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CENTRAL ANALGESIC EFFECTS OF THE NON-Steroidal ANTI-INFLAMMATORY DRUGS

A thesis presented in partial fulfilment of the requirements for the degree of Master of Veterinary Science in Veterinary Pharmacology at Massey University, Palmerston North, New Zealand

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

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

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

The involvement of spinal opioidergic and $\alpha_2$-adrenergic mechanisms in the hypoalgesia induced by the intravenous administration of ketoprofen in sheep was investigated. The prior intrathecal administration of naloxone and atipamezole at a dose that had no effect on nociceptive thresholds and reversed the analgesia mediated by intravenous fentanyl and xylazine, respectively, almost completely reversed the antinociceptive effects mediated by intravenous ketoprofen.
These studies confirm that non-steroidal anti-inflammatory drugs can produce hypoalgesia even when inflammation is absent and, although they did not have a direct effect on the spinal cord, their analgesic action appeared to be spinally mediated by opioidergic and adrenergic descending inhibitory systems.
TABLE OF CONTENTS

ABSTRACT........................................................................................................... iii

ACKNOWLEDGEMENTS..................................................................................... xi

ABBREVIATIONS............................................................................................... xiii

LIST OF TABLES................................................................................................. xvii

LIST OF FIGURES............................................................................................. xix

Chapter 1 GENERAL INTRODUCTION.............................................................. 1

Chapter 2 LITERATURE REVIEW....................................................................... 5

2.1. PAIN.............................................................................................................. 5

2.1.1. The nociceptive pathways........................................................................ 7
  2.1.1.1. Peripheral afferent input................................................................. 8
  2.1.1.2. The spinal cord ............................................................................. 10
  2.1.1.3. Ascending spinal pathways.......................................................... 12
  2.1.1.4. The brain ....................................................................................... 14
  2.1.1.5. Descending inhibitory systems..................................................... 14

2.2. GLUTAMATE RECEPTORS......................................................................... 17

2.2.1. Ionotopic glutamate receptors............................................................. 18
  2.2.1.1. AMPA receptors........................................................................... 18
  2.2.1.2. Kainate receptors......................................................................... 20
  2.2.1.3. NMDA receptors.......................................................................... 21
    2.2.1.3.1. Regulation of the NMDA receptor........................................... 21
    2.2.1.3.2. Effects of NMDA receptor activation..................................... 24
# Table of Contents

2.2.1.3.2.1. NMDA receptors and the AA pathway .................................. 26  
2.2.1.3.2.2. NMDA receptors and the NO pathway ................................ 27  
2.2.1.3.2.3. Interactions between the AA and NO pathways ..................... 31  
2.2.1.3.3. NMDA receptors and the release of other neuromediators .......... 32  

2.2.2. Metabotropic glutamate receptors .............................................. 36  
2.2.2.1. Excitatory effects of mGluRs ................................................. 37  
2.2.2.2. Inhibitory effects of mGluRs ................................................ 38  

2.3. THE ARACHIDONIC ACID PATHWAY .................................................. 39  
2.3.1. Leukotrienes .............................................................................. 41  
2.3.2. Prostaglandins ........................................................................... 42  
2.3.2.1. Cyclooxygenases ..................................................................... 43  
2.3.2.1.1. Cyclooxygenase isoforms and functions .................................. 43  
2.3.2.1.2. Cyclooxygenases and the nociceptive process ......................... 45  
2.3.2.1.3. Cyclooxygenase inhibition by NSAIDs ................................... 47  
2.3.2.2. Prostaglandins and the nociceptive process ................................ 48  
2.3.2.3. Prostanoid receptors ............................................................... 52  

2.4. CENTRAL ANALGESIC EFFECTS OF NONSTEROIDAL ANTI-INFLAMMATORY DRUGS ...................................................... 57  
2.4.1 Possible central antinociceptive mechanisms of action for NSAIDs .... 58  
2.4.1.1. NSAIDs and prostanoid synthesis inhibition ................................ 59  
2.4.1.2. NSAIDs and glutamate receptors ............................................. 60  
2.4.1.3. NSAIDs and descending inhibitory mechanisms ......................... 62  
2.4.1.4. NSAIDs and other receptor systems and signal transduction pathways ................................................................. 63  
2.4.1.5. NSAIDs and interference with nuclear related events ................. 64  

2.5. INDUCTION AND ASSESSMENT OF PAIN .................................... 66  
2.5.1. Animal models of acute pain ...................................................... 67  
2.5.2. Animal models of chronic pain ................................................... 68
## Table of Contents

2.6. AIMS OF THE PROJECT ........................................................................................................... 70

Chapter 3 EFFECT OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS ON NMDA-RECEPTOR SYNAPTIC TRANSMISSION IN THE *in vitro* RAT SPINAL CORD PREPARATION .......................................................... 71

3.1. INTRODUCTION ...................................................................................................................... 71

3.2. MATERIALS AND METHODS .................................................................................................. 72
3.2.1. Spinal cord preparation ....................................................................................................... 72
3.2.2. Recording techniques ......................................................................................................... 75
3.2.3. Drugs .................................................................................................................................. 76
3.2.4. Data analysis ...................................................................................................................... 77

3.3. RESULTS .................................................................................................................................. 77

3.4. DISCUSSION .......................................................................................................................... 80

Chapter 4 THE CENTRAL ANALGESIC EFFECTS OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS IN SHEEP ......................................................................................................................... 85

4.1. INTRODUCTION ...................................................................................................................... 85

4.2. MATERIALS AND METHODS .................................................................................................. 86
4.2.1. Animals and intratecal catheterisation .............................................................................. 86
4.2.2. Mechanical Nociceptive Testing ....................................................................................... 90
4.2.3. Drug administration protocol .......................................................................................... 93
4.2.3.1. Intrathecal administration of NSAIDs .......................................................................... 93
4.2.3.2. Intravenous administration of NSAIDs ......................................................................... 94
| 4.2.3.3. Involvement of opioidergic and α-adrenergic systems in the NSAIDs-induced analgesia | 95 |
| 4.2.4 Statistical analysis | 96 |

<table>
<thead>
<tr>
<th>4.3. RESULTS</th>
<th>97</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.3.1. Intrathecal catheterisation of sheep</td>
<td>97</td>
</tr>
<tr>
<td>4.3.2. Intrathecal administration of NSAIDs</td>
<td>105</td>
</tr>
<tr>
<td>4.3.3. Intravenous administration of NSAIDs</td>
<td>111</td>
</tr>
<tr>
<td>4.3.4. Involvement of opioidergic and α&lt;sub&gt;2&lt;/sub&gt;-adrenergic systems in NSAID-induced analgesia</td>
<td>114</td>
</tr>
<tr>
<td>4.3.4.1. Blockade of spinal opioid receptors</td>
<td>114</td>
</tr>
<tr>
<td>4.3.4.2. Blockade of spinal α&lt;sub&gt;2&lt;/sub&gt;-adrenoceptors</td>
<td>115</td>
</tr>
<tr>
<td>4.3.4.3. Influence of spinal opioid and α&lt;sub&gt;2&lt;/sub&gt;-adrenergic receptor blockade on i.v. ketoprofen-induced analgesia</td>
<td>119</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>4.4. DISCUSSION</th>
<th>121</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4.1. Intrathecal catheterisation in sheep</td>
<td>121</td>
</tr>
<tr>
<td>4.4.1.1. Catheter implantation technique</td>
<td>121</td>
</tr>
<tr>
<td>4.4.1.2. Catheter maintenance</td>
<td>121</td>
</tr>
<tr>
<td>4.4.1.3. Catheter replacing and anchoring</td>
<td>122</td>
</tr>
<tr>
<td>4.4.1.4. Assessment of catheter functionality</td>
<td>124</td>
</tr>
<tr>
<td>4.4.1.5. Complications</td>
<td>126</td>
</tr>
<tr>
<td>4.4.2. Mechanical nociceptive testing</td>
<td>127</td>
</tr>
<tr>
<td>4.4.3. Effect of NSAIDs on sheep's nociceptive mechanical thresholds</td>
<td>129</td>
</tr>
<tr>
<td>4.4.3.1. Intrathecal injection of NSAIDs.</td>
<td>130</td>
</tr>
<tr>
<td>4.4.3.1.1. Spinal COX inhibition as analgesic mechanism of action of NSAIDs?</td>
<td>130</td>
</tr>
<tr>
<td>4.4.3.1.2. Protocol and safety of NSAID injected i.t.</td>
<td>139</td>
</tr>
<tr>
<td>4.4.3.2. Intravenous injection of NSAIDs.</td>
<td>140</td>
</tr>
<tr>
<td>4.4.3.2.1. Lack of analgesia with salicylic acid and phenylbutazone</td>
<td>141</td>
</tr>
<tr>
<td>4.4.3.2.2. Supraspinal analgesic action of NSAIDs.</td>
<td>142</td>
</tr>
</tbody>
</table>
4.4.3.2.1.1. Blockage of spinal opioid and $\alpha_2$-adrenergic receptor .................................................. 143
4.4.3.2.1.2. Activation of descending inhibitory mechanisms as analgesic mode of action of NSAIDs ................................................. 148

Chapter 5 GENERAL CONCLUSIONS ................................................................. 155

REFERENCES .................................................................................................... 157
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Intracellular calcium concentration.
Serotonin.
Arachidonic acid.
Acetylcholine.
Artificial cerebral spinal fluid.
α-amino-3-hydroxy-5-methyl-4-isoxalone propionic acid.
Analysis of variance.
Area under the curve.
Area under the threshold change vs. time curve values for 30 minutes.
Area under the threshold change vs. time curve values for 60 minutes.
Brain-derived neurotrophic factor.
Cervical vertebra 5.
Calcium-calmodulin complex.
Calcium/calmodulin-dependent protein kinase II.
3',5'-cyclic guanosine monophosphate.
Calcium-dependent inactivation.
3',5'-cyclic guanosine monophosphate.
Calcitonin gene-related peptide.
Central nervous system.
Cyclooxygenases.
COX-1-like immunoreactivity.
COX-2-like immunoreactivity.
Carbaprostacyclin.
Cerebral spinal fluid.
Dorsal horn neurones.
Dorsolateral funiculus.
Prostaglandin D receptor.
Dorsal root compound action potential.
Dorsal root ganglion.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR-VRP</td>
<td>Dorsal root-ventral root potential.</td>
</tr>
<tr>
<td>e.p.s.p.</td>
<td>Excitatory postsynaptic potential.</td>
</tr>
<tr>
<td>EEA</td>
<td>Excitatory amino acid.</td>
</tr>
<tr>
<td>eNOS</td>
<td>Eendothelial nitric oxide synthase.</td>
</tr>
<tr>
<td>EOX</td>
<td>Epoxide.</td>
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<tr>
<td>EP</td>
<td>Prostaglandin E_2 receptor.</td>
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<tr>
<td>GABA</td>
<td>(\gamma)-Aminobutyric acid.</td>
</tr>
<tr>
<td>HETE</td>
<td>Hydroxyeicosatetraenoic acids.</td>
</tr>
<tr>
<td>i.c.v.</td>
<td>Intracerebroventricular, intracerebroventricularly.</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal, intraperitoneally.</td>
</tr>
<tr>
<td>i.t.</td>
<td>Intrathecal, intrathecally.</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous, intravenously.</td>
</tr>
<tr>
<td>IL-1(\beta)</td>
<td>Interleukin-1(\beta).</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase.</td>
</tr>
<tr>
<td>Ins(1,4,5)(\mathbf{P3})</td>
<td>Inositol-(1,4,5) triphosphate.</td>
</tr>
<tr>
<td>LOX</td>
<td>Lipoxygenases.</td>
</tr>
<tr>
<td>L-PGDS</td>
<td>Lipocalin-type prostaglandin D synthase.</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide.</td>
</tr>
<tr>
<td>LTs</td>
<td>Leukotrienes.</td>
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<tr>
<td>mGluRs</td>
<td>Metabotropic glutamate receptors.</td>
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<td>MSR</td>
<td>Monosynaptic compound action potential.</td>
</tr>
<tr>
<td>N</td>
<td>Newtons.</td>
</tr>
<tr>
<td>NF(\kappa)B</td>
<td>Nuclear factor (\kappa)B.</td>
</tr>
<tr>
<td>NMDA</td>
<td>(N)-methyl-D-aspartate.</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase.</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide.</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase.</td>
</tr>
<tr>
<td>NRM</td>
<td>Nucleus raphe magnus.</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Non-steroidal anti-inflammatory drugs.</td>
</tr>
<tr>
<td>p.o.</td>
<td>(Per\ os, oral) administration.</td>
</tr>
<tr>
<td>PAG</td>
<td>Periaqueductal grey.</td>
</tr>
<tr>
<td>PGs</td>
<td>Prostaglandins.</td>
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<tr>
<td>PKA</td>
<td>Protein kinase A.</td>
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<tr>
<td>PKC</td>
<td>Protein kinase C.</td>
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Abbreviations

PLA$_2$  Phospholipase A$_2$.
PLC  Phospholipase C.
PMNLs  Polymorphonuclear leukocytes.
PTK  Protein tyrosine kynase.
RVM  Rostral ventromedial medulla.
SP  Substance P.
TNF-α  Tumour necrosis factor-α.
trkA  Tyrosine kinase A.
trkB  Tyrosine kinase B
Tx  Thromboxane.
Table 2.1. Ligand-gated ion channels and ionotropic and metabotropic receptors expressed by nociceptive primary afferent neurones.................................................................9

Table 2.2. Distribution and functions of opioid receptors........................................34

Table 2.3. Comparison between COX-1 and COX-2.............................................44

Table 2.4. Functions of COX-1 and COX-2 determined by Ptgs 1 and Ptgs 2 gene depletion in mice, respectively..............................................46

Table 2.5. Properties and distribution of prostaglandin receptors.....................53

Table 3.1. Electrical stimuli applied to the spinal cord preparation and data acquisition parameters............................................................76

Table 4.1. Summary of the intrathecal catheterisation in the sheep...............98

Table 4.2. Evaluation of the analgesic effect of intrathecally and epidurally administered non-steroidal anti-inflammatory drugs in different species............................................................132
Figure 2.1. Division of the spinal cord grey matter according to its cytoarchitecture in laminae I-X .............................................................. 11

Figure 2.2. The role of glutamate receptors and their interaction with other receptor systems in the modulation of nociceptive processing. Refer to the text for details .......................................................... 25

Figure 2.3. Major pathways of the arachidonic acid cascade .................. 40

Figure 3.1. The preparation chamber. The hemicord was placed to the right of the negative stimulating electrode with the dorsal root in contact to the positive stimulating electrode. The ventral root was in contact with the positive recording electrode ........................................ 73

Figure 3.2. The superfusion system. The ACSF was continuously gassed with 95% O2 / 5% CO2 and pumped through a heated water jacket. The ACSF was dripped onto the preparation chamber and then it was collected as waste .............................................................. 74

Figure 3.3. Artefacts obtained after electrical stimulation to evoke the DRCAP (A), the low intensity e.p.s.p. (B), the high intensity e.p.s.p. (C) and the train e.p.s.p (D) in the neonatal rat hemisected spinal cord preparation ............................................................. 78

Figure 3.4. Effect of lignocaine (1 mM) on nerve action potentials from mice sciatic nerve. The threshold of this nerve was 0.5 v (not showed) and a 6 v stimulus was used to evoke both A and C fibre compound action potentials (A), which amplitude was blocked and reduced, respectively, by lignocaine (B). Recovery is shown 25 minutes following return to lignocaine-free medium (C) ..................................... 79
Figure 4.1. Radiograph showing the correct placement of an intrathecal catheter in sheep, with the catheter tip (arrow) at the C5 level..............................88

Figure 4.2. A knot made with the intrathecal catheter itself (A) and a silicon tubular drain (B) were used to anchor the intrathecal catheter. In both cases, the anchoring element, knot or drain tube, was kept subcutaneously (A and C).................................................................89

Figure 4.3. Mechanical nociceptive device. The 2 mm diameter blunted pin attached to the plunger was pushed against the sheep's leg.........................91

Figure 4.4. Mechanical device's calibration curve. The X axis shows the pressure applied to elicits the corresponding force at 1 Newton intervals.................................................................92

Figure 4.5. Sheep's head medial section with the spinal cord exposed until the caudal aspect of C2. The Tuohy needle was superimposed on the atlanto-occipital joint to indicate the position where the needle had to be inserted through for the implantation of intrathecal catheters.....................100

Figure 4.6. Effect of 100 µL xylazine (388 and 1940 µM i.t.) on nociceptive thresholds of healthy sheep over two or three hours after administration (time 0). The data is the mean ± s.e.m. for n = 11 catheterisations on 7 sheep for the 388 µM dose and n = 6 catheterisations on 6 sheep for the 1940 µM dose.................................................................102

Figure 4.7. Spinal cord histopathology showing neutrophil accumulation in the dura mater (arrow) of sheep no. 116. Magnification 4x.........................104

Figure 4.8. Effect of intrathecal repetitive administration of 100 µL saline solution (0.9%) on nociceptive thresholds of healthy sheep over 30 minutes after administration (time 0) (A) and their respective AUC30 (B). The data is the mean ± s.e.m. for n = 5 sheep.................................................................106
Figure 4.9. Effect of intrathecal cumulative doses of 100 µL ketoprofen (0.8 – 200 µM) on nociceptive thresholds of healthy sheep over 30 minutes after administration (time 0) (A) and their respective AUC30 (B). The data is the mean ± s.e.m. for n = 4-5 sheep........................................107

Figure 4.10. Effect of intrathecal cumulative doses of 100 µL phenylbutazone (0.375 – 200 µM) on nociceptive thresholds of healthy sheep over 30 minutes after administration (time 0) (A) and their respective AUC30 (B). The data is the mean ± s.e.m. for n = 5 sheep.............108

Figure 4.11. Effect of intrathecal cumulative doses of 100 µL salicylic acid (0.375 – 200 µM) on nociceptive thresholds of healthy sheep over 30 minutes after administration (time 0) (A) and their respective AUC30 (B). The data is the mean ± s.e.m. for n = 5 sheep......................109

Figure 4.12. Effect of intrathecal cumulative doses of 100µL tolfenamic acid (0.375 – 200 µM) on nociceptive thresholds of healthy sheep over 30 minutes after administration (time 0) (A) and their respective AUC30 (B). The data is the mean ± s.e.m. for n = 4-5 sheep.....................110

Figure 4.13. Effect of ketoprofen (3 mg/kg i.v.) and tolfenamic acid (2 mg/kg i.v.) on nociceptive thresholds of healthy sheep over six hours after administration (time 0) compared with saline (3 mL i.v.). The data is the mean ± s.e.m. for n = 5-6 sheep from -20 to 120 minutes and n = 2 sheep from 150 to 360 minutes........................................112

Figure 4.14. AUC60 for i.v. injected NSAIDs. The data is the mean ± s.e.m. for n = 5-6 sheep. * Represents a significant (P = 0.0001, t-test) difference between treatment and saline........................................113

Figure 4.15. Effect of ketoprofen (3 mg/kg i.v.) and tolfenamic acid (2 mg/kg i.v.) on nociceptive thresholds of healthy sheep over two hours after administration (time 0) compared with saline (3 mL i.v.).
The data is the mean ± s.e.m. for \( n = 5-6 \) sheep.

**Figure 4.16.** Effect of fentanyl (10 µg/kg i.v.) and naloxone (100 µL, 5.49 mM i.t.) alone and the combinations i.t. naloxone + i.v. fentanyl and i.t. saline (100 µL) + i.v. fentanyl on nociceptive thresholds of healthy sheep over one hour after administration (time -10 and 0 for i.t. and i.v. treatments, respectively) compared with saline (3 mL i.v.) (A) and their respective AUC60 (B). The data is the mean ± s.e.m. for \( n = 4 \) sheep, except saline i.v. where \( n = 5 \). * Represents a significant \((P = 0.0001, t\)-test\) difference between treatment and saline and ** between treatment and fentanyl i.v. alone.

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

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

**Figure 4.19.** Effect of ketoprofen (3 mg/kg i.v.) alone and the combinations i.t. saline (100 µL) + i.v. ketoprofen, i.t. naloxone (100 µL, 5.49 mM) + i.v. ketoprofen and and i.t. atipamezole (100 µL, 4.03 mM) + i.v. ketoprofen.
on nociceptive thresholds of healthy sheep over one hour after administration (time −10 and 0 for i.t. and i.v. treatments, respectively) compared with saline (3 mL i.v.) (A) and their respective AUC60 (B). The data is the mean ± s.e.m. for $n = 6$ sheep, except saline i.v. where $n = 5$. * Represents a significant ($P = 0.0001$, t-test) difference between treatment and saline and ** between treatment and ketoprofen i.v. alone..........................120
Farm animal welfare has been an important issue during the last few years in governmental agencies and research institutions, and although several recommendations have been proposed to improve the wellbeing of these animals, only minor changes in the way of producing food from animal sources have been observed. Developed countries show the most concern for this topic but not many of them have adopted practices to achieve even the minimum standards of comfort for farm animals. These standards, derived from applied ethology studies, are grouped in a concept known as the "five-freedoms of animals" (Crook & Helder, 1994), which are:

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

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

Lameness is one of the most serious welfare problems in farm animals. Not only is lameness detrimental to the well-being of animals it is, in addition,
detrimental to production. Economic losses arise from poor production because lame animals, in pastoral production systems, are unable to walk around to graze and, therefore, lose condition which negatively affects production parameters. Of course, lameness also affects animals raised in feedlot systems in a very similar way. Therefore, rapid and effective treatment is important.

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

Many analgesics exert their antinociceptive effect, at least, at the level of the spinal cord. Hence, by administering a small amount of drug by the intrathecal (i.t.) route it would be possible to achieve analgesia and reduce drug residues. In this regard, the i.t. injection of α2-adrenoceptor (Waterman et al. 1988; Ley et al. 1989; Kyles et al. 1993), γ-aminobutyric acid (GABA) (Kyles et al. 1995) and, to a lesser extent, opioid receptor agonists (Ley et al. 1989) produced good analgesia in sheep. Other analgesic agents such as the non-steroidal anti-inflammatory drugs (NSAIDs) could represent a good option as well.
Acetylsalicylic acid, dipyrone, flunixin, ketoprofen, phenylbutazone, and tolfenamic acid are the NSAIDs licensed for use in cattle in New Zealand\textsuperscript{1}. Although all of them are indicated for the relief of inflammation and pain associated with musculo-skeletal disorders, scarce data on their analgesic effects on cattle and other farm species are available.

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

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

2.1. PAIN

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

The sensory component of pain, referred to as nociception, corresponds to the physiological responses associated with the stimulation of a subpopulation of sensory neurones, called nociceptors, and the subsequent signalling of the information through specialised nociceptive pathways. However, activity induced in nociceptors and the nociceptive pathways is not pain, which is always a psychological state (Merskey, 1986). At levels of stimulation sufficient enough to activate nociceptors, humans report an awareness of sensation but do not describe it as painful. This is referred to as the nociceptive threshold. Higher levels of stimulation are required to reach the pain threshold, which is the least experience of pain that a subject can recognise. Under controlled conditions, these two thresholds are quite consistent among different subjects. The greatest level of pain that a subject is prepared to tolerate is denominated as the pain tolerance level and is highly variable not only between subjects but even in the same subject at different times. The range of stimulus intensities between the pain threshold and the pain tolerance level is the pain sensitivity range. At the lower end of this range, the cognitive and emotional responses that characterise the stimulus as aversive are weak, and as the stimulus
intensity increases, these responses become stronger. Thus, it is the cognitive and emotional components of pain that contribute to the wide variability in the pain tolerance level (Danneman, 1997).

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

Generally, it is accepted that some kind of pain has a protective function within the organism (Iadarola & Caudle, 1997; Malmberg et al. 1997; Dray et al. 1994). This pain is referred to as acute or physiological pain and serves as a warning to prevent further injury. On the other hand, when severe tissue injury is experienced the development of chronic or pathological pain starts immediately with complex changes in the nociceptive pathways (e.g., cell phenotype, protein expression and neural structure alterations). These changes account for the expression of allodynia [pain perception in response to normally non-painful stimuli (Merskey, 1986)] and hyperalgesia [exaggerated pain sensations to normally painful stimuli (Merskey, 1986)] as constant features (Dray et al. 1994). It is important, therefore, to prevent and/or control this kind of pain.
Different groups of analgesic drugs are utilised in the treatment of pain. Opioid and $\alpha_2$-adrenoceptor agonists are among the most used in veterinary clinical practice, followed by NSAIDs, benzodiazepines and local anaesthetics. Ketamine, an N-methyl-d-aspartate (NMDA) receptor antagonist, is indirectly used to produce analgesia when utilised as an anaesthetic agent. However, all of them produce diverse "levels" of analgesia and side effects, both attributed to their mechanism(s) of action. Therefore, alternative routes and timing of administration, as well as drug combination have been implemented to reduce the side effects of a given drug without affecting, or in the best case increasing, the analgesic effects. Thus, it has been reasoned that by administering an analgesic drug before a noxious stimulus is experienced it would prevent, or at least diminish, the hypersensitisation of spinal cord neurones (see below), a concept known as pre-emptive analgesia which is useful in the pre-operative stage. Its effectiveness, of course, will depend on the drug's pharmacokinetics/pharmacodynamics in the target species. Additionally, there have been attempts to combine analgesic drugs from different pharmacological groups to provide better pain relief with less side effects than if a greater dose of one single drug is used, this concept is referred to as balanced analgesia.

