Characterization of opioid binding sites in spinal cord and other tissues

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CHARACTERIZATION OF OPIOID BINDING SITES IN SPINAL CORD AND OTHER TISSUES

by

Malcolm Stuart Wood

A Doctoral Thesis submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy of the Loughborough University of Technology

May 1988

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I am indebted to the following:

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Last but not least, my friends at The Send Forth Church, Herbert Street, Loughborough for their prayer and financial support over the past years.

Thank you Lord.
Dedication

To my father who never saw the completion of this work, my mother and my wife Viv.
Characterization of Opioid Binding Sites in Spinal Cord and other Tissues.

Wood, M.S.

Keywords: Spinal Cord; Analgesia; Endogenous opioid peptides; k-Opioid receptor; k-Subtypes; Dorsal/ventral horn; Rostro-caudal distribution; Opioid binding sites; [\textsuperscript{\textit{H}}]Opioids.

The binding of [\textsuperscript{\textit{H}}]opioid ligands to homogenates prepared from the spinal cords of rat and other species has been studied. Similar numbers of sites were seen in all areas of the cord when measured in a rostro-caudal direction. There was found to be approximately 2 x higher density of sites in the dorsal half of the cord compared with the ventral half. Binding studies suggested a similar relative distribution of mu, delta and kappa sites in all areas of the cord. The results are discussed in relation to the reported distribution of opioid peptides.

In the above study the kappa binding site was defined as the binding of [\textsuperscript{\textit{H}}]unselective opioids in the presence of cold ligands to suppress binding to mu- and delta-sites. Competitive binding assays, however, suggested this site did not have the properties of a single homogeneous group. Approximately 50% of the apparent kappa binding was consistent with a classical kappa site. Saturated binding assays afforded Bmax values which suggested lower 'true' kappa site numbers than previously supposed, values which were confirmed using the kappa peptide [\textsuperscript{\textit{H}}]Dynorphin A-(1-9), and the kappa selective [\textsuperscript{\textit{H}}]U-69593. Heterogeneity was also seen in other central nervous system tissues.

The heterogeneous nature of the kappa site may be due to different sites, due to interactions at a non-opioid site or may represent different conformations of the same site. The second possibility was discounted since observed binding followed the cellular distribution of the plasma marker Na\textsuperscript{+}/K\textsuperscript{+}-ATPase, was stereoselective for levorphanol over dextorphlan, and fully displaceable by naloxone. The third possibility was investigated by studying the role of Na\textsuperscript{+} and Mg\textsuperscript{2+} ions, which are reported to affect receptor conformation in binding assays employing brain tissues. None of the results obtained suggested that conformational changes were responsible for the observed effects, although the experiments were not exhaustive.
## Abbreviations used in this thesis

<table>
<thead>
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<tr>
<td>Aib</td>
<td>α-Aminoisobutyric acid</td>
</tr>
<tr>
<td>2',3'-CNP</td>
<td>2',3'-Cyclic nucleotide 3'-phosphohydrolase</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-Aminobutyric acid</td>
</tr>
<tr>
<td>GppNHp</td>
<td>5-Guanylylimidodiphosphate</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-Hydroxyethylpiperazine-N-ethanesulphonic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>MAO</td>
<td>Monoamine oxidase</td>
</tr>
<tr>
<td>Na⁺,K⁺-ATPase</td>
<td>Na⁺,K⁺-activated adenosine 5'-triphosphate phosphohydrolase</td>
</tr>
<tr>
<td>NRM</td>
<td>Nucleus raphe magnus</td>
</tr>
<tr>
<td>PAG</td>
<td>Periaqueductal grey</td>
</tr>
<tr>
<td>Rpgl</td>
<td>Nucleus reticularis paragigantocellularis lateralis</td>
</tr>
<tr>
<td>RVM</td>
<td>Rostro ventral medulla</td>
</tr>
<tr>
<td>sem</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
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CHAPTER 1

INTRODUCTION
Historical

The pharmacological properties of the exudate from the unripe seed capsules of the opium poppy, *Papaver somniferum* have been recognized since 4000 B.C. The analgesic properties were later realized and morphine (Figure 1.1) the alkaloid most active in this respect, was isolated in the early 19th century and structurally resolved in 1925 [1].

![Figure 1.1 Morphine](image)

In addition to pain relief however, morphine also produces decreased gastrointestinal motility, hypotension and respiratory depression, but more importantly tolerance and dependence after prolonged use. This prompted the search for a non-addictive analgesic without these unwanted side-effects. Early attempts included diacetylmorphine (heroin; Figure 1.2) which only led to an increase in the incidence of drug abuse.

![Figure 1.2 Heroin](image)
Figure 1.3 Pethidine

Figure 1.4 Methadone

Figure 1.5 Etorphine

Figure 1.6 Buprenorphine
Later compounds included structural modifications of and diversifications from the central morphine nucleus in the search for the ideal analgesic. That compound has not yet been discovered but some of the drugs synthesized during this search have been used in medicine, for example pethidine (Figure 1.3) and methadone (Figure 1.4), and have also found use in the treatment of drug addiction, particularly methadone. Some members of the very potent oripavines [2], most notably etorphine (Figure 1.5) are used in veterinary science to immobilize hoofed wild animals such as rhinoceros, elephant or zebra [3, 4] and more recently buprenorphine (Figure 1.6) has been introduced into clinical medicine for the relief of post-operative pain [5] and chronic intractable pain in cancer patients [6].

Discovery of the endogenous opioids

Arising from these observations was the fundamental question of why receptors should exist in the body for externally applied compounds. Martin [14] proposed that 

'morphine-like compounds might mimic an ongoing biochemical process'

whilst Collier [23] postulated the existence of an 'endogenous neurochemical factor'.

Experimental evidence for these hypotheses came when analgesia produced by electrical stimulation of the periaqueductal grey in the brain stem of rats was found to be reversed by the antagonist naloxone (Figure 1.7) [24], antagonism by low levels of naloxone, that is in the region of 1 mg.kg⁻¹, being taken as evidence for opioid receptor involvement.
In 1975 Kosterlitz and Hughes, along with several other groups, isolated from brain and pituitary substances possessing opioid-like activity [25 - 28]. The name enkephalin [29] was given to the endogenous substance isolated from porcine brain. This was shown to consist of two pentapeptides, differing only in their carboxy-terminal amino acid residue which was either leucine (named leucine-enkephalin) or methionine (named methionine-enkephalin) (Figure 1.8) [30]. These are now represented, according to accepted nomenclature as [Leu⁵]- and [Met⁵]enkephalin respectively. Several carboxy-terminal extensions of the [Met⁵]enkephalin sequence were isolated over the next eight years, as shown within Tables 1.1a and b.

One of the earliest extended [Met⁵]enkephalins to be isolated was the peptide β-endorphin. β-Lipotropin [38], a large peptide whose function was unknown at its time of discovery, was shown to contain [Met⁵]enkephalin as amino acid residues 61 - 65 [30]. Mild trypsin digestion of β-lipotropin released the C-fragment, that is residues 61 - 91 [39], which was also found to exist in porcine pituitary [40], and was shown to have opioid activity more than 30 times that of [Met⁵]enkephalin [41]. The β-lipotropin molecule was itself devoid of opioid-like activity suggesting the need for cleavage at specific points in the molecule to produce smaller, opioid-active units. The name β-endorphin is now generally used in place of "C-fragment" to describe residues 61 - 91 of β-lipotropin [42]. It has become apparent from the relative distributions in the central nervous system, as determined by immunohistochemical methods, that β-endorphin is not the main source of [Met⁵]enkephalin [43].

A further group of endogenous opioid peptides were discovered by Goldstein and colleagues [49]. These compounds showed extremely high potency in the guinea-pig ileum bioassay. Initially a 13-amino acid peptide was identified, named dynorphin A-(1-13), but soon other members of this carboxy-terminally extended [Leu⁵]enkephalin series were isolated including the large peptide dynorphin (1-32) [50 - 59] (Table 1.1c).
Figure 1.8  The enkephalins

When R =

Leucine-enkephalin ([Leu<sup>5</sup>]enkephalin)

Methionine-enkephalin ([Met<sup>5</sup>]enkephalin)
Table 1.1a Pre-proenkephalin products

<table>
<thead>
<tr>
<th>Opioid Peptide</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Met&lt;sup&gt;8&lt;/sup&gt;]enkephalin</td>
<td>Hughes et al [30]</td>
</tr>
<tr>
<td>[Leu&lt;sup&gt;8&lt;/sup&gt;]enkephalin</td>
<td></td>
</tr>
<tr>
<td>[Met&lt;sup&gt;8&lt;/sup&gt;]enkephalyl-Arg&lt;sup&gt;8&lt;/sup&gt;</td>
<td>Huang et al [31]</td>
</tr>
<tr>
<td>[Met&lt;sup&gt;8&lt;/sup&gt;]enkephalyl-Arg&lt;sup&gt;8&lt;/sup&gt;-Phe&lt;sup&gt;8&lt;/sup&gt;</td>
<td>Stern et al [32]</td>
</tr>
<tr>
<td>[Met&lt;sup&gt;8&lt;/sup&gt;]enkephalyl-Arg&lt;sup&gt;8&lt;/sup&gt;-Arg&lt;sup&gt;8&lt;/sup&gt;</td>
<td>Lewis et al [33], Stern et al [34]</td>
</tr>
<tr>
<td>[Met&lt;sup&gt;8&lt;/sup&gt;]enkephalyl-Arg&lt;sup&gt;8&lt;/sup&gt;-Gly&lt;sup&gt;8&lt;/sup&gt;-Leu&lt;sup&gt;8&lt;/sup&gt;</td>
<td>Kilpatrick et al [35]</td>
</tr>
<tr>
<td>[Met&lt;sup&gt;8&lt;/sup&gt;]enkephalyl-Lys&lt;sup&gt;8&lt;/sup&gt;</td>
<td>Lewis et al [33], Stern et al [34]</td>
</tr>
<tr>
<td>[Met&lt;sup&gt;8&lt;/sup&gt;]enkephalyl-Arg&lt;sup&gt;8&lt;/sup&gt;-Arg&lt;sup&gt;8&lt;/sup&gt;-Val&lt;sup&gt;8&lt;/sup&gt;-NH&lt;sub&gt;2&lt;/sub&gt; (Metorphamide)</td>
<td>Weber et al [36]</td>
</tr>
<tr>
<td>Peptide I Ser-Pro-Thr-Leu-Glu-Asp-Glu-His-Lys-Glu-Leu-Gln-Lys-Arg&lt;sup&gt;13&lt;/sup&gt;Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro&lt;sup&gt;22&lt;/sup&gt;Glu-Trp-Trp-Met-Asp-Tyr-Gln-Lys&lt;sup&gt;24&lt;/sup&gt;Arg-Tyr&lt;sup&gt;-16&lt;/sup&gt;Gly-Gly-Phe&lt;sup&gt;-19&lt;/sup&gt;Leu</td>
<td>Jones et al [45]</td>
</tr>
<tr>
<td>Peptide E Peptide I (15-39)</td>
<td>Kilpatrick et al [46]</td>
</tr>
<tr>
<td>BAM-12P Peptide I (15-26)</td>
<td>Mizuno et al [47]</td>
</tr>
<tr>
<td>BAM-20P Peptide I (15-34)</td>
<td>Mizuno et al [48]</td>
</tr>
<tr>
<td>BAM-22P Peptide I (15-36)</td>
<td>Mizuno et al [48]</td>
</tr>
<tr>
<td>Fragment</td>
<td>Amino Acid Residues of β-Lipotropin</td>
</tr>
<tr>
<td>---------------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>C-fragment/α-endorphin</td>
<td>61-91</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>C'-fragment</td>
<td>61-87</td>
</tr>
<tr>
<td>γ-endorphin</td>
<td>61-77</td>
</tr>
<tr>
<td>α-endorphin</td>
<td>61-76</td>
</tr>
</tbody>
</table>

β-endorphin is:

\[ ^{1} \text{ Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-} \]
\[ ^{2} \text{ Pro-Leu-Val} \]
\[ ^{3} \text{ Thr} \]
\[ ^{4} \text{ Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys Asn-Ala} \]
\[ ^{5} \text{ His-Lys-Lys-Gly} \]
\[ ^{6} \text{ Gln} \]
<table>
<thead>
<tr>
<th>Opioid Peptide</th>
<th>Amino Acid Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Leu&lt;sup&gt;5&lt;/sup&gt;]enkephalin</td>
<td></td>
<td>Lewis et al [33], Stern et al [34]</td>
</tr>
<tr>
<td>α-neo-endorphin</td>
<td>Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro</td>
<td>Minamino et al [51]</td>
</tr>
<tr>
<td>α-neo-endorphin</td>
<td>Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys</td>
<td>Kangawa et al [52]</td>
</tr>
<tr>
<td>Dynorphin A-(1-17)</td>
<td>Sequence 1-17</td>
<td>Goldstein et al [49,50]</td>
</tr>
<tr>
<td>Dynorphin B</td>
<td>Sequence 20-32</td>
<td>Fischli et al [54], Kilpatrick et al [55,56]</td>
</tr>
<tr>
<td>Dynorphin A-(1-8)</td>
<td>Sequence 1-8</td>
<td>Minamino et al [57]</td>
</tr>
<tr>
<td>Leumorphin</td>
<td>Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr-Arg-Ser-Gln-Glu-Asp-Pro-Asn-Ala-Tyr-Tyr-Glu-Glu-Leu-Phe-Asp-Val</td>
<td>Kakidani et al [58], Suda et al [59]</td>
</tr>
<tr>
<td>Opioid Peptide</td>
<td>Amino Acid Sequence</td>
<td>Reference</td>
</tr>
<tr>
<td>---------------</td>
<td>-----------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>Kyotorphin</td>
<td>Tyr-Arg</td>
<td>Takagi et al [60]</td>
</tr>
<tr>
<td>Dermorphin</td>
<td>Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH₂</td>
<td>Montecucchi et al [61]</td>
</tr>
</tbody>
</table>

**Exogenous Opioids**

<table>
<thead>
<tr>
<th>Opioid Peptide</th>
<th>Amino Acid Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta$-casomorphin</td>
<td>Tyr-Pro-Phe-Pro-Gly-Pro-Ile</td>
<td>Henschen et al [62]</td>
</tr>
<tr>
<td>Morphiceptin</td>
<td>Tyr-Pro-Phe-Pro-NH₂</td>
<td>Chang et al [63]</td>
</tr>
</tbody>
</table>
Research into the origins of these different opioid peptides showed that three large precursors, pre-proenkephalin A [64], pre-proopiomelanocortin [65] and pre-prodynorphin (pre-proenkephalin B) [64] were the materials from which the enkephalins, β-endorphin and the dynorphins were released. After transport across the membranes of the endoplasmic reticulum, cleavage of a signal peptide yields the pro-peptide which, upon further cleavage at basic amino acid pairs such as Lys-Arg by processing enzymes yields the opioid peptides [64].

Other peptide molecules with opioid activity have also been isolated, although some of these are exogenous, originating from pepsin hydrolysates of food proteins (Table 1.1d).

Studies employing opioid peptides in both in vivo and in vitro systems have been hampered by the ready degradation of these compounds, resulting in a reversal of effect [26]. This problem has been partly overcome using two approaches. In the enkephalin series, substitution of the glycine residue at position 2 by D-alanine [73] and leucine or methionine at position 5 by their respective D-isomer [74] produced an increase in potency but no change in receptor selectivity [73, 74]. However, in the dynorphin series, the position 2 glycine appears to be important for receptor selectivity [69]. An alternative approach is the use of specific enzyme inhibitors to reduce proteolysis. This has been shown to be successful with both enkephalin [75] and dynorphin [72] peptides.

**Opioid receptors**

Chemical manipulations of the morphine molecule produced compounds such as nalorphine [7] (Figure 1.9) and naloxone [8] which antagonized the effects of morphine [9, 10]. Nalorphine, in spite of its antagonist properties also produced analgesia [11] in addition to tolerance and dependence [12], although the abstinence syndrome differed from that in morphine-dependent subjects [12, 13].
Martin [14] considered these effects and proposed that morphine- and nalorphine- like drugs were acting via different receptor systems. This hypothesis supported earlier suggestions by Portoghese [15] which were based upon the structural and stereochemical requirements of the opioid alkaloids for their biological activity outlined by Beckett and Casey [16].

Martin and colleagues [17 - 20] later described three different receptor classes following work on the naive- and opioid-dependent chronic spinal dog preparation. Differences in i) behaviour, ii) sensitivity to naloxone and naltrexone antagonism (see Figure 1.10 for the structure of naltrexone) and iii) cross-tolerance selectivity (as evidenced by the ability or inability to suppress abstinence in dependent animals caused by the agonists morphine, ketocyclazocine (Figure 1.11) and SKF 10047 (Figure 1.12), were ascribed to actions at three receptor types termed μ (morphine), k (ketocyclazocine) and σ (SKF 10047).
Additional evidence for the existence of separate μ- and κ-receptors came from in vitro work on isolated tissue preparations such as the guinea-pig ileum longitudinal muscle and mouse vas deferens, developed by Kosterlitz and colleagues. These workers found that some benzomorphanes including ketocyclazocine, which did not substitute for morphine in the morphine-dependant monkey [21] but were good analgesics in the rat, had only one quarter of the agonist potency in the mouse vas deferens preparation as in the guinea-pig ileum longitudinal muscle preparation, when compared to normorphine (Figure 1.13).
These benzomorphans also required between three and seven times more naloxone to antagonize their effects than did normorphine [22]. The rank order of potency for opioid agonists to inhibit the electrically evoked twitch in the two preparations was also different, indicative of different receptor types. However, the actions of the newly discovered opioid peptides, namely the enkephalins in the myenteric plexus of the guinea-pig ileum longitudinal muscle and the mouse vas deferens could not be explained solely by interaction at the putative μ-, κ- or δ-receptors as defined by Martin. For example, [Met⁸]enkephalin was 20 times more potent than normorphine at inhibiting the electrically evoked twitch in the mouse vas deferens, whilst the two compounds were equipotent in the guinea-pig ileum preparation [30].

In addition it was shown that antagonism of the inhibitory action of the enkephalins in the mouse vas deferens required 10 times more naloxone than did morphine, but in the guinea-pig ileum the amount of naloxone needed was the same as for morphine [66]. Kosterlitz and co-workers proposed two types of receptor for opioid peptides; the μ-receptor with which morphine preferentially interacts and which predominates in the guinea-pig ileum and the enkephalin-preferring δ-receptor, found to predominate in the mouse vas deferens. Also present in the guinea-pig ileum is the k-receptor for which the benzomorphan drugs show selectivity [66, 67]. Further studies have shown the mouse vas deferens to contain μ-, δ- and k-receptors [137]. Extension of the above experimental rationale and the use of the rabbit vas deferens, a tissue containing exclusively functional k-receptors [68], demonstrated the activity of some opioid peptides, especially those derived from prodynorphin, at the k-receptor [49, 50, 54, 69 - 72].

This work on isolated tissues was complemented by binding studies. The interaction of an opioid compound with the recognition site of a receptor, produces a translation into biochemical events by an intermediate factor, which leads ultimately to the observed biological responses. Binding assays are concerned with the investigation of these recognition sites.
Early work was unsuccessful in demonstrating specific binding sites for radiolabelled opiates mainly due to the low specific activity and high concentrations of radioligands available. Goldstein and colleagues [76] showed stereospecific binding of radiolabelled levorphanol (Figure 1.14), but this only represented 2% of the total binding to mouse brain homogenates. Later the use of nanomolar concentrations of opiates labelled to high specific activity afforded high specific binding and enabled a more detailed study of opiate binding sites and their properties [77 - 81].

Stereospecific, saturable binding was shown to be reduced by proteolytic enzymes and sulphydryl reagents such as N-ethylmaleimide (Figure 1.15) indicating the recognition site to be proteinaceous with important sulphydryl groups in close proximity [77].
Temperature and pH also influence opiate binding [78]. Subcellular fractionation of brain tissues showed the binding sites to be concentrated in the synaptosomal fraction [81, 84] suggesting that they are located on nerve terminals. This binding could be displaced from brain and intestine homogenates by unlabelled opiates and opioids, with potencies which in many cases were close to those found in vivo for the same compounds [77 - 80].

Importantly it has been found possible to discriminate between agonists and antagonists based upon the influence of monovalent cations such as sodium [77, 78, 83]. Agonists are less potent at displacing \(^{[3H]}\) agonist binding in the presence of 100 mM sodium chloride. This is known as the "sodium shift". However, antagonists such as naloxone are equipotent or more potent under these same conditions [83].

Opioid compounds with a high potency in the isolated guinea-pig ileum preparation were found to be better displacers of \(^{[3H]}\) naloxone, whereas compounds showing higher activity in the mouse vas deferens preparation are better displacers of \(^{[3H]}\)[Leu\(^{5}\)] enkephalin, in guinea-pig brain homogenates [66, 67]. \(^{[3H]}\) Naloxone was therefore proposed to be binding at \(\mu\)-sites and \(^{[3H]}\)[Leu\(^{5}\)] enkaphalin to \(\delta\)-sites in guinea-pig brain. The existence of \(\mu\)- and \(\delta\)-sites has also been confirmed in rat brain [87] by studying the displacement of \(^{[3H]}\) naloxone, \(^{[3H]}\) dihydromorphine and \(^{[125I]}\) [D-Ala\(^{2}\), D-Leu\(^{5}\)] enkephalin by unlabelled opiates and opioid peptides.

The structural modification of the enkephalin nucleus has also been used to improve \(\mu\)- or \(\delta\)-selectivity [74, 89]. For example, the amidation or reduction to hydroxyl of the C-terminal carboxyl group in [Met\(^{5}\)] enkephalin produces an increase in \(\mu\)-selectivity [67, 74]. More extensive modifications have been made, most notably Tyr-D-Ala-Gly-MePhe-Gly-ol which has 220 times greater activity at \(\mu\)- than at \(\delta\)-sites [90, 91], the highly \(\delta\)-selective bis-penicillamine derivatives, including [D-Pen\(^{2}\), D-Pen\(^{2}\)] enkephalin (Figure 1.16) [92], and [D-Thr\(^{2}\), L-Leu\(^{5}\)] enkephalyl-Thr\(^{8}\), 'deltakephalin' (DTLET) [93].
Thus, selective modifications to the enkephalin structure in particular, have been used to produce compounds with an increased resistance to proteolysis and compounds showing an increased selectivity at different opioid binding sites.

The availability of radiolabelled ethylketocyclazocine, a putative k-agonist in the behavioural studies of Martin enabled the demonstration of k-binding sites in the brains of guinea-pig [94 - 96] and rat [97, 98]. Other radiolabelled k-compounds such as bremazocine (Figure 1.17) [101] were shown to have a high affinity for this k-site, but as with ethylketocyclazocine, demonstrated a high degree of cross reactivity with μ- and δ-sites [94 - 99].
By using appropriate concentrations of unlabelled compounds, such as morphiceptin or \([\text{D-Ala}^2,\text{MePhe}^\beta,\text{Gly-ol}^\delta]\)enkaphalin with \([\text{D-Ala}^2,\text{D-Leu}^\delta]\)enkaphalin to suppress the binding at \(\mu\)- and \(\delta\)-sites respectively, more specific study of the \(k\)-site was possible [96 - 98]. Endogenous peptides known to possess \(k\)-selectivity [69 - 72, 102 - 105] were used to study \(k\)-binding sites but here the inhibition of proteolytic enzymes was necessary [72, 104]. Compounds have been synthesized in an attempt to improve \(k\)-specificity. Two compounds which have improved \(k\)-selectivity are tifluadom (Figure 1.18) [106], a benzodiazepine derivative and U-50488H (trans-3,4-dichloro-N-methyl-N-[2-(pyrrolidinyl)cyclohexyl]-benzeneacetamide; Figure 1.19) [107, 109]. Both are interesting since they depart from the opiate or enkephalin nucleus.

![Figure 1.18 Tifluadom](image)

![Figure 1.19 U-50488H](image)
Some compounds which in vivo were dysphoric and psychomimetic [17] apparently bound to a site which was 'inaccessible' to compounds such as naloxone or etorphine [110 - 112], but could be displaced by (+)-SKF 10047 and non-opioids, including haloperidol (Figure 1.20) [111, 112]. Similarly phencyclidine (Figure 1.21) also bound to a naloxone inaccessible site in rat spinal cord [112].

The above observations, combined with the low affinity of opioids for these 'inaccessible' sites imply that they should not be considered as classically opioid but may correspond to the recognition site of the $\sigma$-receptor proposed by Martin [17, 113].
**The distribution of opioid peptides and binding sites in the central nervous system**

i) **General**

Early work showed a non-homogeneous distribution of opioid binding sites in the central nervous system of the rat, monkey and in man. High levels of binding were found in the striatum, lowest levels were in the cerebellum and significant amounts were present in the spinal cord [79, 114]. The subsequent development of immunology-based techniques [117] and autoradiography [115, 116, 136] enabled detailed distribution analysis of the endogenous opioid peptides and their binding sites. Although widely distributed throughout the central nervous system, both opioid peptides and opioid binding sites have been seen most often in association with sensory, limbic or neuroendocrine function.

Focus will be on the sensory system and a proposed model for endogenous pain control.

ii) **The sensory system**

The brain and spinal cord may be divided into specific cytoarchitectural regions as shown in Figures 1.22 and 1.23.

Opioid binding sites are concentrated in three regions of the central nervous system associated with sensory function; i) the midbrain periaqueductal grey (PAG), ii) several nuclei of the rostral ventral medulla (RVM), specifically the midline nucleus raphe magnus (NRM) and in the adjacent reticular nuclei and iii) the dorsal horn of both the spinal cord and the trigeminal (Vth) nerve, particularly the marginal cells of lamina I and the substantia gelatinosa of lamina II [114, 115, 119]. Small diameter primary afferents, comprising unmyelinated C-fibres and thinly myelinated Aδ-fibres which project to the cord and trigeminal nucleus also contain significant levels of opioid binding sites [114, 115, 119].
Figure 1.22  Brain of the rat

KEY:  
1  Rhombencephalon  
2  Cerebellum  
3  Mesencephalon  
4  Thalamus  
5  Hypothalamus  
6  Striatum  
7  Hippocampus  
8  Neocortex  
9  Olfactory peduncle  
10  Olfactory bulb  

(from Nauta & Feirtag [88])
Figure 1.23 The laminae of Rexed, L7-Cat spinal cord
(from Rexed [135])
The spinal and supraspinal regions are linked by complex circuitry to form the major components of the endogenous pain control system.

Within this system, the opioid peptides have distinct anatomical distributions depending upon the precursor molecule from which they are cleaved. For example, β-endorphin is concentrated in the basal hypothalamus and has axons which project caudally towards the PAG [121]. Immunoreactive prodynorphin- and proenkephalin-derived peptides are found in the PAG, dynorphin being more ventrally distributed. In the spinal cord and trigeminal nucleus, enkephalins are concentrated in the marginal cells of lamina I and in lamina V whilst dynorphins are concentrated almost exclusively in the marginal layer of lamina I [122, 123].

Other proposed neuromodulators have been identified in areas associated with nociceptive processing. 5-Hydroxytryptamine has been demonstrated within neurones of the NRM, pallidus and the adjacent nucleus reticularis paragigantocellularis lateralis (Rpgl) and the spinal cord.

Substance P (Figure 1.24) has been demonstrated in the PAG, rostral medulla (where it is also co-localized with enkephalins [134]) and spinal cord. The highest concentrations in the spinal cord are localized in lamina II.

Noradrenaline-containing axons are found in the rostral medulla and neurotensin (Figure 1.25) has been shown in axons which project from the PAG to the RVM [131].

Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂

**Figure 1.24** Substance P

pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu

**Figure 1.25** Neurotensin
The role of the spinal cord in endogenous pain control

A potential model, based upon physiological and biochemical evidence, for the role of these peptides in endogenous pain control is shown in Figure 1.26.

The modulation of nociceptive information is believed to involve spinal and supraspinal regions. At the spinal level, substance P released from primary afferents in the dorsal horn, stimulates post-synaptic receptors on second-order neurones which then transmit the information via the spinothalamic tract to specific areas of the brain. The release of enkephalins within the dorsal horn inhibits this substance P release [124, 125]. Although initial evidence suggested that this inhibition may be via a presynaptic mechanism [124], ultrastructural analysis has failed to show enkephalin- or opioid peptide-containing neurones synapsing directly onto substance P-containing fibres [126]. The inhibition may be a non-synaptic mechanism or alternatively, the action of substance P could be antagonized by the endogenous opioids acting at post-synaptic receptors. In support of this, immunoreactive enkephalinergetic fibres have been demonstrated synapsing onto spinothalamic projecting neurones [127]. Additional modulation of pain transmission at the spinal level involves complex feedback mechanisms from supraspinal centres.

Electrical stimulation [24], in addition to microinjections of opiates and substance P [128, 129] has been shown to elicit naloxone-reversible analgesia. However, substance P is reportedly excitatory [124] and opioids inhibitory [130] on neuronal transmission. To reconcile these observations it is suggested that substance P and opioids act at different neurones within the PAG. Since substance P does not bind at opiate receptors [129] it has been proposed to stimulate an opioidergic neurone which then inhibits an inhibitory (possibly γ-aminobutyric acid (GABA)) neurone. Opiates on the other hand, interact with opioid receptors to directly inhibit the inhibitory neurone (Figure 1.26; PAG section). Thus, both compounds can produce a nett stimulation of the PAG.
Figure 1.26 A proposed model for endogenous pain control.

KEY:

i) Circuitry

- Endogenous opioid peptide neurone
- Inhibitory neuromodulator
- Excitatory neuromodulator

ii) Proposed Neuromodulators

- SP - Substance P
- 5-HT - 5-Hydroxytryptamine
- NA - Noradrenaline
- NT - Neurotensin
- GABA - γ-Aminobutyric acid
- ? end. - Putative endogenous opioid

iii) Anatomical region

- PAG - Periaqueductal grey
- NRM - Nucleus raphe magnus
- Rpgl - Nucleus reticularis paragigantocellularis lateralis

(from Basbaum & Fields [134]).
Projection of neurotensin-containing fibres from the PAG to the RVM suggests that these may be involved in linking the two centres. Intracisternal neurotensin has been shown to produce analgesia [132] supporting a possible neuromodulator role for this peptide in the descending pain control mechanism. Activation of the PAG subsequently activates the RVM, a critical link in descending pain control, from which projections pass to the dorsal horn of the spinal cord via the dorsal part of the lateral funiculus. In particular, cells from the NRM and Rpgl, which are thought to be regulated by excitatory neuromodulators and inhibitory compounds such as noradrenaline [133], are believed to be involved in the control of this descending pathway. At the spinal level, 5-hydroxytryptamine inhibits both spinothalamic-projecting neurones and enkephalinergic neurones. The inhibition of the spinothalamic neurones reduces pain transmission to the supraspinal centres. The synapse with an inhibitory enkephalinergic neurone is less easy to explain, unless the enkephalinergic fibre in-turn synapses with a inhibitory (possibly GABA) neurone. Co-localisation of substance P and 5-hydroxytryptamine [126] may imply a duality of descending control via excitatory and inhibitory pathways.

Endogenous pain control mechanisms are not yet fully understood. However, it is known that the spinal level is an important site for the modulation of pain and that the interpretation of pain at the higher centres involves the limbic system. The use of synthetic opioids, opiates and opioid peptides at the spinal level in particular, has allowed the treatment of post-operative and terminal illness pain, often with fewer side-effects than intravenously administered drugs. The understanding of such pain mechanisms is therefore important for the development of new treatments with lower dependence liability and fewer side effects.
Aims of present work

The pharmacological profiles of opioids after their intrathecal administration are well characterized in a variety of species and in man. Opiate receptors are known to mediate the effects of these compounds and some attempts have been made to relate receptor-type to physiological function. However, the characterization of the receptor binding sites by in vitro studies has produced much controversy.

It is the aim of this study to characterize more fully both the types of binding site present and their distribution throughout the spinal cord. Special emphasis will be on the spinal cord of the rat, but other species will be employed. The possible heterogeneity of k-sites proposed by some authors will also be investigated in the cord. Brain tissue will also be studied for comparative purposes.
CHAPTER 2

MATERIALS AND METHODS
1. Animals

Male animals were used for experiments described in Chapters 4 to 7.

MF1 mice (25 - 30g) and Wistar rats (200 - 250g) were supplied by the Animal Unit, University of Nottingham, Sutton Bonnington. Alderley Park Wistar rats (250 - 350g) were kindly provided by ICI Pharmaceuticals plc, Alderley Park, Macclesfield. The latter were used for all rat studies unless otherwise detailed.

Dunkin-Hartley guinea-pigs (300 - 500g) were supplied by David Hall, Newchurch, Burton-upon-Trent.

For experiments described in Chapter 8, female Swiss-Webster outbred mice (25 - 40g) supplied by Bantin and Kingman Ltd., Grimston Aldlborough, Hull were used.

Porcine spinal cords were kindly provided by Barretts and Baird (Wholesale) Ltd., West Bromwich and bovine spinal cords by E. Coxon and Son, Ashby-de-la-Zouch.

2. Chemicals

a) General

Scintillation cocktails:
Unisolve E was purchased from Koch Light, Suffolk, U.K., PCS (Phase Combining System) was purchased from Amersham International plc, U.K., Ecoscint was purchased from National Diagnostics, Somerville, New Jersey, U.S.A.
Trizma Base (Tris (hydroxymethyl)aminomethane) and HEPES (N-2-Hydroxyethylpiperazine-N'-ethanesulphonic acid) were purchased from Sigma, Poole, U.K. All other reagents used were of analytical grade.
b) **Drugs and peptides**

\[D-Ala^2, D-Leu^5\]enkephalin and \[D-Ala^2, MePhe^4, Gly-ol^5\] enkephalin were purchased from Cambridge Research Biochemicals, Cambridge, U.K.

The following were kindly donated:

Diprenorphine hydrochloride (Reckitt and Colman Ltd., Hull, U.K.), levorphanol and dextrorphan tartrates (Roche Products Ltd, Welwyn Garden City, U.K.), tifluadom base (Roche Products, Switzerland), xorphanol mesylate (H. Parrs, New York, U.S.A.), naloxone hydrochloride (Endo Laboratories, New Jersey, U.S.A.), (-)-bremazocine hydrochloride (Sandoz, Basel, Switzerland), MR 2266 base \((-)-4-5,9-diethyl-2-(3-furylmethyl)-2'-hydroxy-6,7-benzomorphan; Boehringer Ingelheim, F.R.G.), U-50488H base (trans-3,4-dichloro-N-methyl-N-(2-(1-pyrrolidinyl)cyclohexyl) benzeneacetamide) and U-69593 base \((5\alpha, 7\alpha, 8\beta)-(+)-N-methyl-N-(7-(1-pyrrolidinyl)-1-oxaspiro[4,5] dec-8-yl) benzeneacetamide; The Upjohn Company, Kalamazoo, U.S.A.), dynorphin A-(1-17) (Parke-Davis, Cambridge, U.K.), and ICI 174864 (N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH) free acid or arginine salt (Aib = \(\alpha\)-aminoisobutyric acid) (ICI Pharmaceuticals plc, Alderley, U.K.).

