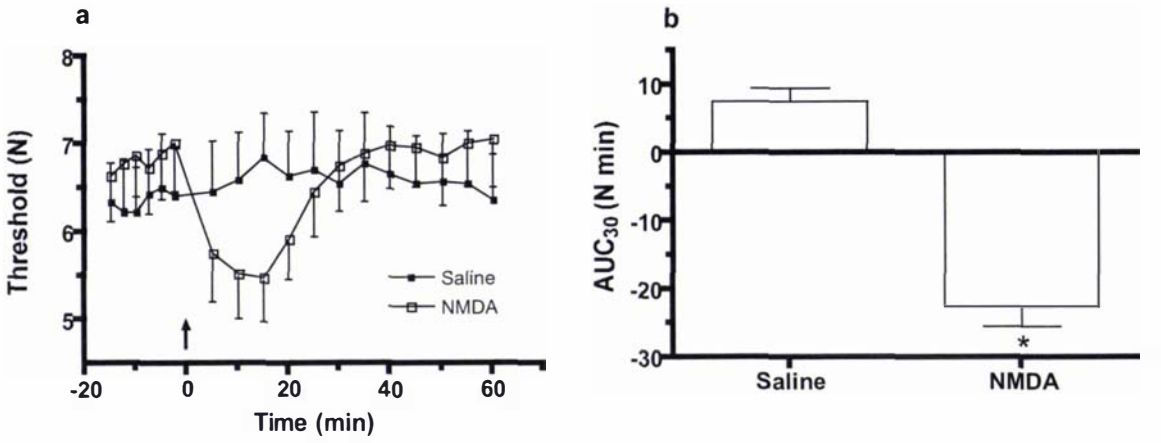

Figure 8.11 *(a) Time course showing the effect of intrathecal administration of NMDA (2 mM, 100 μl) and saline (0.9%, 100 μl) on mechanical nociceptive thresholds in sheep. The arrow represents the time treatments were given. (b) NMDA significantly reduced the area under the curve (AUC) for 30 min post-treatment as compared to saline. * Represents significant difference between NMDA and saline (P < 0.0001). Data are the mean ± s.e.m. of 7 sheep.*

Intrathecal NMDA followed by ketamine, ketoprofen, and ketamine plus ketoprofen

Analysis of variance showed a significant effect of treatment on AUC_{30} values ($P = 0.0010$). As before, NMDA significantly decreased the AUC_{30} as compared to saline (4.61 ± 2.44 *vs.* -24.23 ± 1.30, $P < 0.001$), and this enhanced pain sensitivity was prevented by either ketoprofen (-4.78 ± 6.69, $P < 0.05$), ketamine (-3.88 ± 5.58, $P < 0.05$), or a combination of both (0.22 ± 1.42, $P < 0.01$) (Figure 8.12).

Every time that xylazine was i.t. injected at the end of an experiment, it markedly raised nociceptive thresholds (data not shown).

Behavioural effects

No signs of distress, excessive salivation, altered chewing, changes on movement patterns, head positioning, and respiratory patterns were observed after any treatment.