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

2.1.1. The nociceptive pathways

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

2.1.1.1. Peripheral afferent input

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

<table>
<thead>
<tr>
<th>Receptor/Channel</th>
<th>Major subtypes</th>
<th>Ligand</th>
<th>Expression (cell size/type)</th>
<th>Notes</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ligand-gated ion channels</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium TTXs</td>
<td>PN1, PN4, rB(I-III)</td>
<td>N/A</td>
<td>All cells</td>
<td>NGF regulated</td>
<td>EMC</td>
</tr>
<tr>
<td>Sodium TTXr</td>
<td>SNS/PN3, SNS/NaN</td>
<td>N/A</td>
<td>Small</td>
<td>PKA and PKC substrate; NGF regulated</td>
<td>EMC</td>
</tr>
<tr>
<td>Calcium voltage-gated channels</td>
<td>T- (low threshold)</td>
<td>N/A</td>
<td>T-, Small/medium</td>
<td>Secondary calcium currents mediated by</td>
<td>SyMC</td>
</tr>
<tr>
<td></td>
<td>L-, N- (high threshold)</td>
<td></td>
<td>L-, N-, Mainly small</td>
<td>G-protein-coupled receptors; enhancement of high threshold current by PKC</td>
<td></td>
</tr>
<tr>
<td><strong>Ionotropic receptors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanilloid</td>
<td>VR1</td>
<td>Heat, capsaicin</td>
<td>Small</td>
<td>Sensitised by heat; proton-gated; NGF-regulated</td>
<td>ST, IMR</td>
</tr>
<tr>
<td>Purine</td>
<td>P2X2, P2X3</td>
<td>ATP, ADP, adenosine</td>
<td>P2X3, IB4 positive cells</td>
<td>P2X2 modulated by protons; P2X3 BDNF regulated</td>
<td>IMR, SyMR, AR</td>
</tr>
<tr>
<td>Serotonin</td>
<td>5-HT3</td>
<td>Serotonin</td>
<td>Small</td>
<td>Controls SP release</td>
<td>SyMR, AR</td>
</tr>
<tr>
<td>NMDA</td>
<td>NR1, NRgbs</td>
<td>Glutamate/aspartate</td>
<td>Small/large</td>
<td></td>
<td>SyMR, AR</td>
</tr>
<tr>
<td>AMPA</td>
<td>GluR1-3</td>
<td>Glutamate</td>
<td>R1, Small; R2-3, Small/large</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kainate</td>
<td>GluR5</td>
<td>Glutamate</td>
<td>Small</td>
<td>Controls neurotransmitter release</td>
<td>SyMR, AR</td>
</tr>
<tr>
<td><strong>Metabotropic receptors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IP</td>
<td>IP</td>
<td>PGI2</td>
<td>Mainly small</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histamine</td>
<td>H1</td>
<td>Histamine</td>
<td>Some small</td>
<td>Itch mediator</td>
<td>IMR</td>
</tr>
<tr>
<td>Serotonin</td>
<td>5-HT1A, 5-HT2A, 5-HT4</td>
<td>Serotonin</td>
<td>5-HT1A/5-HT4, Small. 5-HT2A, Most</td>
<td>5-HT4 increases TTXr small cell currents via PKA/C.</td>
<td>IMR</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>B1, B2</td>
<td>Bradykinin</td>
<td>B1, Small/large; B2, Small</td>
<td>B1 sensitises small cells to heat via PKA. B2 increases the number of cells that respond to capsaicin and protons.</td>
<td>IMR</td>
</tr>
<tr>
<td>Cannabinoid</td>
<td>CB1-2</td>
<td>Anandamide</td>
<td>Unspecified</td>
<td>Inhibits peripheral activation</td>
<td>IMR(in)</td>
</tr>
<tr>
<td>Tachykinin</td>
<td>NK1</td>
<td>SP, NKA</td>
<td>Small</td>
<td></td>
<td>SyMR, AR</td>
</tr>
<tr>
<td>Opioid</td>
<td>µ, δ, κ</td>
<td>Enkephalins, dynorphins, β-endorphin</td>
<td>Small</td>
<td>Inflammatory cells release endogenous opioids. Inhibits peripheral activation.</td>
<td>SyMR, IMR(in)</td>
</tr>
</tbody>
</table>

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

Modified from Woolf & Costigan, 1999.
In chronic pain states, the low-threshold Aβ fibres (highly myelinated axons), which normally transmit innocuous tactile stimuli, also acquire the capacity to increase neural excitability. This enhanced excitability is due, at least in part, to the phenotypic switch in a subpopulation of these fibres so that they, like C fibres, can also express the neuropeptide substance P (SP) (Neumann et al. 1996). Thus, Aδ, C and Aβ fibres contribute to the hyperexcitability observed in chronic pain states by enhancing synaptic transmission in sensory pathways, and Aβ fibres do it by switching their phenotype to one resembling pain fibres. This diversity of changes in the peripheral, as well as central (see below), nociceptive pathways may contribute to the apparent loss of the biological significance of pain signalling as an organic protective measure (Dray et al. 1994).

2.1.1.2. The spinal cord

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

It is generally agreed, on the one hand, that non-NMDA receptor spinal cord mechanisms are involved in the transmission of acute nociceptive messages evoked by C and Aδ fibre activity. On the other hand, states of chronic pain, evoked by Aβ fibres too, may lead to NMDA receptor activation that, in turn, results in facilitation of second order neurones (Eisenach, 1997; Calcutt &
Chaplan, 1997; Sorkin, 1997; Liu et al. 1994; Dray et al. 1994). Therefore, it seems that the development and maintenance of central hyperexcitability, which is clinically manifested as allodynia and hyperalgesia (Kaneko & Hammond, 1997; Liu et al. 1997; Ilkjaer et al. 1998), requires the activation and continuous activation, respectively, of both non-NMDA and NMDA receptors (Neugebauer et al. 1993). Nevertheless, this assumption may be an oversimplification, since a wider variety of receptors and agents are present in the spinal cord and they may interact in a much more complex way to produce central sensitisation. In this regard, some of the same primary afferent nociceptors that release glutamate can also synthesise and co-release neuropeptides [e.g., SP, neurokinin A, and calcitonin gene-related peptide (CGRP)] within the spinal cord, where their respective receptors are present and have been implicated in chronic pain states (Iversen, 1998; Cao et al. 1998; Zamponi et al. 1997).

![Figure 2.1. Transversal section of a sheep's spinal cord at the C5 vertebra showing the ten different laminae of the grey matter.](image-url)
Central sensitisation, in a similar way to peripheral sensitisation, is also accompanied by prolonged alterations in the response properties, neurochemistry, genotype and phenotype of DHN (Kaneko & Hammond, 1997; Iadarola & Caudle, 1997; Liu et al. 1997; Liu et al. 1994; Lascelles, 1996). For example, both electrical activation of C and Aδ fibres and carrageenan injection into the hindpaw of rats enhanced spinal c-Fos protein expression [a nuclear protein encoded by the immediate-early gene c-fos which is widely used as an indirect marker of neurones involved in spinal nociceptive processes (Buritova & Besson, 1998)] in superficial (I-II) and deep (V-VI) laminae of the spinal cord dorsal horn (Buritova et al. 1996). These cellular changes, which take place immediately after peripheral afferent stimulation, may persist for long after the stimulus is terminated, blocked or interrupted. There is little information about how long these changes remain for, but in cattle and sheep suffering from lameness, a hyperexcited state for up to 28 and 90 days, respectively, after apparently successful treatment of the lameness causing lesion has been observed (Whay et al. 1997; Whay et al. 1998; Ley et al. 1989; Ley et al. 1995). Even less clear, but not less important, is the time course of all these changes. Undoubtedly, a better understanding of the timing of induction and the precise role of both excitatory and inhibitory elements in the nociceptive pathway might help to identify new therapeutic targets and establish better administration time protocols for analgesic drugs.

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

### 2.1.1.3. Ascending spinal pathways

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

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

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

The spinocervical tract and the second-order dorsal column pathway ascend through the dorsal aspect of the spinal cord white matter. The neurones of origin of both pathways are primarily located in laminae IV of the spinal cord. The spinocervical tract ascends ipsilaterally to the end of the lateral cervical nucleus which in turn projects to the ventral posterior lateral nucleus of the thalamus. The second-order dorsal column pathway projects ipsilaterally in the
dorsal funiculus and the dorsal part of the lateral funiculus, terminating into the dorsal column nuclei which in turn projects to the ventral posterior lateral nucleus of the thalamus (Willis, 1983; Willis, 1989).

2.1.1.4. The brain

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

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

2.1.1.5. Descending inhibitory systems

The descending inhibitory systems regulate the ongoing processing of nociceptive afferent input. Several different tracts have been identified and, like the ascending spinal pathways, species differences also exist. Some of the most important long descending tracts, which are found only in the lateral and ventral aspects of the spinal cord white matter, are the corticospinal pyramidal tract, coming from the sensorimotor cortex; the rubrospinal tract, originated from
the red nucleus in the midbrain; the reticulospinal tract, which fibres arise from
the pontomedullary reticular formation; and the vestibulospinal fibres, which
originate from the lateral vestibular nucleus of the medulla oblongata (Bowsher,
1975). Even though these pathways were anatomically identified several years
ago, their physiopathological role is not well understood yet.

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

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

Modulation of nociceptive transmission by bulbospinal pathways is largely
attributed to the biogenic amines noradrenaline, 5-HT and dopamine, as well as
to adenosine, which mainly exert inhibitory effects both in the brain and the
spinal cord. Noradrenergic inputs are sent by the locus coerules to the PAG,
which connects to noradrenergic pontomedullary cell groups in the RVM (Fields & Basbaum, 1989). Thus, activation of PAG neurones inhibited noxious responses of sacral DHN through a presynaptic $\alpha_2$-adrenoceptor mechanism (Budai et al. 1998) and, interestingly, excitatory effects were also observed but these were mediated by $\alpha_1$-adrecoceptors (Budai et al. 1998). Dopaminergic descending mechanisms seem to be also implicated in the nociceptive process and a pathway descending from diencephalic neurones and projecting into the spinal cord, where activation of dopamine D2 receptors produced analgesia, might play an important role (Barasi et al. 1987). Adenosine also mediates antinociception via the descending DLF; when the locus coeruleus was electrically stimulated a suppressing spiking response from parafascicular neurones was observed and this inhibitory effect was reversed by the i.t. administration of the adenosine receptor antagonists phentolamine and aminophylline (Zhao et al. 1999).

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

Many other neuroactive agents (e.g., cholecystokinin, prolactin, SP) are also produced in the brain and transported by diverse descending pathways to finally
act in the spinal cord; however, in most cases, their contribution to the modulation of sensory transmission is not completely understood. A more detailed overview of these agents surpasses the objectives of this work.

2.2. GLUTAMATE RECEPTORS

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

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

Although all different glutamate receptors can be distributed within the same glutamatergic synapse and may share a similar structure for their agonist recognition domain (Conn & Pin, 1997), their functions are completely different (see below). This could be explained, at least in part, by the different receptor distribution within the same synapse and the differential regulation of by synaptic proteins that physically couple to them. Thus, these adaptor or anchor proteins (e.g., GRIP, PSD-95 and Homer proteins coupled to AMPA, NMDA and kainate, and mGluRs, respectively) may partially regulate the downstream
signalling pathways for each receptor (Sheng, 1997; Dingledine et al. 1999). These pathways are discussed below.

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

2.2.1. Ionotropic glutamate receptors

2.2.1.1. AMPA receptors

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

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

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

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

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

### 2.2.1.2. Kainate receptors

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

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

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

2.2.1.3. NMDA receptors

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

2.2.1.3.1. Regulation of the NMDA receptor

The NMDA receptors, in contrast to most other ion channels, are blocked by Mg^{2+} in a voltage-dependent manner, and are allosterically regulated by glycine and polyamines (Dumuis et al. 1988; Jin & Fredholm, 1997; Ghosh & Greenberg, 1995; Reynolds & Miller, 1990; Woodburn & Woodruff, 1994; Gu et
al. 1996). The voltage dependence of the channel arises from the fact that at resting potential the channel is blocked by extracellular Mg$^{2+}$ ions (Reynolds & Miller, 1990). Stimulation of primary afferents removes the Mg$^{2+}$ block by activating postsynaptic non-NMDA receptors and activation exceeding a defined threshold depolarises the membrane removing the Mg$^{2+}$ ion from the NMDA receptor, permitting the opening of the channel and the influx of Ca$^{2+}$, Na$^+$ and K$^+$ (Chaplan et al. 1997; Ghosh & Greenberg, 1995; Woodburn & Woodruff, 1994). Hence, the non-NMDA and the NMDA receptors might operate in succession (Neugebauer et al. 1993). In fact, Na$^+$ influx through either subtype of ionotropic glutamate receptor or voltage-gated Na$^+$ channels potentiated the activity of NMDA receptors (Yu & Salter, 1998). It seems that once Na$^+$ enters the postsynaptic neurone it functions as a signalling ion acting directly on the NMDA channel or on a closely associated regulatory site, which agrees with Na$^+$ sensitivity of NMDA receptors being set by a Src kinase associated with the channel (Yu & Salter, 1998).

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

In addition, NMDA receptors are also regulated by protein phosphorylation at serine, threonine and tyrosine residues (Yu et al. 1997). Activation of PKC potentiates the NMDA receptor function in central neurones (Schoepp & Conn, 1993; Malmberg et al. 1997; Ghosh & Greenberg, 1995; Woodburn & Woodruff, 1994) by phosphorylating serine and threonine residues (Yu et al. 1997) on the NR1 subunit (Ghosh & Greenberg, 1995). The Src-family PTKs phosphorylate NMDA receptor subunits NR2A and NR2B (Yu et al. 1997; Tezuka et al. 1999) and the anchor protein PSD-95 may play an important role in this process (Tezuka et al. 1999). Furthermore, BDNF, acting through the trkB receptor, elicits, at the NR1 and NR2B subunit level, phosphorylation-dependent enhancement of the NMDA channel functional activity (Levine et al. 1998). Interestingly, nerve grow factor treatment by acting in neurones expressing tyrosine kinase A (trkA) neurotrophin receptors, upregulates BDNF which, besides enhancing NMDA receptor phosphorylation, also induces changes in NR2A and NR2B receptor subunit expression (Thompson et al. 1999). CaMKII, when activated by Ca$^{2+}$ influx through the NMDA receptor, phosphorylates the NR1 and NR2B subunits, thereby increasing Ca$^{2+}$ influx too (Leonard et al. 1999). Phosphorylation of NMDA receptors by PKA also enhances the receptor function (Dingledine et al. 1999; Westphal et al. 1999) and this effect is even more evident when the NMDA receptor is composed of NR1A subunits. This receptor subunit is coupled to the associated protein yotiao which anchors PKA and type I protein phosphatase to NMDA receptors and PKA activation increases the receptor currents in comparison to NMDA receptors not expressing yotiao whereas type I protein phosphatase negatively regulates NMDA receptor currents (Westphal et al. 1999).
Interestingly, the development of NMDA receptor-dependent central sensitisation can be physiologically stopped by the increase in intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]i; e.g., Ca\textsuperscript{2+} entry through NMDA receptors, voltage-gated Ca\textsuperscript{2+} channels, Ca\textsuperscript{2+} permeable AMPA receptors, Ca\textsuperscript{2+} release from intracellular stores or Ca\textsuperscript{2+} entry induced by action potentials), a mechanism denominated Ca\textsuperscript{2+}-dependent inactivation (CDI) of NMDA receptors, which may act as a brake on the recruitment of NMDA receptors during sensory synaptic input to DHN, constituting a potent inhibitory control of NMDA currents (Kyrozis et al. 1996). In fact, L-, P/Q-, and R-type Ca\textsuperscript{2+} channels also suffer Ca\textsuperscript{2+}-mediated inactivation, and facilitation, by action of calmodulin (Ehlers & Augustine, 1999; Lee et al. 1999; Zühlke et al. 1999) which, also, desphosphorylates the NMDA receptor resulting in a negative feedback of NMDA receptor synaptic activation (Tong et al. 1995). Thus, NMDA receptors may fluctuate between phosphorylated and desphosphorylated forms, depending on the rate of synaptic stimulation and the magnitude of the associated Ca\textsuperscript{2+} influx through them, and calmodulin may act as a Ca\textsuperscript{2+} sensor in these and other channels, contributing, therefore, to modulate the cellular response. Nevertheless, activation of NMDA receptors responds almost immediately with stimulation of different metabolic pathways and the formation of retrograde messengers, which may account for the NMDA receptor-mediated excitability.

2.2.1.3.2. Effects of NMDA receptor activation

Activation of NMDA receptors permits the entry of Ca\textsuperscript{2+} into the cytoplasm, which triggers different metabolic pathways activating a number of enzymes and ultimately immediate-early genes (Figure 2.2) (Ghosh & Greenberg, 1995; Coderre et al. 1990; Woodburn & Woodruff, 1994). Retrograde second messengers resulting from this cascade have been posited to cause presynaptic amplification of neurotransmitter release; candidates include the neuromediators AA, PGs and NO, which may diffuse back to the primary afferent terminal (Chaplan et al. 1997; Dumuis et al. 1988; Nicholls, 1992; Schoepp & Conn, 1993; Dawson & Dawson, 1995; Woodburn & Woodruff,
Figure 2.2. The role of glutamate receptors and their interaction with other receptor systems in the modulation of nociceptive processing. Refer to the text for details.
This hypothesis could be true for NO because of its physical properties [e.g., small, diffusible, membrane permeable, reactive (Dawson & Dawson, 1995)]; however, it may not apply for the PGs. Although these lipidic acids are often assumed to traverse membranes by simple diffusion, they are charged anions at physiological pH and diffuse poorly across model biological membranes. This limited simple diffusion appears to be augmented, at least in some circumstances, by a specific carrier-mediated transport (PG transporter) and AA is probably not transported to a significant extent by this transporter (Kanai et al. 1995). Nevertheless, both AA and PGs are involved in the process of pain signal amplification (see below).

2.2.1.3.2.1. NMDA receptors and the AA pathway

The resulting increase of [Ca$^{2+}$]i, preceded by NMDA receptor activation, activates phospholipases. Activation of phospholipase A$_2$ (PLA$_2$) can increase the susceptibility of membrane phospholipids to hydrolytic processes that are associated with an increasing level of free AA, which, in turn, may be converted by cyclooxygenases (COX, also known as prostaglandin H synthase or prostaglandin endoperoxidase synthase) to prostanoids, including the PGs (Malmberg & Yaksh, 1992b; Buritova et al. 1996; Yaksh & Malmberg, 1993; Woodburn & Woodruff, 1994; Miller et al. 1992) (Figure 2.2). In agreement with this model, administration of glutamate and NMDA increased [$^3$H]AA release from cerebellar granule cells (Viu et al. 1998), activation of high-threshold nociceptive afferent inputs, which activate NMDA receptors, released PGs within the spinal cord (Ramwell et al. 1966), and i.t. administration of NMDA evoked spinal increased release of PGE$_2$ and thromboxane (Tx) B$_2$ (Sorkin, 1997). Hence, a clear interaction between NMDA receptors and the AA pathway exists.

Arachadionic acid directly or indirectly potentiates NMDA currents, increases the release of glutamate from presynaptic terminals and impairs its glial reuptake (Dumuis et al. 1988; Nicholls, 1992). Amplification of NMDA receptor
currents by AA itself is probably due to its binding to a different site than the glutamate, glycine, Mg$^{2+}$, Zn$^{2+}$ or polyamine binding sites, or by altering the receptor's lipid environment (Miller et al. 1992); however, the exact mechanism remains unknown. Glutamate increased release and decreased reuptake seem to be mediated by products downstream than AA in the biochemical pathway (Bezzi et al. 1998) but the effects and mechanisms of action of these AA metabolic products in the nociceptive process will be approached in further detail later in this work.

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

2.2.1.3.2.2. NMDA receptors and the NO pathway

In addition to activation of phospholipases, Ca$^{2+}$ entering though the NMDA receptor can also activate the enzyme nitric oxide synthase (NOS). Once in the cytoplasm, Ca$^{2+}$ binds to calmodulin forming the Ca$^{2+}$-calmodulin complex (CaM), which activates NOS, which in turn, in the presence of O$_2$ and NADPH, is critical for the conversion of L-arginine to NO and L-citrulline (Dawson & Dawson, 1995; Machelska et al. 1997; Woodburn & Woodruff, 1994; Appleton et al. 1996; Knowles et al. 1989). This coupled NO production to NMDA receptor activation is thought to be mediated by a signalling pathway mediated by the PDZ-PDZ protein interaction domains between the NMDA receptor anchor protein PSD-95 and NOS (at least the neuronal isoform; see below) (Hillier et al. 1999; Sattler et al. 1999). NOS has to be in desphosphorylated form to be functionally active and when phosphorylated by PKC the enzyme catalytic activity is reduced (Dawson & Dawson, 1995; Snyder et al. 1998); desphosphorylation of NOS can be achieved by calcineurin (Snyder et al. 1998), which, in turn, is activated by Ca$^{2+}$ entering through NMDA receptors and/or L-type Ca$^{2+}$ channels (Genazzani et al. 1999). In addition to its
dephosphorylation action on NOS, calcineurin can also dephosphorylate the inositol-(1,4,5) tri phosphate [Ins(1,4,5)P3] receptor and increase Ins(1,4,5)P3 receptor mRNA and protein levels in cultured cerebellar granule cells via an unidentified pathway (Genazzani et al. 1999), regulating, therefore, Ca\(^{2+}\) efflux from intracellular stores and contributing to the hyperexcited neuronal state.

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

In the peripheral nervous system, NO is released by a widespread network of nerves which mediate some forms of neurogenic vasodilatation and regulate certain gastrointestinal, respiratory and genitourinary functions, presumably by direct action of NO (Moncada et al. 1997). In the CNS, NO synthesis can be mediated by either NOS isoform (Dawson & Dawson, 1995). Specifically in the spinal cord, the enzyme is found predominantly in the superficial layers of the dorsal horn and around the central canal (the intermediolateral cell column) (Machelska et al. 1997; Sakai et al. 1998), conferring a nocimodulator role to this gas in the CNS. In addition, NO modulates, among other functions in the CNS, the formation of memory and the coordination between neuronal activity and blood flow (Moncada et al. 1997). These physiological actions of NO are
mediated by activation of the soluble guanylate cyclase and consequently increase the concentration of 3’,5’-cyclic guanosine monophosphate (cGMP) in target cells (Moncada et al. 1997; Knowles et al. 1989; Bredt et al. 1990).

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

All of the above support the neuromodulator role of NO on the nociceptive process acting as a retrograde messenger between neurones, as a modulator of postsynaptic function, and/or as a paracrine mediator in adjacent neurones or glia. Further support comes from the inhibitory effect of the nociceptive process by inhibiting the enzyme NOS with L-arginine analogues (Morgan et al. 1992;
Machelska et al. 1997; Moore et al. 1991; Moncada et al. 1997; Sakurada et al. 1996; Bulmer et al. 1998). However, the level within the CNS where NOS inhibitors exert their antinociceptive effects is still a matter of debate (Sakurada et al. 1996). Despite where in the CNS NOS inhibitors exert their action, it is possible that these agents have a dual antinociceptive action by inhibiting the synthesis of NO and by augmenting the levels of L-arginine. When the amino acid was administered in mice submitted to the carrageenan paw test, antinociception by an opioidergic mechanism independent of the NO pathway was observed (Kawabata et al. 1992). Interestingly, metabolism of arginine can occur by more than one pathway: by NOS to NO and citrulline, by arginase to ornithine and urea, and by arginine decarboxylase to agmatine. Ornithine and agmatine, in turn, can be converted into the polyamines putrescine, spermidine and spermine (Reis & Regunathan, 2000). Since agmatine and polyamines can modulate NMDA receptors (Appleton et al. 1996; Reis & Regunathan, 2000), they could also have contributed to L-arginine's antinociceptive action. Nonetheless, hyperalgesic actions have also been observed after epidural administration of L-arginine in rats (Masue et al. 1999).

