CHARACTERIZATION OF δ OPIOID RECEPTOR FUNCTION IN RAT BRAIN BY PHARMACOLOGICAL AND ANTISENSE TECHNIQUES

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Abstract

The opioid family of G-protein coupled receptors comprises four known receptor subtype genes (δ, μ, κ, ORL_1) and further receptor heterogeneity within each opioid receptor subfamily has been proposed. All four genes are expressed throughout the central nervous system and are believed to modulate a variety of behavioural responses including analgesia. Opiate drugs such as morphine that are selective for the μ receptor subtype are effective analgesics, but their chronic use is limited by the appearance of side effects such as respiratory depression, constipation and dependence. Consequently, the analgesic potential of agonists selective for other opioid receptors is under investigation. In this regard, previous studies suggest that δ agonists mediate antinociception, yet produce fewer adverse effects than μ agonists. To further investigate the cloned δ opioid receptor (DOR) as a target for novel analgesics, the pharmacological role of DOR in brain was evaluated in rats.

First, we characterized δ agonist binding sites and receptor activation in rat brain membranes. We also introduced a novel antagonist radioligand, [^{125}I]AR-M100613, to label tissues with low δ opioid receptor expression in order to support follow-up studies where radioligand binding was performed on rat brain membranes following antisense treatment. Second, we examined the behavioural response to δ agonists in rats. Deltorphin II and SNC80 (*i.c.v.*) were shown to induce antinociception in acute pain assays, and to reverse hyperalgesia following tissue inflammation induced by Freund's adjuvant with even greater potency. These findings indicate that δ receptors play an enhanced role in the modulation of descending pain pathways following tissue injury. Deltorphin II and SNC80 (*i.c.v.*) were also shown to induce hyperlocomotor activity. Third we used antisense studies to demonstrate that the antinociceptive and locomotor stimulant effects of δ agonists are modulated by the cloned δ opioid receptor (DOR). In contrast to other δ agonists, the antinociceptive effects of DPDPE were not modulated by DOR antisense treatment but rather were blocked by a selective μ antagonist (CTOP) suggesting that DPDPE may activate μ sites in the brain rather than an alternate δ receptor subtype. Finally, we demonstrated that peptide nucleic acids (PNA, *i.c.v.*) can act as target-specific and sequence-selective antisense agents. In total, these findings demonstrate that DOR is an appropriate target for the development of novel analgesics

and that PNA can serve as effective antisense agents for the determination of gene function for CNS targets.

Abrégé

La famille des récepteurs opiacés couplés à la proteine G comprend quatre gènes de récepteurs connus (δ, μ, κ, ORL_1) et une plus grande hétérogénéité de récepteurs a été proposée à l'intérieur de chaque sous-famille de récepteurs opiacés. Les quatre gènes sont exprimés dans le système nerveux central et on croit qu'ils affectent une variété de réponses comportementales incluant l'analgésie. Les opiacés spécifiques au récepteur de sous-type μ tels que la morphine sont des analgésiques efficaces. Par contre, leur utilisation à long terme est limitée par l'apparition d'effets indésirables tels que la dépression du système respiratoire, la constipation et la dépendence. Pour cette raison, le potentiel analgésique des agonistes spécifiques aux autres récepteurs opiacés est étudié. Plusieurs études suggèrent que les agonistes δ auraient des effets antinociceptifs avec moins d'effets indésirables que les agonistes μ. Afin d'étudier cette hypothèse, le rôle pharmacologique du récepteur opiacé cloné δ (DOR) a été évalué chez le cerveau de rat afin de valider le DOR comme cible potentiel pour le développement de nouveaux analgésiques.

Premièrement, nous avons caractérisé les sites de liason des agonistes δ et l'activation du récepteur dans les membranes de cerveau du rat. Nous avons aussi développé un nouveau radioligand antagoniste, [^{125}I]AR-M100613, servant à marquer les tissus à faible expression de récepteurs opiacés δ tels que les membranes de cerveaux de rats traités à l'antisense. Deuxièmement, nous avons examiné le comportement des rats traités aux agonistes δ. Deltorphin II et SNC 80 (*i.c.v.*) ont induit l'antinociception chez des modèles de douleur aiguë et ont inhibé de façon plus efficace l'hyperalgésie due à l'inflammation de tissus causée par l'injection de l'adjuvant de Freund. Ces résultats indique que les récepteurs δ jouent un rôle dans la régulation accrue des voies descendantes de la douleur suivant une lésion des tissus. Deltorphin II et SNC 80 (*i.c.v.*) augmentent aussi l'activité locomotrice. Troisièmement, nos études d'antisense ont démontré que l'antinociception et les effets stimulants sur l'activité locomotrice des agonistes δ sont régulée par le récepteur cloné de l'opiacé δ (DOR). Lors de ces études, DPDPE était une exception. Des études supplémentaires ont démontré que les effets de DPDPE, contrairement aux autres agonistes δ, sont bloqués par l'antagoniste sélectif μ (CTOP). Ceci suggère que le DPDPE active les sites μ au cerveau plutôt qu'un autre sous-type de récepteur δ. Finalement, nous avons démontré que les acides nucléiques

peptidiques (PNA, *i.c.v.*) sont des agents d'antisense spécifiques pour la séquence et la cible. En somme, ces résultats démontre que le DOR est une cible appropriée pour le développement de nouveaux analgésiques et que les PNA peuvent servir d'agents antisense efficaces pour déterminer la fonction des gènes au système nerveux central.