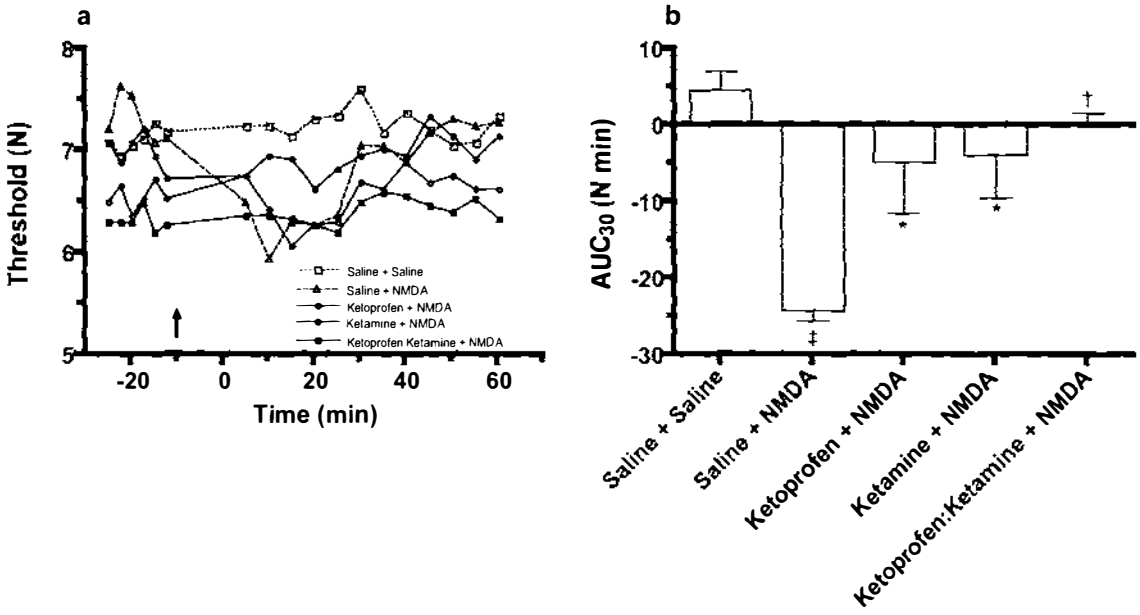

Figure 8.12 *(a) Time course showing the effect of intrathecal administration of saline (0.9%, 100 µl), ketoprofen (3200 µM, 100 µl), ketamine (400 µM, 100 µl), and ketoprofen : ketamine (0.955 : 0.045; 3350.78 µM, 100 µl) followed by NMDA (2 mM, 100 µl) on mechanical nociceptive thresholds in sheep. The arrow represents the time treatments were given before administering NMDA (time 0). (b) NMDA significantly reduced the area under the curve (AUC) for 30 min post-treatment as compared to saline, and this enhanced pain sensitivity was prevented by ketoprofen, ketamine, and a combination of both. * and † Represent significant differences between treatment and saline + NMDA (P < 0.05 and P < 0.01, respectively), ‡ represents significant difference between treatment and saline + saline (P < 0.001). Data are the mean ± s.e.m. of 5 sheep; s.e.m. are not shown in (a) for clarity.*

8.5 Discussion

This study demonstrated that despite inducing no mechanical hypoalgesia, ketoprofen and ketamine alone or in combination prevented the mechanical hypersensitive action of NMDA after i.t. administration in sheep.

Intrathecal catheterisation

Catheterisation of the subarachnoid space is a well-established technique in sheep (Kyles *et al.,* 1992). In this study, placement of funcitional i.t. catheters was achieved 52% of the attempts, which was similar to previously reported values: 58.82% (Kyles *et al.,* 1992) and 54.84% (Lizarraga, 2000). However, the time that catheters remained functional for in this study (110 days) was in between previous published times: ~72 days (Lizarraga, 2000) and ~210 days (Kyles *et al.,* 1992). The reasons for the great variability on persistency of functional catheters are not known. Over 30% of sheep in the current study pulled out their catheters at different post-catheterisation times (Table 8.2). This happened despite catheters producing no obvious discomfort to sheep and being anchored subcutaneously. Firmer anchoring techniques may result in prolonged persistency of functional i.t. catheters.

Since catheters were placed into the spinal canal, the risk of infection through this route was always present. Fibrinosupurative meningitis occurred in 12% of sheep. This happened despite catheter implantation being carried out under standard aseptic conditions and after a single, prophylactic dose of cephazolin, a broad-spectrum antibiotic that penetrates the blood-brain barrier (Radouane *et al.,* 1996). Postoperative wound infection and development of meningitis have previously been noticed in sheep following i.t. cervical catheterisation (Kyles *et al.,* 1992; Lizarraga, 2000). Similarly, bacterial contamination was detected in 18% of the needles used for spinal-epidural anaesthetic procedures in humans, even after aseptic skin preparation (Raedler *et al.,* 1999). Although strict aseptic procedures are followed, it seems that the risk of inducing infection by introducing a foreign body into the spinal canal is always present, and this has to be considered when using these techniques. A course of treatment with antibacterial drugs immediately after catheter implantation may be limited by the potential alteration of microorganisms in the rumen of sheep.

Another complications after catheter implantation were signs of discomfort experienced by 16% of sheep every time that their catheters were flushed. In one of these sheep, it was confirmed that the catheter was unintentionally implanted into the spinal cord parenchyma. Based on the similarity of signs experienced by the other sheep, it is highly likely that their catheters were also misplaced within the spinal cord. Misplacement of an i.t. infusion system into the spinal cord parenchyma in a human being induced intractable pain, which decreased by reducing the pump flow and was resolved when the pump was turned off (Huntoon *et al.,* 2004). The authors suggested that a pressure/volume effect may have induced these symptoms, and this is consistent with duration and intensity of signs in sheep being related to the injected volume. Migration of the catheter from the subarachnoid space into the spinal cord parenchyma seems very unlikely; it would appear that the catheters were misplaced within the spinal cord in the first place. This was despite obtaining good CSF flow from the distal catheter tip. If a sheep shows signs of discomfort while and soon after flushing the catheter, this may be indicative of misplacement of the catheter into the spinal cord parenchyma and removal of the catheter is recommended.

Intrathecal xylazine

As expected (Waterman *et al.,* 1988), i.t. administration of xylazine (1.95 mM; 100 µl) produced mechanical hypoalgesia in sheep, confirming that the catheters were functional. However, the intensity and duration of hypoalgesia, as assessed by the AUC_{60} and AUC_{180}, varied in individual sheep. This has been previously demonstrated in sheep given xylazine intravenously (25 µg/kg), but not i.t. (3.9 mM; 100 µl) (Kyles *et al.,* 1993). These data suggest that at higher concentrations than the one used here, xylazine may produce homogeneous degree of analgesia amongst sheep after i.t. administration. It is possible that the number of receptors available for the drug to exert its effects, the catheter tip positioning within the

i.t. space (*i.e.* dorsal, ventral, lateral), and the spread of the drug through the neuraxis may contribute to the individual variation to i.t. xylazine (Hocking and Wildsmith, 2004; Yaksh, 2005).