Another pharmacological group that is also able to inhibit NOS are the anti-inflammatory steroids (also known as glucocorticoids), but they only affect the induction of iNOS and have no effect on the expression of either constitutive NOS (Salvemini et al. 1993; Salvemini et al. 1994). This inhibition, at least for dexamethasone, seems to involve enhanced lipocortin release (Appleton et al. 1996; Minghetti et al. 1999), a potent endogenous mediator of the anti-inflammatory actions of glucocorticoids. In fact, a lipocortin 1-derived N-terminus peptide (peptide Ac2-26) inhibited PGE₂ and NO production from lipopolysaccharide (LPS)-stimulated microglial cells in a similar way to dexamethasone and these effects were largely due to down-regulation of COX-2 and iNOS (Minghetti et al. 1999).
2.2.1.3.2.3. Interactions between the AA and NO pathways

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

On the other hand, stimulatory and inhibitory actions of NO on PG production may be explained by the relative concentrations of NO. Low levels of NO may stimulate PG formation and thus act as a pronociceptive agent, whereas high levels of NO may inhibit PG production, therefore acting as an antinociceptive agent (Machelska et al. 1997; Appleton et al. 1996). Interestingly, studies \textit{in vitro} using mouse macrophage cells (Salvemini et al. 1993), and \textit{ex vivo} utilising a rabbit hydronephrotic kidney model (Salvemini et al. 1994), have demonstrated a NO-mediated increase in PG production, supporting a target role of COX for NO. In human cultured astroglial cells incubated with interleukin-1\(\beta\) (IL-1\(\beta\)) and tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)), the involvement of NO in the increase of PGE\textsubscript{2} has also been reported (Mollace et al. 1998). This effect was attributed to the activation of iNOS and subsequent release of NO which interacted with COX-2 to enhance the formation of PGE\textsubscript{2}. However, reduced PG
production in human vascular smooth muscle cells was attributed to inhibition of COX-2 activity via activation of the NO-soluble guanylate cyclase pathway (Gitlin et al. 1999). Even more, lack of interaction between iNOS and COX-2 products in the plasma of LPS-treated rats was observed (Hamilton et al. 1998). Therefore, it may be possible that the interactions between these pathways could be tissue or species specific, or both.

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

2.2.1.3.3. NMDA receptors and the release of other neuromediators

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

In general, direct NMDA receptor-mediated events are considered to be postsynaptic (Buritova et al. 1996). However, it was recently demonstrated the existence of active presynaptic NMDA receptors (autoreceptors) in the spinal cord dorsal horn (Liu et al. 1994) which seem to contribute to the release of glutamate (Conway et al. 1997; Robert et al. 1998) and SP (Liu et al. 1997; Malcangio et al. 1998). Interestingly, the neurokinin NK-1 receptor, to which SP primarily couples, can also be located either post- or presynaptically (Urban & Nagy, 1997). Thus, both glutamate and SP, in addition to depolarising the postsynaptic terminal, can also act on presynaptic autoreceptors and may modulate neurotransmitter release. Nevertheless, controversy regarding the role of presynaptic NMDA receptors in nociception exists (Urban & Nagy, 1997).
Under certain conditions of afferent stimulation, GABA, the main inhibitory neurotransmitter in the mammalian CNS, is released in sufficient amounts to cause hyperpolarisation of DHN, thereby limiting the NMDA-mediated development of central sensitisation (Lorenzetti & Ferreira, 1996). In this regard, it is not surprising that GABAergic and glutamatergic synapses often exist in close apposition on DHN and so are stimulated to effectively modulate local membrane potentials (Kaneko & Hammond, 1997). GABA acts on three different receptor types: GABA_A, GABA_B and GABA_C. The GABA_A receptor, located postsynaptically, is a ligand-gated ion channel that, when activated by two molecules of GABA, allows an influx of Cl^- ions hyperpolarising the cell membrane and producing a cessation of the cell's electrical discharge. The GABA_B receptor, which exists pre- and postsynaptically, is linked to G-proteins and when activated by one molecule of GABA, the receptor then couples to associated G-proteins. One of these (G_i) inhibits adenylate cyclase, probably through its α-subunit, and opens a K_+ channel, probably through its βγ subunits. Another G-protein (G_o) retards the opening of the Ca^{2+} channel, probably through its βγ subunit (Bowery & Brown, 1997), hyperpolarising the cell membrane, therefore, by a dual mechanism. The GABA_C receptor is an ionotropic receptor, which mainly localises in mammalian retina cells (Bormann, 2000).

Stimulation of NMDA receptors also seems to be interrelated with the opioidergic and adrenergic pathways. Thus, noxious stimulation enhances the release of endogenous opioid peptides which, by acting on presynaptic receptors (Table 2.2), reduce the release of glutamate and SP from the primary afferent (Dhawan et al. 1996). Increased noradrenaline release after spinal NMDA receptor stimulation also has been observed (Nakai et al. 1999) and activation of α2- subtype receptors mediates its antinociceptive effects. In this regard, α2-adrenoceptor agonists depressed a NMDA receptor-mediated response of nociceptive neurones in the spinal cord (Faber et al. 1998; Zhang et al. 1998), an effect observed with synthetic opioids too (Faber et al. 1997a).
Enhanced release of dopamine (Jin & Fredholm, 1997), ACh (Jin & Fredholm, 1997) and adenosine (Jin & Fredholm, 1997; Conway et al. 1997) can also occur after NMDA receptor activation. Dopamine produces antinocicepcion at the spinal level by activation of dopamine D2 receptors (Barasi et al. 1987) and, interestingly, probably by blocking the NMDA receptor itself (Castro et al. 1999), whereas ACh does it by acting on muscarinic M2 receptors (Gomeza et al. 1999). Adenosine, in turn, inhibits NMDA-receptor mediated hyperalgesia and the concurrent spinal glutamate release by acting on adenosine A1 receptors (Kakinohana et al. 1998).

Table 2.2. Distribution and functions of opioid receptors.

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

Elaborated with information from Dhawan et al. 1996.
Increased IL-1β expression has also been observed after NMDA receptors activation and it seems that this cytokine releases some neurotransmitters (e.g., noradrenaline, dopamine) or retrograde messengers (e.g., AA, NO, platelet-activating factor), or both (Schneider et al. 1998). In this regard, cultured astroglial cells treated with IL-1b increased mRNA P2Y₂ expression (John et al. 1999) and activation of a P2Y receptor in cultured astrocytes resulted in increased COX-2 expression (Brambilla et al. 1999). Moreover, increased NO and subsequent PGE₂ formation in cultured astroglial cells incubated with IL-1β and TNF-α has been reported (Mollace et al. 1998), as well as enhanced SP release from spinal cord slices, presumably through the generation of endogenous prostanoids by the cytokine (Malcangio et al. 1996). Together, these data suggest the involvement of IL-1β in the nociceptive processing, an effect supported by an enhanced Aδ fibre-evoked response of rat spinal neurones after i.t. administration of the cytokine, and by inhibition of both Aδ and C fibre-evoked responses after i.t. application of the IL-1 converting enzyme inhibitor SDZ-224-015 (Reeve et al. 1998).

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

In summary, activation of NMDA receptors permits Ca²⁺ influx which in turns activates the AA and the NO pathways, resulting in the production of retrograde messengers that could act on presynaptic neurones, as well as non-neural structures or postsynaptic neurones. The effects of these molecules include the release of neurotransmitters or neuropeptides, or both, from the presynaptic site and inhibitory interneurones, as well as the formation and expression of new receptors. All these complex mechanisms serve to modulate pain transmission.
Pharmacological inhibition of NMDA receptors, hence, results in attenuation of nociceptive processing (Chaplan et al. 1997; Ilkjær et al. 1998; Dupont et al. 1998). However, mechanisms other than or complementary to purely NMDA receptor antagonism may contribute to the analgesic effect of these receptor blockers. For example, the analgesic effect of intraperitoneal (i.p.) dizocilpine assessed by the hot plate test in rats was partially reversed by i.p. injection of the opioid receptor antagonist naloxone, and the dopamine D1 and D2 receptor antagonists SCH 23390 and sulpiride, respectively (Forman, 1999). Because co-administration of naloxone plus SCH 23390 or sulpiride did not completely abolish dizocilpine analgesia, contribution of serotonergic, α-adrenergic, and muscarinic cholinergic mechanisms was proposed (Forman, 1999). In fact, enhanced [3H]noradrenaline release evoked by electrical stimulation of rat spinal cord slices was observed with dizocilpine (Nakai et al. 1999), whereas increased dopamine and 5-HT release in the rat striatum was detected after i.p. and intra-striatum administration of the NMDA receptor antagonist D-CPPene (Potschka et al. 1999). Thus, it could be possible that NMDA receptor antagonists produce antinociception by partially increasing the release of natural occurring inhibitory mediators.

2.2.2. Metabotropic glutamate receptors

In the same synapses at which glutamate elicits fast excitatory responses, it can modulate fine-tune activity by activation of mGluRs which are coupled to effector systems through G-proteins. These G-protein-linked receptors are divided into three groups (Group I-III) based on their sequence homologies, the selectivity/potency of mGluR agonist and the signal transduction pathways to which each receptor preferentially couples. Group I receptors, which include the splice variants of mGluR1 and mGluR5, stimulate phosphoinositide hydrolysis by stimulating phospholipase C (PLC) to generate Ins(1,4,5)P3 and DAG; whereas Group II (mGluR2 and 3) and III (mGluR4, 6, 7 and 8) receptors evoke an inhibition of forskolin-stimulated 3′,5′-cyclic guanosine monophosphate (cAMP) formation by negatively coupling to adenyl cyclase (Akam et al. 1997;
Cartmell et al. 1997; Pinkney et al. 1997; Woodburn & Woodruff, 1994; Wigmore & Lacey, 1998; Conn & Pin, 1997).

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

2.2.2.1. Excitatory effects of mGluRs

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

In addition to the postsynaptic excitatory action, a presynaptic excitatory action for group I mGluRs has been suggested also. In this model, inhibition of K\(^+\) influx is due via activation of a sensitised PKC by AA diffused back from the postsynaptic terminal (Dumuis et al. 1988; Nicholls, 1992) potentiating, thus,
action potentials and neurotransmitter release. Similarly, increased glutamate release through a Ca\(^{2+}\)-dependent process mediated by PGs was observed after co-activation of group I mGluRs and AMPA-kainate receptors in astrocytes (Bezzi et al. 1998). Furthermore, a decrease in dopamine levels after presynaptic stimulation of group III, and to a lesser extent group II, mGluRs was noticed (Hu et al. 1999) as well as those for GABA, but the specific mGluRs involved in this process are not mentioned (Conn & Pin, 1997). Thus, mGluRs are capable of enhancing the excitatory synaptic transmission by multiple mechanisms that may include potentiation of excitatory neurotransmitter and neuropeptide release, decreased glutamate reuptake, and/or suppression of inhibitory neurotransmitter release.

Recent studies from Kubo et al. (1998) have raised the possibility that mGluRs may also function as ionotropic receptors. They found that continuous stimulation of oocytes expressing mGluR1\(\alpha\) by 2 mM Ca\(^{2+}\) solution, increased the concentration of Ins(1,4,5)P\(3\) in chinese hamster ovary cells. Thus, extracellular physiological Ca\(^{2+}\) concentrations could represent another mechanism of mGluR-mediated excitatory transmission.

2.2.2.2. Inhibitory effects of mGluRs

Reduction of transmission by mGluRs is mediated typically by presynaptic autoreceptors that reduce glutamate release. Depression of glutamatergic transmission attributable to presynaptic group II and III mGluR activation has been observed at several synapses including those in the spinal cord (Wigmore & Lacey, 1998; Scanziani et al. 1997; Conn & Pin, 1997); however, the molecular mechanisms involved in this process are still to be determined. Reduction of voltage-dependent Ca\(^{2+}\) currents seems to be a possible mechanism. Recently, Takahashi et al. (1996) described a presynaptic inhibition of a high voltage-activated P/Q type Ca\(^{2+}\) conductance mediated by group II or III mGluR activation and postulated that these receptors can inhibit transmitter release by targeting ion channels.
Other likely mechanisms for inhibition of synaptic transmission could include the reduction of cGMP accumulation and the increased production of adenosine. The former mechanism was observed after group III mGlur activation in retinal cells (Conn & Pin, 1997); if this also happens in nociceptive neurones, the inhibition of this product of the NO pathway may represent an important mechanism in the modulation of synaptic neurotransmission, because it contributes to the release of neurotransmitters (Fukuto & Chaudhuri, 1995). On the other hand, increased adenosine production was ascribed to co-activation of group II mGlur and β-adrenergic receptors which produced cAMP and subsequently adenosine (Conn & Pin, 1997) that, in turn, may inhibit spinal glutamate release and hyperalgesia by acting on presynaptic adenosine A1 receptors (Kakinohana et al. 1998).

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

2.3. THE ARACHIDONIC ACID PATHWAY

This section intends to review the biochemical steps that lead to the formation of some of the most important autacoids in the AA pathway as well as their effects on the nociceptive process. The AA pathway (Figure 2.3) begins with the release of polyunsaturated fatty acids from the cell membrane’s phospholipids which are converted to AA by action of different phospholipases, although PLA₂.
Figure 2.3. Major pathways of the arachidonic acid cascade.
seems to be the most important. AA is released into the cell and acts as a substrate for enzymes, lipoxygenases (LOX), COX and epoxygenases (EOX), which produce intermediate and final products. LOX are responsible for the production of hydroxyeicosatetraenoic acids (HETE), and specifically 5-LOX for that of leukotrienes (LTs); COX have a role on the synthesis of PGs and Txs; whereas EOX are involved in the formation of epoxyeicosatrienoic acids. The EOX biochemical pathway is not as well characterised as that for COX or LOX, and the effects of their products on nociception have hardly been studied.

2.3.1. Leukotrienes

The LTs are metabolites derived from lipoxygenation of AA. They are potent smooth muscle contracting agents (Freedman et al. 1993) involved in inflammatory and painful reactions; however, these effects seem to be indirectly mediated by producing microvascular changes on the blood flow and increasing the vascular permeability (Bisgaard et al. 1982; Bisgaard, 1987; Ford-Hutchinson, 1985). Their contribution to the pain process, therefore, comes from sensitisation of cutaneous nociceptors rather than producing a direct effect on them (Madison et al. 1992) and this is expressed as a delay in the onset of hyperalgesia after their experimental administration (Taiwo et al. 1987). This sensitising effect of LTs seems to be specific for different agents; for example, LTB₄ sensitised nociceptive fibres to become hyperalgesic to the action of PGE₂ (Martin et al. 1987) but not PGE₁, which was affected by LTD₄ (Rackham & Ford-Hutchinson, 1983). Furthermore, the induction of hyperalgesia by LTB₄, a potent chemotactic factor for polymorphonuclear leukocytes (PMNLs) (Ford-Hutchinson, 1985), might also depend on the generation by cutaneous PMNLs of mediators derived from the 15-lipoxygenation of AA, which modulate primary afferent functions and contribute to inflammatory hyperalgesia (Levine et al. 1986). All these studies illustrate LT-mediated nociceptive effects at the peripheral level and, because both centrally administered LTC₄ and LTD₄ had no effect on thermal pain sensation in mice (Brus et al. 1986), it seems that the effects of these agents on nociception are restricted to this level.
Together, the above data suggest that LTs may indirectly modulate pain responses by sensitising peripheral nociceptors to the action of PGs or by increasing the recruitment of vascular agents that may enhance pain transmission, or both. Inhibition of the LOX pathway, therefore, was postulated as an advantageous adjunct to the effect of the NSAIDs (Bisgaard & Kristensen, 1985). Interestingly, the 5-LOX/COX dual inhibitor SK&F 105809 reduced hyperalgesia as assessed by the phenyl-benzoquinone-induced writhing test in mice (Griswold et al. 1991); however, the antinociceptive importance of inhibiting LT production remains uncertain. In the same nociceptive test, the 5-LOX inhibitors BW A4C and BW A797C had no analgesic effect (Higgs et al. 1988) but the COX inhibitors indomethacin, naproxen, and ibuprofen did produce analgesia (Griswold et al. 1991). More research is necessary to establish the analgesic importance of inhibiting the LOX pathway.

2.3.2. Prostaglandins

The PGs are polyunsaturated fatty acid derivatives, which basic structural unit is a 20-carbon unsaturated carboxylic acid with a characteristic cyclopentane ring structure. The term was first used to describe the presence of these substances in semen and the belief that they came from the prostate; nevertheless, they appear to be present in almost all tissues. Their synthesis is due by oxygenation of AA by COX, which produces PGG₂, unstable peroxide that is converted to PGH₂. PGH₂, in turn, is converted via distinct synthases to PGD₂, PGE₂, PGF₂α, PGI₂, or TxA₂, generally only one prostanoid is formed in abundance and this effect is cell specific. None of these agents are stored within cells but rather are synthesised in demand, and once formed, all are rapidly metabolised with biological half-lives of approximately 1 minute (Appleton et al. 1996). Some of the most important metabolites of these prostanoids are shown in Figure 2.3.

Besides catalysing the formation of PGG₂, COX has a peroxidase activity, reducing the 15-hydroperoxyl group of PGG₂ to PGH₂. These two reactions occur on the same enzyme but the active sites differ and COX inhibition by NSAIDs has no effect on peroxidase activity (Appleton et al. 1996).
Interestingly, PGs, to some extent, can modulate COX activity (Appleton et al. 1996; Kurumbail et al. 1996). Different COX isozymes have been identified and an overview of their pathophysiological functions as well as their expression and regulation is next presented. For those interested in this particular subject various recent reviews have been published elsewhere (Smith & Dewitt, 1996; Vane et al. 1998; Cryer & Dubois, 1998; Mitchell & Warner, 1999; Dubois et al. 1998).

2.3.2.1. Cyclooxygenases

2.3.2.1.1. Cycoolxygenase isoforms and functions

For many years it was thought that COX was a single enzyme present constitutively in most tissues and that inhibition of this enzyme led to decreased production of pro-inflammatory as well as physiological PGs. However, now it is known the existence of at least two COX isozymes: COX-1 and COX-2. Both isoforms use the same endogenous substrate, AA, and form the same products by the same catalytic reaction (Langenbach et al. 1995; Morham et al. 1995); this is due to the highly conserved homology (>90%) of the COX and peroxidase enzyme regions between the two isozymes (Cryer & Dubois, 1998). Their major difference lies in their pathophysiological functions. On the one hand, COX-1 is constitutively expressed in most tissues at a different constant level, and is believed to be responsible for the formation of PGs with physiological functions. COX-2, on the other hand, is normally undetectable in most tissues, but it can be induced at high levels in a variety of cells by pro-inflammatory stimuli (Geisslinger & Schaible, 1996; Yamamoto & Nozaki-Taguchi, 1996; Appleton et al. 1996; Seibert et al. 1994; Kurumbail et al. 1996; Vane et al. 1998; Langenbach et al. 1995; Morham et al. 1995; Mahler et al. 1996). This activation of COX-2 by pro-inflammatory stimuli lead to the suggestion that the NSAIDs' therapeutic effects are mediated via the inhibition of COX-2 whereas their side effects are due to inhibition of COX-1 (Geisslinger & Schaible, 1996; Seibert et al. 1994; Langenbach et al. 1995), an hypothesis that has created a lot of controversy (see below). Furthermore, important
structural peculiarities in the regulation of both COX isozymes exist (Table 2.3). Of great importance is the presence of a glucocorticoid regulatory region on the COX-2 gene which accounts for cytokine or endotoxin induction of COX-2 being inhibited in vitro and in vivo by glucocorticoids such as dexamethasone, whereas COX-1 is unaffected (Appleton et al. 1996; Seibert et al. 1994; Salvemini et al. 1993; Salvemini et al. 1994; Vane et al. 1998; Langenbach et al. 1995; Morham et al. 1995). Together, all these differences suggest that the two isozymes can act independently.

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

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


Although most of our knowledge on the functions of both COX isozymes comes from studies of pharmacological inhibition with NSAIDs and COX-2 selective inhibitors, the development of *Ptgs* 1 and *Ptgs* 2 gene knockout mice (Langenbach et al. 1995; Morham et al. 1995; Mahler et al. 1996) showed interesting findings (Table 2.4) about these two isozymes. For example, COX-1, to some degree, contributes to inflammation, parturition and luteolysis, and its absence alone is not sufficient to cause gastric ulceration. On the other hand, COX-2 is not essential for an inflammatory response and may be involved in cell differentiation, mitogenesis and specific reproductive functions. Therefore, it is highly likely that both COX isozymes have physiological and pathophysiological functions and that inhibition of COX-2 is not devoid of side
effects. Thus, COX-1, besides its well-known role in gastroprotective functions, plays a part in the inflammatory process also (Smith et al. 1998; Wallace, 1999; Wallace et al. 1999). On the other hand, up-regulation of COX-2 has a homeostatic role in the inflamed tissue surrounding gastrointestinal ulcers and PGs produced via this isozyme may be responsible for the beneficial effects on ulcer healing (Wallace, 1999; Wallace & Cirino, 1994; Shigeta et al. 1998). Therefore, when COX-2 selective inhibitors were used in rodents suffering from induced gastric or colonic ulceration there was a significant reduction in mucosal PG synthesis and a marked increase in the severity of the organ damage, delaying the healing (Wallace, 1999; Shigeta et al. 1998). Other possible side effects could occur in the kidneys, lungs or the CNS, where COX-2 is constitutively expressed (Vane et al. 1998; Ermert et al. 1998; Cryer & Dubois, 1998; Phillips & Pearce, 1999), and data from COX-2 knockout mice (Morham et al. 1995) indicates that reproductive functions might be affected as well. Recently, it was highlighted that COX-2 selective inhibitors reduced systemic and renal synthesis of PGI2 in humans (Catella-Lawson et al. 1999; Cullen et al. 1998), suggesting that more than the predicted side effects could be expected with the use of these compounds. A comprehensive review of possible adverse effects by COX-2 inhibition in these and other body organs and systems (e.g., pancreas, cardiovascular system) has been published elsewhere (Mitchell & Warner, 1999).

2.3.2.1.2. Cycooolxygenases and the nociceptive process

As stated above, in the CNS both COX isozymes are constitutively expressed and their prevalence may depend on the anatomical localisation, the age, and the species. In the rat spinal cord COX-2 is the predominant isoform and it is believed that it's here where the nociceptive process is most influenced by this isozyme (Willingale et al. 1997; Seibert et al. 1994; Vane et al. 1998). Thus, increased immunoreactive staining of COX-2 mRNA and protein, but not for COX-1, in the rat spinal cord resulted from inflammatory hyperalgesia to the paw (Vane et al. 1998), and i.t. administration of the selective COX-2 inhibitor NS-398 produced analgesia in the rat paw formalin test (Yamamoto & Nozaki-Taguchi, 1996). Further support for the relevance of COX-2 on the spinal
nociceptive process comes from anatomical studies of COX-1- and COX-2-like immunoreactivity (COX-1-li and COX-2-li, respectively) in the spinal cord of normal rats (Willingale et al. 1997). On the one hand, COX-1-li was detected in small to medium sized cell bodies in the DRG and the dorsal roots, and to a lesser extent in the spinal cord dorsal horn. On the other hand, although COX-2-li was not present in DRG neurones, it was observed in the superficial dorsal horn (laminae I and II), around the central canal (lamina X), in some neurones in the deep dorsal horn layers (medial aspect of laminae V and VI) and in individual motor neurones in the ventral horn. Therefore, distribution of COX-2 in the dorsal horn is consistent with COX products being involved in the processing of nociceptive input, but COX-1 metabolites may also be involved in stimulating afferent fibres.

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

<table>
<thead>
<tr>
<th>Ptgs 1 gene knockout mice</th>
<th>Ptgs 2 gene knockout mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric ulceration</td>
<td></td>
</tr>
<tr>
<td>Absence of COX-1 alone was not sufficient to cause gastric ulceration. Its absence decreased rather than increase the incidence of indomethacin-induced gastric ulceration. PGE$_2$ levels were about 1% of wild type mice.</td>
<td>No gross or microscopic lesions of the stomach.</td>
</tr>
<tr>
<td>Platelet aggregation</td>
<td></td>
</tr>
<tr>
<td>Reduced.</td>
<td>Not determined.</td>
</tr>
<tr>
<td>Inflammation response</td>
<td></td>
</tr>
<tr>
<td>Decreased response to AA*, but not to TPA*.</td>
<td>No decreased response to AA, TPA or carrageenan. Normal response to bacterial invasion of the peritoneum.</td>
</tr>
<tr>
<td>Kidneys</td>
<td></td>
</tr>
<tr>
<td>No deleterious effects.</td>
<td>Development of nephropathy that becomes more severe and complex with the age.</td>
</tr>
<tr>
<td>Fertility</td>
<td></td>
</tr>
<tr>
<td>Matings between Ptgs 1 deficient females and mutant males resulted in reduced fertility and prolonged gestation length (1-2 d). Matings between heterozygous and wild type females and mutant males increased fertility and number of live pups in comparison to matings between mutant mice.</td>
<td>Mutant males were fertile. Homozygous mutant females were infertile due to ovulatory failure.</td>
</tr>
</tbody>
</table>

* AA, arachidonic acid; TPA, tetradecanoyl phorbol acetate.