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Abbreviations & Glossary

ANOVA: analysis of variance

AP: anterior-posterior

CFA: complete Freund's Adjuvant

CGRP: calcitonin gene-related peptide

CTOP: D-Phe-c[-Cys-Tyr-D-Trp-Orn-Thr-Pen]-Thr-NH_2

DAMGO: [D-Ala^2, N-Me-Phe^4, $Gly\text{-}ol^5$]-enkephalin

Deltorphin II: [D-Ala^2,Glu^4]deltorphin

DPDPE: [D-$Pen^{2,5}$]-enkephalin

DOR & DOR-1: cloned δ opioid receptor

DRG: dorsal root ganglia

DV: dorsal-ventral

E_{max}: maximal measured response to drug

HLA: horizontal locomotor activity

i.c.v.: intracerebroventricular

i.pl.: intraplantar

ML: medial-lateral

MOR: cloned μ opioid receptor

MPE: maximum possible effect

ODN: oligodeoxynucleotide

PNA: peptide nucleic acid

pCl-DPDPE: [D-Pen^2, pCl-Phe^4, D-Pen^5]enkephalin

S.E.M.: standard error of the mean

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I thank many of my colleagues at AstraZeneca R&D Montreal for their ideas, support and assistance (where applicable) in completing this thesis work. Special thanks to Drs. Martin Perkins, Kemal Payza, Ralf Schmidt, Andy Dray, Julie Ducharme and Daniel Ménard as well as Geneviève-Anne Gaudreau, Danielle Nicol, Robert Faltus, Maryse LaBarre, Claude Godbout, Joanne Butterworth and Stéphane St-Onge.

Finally, I thank Julie Rémillard for her assistance, support and sympathy throughout the duration of this thesis work.

Guidelines for Thesis Preparation

As stated in the "Guidelines Concerning Thesis Preparation" of the Faculty of the Graduate Studies and research, McGill University:

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Contribution of Authors

The following people have contributed to the research included in the present thesis. Dr. Paul B.S. Clarke is my research and thesis supervisor and therefore is an author on all of the research manuscripts. Dr. Claes Wahlestedt was my thesis supervisor when much of the antisense studies were conceived and performed. Thus, he is the senior author on all research or review articles pertaining to antisense technology.

Almost all of the research described in this thesis was performed on the premises of AstraZeneca R&D Montreal (AZRDM). Dr. Martin N. Perkins was my direct supervisor at AZRDM and is included as a co-author where appropriate.

Original Research Articles

a) **Characterization of [^{125}I]AR-M100613, a high affinity radioligand for a δ opioid receptors.** Graeme L. Fraser, Maryse Labarre, Claude Godbout, Joanne Butterworth, Paul B.S. Clarke, Kemal Payza and Ralf Schmidt (1999) *Peptides* **20**: 1327-1335.

G.L. Fraser conceived this study, wrote the manuscript, and performed all of the experiments and data analyses described in the paper with the exception of the items listed below.

M. Labarre performed the [^{35}S]GTPγS experiments (Figure 4.6 and Table 4.3).

C. Godbout provided technical assistance in the radioligand binding and [^{35}S]GTPγS experiments.

J. Butterworth was responsible for iodinating the radioligand, [^{125}I]AR-M100613.

K. Payza is the Associate Director of *In Vitro* Pharmacology at AZRDM. These experiments were conducted in a laboratory under his direction.

R. Schmidt is the peptide chemist at AZRDM who first synthesized AR-M100613.

b) **The effects of δ agonists on locomotor activity in habituated and non-habituated rats.** Graeme L. Fraser, Heli Parenteau, Thé-Minh Tu, Julie Ducharme, Martin N. Perkins and Paul B. S. Clarke (2000) *Life Sciences* **67**: 913-922.

G.L. Fraser conceived this study with assistance from P.B.S. Clarke, wrote the manuscript, and performed all of the experiments and data analyses described in the paper with the exception of the items listed below.

H. Parenteau provided technical assistance in the experiment measuring the *in vivo* brain penetration of SNC80 and deltorphin II (Figure 5.6).

T.-M. Tu performed the LC/MS analysis to measure the drug concentrations of δ agonists in the brain (Figure 5.6).