Xylazine is an α_2-adrenoceptor agonist (Kyles *et al.*, 1993) and may have exerted its hypoalgesic effects by blocking presynaptic α_2-adrenoceptors, hence reducing neurotransmitter and/or neuropeptide release from primary afferent terminals. However, at the concentration used in this study, it is possible that part of the hypoalgesic action may be due to a local anaesthetic effect (Chambers, 1993), though no motor effects were observed.

In 18% of sheep on which xylazine was tested, no mechanical hypoalgesia was detected. The reason for the lack of analgesia was not investigated, but it is known that i.t. catheters in sheep can move or break and leak (Kyles *et al.*, 1992). Once removed, water was flushed through to check the integrity of these catheters, and no leak was observed. It is possible that catheters may have pulled out a bit retracting the catheter tip from C5 and thus not permitting proper drug distribution through the intended site of action in the neuraxis.

Intrathecal administration of xylazine produced no signs of sedation, which suggests that the drug did not spread to supraspinal centres. Radiolabelled compounds injected into the cervical subarachnoid space at the same volume used in the current study showed that the bulk of radioactivity was distributed within 2 to 3 spinal segments either side from the catheter tip (Waterman *et al.*, 1988; Kyles *et al.*, 1993). However, hydromorphone was detected in CSF collected from the cisterna magna of sheep after lumbar i.t. administration (Payne *et al.*, 1996). In all these studies, drugs were given in a 100 µl volume. However, factors other than volume also affect the distribution of drugs after i.t. administration (Hocking and Wildsmith, 2004). Among them, the physical properties (*i.e.* density, specific gravity, baricity, viscocity, lipophilicity) and the concentration of the drug may have influenced the spread of drugs in this study, but this was not investigated.

Intrathecal ketoprofen and ketamine alone

When administered through the i.t. route, both NSAIDs and NMDA receptor antagonists produced no analgesia in animal models of acute pain (Malmberg and Yaksh, 1992; Kawamata *et al.*, 2000), including mechanical stimulation in sheep (Dolan and Nolan, 1999; Lizarraga and Chambers, 2006). This was consistent with ketoprofen and ketamine not increasing nociceptive thresholds in the current study. Previously, ketoprofen was shown to not increase mechanical nociceptive thresholds in sheep when administered in cumulative i.t. concentrations (0.75-200 µM) and each concentration studied for 30 min (Lizarraga and Chambers, 2006). To investigate the possibility that subanalgesic concentrations were injected in that study or that not enough time was allowed for the drug concentrations to

exert their effect, in the current study higher concentrations of ketoprofen (200-3200 μM) were administered individually in separate occasions, and nociceptive thresholds were assessed for a longer period of time (180 min). This study also showed no change on mechanical nociceptive thresholds, which gives further support to the notion that ketoprofen seems to produce no direct spinal hypoalgesic effects in sheep (Lizarraga and Chambers, 2006).

The possibility that mechanical stimulation may not be a sensitive enough technique to detect the hypoalgesic action of NSAIDs in sheep is unlikely since flunixin (2.2 mg/kg), dipyrone (25 mg/kg), tolfenamic acid (2 mg/kg), and also ketoprofen (3 mg/kg) increased nociceptive thresholds after intravenous administration in this species (Chambers *et al.,* 1995; Lizarraga and Chambers, 2006). Together, these data suggest that the hypoalgesic effects of NSAIDs in sheep are not directly mediated at the spinal cord level. In fact, supraspinal mechanisms involving opioidergic and α_2-adrenergic systems have been implicated in the central hypoalgesic action of NSAIDs in this species (Chambers *et al.,* 1995; Lizarraga and Chambers, 2006).

Ketamine's lack of hypoalgesic action in sheep cannot be attributed to the nociceptive test used either. Mechanical stimulation in sheep detected short-lived hypoalgesia, though accompanied by severe motor effects, after intravenous, but not intramuscular, administration (Chambers, 1992). Also, intramuscularly administered ketamine significantly increased mechanical hypoalgesia induced by xylazine (25 μg/kg) given intravenously (Chambers, 1992).

The concentrations of ketamine used in this study were similar to those found in CSF from the cisterna magna of sheep at the time of recovery from anaesthesia, 15-20 min after intravenous (11.6 mg/kg) and intracerebroventricular (2 mg/kg) administration: ~2 μg/ml (~7.3 μM) and ~50 μg/ml (~182.5 μM), respectively (Waterman and Livingston, 1978). It is possible that higher concentrations of ketamine may induce mechanical hypoalgesia after i.t. administration in sheep, though the mechanism of action may include a local anaesthetic effect (Iida *et al.,* 1997). It would have to be considered that, despite producing no behavioural and clinical signs of neurotoxicity, as occurred in sheep in this study, spinal histopathological abnormalities can occur after i.t. administration of ketamine (Stotz *et al.,* 1999; Vranken *et al.,* 2005). Further experiments assessing both nociception and histopathological findings of the spinal cord are necessary to test the efficacy and safety of i.t. ketamine in sheep.