All compounds were stored as the dry powder or as 1mM or 10mM stock solutions in water (or for tifluadom and xorphanol mesylate dimethylsulphoxide) at -20°C and diluted as required.

c) **Labelled Ligands**

\([15,16-^3H]\)etorphine (33.1 Ci.mmol\(^{-1}\)), \([N\text{-allyl}-2,3-^3H]\) naloxone (55 Ci.mmol\(^{-1}\)), \([15,16-^3H]\)diprenorphine (31-46.5 Ci.mmol\(^{-1}\)) and \([\text{tyrosyl}-3,5-^3H]\) dynorphin A-(1-9) (26 Ci.mmol\(^{-1}\)) as ethanol solutions, \([\text{tyrosyl}-3,5-^3H]\) \([D-Ala^2, D-Leu^5]\)enkephalin (32.1 - 39.5 Ci.mmol\(^{-1}\)) in 0.05M
acetic acid and [tyrosyl-3,5-'H][D-Ala²,MePhe⁴,Gly-ol⁵] enkephalin (60 Ci.mmol⁻¹) in triethylammoniumphosphate buffer were purchased from Amersham International plc, U.K. [Phenyl-3,4-'H]U-69593 (40 Ci.mmol⁻¹) and [9-'H](-)-bremazocine (30-41.4 Ci.mmol⁻¹) as ethanol solutions were purchased from NEN Research Biochemicals, Stevenage, U.K. [¹H][D-Ala²,D-Leu⁴]enkephalin and [¹H][D-Ala²,MePhe⁴,Gly-ol⁵]enkephalin were stored at +4°C. All other radiolabelled materials were stored at -20°C.

d) Biochemicals

i) Enzymes

Salt fractioned alkaline phosphatase (EC 3.1.3.1) from Escherichia coli, type III-S in 2.5M ammonium sulphate, 10.5 units mg protein⁻¹ was purchased from Sigma, Poole, U.K.

ii) Enzyme inhibitors

Captopril was kindly donated by Squibb, U.S.A. Bestatin was purchased from Cambridge Research Biochemicals, Cambridge, U.K.

iii) Nucleotides

Adenosine 5'-triphosphate as the tris (hydroxymethyl) aminomethane salt, adenosine 2',3'-cyclic monophosphate as the sodium salt and 5-guanylylimidodiphosphate as the sodium salt were purchased from Sigma, Poole, U.K.

All were stored desiccated at -20°C.
3. Procedures

a. Preparation of homogenates for binding assays

i) Tissues

Spinal Cord

Mice and rats were stunned and then decapitated.

Guinea-pigs were decapitated after cervical dislocation. The whole spinal column was removed and the spinal cord displaced from the subarachnoid space by the method of Yaksh & Harty [265].

For dorso-ventral studies, longitudinal transection was performed under a binocular microscope, using a razor blade. For rostro-caudal studies, the spinal cord was divided into cervical, thoracic and lumbo-sacral regions according to the method of Yaksh & Harty [265].

Porcine and bovine spinal cords were rapidly removed, within 30 minutes of slaughter and frozen in liquid nitrogen.

Other central nervous tissues

Brain, minus cerebellum, from rat and guinea-pig and cerebellum from guinea-pig were used where detailed in the text. Tissues were used immediately or stored over liquid nitrogen at -150°C to -180°C.
ii) **Buffers**

Homogenizations and assays were performed in 50mM Tris buffer adjusted to pH 7.4 with 1M HCl, or in 25mM HEPES buffer adjusted to pH 7.4 with ammonium hydroxide. Cations were added as their chlorides to buffer solutions when required.

iii) **Homogenization**

Central nervous tissue was homogenized using a Potter/Elvejham glass/teflon homogenizer (Voss Instruments S30/CB, setting 6) in 10 x w/v buffer for 20 seconds. After centrifugation (MSE 65, 37500g) for 15 minutes at 2°C, the pellet was resuspended in 20 x w/v buffer and incubated at 37°C for 30 minutes to remove endogenous ligands. After a repeat homogenization the pellet was resuspended in 50 x w/v buffer for assay.

For some experiments concentrated homogenates (5 x w/v in buffer) were stored at between -150°C and -180°C in the vapour over liquid nitrogen.

b. **Binding assays**

**Competitive displacement assays**

To the final homogenate, containing approximately 1mg.ml⁻¹ protein, were added solutions of the unlabelled competing ligand and labelled primary ligand to a total volume of 1ml. Where suppression of binding to μ-, δ- or κ-sites was required, unlabelled [D-Ala₂,MePhe⁷,Gly-ol⁸]enkephalin for μ, [D-Ala³,D-Leu⁶]enkephalin or ICI 174864 for δ and U-50488H for κ were added as described.

After incubation at 25°C for 45 minutes (30 minutes for [³H]dynorphin A-(1-9)) or 37°C for 45 minutes, 4ml of
ice-cold Tris-HCl was added and the mixture vortexed then filtered through Whatman GF/C glass filters (particle retention 1.2 μm) under vacuum using a Millipore 1225 sampling Manifold. When [³H]dynorphin A-(1-9) or [³H]U-69593 were used as the labelled primary ligand the glass filters were pre-soaked in buffer containing 0.1% polyethylenimine (Sigma, U.K.) to reduce non-specific binding. The retained material was washed twice with 4ml of ice-cold buffer. Radioactivity retained on the filters was counted after addition of scintillation fluid in a Phillips PW4700 liquid scintillation counter. Chemiluminescence was minimized by incubation at 37°C for 30 minutes prior to counting.

Saturated binding assays

The assay mixture comprised as above but contained varying concentrations of labelled primary ligand. Experiments were performed in the absence and presence of suppression as described under competitive displacement assays.

4. Fractionation studies

The method of Gray and Whittaker [260] was employed (Figure 2.1). Sucrose solutions were made up to 10x w/v in 50mM Tris-HCl, pH 7.4 buffer. Fractions were finally resuspended in further Tris-HCl buffer.

a) Myelin purification

The method of Norton and Poduslo [256] was used to produce samples of purified myelin (Figure 2.2). The resulting pellets were finally resuspended to 5x w/v in Tris-HCl buffer.

The purity of the sub-cellular fractions was assessed using marker-enzymes.
Figure 2.1 Fractionation of CNS tissue

CNS tissue

wash pellet twice

pellet

homogenise to 10% by weight in buffered 0.32M sucrose

spin at 11000g, 10 min, 4°C

pellet

P₁

spin at 37500g, 15 min, 4°C

supernatent

resuspend in buffer

layer onto sucrose gradient

spin at 50,000g, 120 min, 4°C

Crude myelin

0.32M

Synaptosomes

0.8M

Mitochondria

1.2M

store at -150--180°C
Crude myelin $\xrightarrow{\text{resuspend in } 50\times \text{w/v H}_2\text{O}}$ suspension

suspension $\xrightarrow{\text{spin at } 75000\text{g, } 15\text{ min, } 4^\circ\text{C}}$ pellet

pellet $\xrightarrow{\text{resuspend in } 50\times \text{w/v H}_2\text{O}}$ suspension

suspension $\xrightarrow{\text{spin at } 12000\text{g, } 10\text{ min, } 4^\circ\text{C}}$ pellet

layer over 0.85M sucrose

pellet $\xrightarrow{\text{resuspend in } 100\text{ml buffered } 0.32\text{M sucrose}}$ suspension

suspension $\xrightarrow{\text{spin at } 75000\text{g, } 30\text{min, } 4^\circ\text{C}}$ pellet

purified myelin $\xrightarrow{\text{resuspend in } 50\times \text{w/v H}_2\text{O}}$ suspension

suspension $\xrightarrow{\text{spin at } 12000\text{g, } 10\text{ min, } 4^\circ\text{C}}$ pellet

$\xrightarrow{\text{resuspend in buffer}}$ 5% suspension $\xrightarrow{\text{store at } -150\text{--180}^\circ\text{C}}$

Figure 2.2 Myelin purification
b) **Enzyme assays**

i) **Dilutions**

All samples were diluted with Tris-HCl buffer as follows:

- Homogenate: 1:5
- P₁ fraction: 1:20
- S₂ fraction: 1:5
- Myelin fraction: 1:5
- Synaptosomal fraction: 1:10
- Mitochondrial fraction: 1:10

ii) **Na⁺,K⁺-Activated ATP phosphohydrolase (ATPase) [EC 3.6.1.3]: a marker for external membranes**

A method after that of Hosie [257] was used (Figure 2.3).

The following solutions were prepared:

- **A** 0.4M Tris-HCl, pH 7.4 buffer
- **B** 0.02M EDTA in Tris-buffer
- **C** 0.06M Adenosine 5'-triphosphate in H₂O (freshly prepared)
- **D** 0.2M MgCl₂
- **E** 2.0M NaCl
- **F** 0.2M KCl
- **G** 22.5% Trichloracetic acid in H₂O
- **H** 5M H₂SO₄
- **I** 10% Ammonium molybdate in H₂O
- **J** Butan-1-ol: Petroleum ether (100 - 120°C) 4:1 v/v
- **K** 2.0M H₂SO₄ in ethanol
- **L** 0.05% SnCl₂ in 5M H₂SO₄
0.1 ml enzyme

Assay tubes → vortex mix 1s → incubate at 37°C for 10 min.

0.2 ml each soln., H & I

together

vortex mix 1s (on ice)

0.8 ml supernatant

vortex mix 1s

onto ice for 15 min

2.0 ml soln. J

vortex mix 15s

1.4 ml of upper organic layer

vortex mix 1s

1.4 ml soln. K

vortex mix 1s

0.2 ml soln. L

vortex mix 1s

room temperature 15-30 min.

read optical density at 650 nm against soln. J as reference

Figure 2.3  Na⁺,K⁺-ATPase assay method
Assay tubes were prepared as follows:

Set A

0.8ml A, 0.1ml B, 0.1ml C, 0.03ml D, 0.15ml E, 0.2ml F, H₂O to 1.9ml.

Set B

As for set A except 0.35ml H₂O replaced E & F.

A phosphate standard curve (0 - 1.5 μmole) was prepared using KH₂PO₄. 0.8ml solution G was added.

Blanks were prepared in which either the enzyme or ATP was absent.

Enzyme solutions and assay tubes were pre-incubated at 37°C for 5 minutes and then assayed as shown (Figure 2.3).

The activity of the enzyme was determined from the difference in optical density between tubes in set A (Na⁺ and K⁺ present) and set B (Na⁺ and K⁺ absent). The specific activity of the enzyme was defined as μmoles phosphate produced. minute⁻¹. mg protein⁻¹.

iii) 2',3'-Cyclic nucleotide 3'-phosphohydrolase [EC3.1.4.16]: a marker for myelin

A method after that of Kurihara et al [266] was employed.

The following solutions were prepared:

A  0.2M Tris-HCl buffer, pH 7.5
B  1% Sodium deoxycholate
C  4mM 2',3'-Cyclic adenosine monophosphate in H₂O
Assay tubes

- vortex mix
- incubate; 10 min, 30°C
- terminate; 30 s, 100°C
- return to 30°C
- 0.1 ml soln. E + 60 pg soln. F
- incubate; 30 min, 30°C
- assay for inorganic phosphate
- determine optical density at 410 nm

Figure 2.4 2',3'-CNP assay method
D 0.05M Tris-maleate buffer, pH 6.5
E 0.3M Tris-HCl buffer, pH 9.0 containing 21mM MgCl₂
F Alkaline phosphatase
G Butan-1-ol:Toluene 1:1 v/v

Assay tubes were made-up containing 0.2ml C and 0.1ml D.

A phosphate standard curve (0-2 μmoles) was prepared using KH₂PO₄. Blanks contained a volume of 0.32M sucrose equivalent to that of added enzyme.

Solubilisation of the enzyme was achieved by adding 0.2ml A and 0.4ml B to 0.4ml enzyme preparation, vortex mixing for 20 seconds and leaving on ice for 10 minutes.

The assay was performed as shown in Figure 2.4 and the inorganic phosphate liberated determined by the formation of the phosphomolybdic acid complex according to the micro-method of Martin and Doty [267]. Optical density was determined at 410nM (Pye Unicam SP 30) against solution G as the reference.

The specific activity of the enzyme was defined as μmoles inorganic phosphate liberated per minute⁻¹ mg protein⁻¹.

iv) Monoamine oxidase [EC 1.4.3.4]: a marker for mitochondria

A modification of the method employed by McEwen, Jr [268] was used.
Assay tubes

- solubilized enzyme and buffer to 1 ml total volume

- incubate; 37°C

- sample taken at 0, 5, 10, 15, 20 & 30 minutes

- terminate; 5 minutes at 0°C

- determine optical density at 250 nm against buffer as reference

Figure 2.5 MAO assay method
Solubilisation of the enzyme was afforded by the addition of Triton X-100 (Octyl Phenoxy Polyethoxypolyethanol; 1% by volume) Sigma U.K.), vortex mixing for 1 minute then centrifugation (Quickfit Micro Centrifuge, 13000g) for 5 minutes.

Assay tubes contained 100μl freshly redistilled benzylamine in 0.2M (Na₂HPO₄·KH₂PO₄) buffer, 0.1M, final pH 7.2.

The assay is outlined in Figure 2.5.

Specific activity was defined as the change in optical density at 250nm. minute⁻¹. mg protein⁻¹ at 37°C.

(A change of 0.001 absorbance units corresponds to the conversion of 0.25 nmoles benzylamine to benzaldehyde).

5. **Protein estimation**

Protein estimation throughout was according to Lowry [269], as modified by Schacterle and Pollack [270].

6. **In vitro degradation of [³H]dynorphin A-(1-9)**

[³H]Dynorphin A-(1-9) (14nM) was incubated in the presence of rat spinal cord homogenates as used in binding assays at 25°C for 30 minutes. Bestatin (30μM) and captopril (300μM) were added to prevent enzymic degradation by peptidases [197]. The reaction was terminated by addition of phosphoric acid to a final concentration of 50mM.
Metabolic products were analysed by high pressure liquid chromatography on a C₁₈ Altex Ultrasphere ODS reverse-phase column (5μm, 4.6 x 250mm) using a nonlinear gradient between solution A, trifluoroacetic acid (26mM)-triethylamine to give pH 3.0 and solution B, acetonitrile (49%)-trifluoroacetic acid (13mM).

The gradient was 45 - 61% B in 15 minutes, 61 - 62% B in 30 minutes, using a flow rate of 0.5ml. minute⁻¹. [³H]Products were identified by the position of standard marker peptides, collected and radioactivity determined by scintillation counting as previously described.
CHAPTER 3

ANALYSIS OF RESULTS
A. Binding Assays

Determination of radioligand binding constants

The following binding equations are based on the principle of the Law of Mass Action, that is a simple bimolecular interaction between a drug (L) and its binding site (R), to give the complex RL.

At equilibrium

\[ R + L \xrightleftharpoons[k_2]{k_1} RL \quad \text{eqn. 1.} \]

The equilibrium dissociation constant, \( K_D \), provides a measure of the affinity of a ligand for its binding site such that

\[ K_D = \frac{k_2}{k_1} = \frac{[R][L]}{[RL]} \quad \text{eqn. 2.} \]

Saturated binding studies

The \( K_D \) for a labelled ligand may be determined by saturation experiments in which the total concentration of radioligand \([Lt]\) is increased and \([RL]\) is determined at equilibrium. The total binding site population \([Rt]\) is kept constant and \([RL]\) is determined as a function of \([L]\).

Therefore, since \( Rt = [R] + [RL] \) then \([R] = [Rt - RL] \) so

\[ \frac{[Rt - RL][L]}{[RL]} = K_D \quad \text{eqn. 3.} \]

which in terms of \([RL]\) is given by

\[ [RL] = \frac{[Rt][L]}{K_D + [L]} \quad \text{eqn. 4.} \]
It can be seen that when $K_D = [L]$ then

$$[RL] = \frac{[Rt]}{2} \quad \text{eqn. 5.}$$

Therefore, $K_D$ is equal to the concentration of radioligand occupying 50% of the total binding sites.

Equation 4 describes a rectangular hyperbola (Figure 3.1a) and is equivalent to the Langmuir adsorption isotherm which is given by

$$[L] = \frac{r}{1 - r} (K_D) \quad \text{when} \quad r = \frac{[LR]}{[Rt]}$$

That is, $r$ = the proportion of the total potential sites of interaction occupied by the ligand for a given surface onto which adsorption can occur.

Scatchard analysis

The method of Scatchard [217] permits the linearization of equation 4 as follows:

Since

$$\frac{[RL]}{[L]} = \frac{([Rt] - [RL])}{K_D} \quad \text{eqn. 6.}$$

then

$$\frac{[RL]}{[L]} = \frac{[Rt]}{K_D} - \frac{[RL]}{K_D} \quad \text{eqn. 7.}$$

The parameters of equations 6 and 7 are more usually expressed as follows:

$[RL] = B$ (Bound radioligand)

$[L] = F$ (Free or unbound radioligand)

$[Rt] = B_{max}$ (Total number of binding sites)
Figure 3.1  

a) Untransformed and b) transformed data from a saturation analysis, according to the method of Scatchard [217] for a single site and c) two non-interacting sites.
Thus equation 7 may be re-written

\[
\frac{B}{F} = \frac{B_{\text{max}} - B}{K_D} \quad \text{eqn. 8.}
\]

A plot of \( \frac{B}{F} \) versus \( B \) will give a slope of \( \frac{-1}{K_D} \) and an intercept on the abscissa of \( B_{\text{max}} \) (Figure 3.1b).

Bound, that is specifically bound radioligand is given by:

Total counts bound - (counts bound in the presence of excess inhibitor*).

* This represents non-specific binding.

Free, that is unbound radioligand, is given by:

Total counts added - counts specifically bound.

Counts may be converted to a concentration term by dividing the dpm by the specific activity of the radioligand (in dpm).

The advantage of the Scatchard plot is the ease with which \( K_D \) and \( B_{\text{max}} \) may be obtained. However, there are disadvantages. These include:

a) The bound term is present in both the ordinate and abscissa. Therefore, any error in the bound value will be magnified in two dimensions. Scatter of points is therefore increased particularly at either of the axes where the measurement of the bound radioligand is subject to the greatest error [161].

b) The data points which are evenly spaced on a non-transformed, hyperbolic plot (Figure 3.1a) are compressed when transformed, particularly at high concentrations of free ligand. False \( B_{\text{max}} \) values may therefore be obtained because of erroneous judgement that saturation has been achieved, when it has not.
c) The interaction of a ligand with a single class of binding site should produce a linear Scatchard plot. Interaction with more than one site, where the affinity of the radioligand for the separate sites is sufficiently different, will yield curvilinear Scatchard plots (Figure 3.1c). However, a number of methodological artifacts, such as metabolism of the radioligand can produce non-linearity and hence, lead to incorrect conclusions and false heterogeneity where none exists.

d) True heterogeneity of binding sites may not be seen if, for example, the affinity of the radioligand for all sites is similar or if the separate populations of high and low affinity sites are too low. The scatter and number of data points used, or the concentration range of radioligand may also lead to erroneous interpretation of an heterogeneity of sites.

e) Because of the correlated, non-uniform errors present in both co-ordinates of the Scatchard plot, non-weighted linear regression of the data is statistically invalid. Therefore, computer-aided analyses have been developed to help overcome these problems.

Computer-assisted analysis of saturation data

The two main computer-aided analytical systems currently in use are non-linear regression [218] and iterative curve-fitting programmes such as LIGAND [161]. Both have greater statistical validity than simple linear regression of the data. In particular, LIGAND, when used appropriately represents a powerful tool for the estimation of $K_D$ and $B_{max}$ values. Several advantages are apparent with this method of analysis:

a) The total binding rather than specific binding is examined. Thus, additional errors which may be added by subtraction of non-specific binding are eliminated.
b) The data points at the extremes of the curve are not equally weighted. Therefore, the non-uniformity of variance of the dependent variable B is compensated for.

c) Incorporated within the analysis of single or multiple site models are statistical methods which allow the validity of each model to be assessed.

The major disadvantage with computer techniques is that because of the complexity and power such methods possess, the experimenter may be tempted to unquestioningly accept the results produced. Large errors are possible where the scatter of data points is great, if the maximum concentration of radioligand used is not sufficient to occupy 90% of the binding sites, that is 9 to 10 times the $K_D$ value, and when too few data points are used. In the latter case 6 to 10 points are sufficient to describe a single site model but twice these should be used when multiple site models are being analysed [213]. The requirements for Scatchard analysis and the sources of error from assay or methodological artifacts also apply to such analyses. Thus, it is the quality of the initial data which determines the precision and accuracy of an analysis, whether that analysis is performed manually or with the aid of computational techniques.

**Competitive inhibition studies**

The affinity of an unlabelled competing ligand for an opioid binding site may be determined from competitive inhibition curves. With such an experiment the binding site concentration [$R_t$] and radioligand concentration [$L$] are kept constant, whilst the concentration of an unlabelled competing ligand [$I$] is varied. Therefore, the concentration of radioligand bound [$RL$] at equilibrium is a function of [$I$]. Such a reaction is described by:
\[ [RL] = \frac{[L][Rt]}{K_D (1 + [I]/K_i) + [L]} \quad \text{eqn. 9.} \]

where \([I]\) = concentration of free unlabelled inhibitor.

\(K_D\) = equilibrium dissociation constant for the interaction of the radioligand with the binding site.

\(K_i\) = equilibrium dissociation constant for the interaction of the inhibitor \(I\) with the binding site, defined by \(K_i = \frac{[R][I]}{[RI]}\).

The concentration of inhibitor which displaces 50\% of the bound radioligand, the IC\(_{50}\), can be determined graphically when the % inhibition of binding is plotted against log concentration of inhibitor. Interpolation to the abscissa provides log IC\(_{50}\) for the competing ligand (Figure 3.2a).

The % inhibition of binding by an inhibitor may be calculated experimentally by:

\[
\left( \frac{\text{specific binding in the absence of inhibitor} - \text{specific binding in the presence of inhibitor}}{\text{specific binding the absence of inhibitor}} \right) \times 100\%
\]

It is possible to linearize the displacement curve by the Hill transformation [219].

\[ [RL] = \frac{[Rt][L]}{K_D + [L]} \quad \text{eqn. 4.} \]

therefore,

\[ \frac{[RL]}{[Rt]} = \frac{[L]}{K_D + [L]} \quad \text{eqn. 10.} \]
Figure 3.2  Analysis of competitive displacement data
a) % inhibition - log concentration inhibitor
curve and b) Hill plot. Figure 3.2c shows
Hill slopes for co-operativity of inhibitor
binding (nH = 2), displacement from an homogeneous
population (nH = 1) and displacement from an
heterogeneous population (nH = 0.5) of sites.
[RL] is the proportion of binding sites occupied by the radioligand and may be given the term r. Substitution and rearrangement of equation 9 gives

\[
[L] = \frac{r}{1 - r} (K_D)
\]
eqn. 11.

This relationship holds if one molecule of radioligand interacts with a single binding site. If two molecules interact simultaneously with a single binding site then:

\[
[L]^2 = \frac{r}{1 - r} \cdot K
\]
eqn. 12.

and in general

\[
[L]^n = \frac{r}{1 - r} \cdot k
\]
eqn. 13.

where \( n \) = number of molecules of radioligand interacting with a single binding site.

\( k \) = constant.

If \( \log (r/(1-r)) \) is plotted against \( \log [L] \) then a straight line with slope \( n \), often designated \( n_H \) (the Hill coefficient) is obtained. For competitive binding assays it is more usual to plot \( \log (I/ (100-I)) \) against \( \log \) [inhibitor], where I represents % inhibition of radioligand binding by the inhibitor. The \( IC_{50} \) for the inhibitor is calculated by interpolation from \( \log (I/ (100-I)) = 0 \) (Figure 3.2b).

A slope of unity is given when the competing ligand displaces the bound radioligand from a single class of binding site or multiple classes of site for which the competing ligand has equivalent affinity.
Low or shallow Hill slopes, that is, less than unity may be obtained when multiple binding sites exist for which the competing ligand has different affinities. Alternatively the low slopes may represent interconverting forms of a given class of binding site [213] or negative co-operativity between binding sites. Hill slopes of greater than unity suggest co-operativity of competitor binding (Figure 3.2c).

Having determined the IC<sub>50</sub>, the Ki for the inhibitor may be determined indirectly by the Cheng and Prusoff equation [220], that is,

\[
Ki = \frac{IC_{50} \cdot K_D}{K_D + [L]}
\]

where \([L]\) = free radioligand concentration.

\(K_D\) = equilibrium dissociation constant for the radioligand.

Ki = equilibrium dissociation constant for the competitor.

IC<sub>50</sub> = 50% inhibitory concentration for competitor, expressed as free concentration of ligand.

It is important to note that the concentration used in competition experiment calculations are the free concentrations at equilibrium.

Because it is often difficult to determine the free concentrations of the competitor, it may be assumed that free concentration is equal to the total concentration present, providing that the amount bound is low with respect to the equilibrium dissociation constant. This means that the total binding sites present [Rt] must be much lower than the respective \(K_D\) value of the radioligand and Ki value of the inhibitor. If [Rt] is greater than or equal to the equilibrium dissociation constant, then the estimation of Ki from equation 14 will be too high [161].
Analysis of heterogeneous binding site populations: a) Biphasic displacement of radioligand by an inhibitor with different affinities for the two populations of binding site and b) Resolution of plot (a) into two Hill plots for the determination of binding parameters.
Multiple binding sites

It is possible to study the interaction of ligands with more than one binding site using competitive inhibition studies, although as mentioned previously, the effectiveness of such work is dependent upon the affinity of the competitor for the respective sites. In some cases, the displacement curves may yield low Hill coefficients but no apparent separation of the binding components (Figure 3.2c). However, where there is sufficient difference in the affinity of the competitor for the respective sites, typically 100 to 1000 fold, noticeably bi-phasic plots may be obtained (Figure 3.3a). These displacement curves may be resolved into separate Hill plots and analysed for binding constants (Figure 3.3b). In addition, the proportions of high and low affinity sites labelled at the concentration of radioligand used, may be estimated as shown in Figure 3.3a.

Alternatively, heterogeneous populations of binding sites may be studied using different radioligands with 100 to 1000 fold specificity for a given class of binding site. Competitive inhibition studies and saturated binding analyses may then be performed and analysed simply.

In the present work, the results from competitive inhibition studies have been obtained either directly from the displacement curves or from Hill plots. Displacement curves are represented as mean % inhibition values with error bars expressing the standard error of mean and Hill plots are represented using mean results.

Saturated binding studies were analysed according to the method of Scatchard, using the non-linear regression method of McPherson [218]. Occasionally, where indicated the data has been analysed for site heterogeneity using LIGAND [161]. Single representative plots for Scatchard analyses are presented to avoid problems associated with deriving mean data, including averaging data from assays using different concentrations of radioligand and thus introducing considerable errors in both the bound and bound/free values.
These are in addition to the errors discussed earlier inherent in Scatchard analysis.

B. Enzyme Assays

In the study of the sub-cellular distribution of binding the presence of specific enzyme-markers was used as evidence that the desired fraction had been obtained and to allow assessment of the purity of fractions.

Each fraction was compared by determination of the amount of component per volume of fraction derived, which was equivalent to one gramme of tissue. Thus, the results obtained from the determinations of enzyme activity for each fraction were multiplied by their respective "volume equivalents" to give the number of units per volume equivalent to 1g of tissue.

The concentration of protein per volume equivalent to 1g of material was then calculated and the percentage recovered activity, and percentage recovered protein were calculated using

\[
\text{Recovery for fraction} \times \frac{x}{\text{Sum of recoveries from all fractions}} \times 100\%
\]

where recovery is expressed as units of activity (or mg protein) per volume equivalent to 1g of tissue.

The relative specific concentration, RSC, may then be calculated by:

\[
\frac{\% \text{ Recovered Activity}}{\% \text{ Recovered Protein}}
\]
This term permits the comparison of a series of results, without the large errors produced by random losses due to volume and analytical error which would be present if absolute values, such as activity per mg protein were used to calculate specific concentrations. An RSC value greater than unity indicates an enrichment of the component under study in that fraction, relative to the parent fraction.

C. **Statistical Analysis**

Results are presented as the statistical mean and standard error of mean. Where comparisons have been made between different regions of the spinal cord, analysis of variance (ANOVA) [222] was initially calculated. When a statistical significance was observed further analysis was made using Duncan's multiple range test [222]. This latter test adjusts the size of the critical difference depending upon whether two means are adjacent or whether one or more means fall between those being compared. Both of these tests are based upon the completely randomized design of experiments. Points to consider for such design are:

a) For each subject in the experimental group there is only one score.

b) Similar numbers of subjects should be in each experimental group where possible.

c) The number of groups to be compared may be arbitrary, but more than four or five groups are rarely used.

The binding experiments performed in the following experimental sections were designed to conform as closely as possible with the randomized design criteria. Each assay produced only a single value for $B_{\text{max}}$ or $K_D$. The animals were randomly chosen from a large population.
In some experiments tissues from more than one animal were pooled to provide sufficient for assay, as with spinal cord preparations. In this case spinal segments for cervical, thoracic and lumbo-sacral regions were separated and pooled as individual groups. Material was then randomly selected from the appropriate population for single assay determination.
CHAPTER 4

EXPERIMENTAL CONDITIONS
INTRODUCTION

An extensive research effort has been undertaken over the past two decades to determine the molecular properties and functional roles of opioid receptors. The development of in vitro methodologies, including binding assays [76, 77, 79, 81] has provided confirmation of the results from in vivo studies [14, 17 - 20] and from bioassays [22, 30, 67], which suggests the existence of multiple opioid receptors [66, 67].

The direct analysis of opioid binding site interactions by ligand binding techniques is relatively simple experimentally. However, because of the sensitivity of opioid binding to modifying influences such as temperature [201], pH [79], ions and nucleotides [77, 78] and incubation times [210], it is necessary to define the assay conditions.

In the work described a selection of [³H]opioids and central nervous system tissues have been used and binding studied under a variety of conditions.
MATERIALS & METHODS

Homogenates were prepared and binding studies performed as detailed in Chapter 2 at 37°C for 45 minutes except in experiments where these parameters were being studied. Mouse and rat brain in addition to bovine and porcine spinal cords were used.

[^H]Etorphine, [^H](-)-bremazocine, [^H]diprenorphine, [^H][D-Ala²,D-Leu⁵]enkephalin and [^H][D-Ala²,MePhe⁵,Gly-ol⁵] enkephalin were the labelled ligands. 1μM unlabelled diprenorphine was used to define non-specific binding except when different unlabelled ligands were under study. The chemical formulae of the compounds are as given in Chapter 2.
Non-specific binding

The definition of non-specific binding of \[^{3}H\]diprenorphine in rat brain homogenates was determined using a selection of unlabelled opioid compounds (Table 4.1). At this level of \[^{3}H\]diprenorphine (0.2nM) specific binding represented 95% of the bound total. Ligands displaying selectivity for individual classes of binding site namely U-50488H and to a lesser extent, tifluadom required much higher concentrations to achieve the displacement obtained with non-selective ligands.

Equilibrium binding

Equilibrium binding was achieved for the four \[^{3}H\]opioids studied in different central nervous system tissues from a variety of animal species (Figures 4.1 - 4). The results are summarized in Table 4.2. Equilibrium was achieved more rapidly at the higher temperatures, being reached within 45 minutes for all the ligands studied at 37°C. In comparison \[^{3}H\]etorphine required greater than 90 minutes at 25°C and 60 minutes at 30°C compared with 45 minutes at 37°C (Figure 4.1). \[^{3}H\](-)-Bremazocine reached equilibrium very rapidly at 37°C, within 15 minutes in porcine lumbo-sacral spinal cord. At 25°C it was more difficult to determine the time required from the temperature-time curves in either porcine lumbo-sacral cord (Figure 4.2a) or rat brain (Figure 4.3c) homogenates.
Table 4.1  The ability of unlabelled opioids at high concentrations to displace [\(^3\text{H}\)]diprenorphine (0.2nM) in rat brain homogenates at 37°C.

<table>
<thead>
<tr>
<th>Unlabelled Ligand</th>
<th>Concentration ((\mu)M)</th>
<th>% Displacement*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MR 2266</td>
<td>1</td>
<td>95.33 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>95.30 ± 0.20</td>
</tr>
<tr>
<td>Diprenorphine</td>
<td>1</td>
<td>96.42 ± 0.39</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>94.97 ± 0.51</td>
</tr>
<tr>
<td>(-)-Bremazocine</td>
<td>1</td>
<td>94.97 ± 0.51</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>94.59 ± 0.41</td>
</tr>
<tr>
<td>Naloxone</td>
<td>10</td>
<td>94.58 ± 0.31</td>
</tr>
<tr>
<td>Tifluadom</td>
<td>1</td>
<td>79.58 ± 0.83</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>93.43 ± 0.40</td>
</tr>
<tr>
<td>U-50488H</td>
<td>10</td>
<td>49.18 ± 2.22</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>90.10 ± 0.23</td>
</tr>
</tbody>
</table>

Values represent means ± sem. of 4 separate experiments in duplicate.