J. Ducharme is the Associate Director of Drug Metabolism & Pharmacokinetics at AZRDM. The experiments measuring brain penetration of δ agonists were conducted under her direction.

c) **Antihyperalgesic effects of δ opioid agonists in a rat model of chronic inflammation.** Graeme L. Fraser, Geneviève-Anne Gaudreau, Paul B.S. Clarke, Daniel P. Ménard and Martin N. Perkins (2000) *Br J Pharmacol* **129:** 1668-1672.

G.L. Fraser conceived this study with assistance from Martin Perkins, wrote the manuscript, and performed all of the experiments and data analyses described in the paper with the exception of the second item listed below.

G.-A. Gaudreau provided technical assistance in the testing of deltorphin II in a thermal hyperalgesia model (Figure 6.2B).

D.P. Ménard tested morphine in the tail flick assay (Figure 6.4B).

d) **Supraspinal antinociceptive response to the [D-Pen^{2,5}]-enkephalin (DPDPE) is pharmacologically distinct from that to other δ-agonists in the rat.** G.L. Fraser, A.A. Pradhan, P.B.S. Clarke and C. Wahlestedt (2000) *J Pharmacol Exp Ther* **295:** 1135-1141.

G.L. Fraser conceived this study with assistance from C. Wahlestedt and P.B.S. Clarke, wrote the manuscript, and performed all of the experiments and data analyses described in the paper.

A.A. Pradhan provided technical assistance in evaluating CTOP antagonism of SNC80 and pCl-DPDPE (Figure 7.3).

e) **Antisense inhibition of δ opioid receptor gene function *in vivo* by peptide nucleic acids.** Graeme L. Fraser, Janna Holmgren, Paul B.S. Clarke and Claes Wahlestedt (2000) *Mol Pharmacol* **57:** 725-731.

G.L. Fraser conceived this study with assistance from C. Wahlestedt and P.B.S. Clarke, wrote the manuscript, and performed all of the experiments and data analyses described in the paper.

J. Holmgren provided technical assistance in evaluating the antinociceptive response to SNC80 following antisense treatment (Figure 8.2B).

Claims for Originality

This thesis presents original data with regard to the role of δ opioid receptors in modulating antinociceptive and psychostimulant behaviour, the pharmacological characterization of the cloned δ opioid receptor, and the application of peptide nucleic acids as antisense agents for the determination of gene function *in vivo*. The following elements are specifically identified as original contributions that have advanced the knowledge in the fields of δ opioid receptor pharmacology or antisense technology.

Chapter 4: Characterization of [^{125}I]AR-M100613, a high-affinity radioligand for δ opioid receptors

Radioligands used to label δ opioid receptor have previously been identified. However, these radioligands have high non-specific binding, low specific activity or are agonists and thus their receptor binding is susceptible to the G protein-coupled state of the receptor. This report presents [^{125}I]AR-M100613 as a high affinity antagonist and δ-selective radioligand with high specific activity and low non-specific binding. Ligand association and dissociation curves, saturation isotherms, competition binding and receptor activation assays are used to characterize the pharmacology of [^{125}I]AR-M100613. In total, these results demonstrate that [^{125}I]AR-M100613 has significant advantage over other δ radioligands as a probe to label tissues such as rat brain membranes that have low δ opioid receptor expression. A secondary finding revealed in the [^{35}S]GTPγS assays of receptor activation presented in Figure 4.6A is that SNC80 has higher intrinsic activity than the peptide agonists, DPDPE and deltorphin II, at δ opioid receptors in rat brain membranes.

Chapter 5: The effects of δ agonists on locomotor activity in habituated and non-habituated rats

Previous reports have described the locomotor effects of δ agonists. However, these reports presented discrepant results with respect to whether δ agonists caused hypo- or hyperlocomotor activity. The current study demonstrated that the observation of hypo- or hyperlocomotor effects of δ agonists depends importantly on the previous habituation of the animals to the test apparatus, agonist dose and the δ agonist used. Also, this study was the first to demonstrate the locomotor effects of SNC80 following direct

administration into the brain and the first to directly compare the locomotor effects of SNC80 and deltorphin II. The observed potency difference between deltorphin II and SNC80 in stimulating locomotor activity in habituated rats suggests that these δ agonists may interact with different receptor subsets in rat brain.

Chapter 6: Antihyperalgesic effects of δ opioid agonists in a rat model of chronic inflammation

The efficacy of δ agonists in the treatment of inflammatory pain associated with tissue injury is key to the proposed clinical development of these agents as analgesics. This report provides original data that the administration of δ agonists directly into the brain significantly attenuates hyperalgesic responses associated with persistent tissue inflammation. In addition, this report demonstrates that δ agonists have greater efficacy in an assay of thermal hyperalgesia than in an assay of acute, thermal nociception.