Contrary to the lack of hypoalgesic action in sheep, the lower concentrations of both ketoprofen and ketamine used in this study reduced nociceptive transmission in an *in vitro* spinal cord preparation from neonatal rats (Lizarraga *et al.,* 2004). Differences between species, age and experimental paradigms may have contributed to the different outcomes.

Intrathecal ketoprofen and ketamine

The experiments with keoprofen and ketamine alone were originally designed to further investigate the combination of both drugs for synergism. However, the lack of hypolgesic effect of these drugs precluded us from establishing an additive drug proportion, which would be tested experimentally. Instead, a 0.955:0.045 proportion for ketoprofen and ketamine was adopted from a previous study in which this drug proportion was synergistic to depress nociceptive transmission in an *in vitro* neonatal rat spinal cord preparation (Lizarraga *et al.*, 2004; Chapter 5). Using mechanical noxious stimulation in sheep, this drug proportion produced no hypoalgesia at any of the concentrations tested. This suggests lack of hypoalgesic interaction between ketoprofen and ketamine, at the drug proportion tested, in the normal sheep spinal cord. It is important to notice that, apart from drug proportion, synergism depends on factors such as drug concentration and measured end point (Tallarida, 2000), which, together with species and age, may have contributed to the different results in both experimental paradigms.

Intrathecal NMDA followed by ketamine, ketoprofen, and ketamine plus ketoprofen

The i.t. administration of NSAIDs and NMDA receptor antagonists prevents or reduces allodynia and hyperalgesia in models of persistent pain (Malmberg and Yaksh, 1992; Chaplan *et al.*, 1997; Dirig *et al.*, 1998; Kawamata *et al.*, 2000; Park *et al.*, 2000). A common feature of these pain models is an increased spinal release of glutamate and further activation of NMDA receptors (Yaksh *et al.*, 1999; Vetter *et al.*, 2001). Consistent with this theory, i.t. administration of NMDA enhances pain sensitivity in rodents (Malmberg and Yaksh, 1992; Park *et al.*, 2000) as well as in sheep (Dolan and Nolan, 1999), and NMDA-induced hypersensitivity is reduced by NMDA receptor antagonist and NSAIDs given by the i.t. route (Malmberg and Yaksh, 1992; Dolan and Nolan, 1999; Park *et al.*, 2000). Here it was confirmed that i.t. administration of NMDA produced mechanical hypersensitivity in sheep, which was prevented by ketoprofen and ketamine alone and by a combination of both. However, the combination was not significantly more efficient to prevent this altered pain state than either drug alone. These results contrast with those obtained from experiments in which sheep were not given NMDA through the i.t. catheter (treatments 2 to 4), and suggest that ketoprofen and ketamine may produce spinal analgesia only when NMDA receptors are activated.

This is the first time that the non-selective COX inhibitor ketoprofen is shown to ameliorate NMDA-induced hypersensitivity, and at least part of its spinal antihypersensitive actions seem to be mediated by inhibition of the NMDA receptor system. Similarly, i.t. administration of the COX-2 selective inhibitor DFU (200 nmol, 200 μl) significantly attenuated mechanical hypersensitivity induced by i.t. administration of NMDA in sheep (Dolan and Nolan, 1999).

It is probable that activation of spinal COX enzymes, and in particular that of COX-2, may contribute to the development of mechanical hypersensitivity induced by i.t. injection of NMDA in sheep.

The antihyperalgesic effects of the non-competitive NMDA receptor channel blocker ketamine have previously been shown in sheep. Given intravenously for induction of anaesthesia, ketamine (10 mg/kg i.v.) prevented the development of thermal hyperalgesia induced by ventral midline laparatomy for embryo retrieval in sheep (Welsh and Nolan, 1995). In the current study, the enhanced mechanical pain sensitivity induced by i.t. injection of NMDA was prevented by ketamine administered by the same route. This also has been shown for the NMDA receptor antagonist dizocilpine (100 nmol, 200 μl) (Dolan and Nolan, 1999). Thus, blockade of NMDA receptors, especially those in the spinal cord, may help to prevent the development of hypersensitivity in sheep.

The findings that both ketoprofen and ketamine have spinal antihypersensitive actions could have important clinical implications in farm animals suffering from disorders in which persistent pain is an issue (*i.e.* many types of lameness). However, although no treatment was associated with behaviours suggestive of neurological side effects throughout the study, a comprehensive spinal toxicological investigation is necessary before clinical trials are carried out.

In summary, i.t. cervical catheterisation of sheep is a relatively non-invasive technique [compared to laminectomy (Eisenach and Tong, 1991)] useful for studying the spinal action of drugs, though complications such as meningitis and mechanical damage of the spinal cord may be considered. Although i.t. administration of ketoprofen and ketamine alone or in combination induced no mechanical hypoalgesia in sheep, these treatments prevented the enhancement of mechanical pain sensitivity induced by NMDA administered through the same route. The antihypersensitive actions of these drugs were achieved with very small doses compared to those producing analgesia after systemic administration in this species (*i.e.* approximate values for a 50 kg sheep for ketoprofen: 81.4 μg *vs.* 150 mg (Lizarraga and Chambers, 2006); and ketamine: 11 μg *vs.* 50 mg (Chambers, 1992), respectively). This could have important clinical implications in farm animals suffering from clinical conditions associated with persistent pain at the same time that may reduce the risk of residues.