Elaborated with information from Langenbach et al. 1995; Morham et al. 1995; Mahler et al. 1996.
This last statement agrees with the study from Dirig et al. (1997) who, based on the blockade of paw carrageenan-evoked hyperalgesia by spinal and systemic pre-administration of, as well as systemic, but not spinal, post-administration of S(+)-ibuprofen or SC58125 (a COX-2 selective inhibitor) in rats, concluded that at least spinal COX-2, if not both spinal COX-1 and COX-2, are essential for the initiation of hyperalgesia, whereas non-spinal sources of COX products are important for their maintenance. Using the same pain model but different drugs (SC-560 and celecoxib; COX-1 and COX-2 selective inhibitors, respectively) and drug administration protocols, Smith et al. (1998) concluded something similar. They proposed that, in addition to the role of peripherally produced PGs, there is a critical, centrally mediated component of inflammatory pain transmission mediated in part by COX-2.

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

2.3.2.1.3. Cycolxygenase inhibition by NSAIDs

It has been postulated (Appleton et al. 1996) that the effect of a NSAID will depend on both the relative amount of COX-1 and COX-2 present in a tissue and on its COX-1:COX-2 inhibitory ratio. Moreover, a classification for NSAIDs according to their COX-1:COX-2 inhibitory ratio was proposed by Frölich (1997) who argued that its use would permit a useful prediction of the major effects and side-effects of these drugs, a statement that has been debated (Griswold et al. 1997). One of the major conflicts in establishing such a classification is the direct comparison of results obtained from different assay systems or conditions in different laboratories which can produce widely disparate results for the COX1:COX-2 inhibition ratio and even change the precise ranking of a drug from model to model (Griswold et al. 1997; Cryer & Dubois, 1998).
Numerous methodological details, including the source (endogenous or exogenous) and concentration of substrate (AA), the use of intact cells or purified enzymes, the protein concentration in the incubation mixture and the time of incubation, are contributing factors to the great range of reported values for most NSAIDs (Fröhlich, 1997; Ricketts et al. 1998). Therefore, the relative ranking of compounds in a single assay system has more meaning than comparisons between several assay systems (Ricketts et al. 1998). However, even comparing results from different laboratories that use the same assay system, small differences in small parameters (e.g., pH, buffer solutions) can cause considerable differences in COX kinetics (Cryer & Dubois, 1998). In addition, important species differences may exist among COX structures and they may account for the different inhibition of COX isozymes by NSAIDs; hence, it is recommended to use enzymes derived from the target species when attempting to predict efficacy and toleration (Ricketts et al. 1998; Cryer & Dubois, 1998).

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

2.3.2.2. Prostaglandins and the nociceptive process

Within the nociceptive pathway, PGs are very well known for their sensitising action on peripheral nociceptors (Ferreira, 1972; Appleton et al. 1996); however, their function(s) in the CNS is still debated and far from being well understood. They can be released from the spinal cord by activation of high-
threshold nociceptive afferent inputs (Ramwell et al. 1966), by direct administration of neurotransmitters (Chaplan et al. 1997; Malmberg & Yaksh, 1993; Dirig & Yaksh, 1999) or by peripheral inflammatory stimuli (Smith et al. 1998; Muth-Selbach et al. 1999). This suggests that a certain level of repetitive stimulation of the primary afferent is required for their release (Buritova et al. 1996), which agrees with the NMDA receptor-mediated generation of PGs (see above). Nonetheless, their generation also can be stimulated by co-activation of AMPA/kainate and mGluRs (Bezzi et al. 1998; Conn & Pin, 1997) which activation does not necessarily require repetitive stimulation. The origin of spinal prostanoids is not well established either, and release into the extracellular space may result not only from neuronal but also glial structures (Buritova et al. 1996; Willingale et al. 1997; Bezzi et al. 1998; Phillips & Pearce, 1999).

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

In addition to their presynaptic actions, these lipidic acids also may influence postsynaptic terminals. For example, it was shown that activation of the AA pathway is required for NMDA receptor-dependent induction of c-Fos protein in the spinal cord (Buritova et al. 1996), suggesting a signalling role for products of this pathway from the cell membrane/cytoplasm to the nucleus, where prostanoid receptors (see below) are present (Bhattacharya et al. 1998). In this
regard, PGF$_{2\alpha}$ is necessary, but not sufficient, for NMDA receptor-induced expression of c-Fos protein (Kaufmann et al. 1997).

Recently, PGD$_2$ and PGE$_2$ were identified as the predominant PGs in the normal rat spinal cord, while 6-keto-PGF$_{1\alpha}$, the stable metabolite of PGI$_2$, and PGF$_{2\alpha}$ were detected in minor concentration (Willingale et al. 1997). Most prostanoids, to some extent, have been investigated for their effects on the pain signalling process, but special interest has been put on the four PGs mentioned above. PGE$_2$, the major studied prostanoid, partially because is released from the spinal cord in response to noxious stimuli (Dirig & Yaksh, 1999) and because it can also release neurotransmitters (Bezzi et al. 1998) and neuropeptides (Yamamoto & Nozaki-Taguchi, 1996; Yaksh & Malmberg, 1993), has been adopted as the major PG involved in hyperalgesia. Nevertheless, when a comparison was made between the effect of PGI$_2$, PGE$_2$, and some analogues on sensory neurones, PGI$_2$ analogues were equally or more effective than those of PGE$_2$ as hyperalgesic or sensitising agents (Bley et al. 1998; Smith et al. 1998). However, this was not true for the paintbrush test in mice where the PGI$_2$ analogue cicaprost produced a smaller allodynic effect than various PGE$_2$ analogues (Minami et al. 1994b). Together, these data suggest that allodynia and hyperalgesia may be mediated by action of different PGs.

Interestingly, the spinal glutamatergic system has been implicated in both PGE$_2$- and PGF$_{2\alpha}$-evoked allodynia but subtypes of glutamate receptors involved may differ between them. Allodynia, assessed by the paintbrush test, was evoked by i.t. injection of PGE$_2$ or PGF$_{2\alpha}$ in conscious mice. The NMDA receptor antagonists MK-801 and D-AP-5 blocked PGE$_2$- but not PGF$_{2\alpha}$-induced allodynia; the non-NMDA receptor antagonists GAMS and CNQX inhibited both PGE$_2$- and PGF$_{2\alpha}$-induced allodynia; and L-AP-3 and L-AP-4, mGluR antagonists, reduced PGF$_{2\alpha}$- but not PGE$_2$-induced allodynia (Minami et al. 1994a). Therefore, it may be feasible that PGE$_2$-induced allodynia is mediated by co-activation of AMPA and NMDA receptors, whereas PGF$_{2\alpha}$-induced allodynia is due to co-activation of AMPA and mGluRs. In addition, the NO pathway may be involved in the PGE$_2$-induced allodynia because PGE$_2$
stimulated NO release from the spinal cord by activation of NMDA receptors; therefore, NO may be implicated in allodynic responses induced by PGE₂ (Sakai et al. 1998). Even more, recent studies on lipocalin-type PGD synthase (L-PGDS; responsible enzyme for the conversion of PGH₂ to PGD₂ in the CNS) knockout mice (Eguchi et al. 1999) showed a possible spinal interaction between PGD₂ and PGE₂ through the GABA_A receptor on the PGE₂-evoked allodynia. This conclusion was driven from the fact that i.t. injection of PGE₂ evoked allodynia in wild-type mice but not in L-PGDS deficient mice and i.t. co-administration of PGD₂ or BW 245C, a PGD receptor (DP) agonist, plus PGE₂ or bicuculline (GABA_A antagonist) evoked allodynia in L-PGDS⁻/⁻ mice similarly to that observed in wild-type mice by PGE₂ and bicuculline alone. Furthermore, the DP antagonist BW A868C reversed the PGD₂ plus PGE₂⁻ or bicuculline-induced allodynia in L-PGDS⁻/⁻ mice, as well as that evoked by PGE₂ or bicuculline in wild-type mice. This interaction between PGD₂ and PGE₂ at the GABA_A receptor level seems to be PG-specific because the i.t. injection of PGF₂α induced allodynia in both wild-type mice and L-PGDS deficient mice (Eguchi et al. 1999), suggesting that its allodynic effect does not depend on PGD₂.

Contrary to the contribution of PGD₂ to evoke allodynic responses, there are reports suggesting that this PG, by acting on DP receptors, might play an inhibitory role in the appearance of this pathologic process. For instance the i.t. injection of PGD₂ in mice blocked both nociceptin- and PGE₂-induced allodynia (Minami et al. 1997; Minami et al. 1996) whereas in rat spinal cord slices, PGD₂ inhibited the PGE₂-evoked NO release (Sakai et al. 1998).

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

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

As mentioned above, prostanoids act as extracellular and intracellular messengers. After being synthesised, they are released immediately acting, on cell membrane receptors to elicit their actions (Oida et al. 1995; Vane et al. 1998) (Table 2.5). It is known that prostanoid receptor-mediated sensitisation of sensory nerve fibres is a key contributor to the generation of hyperalgesia (Bley et al. 1998; Smith et al. 1998), probably by facilitating neurotransmitter/neuropeptide release from the primary afferent (Geppetti et al. 1991; Malcangio et al. 1996; Buritova et al. 1996; Yaksh & Malmberg, 1993; Andreeva & Rang, 1993; Bezzi et al. 1998). Indications for an intracellular function of prostanoids have been strengthened by the demonstration that PGD₂, PGJ₂, and PGI₂ can activate the nuclear peroxisome proliferator-activated receptors and, thus, alter transcription factors (Vane et al. 1998; Bhattacharya et al. 1998). Interestingly, increased c-Fos protein expression through some AA pathway elements signalling from the cell membrane/cytoplasm to the nucleus, where the four different PGE₂ receptor subtypes (EP₁-EP₄) were recently demonstrated (Bhattacharya et al. 1998), has been suggested (Kaufmann et al. 1997; Buritova et al. 1996). In fact, activation of the EP₁ nuclear receptor with the EP₁ agonist 17-phenyltrinor PGE₂ increased \([\text{Ca}^{2+}]\) concentration and c-fos transcription (Bhattacharya et al. 1998). However, to date the knowledge about these intracellular receptors is small.

The diversity of actions of PGE₂ is ascribed to PGE receptor subtypes EP₁, EP₂, EP₃ and EP₄ coupled to different signal transduction pathways. While the EP₁
### Table 2.5. Properties and distribution of prostaglandin receptors.

<table>
<thead>
<tr>
<th>Receptor / Subtype</th>
<th>Distribution</th>
<th>Biological Functions</th>
<th>Transduction System</th>
<th>Observations / Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP</td>
<td>Platelets; vascular, gastrointestinal, uterine, and airway smooth muscles. Nervous tissue including CNS.</td>
<td>Inhibitory responses.</td>
<td>↑ cAMP via Gs.</td>
<td>Distribution highly species specific. Rich population in human platelets, and few in lab animals if any; in uterine smooth muscle appear to be confined to humans. In afferent sensory nerves they can induce hyperalgesia.</td>
</tr>
<tr>
<td>EP</td>
<td>Smooth muscle of trachea, GIT, uterus and bladder.</td>
<td>Contraction and relaxation of smooth muscle; immunoregulation; inhibition and enhancement of neurotransmitter release and non acid (water) secretion; inhibition of lipolysis, gastric acid secretion, inflammatory mediator release, immunoglobulin expression; etc.</td>
<td>↑ Intracellular Ca²⁺ via an IP₃-independent mechanism.</td>
<td>Although are more sparse in non-rodent species, they do exist in superior species including humans.</td>
</tr>
<tr>
<td>EP₂</td>
<td>Smooth muscle. Epithelial cells. Inflammatory cells (mast cells and macrophages). Sensory afferent nerves.</td>
<td>Activation. Contraction.</td>
<td>↓ cAMP via Gi. ↑ PI turnover via Gq.</td>
<td>Development of EP₃ receptor agonist as gastric antisecretory agents. In nervous tissue the mRNA is expressed only in neurones and highly expressed in dorsal root ganglion and brain regions such as hippocampus, preoptic area, hypothalamus, maxillary body, locus coeruleus, and raphe nuclei.</td>
</tr>
<tr>
<td>EP₄</td>
<td>Piglet saphenous vein; rabbit jugular and saphenous veins; rat trachea; hamster uterus.</td>
<td></td>
<td>↑ cAMP via Gs?</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.5. Properties and distribution of prostaglandin receptors. (Cont.)

<table>
<thead>
<tr>
<th>Receptor / Subtype</th>
<th>Distribution</th>
<th>Biological Functions</th>
<th>Transduction System</th>
<th>Observations / Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>FP</td>
<td>Corpus luteum. Rodents myometrium. Cat and dog iris sphincter and airway smooth muscle.</td>
<td>Luteolysis. Contraction. Contraction.</td>
<td>↑ PI turnover via Gq.</td>
<td>PGF&lt;sub&gt;2α&lt;/sub&gt; and analogues serve to synchronise the oestrus cycles of farm animals. They are also effective in lowering intraocular pressure in various species, including human, and are used to treat glaucoma. In dogs and cats FP agonists are lethal. There is no convincing evidence that IP receptors can be subclassified; the only differences that exist probably result from species variants rather than from true subtypes. They appear to regulate the closure of umbilical vessels at birth, and thymocyte differentiation and development. There is controversy regarding different subtypes.</td>
</tr>
<tr>
<td>IP</td>
<td>Platelets, arterial smooth muscles. Sensory afferent nerves.</td>
<td>Inhibition of arterial tone and platelet aggregation. Excitation.</td>
<td>↑ cAMP via G&lt;sub&gt;s&lt;/sub&gt;. ↑ PI turnover.</td>
<td></td>
</tr>
</tbody>
</table>

cAMP = cyclic AMP; PI = phosphatidylinositol; G<sub>s</sub> = stimulatory G-protein; G<sub>i</sub> = inhibitory G-protein; G<sub>q</sub> = pertussis toxin-insensitive G-protein.

Elaborated with information from Coleman et al. 1994.
receptor is coupled to Ca\textsuperscript{2+} mobilisation, EP\textsubscript{2} and EP\textsubscript{4} receptors stimulate adenylate cyclase and the EP\textsubscript{3} receptor inhibits this enzyme (Coleman et al. 1994). Using different approaches (e.g., radio-labelled markers, hybridisation studies and pharmacological screening), EP receptors have been identified in diverse areas of the CNS. For example, binding sites for [\textsuperscript{3}H]iloprost, an EP\textsubscript{1} selective agonist, were observed in DRG, the spinal cord dorsal horn, nodose ganglia, and the nucleus tractus solitarius of rats (Bley et al. 1998), and EP\textsubscript{1}, EP\textsubscript{3} and EP\textsubscript{4} receptor mRNAs were expressed in about 30\%, 50\% and 20\%, respectively, of mice DRG neurones (Oida et al. 1995). These findings match with the spinal EP receptor system being involved in pain transmission on high and low threshold afferents in interaction with neurotransmitters and neuropeptides. In this regard, allodynia induced by i.t. administered PGE\textsubscript{2} in mice was attributed to stimulation of the EP\textsubscript{1} receptor and PGE\textsubscript{2}-evoked hyperalgesia through EP\textsubscript{2} and EP\textsubscript{3} receptors (at high and low doses of PGE\textsubscript{2}, respectively) (Minami et al. 1994b). Furthermore, activation of the EP\textsubscript{1} receptor by PGE\textsubscript{2} enhanced NO release, via the NMDA receptor-nNOS pathway, from rat spinal cord slices (Sakai et al. 1998), suggesting that PGE\textsubscript{2} may stimulate nNOS and thus release NO following NMDA receptor activation via EP\textsubscript{1} receptors and, thus, evoke allodynic responses. However, EP\textsubscript{1}-mediated allodynia may need the presence of PGD\textsubscript{2} because L-PGDS deficient mice did not show PGE\textsubscript{2}-induced allodynia whereas wild type mice did (Eguchi et al. 1999).

The presence of DP receptor mRNA transcripts has been demonstrated, by in situ hybridisation reactions, in the periphery of the rat brain (leptomeninges and choroid plexus) and in both dorsal and ventral horns of the lumbar, but not cervical or thoracic, spinal cord (Wright et al. 1999). Contrary to the need of PGD\textsubscript{2} for the development of PGE\textsubscript{2}-induced allodynia (Eguchi et al. 1999) is the inhibition of the allodynic response by PGD\textsubscript{2}, which was reversed by the DP receptor antagonist BW A868C (Minami et al. 1996). Therefore, it seems that activation of DP receptors may either participate in the development of or reversal of PGE\textsubscript{2}-induced allodynia. Even more, the solely blockage of spinal DP receptors with BW A868C in mice produced allodynia (Minami et al. 1996).
Together, these results suggest important roles for EP and DP receptor systems in nociception as well as complex interactions between them and other nociceptive systems.

Prostacyclin receptors may also play a crucial role in nociception and, although they were very little labelled in the mouse spinal cord, 40% of DRG neurones expressed IP receptor mRNA. Both small- and large-sized cell bodies, but not glial structures, were labelled. Furthermore, expression of preprotachykinin A (a SP precursor) mRNA was noted in 30% of total DRG neurones and 70% of these neurones co-expressed IP receptor mRNA, which corresponded to C and Aδ fibres (Oida et al. 1995). Hence, it is likely that IP receptors contribute to nociception by facilitating SP release from the afferent terminals. However, other sensory mechanisms may also be modulated by these prostanoid receptors because half of the IP receptor mRNA-positive DRG neurones did not express preprotachykinin A mRNA, and 25%, 41% and 34% of the IP receptor mRNA-positive DRG neurones co-expressed EP₁, EP₃ and EP₄ receptor mRNAs, respectively (Oida et al. 1995). Whatever the mechanism by which IP receptors modulated nociception, it was biochemically [cAMP and Ins(1,4,5)P₃ accumulation in cultured sensory neurones] and electrophysiologically (isolated vagus nerve preparation) demonstrated that responses to IP receptor agonists [cicaprost and carbaprostacyclin (cPGl₂)] were greater than those of other endogenous prostanoids (PGE₁, PGE₂, PGD₂, TXB₂, PGF₂α) (Smith et al. 1998). Interestingly, in the vagus nerve preparation, sustained applications of either cPGI₂ or PGI₂ caused receptor desensitisation. Furthermore, pre-treatment with cPGI₂ and PGI₂ attenuated the response to a subsequent application of PGE₂, possibly by depolarising actions of these three agents through action at a single receptor, because high K⁺-evoked nerve depolarisation after PGI₂ or cPGI₂ pre-treatment still occurred. However, the possibility that PGI₂ and cPGI₂ had caused heterologous receptor desensitisation was not excluded (Smith et al. 1998). Together, these results suggest that IP receptor activation may modulate the release of neuropeptides from afferent terminals by a process that involve the adenylate cyclase and PLC.
pathways, and that some interaction or cross-talk between IP and EP receptors may also exist.

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

2.4. CENTRAL ANALGESIC EFFECTS OF NONSTEROIDAL ANTI-INFLAMMATORY DRUGS

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

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

2.4.1 Possible central antinociceptive mechanisms of action for NSAIDs

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

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

Spinal prostanoid synthesis inhibition has been postulated as NSAIDs' major analgesic mechanism of action. Support to this hypothesis comes from the antinociception observed after i.t. administration of various NSAIDs in rats submitted to the paw formalin test and, according to the authors, the analgesic potency of these drugs matched with COX inhibition values (Malmberg & Yaksh, 1992b). Furthermore, the sole inhibition of PGE$_2$ seems to be sufficient to mediate their spinal antinociceptive effects since i.t. injection of the EP receptor antagonists SC-5108 and SC-51234A produced analgesia in rats submitted to the same nociceptive test (Malmberg et al. 1994). Nevertheless, oral dipyprone administered to patients suffering from neurological diseases did not decrease PGE$_2$ levels in the CSF as compared to that in patients suffering from neurological diseases not receiving the NSAID; however, reduction of TxB$_2$ was observed (Levy et al. 1998).

In addition to those three reports, there is the suggestion that paracetamol's analgesic effects are due to selective PG synthesis inhibition in the CNS (Flower & Vane, 1972), which is supported by the PGE$_2$ spinal inhibition, but lack of effect on PGE$_2$ PGF$_{2\alpha}$ and 6-keto-PGF$_{1\alpha}$ urinary excretion levels, after i.p. administration of paracetamol in rats at the same time that produced antinociception in the formalin test (Muth-Selbach et al. 1999). However, failure to demonstrate tissue specific differences in COX sensitivity to paracetamol and other NSAIDs is also reported (McCormack, 1994a). Even more, a recent study demonstrated that inhibition of central COX might not be involved in the antinociceptive effects of paracetamol (Pelissier et al. 1996), suggesting that NSAIDs' analgesic effects are mediated by additional mechanisms to central prostanoid synthesis inhibition.

To try to clarify if NSAIDs-mediated analgesia is due to inhibition of prostanoid production or not, anti-inflammatory steroids have been used for comparison. Because anti-inflammatory steroids indirectly inhibit PLA$_2$ and, therefore, prostanoid synthesis in an earlier step within the AA pathway (Figure 2.3), it could be reasoned that these drugs might produce similar analgesia to that
observed with NSAIDs. By comparing the antinociceptive effect of i.t. injected NSAIDs (Malmberg & Yaksh, 1992a; Malmberg & Yaksh, 1992b; Malmberg & Yaksh, 1993) and anti-inflammatory steroids (Abram et al. 1992) in the formalin test in rats, it is clear that a single injection of a NSAID reduced the nociceptive behaviour observed in the second phase of this test, whereas chronic administration of the anti-inflammatory steroid triamcinolone was required to produce similar analgesia to that of the NSAIDs. Even more, the single i.t. injection of another anti-inflammatory steroid, methylprednisolone, produced allodynia (Abram et al. 1992). Although the analgesic effect of both NSAIDs and anti-inflammatory steroids was quite different, the authors attributed it in both cases to changes on spinal prostanoid levels. However, Chambers et al. (1993; 1995) demonstrated no increase on mechanical nociceptive thresholds of sheep when the anti-inflammatory steroid dexamethasone was intravenously (i.v.) administered, whilst flunixin or dipyrene, both given i.v. too, did increase them. Therefore, comparison of the analgesic action of NSAIDs and anti-inflammatory steroids suggests that the former compounds are highly likely to have central antinociceptive mechanisms of action other than or additional to prostanoid synthesis inhibition.

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

2.4.1.2. NSAIDs and glutamate receptors

Because NSAIDs blocked the hyperalgesia elicited by i.t. injection of NMDA, AMPA or SP (Malmberg & Yaksh, 1992a; Yaksh & Malmberg, 1993; Buritova et
al. 1996; Björkman et al. 1996; Kakinohana et al. 1997; Sorkin, 1997; Dolan & Nolan, 1999), the spinal antinociceptive mechanism of these drugs may involve glutamatergic synapses, where the AA pathway plays an important role and the possible actions of the NSAIDs on this cascade were already discussed. The NO pathway is another important biochemical pathway modulated by glutamate receptors and because its interaction with the AA pathway was previously discussed in this work, here will only be exposed the potential analgesic mechanisms of NSAIDs related to this signal transduction pathway.

Interaction of NSAIDs with the NO pathway could also represent a possible antinociceptive mode of action for these drugs; however, this interaction is far too complex since stimulation and inhibition of NO production seems to regulate NSAID-induced analgesia. Thus, diclofenac and S(+)- but not R(-)-ibuprofen, inhibited the spinal NMDA-induced behavioural response in rats and pre-treatment with L-arginine, but not D-arginine, antagonised their analgesic effect (Björkman et al. 1996). Whereas i.t. administered dipyrone attenuated the rat paw PGE$_2$-induced hyperalgesia, and antinociception was abolished by pre-treating the paws with L-NMMA (a NOS inhibitor) or methylene blue (an inhibitor of soluble guanylate cyclase) (Lorenzetti & Ferreira, 1996).

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

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

2.4.1.3. NSAIDs and descending inhibitory mechanisms

Supraspinal mechanisms where NSAIDs may exert descending inhibitory influences on the spinal transmission of nociceptive inputs have also been proposed. These effects are probably mediated by activation of on- and off-cells of the RVM, which receive an input from the PAG and project to the spinal dorsal horn, facilitating and depressing the spinal nociceptive transmission, respectively (Tortorici et al. 1996). Thus, PAG-micoinjection of NSAIDs may depress on-cell activity, facilitate off-cell activity and, therefore, depress excitation of spinal sensory neurones (Vanegas et al. 1997; Tortorici et al. 1996). In this regard, descending opioidergic (Chambers et al. 1993; Chambers
et al. 1995; Akman et al. 1996), noradrenergic (Chambers et al. 1992; Chambers, 1995), serotonergic (Pelissier et al. 1996; Björkman et al. 1996) and muscarinic cholinergic (Stets & Slivko, 1989) inhibitory mechanisms have been implicated in NSAIDs-mediated analgesia.