* % Displacement represents displacement of total, that is, specific plus non-specific [\(^3\text{H}\)]diprenorphine binding by unlabelled ligand.
Figure 4.1  The influence of temperature on specific [\(^{3}H\)]etorphine binding against time in mouse brain homogenates (concentration of radioligand used = 1.93nM). See Table 4.2 for equilibrium times.
The influence of temperature on specific [$^3$H](−)-bremazocine binding against time in porcine lumbo-sacral spinal cord homogenates, using a radioligand concentration of 0.50 nM. See Table 4.2 for equilibrium times.
Figure 4.3  Specific binding against time for a) \(^{3}H\)[D-Ala\(^{2}\), D-Leu\(^{5}\)]enkephalin (0.46nM) (△), b) \(^{3}H\)[D-Ala\(^{2}\), MePhe\(^{5}\),Gly-ol\(^{5}\)]enkephalin (0.46nM) (▲) and c) \(^{3}H\)(-)-bremazocine (0.44nM) (□) at 25°C in rat brain homogenates. See Table 4.2 for equilibrium times.
Specific binding against time for a) $[^3\text{H}][\text{D-Ala}^2, \text{MePhe}^4, \text{Gly-ol}^5] \text{enkephalin} (0.30\text{nM}) (\Delta)$ and b) $[^3\text{H}](\text{C})-\text{bremazocine} (0.42\text{nM})(\square)$ at 37°C in rat brain homogenates. See Table 4.2 for equilibrium times.
<table>
<thead>
<tr>
<th>Labelled Ligand</th>
<th>Tissue</th>
<th>Temperature (°C)</th>
<th>Time to reach equilibrium (min.)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>[³H]Etorphine</td>
<td>Mouse Brain</td>
<td>25</td>
<td>&gt;90</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>70</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>40</td>
<td>2</td>
</tr>
<tr>
<td><a href="-">³H</a>-Bremazocine</td>
<td>Porcine lumbo-sacral</td>
<td>25</td>
<td>&lt;15</td>
<td>1*</td>
</tr>
<tr>
<td></td>
<td>spinal cord</td>
<td>37</td>
<td>&lt;15</td>
<td>1*</td>
</tr>
<tr>
<td></td>
<td>Rat Brain</td>
<td>25</td>
<td>30</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>30</td>
<td>2</td>
</tr>
<tr>
<td>[³H][D-Ala²,D-Leu²]enkephalin</td>
<td>Rat Brain</td>
<td>25</td>
<td>25</td>
<td>2</td>
</tr>
<tr>
<td>[³H][D-Ala²,MePhe⁴,Gly-ol⁵]enkephalin</td>
<td>Rat Brain</td>
<td>25</td>
<td>45</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>45</td>
<td>2</td>
</tr>
</tbody>
</table>

Results are observations from n separate experiments in duplicate (or * triplicate).
Protein concentration

When 1nM [³H]etorphine was used, the amount specifically bound, as fmoles increased linearly with protein concentrations of up to 1mg.ml⁻¹ in mouse brain homogenates and up to 1.5mg.ml⁻¹ in bovine lumbo-sacral spinal cord homogenates, reaching a plateau of 3mg.ml⁻¹ in bovine cord (Figure 4.5). Concentrations of greater than 2mg.ml⁻¹ protein were not tested with the mouse brain preparation. The ligand specifically bound, when expressed as the % of total activity added did not exceed 6% at protein concentrations up to approximately 6mg.ml⁻¹ in bovine lumbo-sacral spinal cord, but was greater than 10% for protein concentrations of approximately 1mg.ml⁻¹ or greater in mouse brain homogenates (Table 4.3).

Post-filtration washing

The percentage of [³H](-)-bremazocine specifically bound in rat brain homogenates reached a maximum plateau after three 4ml washings with ice-cold buffer and up to three extra washings with 4ml aliquots of ice-cold buffer did not affect the specifically bound [³H](-)-bremazocine (Figure 4.6). A radioligand concentration of 0.52nM was used for the binding assays.
Figure 4.5

Influence of protein concentration on specifically bound [3H]etorphine in homogenates of mouse brain (●) and bovine lumbo-sacral spinal cord (○). An incubation time of 45 minutes at 37°C was used. Concentration of radioligand was 1.0nM. See Table 4.3 for data.
Table 4.3 Binding characteristics of \[^{3}H\]etorphine (1.0nM) at different protein concentrations.

<table>
<thead>
<tr>
<th>Species &amp; Tissue</th>
<th>Protein Conc. (mg.ml(^{-1}))</th>
<th>% Total Added Activity Specifically Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse Brain</td>
<td>0.16</td>
<td>2.43, 4.69</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>3.68, 4.16</td>
</tr>
<tr>
<td></td>
<td>0.34</td>
<td>5.37, 6.13</td>
</tr>
<tr>
<td></td>
<td>0.49</td>
<td>7.09, 8.93</td>
</tr>
<tr>
<td></td>
<td>0.58</td>
<td>9.17, 8.90</td>
</tr>
<tr>
<td></td>
<td>0.99</td>
<td>10.17, 12.72</td>
</tr>
<tr>
<td></td>
<td>1.98</td>
<td>14.93, 22.51</td>
</tr>
<tr>
<td>Bovine lumbo-sacral spinal cord</td>
<td>0.49</td>
<td>2.10, 1.92</td>
</tr>
<tr>
<td></td>
<td>0.98</td>
<td>3.11, 2.98</td>
</tr>
<tr>
<td></td>
<td>1.49</td>
<td>3.93, 3.96</td>
</tr>
<tr>
<td></td>
<td>2.93</td>
<td>5.40, 5.18</td>
</tr>
<tr>
<td></td>
<td>5.87</td>
<td>5.38, 5.25</td>
</tr>
</tbody>
</table>

Results expressed as individual values from 2 separate experiments in duplicate.

% Added activity totally bound, that is specific plus non-specific, represented 25% in mouse brain and 6% in bovine spinal cord at the highest protein concentration studied in each species.
Figure 4.6 Influence of washing on specific binding of [3H](-)-bremazocine to material retained on filter. Homogenates of rat brain were incubated with 0.52nM radioligand for 45 minutes at 37°C prior to filtration. Protein concentration was <1 mg.ml⁻¹. Mean of two experiments in triplicate.
DISCUSSION

All of the unlabelled opioids tested displaced greater than 90% of the total bound \[^{3}H\]diprenorphine, when the concentration of radioligand used was close to the reported $K_D$ values in guinea-pig lumbo-sacral spinal cord [152] and rat brain [98]. However, U-50488H and tifluadom required higher concentrations to displace the specifically bound \[^{3}H\]diprenorphine, indicative of their selectivity for $k$-sites [106, 109] and hence, reduced affinities at other opioid binding sites. Previous reports [97, 211] have proposed that the inhibitor which is used to define non-specific binding should have a high affinity for the radioligand binding site and have a chemical structure which differs from that of the radioligand. This competing ligand should also be used at a concentration sufficient to occupy all specific binding sites but not high enough to displace non-specific binding. Were the latter to occur, the saturation isotherm may yield biphasic Scatchard plots which could be mistakenly interpreted as heterogeneity of binding sites [211]. A concentration of approximately 100 times the $K_D$ concentration of the inhibitor is commonly used [212].

The incubation temperature used for binding assays has been shown to be an important variable. The results in this present study show that the time to reach equilibrium by a selection of \[^{3}H\]opioids decreases as the temperature increases. Stability of the ligands is not a problem when using compounds this stable, but early experiments with the enkephalins [89] were performed at 0-4°C to reduce enzymatic breakdown of the peptides. However, because of the reduction in the radioligand-binding site association rate at these lower temperatures, much longer assay times were required to allow equilibrium binding to be achieved [211]. Furthermore the pharmacological characteristics of opioid binding sites at these low temperatures were reported in an early study [201] to change to a state favouring antagonist binding, and the number of binding sites is reported to decrease at low temperatures. For example, the $B_{max}$ for \[^{3}H\](-)-bremazocine in guinea-pig brain increases by 1.5 times when the assay is performed at 25°C rather than 0°C [197].
Other ligands also show an increase over the same temperature range, although the differences are not as great as with \[^{\text{\text{\text{\[H\]}}}\text{(-)-bremazocine [89]. The use of more physiologically relevant temperatures such as 37°C allow more direct comparisons to be made between the results from binding studies and those gained from bioassays. However, some opioid peptides such as the naturally occurring enkephalins and the smaller dynorphin fragments are unstable at these higher temperatures [74, 197]. Therefore, the use of these peptides whether radiolabelled or unlabelled is not possible unless the temperatures are reduced and/or enzyme inhibitors are used [197]. It is for such reasons that many workers perform their experiments at 22°C or 25°C [74, 152] under conditions which allow a more direct comparison between peptide and non-peptide radiolabelled opioids to be made.

A further factor to be considered is that prolonged incubation at 37°C or the use of temperatures in excess of 40°C may reduce the amount of radioligand bound, possibly due to denaturation of the protein in the binding site [78]. However, in the present work changes in Bmax at different temperatures were not evident. For example, in the thoracic spinal cord of the rat \[^{\text{\text{\text{\[H\]}}}\text{(-)-bremazocine afforded Bmax values of 70.3 and 68.3 fmol.mg\(^{-1}\) at 25°C and 72.3 and 70.1 fmol.mg\(^{-1}\) at 37°C, both after 45 minutes incubation.

Specifically bound \[^{\text{\text{\text{\[H\]}}}\text{etorphine increased linearly up to 1mg.ml\(^{-1}\) in mouse brain and 1.5mg.ml\(^{-1}\) in bovine lumbo-sacral spinal cord homogenates, when 1nM radioligand was used. After this the relationship between specifically bound \[^{\text{\text{\text{\[H\]}}}\text{etorphine and protein concentration deviated from linearity. It is unlikely that this was the result of the available \[^{\text{\text{\text{\[H\]}}}\text{etorphine being totally bound to specific or non-specific sites since even at high levels of protein the total bound, that is specific plus non-specific binding represents only a relatively low % of the added radioligand. The concentration of opioid binding sites present represents only a small fraction of the total protein. The free ligand concentration in an assay system is directly proportional to the concentration of opioid binding sites present. At low opioid binding site concentrations, that is when concentrations are low compared to the \(K_D\) of the radioligand being used, opioid binding has little effect on the free ligand
concentrations. Therefore, the calculation of pharmacological constants is straightforward. However, when the binding site concentration is less than 10 times lower than the true affinity constant, the apparent $K_D$ obtained from direct concentration binding curves will increase as a linear function of binding site concentration [213]. Therefore, when high levels of binding sites are present in the assay mixture, the free ligand concentrations may be so reduced that saturability becomes difficult to demonstrate. The specific activity of the ligands being used must be taken into account when assessing protein concentrations. If only low specific activity ligands are used then the specific to non-specific ratio will be low, particularly at the higher protein concentrations. However, by using high affinity ligands that is, those with a $K_D$ of less than 10nM, a high specific to non-specific ratio will be obtained and the lower will be the level of detection for the amount of radioligand specifically bound. In future experiments the protein levels used were such that the bound radioligand did not exceed 10% of the total added activity.

Post-filtration washing of the homogenates retained on the filter is another experimental step where close control of conditions is necessary. The major consideration for this procedure is the rate of dissociation of the ligand from the opioid binding sites. If the rate is slow then more extensive washing to remove excess, unbound radioligand is possible without disturbing the equilibrium established during assay. For many opioid compounds the dissociation rate is high from non-specific sites [212], but because it is generally found that the rate of dissociation is inversely proportional to the $K_D$, those ligands with higher affinity for the binding sites will dissociate more slowly from specific sites [212]. Therefore, extensive washing will greatly increase the ratio of specific to non-specific binding (Table 4.4). Two approaches have been used for filtration of the assay mixture at the end of incubation. The first is to cool the mixture on ice to 4°C, decreasing the dissociation rate from the binding site, and then filtering. This method has
Table 4.4 Relationship between steady-state dissociation constant \( (K_D) \) and allowable separation time.

<table>
<thead>
<tr>
<th>( K_D ) (M)</th>
<th>Allowable Separation Time ((0.15 \ t^a))</th>
</tr>
</thead>
<tbody>
<tr>
<td>( 10^{-12} )</td>
<td>1.2 days</td>
</tr>
<tr>
<td>( 10^{-11} )</td>
<td>2.9 hours</td>
</tr>
<tr>
<td>( 10^{-10} )</td>
<td>17 minutes</td>
</tr>
<tr>
<td>( 10^{-9} )</td>
<td>1.7 minutes</td>
</tr>
<tr>
<td>( 10^{-8} )</td>
<td>10 seconds</td>
</tr>
<tr>
<td>( 10^{-7} )</td>
<td>0.10 seconds</td>
</tr>
<tr>
<td>( 10^{-6} )</td>
<td>0.01 seconds</td>
</tr>
</tbody>
</table>

\( t^a \) Calculations of \( t^a \) (half-life for dissociation) assume an association rate constant of \( 10^4 \ \text{M}^{-1} \text{S}^{-1} \).

(From Yamamura, H.I. et al [212]).
the inherent disadvantage that the conformation of the binding sites may be changed at these lower temperatures. Alternatively, the assay mixture is passed directly through the filters and then washed with ice-cold buffer. This latter method has been adopted in the present work. The levels of specific binding in terms of the amount bound and the percentage bound was high and appeared to be unaffected by washing with up to six aliquots of 4ml ice-cold buffer, confirming the low dissociation of [³H](-)-bremazocine. It should be remembered that the filtration technique does lead to a loss of material which passes straight through the filter, and that non-specific binding of the [³H]opioid may occur to the filter material [86]. This is seen particularly with the peptides [197] and with compounds such as U-69593 [198]. During assay of these materials use was made of the anti-adsorbent polyethyleneimine at a concentration of 0.1%.

Although the factors discussed are the major influences on the results obtained from binding studies, other potential sources of error should be considered.

The radiochemical purity of ligands is essential to ensure accurate results. Although many radiochemicals are prepared free of radioactive contaminants they may undergo decomposition upon storage. These contaminants may then interfere with calculations concerning the bound and unbound ligand concentrations or even compete for the binding sites, leading to errors in the estimation of binding parameters. Such errors may lead to unnecessarily complicated Scatchard plots from saturation studies, suggesting apparent heterogeneity [213]. The purification of radiolabelled ligands by chromatographic techniques prior to use is necessary.

Metabolism of the radioligand in the assay system to inactive fragments, a problem especially with peptides, will lead to non-equilibrium binding conditions in addition to an over estimation of the concentration of free ligand. The use of specific enzyme inhibitors has been shown to reduce these effects [75, 197] but care must be taken when using such agents since they may have additional actions on the tissue which alter the binding site properties [214].
Additionally such compounds may be adsorbed onto either contaminating cellular components in the assay system or vessel walls in which the assay mixture is contained [197]. This will lower the free ligand concentration at the binding site surface and may go undetected because of rapid dissociation from these non-specific sites upon filtration. Such adsorptive effects may lead to the underestimation of the absolute potency and the potency relative to other ligands where such losses are less prevalent [215, 216].

A major difference between binding assay systems and bioassay or in vivo systems is the medium in which the binding site or receptor preparation is bathed. Although it would be desirable to study binding under physiological conditions so that more direct comparisons may be made between binding and intact tissue systems, problems arise which prevent such studies. For example, in the presence of physiological buffers containing high levels of sodium ions opioid binding sites undergo conformational changes such that antagonist binding is promoted [78]. Since many of the radiolabelled opioids available are agonists, then binding under physiological conditions is very low [78]. It is for reasons such as this that low ionic strength buffers are employed. However, an advantage of these buffers is that they do allow close monitoring of parameters such as pH and the concentrations of ions and nucleotides. Thus, pH which is known to be an important factor affecting opioid binding [77, 78] can be maintained constant throughout the duration of the experiment. Finally, binding studies performed using homogenates expose both the inner and outer surfaces of the membrane to the same conditions. An alternative to these techniques is the study of intact cells, although receptor and ligand internalization would disrupt equilibrium binding and therefore complicate interpretation of the data. If these problems were overcome, the method would have the inherent advantage of studying the intact cell system and the characteristics of the opioid receptor within that environment.
In conclusion, the experimental conditions for binding assays must be accurately defined. Many factors can influence the results obtained using such studies, an understanding of which is important when interpreting results.

From these preliminary investigations experimental conditions were chosen such that assays were performed at 37°C for 45 minutes using less than 1mg.ml⁻¹ protein. Following the separation of bound and free ligand by filtration, the retained material was washed three times with 4ml aliquots of ice-cold buffer. Non-specific binding was defined using an unlabelled non-selective opioid with dissimilar chemical structure to that of the radioligand at 100 x Kᵰ.

Any modification of these conditions, for example, when using labile peptides is detailed in the relevant methods section.
CHAPTER 5

DISTRIBUTION OF OPIOID BINDING SITES

IN THE SPINAL CORD
INTRODUCTION

The dorsal horn of the spinal cord is an important site of action for narcotic analgesics [138]. The intrathecal administration of morphine-like compounds [139] or opioid peptides [140-2] produces a profound dose-dependent, naloxone-reversible analgesia which implies the involvement of opioid receptors at this level. Autoradiographic studies using a variety of $[^{3}H]$opioid ligands have shown opioid binding sites to be concentrated in the marginal zone of lamina I and the substantia gelatinosa of lamina II [115, 119, 143, 147]. Lamina $\overline{V}$ cells which respond to painful stimuli via dendritic processes extending into the substantia gelatinosa [144] contain lower numbers of binding sites [119, 145, 146]. In addition immunohistochemical analysis shows prodynorphin- and proenkephalin A-derived peptides to be concentrated in these areas [134].

Opioid peptides [147, 149-51] and binding sites [119, 146, 152] are also found in lower concentrations within the ventral horn of the spinal cord, an area associated mainly with motor function [153].

Studies along the rostro-caudal axis show inconsistencies. Proenkephalin A and prodynorphin products vary by up to six-fold between cervical, thoracic and lumbo-sacral regions, depending upon the peptide and species [149-51]. Binding studies are not consistent and sites are reported to be homogeneously [146, 154] or heterogeneously [155] distributed throughout these same regions.

Therefore, work was performed in which the dorso-ventral and rostro-caudal distributions of opioid binding sites were studied, both in terms of the total number and types of sites present. The results have been compared to the reported distributions of endogenous opioid peptides.
MATERIALS & METHODS

Whole spinal cords were removed as detailed in Chapter 2 and then sectioned into cervical, thoracic and lumbo-sacral regions. Further divisions into dorsal and ventral halves were then made. Homogenates were prepared and binding assays performed as detailed in Chapter 2.

\[^{1}H\](-)-Bremazocine, \[^{3}H\]etorphine, \[^{3}H\][D-Ala\textsuperscript{2},D-Leu\textsuperscript{5}]enkephalin and \[^{3}H\][D-Ala\textsuperscript{2},MePhe\textsuperscript{5},Gly-\text{o1}\textsuperscript{5}]enkephalin were the radioligands used to define opioid binding sites. 1\,\mu M unlabelled diprenorphine was used to define non-specific binding.
RESULTS

a) **Rostro-caudal distribution of opioid binding sites**

High affinity, saturable binding was demonstrated for the unselective opioids ['H]etorphine [96] in bovine cord and for ['H](-)-bremazocine [96] in the cords from the rat, guinea-pig and farmyard pig (Figures 5.1-4; Tables 5.1 & 5.2).

['H]Etorphine bound to specific sites in the cervical, thoracic and lumbo-sacral regions of bovine cord. The affinity in the cervical region was significantly higher (p<0.05). ['H](-)-Bremazocine bound with a similar affinity to specific sites in each of these three anatomical regions of rat spinal cord.

By using 1nM each of [H][D-Ala²,MePhe⁵,Gly-ol³]enkephalin to define \( \mu \)-sites, [H][D-Ala²,D-Leu⁵]enkephalin in the presence of 25nM [D-Ala²,MePhe⁵,Gly-ol³]enkephalin, to define \( \delta \)-sites and [H](-)-bremazocine in the presence of 200nM each of [D-Ala²,MePhe⁵,Gly-ol³]enkephalin and [D-Ala²,D-Leu⁵]enkephalin to define \( \kappa \)-sites, \( \mu \)-, \( \delta \)- and \( \kappa \)-binding sites were shown to be present in cervical, thoracic and lumbo-sacral spinal cord of the rat (Figure 5.5; Table 5.3). K-Binding was predominant in all three regions. The percentages of each site were equivalent along the rostro-caudal axis. The cervical: thoracic: lumbo-sacral ratios were, for \( \mu \) 0.68: 0.72: 1, for \( \delta \) 0.89: 1.03: 1 and for \( \kappa \) 0.89: 0.69: 1 respectively.
Saturation analysis for [3H]etorphine in bovine cervical spinal cord homogenates, using a concentration range of 0.017 - 33.55nM labelled ligand. Representative plot from 5 experiments. See Table 5.1 for data.
Saturation analysis for [³H]etorphine in bovine thoracic spinal cord homogenates, using a concentration range of 0.080 - 8.53nM labelled ligand. Representative plot from 4 experiments.
See Table 5.1 for data.
Figure 5.1c Saturation analysis for [\textsuperscript{3}H]etorphine in bovine lumbo-sacral spinal cord homogenates, using a concentration range of 0.019 – 10.22nM labelled ligand. Representative plot from 7 experiments. See Table 5.1 for data.
Saturation analysis for [\textsuperscript{\textit{4}}\textit{H}](-)-bremazocine in rat cervical spinal cord homogenates, using a concentration range of 0.018 - 7.32nM radioligand. Representative plot from 3 experiments. For data see Table 5.1.
Figure 5.2b Saturation analysis for [³H](-)-bremazocine in rat thoracic spinal cord homogenates, using a concentration range of 0.018 - 7.32nM radioligand. Representative plot from 3 experiments. For data see Table 5.1.
Saturation analysis for [\(^{3}\)H\)](-)-bremazocine in rat lumbo-sacral spinal cord homogenates, using a concentration range of 0.018 - 7.58nM radioligand. Representative plot from 3 experiments. For data see Table 5.1.
Figure 5.3  Saturation analysis for $[^3]H(\text{-})$-bremazocine in guinea-pig lumbo-sacral spinal cord homogenates, using a concentration range of 0.016 - 12.42nM labelled ligand. Representative plot from 3 experiments. See Table 5.1 for data.
Saturation analysis for [³H](-)-bremazocine in porcine lumbo-sacral spinal cord homogenates, using a concentration range of 0.086 - 22.01nM radioligand. Representative plot from 3 experiments. For data see Table 5.1.
Table 5.1 Saturated binding analysis for \([^3H]\)etorphine in bovine spinal cord and \([^3H]\)(-)-bremazocine in rat, guinea-pig and porcine spinal cord homogenates at 37°C (Figures 5.1 - 5.4).

<table>
<thead>
<tr>
<th>Species</th>
<th>Region</th>
<th>Bmax (fmol.mg(^{-1}))</th>
<th>(K_D) (nM)</th>
<th>(r)</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow</td>
<td>Cervical</td>
<td>25.40 ± 6.97</td>
<td>0.77 ± 0.08*</td>
<td>0.837 - 0.967</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Thoracic</td>
<td>29.64 ± 4.77</td>
<td>1.56 ± 0.45</td>
<td>0.800 - 0.901</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Lumbo-sacral</td>
<td>32.24 ± 2.55</td>
<td>2.20 ± 0.43</td>
<td>0.789 - 0.930</td>
<td>7</td>
</tr>
<tr>
<td>Rat</td>
<td>Cervical</td>
<td>75.98 ± 18.48</td>
<td>1.19 ± 0.15</td>
<td>0.823 - 0.890</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Thoracic</td>
<td>76.27 ± 18.73</td>
<td>1.39 ± 0.01</td>
<td>0.861 - 0.944</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Lumbo-sacral</td>
<td>95.91 ± 6.34</td>
<td>1.44 ± 0.28</td>
<td>0.862 - 0.892</td>
<td>3</td>
</tr>
<tr>
<td>Guinea-Pig</td>
<td>Lumbo-sacral</td>
<td>65.62 ± 10.44</td>
<td>0.88 ± 0.16</td>
<td>0.950 - 0.981</td>
<td>3</td>
</tr>
<tr>
<td>Pig</td>
<td>Lumbo-sacral</td>
<td>20.63 ± 1.56</td>
<td>0.64 ± 0.07</td>
<td>0.896 - 0.946</td>
<td>3</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem of \(n\) separate experiments in duplicate (triplicate for the pig).

\(r\) = Correlation coefficient from non-linear regression.

* \(K_D\) Significantly different from thoracic and lumbo-sacral regions (\(P<0.05\)).

No significant difference between Bmax or \(K_D\) values for different regions except where indicated.

Non-specific binding defined using 1μM unlabelled diprenorphine.
Table 5.2 Specific: non-specific binding ratios for \(^{3}H\)opioids in selected spinal cord regions from different species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Anatomical Region</th>
<th>Specific: Non-Specific Ratio</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.1nM</td>
<td>5nM</td>
</tr>
<tr>
<td>[^{3}H^]Etorphine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cow</td>
<td>Cervical</td>
<td>2.01 ± 0.44</td>
<td>0.60 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>Thoracic</td>
<td>1.31 ± 0.12</td>
<td>0.40 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>Lumbo-sacral</td>
<td>2.53 ± 0.62</td>
<td>0.50 ± 0.05</td>
</tr>
<tr>
<td>[^{3}H^](-)-Bremazocine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>Cervical</td>
<td>7.82 ± 2.44</td>
<td>0.65 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>Thoracic</td>
<td>5.56 ± 1.33</td>
<td>0.65 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>Lumbo-sacral</td>
<td>8.04 ± 2.29</td>
<td>0.83 ± 0.10</td>
</tr>
<tr>
<td>Guinea-Pig</td>
<td>Lumbo-sacral</td>
<td>6.36 ± 0.93</td>
<td>1.05 ± 0.09</td>
</tr>
<tr>
<td>Pig</td>
<td>Lumbo-sacral</td>
<td>3.97 ± 0.77</td>
<td>0.46 ± 0.10</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem.
n = Number of separate experiments.
All assays performed at 37°C.
"Non-specific" defined as binding in presence of 1\(\mu\)M unlabelled diprenorphine.
Distribution of k-, μ- and δ-binding sites in rat spinal cord. μ-Sites were labelled with 1nM [3H][D-Ala², MePhe⁴, Gly-ol⁵]enkephalin, δ-sites with 1nM [3H][D-Ala², D-Leu⁵]enkephalin in the presence of 25nM [D-Ala², MePhe⁴, Gly-ol⁵]enkephalin and k-sites with 1nM [3H](-)-bremazocine in the presence of 200nM each of [D-Ala², D-Leu⁵]enkephalin and [D-Ala², MePhe⁴, Gly-ol⁵]enkephalin. See Table 5.3 for results.
Table 5.3 The rostro-caudal distribution of μ-, δ- and κ-binding sites in rat spinal cord, as identified by 1nM [³H]-ligands at 37°C (Figure 5.5).

<table>
<thead>
<tr>
<th>Labelled Ligand</th>
<th>Cervical</th>
<th>Thoracic</th>
<th>Lumbo-sacral</th>
</tr>
</thead>
<tbody>
<tr>
<td>[D-Ala²,MePhe⁵,Gly-ol⁵]enkephalin (0.78 - 0.95nM)</td>
<td>0.15 ± 0.07</td>
<td>0.16 ± 0.06</td>
<td>0.22 ± 0.09</td>
</tr>
<tr>
<td>[D-Ala²,D-Leu⁵]enkephalin * (0.90 - 1.01nM)</td>
<td>0.080 ± 0.014</td>
<td>0.094 ± 0.034</td>
<td>0.091 ± 0.033</td>
</tr>
<tr>
<td>(-)-Bremazocine ** (0.96 - 1.21nM)</td>
<td>0.79 ± 0.12</td>
<td>0.62 ± 0.12</td>
<td>0.89 ± 0.06</td>
</tr>
</tbody>
</table>

Results are expressed as means ± sem for 5 separate experiments in duplicate.

* [³H][D-Ala²,D-Leu⁵]Enkephalin was suppressed with 25nM unlabelled [D-Ala²,MePhe⁵,Gly-ol⁵]enkephalin to 1.0nM labelled ligand to inhibit μ-site binding.

** [³H](-)-Bremazocine was suppressed with unlabelled [D-Ala²,D-Leu⁵]enkephalin and [D-Ala²,MePhe⁵,Gly-ol⁵] enkephalin each in the ratio 100nM to 0.5nM labelled ligand, to inhibit μ- and δ- site binding.

Non-specific binding defined using 1μM unlabelled diprenorphine.
b) **Dorso-ventral distribution of opioid binding sites**

High affinity, saturable binding was demonstrated for \[^3H\]etorphine and \[^3H\](-)-bremazocine in the dorsal and ventral halves of bovine lumbo-sacral spinal cord (Figure 5.6). The dorsal: ventral ratio of Bmax values was approximately 2:1 for both ligands (Table 5.4). The specific binding for \[^3H\]etorphine was similar in both halves but for \[^3H\](-)-bremazocine was higher in the dorsal half (Table 5.6). Single experiments using \[^3H\](-)-bremazocine in cervical and thoracic regions of bovine cord afforded similar Bmax and K_d values to those seen in the lumbo-sacral region (Table 5.5).

\[^3H\](-)-Bremazocine labelled specific sites in a saturable manner in the dorsal and ventral halves of lumbo-sacral spinal cords from two strains of rat (Figure 5.7). The dorsal: ventral ratio of Bmax values was approximately 2:1 and generally higher specific binding was seen in rat cord when compared with bovine cord (Table 5.6).

\(\mu\), \(\delta\) and \(\kappa\)-Sites were all shown to be present in the dorsal and ventral halves of lumbo-sacral spinal cord of the rat (Figure 5.8; Table 5.7). The dorsal: ventral ratios were for \(\kappa\) 1.85:1, \(\mu\) 1.73:1 and \(\delta\) 1.42:1 respectively.
Saturation analysis for [\(^{3}H\)]etorphine in bovine lumbo-sacral spinal cord homogenates. Dorsal horn opioid sites (●) were labelled using a concentration range of 0.050 – 8.78nM radioligand and ventral sites (○) were labelled using a concentration range of 0.050 – 18.37nM radioligand. Representative plots from 3 experiments. See Table 5.4 for data.
Saturation analysis for [\(^{3}H\)](-)-bremazocine in bovine lumbo-sacral spinal cord homogenates. Dorsal horn binding sites (●) were labelled using a concentration range of 0.091 - 14.60nM radioligand and ventral horn sites (○) were labelled using a concentration range of 0.091 - 6.42nM radioligand. Representative plots from 3 experiments. See Table 5.4 for data.
Saturation analysis for \[^3H\](-)-bremazocine in Alderley Park rat lumbo-sacral spinal cord homogenates. Dorsal horn sites (●) and ventral horn sites (○) were labelled using a concentration range of 0.025 - 5.82nM radioligand. Representative plots from 3 experiments. See Table 5.4 for data.
Saturation analysis for [\(^3\)H](-)-bremazocine in Sutton Bonnington Wistar rat lumbo-sacral spinal cord homogenates. Dorsal horn sites (●) and ventral horn sites (○) were labelled using a concentration range of 0.040 - 6.06nM radioligand. Representative plots from 3 experiments. See Table 5.4 for data.
Table 5.4  Saturated binding analyses for \([^3H]\)etorphine and \([^3H](-)-bremazocine\) in the dorsal and ventral halves of bovine and rat lumbo-sacral spinal cord at 37°C (Figures 5.6 and 5.7).

<table>
<thead>
<tr>
<th>Species</th>
<th>Anatomical Region</th>
<th>Bmax (fmol.mg(^{-1}))</th>
<th>(K_D) (nM)</th>
<th>(r)</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>([^3H])Etorphine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cow</td>
<td>Dorsal</td>
<td>18.99 ± 3.34</td>
<td>0.67 ± 0.27</td>
<td>0.887 - 0.937</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Ventral</td>
<td>12.88 ± 1.73</td>
<td>0.58 ± 0.13</td>
<td>0.865 - 0.969</td>
<td>3</td>
</tr>
<tr>
<td>(<a href="-">^3H</a>-Bremazocine)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cow</td>
<td>Dorsal</td>
<td>29.68 ± 7.23</td>
<td>0.72 ± 0.11</td>
<td>0.916 - 0.979</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Ventral</td>
<td>17.41 ± 5.46</td>
<td>1.13 ± 0.56</td>
<td>0.861 - 0.980</td>
<td>3</td>
</tr>
<tr>
<td>* Wistar Rat</td>
<td>Dorsal</td>
<td>96.81 ± 5.60</td>
<td>0.80 ± 0.09</td>
<td>0.948 - 0.980</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Ventral</td>
<td>46.23 ± 0.48</td>
<td>0.59 ± 0.05</td>
<td>0.894 - 0.976</td>
<td>3</td>
</tr>
<tr>
<td>Alderley Park Rat</td>
<td>Dorsal</td>
<td>75.45 ± 16.82</td>
<td>1.10 ± 0.08</td>
<td>0.846 - 0.924</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Ventral</td>
<td>37.38 ± 5.63</td>
<td>1.41 ± 0.34</td>
<td>0.838 - 0.893</td>
<td>3</td>
</tr>
</tbody>
</table>

* Sutton Bonnington Wistar.

Results expressed as means ± sem of \(n\) separate experiments in duplicate.

\(r\) = Correlation coefficient from non-linear regression.

Non-specific binding defined in the presence of 1\(\mu\)M unlabelled diprenorphine.
Table 5.5 The dorso-ventral distribution of opioid binding sites in bovine spinal cord: saturation analysis for \([^3]H\)(-)bremazocine; single assays at 37°C.

<table>
<thead>
<tr>
<th>Rostro-caudal Region</th>
<th>Dorso-Ventral Region</th>
<th>Bmax (fmol.mg⁻¹)</th>
<th>(K_D) (nM)</th>
<th>(r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cervical</td>
<td>Dorsal</td>
<td>29.89</td>
<td>1.63</td>
<td>0.848</td>
</tr>
<tr>
<td></td>
<td>Ventral</td>
<td>15.01</td>
<td>0.96</td>
<td>0.903</td>
</tr>
<tr>
<td>Thoracic</td>
<td>Dorsal</td>
<td>25.96</td>
<td>2.08</td>
<td>0.942</td>
</tr>
<tr>
<td></td>
<td>Ventral</td>
<td>10.24</td>
<td>0.62</td>
<td>0.804</td>
</tr>
</tbody>
</table>

Assays performed in triplicate.

\(r\) = Correlation coefficient from non-linear regression.

Concentration ranges used for \([^3]H\)(-)bremazocine were:
- Cervical: (0.074 - 16.57nM)
- Thoracic: (0.125 - 24.00nM)

Non-specific binding defined using \(1\mu M\) unlabelled diprenorphine.
Table 5.6 Specific: non-specific binding ratios for [³H]opioids in dorsal and ventral halves of lumbo-sacral spinal cord from different species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Anatomical Region</th>
<th>Specific: Non-Specific Ratio</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.1nM</td>
<td>5nM</td>
</tr>
<tr>
<td>[³H]Etorphine</td>
<td>Dorsal</td>
<td>1.28 ± 0.22</td>
<td>0.32 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>Ventral</td>
<td>1.43 ± 0.41</td>
<td>0.39 ± 0.04</td>
</tr>
<tr>
<td><a href="-">³H</a>-Bremazocine</td>
<td>Dorsal</td>
<td>7.42 ± 1.67</td>
<td>0.54 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>Ventral</td>
<td>2.65 ± 0.55</td>
<td>0.32 ± 0.10</td>
</tr>
<tr>
<td>Wistar Rat</td>
<td>Dorsal</td>
<td>11.67 ± 2.12</td>
<td>1.54 ± 0.22</td>
</tr>
<tr>
<td></td>
<td>Ventral</td>
<td>10.58 ± 1.73</td>
<td>0.70 ± 0.10</td>
</tr>
<tr>
<td>Alderley Park Rat</td>
<td>Dorsal</td>
<td>7.63 ± 0.38</td>
<td>1.06 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>Ventral</td>
<td>4.48 ± 0.14</td>
<td>0.42 ± 0.02</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem.

n = Number of separate experiments performed in duplicate at 37°C.