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9. General discussion and conclusions

This thesis describes the spinal antinociceptive interactions between non-steroidal anti-inflammatory drugs (NSAIDs) and *N*-methyl-D-aspartate (NMDA) receptor systems. Using *in vitro* electrophysiological techniques and an *in vivo* behavioural paradigm, drugs and drug combinations were assessed with the objective of identifying synergistic spinal antinociceptive interactions with potential clinical application for the control of pain. The rational behind this was the importance of spinal NMDA receptors in the processing of nociceptive information and the fact that synergism allows the use of smaller amounts of the constituent drugs and hopefully less side effects. Experiments were focussed on the NMDA receptor channel and the cyclo-oxygenase (COX) and nitric oxide (NO) pathways.

9.1 *In vitro* sciatic nerve-dorsal root preparation

Electrophysiological studies were carried out using an *in vitro* neonatal rat spinal cord preparation (Otsuka and Konishi, 1974). This is a robust preparation that allows several hours of recording that was possible to carry out under the conditions in the laboratory. In this preparation, NMDA receptor-mediated reflexes can be evoked (Brockmeyer and Kendig, 1995; Thompson *et al.,* 1995; Faber *et al.,* 1997). From previous reports, it was noticed that this technique has been used in a relatively wide-range of ages, mainly preparations from pups aged 0 to 12 days old (Brockmeyer and Kendig, 1995; Thompson *et al.,* 1995; Kurihara and Yoshioka, 1996; Faber *et al.,* 1997). The first action taken was to determine animals from what age range would be used for the experiments. For this purpose, *in vitro* sciatic nerve-L4/L5 dorsal root preparations from 0- to 12-day-old rats were used to assess various physiological and pharmacological properties of both A- and C-primary afferent fibres.

Using single square-wave pulses, thresholds for A- and C-fibres were found. Low-level electrical stimulation of the sciatic nerve evoked the appearance of the first discernible response recorded from the dorsal root, the A-fibre threshold. Not surprisingly, higher levels of stimulation were always required to evoke a dorsal root compound action potential (DRCAP) containing a C-fibre waveform, the C-fibre threshold. However, there were marked developmental changes in both A- and C-fibre thresholds. It was noticed that within the first 5 postnatal days, stimulus intensities required to evoke A-thresholds were significantly higher that those for the rest of studied ages. In contrast, stimulus intensities required for evoking C-fibre thresholds in preparation from 2-day-old rats and younger were significantly lower than for any other age. A-fibre thresholds had a trend to decrease with age, whereas those for C-fibres a slight trend to increase.

In regards to conduction velocities, A-fibre waveforms had a trend to increase with age. There was a good positive correlation between thresholds and conduction velocities of A-fibre waveforms. Although conduction velocities of C-fibre waveforms were always below 1 m/s, they had a slight trend to increase within the first postnatal week. No significant correlation between thresholds and conduction velocities of C-fibre waveforms was found.

The depressant actions of the local anaesthetic lignocaine were also developmentally regulated. A-fibre waveforms in preparations from 4-day-old rats and younger were more resistant to the depressant actions of this local anaesthetic. Although significant differences on depressant actions of lignocaine in C-fibre waveforms were found amongst some ages, most median inhibitory concentration values were between 100 and 200 mM.

Based on thresholds and conduction velocities, C-fibre waveforms seemed to be due to activation of C-fibres. To assess this, capsaicin, a neurotoxic substance relatively selective for C-fibres, was used. Irrespective of age, capsaicin depressed C-fibre waveforms in all preparations. Surprisingly, capsaicin also depressed A-fibre waveforms, but only in preparations from 6-day-old rats and younger. These findings were interpreted as A- and C-fibre waveforms being due to activation of immature A- and mature C-fibres, respectively.

Myelination, assessed as percentage transverse area of myelin and myelinated axons, increased with age in dorsal roots from 0- to 12-day-old rats. Myelination was positively correlated with conduction velocities of A-fibres, whereas it was negatively correlated to threshold. These data suggested that as rats aged, myelination and conduction velocities increased and threshold decreased, confirming that A-fibre waveforms were due to activation of immature A-fibres.

It has to be considered that rats are born immature. In terms of neurological development, rats used in this project are considered to be about 25 to 30 weeks gestational post-conceptional age in human terms (Fitzgerald, 1991). In fact, premature infants within this age range require higher doses of local anaesthetics for spinal anaesthesia than adults do (Shenkman *et al.,* 2002). Although data from the current *in vitro* experiments do not explain why these differences arise, they resemble what it is observed *in vivo*. It may be possible to use this preparation as a model for further studies and elucidate the mechanisms involved in such differences. Establishing the time during development when functional nociceptive and analgesic modulatory mechanisms emerge may have important clinical implications since adequate analgesia in neonates may help prevent further hypersensitivity to pain later in life (Lidow, 2002).