Chapter 7: Supraspinal antinociceptive response to [D-Pen^{2,5}]-enkephalin (DPDPE) is pharmacologically distinct from that to other δ-agonists in the rat

DPDPE is reported to have a distinct pharmacology from that of the other prototypical δ agonist, deltorphin II, based on the results of previous studies performed with selective antagonists and antisense studies in mice targeting the cloned δ opioid receptor. The current study extended these findings by demonstrating that antisense targeting the cloned δ opioid receptor does not inhibit the supraspinal antinociceptive response to DPDPE in the rat, despite attenuating the response to the other δ agonists tested, SNC80, deltorphin II and pCl-DPDPE. In addition, the selective μ antagonist, CTOP, completely inhibited the antinociceptive response to DPDPE, but not the response to SNC80 and deltorphin II. These data confirm that the cloned δ opioid receptor modulates supraspinal antinociception in the rat and suggests that the unique pharmacology of DPDPE may be due to non-selective interactions at the μ opioid receptor.

Chapter 8: Antisense inhibition of δ-opioid receptor gene function *in vivo* by peptide nucleic acids

Peptide nucleic acids (PNA) have distinct chemical properties that may confer significant advantages over traditional oligonucleotide molecules with regard to their use as

antisense agents. However, PNA antisense effects *in vivo* had not been demonstrated prior to the initiation of the current study. This research demonstrated that a PNA sequence complementary to a region of the cloned δ opioid receptor attenuated δ opioid receptor function *in vivo* in a target-specific, sequence-specific and reversible manner consistent with an antisense mechanism. Also, this study demonstrated that the [^{35}S]GTPγS assay of receptor activation appears to provide a more sensitive measure of antisense efficacy *in vitro* than traditional saturation binding assays. This work comprised the third original research article demonstrating the use of unmodified antisense PNA *in vivo*.

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Introduction

1 Opioid Receptor Pharmacology

Opium has been used as an analgesic for centuries (Dhawan *et al.*, 1996). Morphine (isolated by Sertürner in 1803) was determined to be the primary analgesic component of crude opium extracts and, to this day, morphine (including morphine analogues, e.g. codeine, fentanyl) continues to be the most prescribed analgesic in the world for the treatment of chronic pain (Pan *et al.*, 1999).

The rigid structural and stereochemical characteristics required for opiate agonists such as morphine to maintain their analgesic efficacy lead to the suggestion that opiates exert their effects via interactions at specific receptors (Beckett & Casy, 1954). This hypothesis was validated when opioid binding sites were first detected in rat brain specimens in 1973 following the advent of specific opioid radioligands (Pert & Snyder, 1973; Terenius, 1973; Simon *et al.*, 1973). The consequent development of both binding and bioassays soon led to the elucidation of endogenous opioid receptor ligands and the pharmacological characterization of opioid receptor subtypes. To date, four opioid receptors (μ, δ, κ, ORL_1) have been cloned (Evans *et al.*, 1992; Kieffer *et al.*, 1992; Chen *et al.*, 1993; Minami *et al.*, 1993; Mollereau *et al.*, 1994) and further receptor heterogeneity has been postulated (Dhawan *et al.*, 1996). This section aims to provide an overview of opioid pharmacology with an emphasis on the δ opioid receptor, in keeping with the primary focus of this thesis.

1.1 Endogenous ligands

The first two classes of mammalian endogenous opioid peptides, the enkephalins and β-endorphin, were discovered in the mid-1970s (Hughes *et al.*, 1975; Bradbury *et al.*, 1976; Cox *et al.*, 1976; Li & Chung, 1976; Pasternak *et al.*, 1976), with a third class, the dynorphins, isolated and sequenced shortly thereafter (Goldstein *et al.*, 1981). These peptides are derived from pro-enkephalin, pro-opiomelanocortin and pro-dynorphin, respectively (see Table 1.1). With the notable exceptions of dynorphin A and dynorphin B (κ-selective), these peptides are not particularly selective for the μ, δ and κ receptor subtypes (Garzón *et al.*, 1983; Leslie, 1987). Also, the value of these peptides as pharmacological agents is limited by their rapid enzymatic degradation (Hambrook *et al.*, 1976). However, these endogenous peptides have served as a template for the design of synthetic peptides with improved opioid receptor selectivity and metabolic stability

(Schiller, 1991). For example, the μ selective agonist DAMGO (D-Ala^2, MePhe^4, Gly-ol^5-enkephalin) (Handa *et al.*, 1981), the δ selective agonist DPDPE (D-Pen^2, D-Pen^5-enkephalin) (Mosberg *et al.*, 1983) and the κ selective ligand DAKLI ([Arg^{11,13}]dynorphin) (Goldstein *et al.*, 1988) were all derived from the endogenous mammalian enkephalin or dynorphin peptides. In addition, amphibian endogenous opioid peptides, namely the dermorphin (μ selective) and deltorphin (δ selective) peptide classes (Esparmer *et al.*, 1989), have also provided useful pharmacological tools for the characterization of opioid receptors.