Non-specific binding defined in the presence of 1μM unlabelled diprenorphine.
Figure 5.8 Distribution of k-, μ- and δ-binding sites in dorsal (D) and ventral (V) halves of rat lumbo-sacral spinal cord. μ-Sites were labelled with 1nM [3H][D-Ala²,MePhe⁵,Gly-ol⁶]enkephalin, δ-sites with [3H][D-Ala²,D-Leu⁴]enkephalin in the presence of 25nM [D-Ala²,MePhe⁵,Gly-ol⁶]enkephalin and k-sites with [3H](-)-bremazocine in the presence of 200nM each of [D-Ala²,D-Leu⁴]enkephalin and [D-Ala²,MePhe⁵,Gly-ol⁶]enkephalin. Results shown in Table 5.7.
Table 5.7  The dorso-ventral distribution of μ-, δ- and κ-binding sites in rat lumbo-sacral spinal cord, as identified by 1nM [³H]ligands at 37°C (Figure 5.10).

<table>
<thead>
<tr>
<th>Labelled Ligand</th>
<th>Specifically Bound (pmol.g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dorsal Horn</td>
</tr>
<tr>
<td>[D-Ala²,MePhe³,Gly-ol⁵]enkephalin</td>
<td>0.19 ± 0.05</td>
</tr>
<tr>
<td>[D-Ala²,D-Leu⁵]enkephalin *</td>
<td>0.17 ± 0.06</td>
</tr>
<tr>
<td>(-)-Bremazocine **</td>
<td>0.98 ± 0.19</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem of 4 separate experiments in duplicate.

* [³H][D-Ala²,D-Leu⁵]Enkephalin suppressed with 25nM unlabelled [D-Ala²,MePhe³,Gly-ol⁵]enkephalin for each 1.0nM labelled ligand, to inhibit binding at μ-sites.

** [³H](-)-Bremazocine suppressed with unlabelled [D-Ala²,D-Leu⁵]enkephalin and [D-Ala²,MePhe³,Gly-ol⁵] enkephalin each in the ratio 100nM to 0.5nM labelled ligand, to inhibit binding at μ- and δ- sites.

Non-specific binding was defined using 1μM unlabelled diprenorphine.
DISCUSSION

a) Rostro-caudal distribution of opioid binding sites

Higher levels of binding were evident in the spinal cords from laboratory species when compared to those from farmyard species, findings which are in agreement with a previous study in brain tissues [156]. Such species differences may also help to explain the anomalies for the $K_D$ values along the rostro-caudal axis in spinal cords from the rat and cow. The relatively uniform distribution of the number of opioid binding sites along the rostro-caudal axis agrees with published work using [$^3$H]etorphine in the rat [155] but is contrary to results in man, where [$^3$H]diprenorphine sites increased from rostral through to caudal sections [152]. However, recent work using quantitative autoradiography for [$^3$H]diprenorphine binding in human spinal cord suggests an homogeneous rostro-caudal distribution [146].

In the current work [$^3$H]opioids displayed a lower affinity for spinal binding sites than has been demonstrated previously in the brain [97], which refutes the work of Mack and colleagues [193] who found no such discrepancy between spinal cord and brain affinities in the rat.

The lower affinity may be caused by the large amounts of myelin present in the cord. Entrapment of [$^3$H]opioid within liposomes would reduce the free ligand concentration at the level of the binding site and hence reduce the observed affinity. Additionally adsorption of the [$^3$H]ligands onto the myelin or other subcellular contaminants [215] may also account for the higher levels of non-specific binding seen in spinal cord binding assays especially at higher concentrations of labelled ligand. This problem has been encountered by other workers [154, 158-60].
By using a non-linear curve-fitting programme [161] the data for all experiments fitted best a single site model. This was not unexpected in view of the similar affinities at \( \mu \)-, \( \delta \)- and \( \kappa \)-sites for etorphine and bremazocine [96]. All three binding site types have previously been found in cervical, thoracic and lumbo-sacral regions of rat [158, 162] and guinea-pig [154] and in thoracic through to sacral regions in the human [152]. However, several studies by Gouarderes and co-workers failed to identify \( \mu \)- or \( \delta \)-binding sites in lumbo-sacral spinal cords from rat [155, 163, 164], guinea-pig, monkey or man [163], or \( \delta \)-sites in the thoracic region of the rat [155]. This was partially supported by evidence from autoradiographical studies in rat and guinea-pig cord [165].

Recent work in rat spinal cord [158] has shown constant cervical: thoracic: lumbo-sacral ratios for \( \mu \)-, \( \delta \)- and \( \kappa \)-sites, but suggested a proliferation of \( \mu \)-sites in contrast to the data presented in this chapter. The high level of \( \kappa \)-binding in the present studies agrees with previous work from this laboratory in the rat [160, 192] and with results of other workers in the guinea-pig [154] and in man [152]. Note should be taken that for the data in Table 5.3, the concentrations of \( \mu \)- and \( \delta \)-ligands were close to their \( K_D \) values, whilst the concentration of \([^3H](-)-bremazocine\) was considerably greater than its \( K_D \) values (as determined in rat brain [97]). Therefore, the proportions of \( \mu \)- and \( \delta \)-sites are underestimated compared to \( \kappa \)-sites.

An extensive literature has shown the presence of many different endogenous opioid peptides in spinal cords from various species. These include prodynorphin-derived [118, 151, 166, 167] and proenkephalin A-derived [118, 150, 168, 169] peptides but appear to exclude the pro-opiomelanocortin-derived peptide \( \beta \)-endorphin [121, 151].
However, Haynes et al. [170] demonstrated the existence of β-endorphin immunoreactivity in neonatal rat spinal cord up to the 28th postnatal day, after which all immunoreactivity abruptly disappeared, suggesting a β-endorphine-like peptide is synthesised in the spinal cord during development.

The presence of high numbers of k-sites in the rat cord complements the presence of prodynorphin-derived peptides [118, 148, 166, 167], many of which have been shown to possess potent activity at the k-receptor [71, 72, 171]. However, reports concerning their rostra-caudal distribution are inconsistent. For example Cox et al. [172] found a relatively homogeneous distribution of immunoreactive dynorphin A-(1-17), whilst Zamir and co-workers [173] suggested much higher concentrations of immunoreactive dynorphin B in the cervical region which decreased caudally. These results suggest differential processes since both dynorphin A-(1-17) and dynorphin B are cleaved from the same precursor molecule [58]. Przewlocki and co-workers [151] showed a six-fold increase in levels of immunoreactive dynorphin A-(1-17) and a three-fold increase for immunoreactive α-neo-endorphin in the sacral region over the cervical region in human spinal cord.

Proenkephalin A-derived peptides show similarly inconsistent results for their rostro-caudal distribution. [Leu⁴]Enkephalin has been shown to be homogeneously distributed [169] and [Met⁵]enkephalin concentrations to increase caudally [150, 151]. [Met⁵]Enkephalyl-Arg⁶-Phe⁷ and [Met⁵]enkephalyl-Arg⁶-Gly⁷-Leu⁸, two extended enkephalins showing increased selectivity for k-receptors [103-5, 174] both increase in a caudal direction [149, 150].

The binding site distribution thus appears to show little correlation with many of the reports on the rostro-caudal distribution of endogenous peptides.
b) Dorso-ventral distribution of opioid binding sites

Results presented in this chapter for the dorso-ventral distribution of opioid binding sites in spinal cord support existing data in the rhesus monkey [145] and in man [152]. High dorsal cord binding has been reported for [^3H]naloxone, [^3H]dihydromorphine and [^3H]buprenorphine in the rat [175] and for [^3H]β-endorphin in the rabbit [176]. Binding data is also supported by a wealth of autoradiographical evidence. Reports for the rat [115, 116, 165, 177, 178], guinea-pig [165], monkey [119, 143] and in humans [146, 179, 180] all show a high dorsal:ventral ratio for [^3H]opioid binding sites. A similar pattern has been demonstrated in the cervical, thoracic and lumbo-sacral regions of spinal cords from different species [115, 119, 146, 165, 179] supporting the findings in bovine cord in this current work.

The results for the dorso-ventral distribution of µ-, δ- and k-binding sites agree with findings in rat, guinea-pig [165] and in human [152] spinal cord. The dorsal:ventral ratios of µ-, δ- and k-sites in the lumbo-sacral region of human cord (Table 5.8) were similar to those seen with rat cord in the present work (Table 5.7).

Results from autoradiography show a distinct localization for opioid binding sites in the superficial laminae of the dorsal horn. This area is known to be important for the modulation of responses to nociceptive stimuli. High binding in the dorsal horn is not therefore unexpected. High levels of binding in the ventral cord are less easy to explain. Such sites, which are not present in distinct bands, may be on sensory fibres [153] or may be associated with motor-function [169, 181].

The binding pattern for [^3H]opioids correlates well with the dorso-ventral distribution of endogenous opioid peptides and peptides associated with pain mechanisms. For example,
Table 5.8  Distribution of opioid binding sites in human spinal cord.
(From Czlonkowski, A. et al [152]).

<table>
<thead>
<tr>
<th>Regions</th>
<th>Classes of binding site (pmol.g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µ</td>
</tr>
<tr>
<td>Sacral dorsal</td>
<td>1.95 ± 0.31</td>
</tr>
<tr>
<td>Sacral ventral</td>
<td>1.70 ± 0.19</td>
</tr>
<tr>
<td>Lumbar dorsal</td>
<td>1.76 ± 0.12</td>
</tr>
<tr>
<td>Lumbar ventral</td>
<td>1.42 ± 0.28</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem for 4 separate experiments.
Assays performed at 25°C for 90 minutes.

µ-, δ- and k-sites were labelled using 0.5nM [³H]diprenorphine. µ-binding was then displaced using 10μM β-casomorphin-4-amide, δ-binding was then displaced using 0.1μM [D-Ala², D-Leu⁵]enkephalin and k-binding represented the remaining binding.
Substance P is released from primary afferents upon noxious stimulation and can be inhibited by opioid compounds [124]. The superficial laminae of the dorsal horn is believed to contain a large number of these synapses. Immunohistochemical studies have shown a close association between substance P-containing and enkephalinergic fibres, many of which are concentrated in this area [146, 169, 182-4]. It has been proposed that enkephalins or endogenous compounds are released to prevent the release or interaction of substance P with its receptor, thus modulating nociceptive information [124]. Binding studies with [3H]substance P in rat thoracic spinal cord [185] have shown a concentration of binding sites in the substantia gelatinosa of lamina II. Quantification of immunoreactive peptides shows that dorsal horn concentrations of the proenkephalin A-derived peptides [Met⁵]enkephalyl-Arg⁶-Phe⁷ [150] and [Met⁴]enkephalyl-Arg⁶-Gly⁷-Leu⁸ [149] are approximately two times those found in the ventral horn of rat cord.

Of the immunoreactive prodynorphin-derived peptides present in rat spinal cord, dynorphin B was found almost exclusively in the marginal layer of lamina I in the dorsal horn [186]. Quantitative studies in human cord [151] show the prodynorphin products e-neo-endorphin and dynorphin A-(1-17) to be much higher in dorsal than ventral laminae rostro-caudally.

The differential distribution of the proenkephalin A- and prodynorphin-derived peptides suggests that they may have different roles in spinal function, not necessarily associated with pain systems. For example, substance P, although related to nociceptive transmission also appears to be involved with ventral motor function [184].

The current results support analgesia in intrathecal dosing studies with animals, using highly selective μ-, δ- and k-compounds against different nociceptive stimuli [191].
Additionally, the present work supports evidence for spinal \(\mu\)-, \(\delta\)- and \(\kappa\)-receptors which has come from clinical studies, where post-operative and intractable cancer pain has been treated by, amongst others, \(\kappa\)-selective dynorphin A-(1-17) [187], \(\mu\)-selective morphine [188] and relatively \(\delta\)-selective \([D-Ala^2, D-Leu^5]enkephalin\) [189].

In conclusion, this work demonstrates that spinal opioid sites are widely distributed throughout the cords of different species. The distribution of opioid sites along the rostro-caudal axis does not correlate well with reported distributions of endogenous opioid peptides. However, the dorso-ventral distribution of opioid sites correlates well with proposed endogenous opioids and neuromodulators associated with nociceptive transmission. Biochemical data in this chapter lends further support for the involvement of \(\mu\)-, \(\delta\)- and \(\kappa\)-receptors in spinally mediated analgesia.
CHAPTER 6

CHARACTERIZATION OF SPINAL K-OPIOID BINDING SITES
INTRODUCTION

Opioid receptors were first classified into varying types following *in vivo* work in the spinal dog by Martin and colleagues [17]. On the basis of their work μ-, κ- and σ-types were proposed of which μ- and κ-types were associated with antinociception. The δ-receptor was later proposed by Kosterlitz and co-workers [66, 67] after ligand specificity in the isolated mouse vas deferens preparation was found to be different to that in the guinea-pig ileum preparation.

The refinement of ligand binding techniques and the development of autoradiography showed opioid binding sites to be differentially distributed in the brain [116] and spinal cord [115]. During the search for endogenous substances which could interact with the opioid receptors, it became apparent that three main groups of opioid peptides existed. β-Endorphin was cleaved from proopiomelanocortin [64], the enkephalins were derived from the precursor proenkephalin A [64] and the cleavage of prodynorphin (also called proenkephalin B) [65] yielded the dynorphins. The concomitant development of immunohistochemical techniques [117] enabled study of the location of endogenous opioid peptides within the central nervous system and their relationship to opioid receptor distribution.

Of particular interest to this present work are the dynorphins which have been shown to display high potency at the κ-receptor [49, 50, 72]. These peptides were identified in regions of the spinal cord associated with pain transmission and where high levels of opioid binding sites were also found [134]. In support of a role for dynorphins in antinociception at the spinal level, intrathecal dosing studies have shown that these peptides, in addition to other κ-selective agents are effective in selectively reducing low grade nociceptive stimuli [191]. They appear to have little effect on high threshold stimuli such as cutaneous-thermal pain [191], although there is some evidence to the contrary [142, 202].
Whilst the presence of μ- and δ-sites in the spinal cord is accepted, inconsistencies have arisen during the attempted classification of k-opioid sites. Analysis of the k-population has produced results which suggest more than a single site [195] and the proposal of k-subtypes has been made [196].

The aim of this study has been to investigate the nature of the spinal k-opioid sites in different species but with particular emphasis on the rat.
Lumbo-sacral spinal cords were removed, homogenates prepared and binding assays performed as detailed in Chapter 2. The radioligands used to label opioid sites were [³H](−)-bremazocine, [³H]diprenorphine, [³H]dynorphin A-(1-9) and [³H]U-69593. An incubation temperature of 25°C for 30 minutes was used for experiments involving [³H]dynorphin A-(1-9) and the proteolytic degradation of this ligand was inhibited by including 300μM captopril and 30μM bestatin in the assay mixture [197]. The chemical structures of competing ligands are described in Chapter 2. Non-specific binding was defined in the presence of 1μM unlabelled diprenorphine, or 10μM unlabelled naloxone when [³H]diprenorphine was the labelled primary ligand. Methods of analysis are given in Chapter 3.

The proteolytic degradation of [³H]dynorphin A-(1-9) was monitored by HPLC analysis as detailed in Chapter 2.
RESULTS

a) Displacement studies

After suppression of binding to \(\mu\)- and \(\delta\)-sites using 100nM each of [D-Ala\(^2\),D-Leu\(^5\)]enkephalin and [D-Ala\(^2\), MePhe\(^\ast\), Gly-ol\(^\ast\)]enkephalin, \([^3H](-)-bremazocine\) bound specifically in homogenates of lumbo-sacral spinal cord from the rat. This binding was completely displaced by 1\(\mu\)M naloxone, and levorphanol was approximately 2400 times more potent than dextrophan at these sites (Figure 6.1; Table 6.1). Non-selective opioids displaced the bound material with Hill slopes of close to unity (Figure 6.4), but ligands showing selectivity for individual opioid site-types afforded slopes of less than unity (Figure 6.2). In particular the displacement profile for [D-Ala\(^2\),D-Leu\(^5\)] enkephalin was noticeably biphasic. Similar results obtained in our laboratory afforded a \(K_i\) 8945 ± 823nM and Hill coefficient 0.61 ± 0.07 (Figure 6.3; J. R. Traynor personal communication) which could be resolved into two Hill plots. The high affinity component afforded a \(K_i\) 321 ± 68nM and slope 1.26 ± 0.35 and the lower affinity component \(K_i\) 25890 ± 5140nM and slope 0.89 ± 0.11 in line with its reported k-affinity [95].

\([^3H](-)-Bremazocine\) was displaced by the k-selective opioids U-50488H and U-69593 in homogenates of porcine lumbo-sacral spinal cord with low affinities. When \(\mu\)- and \(\delta\)-binding was suppressed U-50488H, but not U-69593 showed an increase in slope and affinity (Figure 6.5; Table 6.2). A single experiment in guinea-pig lumbo-sacral spinal cord homogenate afforded a similarly biphasic curve for U-50488H. The high affinity component afforded a \(K_i\) 321 ± 68nM and slope 1.26 ± 0.35 and the lower affinity component \(K_i\) 25890 ± 5140nM and slope 0.89 ± 0.11 in line with its reported k-affinity [95].

It can be seen from Figure 6.3 that 5\(\mu\)M [D-Ala\(^2\),D-Leu\(^5\)]enkephalin displaces all of the higher affinity component for \([^3H](-)-bremazocine\) binding in lumbo-sacral spinal cord homogenates from the rat, leaving the lower affinity site (dotted line) which appears to be a "classical k" binding component [196].
Under these conditions naloxone displaced all the remaining specific binding (97.5 ± 1.5%) though with a low affinity ($IC_{50} = 79.46 ± 1.49\text{nM}; nH = 0.88 ± 0.05; n = 3$) (Figure 6.7), and the $\mu$- and $\delta$-selective peptides and U-50488H displaced with slopes closer to unity (Figure 6.6; Table 6.3). Similar results were obtained in a second strain of rat (Table 6.3).
Figure 6.1 Displacement of $[^3H](-)$-bremazocine (0.14-0.26nM) in the presence of 100nM each [D-Ala$^2$,D-Leu$^5$]enkephalin and [D-Ala$^2$,MePhe$^4$,Gly-ol$^5$]enkephalin to 0.5nM labelled ligand, by levorphanol (●), dextrorphan (◇) and naloxone (▲) in rat lumbo-sacral spinal cord homogenates. See Table 6.1 for data.

Figure 6.2 Displacement of $[^3H](-)$-bremazocine (0.14-0.26nM) by U-50488H (■), [D-Ala$^2$,D-Leu$^5$]enkephalin (○) and [D-Ala$^2$,MePhe$^4$,Gly-ol$^5$]enkephalin (●) in rat lumbo-sacral spinal cord homogenates, under conditions described in Figure 6.1. See Table 6.1 for data.
Figure 6.3 Displacement of [3H](-)-bremazocine (0.5nM), in the presence of 100nM each [D-Ala²,D-Leu⁵]enkephalin and [D-Ala²,MePhe⁴,Gly-ol³]enkephalin to 0.5nM labelled ligand by [D-Ala²,D-Leu⁵]enkephalin in rat lumbo-sacral spinal cord homogenates; a) displacement curve, b) resolution into separate Hill plots.
Displacement of $[^3H](-)$-bremazocine (0.14–0.26nM), in the presence of 100nM each $[D-	ext{Ala}^2,D-	ext{Leu}^5]$enkephalin and $[D-	ext{Ala}^2,	ext{MePhe}^4,	ext{Gly-ol}^5]$enkephalin to 0.5nM labelled ligand by $(-)$-bremazocine (■), diprenorphine (▲) and tifluadom (●) in rat lumbo-sacral spinal cord homogenates. See Table 6.1 for data.
Table 6.1  
<table>
<thead>
<tr>
<th>Unlabelled Ligand</th>
<th>$K_i$ (nM)</th>
<th>Hill Coefficient</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-Bremazocine</td>
<td>0.73 ± 0.11</td>
<td>1.07 ± 0.16</td>
<td>3</td>
</tr>
<tr>
<td>Diprenorphine</td>
<td>1.47 ± 0.42</td>
<td>0.91 ± 0.13</td>
<td>3</td>
</tr>
<tr>
<td>Tifluadom</td>
<td>16.35 ± 3.80</td>
<td>1.08 ± 0.05</td>
<td>5</td>
</tr>
<tr>
<td>U-50488H</td>
<td>145.6 ± 44.7</td>
<td>0.45 ± 0.05</td>
<td>3</td>
</tr>
<tr>
<td>Naloxone *</td>
<td>13.35 ± 1.60</td>
<td>0.86 ± 0.07</td>
<td>4</td>
</tr>
<tr>
<td>Levorphanol</td>
<td>8.60 ± 1.73</td>
<td>1.08 ± 0.16</td>
<td>3</td>
</tr>
<tr>
<td>Dextrorphan</td>
<td>20424 ± 6188</td>
<td>0.97 ± 0.09</td>
<td>5</td>
</tr>
<tr>
<td>[D-Ala$_2$,D-Leu$_3$]enkephalin</td>
<td>4511 ± 469</td>
<td>0.59 ± 0.05</td>
<td>3</td>
</tr>
<tr>
<td>[D-Ala$_2$,MePhe$_3$,Gly-ol$_3$]enkephalin **</td>
<td>1338 ± 309</td>
<td>0.83 ± 0.04</td>
<td>3</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem; $n = $ number of separate experiments performed in duplicate.

Specific binding, after suppression of binding to $\mu$- and $\delta$- sites was greater than 60% and linear correlation coefficient greater than 0.961 (0.928 for U-50488H).

* Specific binding was completely displaced (100.6 ± 1.7%) by 1$\mu$M unlabelled naloxone.

** J. R. Traynor; personal communication.

$\mu$- and $\delta$-Suppression represented 51 ± 2% ($n = 24$) of the total [$^3$H](-)-bremazocine specifically bound.

Non-specific binding was defined using 1$\mu$M unlabelled diprenorphine.
Figure 6.5 Displacement of [³H](-)-bremazocine (0.44-0.54nM), in the absence (open symbols) and presence (closed symbols) of 100nM each of [D-Ala², D-Leu⁵]enkephalin and [D-Ala², MePhe⁵, Gly-ol⁵]enkephalin to 0.5nM labelled ligand, by U-50488H (◊) and U-69593 (○) in porcine lumbo-sacral spinal cord homogenates. See Table 6.2 for data.
Table 6.2

<table>
<thead>
<tr>
<th>Unlabelled Ligand</th>
<th>Ki (nM)</th>
<th>Hill Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>U-50488H</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Unsuppressed)</td>
<td>93.93 ± 33.00</td>
<td>0.49 ± 0.01</td>
</tr>
<tr>
<td>(Suppressed)</td>
<td>21.05 ± 5.40</td>
<td>0.73 ± 0.03</td>
</tr>
<tr>
<td>U-69593</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Unsuppressed)</td>
<td>87.86 ± 12.55</td>
<td>0.95 ± 0.08</td>
</tr>
<tr>
<td>(Suppressed)</td>
<td>95.79 ± 23.85</td>
<td>0.91 ± 0.18</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem for 3 separate experiments in triplicate.

Correlation coefficients from linear regression were greater than 0.968 for U-50488H and U-69593 for all assays.

Specific binding was greater than 50% for all assays.

Non-specific binding was defined in the presence of 1μM unlabelled diprenorphine.
Displacement of $[^3H](-)$-bremazocine (0.28-0.62nM) in the presence of 5μM [D-Ala$^2$, D-Leu$^5$]enkephalin, by U-50488H (■), [D-Ala$^2$, D-Leu$^5$]enkephalin (○) and [D-Ala$^2$, MePhe$^5$, Gly-$\alpha$-$\delta$]enkephalin (△) in rat lumbo-sacral spinal cord homogenates. See Table 6.3 for data.

Displacement of $[^3H](-)$-bremazocine (0.45nM) by naloxone in rat lumbo-sacral spinal cord homogenates, under conditions described in Figure 6.6.
Table 6.3

Potencies of opioid compounds to displace $[^3H]$(-)-bremazocine in the presence of 5μM unlabelled $[\text{D-Ala}^2, \text{D-Leu}^5]$enkephalin to 0.5nM radiolabelled ligand, in lumbo-sacral spinal cord homogenates from two strains of rat at 37°C. (Figures 6.6 and 6.7).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Unlabelled Ligand</th>
<th>Ki (nM)</th>
<th>Hill Coefficient</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alderley Park</td>
<td>$[\text{D-Ala}^2, \text{D-Leu}^5]$enkephalin</td>
<td>35394 ± 1929</td>
<td>0.84 ± 0.10</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>$[\text{D-Ala}^2, \text{MePhe}^6, \text{Gly-ol}^7]$enkephalin</td>
<td>8983 ± 1737</td>
<td>1.13 ± 0.09</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>U-50488H</td>
<td>22.80 ± 3.44</td>
<td>0.77 ± 0.08</td>
<td>3</td>
</tr>
<tr>
<td>Sutton Bonnington</td>
<td>$[\text{D-Ala}^2, \text{D-Leu}^5]$enkephalin</td>
<td>2457.5</td>
<td>1.00</td>
<td>1</td>
</tr>
<tr>
<td>Wistar</td>
<td>$[\text{D-Ala}^2, \text{MePhe}^6, \text{Gly-ol}^7]$enkephalin</td>
<td>3223, 2744</td>
<td>0.94, 0.77</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>U-50488H</td>
<td>18.00</td>
<td>0.80</td>
<td>1</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem; n = number of separate experiments in duplicate. Correlation coefficient for linear regression was greater than 0.970 for all assays. $[^3H]$(-)-Bremazocine concentrations used were: Alderley Park 0.28 - 0.62 nM, Sutton Bonnington Wistar 0.45 - 0.48 nM.

Specific binding was greater than 60% after suppression for all assays, and was displaced by 1μM unlabelled naloxone (97.5 ± 2.0%, n = 3; Figure 6.7). Non-specific binding was defined in the presence of 1μM unlabelled diprenorphine.
b) Saturation analysis

[^H](-)-Bremazocine bound to high affinity sites in a saturable manner in lumbo-sacral spinal cord homogenates from the rat (Figure 6.8a). In the presence of 100nM each of unlabelled [D-Ala²,D-Leu⁵]enkephalin and [D-Ala²,MePhe⁴,Gly-ol⁵]enkephalin to 0.5nM labelled ligand, [^H](-)-bremazocine displayed specific, high affinity, saturable binding in lumbo-sacral spinal cord homogenates from the rat, guinea-pig and farmyard pig (Figures 6.8b, 6.10, 6.11). The Kp values were similar in all species (range 0.40 - 0.72nM) but the number of sites was lower in the farmyard pig (~9 fmol.mg⁻¹) than in the laboratory species (~30 fmol.mg⁻¹) (Table 6.4). For each of the species studied, the number of sites was reduced in the presence of µ- and δ-suppression when compared to the total binding of [^H](-)-bremazocine in the same tissue (Table 6.4). From these findings the percentages of apparent k-binding showed little variation between species. Similar results were obtained for whole spinal cord preparations from a second strain of rat (Figure 6.9; Table 6.4).

The non-selective opioid antagonist [^H]diprenorphine also displayed high affinity, saturable binding in lumbo-sacral spinal cord homogenates from the rat and guinea-pig (Figures 6.12 & 6.13). High specific binding was obtained with this radioligand in both species (Table 6.5). In the rat, the sites remaining after µ- and δ-suppression for which the radioligand showed an increased affinity, represented 28% of the total sites. In the guinea-pig the remaining sites represented 43% of the total sites (Table 6.6) but there was no apparent increase in the affinity of [^H]diprenorphine for these sites.

The total binding in rat cord was approximately twice that in guinea-pig cord. It should be noted that more sites were labelled by [^H]diprenorphine in both species than were labelled by [^H](-)-bremazocine.

In the presence of 5µM [D-Ala²,D-Leu⁵]enkephalin 0.5nM⁻¹ labelled ligand, [^H](-)-bremazocine bound to a small population,
$B_{\text{max}} = 13.60 \pm 1.32 \text{ fmol.mg}^{-1}$ of high affinity sites, $K_D = 0.26 \pm 0.07 \text{nM} \quad (n = 3, \ r = 0.938 - 0.969)$, in rat lumbo-sacral spinal cord homogenates. These sites represented $15.7 \pm 0.3\%$ of the total sites labelled by $[^3\text{H}](\text{--})$-bremazocine (Figure 6.14).

Opioid sites in lumbo-sacral spinal cord homogenates from the guinea-pig and rat were labelled in a saturable manner by $[^3\text{H}]$dynorphin A-(1-9) (Figures 6.15-17; Table 6.7). The total number of sites labelled in the guinea-pig by this radioligand was similar to the levels labelled by $[^3\text{H}](\text{--})$-bremazocine and approximately twice that seen in the rat. The $B_{\text{max}}$ was reduced in both species when $\mu$- and $\delta$-suppression was present, indicating cross reactivity. The $B_{\text{max}}$ seen in the rat for $[^3\text{H}]$dynorphin A-(1-9) under these suppressing conditions was similar to that obtained using $[^3\text{H}](\text{--})$-bremazocine in the presence of $5\mu\text{M} \ [D-\text{Ala}^2,\text{D-Leu}^5]$enkephalin. The specific binding for $[^3\text{H}]$dynorphin A-(1-9) was low (~50%) in the above experiments (Table 6.8) making accurate interpretation of the data difficult.

The $\kappa$-selective opioid $[^3\text{H}]\text{U-69593} \ [198]$ afforded $B_{\text{max}}$ and $K_D$ values of $24.08 \pm 1.87 \text{ fmol.mg}^{-1}$ and $8.45 \pm 1.44 \text{nM}$ respectively ($n = 3, \ r = 0.887 - 0.931$; Figure 6.18) in rat lumbo-sacral spinal cord homogenates. Once more, the very low levels of specific binding (~12%) for the radioligand made the results subject to error (Table 6.8).
Saturation analysis for $[^3]$H(−)-bremazocine in rat lumbo-sacral spinal cord homogenates, using a concentration range of 0.068 - 9.51nM labelled ligand. Representative plot from 3 experiments. See Table 6.4 for data.
Saturation analysis for [³H](-)-bremazocine, in the presence of 100nM each [D-Ala²,D-Leu⁴] enkephalin and [D-Ala²,MePhe⁴,Gly-ol⁵]enkephalin to 0.5nM labelled ligand, in rat lumbosacral spinal cord homogenates. A concentration range of 0.068 - 5.36nM labelled ligand was used. Representative plot from 3 experiments. See Table 6.4 for data.
Saturation analysis for $[^3H](-)$-bremazocine, in the presence of 100nM each $[D-Ala^2, D-Leu^5]$ enkephalin and $[D-Ala^2, MePhe^4, Gly-ol^5]$enkephalin to 0.5nM labelled ligand, in Sutton Bonnington Wistar rat whole spinal cord homogenates. A concentration range of 0.025 - 7.95nM labelled ligand was used. Representative plot from 3 experiments. See Table 6.4 for data.
Saturation analysis for [\(^{3}H\)(-)-bremazocine, in the presence of 100nM each [D-Ala\(^2\),D-Leu\(^5\)] enkephalin and [D-Ala\(^2\),MePhe\(^*\),Gly-ol\(^3\)]enkephalin to 0.5nM labelled ligand, in guinea-pig lumbo-sacral spinal cord homogenates, using a concentration range of 0.056 - 12.22nM labelled ligand. Representative plot from 3 experiments. See Table 6.4 for data.
Saturation analysis for [3H](-)-bremazocine, in the presence of 100nM each [D-Ala²,D-Leu⁵] enkephalin and [D-Ala²,MePhe⁶,Gly-ol⁷]enkephalin to 0.5nM labelled ligand, in porcine lumbo-sacral spinal cord homogenates, using a concentration range of 0.035 – 22.07nM labelled ligand. Representative plot from 3 experiments. See Table 6.4 for data.
<table>
<thead>
<tr>
<th>Species</th>
<th>Bmax (fmol. mg⁻¹)</th>
<th>Kᵰ (nM)</th>
<th>r</th>
<th>% K</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>33.59 ± 3.77</td>
<td>0.72 ± 0.14</td>
<td>0.963 - 0.977</td>
<td>38.7 ± 1.3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>(85.42 ± 7.94)</td>
<td>(1.27 ± 0.21)</td>
<td>(0.890 - 0.960)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sutton Bonnington</td>
<td>31.27 ± 1.12</td>
<td>0.61 ± 0.06</td>
<td>0.937 - 0.976</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Wistar Rat **</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guinea-Pig</td>
<td>27.56 ± 3.58</td>
<td>0.40 ± 0.05</td>
<td>0.937 - 0.976</td>
<td>46.1 ± 9.2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>(65.62 ± 10.44)</td>
<td>(0.88 ± 0.16)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pig</td>
<td>8.93 ± 0.61</td>
<td>0.56 ± 0.14</td>
<td>0.808 - 0.944</td>
<td>43.3 ± 0.14</td>
<td>3*</td>
</tr>
<tr>
<td></td>
<td>(20.63 ± 1.56)</td>
<td>(0.64 ± 0.07)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results expressed as means ± sem; r = correlation coefficient from non-linear regression. 
n = Number of separate experiments performed in duplicate (* triplicate for the pig). 
% K = % K-Sites from saturation analysis. ** Data obtained using whole spinal cord homogenates. 
Figures in parentheses represent total binding of [³H](-)-bremazocine: + See Table 5.1 
++ See Figure 6.8a

Non-specific binding defined in the presence of 1μM unlabelled diprenorphine.
Figure 6.12 Saturation analysis for \(^{3}H\)diprenorphine in rat lumbo-sacral spinal cord homogenates. A concentration range of 0.051 - 17.52nM radioligand was used in the absence of suppression (●) and 0.031 - 10.15nM radioligand was used in the presence of 100nM each [D-Ala\(^2\),D-Leu\(^5\)] enkephalin and [D-Ala\(^2\),MePhe\(^\beta\),Gly-ol\(^\beta\)]enkephalin to 0.45nM labelled ligand (○). Representative plots from 3 experiments. See Table 6.6 for data.
Figure 6.13 Saturation analysis for [³H]diprenorphine in guinea-pig lumbo-sacral spinal cord homogenates. A concentration range of 0.042 - 10.01 nM radioligand was used in the absence of suppression (●) and 0.022 - 5.41 nM radioligand was used in the presence of 100 nM each [D-Ala²,D-Leu⁵]enkephalin and [D-Ala²,MePhe⁴,Gly-ol⁵]enkephalin to 0.45 nM labelled ligand (○). Representative plots from 3 experiments. See Table 6.6 for data.
Table 6.5

$[^3H]$Diprenorphine binding to lumbo-sacral spinal cord homogenates from rat and guinea-pig, in the absence and presence of $\mu$- and $\delta$-suppression, at selected low and high ligand concentrations at 37°C.