In addition to providing information on developmental changes in primary afferents, this study helped to determine the age of rats to be used in further experiments. Based on

thresholds, conduction velocities, and effects of lignocaine, 5- to 7-day-old rats were used in further *in vitro* experiments. Preparations from rats within this age range were very similar to each other in terms of these parameters.

9.2 *In vitro* spinal cord preparation

The effects of blocking the NMDA receptor channel and inhibiting COX and NO pathways on spinal nociceptive transmission were assessed. For these purposes, the NMDA receptor channel blocker ketamine, the non-steroidal anti-inflammatory drugs (NSAIDs, which block COX activity) ketoprofen and salicylic acid, and the nitric oxide synthase (NOS) inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) were infused onto *in vitro* neonatal rat hemisected spinal cord preparations. The electrical stimulus intensity applied to L4 or L5 dorsal root that produced a discernable response in the ipsilateral ventral root was found. Two reflexes were evoked at stimulus intensities 3 times threshold: the monosynaptic response (MSR) and the low intensity excitatory postsynaptic potential (epsp). One further reflex was evoked at 16 times threshold, the high intensity epsp. All three reflexes contain glutamate receptor components. The MSR has non-NMDA receptor components (kainite and α-amino-3-hydroxy-5-methyl-4-isoxalone propionic acid receptor components) (Long *et al.,* 1990), whereas the low and high intensity epsps contain NMDA receptor components (Brockmeyer and Kendig, 1995; Faber *et al.,* 1997). In addition, the high intensity epsp contains peptidergic components (Akagi *et al.,* 1985; Woodley and Kendig, 1991).

9.2.1 Development of spinal NMDA receptor systems

Although stimulus intensities for evoking both the MSR and the low intensity epsp activated peripheral A-fibres only and those for the high intensity epsp also activated C-fibres (Lizarraga *et al.,* 2005; Chapter 3), it has to be considered that, despite the ability of nociceptors to signal noxious events in the periphery, central nociceptive processing is immature in the postnatal period. In rats, neuropeptide containing (substance P, calcitonin gene-related peptide, galanin, somatostatin), TrK receptor-expressing C-fibre terminals are observed in lamina II until embryonic days 18-19, whereas the IB4^{+} subset of C-fibre synaptic terminals are observed until postnatal day 5 (Fitzgerald, 2005). Since spinal cord preparations from 5- to 7-day-old rats were used in the current project, proliferation of C-fibre synaptic input in the superficial dorsal horn may still have been under way. In addition, immature Aβ-fibres innervate lamina I and II and transmit nociceptive information in neonatal rats (Fitzgerald and Gibson, 1984). Thus, for some time during the early postnatal period, both A- and C-fibre terminals occupy the superficial laminae of the dorsal horn.

Also in newborn rats, marked developmental changes in the spinal distribution of NMDA

receptors and NOS enzymes are observed. NMDA receptors are expressed in higher concentration than that of adult rats and are distributed throughout the whole spinal cord grey matter (Kalb *et al.,* 1992). Subunit expression of NMDA receptors is also developmentally regulated and by 21 days of age resembles that of adult animals (Fitzgerald, 2005). Both receptor activity for NMDA and NMDA-evoked Ca^{2+} influx decline with postnatal age (Hori and Kanda, 1994). In contrast, spinal NOS is expressed later in postnatal development than NMDA receptors. In the dorsal horn of the spinal cord, the enzyme is absent in newborn rats and progressively reaches a similar pattern, which resembles that of adults by postnatal day 20 (Soyguder *et al.,* 1994; Vizzard *et al.,* 1994).

Less is known about ontogenic changes in COX distribution and activity both in the periphery and the spinal cord. In the periphery, prostaglandins sensitise afferent neurones to increase depolarisation of lumbar ventral roots in neonatal rat isolated spinal cord-tail preparations (Yanagisawa *et al.,* 1986; Rueff and Dray, 1993). Although this may suggest that the COX pathway is at lest partially functional in the periphery during the immediate postnatal period, it has been reported that the analgesic effects of COX inhibitors are developmentally regulated (Ririe *et al.,* 2004). Further studies on developmental expression and activity of COX enzymes in the spinal cord and periphery will be important in elucidating mechanisms for differences in response to analgesics as a function of age. Ontogenic changes in mediators other than COX enzymes in the COX signaling pathway also need to be considered. In this regard, PGE_2 receptors EP_1-EP_4 underwent a transitory postnatal increase in transcript levels in the brainstem of 5- and 15-day-olds followed by a decrease to foetal levels by adulthood (Tai *et al.,* 1998).

In addition to spinal inhibitory mechanisms not being developed yet, all abovementioned ontogenic processes make neonates to have an increased excitatory action in the spinal cord in comparison to adults (Fitzgerald, 2005). This may explain the long-lasting intersegmental reflexes evoked in the ventral root after stimulation of the dorsal root.