Recently, two additional endogenous opioid peptide classes have been identified (Table 1.1). The first class currently comprises nociceptin (also known as orphanin FQ), the endogenous ligand for the ORL_1-receptor. This peptide has low affinity for the μ, δ, and κ opioid receptors. However, its precursor, pro-nociceptin, shares significant homology with pro-dynorphin indicative of a possible evolutionary link between these opioid peptide classes (Mollereau *et al.*, 1996; Nothacker *et al.*, 1996; Meunier *et al.*, 1995; Reinscheid *et al.*, 1995).

The second recently identified class of endogenous peptides currently comprises the endomorphins (endomorphin-1, endomorphin-2). These are amidated tetrapeptides that appear to be structurally unrelated to all other known endogenous opioid peptides (Zadina *et al.*, 1997). These peptides are highly selective for the μ opioid receptor and appear to be highly localized to discrete regions of the brain and spinal cord known to contain high concentrations of μ receptors (Zadina *et al.*, 1997; Zadina *et al.*, 1999). The precursor for the endomorphin class of peptides has not yet been elucidated.

1.2 Opioid Receptor Subtypes

Portoghese first proposed the existence of opioid receptor subtypes in 1965 to explain the mixed actions of the various opioid ligands then known (Portoghese, 1965). However, it was not until 1976 that multiple receptors (μ, κ, σ) were classified (Martin *et al.*, 1976) based on the distinct physiological syndromes elicited by different opioid agonists in spinalized dogs (note: σ is no longer considered an opioid receptor (Quirion *et al.*, 1987)). An additional opioid receptor, δ, was identified shortly thereafter to explain the *in vitro* activity of the enkephalins in various bioassays (Lord *et al.*, 1977). The μ, δ, and κ opioid receptors have since been cloned (Evans *et al.*, 1992; Kieffer *et al.*, 1992; Chen

et al., 1993; Minami *et al.*, 1993; Mollereau *et al.*, 1994) and, together with the recently cloned and pharmacologically distinct ORL_1 (Mollereau *et al.*, 1994; Meunier *et al.*, 1995; Reinscheid *et al.*, 1995), comprise the opioid receptor family. All four opioid receptor genes share 50-70% homology. Additional receptor heterogeneity within each opioid receptor class is predicted based on the diverse pharmacology of various subtype-specific opioid ligands (Dhawan *et al.*, 1996).

Precursor	Endogenous peptide	Amino acid sequence	Selectivity
Pro-opiomelacortin	β-Endorphin	YGGFMTSEKSQTPLVTLFKNAIIKNAYKKGE	μ-preferring
Pro-enkephalin	[Met]enkephalin	YGGFM	
	[Leu]enkephalin	YGGFL	
		YGGFMRF	δ
		YGGFMRGL	
		YGGFMRRV-NH_2	
Pro-dynorphin	Dynorphin A	YGGFLRRIRPKLKWDNQ	
	Dynorphin A (1-8)	YGGFLRRI	
	Dynorphin B	YGGFLRRQFVVT	κ
	α-neoendorphin	YGGFLRKYPK	
	β-neoendorphin	YGGFLRKYP	
Pro-nociceptin/OFQ	Nociceptin (OFQ)	FGGFTGARKSARKLANQ	ORL_1
Pro-endomorphin *(presumed to exist)*	Endomorphin-1	YPWF-NH_2	μ
	Endomorphin-2	YPFF-NH_2	

Table 1.1 Mammalian endogenous opioid ligands

1.2.1 Evidence for δ Receptor Subtypes

1.2.1.1 Radioligand binding in brain tissue

Although only a single δ opioid receptor (DOR) has been cloned to date (Evans *et al.*, 1992; Kieffer *et al.*, 1992), discrepancies in the activity of various δ ligands in radioligand binding, cell signaling and behavioural assays has led to the proposed existence of δ opioid receptor subtypes. δ Receptor heterogeneity was first postulated on the basis of radioligand binding studies in both guinea pig cortical membranes and in the δ-rich NG108-15 rodent hybrid neuroblastoma cell line. In both tissues, the binding of the non-selective ligand [^{3}H]diprenorphine was inhibited biphasically by DSLET (δ selective) even in the presence of GTP (Werling *et al.*, 1988). Similarly, radioligand binding studies using ligands such as DPDPE, DADLE, DSLET, deltorphin and naltrindole have also supported the existence of δ receptor subtypes in rat, mouse and human brain membranes (Negri *et al.*, 1991b; Fang *et al.*, 1994; Kim *et al.*, 2001). [^{3}H]DSLET labels 40% more sites than those labeled by [^{3}H]DPDPE in rat brain membrane homogenates (Sofuoglu *et al.*, 1992) and, more dramatically, the δ selective antagonist 7-benzlidenenaltrexone (BNTX) inhibited the binding of these radioligands with a 100-fold higher potency (based on comparison of the K_i values) at [^{3}H]DPDPE labeled sites (Portoghese *et al.*, 1992). Also, quantitative autoradiographic evaluation of [^{3}H]DPDPE and [^{3}H]DSLET binding suggests that these radioligands label distinct regions of rat brain. (Hiller *et al.*, 1996). Finally, in a series of experiments where site-directed acylating agents were used to deplete μ sites, Rothman et al. have suggested the existence of up to four δ subtype binding sites, or one receptor with four affinity states, on the basis of complex radioligand binding experiments using combinations of δ selective radioligands to inhibit [^{3}H]DADLE binding from rat brain membranes (Xu *et al.*, 1992; Xu *et al.*, 1993).