<table>
<thead>
<tr>
<th>Species</th>
<th>Assay Conditions</th>
<th>Approx. Conc./nM</th>
<th>Counts (dpm.mg$^{-1}$)</th>
<th>% Specific</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total</td>
<td>Saturated</td>
<td>Specific</td>
</tr>
<tr>
<td>Rat</td>
<td>Unsuppressed</td>
<td>0.1</td>
<td>1248 ± 134</td>
<td>257 ± 40</td>
<td>991 ± 153</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0</td>
<td>9461 ± 597</td>
<td>1237 ± 28</td>
<td>8224 ± 610</td>
</tr>
<tr>
<td></td>
<td>*Suppressed</td>
<td>0.1</td>
<td>547 ± 10</td>
<td>93 ± 9</td>
<td>454 ± 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0</td>
<td>4341 ± 255</td>
<td>1673 ± 137</td>
<td>2668 ± 147</td>
</tr>
<tr>
<td>Guinea Pig</td>
<td>Unsuppressed</td>
<td>0.1</td>
<td>1097 ± 186</td>
<td>75 ± 14</td>
<td>1022 ± 136</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0</td>
<td>6764 ± 768</td>
<td>1486 ± 543</td>
<td>5278 ± 402</td>
</tr>
<tr>
<td></td>
<td>*Suppressed</td>
<td>0.1</td>
<td>695 ± 212</td>
<td>83 ± 16</td>
<td>612 ± 213</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0</td>
<td>4130 ± 559</td>
<td>1779 ± 506</td>
<td>2351 ± 231</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem of n separate experiments in duplicate.
Counts expressed as dpm.mg$^{-1}$ protein.
*Suppressed at 100nM $[D-Ala^2,D-Leu^5]$enkephalin and $[D-Ala^2,MePhe^4,Gly-ol^5]$enkephalin $0.45nM^{-1}$ labelled ligand.
Non-specific binding defined in the presence of 10μM unlabelled naloxone.
Table 6.6 Saturated binding analysis in rat and guinea-pig lumbo-sacral spinal cord homogenates for \([^{1}H]\)diprenorphine, in the presence and absence of 100nM each of [D-Ala\(^4\),D-Leu\(^4\)]enkephalin and [D-Ala\(^4\),MePhe\(^4\),Gly-ol\(^5\)]enkephalin to 0.45nM labelled ligand, at 37°C. (Figures 6.12 & 6.13).

<table>
<thead>
<tr>
<th>Species</th>
<th>Assay</th>
<th>Bmax (fmol.mg(^{-1}))</th>
<th>(K_p) (nM)</th>
<th>r</th>
<th>% K</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>Unsuppressed</td>
<td>153.3 ± 5.26</td>
<td>1.03 ± 0.29</td>
<td>0.871 - 0.974</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Suppressed</td>
<td>43.41 ± 3.61</td>
<td>0.50 ± 0.04</td>
<td>0.944 - 0.962</td>
<td>28.2 ± 1.6</td>
<td>3</td>
</tr>
<tr>
<td>Guinea Pig</td>
<td>Unsuppressed</td>
<td>83.14 ± 4.48</td>
<td>0.40 ± 0.02</td>
<td>0.942 - 0.969</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Suppressed</td>
<td>33.64 ± 2.97</td>
<td>0.40 ± 0.16</td>
<td>0.907 - 0.940</td>
<td>42.7 ± 1.5</td>
<td>3</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem; \(r\) = correlation coefficient from non-linear regression.

\(n\) = Number of separate experiments performed in duplicate.

\(\% K\) = \% K-Sites from saturation analysis.

Non-specific binding defined in the presence of 10 \(\mu\)M unlabelled naloxone.
Figure 6.14  Saturation analysis for \[^{3}H\](+) -bremazocine, in the presence of 5μM [D-Ala^2,D-Leu^5]enkephalin to 0.5nM labelled ligand, in rat lumbo-sacral spinal cord homogenates. A concentration range of 0.068 - 2.70nM radioligand was used. Representative plot from 3 experiments.
Figure 6.15  Saturation analysis for [³H]dynorphin A-(1-9) in rat lumbo-sacral spinal cord homogenates, using a concentration range of 0.014 - 4.56nM radioligand. Representative plot from 3 experiments. See Table 6.8 for data.
Figure 6.16  Saturation analysis for $[^3]$H]dynorphin A-(1-9), in the presence of 100nM each [D-Ala²,D-Leu⁵] enkephalin and [D-Ala²,MePhe⁶,Gly-ol⁷] enkephalin to 0.1nM labelled ligand, in rat lumbo-sacral spinal cord homogenates. Results shown as individual results from 2 experiments. See Table 6.8 for data.
Saturation analysis for [³H]dynorphin A-(1-9) in guinea-pig lumbo-sacral spinal cord homogenates. A concentration range of 0.018 - 3.50 nM radioligand was used in the absence of suppression (●) and 0.010 - 2.17 nM radioligand was used in the presence of 100 nM each [D-Ala²,D-Leu⁴]enkephalin and [D-Ala²,MePhe⁵,Gly-ol³]enkephalin to 0.1 nM labelled ligand (○). Representative plots from 3 experiments. See Table 6.8 for data.
Saturation analysis for \([^3H]U-69593\) in rat lumbo-sacral spinal cord homogenates, using a concentration range of 0.45 - 31.67 nM radioligand. Representative plot from 3 experiments. See Table 6.8 for data.
Table 6.7  Saturated binding analysis for \([^3H]U-69593\) in rat and \([^3H]dynorphin\ A-(1-9)\) in rat and guinea-pig lumbo-sacral spinal cord homogenates at 37°C and 25°C respectively. (Figures 6.15 - 6.18).

<table>
<thead>
<tr>
<th>Species</th>
<th>Assay Conditions</th>
<th>Bmax (fmol.mg(^{-1}))</th>
<th>KD (nM)</th>
<th>r</th>
<th>% K</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>([^3H]U-69593)</td>
<td>Unsuppressed</td>
<td>24.08 ± 1.87</td>
<td>8.45 ± 1.44</td>
<td>0.887 - 0.931</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Suppressed</td>
<td>7.95, 11.78</td>
<td>0.14, 0.29</td>
<td>0.920, 0.896</td>
<td>22.7, 30.4</td>
<td>2</td>
</tr>
<tr>
<td>([^3H]Dynorphin\ A-(1-9))</td>
<td>Unsuppressed</td>
<td>34.65 ± 2.46</td>
<td>0.71 ± 0.11</td>
<td>0.942 - 0.979</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Suppressed</td>
<td>18.60 ± 2.39</td>
<td>0.56 ± 0.06</td>
<td>0.846 - 0.953</td>
<td>24.6 ± 0.7</td>
<td>3</td>
</tr>
<tr>
<td>Guinea-Pig</td>
<td>Unsuppressed</td>
<td>75.39 ± 8.52</td>
<td>0.81 ± 0.08</td>
<td>0.883 - 0.900</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Suppressed</td>
<td>18.60 ± 2.39</td>
<td>0.56 ± 0.06</td>
<td>0.846 - 0.953</td>
<td>24.6 ± 0.7</td>
<td>3</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem; \(r\) = correlation coefficient from non-linear regression.
\(n\) = Number of separate experiments; \(\% K\) = \% K-sites from saturation analysis.
Unsuppressed = Total number of sites
Suppressed = Binding in the presence of 100nM each of unlabelled [D-Ala\(^2\),D-Leu\(^3\)]enkephalin and [D-Ala\(^2\),MePhe\(^4\),Gly-ol\(^5\)]enkephalin to 0.1nM labelled ligand.
Non-specific binding defined in the presence of 1\(\mu\)M unlabelled diprenorphine.
<table>
<thead>
<tr>
<th>Radiolabelled Ligand</th>
<th>Species</th>
<th>Approx. Conc. (nM)</th>
<th>Counts (dpm.mg⁻¹)</th>
<th>% Specific</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>[³H]dynorphin A-(1-9)</td>
<td>Guinea-Pig</td>
<td>0.1</td>
<td>355 ± 73</td>
<td>156 ± 31</td>
<td>199 ± 44</td>
</tr>
<tr>
<td>*(Suppressed)</td>
<td></td>
<td>2.0</td>
<td>3018 ± 432</td>
<td>2204 ± 362</td>
<td>814 ± 70</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td>0.1</td>
<td>399, 363</td>
<td>170, 167</td>
<td>229, 194</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0</td>
<td>6026, 6953</td>
<td>5598, 6350</td>
<td>428, 603</td>
</tr>
<tr>
<td>[³H]U-69593</td>
<td>Rat</td>
<td>0.2</td>
<td>461 ± 32</td>
<td>406 ± 25</td>
<td>55 ± 12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15.0</td>
<td>19549 ± 249</td>
<td>18118 ± 208</td>
<td>1431 ± 42</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem; n = number of separate experiments in duplicate.
Counts expressed as dpm.mg protein⁻¹.
*Suppressed at 100nM [D-Ala²,D-Leu⁵]enkephalin and [D-Ala²,MePhe⁵,Gly-ol⁶]enkephalin to 0.1nM labelled ligand.
Non-specific binding defined in the presence of 1μM unlabelled diprenorphine.
In vitro metabolism of $[^3H]$dynorphin A-(1-9)

$[^3H]$Dynorphin A-(1-9) was sensitive to proteolysis when incubated with rat spinal cord homogenates at 25°C for 30 minutes, affording products which were separated by HPLC using reference standards (Figure 6.19). Metabolism occurred even in the presence of 300μM captotril and 30μM bestatin. Under these conditions the amino-terminal fragments, that is tyrosine, tyrosyl-glycine and tyrosyl-glycyl-glycine constituted the major metabolites and only 41% of the nonapeptide $[^3H]$dynorphin A-(1-9) remained. Two other major peaks corresponded to dynorphin A fragment 1-5 ([Leu$^5$] enkephalin) and dynorphin A-(1-8). The amino terminal tetrapeptide tyrosyl-glycyl-glycyl-phenylalanine was not detected (Figure 6.20).
Figure 6.19

Elution profile of authentic peptide standards using reverse-phase HPLC on a C₁₈ Altex Ultrasphere ODS reverse-phase column.

Solution A = Trifluoroacetic acid (26mM) - triethylamine to give pH 3.0.

Solution B = Acetonitrile (49%) - trifluoroacetic acid (13mM).

Flow rate = 0.5 ml. minute⁻¹.

Key to abbreviations

Inj. = Sample injected onto column.

"Tyr" = Tyrosine fragments; Tyr, Tyr-Gly, Tyr-Gly-Gly.

YGGF = Tyr-Gly-Gly-Phe.


dyn 1-6 = dynorphin A-(1-6).

dyn 1-7 = dynorphin A-(1-7).

dyn 1-8 = dynorphin A-(1-8).

dyn 1-9 = dynorphin A-(1-9).

Dynorphin A-(1-9) is:

Tyr-Gly-Gly-Phe-⁵Leu-⁶Arg-⁷Arg-⁸Ile-⁹Arg
<table>
<thead>
<tr>
<th>No.</th>
<th>Metabolic Product</th>
<th>% Recovered Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tyr, Tyr-Gly, Tyr-Gly-Gly</td>
<td>25.9 ± 0.7</td>
</tr>
<tr>
<td>2</td>
<td>Tyr-Gly-Gly-Phe</td>
<td>N.D.</td>
</tr>
<tr>
<td>3</td>
<td>Tyr-Gly-Gly-Phe-Leu</td>
<td>16.7 ± 1.3</td>
</tr>
<tr>
<td>4</td>
<td>Tyr-Gly-Gly-Phe-Leu-Arg</td>
<td>1.44 ± 0.11</td>
</tr>
<tr>
<td>5</td>
<td>Tyr-Gly-Gly-Phe-Leu-Arg-Arg</td>
<td>0.60 ± 0.05</td>
</tr>
<tr>
<td>6</td>
<td>Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile</td>
<td>14.3 ± 0.2</td>
</tr>
<tr>
<td>7</td>
<td>Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg</td>
<td>40.9 ± 1.6</td>
</tr>
</tbody>
</table>

Total Recovery 99.84 ± 1.99

Results expressed as means ± sem of 3 separate experiments.

All products were analysed against authentic standards on a C_{18} Altex Ultrasphere C18 reverse-phase column.

N.D. = no product detectable.

**Figure 6.20** Metabolism of [³H]dynorphin A-(1-9) by rat lumbo-sacral spinal cord homogenates, in the presence of 30μM bestatin and 300μM captopril, at 25°C.
Both \[^{3}H\](++)-bremazocine and \[^{3}H\]diprenorphine (Figure 6.21) labelled a high number of sites in spinal cord homogenates. \[^{3}H\]Diprenorphine afforded higher Bmax values than \[^{3}H\](++)-bremazocine which may be indicative of ligand differences. The low non-specific binding and high total binding obtained using \[^{3}H\]diprenorphine, especially at higher radioligand concentrations may make this ligand a good choice for spinal cord binding assays, which are made difficult by the relatively low numbers of sites and the low ratio of nervous tissue:lipid material.

\[\text{Figure 6.21 Diprenorphine}\]

\[^{3}H\](++)-Bremazocine, in the presence of 100nM each of [D-Ala\(^2\),D-Leu\(^4\)] enkephalin and [D-Ala\(^2\),MePhe\(^\*\),Gly-ol\(^\*\)]enkephalin to suppress binding to \(\delta\)- and \(\mu\)-sites, labelled a population of sites, which were initially presumed to be of the \(k\)-type in rat lumbo-sacral spinal cord homogenates. These sites represented 39% of the total number labelled by \[^{3}H\](++)-bremazocine. Similar levels were seen in guinea-pig (46%) and porcine (43%) lumbo-sacral cords. These values are close to those previously reported for the levels of \(k\)-sites seen in lumbo-sacral cords of rat [152, 155, 158, 195], guinea-pig [152, 154], frog, pigeon and man [152].

In the present work, the remaining bound radioligand was completely displaced by naloxone, and levorphanol was 2400 times more potent than dextrorphan. These results confirm the opioid nature of the sites and suggest that \[^{3}H\](++)-bremazocine is not labelling any non-opioid sites such as the \(\sigma\)-site reported in rat spinal cord by Tam [112].
However, the system is not straightforward as shallow Hill plots obtained from displacement studies with selective opioids suggest that heterogeneity exists within these k-sites. Indeed the displacement curve for \([D\text{-}Ala^2, D\text{-}Leu^5]\)enkephalin could be resolved into two Hill plots, affording a high affinity component for which the competing ligand displayed an affinity atypical for interaction with \(\mu\)- or \(\delta\)-sites [97] and a low affinity phase, with a Ki value in the typical range reported for interaction of the peptide with k-sites [95, 97]. Theoretical calculations based on the method of Ariens [221] for the interaction of more than one ligand at a binding site confirm that \(\mu\)- and \(\delta\)-sites are unlikely to be responsible for this heterogeneity since a sum total of less than 4% of these sites remain in the presence of 100nM each of \([D\text{-}Ala^2, D\text{-}Leu^5]\) enkephalin and \([D\text{-}Ala^2, MePhe^4, Gly\text{-}ol^5]\)enkephalin. The theoretical curve and data used to derive this are shown in Figure 6.22.

It can be seen from the displacement of \([^3H](\text{-})\text{-bremazocine by [D\text{-}Ala^2, D}\text{-}Leu^5]\)enkephalin (Figure 6.3) that a concentration of 5\(\mu\)M - 10\(\mu\)M unlabelled ligand is sufficient to displace binding of the radioligand from the higher affinity sites. In the presence of 5\(\mu\)M \([D\text{-}Ala^2, D\text{-}Leu^5]\)enkephalin, the remaining binding was displaced by \(\mu\)- and \(\delta\)-selective compounds with affinities in the range reported for interaction at k-sites [97], and slopes of approximately unity. The k-selective opioid U-50488H also displaced the bound \([^3H]\)material with an affinity typical for interaction at k-sites [109] but the slope was still apparently shallow. The visually biphasic curve afforded a linear correlation coefficient of 0.970.

The remaining sites in the presence of 5\(\mu\)M \([D\text{-}Ala^2, D\text{-}Leu^5]\)enkephalin represented only 16% of the total labelled by \([^3H](\text{-})\text{-bremazocine. These sites have the properties of k-sites but represent just 40% of the k-population defined by the usual criterion of binding, that is \([^3H](\text{-})\text{-bremazocine in the presence of 100nM each of [D\text{-}Ala^2, D\text{-}Leu^5]\) enkephalin and [D\text{-}Ala^2, MePhe^4, Gly\text{-}ol^5]\)enkephalin, in rat lumbar-sacral cord. When combined, the results from the competition assays and saturation binding isotherms support the existence of a population of sites in addition to \(\mu\)-, \(\delta\)- and "classical k" sites in rat lumbo-sacral spinal cord.
Theoretical displacement profiles for [D-Ala²,D-Leu²] enkephalin (O) and [D-Ala²,MePhe³,Gly-ol⁴]enkephalin (●) at δ- and μ-sites respectively in rat brain, after the method of Ariens [221], using experimentally derived data [97] given in table below.

### Binding characteristics of opioids

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Affinity at opioid sites (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-Bremazocine</td>
<td>μ</td>
</tr>
<tr>
<td>[D-Ala²,D-Leu²]enkephalin</td>
<td>0.32</td>
</tr>
<tr>
<td>[D-Ala²,MePhe³,Gly-ol⁴]enkephalin</td>
<td>12.6</td>
</tr>
</tbody>
</table>

% Sites Present

|                  | 46 | 42 | 12 |

Concentration [³H](-)-bremazocine - 0.2nM.

At 100nM [D-Ala²,D-Leu²]enkephalin 2.2% δ-sites remaining.
At 100nM [D-Ala²,MePhe³,Gly-ol⁴]enkephalin 1.7% μ-sites remaining.
Preliminary evidence also suggests an heterogeneity of the k-population in guinea-pig and porcine lumbo-sacral spinal cord. Although the results are more difficult to interpret it would appear that U-50488H recognizes the two binding components with more selectivity than U-69593 (structure given in Figure 6.23). The paucity of sites and low specific binding achieved in porcine cord (Chapter 5; Table 5.2) are problems with this tissue which may be overcome by the use of ligands labelled to higher specific activity.

The existence of k-heterogeneity in spinal cord tissue has previously been reported. Early studies by Gouarderes and co-workers [163] failed to identify μ- and δ-sites, but demonstrated two classes of site, to which [3H]ethylketocyclazocine bound, in the lumbo-sacral spinal cords of rat, guinea-pig, monkey and man. Further work in the guinea-pig cord [196] which was based on the sensitivity of this binding to displacement by [D-Ala²,D-Leu⁵]enkephalin, led to the proposal of K₁ and K₂ subtypes. However, since the same group [165] and other workers [151, 160, 193] later demonstrated the presence of μ-, δ- and k-sites in spinal cords from these same species it seems likely that the affinities proposed to represent K₂-sites, were actually composite affinities made up of interactions at all three binding site types. On the other hand, other workers [195] have been able to demonstrate that even after the suppression of binding to μ- and δ-sites, a population of binding sites remain which are heterogeneous.

Similarly a sub-classification of k-sites in brain, based on displacement studies using reportedly k-selective peptides has been attempted by other workers.
When binding to \( \mu \)- and \( \delta \)- sites was suppressed, Pfeiffer and colleagues [199] were able to demonstrate two binding components for the displacement of \([^{3}H]diprenorphine\) by dynorphin A-(1-17) in homogenates of human amygdala. Also, Su [200] showed two components for the displacement of \([^{3}H]ethylketocyclazocine\), in the presence of \( \mu \)-, \( \delta \)- and \( \alpha \)-suppression by dynorphin A-(1-13) in homogenates of guinea-pig brain.

The observed heterogeneity of the \( k \)-opioid receptor in the current work may be the result of different types of receptor, or high and low affinity forms of a single type of receptor. For example, the existence of high and low affinity forms of the \( \mu \)-receptor has been proposed by Wolozin and Pasternak [208]. Other neuromodulators such as dopamine have been shown to interact with high and low affinity states of the dopamine receptor [209]. In such cases the high affinity state is generally recognized by agonists, but antagonists recognize both states equally well.

Much of the literature precedent has used non-selective \([^{3}H]\) opioids in the presence of the suppression of binding to \( \mu \)- and \( \delta \)- sites, to define \( k \)-binding sites. In an attempt to improve on this approach, use was made of the more \( k \)-selective ligands \([^{3}H]dyorphin A-(1-9)\) and \([^{3}H]U-69593\). Low numbers of sites were labelled by \([^{3}H]dyorphin A-(1-9)\) in the presence of unlabelled \( \mu \)- and \( \delta \)-selective peptides as described previously, and by \([^{3}H]U-69593\) in rat lumbo-sacral spinal cord homogenates. The low levels of specific binding seen particularly with \([^{3}H]U-69593\) may have introduced significant error into the results. Indeed Lahti and colleagues [198] were unable to identify any specific binding for \([^{3}H]U-69593\) in rat spinal cord. A similar number of sites were labelled by the \( k \)-selective opioids under the conditions described above and by \([^{3}H](-)-bremazocine\) in the presence of 5\( \mu M \) \([D-Ala^2, D-Leu^5]enkephalin\). These results add support to the existence of "classical \( k \)" sites in rat lumbo-sacral spinal cord, but at very low levels.

The higher total sites labelled by \([^{3}H]dyorphin A-(1-9)\) in the guinea-pig cord and the increase in the affinity of the radioligand for the sites remaining after the suppression of binding to \( \mu \)- and \( \delta \)-sites in rat but not in guinea-pig cord, suggest differences in spinal receptor populations between the two species.
However, the difficulties of working with [\(^{1}H\)]dynorphin A-(1-9) must be emphasised and taken into account in assessing the significance of the results. The low affinity of [\(^{1}H\)]dynorphin in the absence of suppression may reflect cross-reactivity with \(\mu\)- and \(\delta\)-sites [72]. Additionally adsorption onto experimental vessels and subcellular contaminants [215, 216] is known to reduce the free ligand concentration and hence produce an underestimate of the absolute potency. The metabolism of [\(^{1}H\)]dynorphin A-(1-9) to [\(^{1}H\)]fragments with high affinities for opioid binding sites is a very real source of error, especially in the unsuppressed assays where such metabolites can compete for \(\mu\)-, \(\delta\)- and possibly \(\kappa\)-sites. For example, [\(^{1}H\)](Leu\(^{5}\)) enkephalin, [\(^{1}H\)](Leu\(^{5}\))enkephalyl-Arg\(^{6}\), [\(^{1}H\)](Leu\(^{5}\))enkephalyl-Arg\(^{6}\)-Arg\(^{7}\) and [\(^{1}H\)]dynorphin A-(1-8) are all metabolites with activity at \(\mu\)- and \(\delta\)-sites. The extended (Leu\(^{5}\))enkephalins, especially [\(^{3}H\)]dynorphin A-(1-8) also show activity at the k-site [72, 174, 197].

Gillan and her colleagues [197] have demonstrated that [\(^{1}H\)]dynorphin A-(1-9) in the presence of 30\(\mu\)M bestatin and 300\(\mu\)M captopril, was metabolized in guinea-pig brain homogenates such that only 17.5\% of the recovered radioactivity represented the nonapeptide. When L-leucyl-L-arginine was included, further stabilization of the Leu\(^{5}\)-Arg\(^{6}\) bond was afforded so that the [\(^{1}H\)]dynorphin A-(1-9) fraction increased to almost 50\% of the total recovered activity. In the current work 40\% of the original nonapeptide remained when metabolism by rat lumbo-sacral spinal cord homogenates was inhibited by 30\(\mu\)M bestatin and 300\(\mu\)M captopril.

It is noteworthy that the present studies, like the work of Gillan and colleagues analysed only the free metabolites and not those bound at the k-site. Evidence suggests that bound peptides are less susceptible to metabolism than those in the biophase [214], but further proof of this proposal requires separation of the bound and unbound peptides [197]. The suppression of binding to \(\mu\)- and \(\delta\)-sites in the current work will have reduced cross-reactivity by the metabolites at these sites. Since the free ligand concentration of [\(^{1}H\)]dynorphin A-(1-9) was reduced the observed affinity is likely to have been underestimated. However the Bmax is most probably a true estimate since saturation equilibrium binding was achieved in nearly all assays, a possible exception being the guinea-pig (Figure 6.17).
However, in addition recent results from our laboratory have shown that bestatin, captopril and dipeptides such as L-leucyl-L-leucine reduce the specific binding of both \[^{3}H\]dynorphin A-(1-8) and \[^{3}H\]dynorphin A-(1-9) in rat brain homogenates (D. Dixon and J. R. Traynor unpublished observations). Such inhibition of binding could have reduced the observed Bmax in the present spinal cord experiments.

The presence of spinal opioid binding sites has prompted investigations into their physiological relevance and in particular, the roles of the corresponding receptors in the modulation of nociceptive stimuli. Compounds with reported \(\mu\)-, \(\delta\)- and \(k\)-selectives have been tested in both animal models and clinical situations. Yaksh and colleagues [190, 194] demonstrated that compounds with reported \(\mu\)-selectivity produce a powerful suppression of responses to cutaneous-thermal and visceral-chemical pain in rats. \(\delta\)-Selective compounds were reported to be effective only against cutaneous-thermal pain. Although some of the compounds used in these studies are not as selective as initially proposed, the results have been substantiated by further work using more selective ligands [191]. \(k\)-Selective compounds were proposed to selectively block visceral-chemical pain [191].

Particular interest has been focussed on the \(k\)-receptor since interaction at this receptor produces analgesia, but fewer of the unwanted side-effects such as respiratory depression, dependence liability and constipation. Many studies have employed the endogenous \(k\)-ligands, the dynorphins in addition to synthetic \(k\)-selective agonists. For example, Jhamandas and colleagues [202] studied analgesia induced by U-50488H and two dynorphin fragments, dynorphin A-(1-8) and dynorphin A-(1-13) using the rat tail-flick test. U-50488H required higher doses of naloxone for reversal than the dynorphins. The latter also reduced the spontaneous urine output in rats but U-50488H was without effect. These workers concluded that the two classes of compound appear to act via different receptors and that the dynorphins may interact with more than just the \(k\)-receptor. It should be noted that U-50488H-induced analgesia was not maximal until 60 minutes post injection which may indicate redistribution to higher centres, rather than a local spinal effect.
In contrast Stevens and Yaksh [203] found that k-receptor mediated analgesic activity for intrathecally administered U-50488H was limited to visceral-chemical stimuli in the rat, whilst dynorphin A-(1-17) produced no antinociceptive responses at doses below those which produced motor dysfunction. This is believed to be mediated by des-tyrosyl fragments and is a phenomenon which is not uncommon with high doses of dynorphin peptides [204, 205]. The authors therefore proposed that the high potency of the dynorphins at the k-receptor in vitro was not seen in vivo. In support of this a recent report by Leighton and co-workers [276] failed to show any evidence for k-receptor mediated analgesia at the spinal level with U-50488H, U-69593 or PD117302 ((±)-trans-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]benzo[b]-thiophene-4-acetamide) [277], using the paw pressure and tail-flick tests. These are animal models used for higher grade pain which require higher efficacy compounds than visceral-chemical stimuli.

Thus although some apparently conflicting evidence exists over the receptors through which compounds with reported k-selectivities act, there is general agreement that k-receptors mediate antinociceptive responses to lower grade visceral pain at the spinal level. Indeed in spite of the controversy surrounding the opioid nature of the analgesia induced by the dynorphins in animal models, these compounds have been shown to be clinically effective in the management of intractable cancer pain after intrathecal administration [187]. In these studies, analgesia was effected at doses which did not cause motor dysfunction and very few other side effects were seen.

In conclusion, the present work has demonstrated an heterogeneity of the k-population in the lumbo-sacral spinal cord of the rat. Such heterogeneity may extend to other species. Only a small proportion of these sites in the rat appear to be of the "classical k" type since the additional sites do not appear to bind [3H]dynorphin A-(1-9), or the k-selective opioid [3H]U-69593. Although the remaining sites require further definition the results do lend support for the involvement of k-receptors in antinociceptive responses at the spinal level.
CHAPTER 7

CHARACTERIZATION OF BRAIN K-OPIOID BINDING SITES

IN THE RAT AND GUINEA-PIG
INTRODUCTION

The existence of \(\mu\)-, \(\delta\)- and \(k\)-opioid binding sites has been demonstrated in the spinal cord [152, 154, 192] and in brain [157, 248]. Work in the spinal cord suggested an apparently heterogeneous \(k\)-population [163, 196] and an absence of \(\mu\)- and \(\delta\)-sites. Although the same research group later demonstrated the presence of \(\mu\)- and \(\delta\)-sites in spinal cord tissues [165], suggesting that the apparent heterogeneity actually represented \(\mu\)-, \(\delta\)- and \(k\)-sites, other workers [195] were able to demonstrate that even after binding to \(\mu\)- and \(\delta\)-sites was suppressed, the residual \(k\)-population was apparently heterogeneous. An heterogeneity of \(k\)-sites has also been demonstrated in brain tissues [199, 200].

Heterogeneous binding may be real. On the other hand such binding characteristics may be seen if both high and low affinity states of the same receptor are present [213]. When monovalent cations such as \(Na^+\) are used alone, or in combination with guanyl nucleotides [241, 245], it is possible to almost abolish agonist binding whilst leaving antagonist binding relatively unaffected. Alternatively divalent cations, for example \(Mg^{2+}\) may be employed to promote high affinity binding for some agonists [245], particularly to \(\delta\)-sites [245, 254]. However, it is known that mono- and divalent cations differentially affect the binding of \(^{1}H\)opioids to \(\mu\)-, \(\delta\)- and \(k\)-sites [245, 254, 255]. Therefore, a careful selection of the levels of ligand needed to effect \(\mu\)-, \(\delta\)- or \(k\)-suppression is necessary for each experimental condition.

Apparent heterogeneity of binding may also arise from \(^{1}H\)opioid interaction with low affinity specific sites on lipids [85] or even glassfibre filters [86]. Myelin is composed of a high proportion of phospholipid and galactolipid [256] and may constitute a potential target for these interactions. \(Na^+\), \(K^+\)-activated adenosine 5'-triphosphate phosphohydrolase (\(Na^+\), \(K^+\)-ATPase; EC 3.6.1.3), is an enzyme-marker for the external membranes of nerve terminals [257]. Monoamine oxidase (MAO; EC 1.4.3.4) is localised on the inner membrane of mitochondria [258] and constitutes a useful marker enzyme for mitochondria. 2',3'-Cyclic nucleotide 3'-phosphohydrolase
(2',3'-CNP; EC 3.1.4.16) is a myelin-specific enzyme [256]. Therefore, the subcellular components may be identified after their separation by differential centrifugation.

The aim of this work was to characterize k-binding in brain tissue, a relatively abundant source of opioid binding sites, under carefully chosen experimental conditions.
MATERIALS & METHODS

Homogenates of rat or guinea-pig brain or guinea-pig cerebellum were prepared and binding assays were performed using a selection of different buffers as detailed in Chapter 2.

Binding assays were performed at 25°C for 45 minutes (30 minutes for \[^3H\]dynorphin A-(1-9)).

\[^3H\](-)-Bremazocine, \[^3H\]dynorphin A-(1-9) and \[^3H\]diprenorphine were the radiolabelled ligands used. Non-specific binding was defined using 1µM diprenorphine for \[^3H\](-)-bremazocine and \[^3H\]dynorphin A-(1-9) or 10µM naloxone for \[^3H\]diprenorphine. All other chemicals used were as described in Chapter 2.

All preparations and procedures for the subcellular distribution of opioid binding were performed as described in Chapter 2 (pp. 32 - 41).
RESULTS

Binding studies in 50mM Tris-HCl, pH 7.4 buffer

a) Competition assays

[³H](-)-Bremazocine was displaced from rat (Figure 7.1) and guinea-pig (Figure 7.2) brain homogenates by μ- and δ-selective opioid peptides. In the rat a biphasic curve was obtained for the μ-selective peptide [D-Ala³,MePhe⁴,Gly-ol⁵] enkephalin affording a high affinity component indicative of binding to a μ-site [90, 91] and a low affinity component. The former represented 45 - 50% of the specifically bound radioligand. The displacement profile for [D-Ala³,D-Leu⁵] enkephalin was apparently monophasic up to 10μM (Figure 7.1). However, the Hill coefficient was low and there was a discernable inflexion of the displacement curve at approximately 100nM, indicative of interaction with more than one type of binding site. The δ-antagonist ICI 174864 displaced 35% of the specifically bound [³H](-)-bremazocine with an IC₅₀ in line with its reported δ-affinity [238] and a Hill slope close to unity (Table 7.1).

In the guinea-pig, all three unlabelled competing ligands displaced [³H](-)-bremazocine in a biphasic manner (Figure 7.2), although the higher affinity component for each curve represented a much lower proportion of the bound material than was seen with the rat. Indeed ICI 174864 displaced only 10% of the bound radioligand. The plateaux between the binding components were also larger for each of the ligands used. The calculations of the IC₅₀ values and Hill coefficients in guinea-pig brain may contain significant errors due to the low levels of displacement obtained in this tissue, but were in line with reported values [90, 157, 238] (Table 7.1).

When [³H](-)-bremazocine binding to μ- and δ-sites was suppressed using unlabelled [D-Ala³,MePhe⁴,Gly-ol⁵]enkephalin and [D-Ala³,D-Leu⁵]enkephalin each in the ratio 100nM to 0.2nM radioligand, different displacement profiles for the remaining K-population were obtained in the rat and guinea-pig.
In the rat, [D-Ala²,MePhe⁴,Gly-ol³]enkephalin displaced the specifically bound radioligand with low affinity and a slope close to unity (Figure 7.3; Table 7.2). [D-Ala²,D-Leu⁵]enkephalin and U-50488H both displaced [³H](-)-bremazocine with low affinities and afforded Hill coefficients of less than unity (Figure 7.3; Table 7.2). There was no biphasic nature visually apparent in either of these curves.