Nevertheless, indications against a supraspinal antinociceptive action for the NSAIDs come from the lack of analgesia in the rat paw PGE$_2$-induced hyperalgesia test (Lorenzetti & Ferreira, 1996) and the failure to depress a C fibre reflex in anaesthetised rats (Bustamante et al. 1997) by intracerebroventricular (i.c.v.) administered NSAIDs.

2.4.1.4. NSAIDs and other receptor systems and signal transduction pathways

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

The possibility that NSAIDs exert their analgesic action, at least in part, by an inhibitory action via vanilloid receptors has been raised due to the inhibition of capsaicin-induced current in rat DRG neurones (Buritova & Besson, 1998) and attenuation of capsaicin-induced pain and allodynia in human volunteers (Szallasi & Blumberg, 1999) by NSAIDs. Similarly, inhibition of the cAMP second messenger system, which is involved in the modulation of voltage- and ligand-gated ion channels in sensory neurones (Bley et al. 1998), by a mechanism unrelated to COX inhibition was suggested for indomethacin-mediated analgesia (Yamamoto & Nozaki-Taguchi, 1996). However, those both inhibitory actions, vanilloid receptors and cAMP cascade, could still be mediated
through direct PG synthesis inhibition, because some of these lipidic acids stimulate the cAMP/PKA cascade and phosphorylation of the capsaicin receptor by PKA increases its conductance or open probability (Bley et al. 1998; Kress & Zeilhofer, 1999).

Blockade of L- and T-type Ca\(^{2+}\) currents by the COX-2 selective inhibitor nimesulide was observed in cultured human myometrial myocytes (Knock & Aaronson, 1999); if this is also true in neuronal tissue, this mechanism might reduce neurone depolarisation and represent a potential analgesic mode of action for NSAIDs. On the other hand, neurone hyperpolarisation through an outward K\(^{+}\) conductance has been ascribed to the antinociceptive action of some NSAIDs (Geisslinger & Schaible, 1996). Together, these results suggest that NSAIDs could produce analgesia by interacting, directly or indirectly, with ion channels. Interestingly, direct opposite effects of mefenamic acid on different types of \(\beta\) human recombinant GABA\(_{A}\) receptor subunits have recently been identified. Interaction of the NSAID with \(\beta_{23}\) subunits produced excitatory responses and with \(\beta_{1}\) either zero or inhibitory responses (Halliwell et al. 1998). Thus, it seems that NSAIDs could produce analgesia by direct activation of GABA\(_{A}\) receptors; however, this could also be linked to some side effects (e.g., convulsions and coma) observed with these drugs.

2.4.1.5. NSAIDs and interference with nuclear related events

The NSAIDs have been shown to down- or up-regulate the level of diverse transcription factors, enzymes and even receptors that could be involved in the nociceptive process. Interference of these processes, which take place at the nuclear level, may represent very important mechanisms of analgesia, because long-lasting neuronal responses may depend on them; however, they are poorly understood. Thus, as mentioned above, NSAIDs are able to decrease c-Fos protein expression in spinal cord neurones (Buritova et al. 1996; Buritova & Besson, 1998) and this transcription factor, although its function within the nociceptive process is not known, is used as an indirect marker of neurones
involved in spinal nociceptive processes (Buritova & Besson, 1998). Another transcription factor inhibited by acetylsalicylic acid and salicylic acid is NFκB (Xu et al. 1999; Cronstein et al. 1999). These two salicylates, but not indomethacin, also inhibit the transcription factor Erk, which activation was reduced in neutrophils, but only at the mM range concentration of the drugs, by a COX-independent inhibitory mechanism upstream of Erk (Pillinger et al. 1998). If this effect by salicylates also occurs in neurones, nNOS could be involved in this process. Inhibition or depletion of nNOS, and therefore NO diminished production, in mice primary cortical neurones prevented activation of the Ras pathway and thereby the transcription factor Erk by an independent action in cGMP increase (Yun et al. 1998). Thus, acetylsalicylic acid and salicylic acid could inhibit nNOS and this could be a possible analgesic mode of action for these drugs; in fact inhibition of nNOS has been demonstrated for high-dose ketoprofen (Schubert et al. 1999).

Further to the information mentioned above about acetylsalicylic acid and salicylic acid, these salicylates, but not indomethacin nor the COX-2 selective inhibitor NS398, reduced in vitro and in vivo both COX-2 mRNA and protein expression (Xu et al. 1999). Regarding receptor levels, in human mononuclear cells, acetylsalicylic acid increased both ex vivo and in vitro secretion of soluble IL-1 type II receptor, which is believed not to transduce signals (Daun et al. 1999). Because IL-1β is present in the CNS (Schneider et al. 1998), enhances SP release from the spinal cord (Malcangio et al. 1996) and NO and PGE₂ from astroglial cells (Mollace et al. 1998), and is involved in the processing of noxious inputs in the spinal cord dorsal horn (Reeve et al. 1998), it follows that inactivation of the cytokine by coupling to the soluble IL-1 type II receptor may contribute to the analgesic effects of acetylsalicylic acid, only if the same increment in receptor level happens in neuronal tissue.

Thus, interference with diverse biochemical functions in glutamatergic synapses and non-neuronal structures, interaction with natural occurring inhibitory systems as well as modulation of other receptors, transduction signal pathways and even nuclear events related to nociceptive signals represent a potential explanation for the central analgesic effects of NSAIDs. However, the current
knowledge on their actions at the molecular level is incomplete and, therefore, far to be well understood.

2.5. INDUCTION AND ASSESSMENT OF PAIN

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

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

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

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

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

2.5.2. Animal models of chronic pain

Contrary to acute pain models, chronic pain tests cause inescapable pain that may last up to several weeks and is always accompanied with obvious tissue damage. This tissue injury is generally directed to specific areas within the organism, giving us the opportunity to investigate chronic pain associated with different conditions. Thus, inflammatory, arthritic, visceral and neuropathic models of chronic pain have been developed. Models of inflammatory pain and hyperalgesia, such as the formalin and the carrageenan tests, are widely used. In the former, formalin is injected in the hind paw of rats or mice and two behavioural phases are observed. The first one, denominated phase 1, is thought to correspond to acute pain, stars immediately after the formalin injection and lasts about 5 minutes. Phase 2, which is believed to represent chronic pain, begins after 10 minutes, peaks at around 25 to 35 minutes and diminishes at about 45 to 60 minutes (Malmberg & Yaksh, 1992b; Coderre et al. 1990). The carrageenan test involves the s.c. administration of carrageenan into
the hind paw, developing inflammation with localised hyperthermia and oedema in less than 3 hours (Portanova et al. 1996; Zhang et al. 1997; Traub, 1996; Ochi et al. 1999). Alternatively, injection of capsaicin in the hind paw has also been used (Sakurada et al. 1996). Different scoring systems and methodologies are used to evaluate nociception in these tests.

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

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

Large number of experimental models of neurophatic pain, one of the most troublesome and poorly understood forms of pain, have been developed. Among them, gentle ligation of the sciatic nerve (Liu et al. 1994) or lumbar dorsal roots (Chaplan et al. 1997; Liu et al. 1994) of rats are some of the most commonly used. These two tests produce partial peripheral denervation because of demyelination of large fibres and destruction of some small
unmyelinated axons, which is shown as allodynia, hyperalgesia and spontaneous pain. Furthermore, nerve injury models associated with systemic disease as the streptozotocin-induced painful diabetic neuropathy in rats (Calcutt & Chaplan, 1997), are also of great value.

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

2.6. AIMS OF THE PROJECT

The aims of this research were to evaluate the spinal effect of NSAIDs as well as their interaction with opioidergic and $\alpha_2$-adrenergic mechanisms with the hope to elucidate the central analgesic mode of action of these drugs. For these purposes, a neonatal rat spinal cord preparation and sheep with indwelling cervical i.t. catheters submitted to mechanical nociceptive stimulation of the left radius were utilised.
Chapter 3
EFFECT OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS ON NMDA-RECEPTOR SYNAPTIC TRANSMISSION IN THE in vitro RAT SPINAL CORD PREPARATION

3.1. INTRODUCTION

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

In this preparation, supramaximal electrical stimulation of a lumbar dorsal root evokes a reflex in the ipsilateral ventral root (the high intensity excitatory postsynaptic potential). This reflex is due to the activation of C fibres and therefore is considered to reflect a nociceptive reflex (Faber et al. 1997a; Akagi et al. 1985). It can be depressed by opioids (Faber et al. 1997a) and $\alpha_2$-adrenoceptor agonists (Faber et al. 1997a; Faber et al. 1997b; Faber et al. 1998). In addition, stimulating the dorsal root at low intensities can evoke an A fibre-mediated response (the low intensity excitatory postsynaptic potential), which is also sensitive to these drugs (Faber et al. 1997a; Faber et al. 1998).

Both the high and low intensity excitatory postsynaptic potentials have NMDA receptor-mediated components which must be mediated by the enhanced glutamate release after electrical stimulation of A fibres (Suzuki et al. 1998) and the enhanced glutamate and SP and CGRP release after C fibre stimulation (Akagi et al. 1985; Andreeva & Rang, 1993; Malcangio et al. 1998; Suzuki et al. 1998). NMDA receptor-mediated synaptic excitation seems to be depressed by
centrally acting analgesic (Faber et al. 1997a). Therefore, if NSAIDs produce analgesia at the spinal level, they should depress the NMDA receptor-mediated reflexes of the in vitro neonatal rat hemisected spinal cord preparation.

3.2. MATERIALS AND METHODS

3.2.1. Spinal cord preparation

Minor modifications were made to the method described by Otsuka and Konishi (1974), which allows a stable recording and good reflex responses for at least several hours. After approval from the Massey University Animal Ethics Committee, neonatal Sprague-Dawley rats [3–9 days old, unsexed, 9–16 g; preparations from older pups cannot be maintained in vitro (Wong et al. 1998)] (Small Animal Production Unit, Massey University, Palmerston North, New Zealand) were cervically dislocated and decapitated. The abdomen ventral skin was removed and an incision was performed on the thoracic cage and the ventral abdomen wall, all thoracic and abdomen internal organs were removed and the vertebral canal was opened on the ventral side. The dura mater covering the ventral side of the spinal cord was removed and the spinal cord was carefully dissected out of the dorsal part of the vertebral column together with attached L4 or L5 dorsal and ventral roots. The spinal cord was placed in a petri dish containing artificial cerebral spinal fluid (ACSF) containing the following (mM): NaCl 118, NaHCO3 24, C6H12O6 12, CaCl2 1.5, KCl 3 and MgSO4·7H2O 1.25 (all reagents purchased from BDH Laboratory Supplies, Poole, England). The ACSF was gassed with 95% O2 and 5% CO2, (Carbogen 5; BOC Gases, New Zealand) maintaining the solution at pH 7.4, and was kept at a temperature of 27 ± 1°C, normal temperature for pups of this age when they are not under the mother (Wong et al. 1998).

The roots, but not from L4 or L5, were removed along with the dura mater covering the dorsal side of the spinal cord. The spinal cord was hemisected sagittally by making a small incision at the centre of the cord at the cranial end with a scalpel and gently tearing the two halves apart. The ventral and dorsal
roots were separated at the DRG, leaving the dorsal root intact with the DRG. Then, the hemisected spinal cord was placed in a chamber (made up from Perspex glass; Figure 3.1) with L4 or L5 dorsal root and attached DRG in contact with the stimulating silver – silver chloride wire electrode, which was bent into the shape of a hook in order to hold the spinal cord in place on the preparation chamber. The corresponding ventral root was in contact with the recording silver wire electrode. Electrical isolation of the electrodes was made by using grease gaps made of vaseline and liquid paraffin (5:4 v/v).

Figure 3.1. The preparation chamber. The hemicord was placed to the right of the negative stimulating electrode with the dorsal root in contact to the positive stimulating electrode. The ventral root was in contact with the positive recording electrode.
Once in the chamber, the spinal cord preparation was superfused with ACSF as it is shown in Figure 3.2. The ACSF, contained in a glass reservoir and gased with 95% O₂ / 5% CO₂, was pumped (H.R. Flow Inducer, model MHRE 22, Watson-Marlow Ltd., Cornwall, England) at a rate of 2 mL/minute into polyethylene tubes (2 mm diameter; SMA Flow Rated Pump Tubes, model 116-0549P12, Technicon Instruments Corporation, Tarrytown, N.Y., U.S.A.) which passed by a heated water jacket ensuring a temperature of 27 ± 1°C. The chamber was placed with an inclination of about 30° to maintain constant flow of ACSF over the hemisected spinal cord, which ensures a stable baseline of recordings. The ACSF was dripped off the chamber and collected as waste. The preparations were allowed to equilibrate for at least 60 minutes before recordings were performed and they were only accepted if two subsequent control measurements, 15 minutes apart, differed in amplitude by less than 10%. Drugs were applied to the preparation in known concentrations by adding them to the superfusate. The distance between the superfusion reservoir and the chamber was 75 cm, approximately. The polyethylene tubes were cleaned for each experiment by flushing them through with HCl (37% diluted by a factor of two in distilled water; BDH Laboratory Supplies, Poole, England) and rinsing with distilled water.

Figure 3.2. The superfusion system. The ACSF was continuously gassed with 95% O₂ / 5% CO₂ and pumped through a heated water jacket. The ACSF was dripped onto the preparation chamber and then it was collected as waste.
Following the technique described above, and to assess the dorsal root compound action potential (DRCAP), the sciatic nerve was also isolated together with the hemisected spinal cord to be used for the experiment. In these experiments the nerve laid across the preparation chamber with the peripheral end of the sciatic nerve in contact with the stimulating electrode and the dorsal root in contact with the recording electrode. Both electrodes were isolated with grease gaps as described above.

3.2.2. Recording techniques

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

The A fibre-mediated MSR and the low intensity e.p.s.p. were evoked by a single impulse 0.5 ms in duration at three times the threshold (the threshold being the intensity at which a discernible response first appears in the ventral root). From previous experiences (Faber et al. 1997a), it is known that approximately one volt (v) is required to evoke a threshold. The MSR was measured over 120 ms at a sampling rate of 10 kHz and the low intensity e.p.s.p. was measured over 1200 ms at 1kHz. The high intensity e.p.s.p. was
evoked by a single pulse 0.5 ms in duration at 16 times the threshold, this response was measured over 60 seconds at 20 Hz. The train e.p.s.p. was evoked with a train of 5 stimulus (20 Hz) at 2 times the threshold and was measured over 25 seconds at 40 Hz. In order to elucidate which primary afferent fibres (A or C) are activated following stimulation of the dorsal root at different intensities, the DRCAP was evoked in the central end of the sciatic nerve following a single pulse of 0.5 ms duration and was measured over 60 ms at 10 kHz (Table 3.1).

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

<table>
<thead>
<tr>
<th>Electrical stimulation</th>
<th>Data acquisition parameters</th>
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<tbody>
<tr>
<td><strong>Stimulus</strong></td>
<td><strong>Duration</strong></td>
</tr>
<tr>
<td>DRCAP</td>
<td>0.5 ms</td>
</tr>
<tr>
<td>MSR</td>
<td>0.5 ms</td>
</tr>
<tr>
<td>Low intensity e.p.s.p.</td>
<td>0.5 ms</td>
</tr>
<tr>
<td>High intensity e.p.s.p.</td>
<td>0.5 ms</td>
</tr>
<tr>
<td>Train e.p.s.p.</td>
<td>5 stimuli of 20 Hz</td>
</tr>
</tbody>
</table>

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

3.2.3. Drugs

Bupivacaine HCl (Marcain), Astra Pharmaceutics Pty Ltd, N. Ryde, NSW, Australia.
Ketamine HCl (Ketamine Injection), Parnell Laboratories NZ Limited, East Tamaki, New Zealand.
Lignocaine HCl (Lopaine), Ethical Agents Ltd, Auckland, New Zealand.
Mepivacaine HCl (Mepivacaine), Nature Vet Pty Limited, Agnes Banks, NSW, Australia.
Morphine sulphate (Morphine Sulphate Injection), David Bull Laboratories, Victoria, Australia.
Xylazine HCl (Phoenix Xylazine 2% Injection), Phoenix Pharm Distributors Ltd, Auckland, New Zealand.
3.2.4. Data analysis

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

3.3. RESULTS

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

In another set of experiments, isolated sciatic nerves from mice were electrically stimulated to evaluate the performance of the equipment. They were laid on the same chamber used for the spinal cord, with the peripheral end in contact with the stimulating electrode and the proximal end in contact with the recording one. Not all the preparations displayed consistent responses. However, in some of them, nerve action potentials were recorded and conduction blockade was observed with the local anaesthetic lignocaine when used at 1 mM concentration (Figure 3.4).
Figure 3.3. Artefacts obtained after electrical stimulation to evoke the DRCAP (A), the low intensity e.p.s.p. (B), the high intensity e.p.s.p. (C) and the train e.p.s.p (D) in the neonatal rat hemisected spinal cord preparation.
Figure 3.4. Effect of lignocaine (1 mM) on nerve action potentials from mice sciatic nerve. The threshold of this nerve was 0.5 v (not showed) and a 6 v stimulus was used to evoke both A and C fibre compound action potentials (A), which amplitude was blocked and reduced, respectively, by lignocaine (B). Recovery is shown 25 minutes following return to lignocaine-free medium (C).
3.4. DISCUSSION

The hemisected neonatal rat spinal cord preparation is a robust in vitro preparation that permits the study of the spinal actions of centrally acting analgesics (Faber et al. 1997a; Faber et al. 1998; Brockmeyer & Kendig, 1995). However, in this trial, proper setting up of this technique was not possible and all records obtained from this preparation were identified as artefacts. Several lines conveyed us to this conclusion. Firstly, none of the analgesic and anaesthetic drugs used in this study depressed these “responses”. The opioid agonist morphine, known to depress both low and high intensity e.p.s.p. with EC\textsubscript{50} values of 99 nM and 79 nM, respectively (Faber et al. 1997a), produced no change in the records even when it was administered at concentrations of 1–10 µM. Similarly the α\textsubscript{2}-adrenoceptor agonist xylazine, which inhibits both spinal reflexes with EC\textsubscript{50} values of 760 nM and 910 nM, respectively (Faber et al. 1998), did not depress the obtained records after infusing the drug at 1–500 µM concentration. The NMDA receptor antagonist ketamine, which has concentration-dependent (1–50 µM) depressant actions on both spinal reflex components (Brockmeyer & Kendig, 1995), did not modify the records either when a 150 µM concentration was used. The local anaesthetic lignocaine also depresses the high intensity e.p.s.p. with an EC\textsubscript{50} values of 0.15 µM (J.P. Chambers, unpublished observations) but here, when used at 0.5–1 µM, it did not alter the recorded responses. The same results were observed with the more potent local anaesthetics mepivacaine (0.2 µM) and bupivacaine (0.2 µM).

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

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

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

The lumbar dorsal root was placed in contact with the stimulating electrode whereas the ipsilateral ventral root was in contact with the recording electrode. Differentiation between dorsal and ventral roots is not difficult. Under the microscope, the dorsal root looks like a continuation of the DRG. The ventral root, on the other hand, looks like an "independent" structure from the DRG. To confirm this, black ink was injected in the ventral aspect of the cranial spinal cord of some preparations, allowing us to identify the ventral aspect of the spinal cord. This process validated the differentiation as stated above. Electrical isolation of the electrodes was achieved by utilising grease gaps that prevented the ACSF from flowing freely through the entire chamber. Hence, inappropriate setting up of the spinal cord on the chamber does not seem to be a likely cause for the recording of artefacts. Furthermore, on several different occasions placing of the spinal cord on the chamber was carried out by more than one person and the results were the same, artefacts.
In order to reduce the possibility of having contaminants in contact with the spinal tissue, the material where the neonatal rat and the spinal cord were dissected as well as the flasks and the tubing system in which the ACSF was contained and pumped through were properly cleaned with HCl and then thoroughly rinsed with distilled water before each experiment. Moreover, they were replaced by new material at some stage and still no physiological responses were recorded. Thus, the chance of having a nasty substance in contact with the spinal tissue, which may have affected its viability, seems unlikely. Pieces of absorbent cloth were used to keep the spinal tissue in contact with the electrodes, and different medical materials were tried (e.g. masks, hats, shoe covers, etc.) without producing different results. The preparation chamber was the only material in contact with the spinal cord that was not replaced. It was made from previously used Perspex glass and although it was washed, the possibility that a toxic substance had remained on it cannot be excluded.

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

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

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

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

The NSAIDs are widely used for their analgesic, anti-inflammatory and sometimes antipyretic and antithrombotic effects. In spite of their structural diversity, it is accepted that all NSAIDs inhibit COX to a greater or lesser extent and it has been assumed that this action causes analgesia by preventing the sensitisation of peripheral nerve endings by PGs, particularly PGE\textsubscript{2} and PGI\textsubscript{2} (Ferreira, 1972). However, for some NSAIDs there is only a poor correlation between their ability to inhibit COX and their analgesic efficacy (McCormack, 1994a; McCormack, 1994b), implying that mechanisms other than or in addition to COX inhibition may be involved in the analgesic action of these drugs.

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

This study was undertaken to determine both the site and the mechanisms of action on/by which NSAIDs produce analgesia. The technique of mechanical noxious stimulation of a sheep's leg has demonstrated to be sensitive to the analgesia produced by NSAIDs in this species (Chambers et al. 1993; Chambers et al. 1995; Welsh & Nolan, 1994) and the i.t. cervical catheterisation of sheep has been useful to determine the spinal actions of different analgesic drugs (Waterman et al. 1988; Ley et al. 1989; Kyles et al. 1993) but not the
NSAIDs. Therefore, ketoprofen, phenylbutazone, salicylic acid and tolfenamic acid were i.t. and i.v. injected to non-lame, healthy sheep implanted with indwelling cervical catheters (Kyles et al. 1992) and submitted to mechanical noxious stimulation of one forelimb. It was reasoned that if NSAIDs-mediated analgesia is due to spinal COX inhibition, increase in nociceptive thresholds should be observed after injecting these drugs into the subarachnoid space at concentrations high enough to inhibit the activity of these enzymes (Laneuville et al. 1994; Ricketts et al. 1998; Giuliano & Warner, 1998). In a similar way, it was assumed that if NSAIDs induce analgesia by activating descending inhibitory mechanisms then the i.t. injection of the opioid receptor antagonist naloxone (Emmerson et al. 1994) and the \( \alpha_2 \)-adrenoceptor antagonist atipamezole (Schwartz & Clark, 1998) should decrease the analgesic actions of i.v. administered NSAIDs.

4.2. MATERIALS AND METHODS

4.2.1. Animals and intratecal catheterisation

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

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

Because several placed catheters were lost and others migrated rostrally, it was decided that in the future they should be anchored. Hence, before passing the catheter subcutaneously a knot made with the catheter itself was tried (Figure 4.2). However, the knot became so tight that it blocked the catheter and therefore this technique was abandoned and replaced by advancing the catheter in a loop manner through a Yates draining tube approximate 1 cm long consisting of four catheter lumens joined together, each of 3 mm diameter (Flexi-Drain; Obex Medical Ltd., Auckland, New Zealand) (Figure 4.2). This
Figure 4.1. Radiograph showing the correct placement of an intrathecal catheter in sheep, with the catheter tip (arrow) at the C5 level.
Figure 4.2. A knot made with the intrathecal catheter itself (a) and a silicon tubular drain (a) were used to anchor the intrathecal catheter. In both cases the anchoring element, knot or drain tube, was kept subcutaneously (a and c).
Sheep implanted with i.t. catheters proved to be more efficacious in keeping in place a functional i.t. catheter. The knot or the draining tube was placed subcutaneously at the level of the skin incision creating a physical barrier between the catheter inside the sheep's body and the external part of the catheter.

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

4.2.2. Mechanical nociceptive testing

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

Figure 4.3. Mechanical nociceptive device. The 2 mm diameter blunted pin attached to the plunger was pushed against the sheep's leg.
equivalent to 20 Newtons (N), the stimulus was terminated to prevent tissue damage and this value recorded.

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

![Mechanical device's calibration curve. The X axis shows the pressure applied to elicits the corresponding force at 1 Newton intervals.](image)

During the experiments, the sheep were placed individually in steel, mobile crates (86 cm high x 112 cm long x 53 cm wide), which were constructed in such a way to allow the sheep to see each other easily, have room to move slightly without turning round and give easy access to their lower legs. The sheep were content to stand in these crates for long periods (up to 7 hours) and
always were kept in pairs since individual sheep become distressed when isolated from the flock. A portable radio was used to provide background noise to prevent the sheep from being distracted by events outside. The skin on the cranial aspect of the left antebrachium was clipped and the device, to which sheep rapidly became accustomed, was attached to the lower end of the radius. The sheep were given a 15 to 20 minute period to acclimatise to the new environment, then at least four baseline readings were taken. After administration of the drug being tested, readings were recorded every 5 minutes for the first hour, every 10 minutes for the second hour and every 30 minutes for up to 6 hours, or until values returned to pre-administration baseline levels.