1.2.1.2 Whole animal studies

Behavioural studies in mice and rats also support the pharmacological heterogeneity of δ receptor subtypes. Thus, independent laboratories have demonstrated that the supraspinal antinociceptive activity of the δ agonist DPDPE can be selectively antagonized by 7-benzlidenenaltrexone (BNTX) or DALCE (Jiang *et al.*, 1991; Vanderah *et al.*, 1994; Thorat & Hammond, 1997; Sofuoglu *et al.*, 1993) whereas the antinociceptive activity of deltorphin II (or DSLET) is selectively reversed by naltriben or naltrindole 5'-

isothiocyanate (NTII) (Jiang *et al.*, 1991; Sofuoglu *et al.*, 1991b; Vanderah *et al.*, 1994; Thorat & Hammond, 1997). Furthermore, while mice develop tolerance to the antinociceptive effects of DPDPE or deltorphin II following repeated injections, cross-tolerance between these δ agonists was not observed at supraspinal sites (Mattia *et al.*, 1991).

In total, the radioligand binding and behavioural data provide strong evidence for δ receptor heterogeneity in brain (Table 1.2). The putative δ_1 receptor is stimulated preferentially by DPDPE and antagonized by BNTX and DALCE whereas the putative δ_2 receptor is stimulated preferentially by deltorphin II and selectively antagonized by naltriben and 5'-NTII (Zaki, 1996). The cloned δ opioid receptor (DOR) appears to correspond to the δ_2 subtype based on studies where antisense directed against DOR inhibited the supraspinal antinociceptive effects of deltorphin II, but not the effects of DPDPE in mice (Bilsky *et al.*, 1996; Rossi *et al.*, 1997) and rats (Fraser *et al.*, 2000).

Receptor subtype	Agonist		Antagonist			
		Selectivity δ/μ	Competitive	Selectivity δ/μ	Non-equilibrium	Selectivity δ/μ
δ_1	DPDPE	110	BNTX	1.0	DALCE	N.D.†
	DADLE	8.0				
δ_2	Deltorphin II	160	Naltriben	60	5'-NTII	N.D.
	DSLET	28				
δ (combined)	SNC80	300	Naltrindole	4.5		
			ICI-174,864	190		

Table 1.2 Putative δ-receptor subtype specific ligands
These values were determined from competitive binding assays performed on isolated preparations of cloned human μ and δ receptors (Payza *et al.*, 1996). μ/δ selectivity ratios were not determined (N.D.) for the non-equilibrium antagonists. †DALCE has previously been reported to interact non-selectively with μ receptors (Bowen *et al.*, 1987).

1.2.1.3 δ Receptor subtypes in spinal cord

The pharmacological evidence for δ subtypes at the level of the spinal cord is controversial. In the mouse, cross-tolerance was not observed between deltorphin II and

DPDPE (Sofuoglu *et al.*, 1991a). Also, some investigators have reported that BNTX and naltriben selectively antagonised the effects of DPDPE and deltorphin II, respectively, at the spinal level (Sofuoglu *et al.*, 1993) whereas others have demonstrated that the effects of DPDPE and deltorphin II are antagonised by 5'-NTII, but not DALCE, suggesting that the δ_2 receptor alone mediates antinociception in the mouse spinal cord (Mattia *et al.*, 1992). Critically, the latter findings are supported by studies where antisense treatment targeting DOR in the mouse spinal cord blocked the antinociceptive effects of both DPDPE and deltorphin II, but not the effects of agonists at other opioid receptors (Bilsky *et al.*, 1994; Tseng *et al.*, 1994).

In the rat, the existence of δ subtypes at the level of the spinal cord is also inconclusive. Thus, intrathecal administration of naltriben selectively antagonized both the spinal antinociceptive (Stewart & Hammond, 1993) and antihyperalgesic (Stewart & Hammond, 1994) effects of deltorphin II, but not of DPDPE or μ agonists. These findings are further supported by electrophysiological studies conducted in the rat spinal cord where electrically evoked postsynaptic currents were partly reduced by deltorphin II in a naltriben-reversible manner. In comparison, DPDPE and DAMGO (μ agonist) fully reduced these evoked postsynaptic currents in a naltriben-insensitive manner (Glaum *et al.*, 1994). Although these studies suggest that deltorphin II and DPDPE mediate spinal antinociception via different receptors, it is not clear whether the response to DPDPE was δ receptor-mediated because no attempt was made to block this effect by using alternate δ antagonists.