In the guinea-pig, [D-Ala²,MePhe⁴,Gly-ol³]enkephalin and U-50488H produced shallow Hill slopes. The affinity for U-50488H was markedly higher than in the rat under the same conditions, and close to reports for interaction of this ligand with K-sites [108, 109] (Figure 7.4; Table 7.2).
Displacement of $[^3H](-)$-bremazocine by [D-Ala$^2$,D-Leu$^5$]enkephalin (■) (0.52–0.54nM), [D-Ala$^2$,MePhe$^5$, Gly-ol$^5$]enkephalin (○) (0.40–0.42nM) and ICI 174864 (◆) (0.54–0.71nM) in rat brain homogenates prepared in 50mM Tris-HCl buffer. Values in parentheses show concentration ranges of radioligand. See Table 7.1 for data.
Displacement of $[^3]$H(−)-bremazocine by [D-Ala$^2$,D-Leu$^5$]enkephalin (■) (0.46-0.52nM), [D-Ala$^2$,MePhe$^4$, Gly-ol$^6$]enkephalin (○) (0.51-0.52nM) and ICI 174864 (◆) (0.53-0.58nM) in guinea-pig brain homogenates prepared in 50mM Tris-HCl buffer. Values in parentheses show concentration ranges of radioligand. See Table 7.1 for data.
Table 7.1  Potencies of \( \mu \)- and \( \delta \)-selective opioid peptides to displace \([^3H](-)-bremazocine (0.40 - 0.71\text{nM})\) in rat and guinea-pig brain homogenates at 25°C in Tris-HCl buffer (Figures 7.1 & 7.2).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Competing Ligand</th>
<th>IC(_{50}) nM</th>
<th>nH</th>
<th>Min. r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat brain</td>
<td>[D-Ala(^2),MePhe(^4),Gly-ol(^5)]enkephalin</td>
<td>H 15.84 ± 4.46</td>
<td>0.86 ± 0.04</td>
<td>0.990</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L &gt;5(\mu)M</td>
<td>1.10 ± 0.10</td>
<td>0.993</td>
</tr>
<tr>
<td></td>
<td>[D-Ala(^2),D-Leu(^5)]enkephalin</td>
<td>H 35.93 ± 4.01</td>
<td>0.52 ± 0.04</td>
<td>0.934</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L &gt;80(\mu)M</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ICI 174864</td>
<td>H 849.6 ± 120.6</td>
<td>0.97 ± 0.06</td>
<td>0.987</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L &gt;80(\mu)M</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Guinea-pig brain</td>
<td>[D-Ala(^2),MePhe(^4),Gly-ol(^5)]enkephalin</td>
<td>H 26.14 ± 5.91</td>
<td>1.07 ± 0.13</td>
<td>0.932</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L &gt;6(\mu)M</td>
<td>0.93 ± 0.08</td>
<td>0.993</td>
</tr>
<tr>
<td></td>
<td>[D-Ala(^2),D-Leu(^5)]enkephalin</td>
<td>H 8.47 ± 1.11</td>
<td>0.86 ± 0.20</td>
<td>0.841</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L &gt;60(\mu)M</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ICI 174864</td>
<td>H 215.4 ± 40.1</td>
<td>0.87 ± 0.18</td>
<td>0.830</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L &gt;100(\mu)M</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem of 3 separate experiments in duplicate.

Min. r = Minimum linear correlation coefficient.

H = High affinity binding component.

L = Low affinity binding component.

Non-specific binding was defined using 1\(\mu\)M unlabelled diprenorphine.
Figure 7.3  Displacement of $[^3H](-)$-bremazocine (0.20-0.28nM), in the presence of 100nM each of [D-Ala$^2$,D-Leu$^5$]enkephalin and [D-Ala$^2$,MePhe$^5$,Gly-ol$^5$]enkephalin to 0.2nM labelled ligand, by [D-Ala$^2$,D-Leu$^5$]enkephalin (■), [D-Ala$^2$,MePhe$^5$,Gly-ol$^5$]enkephalin (○) and U-50488H (●) in rat brain homogenates prepared in 50mM Tris-HCl buffer. See Table 7.2 for data.

Figure 7.4  Displacement of $[^3H](-)$-bremazocine (0.20-0.23nM) by [D-Ala$^2$,D-Leu$^5$]enkephalin (■), [D-Ala$^2$,MePhe$^5$,Gly-ol$^5$]enkephalin (○) and U-50488H (●) in guinea-pig brain homogenates under the conditions described in Figure 7.3. See Table 7.2 for data.
Table 7.2  
Potencies of selective opioids to displace $[^{1}H]$(-)-bremazocine in the presence of 100nM each of unlabelled [D-Ala$^{2}$,D-Leu$^{5}$]enkephalin and [D-Ala$^{2}$,MePhe$^{4}$,Gly-ol$^{5}$]enkephalin to 0.2nM labelled ligand in brain homogenates at 25°C in Tris-HCl buffer (Figures 7.3 & 7.4).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Unlabelled Ligand</th>
<th>IC$_{50}$ (nM)</th>
<th>nH</th>
<th>Min. r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat Brain (0.20 - 0.28nM)</td>
<td>[D-Ala$^{2}$,MePhe$^{4}$,Gly-ol$^{5}$]enkephalin</td>
<td>2706 ± 363</td>
<td>0.96 ± 0.04</td>
<td>0.986</td>
</tr>
<tr>
<td></td>
<td>[D-Ala$^{2}$,D-Leu$^{5}$]enkephalin</td>
<td>16509 ± 2954</td>
<td>0.64 ± 0.03</td>
<td>0.925</td>
</tr>
<tr>
<td></td>
<td>U-50488H</td>
<td>178.3 ± 36.5</td>
<td>0.69 ± 0.02</td>
<td>0.988</td>
</tr>
<tr>
<td>Guinea-pig brain (0.20 - 0.23nM)</td>
<td>[D-Ala$^{2}$,MePhe$^{4}$,Gly-ol$^{5}$]enkephalin</td>
<td>4439 ± 397</td>
<td>0.72 ± 0.02</td>
<td>0.991</td>
</tr>
<tr>
<td></td>
<td>[D-Ala$^{2}$,D-Leu$^{5}$]enkephalin</td>
<td>56287 ± 4913</td>
<td>0.90 ± 0.13</td>
<td>0.994</td>
</tr>
<tr>
<td></td>
<td>U-50488H</td>
<td>12.19 ± 0.90</td>
<td>0.74 ± 0.03</td>
<td>0.987</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem of 3 separate experiments in duplicate.

Min. r = Minimum linear correlation coefficient.

Figures in parentheses represent $[^{1}H]$(-)-bremazocine concentration range.

Non-specific binding defined using 1μM unlabelled diprenorphine.
b) **Saturation analysis**

In rat brain, \[^3H\](-)-bremazocine in the presence of 100nM each of [D-Ala²,D-Leu⁴]enkephalin and [D-Ala²,MePhe⁴,Gly-ol³] enkephalin to 0.2nM labelled ligand labelled approximately one tenth of the total site numbers. The affinity in the presence of suppression was higher than when no suppression was present (Figure 7.5; Table 7.3). Both Scatchard plots were linear. \[^3H\]Oiprenorphine labelled a higher total number of sites (Bmax 339.6 ± 26.3 fmol.mg⁻¹; Figure 7.11) with higher affinity (Kᵦ 0.36 ± 0.04nM) than \[^3H\](-)-bremazocine. When experiments were performed in guinea-pig cerebellar homogenates, a tissue containing mainly K-sites [242], both \[^3H\](-)-bremazocine and \[^3H\]dynorphin A-(1-9) under the µ- and δ-suppressing conditions described above, labelled similar numbers of high affinity sites (Figures 7.6 & 7.7; Table 7.3).
Saturation analysis for $[^3]H(-)$-bremazocine in the absence (●) and presence (O) of 100nM each of [D-Ala$^2$,D-Leu$^5$]enkephalin and [D-Ala$^2$,MePhe$^5$,Gly-$\text{ol}^5$]enkephalin to 0.2nM labelled ligand, in rat brain homogenates prepared in 50mM Tris-HCl buffer. See Table 7.3 for data.
Table 7.6  Saturation analysis for [\(^{3}\text{H}\)](-)-bremazocine, in the presence of 100nM each of \([\text{D-Ala}^2,\text{D-Leu}^4]\)enkephalin and \([\text{D-Ala}^2,\text{MePhe}^4,\text{Gly-ol}^5]\)enkephalin to 0.2nM radioligand in guinea-pig cerebellar homogenates prepared in 50mM Tris-HCl buffer. Representative plot from 3 experiments. See Table 7.3 for data.

![Graph](image1)

![Graph](image2)

Figure 7.7  Saturation analysis for \([\text{H}]\)dynorphin A-(1-9), in the presence of 100nM each of \([\text{D-Ala}^2,\text{D-Leu}^4]\)enkephalin and \([\text{D-Ala}^2,\text{MePhe}^4,\text{Gly-ol}^5]\)enkephalin to 0.1nM radioligand in guinea-pig cerebellar homogenates prepared in 50mM Tris-HCl buffer. Representative plot from 3 experiments. See Table 7.3 for data.
Table 7.3 Saturated binding analysis for $[^3H](-)$-bremazocine or $[^3H]$dynorphin A-(1-9) in the presence of 100nM each of unlabelled [D-Ala$^2$,D-Leu$^5$]enkephalin and [D-Ala$^2$,MePhe$^4$,Gly-$\beta$ol$^5$]enkephalin to 0.2nM labelled ligand (0.1nM for $[^3H]$dynorphin A-(1-9)) in CNS tissues from rat and guinea-pig in Tris-HCl buffer at 25°C (Figures 7.5 - 7.7).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Radioligand</th>
<th>Bmax (fmol.mg$^{-1}$)</th>
<th>$K_D$ (nM)</th>
<th>$r$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat Brain</td>
<td>$<a href="-">^3H</a>$-Bremazocine (0.027 - 11.75nM)</td>
<td>*210.3 ± 22.2</td>
<td>1.19 ± 0.12</td>
<td>0.975 - 0.991</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21.18 ± 1.74</td>
<td>0.31 ± 0.05</td>
<td>0.968 - 0.989</td>
</tr>
<tr>
<td>Guinea-pig cerebellum</td>
<td>$<a href="-">^3H</a>$-Bremazocine (0.025 - 10.80nM)</td>
<td>56.13 ± 1.75</td>
<td>0.17 ± 0.01</td>
<td>0.969 - 0.981</td>
</tr>
<tr>
<td></td>
<td>$[^3H]$Dynorphin A-(1-9) (0.045 - 7.35nM)</td>
<td>63.98 ± 12.25</td>
<td>0.30 ± 0.09</td>
<td>0.872 - 0.988</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem of 3 experiments in duplicate.

$r$ = Non-linear correlation coefficient.

Figures in parentheses represent radioligand concentration ranges used.

* Results represent binding in the absence of suppression.

Non-specific binding defined using 1μM unlabelled diprenorphine.
Binding studies in 50mM Tris-HCl buffer containing 25mM NaCl

a) Competition assays

The ability of unlabelled ligands to define non-specific binding was reassessed in the presence of 25mM NaCl (Table 7.4). The non-selective opioid compounds all displaced greater than 96% of the total, that is, specific plus non-specific [\(^1\)H]diprenorphine bound. The antagonist naloxone also displaced greater than 96% when used at higher concentrations. U-50488H and to a lesser extent tifluadom, are two compounds showing \(k\)-binding properties. They are also both agonists. These latter properties are reflected by the low potencies of the compounds in the presence of 25mM NaCl. Thus, only when 200\(\mu\)M U-50488H or 10\(\mu\)M tifluadom is used, does the displacement of bound [\(^1\)H]diprenorphine exceed 80%. These results demonstrate that the ligands used to define non-specific binding in Tris-HCl buffer are also suitable in the presence of 25mM sodium ions.

[\(^1\)H]Diprenorphine displacement in rat brain homogenates by the \(\mu\)-selective [\(\text{D-Ala}^2,\text{MePhe}^4,\text{Gly-ol}^5\)]enkephalin or \(k\)-selective U-50488H afforded low Hill coefficients although neither curves were visually biphasic (Figure 7.8). The \(\delta\)-antagonist ICI 174864 was able to displace only 30% of the bound material. Surprisingly, when lower concentrations of [\(^1\)H]diprenorphine were used, ICI 174864 displaced the bound ligand with a lower affinity and with a shallow slope (Figure 7.9). Data is presented in Table 7.5.

[\(^1\)H]Diprenorphine (0.23nM) binding in guinea-pig cerebellar homogenates was displaced by U-50488H in an apparently biphasic manner (Figure 7.10). The higher affinity component represented approximately 75% of the bound radioligand and afforded IC\(_{50}\) values of 20.90nM and 22.33nM in two experiments.
The Hill coefficients for these curves were 0.61 and 0.57 respectively. The IC₅₀ of the lower affinity component was greater than 10µM.

b) **Saturation analysis**

In Tris-HCl containing 25mM NaCl, the Bmax and Kᵰ values were 315.6 ± 14.3 fmol.mg⁻¹ and 0.28 ± 0.06nM, which were not significantly different from studies in Tris-buffer alone (Figure 7.11). There appeared to be no difference in the shape of the Scatchard plots obtained under both experimental conditions.
Table 7.4 The ability of unlabelled ligands at high concentrations to displace [\textsuperscript{3}H]diprenorphine (0.25nM) binding from rat brain homogenates in Tris-HCl buffer containing 25mM NaCl, at 25°C.

<table>
<thead>
<tr>
<th>Unlabelled Ligand</th>
<th>Concentration ((\mu)M)</th>
<th>% Displacement*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MR 2266</td>
<td>1</td>
<td>96.02 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>96.81 ± 0.44</td>
</tr>
<tr>
<td>Diprenorphine</td>
<td>1</td>
<td>96.81 ± 0.39</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>96.68 ± 0.30</td>
</tr>
<tr>
<td>(-)-Bremazocine</td>
<td>1</td>
<td>96.71 ± 0.28</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>89.42 ± 0.42</td>
</tr>
<tr>
<td>Naloxone</td>
<td>10</td>
<td>96.41 ± 0.35</td>
</tr>
<tr>
<td>Tifluadom</td>
<td>1</td>
<td>68.06 ± 1.03</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>89.42 ± 0.42</td>
</tr>
<tr>
<td>U-50488H</td>
<td>10</td>
<td>37.26 ± 2.89</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>82.07 ± 0.38</td>
</tr>
</tbody>
</table>

Results are means ± sem of 4 experiments in duplicate.

* % Displacement represents the displacement by the unlabelled ligand of total, that is, specific plus non-specific [\textsuperscript{3}H]diprenorphine binding, in the presence of 25mM NaCl.
Displacement of $[^3]H$diprenorphine by U-50488H (○) and [D-Ala²,MePhe³,Gly-ol⁴]enkephalin (□) in rat brain homogenates prepared in 50mM Tris-HCl buffer containing 25mM NaCl. Assays using lower concentrations (-0.2nM) of radioligand are shown in open symbols, and assays using higher radioligand concentrations (-2nM) are shown in solid symbols. See Table 7.5 for data.

Displacement of low (△) and high (▲) concentrations of $[^3]H$diprenorphine by ICI 174864 in homogenates of rat brain prepared in 50mM Tris-HCl buffer containing 25mM NaCl. See Table 7.5 for data.
Table 7.5  Potencies of selective opioid compounds to displace $[^{1}H]$diprenorphine binding in rat brain homogenates in Tris-HCl buffer containing 25mM NaCl at 25°C (Figures 7.8 & 7.9).

<table>
<thead>
<tr>
<th>Competing Ligand</th>
<th>$[^{1}H]$Diprenorphine Concentration (nM)</th>
<th>$IC_{50}$ (nM)</th>
<th>$nH$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>[D-Ala$^4$, MePhe$^6$, Gly-$\alpha$-ol$^8$]enkephalin</td>
<td>0.14 - 0.19</td>
<td>$63.34 \pm 8.71$</td>
<td>$0.45 \pm 0.04$</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1.98 - 2.18</td>
<td>$732.1 \pm 158.6$</td>
<td>$0.59 \pm 0.07$</td>
<td>3</td>
</tr>
<tr>
<td>U-50488H</td>
<td>0.20 - 0.24</td>
<td>$17657 \pm 4463$</td>
<td>$0.63 \pm 0.03$</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>2.05 - 2.31</td>
<td>$41393 \pm 4041$</td>
<td>$0.76 \pm 0.01$</td>
<td>3</td>
</tr>
<tr>
<td>ICI 174864</td>
<td>0.20, 0.24</td>
<td>917.0, 357.3</td>
<td>0.78, 0.75</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1.82, 2.31</td>
<td>249.5, 232.2</td>
<td>0.55, 1.04</td>
<td>2</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem of n experiments in duplicate or as individual results.

$nH$ = Hill coefficient.

Non-specific binding for $[^{1}H]$diprenorphine defined using 10μM unlabelled naloxone.

Hill slopes for ICI174864 were calculated taking the plateau to represent 100% displacement.
Figure 7.10 Displacement of $[^3H]$diprenorphine (0.23nM) by U-50488H in guinea-pig cerebellar homogenates prepared in 50mM Tris-HCl buffer containing 25mM NaCl. Mean of 2 experiments.
Figure 7.11 Saturation analysis for [³H]diprenorphine in rat brain homogenates prepared in 50mM Tris-HCl buffer (O) and 50mM Tris-HCl buffer containing 25mM NaCl (●). A concentration range of 0.027 - 40.02nM labelled ligand was used for all experiments. Representative plots from 3 experiments.
Binding studies in 25mM HEPES buffer containing 10mM MgCl₂, pH 7.4

a) Competition assays

[^H]Diprenorphine displacement in rat brain homogenates by the μ-selective [D-Ala², MePhe⁴, Gly-ol⁴]enkephalin produced a biphasic curve (Figure 7.12). The high affinity component afforded an IC₅₀ value in the typical μ-range [90, 91] and represented 40 - 45% of the labelled sites. A plateau was evident between 20nM and 100nM displacing ligand.

[D-Ala², D-Leu⁴]enkephalin, in the presence of μ-suppression also afforded a biphasic displacement curve (Figure 7.12) with an IC₅₀ value for the high affinity binding component, which represented 25 - 30% of the total labelled sites, in the reported range for interaction with δ-sites [97, 157]. A plateau was discernable between 20nM and 100nM displacing ligand. Results are summarized in Table 7.6.

When U-50488H displaced the specifically bound[^H]diprenorphine in the presence of 100nM each of unlabelled [D-Ala², D-Leu⁴]enkephalin and [D-Ala², MePhe⁴, Gly-ol⁴] enkephalin to suppress binding to δ- and μ-sites respectively, a noticeably biphasic curve was obtained with a pronounced plateau (Figure 7.13; Table 7.6). The high affinity component represented approximately 20% of the bound material and afforded an affinity in line with that reported for interaction with k-sites [108, 109, 242]. It was therefore possible to selectively block μ-, δ- and k-sites in this buffer system and still retain a binding component.

This residual[^H]diprenorphine binding remaining after μ-, δ- and k-suppression was displaced by [D-Ala², D-Leu⁴] enkephalin, [D-Ala², MePhe⁴, Gly-ol⁴] enkephalin and U-50488H with low affinities which were intermediary between those reported for μ-, δ- and k-site interaction (Figure 7.14; Table 7.7). The two peptide analogues also afforded shallow Hill slopes whilst the slope for U-50488H was closer to unity.
This residual binding was also displaced by 1μM naloxone, and levorphanol was approximately 300 times more potent than dextrorphan (Figure 7.15). Displacement of a second non-selective opioid, [3H](-)-bremazocine by [D-Ala²,D-Leu⁵]enkephalin under the same experimental conditions (Figure 7.16) afforded an IC₅₀ and slope similar to those obtained using [3H]diprenorphine (Figure 7.14; Table 7.7).

When the displacements were repeated in guinea-pig brain under the same conditions (Figure 7.17) similar affinities to those seen with rat brain were obtained (Tables 7.7 & 7.8), but the slopes were close to unity. In the light of these observations it was decided to investigate the guinea-pig brain binding sites more closely.
Figure 7.12 Displacement of $[^3]H$ diprenorphine (0.23–0.32nM) by [D-Ala$^2$, MePhe$^5$,Gly-$\beta$-ol$^5$]enkephalin (○) and [D-Ala$^2$,D-Leu$^5$]enkephalin (■) in the presence of 100nM [D-Ala$^2$,MePhe$^5$,Gly-$\beta$-ol$^5$] enkephalin in rat brain homogenates prepared in 25mM HEPES buffer containing 10mM MgCl$_2$. See Table 7.6 for data.

Figure 7.13 Displacement of $[^3]H$ diprenorphine (0.23–0.32nM), in the presence of 100nM each of [D-Ala$^2$,D-Leu$^5$]enkephalin and [D-Ala$^2$,MePhe$^5$,Gly-$\beta$-ol$^5$]enkephalin to 0.2nM radioligand, by U-50488H in rat brain homogenates prepared in 25mM HEPES buffer containing 10mM MgCl$_2$. See Table 7.6 for data.
Table 7.6 Displacement of [³H]diprenorphine in rat brain homogenates by selective opioid ligands in 25mM HEPES buffer containing 10mM MgCl₂ at 25°C (Figures 7.12 & 7.13).

<table>
<thead>
<tr>
<th>Competing Ligand</th>
<th>[³H]Diprenorphine Concentration (nM)</th>
<th>IC₅₀ nM</th>
<th>nH</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>[D-Ala²,MePhe⁴,Gly-ol⁸]enkephalin</td>
<td>0.23 - 0.38</td>
<td>H 3.75 ± 1.36</td>
<td>1.05 ± 0.15</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L 947 ± 207</td>
<td>0.67 ± 0.11</td>
<td>4</td>
</tr>
<tr>
<td>[D-Ala²,D-Leu⁴]enkephalin *</td>
<td>0.23 - 0.38</td>
<td>H 3.44 ± 0.96</td>
<td>1.02 ± 0.04</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L &gt;6μM</td>
<td>0.71 ± 0.01</td>
<td>3</td>
</tr>
<tr>
<td>U-50488H **</td>
<td>0.23 - 0.32</td>
<td>H 3.27, 6.32</td>
<td>0.70, 1.00</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L &gt;5μM</td>
<td>0.72 ± 0.07</td>
<td>3</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem for n separate experiments in duplicate, or as individual results.
nH = Hill coefficient.
* Performed in the presence of 100nM unlabelled [D-Ala²,MePhe⁴,Gly-ol⁸]enkephalin to suppress μ-binding.
** Performed in the presence of 100nM each of unlabelled [D-Ala²,MePhe⁴,Gly-ol⁸]enkephalin and [D-Ala²,D-Leu⁴]enkephalin to suppress μ- and δ-binding.
H = High affinity binding site.
L = Low affinity binding site.
Non-specific binding defined using 10μM unlabelled naloxone.
Figure 7.14 Displacement of $[^3H]$diprenorphine (0.23-0.31nM), in the presence of 100nM each of [D-Ala$^2$,D-Leu$^5$]enkephalin, [D-Ala$^2$,MePhe$^4$,Gly-ol$^5$]enkephalin and U-50488H to 0.2nM labelled ligand, by [D-Ala$^2$,D-Leu$^5$]enkephalin (■), [D-Ala$^2$,MePhe$^4$,Gly-ol$^5$]enkephalin (○) and U-50488H (●) in rat brain homogenates prepared in 25mM HEPES buffer containing 10mM MgCl$_2$. See Table 7.7 for data.

Figure 7.15 Displacement of $[^3H]$diprenorphine (0.21-0.23nM) by naloxone (△), levorphanol (▲) and dextrorphan (▼) in rat brain homogenates under the conditions described in Figure 7.14. Mean results from 2 experiments. See Table 7.7 for data.
Figure 7.16  Displacement of $[^3H]$(-)-bremazocine (0.23–0.28nM), in the presence of 100nM each [D-Ala$^2$,D-Leu$^3$] enkephalin, [D-Ala$^2$,MePhe$^4$,Gly-$\text{ol}^5$]enkephalin and U-50488H to 0.2nM labelled ligand by [D-Ala$^2$, D-Leu$^3$]enkephalin in rat brain homogenates prepared in 25mM HEPES buffer containing 10mM MgCl$_2$. See Table 7.7 for data.
Table 7.7 The displacement of non-selective \(^{[1]}\text{H}\)opioids in the presence of 100nM each unlabelled \([\text{D-Ala}^2,\text{D-Leu}^5]\) enkephalin, \([\text{D-Ala}^2,\text{MePhe}^6,\text{Gly-ol}^8]\)enkephalin and \(\text{U-50488H}\) to suppress \(\delta\), \(\mu\)- and \(\kappa\)-binding respectively, by selective opioids in rat brain homogenates, in 25mM HEPES buffer containing 10mM MgCl\(_2\) at 25°C (Figures 7.14 - 7.16).

<table>
<thead>
<tr>
<th>Radioligand</th>
<th>Radioligand Concentration (nM)</th>
<th>Unlabelled Competing Ligand</th>
<th>IC(_{50}) (nM)</th>
<th>nH</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{[1]}\text{H})Diprenorphine</td>
<td>0.23 - 0.31</td>
<td>([\text{D-Ala}^2,\text{D-Leu}^5])enkephalin</td>
<td>15135 ± 3319</td>
<td>0.66 ± 0.08</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>([\text{D-Ala}^2,\text{MePhe}^6,\text{Gly-ol}^8])enkephalin</td>
<td>3711 ± 351</td>
<td>0.72 ± 0.08</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(\text{U-50488H})</td>
<td>6649 ± 1461</td>
<td>0.88 ± 0.08</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>0.21 - 0.23</td>
<td>(\text{Naloxone})</td>
<td>38.10, 47.52</td>
<td>1.26, 1.30</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(\text{Dextrophan})</td>
<td>17050, 17656</td>
<td>1.01, 1.17</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(\text{Levorphanol})</td>
<td>48.00, 58.50</td>
<td>1.08, 1.12</td>
<td>2</td>
</tr>
<tr>
<td>(^{[1]}\text{H})(-)-Bremazocine</td>
<td>0.23 - 0.28</td>
<td>([\text{D-Ala}^2,\text{D-Leu}^5])enkephalin</td>
<td>9633 ± 1308</td>
<td>0.69 ± 0.11</td>
<td>3</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem of \(n\) separate experiments in duplicate or as individual results.

\(nH = \) Hill Coefficient

Minimum linear correlation coefficient was greater than 0.977 for all assays.

1µM naloxone displaced 94.7% of the specifically bound radioligand.

Non-specific binding defined for \(^{[1]}\text{H}\)diprenorphine using 10µM unlabelled naloxone and for \(^{[1]}\text{H}\)(-)-bremazocine using 1µM unlabelled diprenorphine.
Displacement of $[^3H]$diprenorphine (0.25-0.31nM), in the presence of 100nM each of [D-Ala$^2$, D-Leu$^5$]enkephalin, [D-Ala$^2$,MePhe$^4$,Gly-$\alpha$-$\text{ol}^5$]enkephalin and U-50488H to 0.2nM labelled ligand, by [D-Ala$^2$,D-Leu$^5$]enkephalin (■), [D-Ala$^2$,MePhe$^4$,Gly-$\alpha$-$\text{ol}^5$]enkephalin (○) and U-50488H (●) in guinea-pig brain homogenates prepared in 25mM HEPES buffer containing 10mM MgCl$_2$. See Table 7.8 for data.
Table 7.8  Potencies of unlabelled selective opioids to displace [³H]diprenorphine (0.25 - 0.31nM), in the presence of unlabelled [D-Ala²,D-Leu⁵]enkephalin, [D-Ala²,MePhe⁴,Gly-ol⁵]enkephalin and U-50488H, each in the ratio 100nM to 0.2nM labelled ligand, in guinea-pig brain homogenates in HEPES buffer containing 10mM MgCl₂ at 25°C (Figure 7.17).

<table>
<thead>
<tr>
<th>Unlabelled Ligand</th>
<th>IC₅₀ (nM)</th>
<th>Hill Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>[D-Ala²,D-Leu⁵]enkephalin</td>
<td>16851 ± 3620</td>
<td>0.93 ± 0.09</td>
</tr>
<tr>
<td>[D-Ala²,MePhe⁴,Gly-ol⁵]enkephalin</td>
<td>2607 ± 334</td>
<td>0.98 ± 0.06</td>
</tr>
<tr>
<td>U-50488H</td>
<td>4503 ± 1125</td>
<td>0.97 ± 0.06</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem for 3 separate experiments in duplicate.
Minimum linear correlation coefficient was greater than 0.959 for all assays.
Non-specific binding was defined using 10μM unlabelled naloxone.
[\textsuperscript{3}H]Diprenorphine binding in guinea-pig brain homogenates was displaced by [D-Ala\textsuperscript{2},MePhe\textsuperscript{\textprime},Gly-ol\textsuperscript{\textprime}]enkephalin in a biphasic manner (Figure 7.18). The high affinity site, representing approximately 30% of the bound material had the characteristics of a \(\mu\)-site [90, 91], but the plateau was not very pronounced. The displacement profile for [D-Ala\textsuperscript{2},D-Leu\textsuperscript{\textprime}]enkephalin showed no biphasic nature by visual inspection although the shallow Hill slope was indicative of more than one type of binding site, nor was the IC_{50} for this displacement consistent with a \(\delta\)-site interaction [97, 157] (Table 7.9).

On the other hand the selective \(\delta\)-antagonist ICI 174864 displaced approximately 33% of the specifically bound [\textsuperscript{3}H]diprenorphine with high affinity and a marked plateau was observed. In an attempt to improve the ability of [D-Ala\textsuperscript{2},MePhe\textsuperscript{\textprime},Gly-ol\textsuperscript{\textprime}]enkephalin to separate binding components, displacements were reassessed in the presence of 1\(\mu\)M ICI 174864 (Figure 7.17). However, there was no apparent change in the displacement profile obtained. The displacement of specifically bound [\textsuperscript{3}H]diprenorphine in the presence of \(\delta\)-suppression (1\(\mu\)M ICI 174864) and \(\mu\)-suppression (100nM [D-Ala\textsuperscript{2},MePhe\textsuperscript{\textprime},Gly-ol\textsuperscript{\textprime}]enkephalin) by U-50488H afforded a clearly biphasic curve. Approximately 55% of bound material could be attributed to high affinity binding (k-sites) (Figure 7.19). A second \(k\)-selective ligand dynorphin A-(1-17) [50, 71] displaced the residual [\textsuperscript{3}H]diprenorphine binding with low affinity and a shallow Hill slope (Figure 7.20). Re-calculation of the affinity, taking 65% to represent total displacement gave an increase in affinity but still afforded a shallow slope (Table 7.10). When [\textsuperscript{3}H]diprenorphine binding was additionally suppressed at k-sites, the affinity of dynorphin A-(1-17) was reduced and the slope was less than unity. Only 50% of the bound radioligand was "sensitive" to displacement by concentrations of dynorphin A-(1-17) up to 1\(\mu\)M. When the plateau was chosen to represent 100% displacement of this component, the re-calculated slope was close to unity and the affinity increased, but remained lower than for observations in the presence of only \(\mu\)- and \(\delta\)-suppression.
U-50488H, [D-Ala\(^2\), D-Leu\(^4\)]enkephalin and [D-Ala\(^2\), MePhe\(^3\), Gly-ol\(^5\)] enkephalin were all relatively inactive at displacing the remaining bound \(^{[3]}\text{H}\)ligand (Figure 7.21; Table 7.10).

When \(^{[3]}\text{H}\)diprenorphine in the presence of \(\mu\)- and \(\delta\)-suppression, was displaced from guinea-pig cerebellar homogenates by U-50488H, a biphasic curve was obtained (Figure 7.22a). The high affinity site represented approximately 75% of the bound radioligand. This curve was resolved into two Hill plots (Figure 7.22b) affording a high affinity component, \(IC_{50}\) 1.60 ± 0.36nM and slope 1.04 ± 0.04, typical of a k-site interaction [242] and a low affinity component, \(IC_{50}\) 535.7 ± 70.5nM, slope 0.91 ± 0.12.

b) Saturation analysis

\(^{[3]}\text{H}\)Diprenorphine binding in the presence of \(\mu\)-, \(\delta\)- and \(k\)-suppression was reduced to less than 10% of the total sites labelled in guinea-pig brain in this buffer system (Figure 7.23). The affinity of the radioligand for the remaining sites was high (Table 7.11).
Figure 7.18 Displacement of [3H]diprenorphine by [D-Ala²,MePhe⁴,Gly-ol⁵]enkephalin (○), [D-Ala²,D-Leu⁴] enkephalin in the presence of 100nM [D-Ala²,MePhe⁴,Gly-ol⁵]enkephalin (●), ICI 174864 (◆) and [D-Ala²,MePhe⁴,Gly-ol⁵]enkephalin in the presence of 1µM ICI 174864 (●) in guinea-pig brain homogenates prepared in 25mM HEPES buffer containing 10mM MgCl₂. See Table 7.9 for radioligand concentrations used and results.
Figure 7.19 Displacement of \([^3H]diprenorphine\) (0.22–0.25nM), in the presence of 100nM [D-Ala²,MePhe⁴,Gly-ol⁵] enkephalin and 1µM ICI 174864 to 0.2nM radioligand, by U-50488H in guinea-pig brain homogenates prepared in 25mM HEPES buffer containing 10mM MgCl₂. See Table 7.9 for data.
Table 7.9  Affinities for \( \mu \)-, \( \delta \)- and k-selective ligands to displace \([^3]H\)diprenorphine in guinea-pig brain homogenates in 25mM HEPES buffer containing 10mM MgCl\(_2\) at 25°C (Figures 7.18 & 7.19).

<table>
<thead>
<tr>
<th>Competing Ligand</th>
<th>([^3]H)Diprenorphine Concentration (nM)</th>
<th>IC(_{50}) (nM)</th>
<th>nH</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>[D-Ala(^2),MePhe(^{+}),Gly-ol(^{+})]enkephalin</td>
<td>0.25 - 0.27</td>
<td>H 3.27 ± 0.94</td>
<td>1.07 ± 0.10</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L 81.76 ± 13.7</td>
<td>0.74 ± 0.02</td>
<td>3</td>
</tr>
<tr>
<td>[D-Ala(^2),D-Leu(^3)]enkephalin *</td>
<td>0.26</td>
<td>H 31.62 ± 1.05</td>
<td>0.46 ± 0.05</td>
<td>3</td>
</tr>
<tr>
<td>ICI 174864</td>
<td>0.28 - 0.31</td>
<td>H 48.56 ± 17.04</td>
<td>0.87 ± 0.10</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L &gt;10(\mu)M</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>[D-Ala(^2),MePhe(^{+}),Gly-ol(^{+})]enkephalin **</td>
<td>0.26 - 0.33</td>
<td>H 4.27 ± 1.46</td>
<td>1.16 ± 0.10</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L 294.9 ± 183.9</td>
<td>0.80 ± 0.08</td>
<td>3</td>
</tr>
<tr>
<td>U-50488H ***</td>
<td>0.22 - 0.25</td>
<td>H 2.93 ± 0.24</td>
<td>1.06 ± 0.12</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L 2396 ± 624</td>
<td>0.82 ± 0.07</td>
<td>3</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem for n separate experiments in duplicate.

nH = Hill coefficient.

* Suppressed using 100nM unlabelled [D-Ala\(^2\),MePhe\(^{+}\),Gly-ol\(^{+}\)]enkephalin (\( \mu \)-suppression).

** Suppressed using 1\(\mu\)M unlabelled ICI 174864 (\( \delta \)-suppression).

*** Suppressed using 100nM unlabelled [D-Ala\(^2\),MePhe\(^{+}\),Gly-ol\(^{+}\)]enkephalin and 1\(\mu\)M unlabelled ICI 174864.

H = High affinity site.

L = Low affinity site.