4.2.3. Drug administration protocol

4.2.3.1. Intrathecal administration of NSAIDs

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

The NSAIDs were dissolved in saline and NaOH (not more than 2.4 mM final concentration of NaOH for the highest drug concentration; pH between 7.0 and 7.8 for all different drugs and concentrations). Xylazine was dissolved in saline only (pH 6.0 and 6.5). Once dissolved, drugs were filtered (26 mm, 0.2 µm syringe filter; Sartorius, Minisart CE, Goettingen, Germany) and then injected through a 0.2 µm filter (4 mm filter unit, Phenomenex, Torrance, California, U.S.A.) attached to the luer lock connector affixed to the catheter. All drugs were administered i.t. in a 100 µL volume followed by a wash-in injection of 300 µL (the volume of the system) of saline. The volume of 100 µL was adopted because previous studies (Waterman et al. 1988; Kyles et al. 1993) have shown that the drug distribution is limited to about 3 or 4 vertebral segments either side of the catheter tip when administered in such a volume.
Demonstration of a functional i.t. catheter was made by injecting xylazine (10 and 50 µg/ 100 µL) through it, which is known to produce good analgesia in sheep (Waterman et al. 1988; Kyles et al. 1993). Therefore, xylazine was used as the positive control. Once confirmed that the catheter was working properly, cumulative doses (0.375 to 200 µM) of NSAIDs and repetitive doses of saline solution (10 doses; negative control), were i.t. administered. The sheep received all agents but xylazine in a randomised manner with at least one week interval between successive treatments on each sheep to avoid drug residue interference. The operator was blind to the drug treatment except xylazine in all trials.

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

4.2.3.2. Intravenous administration of NSAIDs

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

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

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

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

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

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

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

atipamezole and yohimbine on the analgesia elicited by an i.v. administered $\alpha_2$-adrenoceptor agonist was evaluated. The analgesic effect of xylazine (20 $\mu$g/kg i.v.) alone and in combination with i.t. atipamezole (100 $\mu$L, 4.03 mM) and yohimbine (100 $\mu$L, 5.11 mM) was studied. In addition, 100 $\mu$L i.t. saline solution was used as control drug in combination with i.v. fentanyl and xylazine. The i.t. antagonist administration took place 10 minutes before the i.v. agonist injection. The effect of i.t. naloxone, atipamezole and yohimbine alone on nociceptive thresholds was also studied.

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

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

4.2.4 Statistical analysis

All the results are expressed as the mean ± s.e.m. An analysis of variance (ANOVA), which assessed the variation produced by each sheep, the drug treatment and time, was used to determine variations in threshold over time. The peak effects of each treatment were compared with pre-drug administration values using a Student's $t$ test. The areas under the threshold change vs. time
curve values for 30 and 60 (AUC30 and AUC60) minutes after i.t and i.v. drug administration, respectively, for individual sheep (with the baseline subtracted) were calculated and compared using a Student's t test. Differences with $P < 0.05$ were considered significant.

4.3. RESULTS

4.3.1. Intrathecal catheterisation of sheep

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

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

Two animals in which the catheter's distal tip migrated rostrally with the tip lying at the level of C3 were reanaesthetised and the catheter was successfully pushed back in at the C5 level on one of them. For the other, a 16G i.v. Teflon
<table>
<thead>
<tr>
<th>Sheep</th>
<th>Attempt</th>
<th>Radiography</th>
<th>Xylazine</th>
<th>Outcome</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>Readjust catheter tip position</td>
<td>Yes</td>
<td>30 days</td>
<td>Knot made with the catheter itself. Postoperative wound infection which reached the spinal canal and produced ataxia. Euthanasia.</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>Readjust catheter tip position</td>
<td>Yes</td>
<td>147 days</td>
<td>Knot made with the catheter itself was too tight and next day silicon draining tube was used for anchoring instead. i.v. catheter over the i.t. one was intended but excessive scar tissue prevented for advancing it further in. A Tuohy needle was inserted and silicon draining tube used for anchoring.</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Catheter tip at C5</td>
<td>Yes</td>
<td>29/2/00</td>
<td>I.t. catheter anchored with silicon draining tube. Rostrally migration with distal tip at C3 level. I.v. catheter passed over the i.t. one. I.t. catheter anchored with silicon draining tube.</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>Catheter tip at C5</td>
<td>Yes</td>
<td>150 days</td>
<td>I.t. catheter anchored with silicon draining tube. Rostrally migration with distal tip at C3 level.</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Readjust catheter tip position</td>
<td>Yes</td>
<td>22/3/00</td>
<td>I.t. catheter anchored with silicon draining tube. Old i.t. catheter unintentionally cut during dissection to pass an i.v. catheter over it. The inner end was not found. Euthanasia.</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>Readjust catheter tip position</td>
<td>Yes</td>
<td>107 days</td>
<td>I.t. catheter anchored with silicon draining tube.</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Unsuccessful surgery</td>
<td>-</td>
<td>-</td>
<td>Pronociceptive problems in both forelimbs. Catheter was pulled out and sheep recovered by itself. I.t. catheter anchored with silicon draining tube. Rostrally migration with distal tip at C3 level. I.v. catheter over the i.t. one was intended but excessive scar tissue prevented for advancing it further in. A Tuohy needle was inserted and silicon draining tube used for anchoring.</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>Readjust catheter tip position</td>
<td>Not tested</td>
<td>Removed</td>
<td>I.t. catheter anchored with silicon draining tube. Rostrally migration with distal tip at C3 level.</td>
</tr>
<tr>
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<td>2</td>
<td>Catheter tip at C5</td>
<td>Yes</td>
<td>56 days</td>
<td>I.t. catheter passed over the i.t. one. I.t. catheter anchored with silicon draining tube.</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Catheter tip at C5</td>
<td>Yes</td>
<td>22/3/00</td>
<td>I.t. catheter passed over the i.t. one. I.t. catheter anchored with silicon draining tube.</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>Readjust catheter tip position</td>
<td>Yes</td>
<td>76 days</td>
<td>I.t. catheter anchored with silicon draining tube. Rostrally migration with distal tip at C3 level. I.v. catheter passed over the i.t. one. I.t. catheter anchored with silicon draining tube.</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Readjust catheter tip position</td>
<td>Yes</td>
<td>21/3/00</td>
<td>I.t. catheter passed over the i.t. one. I.t. catheter anchored with silicon draining tube.</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>Unsuccessful surgery</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>Unsuccessful surgery</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Unsuccessful surgery</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>93</td>
<td>1</td>
<td>Unsuccessful surgery</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Readjust catheter tip position</td>
<td>Yes</td>
<td>55 days</td>
<td>Pulled out.</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Readjust catheter tip position</td>
<td>Yes</td>
<td>60 days</td>
<td>I.t. catheter anchored with silicon draining tube. Pulled out.</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Unsuccessful surgery</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>95</td>
<td>1</td>
<td>Readjust catheter tip position</td>
<td>Not tested</td>
<td>21 days</td>
<td>Pulled out.</td>
</tr>
</tbody>
</table>
Table 4.1. Summary of the intrathecal catheterisation in the sheep. (Cont.)

<table>
<thead>
<tr>
<th>Sheep</th>
<th>Attempt</th>
<th>Radiography</th>
<th>Xylazine</th>
<th>Outcome</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>102</td>
<td>1</td>
<td>Readjust catheter tip position</td>
<td>Not tested</td>
<td>6 days</td>
<td>Pulled out.</td>
</tr>
<tr>
<td>103</td>
<td>1</td>
<td>Catheter tip at C5</td>
<td>Not tested</td>
<td>14 days</td>
<td>Pulled out.</td>
</tr>
<tr>
<td>116</td>
<td>1</td>
<td>Readjust catheter tip position</td>
<td>Yes</td>
<td>17 days</td>
<td>Dead because of lung and liver damage.</td>
</tr>
<tr>
<td>385</td>
<td>1</td>
<td>Unsuccessful surgery</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Unsuccessful surgery</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>404</td>
<td>1</td>
<td>Readjust catheter tip position</td>
<td>No</td>
<td>31 days</td>
<td>Rostrally migration with distal tip at C3 level. Catheter from attempt 1 was pushed back in.</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Readjust catheter tip position</td>
<td>Yes</td>
<td>25 days</td>
<td>I.v. catheter passed over the i.t. one. l.t. catheter anchored with silicon draining tube. Catheter leakage.</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Readjust catheter tip position</td>
<td>Yes</td>
<td>75 days</td>
<td>I.v. catheter passed over the i.t. one. l.t. catheter anchored with silicon draining tube. Catheter was removed because of signs of ataxia.</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Catheter tip at C5</td>
<td>Yes</td>
<td>45 days</td>
<td>-</td>
</tr>
<tr>
<td>1572</td>
<td>1</td>
<td>Readjust catheter tip position</td>
<td>No</td>
<td>21 days</td>
<td>Rostrally migration with distal tip at C3 level. Catheter from attempt 1 was unsuccessfully tried to be pushed back in. Instead, i.v. catheter passed over the i.t. one. Dead because of lung and liver damage.</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Readjust catheter tip position</td>
<td>Yes</td>
<td>33 days</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 4.5. Sheep's head medial section with the spinal cord exposed until the caudal aspect of C2. The Tuohy needle was superimposed on the atlanto-occipital joint to indicate the position where the needle had to be inserted through for the implantation of intrathecal catheters.
catheter (Quick-cath, Travenol Laboratories Inc, Dearfield, IL, U.S.A.) was necessary to reposition a new i.t. catheter. The i.v. catheter was passed over the old i.t. catheter until CSF flowed. This i.t. catheter was replaced with a new one which was freely moved until it reached the C5 level. This procedure was successfully carried out four more times. On two more occasions, however, it was not possible to perform this procedure and the catheter was pulled out to insert a Tuohy needle instead and to place a new catheter. One other time, while dissecting around the old i.t. catheter to pass over the i.v. one, the i.t. catheter was unintentionally cut and the distal part never found. This sheep was killed.

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

In all the cases in which the catheter tip lay at the C5 level, the i.t. injection of 100 µL xylazine produced a marked elevation in the nociceptive thresholds with no obvious supraspinal behaviours such as sedation. This confirmed that the catheters were functional and that the effect of the volume dose was restricted to the spinal area. Two doses of xylazine, 388 and 1940 µM (10 and 50 µg/100 µL, respectively), were used in these experiments and their effect on the nociceptive thresholds seemed to be dose dependent (Figure 4.6). The 388 µM xylazine dose increased the nociceptive thresholds above 5 N from 5 to 60 minutes after drug administration with a peak effect above 8 N at 20 minutes. Thresholds returned to baseline levels after 70 minutes. When the 1940 µM dose was injected, the nociceptive thresholds raised after the 5 minute reading and lasted above the baseline level for up to 3 hours, the time frame of the experiment. From 20 to 90 minutes after xylazine administration the thresholds were above 15 N and at 40 minutes reached almost 19 N. No behavioural changes were observed with any dose of xylazine used in these trials.
Complications after the placement of the i.t. catheter were rarely seen. All animals, but the one that developed pronociceptive problems, appeared completely normal within a few hours of surgery and remained so until the end of the trials. Only two sheep developed signs of ataxia. In one of them antibiotic treatment was instituted at the same time that the i.t. catheter was removed. This sheep clinically recovered showing normal thresholds after two weeks, which persisted for three more, at which time the sheep was moved to a teaching unit. The other sheep developed postoperative wound infection that appeared to respond to antibiotic therapy; however, a few days later, after the therapy had been suspended, severe ataxia was noticed. This sheep was euthanased and at histopathological examination of the cervical spinal cord there was observed marked diffuse oedema of the leptomeninges and necrosis and cavitation of dorsal horns at the level of C1 and C2.
Two more sheep died during these experiments. Both of them showed extensive adhesions between the lungs, diaphragm and thoracic wall as well as between the liver and the diaphragm. Large (4 – 7 cm) abscesses were present in both organs. Fibropurulent pneumonia and septicaemia were the diagnosis. Histopathological examination of the cervical spinal cord showed in both cases extensive accumulation of neutrophils in the dura matter along with neutrophilic infiltration into both the grey and white matter of the cord, supporting a diagnosis of suppurative spinal meningitis (Figure 4.7).

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

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

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

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

Apart from transitory mild excitement after the i.t. administration of tolfenamic acid in some sheep, no behavioural changes were observed with any i.t. injected NSAID under the conditions here described.
Figure 4.8. Effect of intrathecal repetitive administration of 100 µL saline solution (0.9%) on nociceptive thresholds of healthy sheep over 30 minutes after administration (time 0) (A) and their respective AUC30 (B). The data is the mean ± s.e.m. for n = 5 sheep.
Sheep implanted with i.t. catheters

Figure 4.9. Effect of intrathecal cumulative doses of 100 μL ketoprofen (0.8 – 200 μM) on nociceptive thresholds of healthy sheep over 30 minutes after administration (time 0 (A) and their respective AUC30 (B). The data is the mean ± s.e.m. for n = 4-5 sheep.
Figure 4.10. Effect of intrathecal cumulative doses of 100 µL phenylbutazone (0.375 - 200 µM) on nociceptive thresholds of healthy sheep over 30 minutes after administration (time 0) (A) and their respective AUC30 (B). The data is the mean ± s.e.m. for $n = 5$ sheep.
Sheep implanted with i.t. catheters

Figure 4.11. Effect of intrathecal cumulative doses of 100 µL salicylic acid (0.375 - 200 µM) on nociceptive thresholds of healthy sheep over 30 minutes after administration (time 0) (A) and their respective AUC30 (B). The data is the mean ± s.e.m. for n = 5 sheep.
Sheep implanted with i.t. catheters

Figure 4.12. Effect of intrathecal cumulative doses of 100 µL tolfenamic acid (0.375
200 µM) on nociceptive thresholds of healthy sheep over 30 minutes after administration
(time 0) (A) and their respective AUC30 (B). The data is the mean ± s.e.m. for n = 4-
sheep.
4.3.3. Intravenous administration of NSAIDs

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

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

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

Comparison of the AUC60 revealed no significant difference between saline (12.38 ± 2.77 N min), salicylic acid (21.87 ± 9.35 N min), and phenylbutazone (30.65 ± 16.89 N min) (\( P \geq 0.2787 \), t-test). However when ketoprofen and tolfenamic acid's AUC60 (111.31 ± 5.42 N min and 120.38 ± 14.58 N min, respectively) were compared with that of saline, or even any of the other two NSAIDs, a significant difference was found (\( P = 0.0001 \), t-test). No significant difference was observed between ketoprofen and tolfenamic acid's AUC60 (\( P = 0.5519 \), t-test) (Figure 4.14).
Sheep implanted with i.t. catheters

Figure 4.13. Effect of ketoprofen (3 mg/kg i.v.) and tolfenamic acid (2 mg/kg i.v.) on nociceptive thresholds of healthy sheep over six hours after administration (time 0) compared with saline (3 mL i.v.). The data is the mean ± s.e.m. for n = 5-6 sheep from -20 to 120 minutes and n = 2 sheep from 150 to 360 minutes.

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

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

No behavioural changes with any i.v. administered NSAID were noticed during the frame time of each experiment.
Sheep implanted with i.t. catheters

Figure 4.14. AUC60 for i.v. injected NSAIDs. The data is the mean ± s.e.m. for n = 5-6 sheep. * Represents a significant (P = 0.0001, t-test) difference between treatment and saline.

Figure 4.15. Effect of ketoprofen (3 mg/kg i.v.) and tolfenamic acid (2 mg/kg i.v.) on nociceptive thresholds of healthy sheep over two hours after administration (time 0) compared with saline (3 mL i.v.). The data is the mean ± s.e.m. for n = 5-6 sheep.
4.3.4. Involvement of opioidergic and $\alpha_2$-adrenergic systems in NSAID-induced analgesia

In order to establish any possible interaction at the spinal level between the opioidergic and $\alpha_2$-adrenergic systems with the analgesia produced by the i.v. administration of NSAIDs, it was necessary to find a high enough i.t. dose for opioid and $\alpha_2$-adrenergic drug receptor antagonists to block the analgesia produced by i.v. fentanyl and xylazine, respectively, without affecting baseline nociceptive thresholds when these receptor antagonists were i.t. injected alone.

4.3.4.1. Blockade of spinal opioid receptors

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

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

To be sure that the reduced analgesic effect of fentanyl in the combination i.t. naloxone / i.v. fentanyl was due to an antagonist effect on opioid receptors in the spinal cord and not just to a volume effect, i.t. 0.9% saline solution (100 µL) followed by i.v. fentanyl was injected. Saline failed to block the fentanyl-mediated analgesia (Figure 4.16) and an AUC60 equivalent to 87.82% for that
of i.v. fentanyl alone was obtained $(182.12 \pm 25.82$ vs. $207.37 \pm 32.68 \text{ N min}; P = 0.3639$, t-test) (Figure 4.16).

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

4.3.4.2. Blockade of spinal $\alpha_2$-adrenoceptors

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

After it had been established that xylazine produced antinociception in the sheep, yohimbine $(100 \mu L, 5.11 \text{ mM})$ was i.t. preinjected to prevent this hypoalgesic effect. Surprisingly, yohimbine increased, instead of decreasing, the nociceptive thresholds in two sheep of which the drug combination was employed. Nociceptive thresholds were elevated over $13 \text{ N}$ after 10 minutes post-agonist drug administration and returned to baseline levels after 240 minutes (Figure 4.18). In one sheep on which yohimbine alone was injected, no significant effect on nociceptive thresholds was observed over 210 minutes. No further experiments were done with yohimbine.
Sheep implanted with i.t. catheters

Figure 4.16. Effect of fentanyl (10 µg/kg i.v.) and naloxone (100 µL, 5.49 mM i.t.) alone and the combinations i.t. naloxone + i.v. fentanyl and i.t. saline (100 µL) + i.v. fentanyl on nociceptive thresholds of healthy sheep over one hour after administration (time -10 and 0 for i.t. and i.v. treatments, respectively) compared with saline (3 mL i.v.) (A) and their respective AUC60 (B). The data is the mean ± s.e.m. for \( n = 4 \) sheep, except saline i.v. where \( n = 5 \). * Represents a significant \( (P = 0.0001, t\text{-test}) \) difference between treatment and saline and ** between treatment and fentanyl i.v. alone.
Sheep implanted with i.t. catheters

Figure 4.17. Effect of xylazine (20 µg/kg i.v.) and atipamezole (100 µL, 4.03 mM i.t.) alone and the combinations i.t. atipamezole + i.v. xylazine and i.t. saline (100 µL) + i.v. xylazine on nociceptive thresholds of healthy sheep over one hour after administration (time -10 and 0 for i.t. and i.v. treatments, respectively) compared with saline (3 mL i.v.) (A) and their respective AUC60 (B). The data is the mean ± s.e.m. for n = 4 sheep, except saline i.v. where n = 5. * Represents a significant (P = 0.0001, t-test) difference between treatment and saline and ** between treatment and xylazine i.v. alone.
Atipamezole was i.t. injected to prevent i.v. xylazine-mediated analgesia. The selected dose of 100 µL, 4.03 mM inhibited by 88.13% the AUC60 of i.v. xylazine alone, generating an AUC60 of 17.72 ± 13.57 N min for the coadministration i.t. atipamezole / i.v. xylazine ($P = 0.0001$, t-test) (Figure 4.17). Atipamezole alone had no significant effect on the nociceptive thresholds when these were read 10 minutes post-drug administration and then for up to 60 minutes (Figure 4.17), producing an AUC60 of -10.19 ± 9.31 N min ($P = 0.3121$, t-test; when compared to i.v. saline alone) (Figure 4.17).

To demonstrate that atipamezole inhibition of xylazine-mediated antinociception was not due to a mere volume effect, the analgesic effect of i.t. saline solution (0.9%, 100 µL) followed by i.v. xylazine was evaluated. Saline failed to block the increase in nociceptive thresholds induced by the i.v. injection of xylazine. In fact, very similar results in comparison to those from i.v. xylazine alone were obtained (Figure 4.17). The AUC60 for this drug combination (141.85 ± 24.92 N min) was not found significantly different from that of i.v xylazine alone ($P = 0.7479$, t-test) (Figure 4.17).
One to two minutes after xylazine being administered transit short lasting (1-2 minutes) hyperventilation was observed, soon after light sedation for 15-20 minutes was perceived as well. Yohimbine and atipamezole produced no obvious behavioural effects and had no moderating influence on xylazine-induced behavioural changes.

4.3.4.3. Influence of spinal opioid and $\alpha_2$-adrenergic receptor blockade on i.v. ketoprofen-induced analgesia

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

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

4.4.1. Intrathecal catheterisation in sheep

4.4.1.1. Catheter implantation technique

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

4.4.1.2. Catheter maintenance

The catheters were daily flushed with 1 mL sterile 0.9% saline solution to prevent blockage, compared to once a week with 0.35 mL injected by Kyles et al. (1992) in their sheep. These same authors suggested that scar tissue
formation in blocked catheters depends on the length of time the catheter has been in position and the number of injections made through it. We did not find fibrous plugs in catheters that were removed even after being in place and in regular use for up to 150 days. This procedure neither affected the nocicepti

tive thresholds of sheep, since similar baseline levels were obtained every experiment. Therefore, it seems that daily flush of i.t. catheters in sheep is a harmless, adequate procedure that helps to maintain catheters in good operating order.

4.4.1.3. Catheter replacing and anchoring

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

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

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

Needle and catheter placement were successfully carried out on 15 different occasions (sheep no. 1, 2, 3, 4, 5 twice, 6, 93 twice, 95, 102, 103, 116, 404, 1572). However, three of these catheters (sheep no. 95, 102, 103) pulled out completely before even trying xylazine and two more (sheep no. 404, 1572) migrated rostrally and no analgesia was observed when xylazine was administered through them. Thus, to keep catheters in place became a major problem and even when into the subarachnoid space, if the tip was not at the C5 level no analgesic drug effect was perceived with the algesiometric test used here.
A possible reason for this migration could be attributed to the catheter design itself (e.g. wall thickness, fabrication material), which is known to have an impact upon complication-free "survival" of the catheter (Follett & Naumann, 2000). Actually, the manufacturer changed the material of the catheter and this has resulted in chronic kinking and occlusions in different parts of the world. We continued working with the same type of catheters because we had them in stock and we did not realise, until later, that the new material could be causing this problem. A number of different anchoring techniques for the catheters were used, a knot made with the catheter itself was tried twice (sheep no. 1, 2) but blockage of the catheter was noticed. To anchor the catheter, a Yates silicon draining tube (Figure 4.2) was used instead twelve different times (sheep no. 2 twice, 3 twice, 4, 5 twice, 6 twice, 93, 404 twice). This technique was more effective in keeping catheters in the right position for longer period of time than by only suturing the catheter in place where it emerged from the subcutaneous tissue. The occurrence of migration is associated with failure to anchor the catheter, and in humans, a migration incidence of more than 45% for non-anchored catheters was reported (Follett & Naumann, 2000).

4.4.1.4. Assessment of catheter functionality

Catheter insertion in a length of about 25 to 28 cm was estimated as necessary to reach the C5 level in dead sheep. In most experimental animals, this length was enough to position the catheter tip around C4 and C5, as it was assessed radiographically. If catheter tip was not placed at the C5 level, it was repositioned until it got in the right place. In all cases, radiographic confirmation of catheter placement, catheter tip position at the C5 level as well as assessment of the integrity of the catheter was used to validate the catheterisation technique (Figure 4.1). This radiographic procedure has been accurately used by others to assess the functional integrity of the catheters (Kyles et al. 1992). In our study, catheter functionality was evaluated by administering the α2-adrenoceptor agonist xylazine through them and assessing its effect on mechanical nociceptive thresholds.

1 J.P.Chambers 2000, E-mail 27 March.
Xylazine is known to produce good analgesia in sheep when administered through the i.t. route in a 100 µL volume and this effect has been solely attributed to spinal mechanisms because the bulk of radioactivity from $[^3H]$-clonidine (Waterman et al. 1988) and $[^3H]$-idazoxan (Kyles et al. 1993) injected in such a volume was distributed within two to three vertebral segments to either side of the catheter tip and because no detectable behavioural changes such as sedation were perceived after the i.t. injection of xylazine (Waterman et al. 1988; Kyles et al. 1993). The two doses of xylazine used in this study (10 and 50 µg/100 µL; equivalent to 388 and 1940 µM, respectively) produced dose dependent analgesia when the catheter tip was located at the C5 level. Similar results were reported by Waterman et al. (1988). The major difference between the two studies was observed with the 50 µg dose. Waterman and co-workers observed that mechanical nociceptive thresholds returned to baseline level after 120 min of xylazine being administered (Waterman et al. 1988), whereas this study recorded hypoalgesia for up to 180 min, the time frame of the experiment, with thresholds not returning to baseline levels within that time. This longer lasting xylazine-mediated analgesia seems unlikely to be due to a combined spinal-supraspinal action of the drug in our experiments since no sedation was evident. This effect was not investigated further. Nevertheless, this probably reflects different levels, subtypes and distribution of $\alpha_2$-adrenoceptors in the spinal cord of these two distinct sheep populations (Clun-cross and Romney-cross). The antinociceptive effects of i.v. xylazine (50 µg/kg), for instance, varied in different breeds of sheep (Ley et al. 1990) but no conclusive information in this respect is available when xylazine is i.t. injected. Other possible explanations might include differences in the operation technique of the mechanical nociceptive device in both trials and the recovery time between insertion of the i.t. catheter and xylazine testing. Waterman et al. (1988) allowed at least one-week intervals whereas we allowed at least two before the $\alpha_2$-adrenoceptor agonist was administered.