1.2.1.4 In vitro functional experiments

In vitro studies of receptor activation or second messenger systems also tend to support the existence of δ subtypes. For example, an examination of δ receptor mediated increases in intracellular Ca^{2+} in the ND8-47 cell line demonstrated a selective antagonism of DPDPE by BNTX, and deltorphin II by naltriben (Tang *et al.*, 1994). Similarly, DPDPE and deltorphin II-mediated inhibition of basal (Búzás *et al.*, 1994) and forskolin-stimulated (Noble & Cox, 1995) adenylyl cyclase activity in rat nucleus accumbens and caudate putamen were also selectively antagonised by BNTX and naltriben, respectively (Búzás *et al.*, 1994; Noble & Cox, 1995), despite little selectivity of these antagonists for [^{3}H]DPDPE or [^{3}H]deltorphin II labeled sites in competitive binding studies (Búzás *et al.*, 1994). In contrast, in the rat olfactory bulb and striatum,

the effects of both DPDPE and deltorphin II on adenylyl cyclase activity was selectively inhibited by naltriben and 5'-NTII rather than BNTX and DALCE, suggesting that adenylyl cyclase activity in these brain regions are mediated exclusively by the δ_2 subtype (Olianas & Onali, 1995). A recent study of δ agonist mediated G-protein activation in different regions of mouse and rat midbrain, limbic forebrain and striatal membranes demonstrated that in each of these regions the effects of DPDPE were inhibited by BNTX and the effects of deltorphin II were inhibited by naltriben. Unfortunately, cross-antagonism was not performed in this study so δ subtype-specific effects were never definitively demonstrated (Tsuji *et al.*, 1999).

1.2.1.5 Weakness in the pharmacological evidence for δ subtypes

Recent studies have revealed numerous weaknesses in the pharmacological determination of δ subtypes as described above. Firstly, the competitive binding data must be interpreted with the understanding that many of the radioligands used in these assays are agonists. Thus, their biphasic displacement in competitive radioligand binding studies may simply reflect binding at a single receptor modulated by coupling with its G-protein, as has been demonstrated for other G-protein coupled receptors, including opioid receptors (Lutz & Pfister, 1992; Richardson *et al.*, 1992). For example, competitive binding studies utilizing δ agonists reveal biphasic displacement curves in the SK-N-BE human neuroblastoma cell line. However, functional studies in this cell line were not predictive of δ receptor subtypes based on the lack of selective antagonism of DPDPE and deltorphin II mediated inhibition of adenylyl cyclase activity by 'δ_1' and 'δ_2' selective antagonists, respectively (Allouche *et al.*, 2000). In the same study, the possibility that the biphasic inhibition curves could arise from DOR splice variants was ruled out by RT-PCR experiments that revealed a single DOR transcript (Allouche *et al.*, 2000).

A second reason to question the pharmacological evidence for δ subtypes is that several of the δ ligands are of questionable selectivity. For example, DPDPE has been presented as the prototypical δ_1 agonist in spite of behavioural studies in mice that suggest that DPDPE may interact with both δ_1 and δ_2 receptors (Vanderah *et al.*, 1994). Similarly, putative δ subtype selective antagonists such as naltriben and BNTX have shown little potency difference in competitively inhibiting the binding of [^{3}H]DPDPE (δ_1) or

[^{3}H]deltorphin II (δ_2) in rat brain membranes (Búzás *et al.*, 1994). These antagonists also appear to have a narrow window of selectivity *in vivo* (Thorat & Hammond, 1997).

Recent evidence suggests that various δ ligands are, in fact, not particularly δ selective at all. Radioligand binding studies performed on pure populations of the cloned μ, δ or κ opioid receptors expressed in cultured cell lines have demonstrated that many of the agonists and antagonists used to characterize δ subtypes have low δ/μ opioid receptor selectivity (Table 1.2; Payza *et al.*, 1996; Chaturvedi *et al.*, 2000). Thus, it is not surprising that many of these ligands appear to interact with multiple sites in tissues such as rat (or mouse) brain where μ opioid receptors are predominant (Mansour *et al.*, 1995). Also, the low δ/μ selectivity ratio observed for various δ ligands *in vitro* may influence the pharmacological profile of these agents *in vivo*. For example, the prototypical δ_1 selective agonist, DPDPE, has been known to cause μ-like behavioural effects (Cowan & Murray, 1989; Weinger *et al.*, 1996). In addition, the antinociceptive effects of DPDPE in rodents are blocked by the selective μ antagonist analogues, CTAP and CTOP, at the level of the brain (Kramer *et al.*, 1989; Fraser *et al.*, 2000) and spinal cord (He & Lee, 1998). Furthermore, in μ knockout mice, DPDPE-mediated antinociception (Sora *et al.*, 1997; Fuchs *et al.*, 1999; Matthes *et al.*, 1998; Hosohata *et al.*, 2000) and DPDPE-stimulated GTPγS binding activity on -/- brain membrane preparations (Hosohata *et al.*, 2000) were significantly reduced. In total, these findings suggest that DPDPE may interact directly with both δ and μ opioid receptors *in vivo*. This observation provides an alternate hypothesis to 'δ receptor subtypes' to explain the pharmacological differences in the activity of DPDPE and deltorphin II.