Non-specific binding defined using 10\(\mu\)M unlabelled naloxone.
Displacement of $[^3]H$ diprenorphine (0.20-0.21nM) by dynorphin A-(1-17) in guinea-pig brain homogenates prepared in 25mM HEPES buffer containing 10mM MgCl$_2$. Open circles show results obtained in the presence of 100nM [D-Ala$^2$,MePhe$^4$,Gly-$\beta$-ol$^5$]enkephalin and 1μM ICI 174864; closed circles show results obtained in the presence of the above plus additional suppression by 100nM U-50488H. See Table 7.10 for data.
Figure 7.21 Displacement of [³H]diprenorphine (0.19-0.21nM) in the presence of 100nM each of [D-Ala²,MePhe⁴, Gly-ol⁶]enkephalin and U-50488H, and 1µM ICI 174864 to 0.2nM labelled ligand by [D-Ala²,D-Leu⁵]enkephalin (■), [D-Ala²,MePhe⁴,Gly-ol⁶]enkephalin (○) and U-50488H (●) in guinea-pig brain homogenates prepared in 25mM HEPES buffer containing 10mM MgCl₂. See Table 7.10 for data.
Table 7.10
Diplacement of \(^{[3]H}\)diprenorphine by \(\mu\), \(\delta\) and \(k\)-selective opioid compounds in guinea-pig brain homogenates in the presence of 100nM each of unlabelled U-50488H and [D-Ala\(^2\),MePhe\(^\ast\), Gly-ol\(^\ast\)]enkephalin and 1\(\mu\)M unlabelled ICI 174864 (to suppress binding to \(k\)-, \(\mu\)- and \(\delta\)-sites) in 25mM HEPES buffer containing 10mM MgCl\(_2\) at 25\(^\circ\)C (Figures 7.20 & 7.21).

<table>
<thead>
<tr>
<th>Competing Ligand</th>
<th>[^{[3]H})Diprenorphine Concentration (nM)</th>
<th>IC(_s_0) (nM)</th>
<th>(n_H)</th>
<th>Min. (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dynorphin A-(1-17) (^\ast)</td>
<td>0.20 - 0.21</td>
<td>47.09 ± 3.89</td>
<td>0.45 ± 0.03</td>
<td>0.992</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ (13.25 ± 1.54)</td>
<td>(0.82 ± 0.03)</td>
<td></td>
</tr>
<tr>
<td>Dynorphin A-(1-17)</td>
<td></td>
<td>753.0 ± 79.8</td>
<td>0.40 ± 0.06</td>
<td>0.986</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ (36.26 ± 10.11)</td>
<td>(0.93 ± 0.17)</td>
<td></td>
</tr>
<tr>
<td>[D-Ala(^2),D-Leu(^3)]enkephalin</td>
<td>0.19 - 0.21</td>
<td>++ &gt;1(\mu)M</td>
<td>0.53 ± 0.01</td>
<td>0.959</td>
</tr>
<tr>
<td>[D-Ala(^2),MePhe(^\ast),Gly-ol(^\ast)]enkephalin</td>
<td>+++ &gt;10(\mu)M</td>
<td>0.31 ± 0.03</td>
<td>0.917</td>
<td></td>
</tr>
<tr>
<td>U-50488H</td>
<td></td>
<td>6559 ± 519</td>
<td>0.92 ± 0.02</td>
<td>0.962</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem for 3 separate experiments in duplicate.

\(n_H\) = Hill coefficient.

Min. \(r\) = Minimum linear correlation coefficient.

Dynorphin A-(1-17) values for whole curve, except (+) where calculated using plateau as 100%.

\(\ast\) Suppressed using 1\(\mu\)M unlabelled ICI 174864 and 100nM unlabelled [D-Ala\(^2\),MePhe\(^\ast\),Gly-ol\(^\ast\)]enkephalin (\(\mu\)- and \(\delta\)-suppression).

Non-specific binding defined in the presence of 10\(\mu\)M unlabelled naloxone.
Displacement of [³H]diprenorphine (0.22nM), in the presence of 100nM [D-Ala²,MePhe⁴,Gly-ol⁶]enkephalin and 1µM ICI 174864 to 0.2nM radioligand, by U-50488H in guinea-pig cerebellar homogenates prepared in 25mM HEPES buffer containing 10mM MgCl₂: a) Displacement profile. b) Resolution into Hill plots.
Figure 7.23 Saturation analysis for [³H]diprenorphine in guinea-pig brain homogenates prepared in 25mM HEPES buffer, containing 10mM MgCl₂, in the absence (●) and presence (O) of 100nM each of [D-Ala²,MePhe⁶,Gly-ol⁵]enkephalin and U-50488H, and 1μM ICI 174864 to 0.2nM labelled ligand. Representative plots from 3 experiments. See Table 7.11 for radioligand concentration ranges used and results.
Binding studies in 25mM HEPES buffer, pH 7.4

a) Competition assays

The displacement of \(^{3}H\)diprenorphine in rat and guinea-pig brain homogenates by U-50488H was studied (Figure 7.24) using the \(\mu\)- and \(\delta\)-suppressing conditions previously determined. In the rat a high affinity \(k\)-component \([109]\) \((IC_{50} = 5.07 \pm 2.30nM; \text{slope } = 1.16 \pm 0.11)\) represented approximately 21% of the bound material. A low affinity component \((IC_{50} = 8055 \pm 1850nM; \text{slope } = 0.75 \pm 0.13)\) was also evident and a plateau extended from approximately 20nM to 100nM unlabelled ligand. In the guinea-pig the high affinity, \(k\)-component \((IC_{50} = 4.89 \pm 0.56nM; \text{slope } = 1.06 \pm 0.03)\) represented approximately 33% of the bound radioligand. The low affinity component afforded an \(IC_{50}\) value of 6741 \(\pm\) 247nM and a slope 0.91 \(\pm\) 0.17. A plateau was evident from approximately 40nM to 300nM.

b) Saturation analysis

The maximum number of sites labelled by \(^{3}H\)diprenorphine in guinea-pig brain homogenates was reduced by approximately 1.5 times in the absence of magnesium ions. However, the number of sites remaining after \(\mu\)-, \(\delta\)- and \(k\)-suppression was similar in both buffers (Figure 7.25; Table 7.11).

The influence of buffer systems on \(^{3}H\)diprenorphine binding

High specific binding was seen for \(^{3}H\)diprenorphine in all the buffer systems tested in this chapter (Table 7.12). A summary of the influences of ions and buffers on opioid binding sites in different CNS tissues is presented in Table 7.13.
Displacement of \([{}^3\text{H}]\)diprenorphine by U-50488H in rat brain (●) and guinea-pig brain (○) homogenates prepared in 25mM HEPES buffer. Rat brain experiments were performed in the presence of 100nM each of [D-Ala², D-Leu⁵]enkephalin and [D-Ala², MePhe⁴, Gly-ol⁵]enkephalin to 0.2nM radioligand, using a radioligand concentration range of 0.22 - 0.24nM. Guinea-pig brain experiments were performed in the presence of 100nM [D-Ala², MePhe⁴, Gly-ol⁵]enkephalin and 1μM ICI 174864 to 0.2nM radioligand, using a radioligand concentration range of 0.21 - 0.23nM.
Figure 7.25 Saturation analysis for [³H]diprenorphine in guinea-pig brain homogenates prepared in 25mM HEPES buffer, in the absence (●) and presence (○) of 100nM each of [(D-Ala^2,MePhe^4,Gly-ol^5)]enkephalin and U-50488H and 1μM ICI 174864 to 0.2nM labelled ligand. Representative plot from 3 experiments. See Table 7.11 for radioligand concentration ranges used and results.
Table 7.11  Saturated binding analysis for [³H]diprenorphine in guinea-pig brain homogenates in 25mM HEPES buffer and 25mM HEPES buffer containing 10mM MgCl₂, at 25°C (Figures 7.23 & 7.25).

<table>
<thead>
<tr>
<th>Buffer Conditions</th>
<th>Suppression</th>
<th>[³H]Diprenorphine Concentration (nM)</th>
<th>Bmax (fmol.mg⁻¹)</th>
<th>K_D (nM)</th>
<th>% Total Sites</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES</td>
<td>None</td>
<td>0.027 - 10.79</td>
<td>117.8 ± 16.22</td>
<td>0.160 ± 0.004</td>
<td>-</td>
<td>0.886 - 0.973</td>
</tr>
<tr>
<td></td>
<td>* µδ+κ</td>
<td>0.027 - 2.80</td>
<td>22.55 ± 4.44</td>
<td>0.079 ± 0.010</td>
<td>18.9 ± 1.1</td>
<td>0.854 - 0.946</td>
</tr>
<tr>
<td>HEPES/Mg</td>
<td>None</td>
<td>0.121 - 10.80</td>
<td>172.2 ± 11.32</td>
<td>0.201 ± 0.037</td>
<td>-</td>
<td>0.928 - 0.998</td>
</tr>
<tr>
<td></td>
<td>* µδ+κ</td>
<td>0.006 - 2.46</td>
<td>16.42 ± 1.55</td>
<td>0.097 ± 0.021</td>
<td>9.22 ± 0.12</td>
<td>0.860 - 0.975</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem for 3 separate experiments in duplicate.

r - Non-linear coefficient.

* Suppressed using 100nM each of unlabelled [D-Ala²,MePhe³,Gly-ol⁴]enkephalin and U-50488H (µ- & κ-suppression) and 1µM unlabelled ICI 174864 (δ-suppression).

+ HEPES/Mg represents 25mM Hepes buffer containing 10mM MgCl₂.

Non-specific binding defined using 10µM unlabelled naloxone.
Table 7.12 The influence of cations and buffers upon [³H]diprenorphine binding in brain homogenates from rat and guinea-pig, at 25°C.

<table>
<thead>
<tr>
<th>Species &amp; Tissue</th>
<th>Buffer System</th>
<th>[³H]Diprenorphine Concentration (nM)</th>
<th>Counts Bound (dpm)</th>
<th>% Specific</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat brain</td>
<td>Tris/Na</td>
<td>0.20 - 0.24</td>
<td>6176 ± 185</td>
<td>276 ± 18</td>
<td>95.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>HEPES/Mg</td>
<td>0.23 - 0.24</td>
<td>5503 ± 179</td>
<td>334 ± 12</td>
<td>93.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>HEPES</td>
<td>0.22 - 0.24</td>
<td>4537 ± 77</td>
<td>282 ± 13</td>
<td>93.6 ± 0.4</td>
</tr>
<tr>
<td>Guinea-pig brain</td>
<td>HEPES/Mg</td>
<td>0.25 - 0.26</td>
<td>5453 ± 137</td>
<td>352 ± 12</td>
<td>94.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>HEPES</td>
<td>0.21 - 0.23</td>
<td>3062 ± 39</td>
<td>231 ± 12</td>
<td>92.0 ± 0.7</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem of n individual experiments in duplicate.

Total = Specific plus non-specific [³H]diprenorphine binding.
Non-specific = binding of [³H]diprenorphine in the presence of 10μM unlabelled naloxone.

Tris/Na = 50mM Tris-HCl buffer containing 25mM NaCl.
HEPES/Mg = 25mM HEPES buffer containing 10mM MgCl₂.
HEPES = 25mM HEPES buffer.
Table 7.13 The influence of ions and buffers on the high affinity binding displaced by compounds with \(\mu\)-, \(\delta\)- and \(\kappa\)-specificity at 25°C. [D-Ala\(^2\),D-Leu\(^4\)]enkephalin (in the presence of \(\mu\)-suppression) or ICI 174864 displaced from \(\delta\)-sites, [D-Ala\(^2\),MePhe\(^4\),Gly-ol\(^5\)]enkephalin displaced \(\mu\)-binding and U-50488H displaced \(\kappa\)-binding. A summary of the results obtained in Chapter 7.

<table>
<thead>
<tr>
<th>Radioligand</th>
<th>Concentration Range (nM)</th>
<th>Species &amp; Tissue</th>
<th>Buffer System</th>
<th>Competing Ligand</th>
<th>Approximate Proportion of Sites (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>([^3]H)(-)Bremazocine</td>
<td>0.40 - 0.52</td>
<td>Rat Brain</td>
<td>Tris-HCl</td>
<td>[D-Ala(^2),MePhe(^4),Gly-ol(^5)]enkephalin</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>0.54 - 0.71</td>
<td></td>
<td></td>
<td>ICI 174864</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>0.51 - 0.52</td>
<td>GP Brain</td>
<td></td>
<td>[D-Ala(^2),MePhe(^4),Gly-ol(^5)]enkephalin</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>0.53 - 0.58</td>
<td></td>
<td></td>
<td>ICI 174864</td>
<td>10</td>
</tr>
<tr>
<td>([^3]H)Diprenorphine</td>
<td>1.82 - 2.31</td>
<td>Rat Brain</td>
<td>Tris/Na</td>
<td>ICI 174864</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>0.23</td>
<td>GP Cerebellum</td>
<td>HEPES/Mg</td>
<td>U-50488H</td>
<td>75</td>
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<tr>
<td></td>
<td>0.23 - 0.38</td>
<td></td>
<td></td>
<td>[D-Ala(^2),MePhe(^4),Gly-ol(^5)]enkephalin</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>0.23 - 0.38</td>
<td>Rat Brain</td>
<td>HEPES/Mg</td>
<td>[D-Ala(^2),D-Leu(^4)]enkephalin</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>0.23 - 0.32</td>
<td></td>
<td></td>
<td>U-50488H</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>0.26 - 0.33</td>
<td>GP Brain</td>
<td></td>
<td>[D-Ala(^2),MePhe(^4),Gly-ol(^5)]enkephalin</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>0.28 - 0.31</td>
<td></td>
<td></td>
<td>ICI 174864</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>0.22 - 0.25</td>
<td></td>
<td></td>
<td>U-50488H</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>0.22 - 0.24</td>
<td>GP Cerebellum</td>
<td>HEPES/Mg</td>
<td>U-50488H</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>0.21 - 0.23</td>
<td>Rat Brain</td>
<td>HEPES/Mg</td>
<td>U-50488H</td>
<td>21</td>
</tr>
</tbody>
</table>

Tris-HCl = 50mM Tris-HCl buffer.
Tris/Na = 50mM Tris-HCl buffer containing 25mM NaCl.
HEPES/Mg = 25mM HEPES buffer containing 10mM MgCl\(_2\).
HEPES = 25mM HEPES buffer.
Prefix "GP" = guinea-pig.
Subcellular distribution of opioid binding

After the fractionation of rat brain tissues according to the method of Gray and Whittaker [260] (Chapter 2) the mitochondrial enzyme-marker monoamine oxidase (MAO) was concentrated in the mitochondrial pellet, the myelin marker 2',3'-cyclic nucleotide 3'-phosphohydrolase (2',3'-CNP) was concentrated in the crude myelin fraction, though the membrane marker Na⁺,K⁺-activated adenosine 5'-triphosphate phosphohydrolase (Na⁺,K⁺-ATPase) was distributed between the synaptosomal and myelin fractions (Figure 7.26; Table 7.14) suggesting contamination of the myelin fraction. [³H]Diprenorphine binding followed the membrane marker Na⁺,K⁺-ATPase. Using [³H](−)-bremazocine, opioid binding was confirmed in myelin. In the presence of μ- and δ-suppression the specifically bound radioligand was reduced by 10% suggesting that most of the binding was of the k-type. Under these conditions this bound material was displaced by U-50488H (Figure 7.27) affording an IC₅₀ value of 56.23nM, Hill slope 0.55 (means of two experiments in duplicate). Indeed attempts were made to purify the crude myelin (Figure 7.28) but difficulty was experienced in gaining reproducible results, especially for Na⁺,K⁺-ATPase (Table 7.15). However, the activity of Na⁺,K⁺-ATPase did appear to decrease with myelin purification and this was closely followed by a decrease in [³H]diprenorphine binding. The activity of the myelin marker 2',3'-CNP increased approximately four fold during purification.
Figure 7.26

Distribution of marker enzyme activity and [\textsuperscript{3}H]diprenorphine binding in rat brain fractions. Data is given in Table 7.14.

**KEY**

**Markers**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAO</td>
<td>Monoamine oxidase.</td>
</tr>
<tr>
<td>CNP</td>
<td>2',3'-cyclic nucleotide 3'-phosphohydrolase.</td>
</tr>
<tr>
<td>NaK</td>
<td>Na\textsuperscript{+}, K\textsuperscript{+}-activated adenosine 5'-triphosphate phosphohydrolase.</td>
</tr>
<tr>
<td>DIP</td>
<td>[\textsuperscript{3}H]Diprenorphine.</td>
</tr>
</tbody>
</table>

**Brain Fraction**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>p</td>
<td>P\textsubscript{1} fraction.</td>
</tr>
<tr>
<td>y</td>
<td>myelin fraction.</td>
</tr>
<tr>
<td>s</td>
<td>synaptosomal fraction.</td>
</tr>
<tr>
<td>i</td>
<td>mitochondrial fraction.</td>
</tr>
</tbody>
</table>
Table 7.14 Enzyme marker activity and [\textsuperscript{3}H]diprenorphine binding in the subcellular components of rat brain (Figure 7.26).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>% Recovered Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MAO</td>
</tr>
<tr>
<td>P\textsubscript{1}</td>
<td>*2.09 ± 2.09</td>
</tr>
<tr>
<td>Crude Myelin</td>
<td>*2.69 ± 2.69</td>
</tr>
<tr>
<td>Synaptosomal</td>
<td>*8.57 ± 8.57</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>86.1 ± 7.5</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem of 3 separate experiments in duplicate.

MAO = Monoamine oxidase [EC 1.4.3.4].
2',3'-CNP = 2',3'-Cyclic nucleotide 3'-phosphohydrolase [EC 3.1.4.16].
Na\textsuperscript{+},K\textsuperscript{+}-ATPase = Na\textsuperscript{+},K\textsuperscript{+}-Activated adenosine triphosphate phosphohydrolase [EC 3.6.1.3].

* Activity detected in only one out of three experiments.
** No activity detected in all three experiments.

Non-specific binding for [\textsuperscript{3}H]diprenorphine defined in the presence of 10\textmu M unlabelled naloxone.
Displacement of \( ^{[\text{H}]}(-)\)-bremazocine (0.14nM), in the presence of 1\(00\text{nM}\) each of [D-Ala\(^2\), D-Leu\(^5\)] enkephalin and [D-Ala\(^2\), MePhe\(^4\), Gly-ol\(^5\)] enkephalin to 0.2nM labelled ligand, by \( \text{U-50488H} \), in the myelin fraction of rat brain.
Figure 7.28  Marker enzyme activity and [3H]diprenorphine binding in crude and purified myelin. CNP - 2',3'-cyclic nucleotide 3'-phosphohydrolase; DIP - [3H]diprenorphine; NaK - Na⁺,K⁺-activated adenosine 5'-triphosphate phosphohydrolase. See Table 7.15 for data.
<table>
<thead>
<tr>
<th>Myelin Fraction</th>
<th>* 2',3'-CNP</th>
<th>** Na⁺,K⁺-ATPase</th>
<th>*** [³H]Dip.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>3.15 ± 2.29</td>
<td>0.42 ± 0.05</td>
<td>10.0 ± 1.8</td>
</tr>
<tr>
<td>Purified</td>
<td>14.1 ± 1.8</td>
<td>+ 0.12 ± 0.08</td>
<td>+ 4.64 ± 1.7</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem of 4 separate experiments in duplicate.  
* 2',3'-CNP = 2',3'-Cyclic nucleotide 3'-phosphohydrolase.  
** Na⁺,K⁺-ATPase = Na⁺,K⁺-Activated adenosine 5'-triphosphate phosphohydrolase.  
Units of activity:  
* 2',3'-CNP - 1 unit = 1 μmole phosphate liberated. minute⁻¹.  
** Na⁺,K⁺-ATPase - 1 unit = 1 μmole phosphate liberated. minute⁻¹.  
*** [³H]Diprenorphine - 1 unit = 1 fmole specifically bound.  
+ No activity was detectable in two out of the four assays.  
Non-specific binding for [³H]diprenorphine was defined using 10μM unlabelled naloxone.
DISCUSSION

Conditions under which μ- and δ-opioid sites could be selectively blocked were chosen from binding studies in rat and guinea-pig brain homogenates prepared in 50mM Tris-HCl buffer. Under these suppressing conditions, the remaining binding displayed characteristics of an heterogeneous population of opioid sites in both species. In the rat, these sites represented approximately 10% of the total number labelled by \[^{3}H\](-)-bremazocine in line with previous reports for k-sites [97, 193]. However, displacement studies using μ-, δ- and k-selective compounds afforded binding constants which were not typical of interaction at k-sites. In the guinea-pig, the binding constants afforded were in line with those for interaction at k-sites but the shallow Hill plots suggested that the situation may be more complex. It was seen that [D-Ala²,MePhe⁴,Gly-ol⁵]enkephalin afforded shallow Hill slopes in the guinea-pig but not in the rat, whilst [D-Ala²,D-Leu⁵]enkephalin afforded shallow Hill plots only in the rat, which may suggest a different heterogeneity in the rat compared to the guinea-pig. However, the various components of binding were not separable.

When 25mM sodium chloride was included in the Tris-HCl buffer, conditions which have been previously shown to promote the antagonist state of the opioid receptor [253] and confirmed by the shift to lower potency for agonists, it was not possible to discern more than one binding component for U-50488H or [D-Ala²,MePhe⁴,Gly-ol⁵] enkephalin. Further, ICI 174864 displaced \[^{3}H\]diprenorphine with an affinity in the range for interaction with δ-sites [238, 239] only at high concentrations of radioligand (2nM), suggesting that with the level of \[^{3}H\]diprenorphine usually used (0.2nM) few δ-sites were being labelled.

Saturation analysis of \[^{3}H\]diprenorphine showed no change in Bmax or \(K_D\) values in the presence/absence of 25mM sodium chloride supporting previous observations by Childers and Snyder [241], who showed no change for these parameters using the antagonist \[^{3}H\]naloxone in 100mM sodium chloride, although others (Wood and colleagues [248]) have demonstrated an increase in both the affinity and Bmax under similar conditions.
Kosterlitz and colleagues [255] have shown a potentiation of [\(^{3}H\)]diprenorphine binding at the \(\mu\)-site by sodium ions and an apparent inhibition at the \(k\)-site under the same conditions. Therefore, the \(\mu\)-, \(\delta\)- and \(k\)-binding of [\(^{3}H\)]diprenorphine may be differentially affected by sodium ions which may result in the nett effect of no change in the binding parameters observed.

In a buffer system of 25mM HEPES containing 10mM magnesium chloride it was possible to discern high and low affinity binding components separated by a plateau for [D-Ala\(^2\),D-Leu\(^3\)]enkephalin, [D-Ala\(^2\), MePhe\(^4\),Gly-ol\(^5\)]enkephalin and U-50488H displacement of [\(^{3}H\)]diprenorphine in rat brain homogenates. The high affinity components of these curves corresponded to displacement from \(\delta\)-, \(\mu\)- and \(k\)-sites respectively. When suitable concentrations of these unlabelled ligands were chosen to suppress [\(^{3}H\)]diprenorphine binding to \(\delta\)-, \(\mu\)- and \(k\)-sites, a low affinity component still remained which could be studied. The increased potency of levorphanol over dextrorphan and displacement by naloxone showed that the remaining sites were opioid in nature. The apparently low affinities of levorphanol and naloxone suggest that the sites have more in common with the \(k\)-type than with \(\mu\)- or \(\delta\)-types and the shallow Hill slopes suggest that heterogeneity may still exist within the remaining population.

Results in guinea-pig brain homogenates show that after \(\mu\)- and \(\delta\)-suppression, displacements using U-50488H and the \(k\)-selective peptide dynorphin A-(1-17) afforded shallow Hill slopes and lower affinities than previously reported for interaction at \(k\)-sites by these ligands[72, 242]. Indeed after suppression of the \(k\)-component only 50% of the remaining [\(^{3}H\)]diprenorphine bound was sensitive to displacement by dynorphin A-(1-17) suggestive of heterogeneity of the \(k\)-population in guinea-pig brain. Consideration should be given to the fact that the apparently low affinities seen with dynorphin A-(1-17) in these studies may result from adsorptive losses of free ligand onto sub-cellular contaminants and vessels containing the assay mixture [214-16]. The remaining sites represent only a small proportion (<10%) of the total sites labelled by [\(^{3}H\)]diprenorphine.
In agreement with the findings presented in this chapter, Morre and colleagues [262] have shown that after \( \mu \)-, \( \delta \)- and \( \sigma \)-suppression, \([^{1}H]\)ethylketocyclazocine is readily displaced in guinea-pig brain homogenates by non-peptidic opioids but was "resistant" to displacement by dynorphin A-(1-9), dynorphin A-(1-17) and by \( \alpha \)- and \( \beta \)-neo-endorphins. The "dynorphin-sensitive" site was proposed to correspond to the "classical k" site. Similarly Su [200] has proposed an heterogeneity of k-sites in guinea-pig brain homogenates which are readily displaced by tifluadom, U-50488H and dynorphin A-(1-13), but in a biphasic manner. In this work 100nM [D-Ala\(^{2}\), D-Leu\(^{4}\)]enkephalin was used to suppress both \( \mu \)- and \( \delta \)-sites and therefore, some of the apparent heterogeneity may represent interaction with unsuppressed \( \mu \)-sites. An heterogeneous k-population may also exist in human brain. Thus Pfeiffer and co-workers [199] have shown that in the presence of \( \mu \)- and \( \delta \)-suppression \([^{1}H]\)diprenorphine was displaced in a biphasic manner from human amygdala by dynorphin A-(1-17). The k-population was divided into "dynorphin A-(1-17)-sensitive" and "-insensitive" sites.

On the other hand Audigier and colleagues [261] were unable to show any specific binding for \([^{1}H]\)etorphine in guinea-pig striatum after suppression with 5wM unlabelled [D-Ala\(^{2}\), D-Leu\(^{4}\)]enkephalin and demonstrated an homogeneous population of sites at lower levels of \( \mu \)- and \( \delta \)-suppression. This result may reflect regional differences in the distribution of opioid sites.

Reports on heterogeneity of the k-population in rat brain are few presumably because of the low levels of k-sites present in this tissue [97, 193]. However, there is some evidence in the literature that an heterogeneity of k-binding does exist although it has rarely been fully addressed. For example, Mansour and co-workers [263] used [D-Ala\(^{2}\), MePhe\(^{4}\), Gly-ol\(^{5}\)]enkephalin and [D-Pen\(^{2}\), D-Pen\(^{4}\)]enkephalin to suppress \([^{1}H]\)(-)-bremazocine binding to \( \mu \)- and \( \delta \)-sites respectively. Under these conditions, U-50488H displaced the radioligand with an affinity in excess of 650nM. Morris and Herz [264] suppressed \([^{1}H]\)(-)-bremazocine binding to \( \mu \)- and \( \delta \)-sites using 1\( \mu \)M each of [D-Ala\(^{2}\), D-Leu\(^{4}\)]enkephalin and [D-Ala\(^{2}\), MePhe\(^{4}\), Gly-ol\(^{5}\)]enkephalin.
The remaining binding was displaced by U-50488H affording an IC$_{50}$ of approximately 30nM and a slope of less than unity. The higher affinity of U-50488H under these conditions may reflect an over-suppression of µ- and δ-sites and therefore, the removal of some of the low affinity component. In both of the above reports, the autoradiographical distribution of k-sites was studied using the suppressing conditions described above. The distribution of k-sites, which is anatomically distinct from either µ- or δ-sites will therefore include any extra unidentified binding sites. The close correlation of the k-site and the low affinity site distributions may represent indirect evidence that the two are related, possibly representing high and low affinity forms of the same receptor.

When HEPES alone was the buffer system used, the displacement profile for U-50488H in rat brain homogenates remained relatively unchanged from that seen in HEPES containing magnesium ions. In the guinea-pig there was a more pronounced change in the displacement curve, with a marked decrease in the % of the high affinity component and a shift to decreased potency for the low affinity binding component of approximately three-fold, suggesting a differential effect on the high and low affinity U-50488H binding sites by magnesium ions. The proportion of sites remaining after µ-, δ- and k-suppression was approximately 19% of the total labelled by [³H]diprenorphine. This higher percentage was reflected by a decrease in the B$_{max}$ for the total sites labelled in HEPES buffer alone, rather than an increase in the number of sites remaining after suppression. The higher levels of [³H]diprenorphine binding in the presence of magnesium ions may be the result of increased δ-binding [245, 254]. The apparent increase in the high affinity k-component in guinea-pig brain when magnesium ions are included in the HEPES buffer is more difficult to explain, since divalent cations have been shown to reduce binding at the k-site for [³H]dynorphin A-(1-9) [254], [³H](−)-bremazocine [254, 255] and [³H]diprenorphine [255].

The lower levels of binding seen in HEPES only (Table 7.12) may help to explain the low levels of binding seen by Lahti and colleagues [198] for [³H]U-69593 in a variety of CNS tissues.
The heterogeneity of k-sites was explored further using guinea-pig cerebellar homogenates. In 50mM Tris-HCl buffer containing 25mM sodium chloride a biphasic curve was obtained when \[^{3}H\]diprenorphine was displaced by U-50488H. However, since no suppression was present the lower affinity component may represent \(\mu\)- and \(\delta\)-sites. When the displacement was repeated in the presence of \(\mu\)- and \(\delta\)-suppression using homogenates prepared in 25mM HEPES buffer containing 10mM magnesium chloride, a biphasic curve afforded lower IC\(_{50}\) values for both the high affinity component and more especially the lower affinity component, which was five-fold more potent in the cerebellum than in the guinea-pig brain. These results are inconsistent with the work of Robson and her colleagues [242] who demonstrated an homogeneous population of k-sites in the guinea-pig cerebellum by displacing \[^{3}H\](-)-bremazocine, in the presence of \(\mu\)- and \(\delta\)-suppression with U-50488H. One possible explanation for the discrepancy is that these workers used 100nM \(\text{[D-Ala}^2,\text{D-Leu}^5\text{]}\text{enkephalin}\) to suppress \(\delta\)-sites (this present work used 1\(\mu\)M ICI 174864) which may also have suppressed some of the lower affinity component to the extent that it no longer affects the slope of the Hill plot.

The extra sites are unlikely to be interconverting high and low affinity forms of the k-site since shallow Hill plots have been obtained for the displacement of \[^{3}H\](-)-bremazocine by U-50488H in guinea-pig cerebellar homogenates in a selection of different buffers, including more physiologically relevant HEPES-buffered Krebs [246].

Saturation analysis showed that \[^{3}H\]dynorphin A-(1-9) and \[^{3}H\](-)-bremazocine labelled similar numbers of sites in the guinea-pig cerebellum, after \(\mu\)- and \(\delta\)-suppression. These results are similar to those seen in the guinea-pig (but, not the rat) spinal cord (Chapter 6).

Binding data presented in this thesis suggests that the nature of the population of opioid sites remaining after \(\mu\)- and \(\delta\)-suppression may differ between species and may even vary between the CNS regions of the same species (in the case of the guinea-pig). Alternatively, the results may reflect differences in the size of the populations of the high affinity and lower affinity components in the tissues studied.
Following an early report that \[^{1}H\]opioids bind stereospecifically to cerebrosides [85], investigations were performed using \[^{1}H\]diprenorphine to see if binding to myelin could be responsible for the apparent heterogeneity observed in the displacement assays.

The distribution of \[^{1}H\]diprenorphine binding followed that of the plasma-membrane marker \(Na^{+},K^{+}\)-activated ATPase in subcellular fractions of rat brain, suggesting that the extra sites are not the result of radioligand binding to cerebrosides or other myelin components [85]. These results are supported by the findings of Pert and her colleagues [84]. Cross-contamination between fractions was evident which may be a consequence of the preparative technique used, especially the severity of the initial homogenisation [257]. Entrapment of synaptosomes within the \(P_{1}\), crude myelin and mitochondrial fractions [257] is another likely cause of the contamination seen with the present studies. The degree of myelin contamination may also be a function of the age of rats used since it is known that the myelin content of rat brain increases by 1500\% between days 15 and 180 [256]. In an attempt to study possible opioid binding to myelin, the myelin fraction was purified. This afforded inconsistent results and although the binding of \[^{1}H\]diprenorphine appeared to follow \(Na^{+},K^{+}\)-ATPase activity, further work is required to confirm the binding of \[^{1}H\]opioids to myelin.

In conclusion, by using carefully selected assay conditions it has been possible to study a population of opioid binding sites which are not of the \(\mu\)-, \(\delta\)- or \(k\)-type. Heterogeneity of the \(k\)-component was evident in rat and guinea-pig neural tissues although the characteristics of these sites appeared to vary between species and CNS regions. The low affinity of dynorphin A-(1-17) and U-50488H, two \(k\)-selective ligands for the sites remaining after \(\mu\)-, \(\delta\)- and \(k\)-suppression implies that they may not be subtypes of the \(k\)-receptor and preliminary studies suggest that they are not low affinity sites on myelin. However, they may represent low affinity states of the \(\mu\)-, \(\delta\)- or \(k\)-receptor which are not susceptible to the low concentrations of suppressing agonists used. This work provides a method by which further characterization may be performed. In the light of this work, the definition of the \(k\)-opioid binding site requires modification (see Appendix).
CHAPTER 8

OPIOID BINDING STUDIES IN THE SPINAL CORD

OF THE MOUSE, AND SOME BINDING CHARACTERISTICS

OF XORPHANOL
INTRODUCTION

The mouse is a commonly used animal for testing the antinociceptive properties of opioid analgesics. Activity has been demonstrated at the spinal [224-226] and supraspinal levels [223-225] for compounds with different activity profiles at \( \mu \)-, \( \delta \)- and \( \kappa \)-receptors. Although the involvement of these receptors in different antinociceptive responses has been postulated, there appears to have been no attempt to verify the existence of such receptors in the spinal cord by binding assays.

The opioid analgesic xorphanol (Figure 8.1) is a relatively recently introduced compound known to have analgesic properties in different species [227, 229] including the mouse [228, 229] and in man [230, 231].

\[ \text{Figure 8.1 Xorphanol} \]

In vitro work using peripheral tissues known to contain opioid receptors from the guinea-pig [229, 232], rat and rabbit [229] have postulated that xorphanol possesses partial agonist properties at the \( \kappa \)-receptor and antagonist properties at the \( \mu \)-receptor [229]. This work supports observations seen in whole animal studies [228, 229]. The apparently low dependence liability of the compound [232, 233] and few side effects have made xorphanol a useful candidate for analgesia in clinical situations [230, 231]. However, the current literature does not appear to contain any work in which the binding characteristics of xorphanol have been studied.
The aim of this study is to characterize the opioid binding sites in mouse spinal cord, using standard ligand binding techniques employing μ-, δ- and κ-selective compounds. Secondly, xorphanol binding will be studied in mouse cord and then in more readily available central nervous system (CNS) tissues from the guinea-pig.
MATERIALS & METHODS

Whole spinal cords from female Swiss-Webster outbred mice were removed as described in Chapter 2. Homogenates of mouse cord, guinea-pig brain and guinea-pig cerebellum were prepared and binding studies at 25°C were performed as detailed in Chapter 2, except for antagonist binding which was studied using [³H]naloxone or [³H]diprenorphine in the presence of 100mM sodium chloride and 50µM of the stable guanosine triphosphate analogue, 5-guanylylimidodiphosphate (GppNHp).