9.2.2 Single drug studies

Infusion of cumulative concentrations of ketamine (1-50 μM) depressed all three spinal reflexes. As previously (Brockmeyer and Kendig, 1995), the most likely mechanism of action was due to blockade of the ion channel in NMDA receptors, though a local anaesthetic action may have also contributed. Ketoprofen (200-600 μM) depressed both the low and the high intensity epsps, without affecting the MSR. These findings demonstrated for the first time that NSAIDs produce a true antinociceptive action at the spinal cord level. Subdural administration of NSAIDs prevented hypersensitivity induced by i.t. administration of NMDA or SP (Malmberg and Yaksh, 1992; Dolan and Nolan, 1999; Yaksh *et al.,* 2001), which agreed with ketoprofen depressing both the low and the high intensity epsps. However,

the mechanisms by which this depressant action occurred remain unknown. Ketoprofen was infused at concentrations high enough to inhibit COX activity (Warner *et al.,* 1999), but confirmation of this mechanism of action was not possible since salicylic acid was not administered at concentrations sufficient to inhibit these enzymes (Warner *et al.,* 1999). In addition, computed median inhibitory concentrations for ketoprofen were within range to inhibit amidohydrolase activity, the enzyme responsible to metabolise anandamide into arachidonic acid, (Fowler *et al.,* 1997). Anandamide is known to inhibit glutamergic transmission in spinal cord slices from 15- to 25-day-old rats (Morisset and Urban, 2001) and spinal antinociceptive actions of NSAIDs have been associated to an endocannabinod-dependent effect (Gühring *et al.,* 2002; Ates *et al.,* 2003). A cannabinoidmimetic effect was attempted, but not possible, to be studied during this project. Further research is necessary to establish the mechanisms of action by which ketoprofen depressed spinal nociceptive transmission.

L-NAME (1-100 μM) had no significant effect on any of the studied reflexes. This agrees with the lack of effect of NOS inhibitors on the high intensity epsp of 10- to 12-day-old rats (Thompson *et al.,* 1995), but contrasts with the increase in area under the curve for the high intensity epsp in 0- to 2-day-old rats (Kurihara and Yoshioka, 1996) and the reduction of pain hypersensitivity in adult animals when given by the i.t. route (Kawamata and Omote, 1999; Gühring *et al.,* 2000). It seems that the NO pathway may undergo developmental changes from being antinociceptive in newborns, entering a latency phase in young animals, and being pronociceptive in adults. This hypothesis requires confirmation.

9.2.3 Drug combination studies

Drug combinations were analysed for synergism using isobolographic and composite-line analyses, which relate in a quantitative methodology (drug concentration and magnitude of effect) to differentiate from additive responses. The mathematical foundation for studying the effect of drug combinations with statistical analyses has been reviewed elsewhere (Porreca *et al.,* 1990; Tallarida *et al.,* 1997; Tallarida, 2000). Isobolograms allow a distinction between synergism and additivity at one particular effect level, whereas composite additive line extends the analysis to other effects levels.

Using linear-regression of the concentration-effect data for each drug, values for 40% reduction of the maximum effect (IC_{40}) were calculated as this effect level was achieved by drugs that depressed reflexes (ketamine and ketoprofen). These values were used for constructing isobolograms. Using 0.5 fractions for each drug (equieffective concentrations), combinations that were equivalent in producing 40% reduction of the maximum effect were calculated and these lay on the line of additivity. The actual experiments were carried out using a fixed-ratio design, in which the constituents were administered in concentrations that

kept the proportions of each constant. Various concentrations were used to determine the actual concentration to achieve IC_{40}. When the experimental IC_{40} value lay below the line of additivity this meant that lower drug concentrations were needed in the combination to produce the same effect as either dug alone; this was referred as synergism. In contrast, some combinations required concentrations that were greater than the additive amounts, in those cases the experimental IC_{40} value lied above the line of additivity. This was referred as sub-additivity or antagonism. The Student's *t* distribution test was used to distinguish departure from simple additivity. Additivity meant that each constituent contributed to the effect in accord with its own potency, and the less potent drug was merely acting as a diluted form of the other (Tallarida, 2000).

For drugs that lacked or had minor efficacy when given alone, the method described by Porreca *et al.* (1990) was used to quantify and demonstrate synergism. In this case the line of additivity in the isobologram was horizontal. The same principles of synergism and sub-additivity applied. Also a fixed-ratio combination of the two compounds was used. Since only one drug contributed to the effect, the additive total concentration was greater than its own IC_{40} because of the dilution brought about by the presence of the inactive drug.

Composite additive lines were constructed using the relative potency at any effect level for concentration-effect linear regression lines for active drugs (Tallarida *et al.,* 1997). When one of the drugs was inactive, no composite additive curve was constructed. The experimental line was compared to its corresponding composite additive line or to that of the active drug, as appropriate. The F-distribution test was used to assess whether two lines differed significantly (Tallarida, 2000).

All three combinations studied, ketamine and ketoprofen, ketamine and L-NAME, and ketoprofen and L–NAME, were synergistic at depressing NMDA receptor-mediated transmission on the *in vitro* neonatal rat spinal cord preparation. Ketamine also depressed non-NMDA receptor-mediated transmission, but when co-infused with ketoprofen or L-NAME, a sub-additive effect was observed. These results were interpreted as all drug combinations being synergistically antinociceptive with reduced potential side effects. The synergistic reduction of reflexes suggested that these drugs were acting independently one from the other by different mechanisms. The mechanisms by which these drugs induced these interactions were not studied here and further research is required to clarify this.