While in use, catheters were periodically tested to verify that they were working properly. Regular injection of xylazine through the catheter is recommended for this purpose (Kyles et al. 1992) and here a 10 µg dose proved to be very useful.
When no antinociception was achieved after i.t. xylazine injection, catheter replacing was carried out as explained above.

4.4.1.5. Complications

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

Two other sheep (116, 1572) that came from a different farm than the others died of pneumonia and septicaemia with the cervical spinal cord showing histological changes consistent with suppurative spinal meningitis. These
changes in the CNS could be attributed to the septicaemia itself. It seems that a greater chance of infection exists when a surgical procedure is performed in an animal with septicaemia. Therefore, it could be argued that these changes were due more to the animal’s condition than to the implantation technique. However, as mentioned before, the risk of contamination when introducing a foreign object into the spinal canal is always present (Raedler et al. 1999); hence, although less unlikely, spinal infection via the i.t. catheter cannot be ruled out.

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

4.4.2. Mechanical nociceptive testing

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

At the beginning of the trial, the device was calibrated before each experiment; however, this practice was abandoned since very similar responses were obtained each time, and once a month calibrations were carried out instead with no different results. The only difference observed during the entire experiment,
was some variation in the pressure needed to release the plunger which means that a bigger force was required for the blunted pin to make contact with the scale's plate. This was corrected by simply greasing or replacing the plunger. Occasionally, the device was attached to the operator's wrist and thresholds were measured to verify that it was working properly. No problems were detected at any time.

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

The mechanical nociceptive device was simple to use and produced reproducible responses as long as it was utilised by the same operator. When different operators tried the device the same stimulus was difficult to obtain since the rate at which the pressure was applied into the system varied with the rate at which the operator pushed the syringe's plunger. In his more sophisticated mechanical nociceptive apparatus, Chambers (1992) solved this problem by utilising a flow pneumatic system powered by compressed oxygen which allowed entrance into the device to be controlled by valves producing always the same stimulus regardless of the operator. Here, to minimise this variation only one operator, blinded to the treatment the sheep were given to avoid bias, was in charge of taking readings throughout the entire trial. This
allowed baseline thresholds to remain constant from test to test. It has been shown that sheep, in contrast to horses, show no sign of learning to respond before the mechanical stimulus become painful (Chambers, 1992). For instance, the control thresholds stayed constant for at least 5 hours and others have reported this for longer periods of time (Chambers, 1992; Chambers et al. 1995). Furthermore, the nociceptive test has been shown to discriminate between sedation and analgesia. When sheep were systemically administered the phenothiazine derivative acepromazine, sedation but no change on mechanical nociceptive thresholds were observed (Nolan et al. 1987a; Chambers, 1992).

Ambient air temperature has been shown to influence mechanical nociceptive thresholds of sheep. When the temperature was below 8°C thresholds were raised and even reached about 20 N at 0°C. Between 10 and 25°C there was no significant variation on nociceptive thresholds (Chambers, 1992). Hence, a thermometer was put in the experimental room and it was decided that experiments would be carried out only if the temperature was higher than 10°C. All experiments were done between September and April and the lowest registered ambient temperature was 12°C and the highest 24°C; therefore, this environmental factor should have not influenced the results of this study.

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

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

The analgesic effects of NSAIDs have been demonstrated in various species, including human beings. These effects are mainly attributed to peripheral tissue
Sheep implanted with i.t. catheters

COX inhibition which, therefore, prevents sensitisation of peripheral nerve endings by PGs, particularly PGE$_2$ and PGl$_2$ (Ferreira, 1972). This is further supported by the fact that neutralisation of PGE$_2$ with the monoclonal antibody 2B5 prevented and reduced inflammatory hyperalgesia in rats as effectively as indomethacin (Portanova et al. 1996) or ketorolac (Zhang et al. 1997) did. Thus, since antibodies do not cross the blood-brain barrier, it seems that peripherally derived PGs are essential for establishing and sustaining hyperalgesia. Nevertheless, extensive evidence for a central analgesic site of action for the NSAIDs also exists (Bustamante et al. 1997; Malmberg & Yaksh, 1992a; Malmberg & Yaksh, 1992b; Malmberg & Yaksh, 1993; Chambers et al. 1995; Pelissier et al. 1996; Brune et al. 1992; Björkman et al. 1996; Willingale et al. 1997; Piletta et al. 1991; Devoghel, 1983). It is not clear, however, if these central effects are mediated by prostanoid synthesis inhibition, or if these drugs interact with neurones in different ways.

4.4.3.1. Intrathecal injection of NSAIDs

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

The PGs and all the necessary machinery for their synthesis are present in the spinal cord (Willingale et al. 1997). They can be released from the cord by activation of high-threshold nociceptive afferent inputs (Ramwell et al. 1966), by direct administration of neurotransmitters (Chaplan et al. 1997; Malmberg & Yaksh, 1993; Dirig & Yaksh, 1999) or by peripheral inflammatory stimuli (Smith et al. 1998; Dirig & Yaksh, 1999; Muth-Selbach et al. 1999), suggesting that a certain level of repetitive stimulation of the primary afferent is required for their release. This agrees with the involvement of the NMDA receptor system in the generation of PGs, but their synthesis also can be stimulated by co-activation of AMPA/kainate and mGluRs (Bezzi et al. 1998; Conn & Pin, 1997), an activation which does not necessarily require repetitive stimulation. The origin of spinal prostanoids is not well established either, and release into the extracellular space may not only be from neuronal but also glial structures (Buritova et al. 1996; Willingale et al. 1997; Bezzi et al. 1998; Phillips & Pearce, 1999).
Little is known about the functions of the PGs in the spinal cord. In rodents, the i.t. administration of PGs increase nociceptive responses (Minami et al. 1994a; Minami et al. 1994b; Minami et al. 1996; Eguchi et al. 1999) but not all the PGs are pain-evoking and some diminish pain sensitivity (Minami et al. 1996; Minami et al. 1997; Matsumura et al. 1993; Eguchi et al. 1999). The mechanisms by which PGs mediate hyperalgesia at the spinal level are unknown, but it seems likely that they augment the synaptic processing of pain information by enhancing Ca\(^{2+}\)-mediated glutamate (Bezzi et al. 1998), SP (Malcangio et al. 1996) and CGRP (Andreeva & Rang, 1993) release through activation of prostanoid receptors on high and low threshold afferent terminals (Bley et al. 1998; Oida et al. 1995; Wright et al. 1999) and further modulation of cAMP and/or Ins(1,4,5)P\(_3\) transduction cascades. Additionally, it has been suggested that spinal PGs may exert a presynaptic inhibition on noradrenaline release from the bulbo-sympathetic noradrenergic pathway (Taiwo & Levine, 1988). Thus, PG-mediated neurotransmitter/neuropeptide release and noradrenaline release inhibition could be mechanisms for amplification of pain signals. Spinal prostanoid synthesis inhibition, therefore, could represent a mode of action by which NSAIDs exert antinociceptive effects.

The administration of NSAIDs into the spinal canal produced analgesia as assessed by different algesiometric tests (Table 4.2). The possibility that these effects had been mediated by inhibiting spinal COX enzymes is supported by the antinociceptive potency of various i.t. injected NSAIDs matching with COX inhibition values (Malmberg & Yaksh, 1992b). The possibility is also supported by the fact that paracetamol produced analgesia at the same time that inhibited spinal PGE\(_2\) release (Muth-Selbach et al. 1999) and the EP receptor antagonists SC-5108 and SC-51234A generated antinociception after being i.t. injected (Malmberg et al. 1994). The spinal COX isozyme responsible for the formation of these “pain-evoking” PGs is also a matter of debate. It is believed that the nociceptive process in the cord is more influenced by COX-2 rather than COX-1, mainly because COX-2 is the predominant isoform in this organ (Willingale et al. 1997; Seibert et al. 1994; Vane et al. 1998) and, contrary to COX-1, it is up-regulated by inflammatory hyperalgesia (Vane et al. 1998) which
Table 4.2. Evaluation of the analgesic effect of intrathecally and epidurally administered non-steroidal anti-inflammatory drugs in different species.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Species</th>
<th>Nociceptive test</th>
<th>NSAID</th>
<th>Dose</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intrathecal</strong></td>
<td></td>
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<tr>
<td>Bustamante et al. 1997</td>
<td>Rats (anaesthetised)</td>
<td>C fibre reflex recorded from the ipsilateral biceps femoris muscle after electrical stimulation within the area of the sural nerve</td>
<td>Acetylsalicylic acid</td>
<td>100 and 500 µg in 10 µL = 55 and 277 mM</td>
<td>Reduction of the area under the curve by 35 and 80%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Indomethacin</td>
<td>200, 300 and 500 µg in 10 µL = 55, 83 and 139 mM</td>
<td>Reduction of the area under the curve by 36, 62 and 89%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ketoprofen</td>
<td>300 and 500 µg in 10 µL = 112 and 196 mM</td>
<td>Reduction of the area under the curve by 23 and 40%</td>
</tr>
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<td></td>
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<td></td>
<td>Lysine clonixinate</td>
<td>300, 500 and 1000 µg in 10 µL = 73, 122 and 244 mM</td>
<td>Reduction of the area under the curve by 30, 48 and 63%</td>
</tr>
<tr>
<td>Malmberg &amp; Yaksh, 1992a</td>
<td>Rats</td>
<td>Intrathecal NMDA + hot plate test</td>
<td>Acetylsalicylic acid</td>
<td>100 nM</td>
<td>Reversal of thermal hyperalgesia in about 75-90%</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>Ketorolac</td>
<td>27 nM</td>
<td>Reversal of thermal hyperalgesia in about 85-95%</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>tromethamine</td>
<td>27 nM</td>
<td>Reversal of thermal hyperalgesia in about 90-95%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>S(+)-ibuprofen</td>
<td>270 nM</td>
<td>Failure to reverse thermal hyperalgesia</td>
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<td></td>
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<td></td>
<td>R(-)-ibuprofen</td>
<td>270 nM</td>
<td>Reversal of thermal hyperalgesia in about 85-95%</td>
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<td>S(+)-ibuprofen</td>
<td>27 nM</td>
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<td>R(-)-ibuprofen</td>
<td>270 nM</td>
<td>Reversal of thermal hyperalgesia in about 110-130%</td>
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<td></td>
<td></td>
<td></td>
<td>R(-)-ibuprofen</td>
<td>270 nM</td>
<td>Failure to reverse thermal hyperalgesia</td>
</tr>
<tr>
<td>Malmberg &amp; Yaksh, 1992b</td>
<td>Rats</td>
<td>Subcutaneous formalin injection into one hindpaw</td>
<td>Acetylsalicylic acid</td>
<td>27 nM (18-41 nM)*</td>
<td>About 70% inhibition of the phase 2 with the two highest doses</td>
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<td></td>
<td></td>
<td></td>
<td>Indomethacin</td>
<td>1.9 nM (1.2-4.0 nM)*</td>
<td>About 80% inhibition of the phase 2 with the two highest doses</td>
</tr>
</tbody>
</table>
Table 4.2. Evaluation of the analgesic effect of intrathecally and epidurally administered non-steroidal anti-inflammatory drugs in different species. (Cont.)

<table>
<thead>
<tr>
<th>Reference</th>
<th>Species</th>
<th>Nociceptive test</th>
<th>NSAID</th>
<th>Dose</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malmberg &amp; Yaksh,</td>
<td>Rats</td>
<td>Subcutaneous formalin injection</td>
<td>Flurbiprofen</td>
<td>2.1 nM (1.0-4.3 nM)*</td>
<td>About 70% inhibition of the phase 2 with the two highest doses</td>
</tr>
<tr>
<td>1992b</td>
<td></td>
<td>into one hindpaw</td>
<td>Ketorolac</td>
<td>5.2 nM (3.2-8.3 nM)*</td>
<td>About 80% inhibition of the phase 2 with the two highest doses</td>
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<td></td>
<td></td>
<td></td>
<td>tromethamine</td>
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<td></td>
<td></td>
<td></td>
<td>Zomepirac</td>
<td>5.9 nM (3.9-8.9 nM)*</td>
<td>About 75% inhibition of the phase 2 with the two highest doses</td>
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<td></td>
<td></td>
<td></td>
<td>sodium</td>
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<td></td>
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<td></td>
<td>S(+)-ibuprofen</td>
<td>15.7 nM (6.7-36 nM)*</td>
<td>About 60% inhibition of the phase 2 with the two highest doses</td>
</tr>
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<td></td>
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<td></td>
<td>Racemic ibuprofen</td>
<td>18.9 nM (9.3-38 nM)*</td>
<td>About 55% inhibition of the phase 2 with the two highest doses</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Acetaminophen</td>
<td>257 nM (163-405 nM)*</td>
<td>About 50% inhibition of the phase 2 with the two highest doses</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hot plate test (52.5°C)</td>
<td>R(-)-ibuprofen</td>
<td>&gt;270 nM</td>
<td>Failure to inhibit the phase 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Acetylsalicylic acid</td>
<td>100 nM</td>
<td>No significant effect on response latency</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Flurbiprofen</td>
<td>80 nM</td>
<td>No significant effect on response latency</td>
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<td></td>
<td></td>
<td></td>
<td>Ketorolac</td>
<td>80 nM</td>
<td>No significant effect on response latency</td>
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<td></td>
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<td></td>
<td>tromethamine</td>
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<td></td>
<td></td>
<td></td>
<td>Zomepirac</td>
<td>80 nM</td>
<td>No significant effect on response latency</td>
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<td></td>
<td></td>
<td></td>
<td>sodium</td>
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<td></td>
<td></td>
<td></td>
<td>Indomethacin</td>
<td>30 nM</td>
<td>No significant effect on response latency</td>
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<td></td>
</tr>
<tr>
<td>Malmberg &amp; Yaksh,</td>
<td>Rats</td>
<td>Subcutaneous formalin injection</td>
<td>Ketorolac</td>
<td>2.7-80 nM</td>
<td>Dose-dependent inhibition on phase 1 and 2. Maximal inhibition of 52% and 80% for each phase at the highest dose.</td>
</tr>
<tr>
<td>1993</td>
<td></td>
<td>into one hindpaw</td>
<td>tromethamine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pelissier, 1996</td>
<td>Rats</td>
<td>Paw pressure test</td>
<td>Acetaminophen</td>
<td>100 and 200 µg in 10</td>
<td>Increased the area under the nociceptive threshold curve by 6 and 8 times when compared to saline.</td>
</tr>
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<td></td>
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<td></td>
<td>µL = 66 and 132 mM</td>
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<tr>
<td>Carlsson et al. 1986</td>
<td>Intact</td>
<td>Radian heat tail-flick test</td>
<td>Dipyrone</td>
<td>50-400 µg in 5 µL =30-240 mM</td>
<td>Dose-dependent increase of tail withdrawal latencies.</td>
</tr>
<tr>
<td></td>
<td>rats</td>
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<tr>
<td>Reference</td>
<td>Species</td>
<td>Nociceptive test</td>
<td>NSAID</td>
<td>Dose</td>
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<tr>
<td>Carlsson et al. 1986</td>
<td>Spinal rats</td>
<td>Radian heat tail-flick test</td>
<td>Dipyone</td>
<td>400 µg in 5 µL = 240 mM</td>
<td>Failure to modify tail withdrawal latencies.</td>
</tr>
<tr>
<td></td>
<td>Spinal rats</td>
<td>C fibre reflex recorded from the ipsilateral tibialis anterior muscle after electrical stimulation of the sural nerve</td>
<td>Dipyone</td>
<td>100 and 400 µg in 5 µL = 60 and 240 mM</td>
<td>No change on flexor reflex activity with the 100 µg dose but increased it with the 400 µg dose.</td>
</tr>
<tr>
<td></td>
<td>Spinal rats</td>
<td>Ascending axon activity in response to C fibre stimulation of the sural nerve</td>
<td>Dipyone</td>
<td>100 and 200 µg in 5 µL = 60 and 120 mM</td>
<td>About 150% increase in C fibre evoked activity.</td>
</tr>
<tr>
<td>Lorenzetti &amp; Ferreira 1996</td>
<td>Rats</td>
<td>Paw pressure test + paw PGE$_2$ injection</td>
<td>Dipyone</td>
<td>2.5-80 µg in 20 µL = 0.375-12 mM</td>
<td>Dose-dependent reduction in escape behaviours.</td>
</tr>
<tr>
<td>Akman et al. 1996</td>
<td>Mice</td>
<td>Radian heat tail-flick test</td>
<td>Dipyone</td>
<td>50-400 µg in 5 µL = 30-240 mM</td>
<td>Failure to modify tail withdrawal latencies.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hot plate test (55°C)</td>
<td>Dipyone</td>
<td>50-400 µg in 5 µL = 30-240 mM</td>
<td>Failure to modify paw withdrawal latencies.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Abdominal constriction test</td>
<td>Dipyone</td>
<td>100 and 200 µg in 5 µL = 60 and 120 mM</td>
<td>About 50 and 65% reduction in the number of stretches.</td>
</tr>
<tr>
<td>Yamamoto &amp; Nozaki-Taguchi 1996</td>
<td>Rats</td>
<td>Subcutaneous formalin injection into one hindpaw</td>
<td>Indomethacin</td>
<td>0.03-30 µg in 10 µL = 8.3 µM-8.3 mM</td>
<td>Decreased number of flinches in both phase 1 and 2 in a dose-dependent manner.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NS-398</td>
<td>0.03-30 µg in 10 µL = 9.5 µM-9.5 mM</td>
<td>Decreased number of flinches in both phase 1 and 2 in a dose-dependent manner.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hot plate test (52.5°C)</td>
<td>Indomethacin</td>
<td>30 µg in 10 µL = 8.3 µM</td>
<td>Failure to modify paw withdrawal latencies.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NS-398</td>
<td>30 µg in 10 µL = 9.5 µM</td>
<td>Failure to modify paw withdrawal latencies.</td>
</tr>
<tr>
<td>Willingale et al. 1997$^a$</td>
<td>Rats</td>
<td>Mechanical noxious stimulation of the ankle joint</td>
<td>Meclofenamic acid</td>
<td>10 and 100 µM</td>
<td>Failure to modify dorsal hom neurone responses</td>
</tr>
<tr>
<td></td>
<td>(anaesthetised)</td>
<td>Mechanical noxious stimulation of the knee joint</td>
<td>Indomethacin</td>
<td>1 and 100 µM</td>
<td>Failure to modify dorsal hom neurone responses</td>
</tr>
</tbody>
</table>
Table 4.2. Evaluation of the analgesic effect of intrathecally and epidurally administered non-steroidal anti-inflammatory drugs in different species. (Cont.)

<table>
<thead>
<tr>
<th>Reference</th>
<th>Species</th>
<th>Nociceptive test</th>
<th>NSAID</th>
<th>Dose</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Willingale et al. 1997&lt;sup&gt;g&lt;/sup&gt;</td>
<td>Rats (anaesthetised)</td>
<td>C fibre reflex recorded from the ipsilateral biceps femoris muscle after electrical stimulation of the sural nerve</td>
<td>Indomethacin</td>
<td>10 and 100 µM</td>
<td>No change with the low dose. About 67% reduction in the magnitude of C fibre-evoked reflex with the high dose.</td>
</tr>
<tr>
<td>Sorkin 1997</td>
<td>Rats (anaesthetised)</td>
<td>Intrathecal NMDA injection</td>
<td>Ketorolac</td>
<td>30 µg in 10 µL = 7.9 mM</td>
<td>Blockage of PGE&lt;sub&gt;2&lt;/sub&gt; and TxB&lt;sub&gt;2&lt;/sub&gt; increased release by 94% and 119% of basal levels, respectively.</td>
</tr>
<tr>
<td>Dirig et al. 1997</td>
<td>Rats</td>
<td>Radiant thermal stimulus + subcutaneous carragennan injection into one hindpaw</td>
<td>S(+)-ibuprofen</td>
<td>Not specified</td>
<td>Prevented decrease of paw withdrawal latencies in a dose-dependent manner.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R(-)-ibuprofen</td>
<td>Not specified</td>
<td>Failed to prevent decrease of paw withdrawal latencies. Prevented decrease of paw withdrawal latencies in a dose-dependent manner.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SC58125</td>
<td>Not specified</td>
<td></td>
</tr>
<tr>
<td>Devoghel 1983</td>
<td>Humans</td>
<td>Cancer pain sufferers</td>
<td>Lysine-</td>
<td>60 mg in 1 mL = 33 mg acetylsalicylic acid in 1 mL = 5.55 M</td>
<td>Quick (within 15 minutes) and log-lasting (1-22 days) pain relief.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>acetylsalicylate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dolan &amp; Nolan 1999&lt;sup&gt;®&lt;/sup&gt;</td>
<td>Sheep</td>
<td>Mechanical nociceptive thresholds</td>
<td>DFU</td>
<td>200 nM</td>
<td>No modifying effect.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mechanical nociceptive thresholds + intrathecal NMDA</td>
<td>DFU</td>
<td>200 nM</td>
<td>Attenuated reduction on pain thresholds from 57% to 17% only.</td>
</tr>
<tr>
<td><strong>Epidural</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Masue et al. 1999</td>
<td>Rats</td>
<td>Tail-flick test + intrathecal nitroglycerin</td>
<td>S(+)-ibuprofen</td>
<td>52 µg (38-66 µg) /30 µL = 8.4 mM (6.1-10.7 mM)*</td>
<td>Dose-dependent prevention on shortening of tail-flick latencies.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Indomethacin</td>
<td>5 µg (2.8-7.3 µg) /30 µL = 460 µM (258-672 µM)*</td>
<td>Dose-dependent prevention on shortening of tail-flick latencies.</td>
</tr>
</tbody>
</table>
Table 4.2. Evaluation of the analgesic effect of intrathecally and epidurally administered non-steroidal anti-inflammatory drugs in different species. (Cont.)

<table>
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<th>Dose</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Masue et al. 1999</td>
<td>Rats</td>
<td>Tail-flick test + intrathecal nitroglycerin</td>
<td>Diclofenac</td>
<td>6.3 µg (5.7-7.6 µg) /30 µL = 660 µM (597-796 µM)*</td>
<td>Dose-dependent prevention on shortening of tail-flick latencies.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R(-)-ibuprofen</td>
<td>&gt;1000 µg/30µL = &gt;161 mM*</td>
<td>Failure to prevent shortening of tail-flick latencies.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>S(+) ibuprofen</td>
<td>1000 µg/30µL = 161 mM</td>
<td>Suppressed the paw-lifting behaviour in the phase 2.</td>
</tr>
<tr>
<td>Lauretti et al. 1998</td>
<td>Humans</td>
<td>Cancer pain sufferers</td>
<td>Diclofenac</td>
<td>75mg</td>
<td>Complete pain relief (VAS* = 0) for 48 h.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tenoxicam</td>
<td>20mg</td>
<td>Improvement in pain relief (VAS = 2) for 22.5 h</td>
</tr>
</tbody>
</table>

*ID₅₀ values and 95% confidence intervals.
* Administration volume was 10 µL.
* Administration volume was 40-50 µL.
* Administration volume not specified.
* VAS = visual analogue scale.
Sheep implanted with i.t. catheters

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

As presented above, documentation exists for a feasible role of spinal PGs in the nociceptive process and synthesis inhibition of these lipidic acids by NSAIDs. Nonetheless, we did not record analgesia with the i.t. injection of ketoprofen, salicylic acid, phenylbutazone and tolfenamic acid, which were administered in concentrations high enough to inhibit COX activity in in vitro (Laneuville et al. 1994; Ricketts et al. 1998) and ex vivo (Giuliano & Warner, 1996) studies. These differences may be accounted for by algesiometric test and dosage differences. Most papers describing the central analgesic effects of NSAIDs assessed nociception by using models of inflammatory pain and hyperalgesia (Malmberg & Yaksh, 1992a; Malmberg & Yaksh, 1992b; Malmberg & Yaksh, 1993; Lorenzetti & Ferreira, 1996; Akman et al. 1996; Yamamoto & Nozaki-Taguchi, 1996; Sorkin, 1997; Dirig et al. 1997; Dolan & Nolan, 1999; Masue et al. 1999) but failure to produce analgesia was observed when models of acute pain were utilised (Malmberg & Yaksh, 1992b; Akman et al. 1996; Yamamoto & Nozaki-Taguchi, 1996; Willingale et al. 1997; Dolan & Nolan, 1999). It seems, therefore, that NSAIDs produce analgesia at the spinal level only when an ongoing afferent barrage is established (peripheral and central sensitisation). However, a report in which rats were submitted to a paw pressure test without induction of inflammation, shows i.t. paracetamol had an antinociceptive effect (Pelissier et al. 1996), which suggests that these drugs are able to produce analgesia in the absence of spinal facilitated states. This effect was only evident by injecting the drug in very high concentrations (mM range) and when NSAIDs were administered at smaller concentrations, in one or two orders of magnitude, they failed to increase mechanical nociceptive thresholds (Willingale et al. 1997; Dolan & Nolan, 1999), as was observed here. In fact, most NSAID-mediated central analgesic effects have only been reported with the drugs administered in mM concentrations, the only exception are those
papers from Yaksh’s laboratory which reported analgesia in the rat paw formalin test by utilising drug concentrations within the nM range (Table 4.2). Since COX inhibition by NSAIDs only requires these drugs to be present in the medium in nM to µM range concentrations (Laneuville et al. 1994; Ricketts et al. 1998; Giuliano & Warner, 1998), it seems reasonable to suggest that mechanisms other than or complementary to prostanoid synthesis inhibition might mediate NSAIDs-induced central antinociception. Interestingly, detailed reviews of the literature have emphasised an almost complete dissociation between the analgesic efficacy of these agents and their COX inhibition potency (McCormack, 1994a; McCormack, 1994b).