Radiolabelled ligands used to label opioid binding sites were [³H]diprenorphine, [³H][D-Ala²,MePhe⁵,Gly-ol³]enkephalin, [³H][D-Ala²,D-Leu⁵]enkephalin and [³H]naloxone.

Non-specific binding was defined using 1µM unlabelled MR 2266 for [³H]naloxone, 1µM unlabelled diprenorphine for [³H][D-Ala², MePhe⁵,Gly-ol³]enkephalin and [³H][D-Ala²,D-Leu⁵]enkephalin and 10µM unlabelled naloxone for [³H]diprenorphine. All compounds have the chemical structures described in Chapter 2.
RESULTS

Studies in mouse spinal cord homogenates

a) Saturation analysis

The total high affinity, saturable \([^3]H\)diprenorphine binding in whole spinal cord homogenates from the mouse was reduced by 78% when \(\mu\)- and \(\delta\)-suppression was present (Figure 8.2; Table 8.1). The affinity of the radioligand increased upon suppression.

\[^3]H\)[D-Ala\(^2\),D-Leu\(^5\)]Enkephalin, in the presence of \(\mu\)-suppression labelled a total population of high affinity sites which may be attributed to \(\delta\)-sites representing 14% of the total number labelled by \[^3]H\)diprenorphine (Figure 8.3). The levels of specific binding with \[^3]H\)[D-Ala\(^2\),D-Leu\(^5\)] enkephalin were low (Table 8.2) and may therefore contain considerable error.

The \(\mu\)-selective peptide \[^3]H\)[D-Ala\(^2\),MePhe\(^\ast\),Gly-ol\(^\ast\)] enkephalin bound in a saturable manner to high affinity sites (Figure 8.4) which may be attributed to \(\mu\)-sites [90, 91].

From this data the total binding of the putative \(\mu\)-, \(\delta\)- and \(k\)-populations represented only 82 fmol.mg\(^{-1}\), that is 65% of the total sites labelled by \[^3]H\)diprenorphine in mouse spinal cord homogenates (125.4 fmol.mg\(^{-1}\)).
Figure 8.2 Saturation analysis for [³H]diprenorphine in the absence (○) and presence (O) of 100nM [D-Ala²,D-Leu⁵]
enkephalin and [D-Ala²,MePhe⁴,Gly-ol⁵]enkephalin to 0.2nM labelled ligand in homogenates of mouse whole
spinal cord. Concentration range of radioligand used was 0.098 - 10.30nM in the absence of suppression
and 0.098 - 5.05nM for suppressed assays. Representative plots from 3 experiments. See Table 8.1 for data.
Saturation analysis for \(^{3}H\)[D-Ala\(^2\),D-Leu\(^5\)]enkephalin, in the presence of 25nM [D-Ala\(^2\),MePhe\(^4\),Gly-ol\(^5\)] enkephalin to 1nM radioligand in homogenates of mouse whole spinal cord. Concentration range of radioligand used was 0.111 - 10.79nM. Representative plot from 3 experiments. See Table 8.1 for data.
Figure 8.4 Saturation analysis for \[^{3}H\]D-Ala\(^{2}\),MePhe\(^{4}\),Gly-ol\(^{5}\)enkephalin in homogenates of mouse whole spinal cord, using a radioligand concentration range of 0.063 - 9.57 nM. Representative plot from 3 experiments. See Table 8.1 for data.
### Table 8.1
Determination of μ-, δ- and κ-binding sites in mouse whole spinal cord homogenates by saturated binding analysis at 25°C (Figures 8.2 - 8.4).

<table>
<thead>
<tr>
<th>Ligand and Assay Conditions</th>
<th>Bmax (fmol.mg⁻¹)</th>
<th>K_D (nM)</th>
<th>r</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>[³H]diprenorphine (Total)</td>
<td>125.4 ± 2.4</td>
<td>0.59 ± 0.14</td>
<td>0.934 - 0.989</td>
<td>3</td>
</tr>
<tr>
<td>*(Suppressed)</td>
<td>27.71 ± 0.43</td>
<td>0.23 ± 0.03</td>
<td>0.863 - 0.963</td>
<td>3</td>
</tr>
<tr>
<td>[³H][D-Ala²,D-Leu⁴]enkephalin ** (Suppressed)</td>
<td>17.32 ± 3.66</td>
<td>2.44 ± 0.56</td>
<td>0.937 - 0.976</td>
<td>3</td>
</tr>
<tr>
<td>[³H][D-Ala²,MePhe⁴,Gly-ol⁵]enkephalin</td>
<td>36.72 ± 3.02</td>
<td>1.22 ± 0.34</td>
<td>0.966 - 0.972</td>
<td>3</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem for n separate experiments in duplicate.

r = Non-linear correlation coefficient.

* Suppressed at 100nM each unlabelled [D-Ala²,MePhe⁴,Gly-ol⁵]enkephalin and [D-Ala²,D-Leu⁴]enkephalin to 0.2nM [³H]ligand.

** Suppressed at 25nM [D-Ala²,MePhe⁴,Gly-ol⁵]enkephalin to each 1nM labelled ligand.

Non-specific binding defined in the presence of 1μM unlabelled diprenorphine for [³H][D-Ala²,D-Leu⁴]enkephalin and [³H][D-Ala²,MePhe⁴,Gly-ol⁵]enkephalin, and 10μM unlabelled naloxone for [³H]diprenorphine.
### Table 8.2

Levels of $[^3H][D{-}\text{Ala}^2,D{-}\text{Leu}^5]enkephalin$ binding in mouse whole spinal cord homogenates at 25°C.

<table>
<thead>
<tr>
<th>Approximate Radioligand Concentration (nM)</th>
<th>Bound dpm.mg$^{-1}$ protein</th>
<th>% Specific Binding</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Non-Specific</td>
<td>Specific</td>
</tr>
<tr>
<td>0.1</td>
<td>91 ± 22</td>
<td>29 ± 18</td>
<td>63 ± 5</td>
</tr>
<tr>
<td>0.2</td>
<td>214 ± 41</td>
<td>88 ± 20</td>
<td>127 ± 21</td>
</tr>
<tr>
<td>1.0</td>
<td>628 ± 73</td>
<td>288 ± 44</td>
<td>340 ± 36</td>
</tr>
<tr>
<td>3.0</td>
<td>1112, 1761</td>
<td>706, 1125</td>
<td>406, 636</td>
</tr>
<tr>
<td>10.0</td>
<td>4084 ± 967</td>
<td>3604 ± 856</td>
<td>962 ± 210</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem.

n = Number of separate experiments from which data were derived.

Non-specific binding defined in the presence of 1μM unlabelled dioremorphine.

Binding studies were performed in the presence of 25nM unlabelled $[D{-}\text{Ala}^2,\text{MePhe}^3,\text{Gly-ol}^5]enkephalin$ to each 1nM labelled ligand.
b) Displacement studies

Binding at putative k-sites was studied using \(^{\text{[3H]}}\) diprenorphine in the presence of \(\mu\)- and \(\delta\)-suppression (Figure 8.5; Table 8.3). Xorphanol displaced the remaining binding with a slope close to unity and (-)-bremazocine similarly displaced with a slope of unity, but with a relatively higher affinity. The k-selective opioid U-50488H afforded a shallow Hill plot and a very low affinity (IC\(_{50}\) > 10\(\mu\)M). \(^{\text{[3H]}}\) Diprenorphine binding at a level of 0.2nM was reduced by 45% in the presence of \(\mu\)- and \(\delta\)-suppression.

The binding of 1nM \(^{\text{[3H]}}\)[D-Ala\(^2\),D-Leu\(^5\)]enkephalin in the presence of \(\mu\)-suppression represented 75% of the total specifically bound. This binding, taken to represent binding at \(\delta\)-sites, was readily displaced by xorphanol and the \(\delta\)-antagonist ICI 174864 with Hill slopes close to unity (Figure 8.6; Table 8.3).

Both [D-Ala\(^2\),MePhe\(^3\),Gly-ol\(^5\)]enkephalin and xorphanol displaced \(^{\text{[3H]}}\)[D-Ala\(^2\),MePhe\(^3\),Gly-ol\(^5\)]enkephalin from \(\mu\)-sites with Hill slopes of close to unity (Figure 8.7). Comparison studies in guinea-pig brain afforded similar results (Figure 8.8; Table 8.4).
Figure B.5 - Displacement of [3H]diprenorphine (0.18-0.25nM), in the presence of 100nM each of [D-Ala2,D-Leu5]enkephalin and [D-Ala2,MePhe4,Gly-ol5]enkephalin to 0.2nM radioligand, by (-)-bremazocine (▼), xorphanol (▼) and U-50488H (●) in mouse whole spinal cord homogenates. See Table B.2 for data.
Figure 8.6 Displacement of $[^3H][\text{D-Ala}^2, \text{D-Leu}^5]\text{enkephalin}$ (0.98-0.99\text{nM}), in the presence of 25\text{nM} $[\text{D-Ala}^2, \text{MePhe}^4, \text{Gly-ol}^5]\text{enkephalin}$ to 1\text{nM radioligand}, by xorphanol (■) and ICI 174864 (●) in mouse whole spinal cord homogenates. See Table 8.3 for data.

Figure 8.7 Displacement of $[^3H][\text{D-Ala}^2, \text{MePhe}^4, \text{Gly-ol}^5]\text{enkephalin}$ (2.24-2.38\text{nM}) by xorphanol (■) and $[\text{D-Ala}^2, \text{MePhe}^4, \text{Gly-ol}^5]$ enkephalin in mouse whole spinal cord homogenates. See Table 8.3 for data.
Table 8.3  Displacement of binding from putative μ-, δ- and k-binding sites in mouse whole spinal cord homogenates at 25°C (Figures 8.5 - 8.7).

<table>
<thead>
<tr>
<th>Radioligand (concentration range)</th>
<th>Unlabelled Competing Ligand</th>
<th>IC₅₀ (nM)</th>
<th>Hill Coefficient (nH)</th>
<th>Minimum r</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>[^3]H</em>Diprenorphine (0.18 - 0.25nM)</td>
<td>(-)-Bremazocine</td>
<td>2.79 ± 0.41</td>
<td>0.91 ± 0.05</td>
<td>0.935</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>U-50488H</td>
<td>&gt;10000</td>
<td>0.63 ± 0.02</td>
<td>0.978</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Xorphanol</td>
<td>4.71 ± 0.71</td>
<td>1.05 ± 0.08</td>
<td>0.938</td>
<td>3</td>
</tr>
<tr>
<td>**[^3]H][D-Ala²,D-Leu⁸]enkephalin (0.98 - 0.99nM)</td>
<td>ICI 174864</td>
<td>440 ± 130</td>
<td>1.08 ± 0.10</td>
<td>0.930</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Xorphanol</td>
<td>4.05 ± 0.48</td>
<td>1.00 ± 0.12</td>
<td>0.968</td>
<td>3</td>
</tr>
<tr>
<td>[^3]H][D-Ala²,MePhe⁴,Gly-ol³]enkephalin (2.24 - 2.38nM)</td>
<td>[D-Ala²,MePhe⁴, Gly-ol³]enkephalin</td>
<td>13.79 ± 1.00</td>
<td>1.14 ± 0.05</td>
<td>0.981</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Xorphanol</td>
<td>3.51 ± 0.87</td>
<td>1.17 ± 0.11</td>
<td>0.975</td>
<td>4</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem from n separate experiments in duplicate.

r = Correlation coefficient from linear regression.

* Binding to μ- and δ-sites suppressed using 100nM each of unlabelled [D-Ala²,D-Leu⁸]enkephalin and [D-Ala²,MePhe⁴,Gly-ol³]enkephalin to each 0.2nM labelled ligand.

** Binding to μ-sites suppressed using 25nM unlabelled [D-Ala²,MePhe⁴,Gly-ol³]enkephalin to 1nM labelled ligand. Non-specific binding defined in the presence of 1μM unlabelled diprenorphine, except for[^3]Hdiprenorphine where 10μM unlabelled naloxone was used.
Xorphanol studies

Xorphanol showed no selectivity in mouse spinal cord homogenates under the experimental conditions used. The displacement curves all afforded Hill slopes of approximately unity (Table 8.3).

Further characterization of xorphanol was achieved using [³H]ligands under conditions favouring agonist or antagonist states of the receptor. The agonist [³H][D-Ala²,MePhe⁴,Gly-ol⁵]enkephalin, in addition to low concentrations (0.15nM - 0.21nM) of the antagonist [³H]naloxone [87] were used to label μ-sites. Both [³H]ligands were displaced in guinea-pig brain homogenates by unlabelled naloxone and [D-Ala²,MePhe⁴,Gly-ol⁵]enkephalin with affinities indicative of interaction at μ-sites, and by xorphanol (Figures 8.8 - 8.11; Table 8.4 & 8.5). Low levels of specific [³H]naloxone binding under these conditions (Table 8.5) meant that only two out of three experimental results could be used. The slopes of the displacement curves were all close to unity. When [³H]naloxone (0.2nM) was studied in the presence of 100mM sodium chloride and 50μM GppNHp the level of bound radioligand increased and non-specific binding decreased (Table 8.6). Under these conditions the μ-agonist [D-Ala²,MePhe⁴,Gly-ol⁵]enkephalin showed a potency shift of approximately 50 times to lower affinity (Figure 8.9; Table 8.5), naloxone shifted by approximately twice to higher affinity (Figure 8.10) and xorphanol shifted by approximately three-fold to lower affinity (Figure 8.11). It was also seen that the slope of the displacement curves for [D-Ala²,MePhe⁴,Gly-ol⁵]enkephalin and naloxone, but not xorphanol were less than unity. (Table 8.5).

Xorphanol interaction with k-sites was studied using [³H]diprenorphine in the guinea-pig cerebellum under the agonist/antagonist conditions used above (Table 8.5). In the presence of sodium ions and GppNHp the total [³H]diprenorphine bound at 0.2nM appeared to increase (Table 8.6).
Under these conditions the U-50488H displacement curve shifted 10-fold to lower potency (Figure 8.12), the naloxone displacement shifted by 1.5 times to higher affinity (Figure 8.13) and xorphanol was equipotent (Figure 8.13). The Hill slope for U-50488H remained shallow and for xorphanol remained close to unity.
Figure 8.8. Displacement of $[^3H][D-Ala^2,MePhe^4,Gly-ol^5]$enkephalin (2.47-2.54nM) by xorphanol (■), naloxone (▲) and $[D-Ala^2,MePhe^4,Gly-ol^5]$enkephalin (○) in guinea-pig brain homogenates. See Table 8.4 for data.
Table 8.4  Displacement of \([\text{H}]\text{[D-Ala}^2,\text{MePhe}^\bullet,\text{Gly-ol}^\bullet]\)enkephalin (2.47 - 2.54nM) in guinea-pig brain homogenates at 25°C (Figure 8.8).

<table>
<thead>
<tr>
<th>Competing Ligand</th>
<th>IC$_{50}$ (nM)</th>
<th>nH</th>
<th>Minimum r</th>
</tr>
</thead>
<tbody>
<tr>
<td>[D-Ala$^a$,MePhe$^b$,Gly-ol$^c$]enkephalin</td>
<td>11.19, 9.87</td>
<td>0.98, 0.79</td>
<td>0.985</td>
</tr>
<tr>
<td>Naloxone</td>
<td>10.78, 8.36</td>
<td>1.00, 1.01</td>
<td>0.996</td>
</tr>
<tr>
<td>Xorphanol</td>
<td>2.25, 3.99</td>
<td>1.33, 1.32</td>
<td>0.980</td>
</tr>
</tbody>
</table>

Results expressed as individual determinations from 2 experiments in duplicate.

nH = Hill coefficient.

Minimum r = minimum correlation coefficient from linear regression.

Non-specific binding defined using 1µM unlabelled diprenorphine.
Displacement of $[^3]H$naloxone (0.15-0.21nM) by [D-Ala$^2$,MePhe$^4$,Gly-$ol^5$]enkephalin, in the absence (○) and presence (●) of 100mM NaCl and 50μM GppNHp, in homogenates of guinea-pig brain. See Table 8.5 for data.
Displacement of $[^{1}	ext{H}]$naloxone (0.15-0.21nM) by naloxone, in the absence ($\Delta$) and presence ($\Box$) of 100mM NaCl and 50μM GppNHp, in guinea-pig brain homogenates. See Table 8.5 for data.

Displacement of $[^{1}	ext{H}]$naloxone (0.15-0.21nM) by xorphanol, in the absence ($\Box$) and presence ($\blacksquare$) of 100mM NaCl and 50μM GppNHp, in guinea-pig brain homogenates. See Table 8.5 for data.
Displacement of [\(^{3}\)H]diprenorphine (0.24-0.36nM) by U-50488H, in the absence (○) and presence (●) of 100mM NaCl and 50μM GppNHp, in guinea-pig cerebellar homogenates:
a) Displacement curve b) Hill plot. For data see Table 8.5.
Figure 8.13  Displacement of [³H]diprenorphine (0.24-0.36nM) by xorphanol (□) and naloxone (△), in the absence (open symbols) and presence (closed symbols) of 100mM NaCl and 50μM GppNHp in guinea-pig cerebellar homogenates. See Table 8.5 for data.
Table 8.5 Displacement of $[^3\text{H}]$naloxone in guinea-pig brain homogenates and $[^3\text{H}]$diprenorphine in guinea-pig cerebellar homogenates at 25°C, in the absence and presence of 100mM NaCl and 50μM GppNHp (Figures 8.9 – 13).

<table>
<thead>
<tr>
<th>Species &amp; Radioligand</th>
<th>Control</th>
<th>+ 100mM NaCl &amp; 50μM GppNHp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Competing Ligand</td>
<td>IC$_{50}$ (nM)</td>
<td>nH</td>
</tr>
<tr>
<td>$[^3\text{H}]$Naloxone (0.15 – 0.21nM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guinea-pig brain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[D-Ala$^4$,MePhe$^8$,Gly-ol$^9$] enkephalin</td>
<td>13.20, 16.29</td>
<td>0.77, 1.10</td>
</tr>
<tr>
<td>Naloxone</td>
<td>5.45, 10.41</td>
<td>0.88, 0.93</td>
</tr>
<tr>
<td>Xorphanol</td>
<td>0.46, 0.67</td>
<td>1.06, 0.89</td>
</tr>
<tr>
<td>$[^3\text{H}]$Diprenorphine (0.24 – 0.36nM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guinea-pig cerebellum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U-50488H</td>
<td>20.03 ± 2.00</td>
<td>0.73 ± 0.07</td>
</tr>
<tr>
<td>Naloxone</td>
<td>21.06 ± 1.68</td>
<td>0.91 ± 0.11</td>
</tr>
<tr>
<td>Xorphanol</td>
<td>1.09 ± 0.90</td>
<td>1.09 ± 0.10</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem or as individual results for n separate experiments in duplicate.
min. r = minimum correlation coefficient from linear regression.
nH = Hill coefficient.
Non-specific binding for $[^3\text{H}]$naloxone defined using 1μM unlabelled MR 2266. For $[^3\text{H}]$diprenorphine 10μM unlabelled naloxone was used.
Table 8.6  Specific binding of [³H]opioids in the absence and presence of 100mM NaCl and 50μM GppNHp in guinea-pig CNS tissue homogenates at 25°C.

<table>
<thead>
<tr>
<th>Tissue &amp; Radioligand</th>
<th>Conditions</th>
<th>Bound (cpm)</th>
<th>% Specific Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>Non-Specific</td>
</tr>
<tr>
<td>Guinea-pig brain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[³H]Naloxone</td>
<td>Control</td>
<td>452 ± 78</td>
<td>172 ± 18</td>
</tr>
<tr>
<td>(0.15 - 0.21nM)</td>
<td>NaCl + GppNHp</td>
<td>625 ± 64</td>
<td>112 ± 6</td>
</tr>
<tr>
<td>Guinea-pig cerebellum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[³H]Diprenorphine</td>
<td>Control</td>
<td>1289 ± 22</td>
<td>272 ± 18</td>
</tr>
<tr>
<td>(0.24 - 0.36nM)</td>
<td>NaCl + GppNHp</td>
<td>1890 ± 140</td>
<td>251 ± 21</td>
</tr>
</tbody>
</table>

Results expressed as means ± sem.

n = Number of assays from which data was derived.

Figures in parentheses show the concentration ranges of radioligands used.

'Control' represents binding in the absence of NaCl & GppNHp.

Non-specific binding for [³H]naloxone was defined using 1μM unlabelled MR2266, and for [³H]diprenorphine using 10μM unlabelled naloxone.
DISCUSSION

Mouse spinal cord studies.

The total number of sites labelled in mouse spinal cord are in line with those seen in the rat but higher than seen in the guinea-pig (Chapter 6; Table 6.6). Of these sites approximately 29% were of the \( \mu \)-type, a level similar to that seen previously in the rat [192] and guinea-pig [152, 154] cords. When \( \delta \)-sites were labelled using \([^{3}H](\text{D-Ala}^2, \text{D-Leu}^5)\text{enkephalin}\) the presence of \( \mu \)-suppression the selective \( \delta \)-antagonist ICI 174864 [237] displaced the bound radioligand with an affinity in the typical range for interaction with \( \delta \)-sites [238, 239]. The labelled sites represented 14% of the total sites in line with previous reports for \( \delta \)-binding in the spinal cords of rat [152, 192], guinea-pig [152, 154], frog, pigeon and in man [152].

This work thus supports reports from in vivo work which have postulated both spinal and supraspinal activity for \( \mu \)- and \( \delta \)-selective compounds [223, 225, 236] in the mouse. In particular, supraspinal \( \mu \)-receptors appear to modulate thermally-induced pain [223, 225]. Spinal \( \delta \)-receptors have also been proposed to modulate thermal stimuli [224, 225, 235], but may also modulate visceral-chemical stimuli [225, 228] and gastrointestinal transit [224, 226, 236]. Although some of these results are based on studies using the relatively unselective \( \delta \)-ligand \([\text{D-Ala}^2, \text{D-Leu}^5]\text{enkephalin}\) [223], supporting evidence has also been derived using the bis-penicillamine enkephalin analogues [224, 225], compounds which are known to be agonists with high selectivity for the \( \delta \)-receptor [192, 238, 239].

When \([^{3}H]\text{diprenorphine}\) binding was studied in the presence of \( \mu \)- and \( \delta \)-suppression, results which were inconsistent with a \( k \)-population were afforded. Both U-50488H and (-)-bremazocine displaced this binding with lower affinities than reported previously for other tissues [96, 97, 108, 109]. A low Hill slope for U-50488H was indicative of interaction with an heterogeneous population of sites although no visually biphasic nature was apparent. These non \( \mu \)- or \( \delta \)-sites represented 22% of the total labelled sites.

It can be seen that in Swiss-Webster outbred mice, the total...
number of sites labelled by [³H]diprenorphine is greater than the
sum total of the sites discussed so far. This anomaly is difficult
to explain. One possibility is that over-suppression occurred,
that is 100nM each of [D-Ala²,D-Leu⁵]enkephalin and [D-Ala²,MePhe⁴,
Gly-ol⁵]enkephalin removed some of the putative k-component.
Since these conditions have been shown previously to remove only
the μ- and δ-sites [97], this lends further evidence to the non-
μ- or δ-sites in mouse spinal cord being different to k-sites seen
in other tissues.

An extensive literature covers k-opioid-mediated analgesia in the
mouse. k-Selective agents have been shown to be especially
effective against visceral-chemical pain after intrathecal
administration [225, 235] but inactive after intracerebroventricular
injection [223, 225, 229]. The k-selective peptides dynorphin
A-(1-9) [226], dynorphin A-(1-13) and dynorphin A-(1-17) [235] have
also been shown to have antinociceptive activity against cutaneous-
thermal pain. Some of the dynorphin A-(1-17) activity against
cutaneous-thermal pain was shown to be produced by a des-tyrosyl
fragment and hence, was non-opioid in nature, but the same
des-tyrosyl fragment was inactive against visceral-chemical pain
[235]. Therefore, in this latter lower-threshold pain, k-agonists
appear to operate via opioid receptors. Intrathecally-administered
k-agents do not produce the depression of gastrointestinal transit
in mice, seen with μ- and δ-selective opioids [224, 236].

The results of the displacement studies shown in Figure 8.5 suggest
that if the k-binding site exists in mouse spinal cord it is present only as a very low percentage of the total residual
population remaining after μ- and δ-suppression of [³H]diprenorphine
binding. Therefore, the in vivo effects of spinally administered
opioids may be via this small population of k-receptors or
alternatively may be mediated in the mouse via a k-receptor subtype.
It would be interesting to study the displacement profile of dynorphin A-(1-17) at these sites to determine whether the heterogeneity of binding seen in other tissues such as human brain [199] were present.

This study has only been able to partially characterize the non-μ- or δ-component, mainly due to the scarcity of tissue. Further work is necessary in which the ability of naloxone to displace the specifically bound [³H]diprenorphine and the stereoselectivity of the sites should be analysed in addition to displacement studies using a range of opioid compounds with known binding profiles.

Mice have shown strain differences in both their pharmacological response to opioids [74, 240] and binding characteristics of brain opioid binding sites [74, 157]. The results obtained in this current work may therefore, be unique to the Swiss-Webster strain of mice.

In conclusion, the spinal cord from Swiss-Webster outbred mice contains μ-sites (29%) and δ-sites (14%). There is no evidence from these studies of a true k-site in the cord although it is possible that such sites are present in very small numbers, based on evidence from in vivo work. Further characterization of all the sites is necessary.
Xorphanol Studies

The results obtained from binding studies in mouse spinal cord and guinea-pig CNS tissues suggest that xorphanol is a non-selective opioid at \( \mu \)- and \( k \)-sites.

The small shift to lower potency when \( [\text{H}] \)naloxone was displaced in guinea-pig brain by xorphanol in the presence of sodium ions and GppNHp lends evidence to xorphanol being an antagonist at the \( \mu \)-site, supporting in vitro results from the isolated rat vas deferens preparation.[229].

Displacement studies in the guinea-pig cerebellum show that xorphanol has antagonist properties at the \( k \)-site, in agreement with observations made using the field-stimulated guinea-pig ileum preparation where xorphanol displayed a low maximum agonist activity typical of a partial agonist [229]. \( \beta \)-Funaltrexamine, a selective \( \mu \)-receptor antagonist [243] had no effect on the xorphanol agonist activity, but \( \beta \)-chloroaltrexamine, an irreversible antagonist [244], in the presence of \( \mu \)-protection produced a shift to lower potency for xorphanol. These results indicate that the observed xorphanol agonism is mediated via \( k \)-receptors. However, in the rabbit vas deferens, a tissue containing only functional \( k \)-receptors [68] xorphanol is an antagonist [229].

Reports in the literature on xorphanol interactions at \( \delta \)-receptors are scarce. Hayes and co-workers [250] have demonstrated that xorphanol displays better \( \delta \)-antagonist properties than ICI 174864 against \( [D\text{-Ala}^2,D\text{-Leu}^5] \)enkephalin in the hamster vas deferens, a tissue known to contain only functional \( \delta \)-receptors [251].

Xorphanol in vivo has been shown to produce potent antinociceptive activity in the mouse abdominal constriction and guinea-pig paw pressure test but is inactive in the mouse hotplate and rat paw pressure test [228]. This spectrum of activity is characteristic of a low efficacy \( k \)-agonist [229].
Xorphanol is also known to produce no apparent physical dependence in animal tests [232, 233] which makes the compound a potentially useful analgesic clinically. These proposals are supported by preliminary clinical trials [230, 231].
CHAPTER 9

GENERAL CONCLUSIONS
The demonstration of k-sites in the spinal cord lends further evidence for k-mediated, spinal analgesia [190, 203]. Previously, immunohistochemical [134, 186] and autoradiographical [115, 116, 119, 145] work has demonstrated a high concentration of opioid peptides and binding sites in the dorsal horn, most notably in laminae I and II. However, autoradiographical work has been unable to demonstrate any significant binding in the ventral horn [115, 116, 119]. The present work has confirmed the high dorsal levels of opioid binding sites but also proposes that significant levels occur in the ventral horn, in agreement with a previous study which also used ligand binding techniques [152].

One explanation of the apparent discrepancy between methodologies would be that ventral sites are distributed in a diffuse manner, without the definite patterns found in the dorsal horn, and are not therefore visible to autoradiography. Although the function of ventral horn opioid receptors has not been clearly established, such receptors have been proposed to exist on sensory fibres entering the cord via this route [153] or to be involved in the modulation of motor activity [169, 181].

The location of opioid receptors in the dorsal horn was initially proposed to be pre-synaptic [124, 271]. However, following the demonstration by immunohistochemical techniques that enkephalinergic fibres synapsed with spinothalamic-projecting neurones [127], a post-synaptic location was additionally proposed. Attempts were made in the present work to study the location of k-sites using capsaicin (Figure 9.1) a compound known to destroy primary afferents [114, 115, 120, 272]. Unfortunately the low levels of capsaicin used did not affect opioid binding. Analysis of opioid binding after higher doses of capsaicin would indicate the levels to which dorsal horn binding is reduced and may also indicate whether particular site-types are selectively destroyed.

![Figure 9.1 Capsaicin](image-url)
The relatively uniform distribution of \( \mu \)-, \( \delta \)- and \( k \)-sites along the rostro-caudal axis demonstrated by the current studies both supports [146, 155] and refutes [152] previous findings, and does not appear to correlate with reported distributions of endogenous opioid peptides [149-51, 173].

The apparently large population of opioid sites remaining after \( \mu \)- and \( \delta \)-suppression in rat lumbo-sacral spinal cord is heterogeneous. Displacement of this binding by \([D-Ala^2,D-Leu^5]\) enkephalin affords high and low affinity binding components. When the portion of binding "sensitive" to displacement by lower concentrations of \([D-Ala^2,D-Leu^5]\) enkephalin is suppressed, only a small residual population of sites with the binding characteristics of "classical \( k \)" sites remains. Therefore, the levels of \( k \)-binding in spinal cord may be lower than previously reported [160, 195]. Preliminary work in this thesis suggests that an heterogeneity of \( k \)-sites may also occur in the spinal cord of the guinea-pig, mouse and farmyard pig, which is supported by other workers who have identified apparent \( k \)-site heterogeneity in the spinal cords from different species [163, 196]. A similar heterogeneity has also been postulated in guinea-pig [200] and human brain [199]. Indeed, this current work has been able to demonstrate heterogeneity in brain tissues from the rat and guinea-pig where, after careful choice of conditions for the selective suppression of \( \mu \)-, \( \delta \)- and \( k \)-sites, an additional population of opioid sites remained. It is unlikely that these extra sites were on myelin since \([^3H]\)opioid binding followed closely the distribution of the external membrane marker-enzyme \( Na^+,K^+ \)-activated ATPase when brain tissue from the rat was fractioned. They may however, represent a physiologically distinct class of sites or a low affinity state of another class of opioid site. Studies in the guinea-pig cerebellum [246] have shown that U-50488H retains a shallow displacement profile under conditions which promote the agonist or antagonist state of the opioid receptor. This is evidence that the extra sites may represent a distinct class of opioid site, a factor supported by the insensitivity of the sites in brain to displacement by the \( k \)-opioids U-50488H and dynorphin A-(1-17).
Additionally autoradiographical studies [263, 264] have shown that the distribution of sites labelled by $[^3]H\)(-)-bremazocine in the presence of $\mu$- and $\delta$-suppression was distinct from that of $\mu$- or $\delta$-sites. Therefore, since under these conditions $[^3]H\)(-)-bremazocine may also be labelling the additional sites, the apparent correlation with $k$-, but not $\mu$- or $\delta$-sites lends evidence to the extra sites being $k$-subtypes. Alternatively, they may represent a component which is only identified by binding assay techniques and therefore, the use of a physiological correlate is necessary to identify the corresponding receptor in vivo. In this context Wood and colleagues [206] have claimed physiological evidence for the existence of $k$-subtypes from studies where the influence of proposed $k$-agonists on plasma TSH and corticosterone levels was determined. However, not all of the compounds used to evaluate $k$-activity were specific. For example, *MR 2034*, tifluadom and ethylketocyclazocine possess $\delta$-antagonist activity in the isolated hamster vas deferens preparation [250] and ethylketocyclazocine displays $\mu$-receptor antagonist properties in the isolated rat vas deferens preparation [273]. Therefore, the in vivo activity profiles of such compounds may well represent a combination of interactions at different opioid receptors.

Using a different approach Herz and colleagues [274] have claimed the existence of possible $k$-subtypes in peripheral tissues. This work studied cross-tolerance in the isolated mouse vas deferens preparation. For example, vasa deferentia rendered tolerant to MR 2034, were tolerant to MR 2034 and to ethylketocyclazocine but vasa deferentia rendered tolerant to ethylketocyclazocine were highly tolerant to the infused benzomorphan, but displayed only a minor-degree of cross-tolerance to MR 2034. However, the possible cross-reactivity at other receptor types makes interpretation of the results difficult.

Obviously, further work employing more selective agonists such as $\delta$-selective [D-Pen$^2$,D-Pen$^5$]enkephalin, $\mu$-selective [D-Ala$^2$,MePhe$^\beta$, Gly-$\omega^1$]enkephalin or $k$-selective U-50488H and antagonists such as $\delta$-selective ICI 174864 is necessary to resolve these problems. In particular the resolution of the $k$-subtype problem has awaited the development of a $k$-selective antagonist.

* See p.242
Recent work by Portoghese and colleagues [275] has produced bimorphinan compounds with claimed k-selective antagonist properties, which may help to resolve some of the uncertainty surrounding proposed k-heterogeneity.

It will be of interest to see what physiological relevance, if any the extra opioid site possesses and whether this site corresponds to a receptor which is involved in analgesia particularly at the spinal level.

* MR 2034 is: 5,9-dimethyl-2'-hydroxy-2-tetrahydrofuranyl-6,7-benzomorphan.


APPENDIX.

A revised definition of the k-opioid binding site arising from the work contained in chapters 6 and 7 of this thesis.

At the commencement of this thesis the k-opioid binding site was universally recognized as the site to which an unselective [3H]opioid binds, after binding to μ- and δ-sites has been suppressed using selective ligands.

However, the work presented in chapters 6 and 7 of this thesis shows that under the above conditions an heterogeneous population of binding sites is labelled. Work with more selective ligands has allowed the refinement of this definition to the following:-

The k-opioid binding site is the site recognized by the compounds trans-3,4-dichloro-N-methyl-N-[2-(pyrrolidinyl) cyclohexyl]-benzeneacetamide (U-50488H) and (5α, 7α, 8β)-(+)N-methyl-N-(7-(1-pyrrolidinyl)-1-oxaspiro [4,5] dec-8-yl) benzeneacetamide (U-69593), and by dynorphin A-(1-9) and dynorphin A-(1-17) with high affinity.
REFERENCES
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