Synergism is important in clinical situations, as it allows the use of smaller amounts of the constituent drugs and hopefully less side effects. *In vivo* studies to assess spinal analgesic synergism between combinations of ketamine, ketoprofen, and L-NAME are necessary before any recommendations can be done regarding the synergistic combinations reported in this

project. It is also important to determine the effect of these combinations in both neonates and adults as developmental changes may affect the outcome.

9.3 Intrathecal drug administration in sheep

The effects of ketamine and ketoprofen alone and in combination were investigated in further *in vivo* studies in sheep. These drugs were chosen since both decreased spinal NMDA receptor-mediated transmission and they did it in a synergistic way when infused together in previous *in vitro* studies (Lizarraga *et al.,* 2004; Chapter 5). They were also selected since they are used in both veterinary and human medicine, hence the information gained would be of clinical relevance.

Catheterisation of the subarachnoid space provides a convenient means to deliver drugs directly onto the spinal cord. This method has been instrumental to our understanding of spinal mechanisms of analgesia. This technique was originally described in the rat (Yaksh and Rudy, 1976) and involved surgical exposure of the atlanto-occipital membrane. Further modifications also required extensive surgery, including laminectomy (Poon *et al.,* 2005). In both cases, postoperative mortality (13-14%), paraplegia (17-21%), and bladder dysfunction (17-21%) were amongst the most common complications (Poon *et al.,* 2005). An improved catheterisation procedure by means of a hole drilled in T13 seemed to reduce these complications by 5%, 4%, and 0%, respectively (Poon *et al.,* 2005).

In sheep, catheterisation of the subarachnoid space has been performed following bilateral cervical or lumbar laminectomy (Eisenach and Tong, 1991). A less invasive method used a Tuohy needle to penetrate the subarachnoid space at the level of the atlanto-occipital joint (Kyles *et al.,* 1992). However, few data on shortcomings of subarachnoid space catheterisation in sheep are available. Using the latter technique, the current study found paraplegia and infectious meningitis to be the most important complications; both have previously been recognised (Kyles *et al.,* 1992; Lizarraga, 2000). In addition, the success rate for placement of funcitional i.t. catheters seems to be relatively low (52-59%), and the mean time that catheters remain functional seems to vary considerable between studies (72-210 days) (Kyles *et al.,* 1992; Lizarraga, 2000; Chapter 8). Improved procedures to increase the success rate of functional i.t. catheters, anchor i.t. catheters firmer, and reduced mortality and morbility are areas for further development.

The spinal effects of ketamine and ketoprofen were assessed in healthy sheep following mechanical nociceptive stimulation of a forelimb. Neither drug increased mechanical nociceptive thresholds, which is consistent with NMDA receptor antagonists and NSAIDs

producing no analgesia in animal models of acute pain (Malmberg and Yaksh, 1992; Kawamata *et al.,* 2000), including mechanical stimulation in sheep (Dolan and Nolan, 1999; Lizarraga and Chambers, 2006). No hypoalgesia was detected even after administering both drugs in a proportional combination (0.045:0.955) that was synergistic to depress nociceptive transmission in the *in vitro* neonatal rat spinal cord preparation (Lizarraga *et al.,* 2004; Chapter 5). Synergism, however, is not only a property of the drug pair, but also depends in the relative amounts in the combination, drug concentration, and measured end point (Tallarida, 2000). These data suggest that spinal administration of ketamine and ketoprofen alone or in combination induce no hypoalgesia in sheep. Species, developmental, and experimental condition differences may have contributed to the opposite outcomes between *in vitro* and *in vitro* studies in this project.

Activation of spinal NMDA receptors is regarded as an important feature for development of pain hypersensitivity (Bennett, 2000). In keeping with this, i.t. administration of NMDA produced mechanical hypersensitivity in sheep, as previously demonstrated (Dolan and Nolan, 1999). Subarachnoid administration of ketamine and ketoprofen alone or in combination prevented NMDA-induced mechanical pain hypersensitivity. These data confirmed the importance of the NMDA/COX pathway in the spinal nociceptive processing of pain (Svensson and Yaksh, 2002; Chapter 2).

Clinical relevant drugs were used in the current project, which could have important practical implications. Further experiments assessing the potential antihyperalgesic effect of ketoprofen and ketamine in clinical cases of persistent pain, such as that in sheep suffering from interdigital pododermatitis, are necessary. This is a condition that affects the soft, sensitive tissue of the claws and induces pain hypersensitivity (Ley *et al.,* 1989; Ley *et al.,* 1995). Such a study would certainly have relevance for sheep as a species and could serve as departing point for other farm animals, cows in particular. These animals are also known to develop pain hypersensitivity to some types of lameness, and remain so even after apparent resolution of the lameness-causing problem (Whay *et al.,* 1997; Whay *et al.,* 1998).

The objective of the current project of identify drug combinations between NSAIDs and NMDA receptors systems that may be useful in producing analgesic synergism at the spinal cord level was achieved. Preclinical trials were carried out also. Further preclinical, toxicological, and clinical experiments are necessary before making any recommendations.

9.4 References

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