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

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

At concentrations used in this study, the NSAIDs were found to be highly insoluble in normal saline and once dissolved the pH of the solutions was around 2. Therefore, NaOH was used to dissolve them as well as adjust pH. No control solution with NaOH was intended since dilution of this substance in saline at equivalent concentrations to those used with the NSAIDs produced alkaline solutions with pH between 11 and 12, which were considered inappropriate for injection into the subarachnoid space. Other workers have used dimethyl sulfoxide (Yamamoto & Nozaki-Taguchi, 1996) cyclodextrin (Malmberg & Yaksh, 1992b) and ethanol (Malmberg & Yaksh, 1992b) to dissolve NSAIDs for i.t. injection and although they did not find these agents affected motor function, it raises a question about the extent to which these known antinociceptive agents may have affected or contributed to the reported analgesic effect of those NSAIDs. The possibility exists that the interaction between NaOH and the NSAIDs could also have modified the pharmacological properties of the NSAIDs; however, it was not studied here and clarification on this point is required.
No modification in sheep’s general behaviour was observed after the i.t. injection of any NSAIDs used in this study. This is in agreement with studies on rats, in which administration of different NSAIDs into the subarachnoid space did not produce behavioural changes or motor dysfunction (Malmberg & Yaksh, 1992b; Malmberg & Yaksh, 1993; Yamamoto & Nozaki-Taguchi, 1996). However, transit allodynia with squeaking and agitation has been observed in rats after 1 and 3 hours of i.t. indomethacin (28 nM) and ketorolac (27 nM) administration, respectively (Malmberg & Yaksh, 1992b). Alterations in cardiovascular and respiratory parameters were not observed during or after the i.t. injection of NSAIDs in anaesthetised rats (Bustamante et al. 1997; Sorkin, 1997) and, apart from light congestion and oedema, no histopathological lesions were observed with i.t. dipyrone or saline in mice (Akman et al. 1996). Moreover, no sensory, motor and neurovegetative side effects were observed in humans who received i.t. (Devoghel, 1983) or epidural (Lauretti et al. 1998) injections of NSAIDs. Thus, although the spinal administration of these analgesic agents seems to be devoid of major side effects, preclinical scrutiny of especially designed preparations should be carried out before any recommendations are made for these drugs to be administered through this route.

4.4.3.2. Intravenous injection of NSAIDs.

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

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

Clinically, acetylsalicylic acid is almost devoid of anti-inflammatory effects and is considered as weak analgesic. This NSAID decreased serum TxB₂ values in sheep after i.v. administration at a rate of 10 mg/kg (Nolan et al. 1990); however, since acetylsalicylic acid is rapidly metabolised to salicylic acid (Higgs et al. 1987), this effect must be attributed to salicylic acid rather than the produg acetylsalicylic acid. In the present study, i.v. injection of salicylic acid (10 mg/kg) did not increase mechanical pain thresholds of healthy sheep. Thus, if salicylic acid produced in our sheep a similar effect on prostanoid synthesis inhibition to that described for acetylsalicylic acid (Nolan et al. 1990), it is possible to suggest that peripheral COX inhibition is not an analgesic mechanism of action for NSAIDs. Determination of plasma TxB₂ or PGE₂ would have been useful to clarify this point. Furthermore, the possibility that the lack of analgesia observed with salicylic acid had been due to a dose effect rather than an action of the drug itself cannot be ruled out, a dose-response trial would answer this.

Phenylbutazone, a pyrazolone derivative widely used in the equine clinic more for its anti-inflammatory effects than for its analgesic properties, produced no change on nociceptive thresholds of healthy sheep either, even when a quite large dose was used. This contrasts with the reported analgesic action of this drug in sheep submitted to unilateral stifle surgery (Dowd et al. 1998); however, this effect was achieved by administering the NSAID over three days and we only used a single dose. In addition, inflammation must have been present after surgery and an anti-inflammatory action by phenylbutazone may have contributed to the analgesic effect of this drug. Interestingly, dipyrone, another pyrazolone derivative with similar structure and molecular weight to
phenylbutazone and known for having good analgesic but poor anti-inflammatory action, raised the mechanical nociceptive thresholds in healthy sheep (Chambers et al. 1995). However, this analgesic effect was observed after injecting dipyrone in a 3-fold molar equivalent dose of phenylbutazone. The potential toxic effects of phenylbutazone may restrict its evaluation at bigger doses, which also may argue for different mechanisms of action between these two drugs. Thus, it seems that the analgesic effects of NSAIDs, even when classified within the same group, differ for each individual agent. Although the ability to inhibit COX enzymes for these two drugs seems to vary markedly with phenylbutazone and dipyrone being potent and weak COX inhibitors, respectively, it was shown that flunixin (potent COX inhibitor) and dipyrone had similar effects on healthy sheep's nociceptive thresholds and on TxB2 plasma levels (Chambers et al. 1995). It is not clear therefore, whether or not COX inhibition accounts for the analgesic action of NSAIDs.

4.4.3.2.2. Supraspinal analgesic action of NSAIDs

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

Opiodergic (Chambers, 1992; Chambers et al. 1993; Chambers et al. 1995; Akman et al. 1996; Tortorici et al. 1996; Ochi et al. 1999) and adrenergic (Chambers, 1992; Chambers et al. 1995) links have been implicated with the analgesic effects of some NSAIDs. To evaluate the significance of these two systems on the hypoalgesia produced by ketoprofen, drug antagonists at these receptor systems were injected i.t. The opioid receptor antagonist naloxone and the $\alpha_2$-adrenoceptor antagonists yohimbine and atipamezole were used for these purposes.

4.4.3.2.1.1. Blockage of spinal opioid and $\alpha_2$-adrenergic receptors

Firstly, it was confirmed that the selective $\mu$-opioid receptor agonist fentanyl (Emmerson et al. 1994) and the $\alpha_2$-adrenoceptor agonist xylazine (Faber et al. 1998) produced analgesia in sheep when given i.v., as it has been reported by others (Nolan et al. 1987b; Ley et al. 1991; Waterman et al. 1990; Waterman et al. 1991; Kyles et al. 1993; Welsh & Nolan, 1994). Secondly, it was necessary to find an i.t. dose for the opioid receptor and the $\alpha_2$-adrenoceptor antagonists that prevented the rise of mechanical thresholds by fentanyl and xylazine, respectively, and that, when administered alone, had no effect on nociceptive thresholds.

Naloxone has been administered i.t. to laboratory animals in a broad range of doses, such as 0.055 mM (100 ng/ 5 µL) in mice (Akman et al. 1996), 2.75 mM (10 µg/ 10 µL) in rats (Pelissier et al. 1996), and 27.45 mM (1000 µg/ 100 µL) and 68.62 mM (2500 µg/ 100 µL) in sheep (Waterman et al. 1991; Kyles et al. 1990). Although some of these doses blocked the analgesic effects of different opioid receptor agonists without having a significant effect on control readings, Kyles et al. (1990) observed that the "low" naloxone dose used in their study (27.45 mM) increased mechanical thresholds. The onset of analgesia occurred around 50 minutes post-injection and persisted for 60 more minutes with the peak analgesic effect (325% increase from pretest values) seen at about 90 minutes after administration. Contrary to this is the lack of effect of low and high doses of systemically administered naloxone (0.4 and 10 mg, respectively) on
mechanical nociceptive thresholds of humans (Kaiser et al. 1997). Even more, excitatory effects on rat DHN (Budai & Fields, 1998) and increased secondary hyperalgesia in humans (Ilkjaer et al. 1998) have also been reported for naloxone. It seems, therefore, that paradoxical effects can be expected with this antagonist. Here, we found that a 5.49 mM dose of naloxone (200 µg/100 µL) had no effect on mechanical nociceptive thresholds of sheep for at least 70 minutes. Since analgesia was observed in sheep about 50 minutes after naloxone administration (Kyles et al. 1990), it is unlikely that the dose chosen in our study produces hypoalgesia. However, it would be interesting to test this i.t. naloxone dose for longer periods to confirm that it is void of altering nociceptive thresholds in sheep. This was not done here because the paper by Kyles and co-workers (1990) was not available to us at the time of running the experiments.

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

Naloxone inhibition of fentanyl-induced analgesia should be attributed to the blockage of spinal µ-opioid receptors since fentanyl binds to them with high affinity (Emmerson et al. 1994). Although naloxone was injected in mM concentration and this concentration was surely lower by the time the antagonist diffused in to where the receptors are, the possibility of an interaction with κ- and probably δ-opioid receptors cannot be excluded. This is due to naloxone's weak selectivity for µ-opioid receptors which is only 3 times greater
than that for κ-opioid receptors and 79 times greater than that for the δ-subtype (Emmerson et al. 1994).

Alpha₂-adrenoceptor antagonists have also been administered i.t. to laboratory animals. For example, yohimbine was used at a 76 nM (300 pg/10 μL) (Malmberg & Yaksh, 1993) and 7.66 mM concentration (30 µg/10 μL) in rats (Grabow et al. 1999) and at 2.6 mM (102 µg/100 μL) in cats (Chua et al. 1998), whereas idazoxan at a 4.15 mM concentration (100 µg/100 μL) and RX811059 at a 3.84 mM concentration (33 µg/100 μL) were used in sheep (Waterman et al. 1991; Kyles et al. 1993).

The results obtained with i.t. yohimbine were quite surprising, but the number of sheep used in the trials was too small to draw any firm conclusions. The selected 5.11 mM dose of yohimbine (200 µg/100 μL) administered i.t., dramatically increased the magnitude and duration of analgesia by i.v. xylazine (Figure 4.18). Since the yohimbine utilised here was one available for clinical use and it comes dissolved in a substance “X” (not mentioned here because is commercially sensitive information provided to us by the manufacturer in the strictest of confidence) that is known to produce analgesia when spinally injected on its own, these effects could be attributed to this substance. However, a direct effect of substance “X” on nociceptive thresholds seems unlikely since no significant change was observed when yohimbine was i.t. injected alone. This enhanced analgesic effect cannot be attributed to yohimbine blocking α₂-adrenergic receptors either, since the α₂-adrenoceptor antagonist atipamezole did not produce the same effects (this study). It is known that high concentrations of α₂-adrenoceptor agonists produce a local anaesthetic action independent of their effect on α₂-adrenoceptors (Chambers, 1993; Faber et al. 1998) and this has also been observed for some α₂-adrenoceptor antagonists (Chambers, 1993). Most α₂-adrenoceptor agonists are small molecules (MW 220-250) with a similar structure to lignocaine; however, yohimbine is quite large (MW 390) and different in structure to the local anaesthetic, so a local anaesthetic effect seems also unlikely. Molecular structure modification of yohimbine during the extraction technique utilised to separate the α₂-adrenoceptor agonist from substance “X” may also account for
this activity. Thus, by using the pure salt, yohimbine should be thoroughly evaluated to clarify the increased analgesic effect of i.v. xylazine described here.

Atipamezole was utilised instead of yohimbine in all other experiments that required blockage of \( \alpha_2 \)-adrenergic receptors. Although a local anaesthetic action has been described for atipamezole (Chambers, 1993), the i.t. injection of this antagonist in sheep at a 4.03 mM concentration (100 \( \mu \)g/100 \( \mu \)L) was void of effect on mechanical nociceptive thresholds for at least 70 minutes and, contrary to yohimbine, prevented the i.v. xylazine-induced increase in nociceptive thresholds by almost 90%. Also in sheep, the administration of the \( \alpha_2 \)-adrenoceptor antagonists idazoxan (4.15 mM) and RX811059 (3.84 mM) into the subarachnoid space reduced in about 60-65% the analgesic effect of i.v. xylazine (Waterman et al. 1991; Kyles et al. 1993). By comparing these reports with our experiments, it was found that all the antagonists were administered in very similar doses but the dose and time of administration for xylazine and the way in which the AUC was calculated were distinct. In our experiments, 10 minutes after atipamezole injection, all sheep received the same xylazine dose (20 \( \mu \)g/kg) and the AUC was evaluated for the 60 minutes after its administration. When idazoxan and RX811059 were administered, the xylazine dose was individualised for each sheep (12.5 to 41.6 \( \mu \)g/kg) and injected 30 minutes after the i.t. administration of the antagonist and the AUC was appraised for 40 minutes. Therefore, differences on methodological and analytical practices could explain these differences. Breed differences could also account for these analgesic variations (Ley et al. 1990). In this study, Romney-cross sheep were used whereas Swaledale, Swaledale cross Leicester and Clun Forest sheep were represented in the other study (Kyles et al. 1993). However, these breed differences might have been minimised by individualising the dose of xylazine.

The source of atipamezole was a commercial clinical product and, therefore, it came dissolved together with another ingredient, "Y" (not mentioned here for reasons already exposed). The sole administration of "Y" and in combination with xylazine was not performed as a measure of control and it can be argued
that the blockage or the bigger blockage in comparison to idazoxan or RX811059 (Waterman et al. 1991; Kyles et al. 1993) of xylazine-mediated analgesia may be attributed to "Y". Nonetheless, "Y" is a non-active preservative widely used in eye drops (Parfitt, 1999) and, hence, it is unlikely that the antagonistic effects observed with the administration of atipamezole would have been mediated by "Y".

It is possible that atipamezole, apart from acting on $\alpha_2$-adrenergic receptors may have also blocked non-adrenoceptor imidazoline receptors (Sjöhollm et al. 1996), which are localised in the substantia gelatinosa of the spinal cord and other sensory processing centres (Ruggiero et al. 1998). Binding to these sites by idazoxan is also documented (Mallard et al. 1991; Ruggiero et al. 1998) but RX811059 seems to have a low affinity for them (Mallard et al. 1991). Kyles et al. (1993) proposed that the reduction of i.v. xylazine-induced analgesia in sheep by i.t. idazoxan was due to the blockage of $\alpha_2$-adrenoceptors but not non-adrenoceptor imidazoline receptors, since similar reduction was observed with the i.t. injection of RX811059. It would be interesting to compare the effect of atipamezole and RX811059 under the conditions here studied and, thus determine the impact of imidazoline receptor blockage on the attenuation of xylazine-mediated analgesia by atipamezole. However, it is reasonable to anticipate that a similar reduction by both antagonists would be observed because the spinal depressant actions of $\alpha_2$-adrenoceptor agonists do not involve imidazoline receptors (Kyles et al. 1993; Faber et al. 1998). It seems, therefore, that the differences in the reduction of xylazine-induced antinociception by atipamezole (this study) and idazoxan and RX811059 (Kyles et al. 1993) are due to the methodological approaches discussed above.

Precipitation out of solution occurred when the antagonists' pH was adjusted to 7; therefore, they were administered with a slightly acid pH. Because no control experiments with an acid solution injected into the subarachnoid space were carried out, it is possible to argue that the pH of the solutions containing the antagonists used in this study could have had an effect on mechanical nociceptive thresholds of sheep. However, it has been observed that the i.t. injection of a citric acid solution with pH as low as 4.4 produced no evidence of
discomfort and did not affect mechanical nociceptive thresholds in sheep (Kyles et al. 1995). Hence, it seems unlikely that the effects produced by naloxone, yohimbine and atipamezole were due to a merely pH effect.

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

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

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

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

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

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

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

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

Support for the involvement of adrenergic mechanisms in NSAIDs-mediated analgesia come from the blockage of i.v. dipyrone- and flunixin-induced increase of mechanical nociceptive thresholds in sheep by the \(\alpha_2\)-adrenoceptor antagonist atipamezole injected i.v. as well (Chambers, 1992; Chambers et al. 1995). Interestingly, NSAIDs-mediated effects other than analgesia seem to also involve adrenergic mechanisms. Gastrointestinal motor disturbances after lysine-acetylsalicylic acid treatment in sheep were abolished by injecting i.v. the \(\alpha\)-adrenoceptor antagonists phentolamine and tolazoline (Hondre & Bueno, 1984). Thus, it seems that some therapeutic and side effects of the NSAIDs could be attributed to an increase in noradrenaline release. Determination of this neurotransmitter in the sheep's CSF was not carried out either in this experiment; however, its measurement before and after the administration of ketoprofen may serve to give a better understanding of the mode of analgesic action of this NSAID.

The \(\alpha_2\)-adrenoceptor subtype (\(\alpha_{2A}\), \(\alpha_{2B}\), and \(\alpha_{2C}\)) involved in the analgesic action here described was not investigated; however, the \(\alpha_{2A}\)-adrenoceptor subtype is a major candidate since it has been shown to mediate antinociception (Millan et al. 1994). In addition, the possibility that ketoprofen
Sheep implanted with i.t. catheters

had induced an increased release of an endogenous substance that by acting on imidazoline receptors had produced analgesia cannot be excluded since atipamezole, the $\alpha_2$-adrenoceptor antagonist used in this study, has some affinity for these receptors (Sjöholm et al. 1996) and their activation at the spinal level has been implicated with antinociceptive responses (Diaz et al. 1997). It would be interesting to test this hypothesis in sheep by injecting i.t. an imidazoline receptor antagonist [e.g. BU-224 (Diaz et al. 1997)] followed by i.v. ketoprofen.

The mechanisms by which NSAIDs increase the discharge rate of neurones in the PAG (Carlsson et al. 1986; Carlsson & Jurna, 1987) and, thus, supposedly increase the release of endogenous antinociceptive substances are unknown. However, it has recently been suggested that $\mu$-opioid receptor activation in GABAergic presynaptic terminals of the PAG reduces the probability of neurotransmitter release. It uses a mechanism that involves a voltage-dependent $K^+$ conductance via a PLA$_2$/AA/12LOX pathway (Vaughan et al. 1997), with inhibition of COX-1, more than that of COX-2, accounting more for this action (Vaughan, 1998). Thus, by inhibiting COX enzymes in GABAergic presynaptic terminals in the PAG, NSAIDs may make AA more available for conversion to 12-LOX products and these may contribute to hyperpolarise these neurones and reduce GABA release, rendering neurones in the synapse more susceptible to firing and releasing endogenous antinociceptive agents. In fact, microinjection of the GABA$_A$ receptor antagonist bicuculine into the PAG inhibited cutaneous noxious heat-induced responses in spinal cord DHN by releasing endogenous substances that activated $\mu$-opioid (Budai & Fields, 1998) and $\alpha_2$-adrenergic (Budai et al. 1998) receptors on these spinal neurones.

The binding of ketoprofen to opioid and $\alpha_2$-adrenergic receptors was not studied. However, the possibility that this drug had directly interacted with these receptors to produce analgesia seems unlikely. Receptor binding studies have shown no affinity of paracetamol for adrenergic ($\alpha_1$, $\alpha_2$ and $\beta$), opioid ($\mu$, $\delta$ and $\kappa$), muscarinic, adenosine (A1 and A2), dopamine (D1 and D2), 5-HT (5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{2A}$, 5-HT$_{2C}$ and 5-HT$_3$) histamine (H1), benzodiazepine, GABA$_A$, NMDA and NK$_1$ receptors (Pelissier et al. 1996). Flunixin did bind to $\alpha_2$-
Sheep implanted with i.t. catheters

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

Despite all above evidence implicating opioidergic and adrenergic mechanisms in the antinociceptive action of NSAIDs, some reports do not support this theory. For instance, the antinociception observed in the acetic acid abdominal constriction test in mice following s.c. acetylsalicylic acid injection was not reversed by the opioid receptor antagonists naloxone and naltridone nor the enkephalin catabolism inhibitor acetorphan (Gray et al. 1998; Gray et al. 1999) or by the \( \alpha_2 \)-adrenoceptor antagonist RX821002 (Gray et al. 1999), all administered s.c. Because some inflammation may have occurred in this assay, peripheral COX inhibition could be argued as the mechanism of analgesic action in this case. However, in a rat paw pressure test without induction of inflammation, i.v. paracetamol-induced analgesia was not inhibited by i.t. naloxone or i.v yohimbine (Pelissier et al. 1996), which may suggest that not all NSAIDs share the same analgesic mechanisms of action or that these mechanisms may be influenced by the employed nociceptive test, or both.

The possibility that ketoprofen had activated other descending inhibitory mechanism to produce analgesia in sheep cannot be excluded since serotoninergic and cholinergic pathways have also been implicated in NSAIDs-induced analgesia. On the one hand, the i.t. injection of the 5-HT\(_3\) receptor antagonist tropisetron inhibited the antinociceptive action of paracetamol administered systemically and i.t. in rats submitted to a paw pressure test (Pelissier et al. 1996) and the analgesic effect of diclofenac in rats was also attributed to enhancement of descending 5-HT neuronal activity (Björkman et al. 1996). On the other hand, the muscarinic cholinergic blocking agent atropine reversed the analgesic effect of a pyrazolone derivative NSAID as it was assessed in mice by a hot-plate test (Stets & Slivko, 1989). It would be interesting to investigate if these two descending inhibitory mechanisms take part in the antinociceptive action of ketoprofen, even though serotoninergic mechanisms may only be relevant in rats, since large inter-species differences exist in central distribution, pharmacology and allosteric modulation of 5-HT\(_3\) receptors (Fletcher & Barnes, 1998). Other substances that are released by
descending inhibitory mechanisms to modulate nociceptive transmission in the spinal cord include dopamine (Barasi et al. 1987), adenosine (Zhao et al. 1999), GABA and glycine (Xu et al. 1998; Nicoll & Malenka, 1998). However, whether these agents contribute to the analgesic action of NSAIDs remains to be elucidated.

However, there are some reports not supporting an antinociceptive supraspinal site of action for the NSAIDs. For instance, failure to modify rat behavioural responses to a paw PGE$_2$-induced hyperalgesia test (Lorenzetti & Ferreira, 1996) and to depress a C fibre-mediated reflex in anaesthetised rats (Bustamante et al. 1997) was observed after i.c.v. administration of NSAIDs. This may indicate that NSAIDs have no supraspinal analgesic effect on these nociceptive models or that not enough drug diffusion into the PAG, or other specific structure where these drugs exert their supraspinal analgesic action (Tortorici et al. 1996), was achieved. Although some drug may have reached supraspinal structures after the i.t. cumulative administration of NSAIDs in the current study (see above), no analgesia was recorded. However, it is difficult to know the amount of drug that spread into brain structures; therefore, a set of experiments in which known concentrations of ketoprofen are injected through the i.c.v. route in sheep would serve to further confirm a supraspinal site of action for this drug.

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

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

In conclusion, our results showed that although the NSAIDs did not have a direct effect on the spinal cord, their analgesic action appeared to be spinally mediated, probably by activating inhibitory descending opioidergic and adrenergic mechanisms. This raises the possibility of exploring drug combinations of NSAIDs and other agents which act on these spinal neurones with the hope of finding better ways to control pain.
Both in vitro and in vivo experiments were designed to evaluate the central analgesic effects of NSAIDs. For unknown reasons, it was not possible to properly set up the neonatal rat hemisected spinal cord preparation and therefore, experiments using this technique were not carried out.

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

The NSAIDs used in this study were devoid of effect on mechanical nociceptive thresholds of sheep when given i.t. However, i.v. ketoprofen and tolfenamic acid, but not phenylbutazone nor salicylic acid, raised the thresholds. From these results it could be inferred that the nociceptive test employed here is adequate to evaluate the analgesic actions of NSAIDs, since ketoprofen and tolfenamic acid seem to be clinically more potent analgesics than phenylbutazone or salicylic acid. Discrepancies in the analgesic action of NSAIDs have also been reported in humans, with paracetamol producing analgesia but not acetylsalicylic acid (Piletta et al. 1991). Hence, our findings confirm that different NSAIDs have different analgesic potency. They also support the antinociceptive action of these drugs in the absence of inflammation.
(Chambers et al. 1993; Chambers et al. 1995; Pelissier et al. 1996; Ochi et al. 1999).

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

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

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