A number of recent experiments have employed highly selective approaches, such as antisense or knockout techniques, to evaluate δ receptor heterogeneity. These studies have failed to provide consistent conclusions. Antisense oligonucleotides targeting the cloned δ opioid receptor (DOR; pharmacologically similar to δ_2 (Raynor *et al.*, 1994)) inhibited the antinociceptive response to deltorphin II, but not DPDPE, following supraspinal administration of opioid agonists into the brain (Bilsky *et al.*, 1994; Tseng *et al.*, 1994). This finding suggests that DPDPE mediates antinociception through receptors other than DOR, but it does not necessarily predict the existence of δ subtypes. In contrast, antisense mapping studies in mice suggest that distinct pharmacological δ

subtypes may arise from splice variants of the DOR gene (Rossi *et al.*, 1997), although no physical evidence of splice variants has been presented to date.

In comparison to the antisense studies, [^{3}H]DPDPE, [^{3}H]deltorphin II and [^{3}H]naltrindole binding to brain membrane homogenates was completely eliminated in two independent strains of DOR knockout mice which suggests that any δ receptor subtypes must arise from the common DOR gene (Zhu *et al.*, 1999; Filliol *et al.*, 2000). Nonetheless, deltorphin II and DPDPE-mediated supraspinal antinociception was not inhibited in these knockout mice (Zhu *et al.*, 1999). The latter finding seems to indicate that these δ agonists must interact with a non-DOR site in mouse brain (Zhu *et al.*, 1999). Additional studies are required to determine whether this non-DOR site is a δ subtype, or one of the other cloned opioid receptors.

1.2.1.6 Evidence for a μ/δ Receptor Complex

A previous classification of δ subtypes was proposed based on the hypothesis that one type of δ receptor (δ_{cx}) was complexed with μ-receptors whereas a second type was not associated with any receptor complex (δ_{ncx}) (Rothman *et al.*, 1988; Traynor & Elliot, 1993). This hypothesis is supported by neuroanatomical studies demonstrating that μ and δ receptors can be co-expressed on the same neurons (Rogers & Henderson, 1990; Kalyuzhny *et al.*, 1996). For example, μ and δ opioid receptors are co-expressed in pain circuits such as the serotonergic neurons projecting from the rostral ventromedial medulla (RVM) to the spinal cord (Wang & Wessendorf, 1999), small DRG neurons (Wang & Wessendorf, 2001) and the superficial layers of the rat spinal cord (Cheng *et al.*, 1997). Evidence for a μ/δ receptor complex is also supported by behavioural studies where the co-administration of δ and μ agonists caused a synergistic increase in supraspinal (Miaskowski *et al.*, 1991; Negri *et al.*, 1995) and spinal (Malmberg & Yaksh, 1992) antinociception. Also, simultaneous activation of μ and δ opioid receptors mediates a synergistic release of adenosine from spinal cord synaptosomes (Cahill *et al.*, 1996). Although μ and δ receptors do share the same inhibitory G protein (Schoffelmeer *et al.*, 1987), functional cooperativity between these receptors has not been observed *ex vivo* as the intrinsic activity of δ agonists was not affected in brain homogenates prepared from μ opioid receptor knockout mice (Matthes *et al.*, 1998). However, studies on transfected cells co-expressing μ and δ receptors have demonstrated that co-administration of DAMGO and DPDPE resulted in a synergistic increase in both competitive binding at

[^{3}H]DPDPE-labeled sites and in the agonist-induced inhbition of adenylyl cyclase activity (Martin & Prather, 2001). Similarly, co-administration of DAMGO and DPDPE also caused a synergistic increase in cellular metabolic function in SH-SY5Y cells co-expressing native μ and δ receptors (Chen *et al.*, 2001). Thus, δ/μ cooperativity may occur at the receptor level (Martin & Prather, 2001) in keeping with the recent discovery of δ/μ heterodimers (George *et al.*, 2000; Gomes *et al.*, 2000). The δ/μ heterodimer exhibits a distinct pharmacological profile from either the cloned δ or μ receptors and may correspond to the predicted δ_2 subtype (Gomes *et al.*, 2000). The oligomerization of opioid receptors is discussed in greater detail at the end of this section.

In summary, further studies are required to determine whether the pharmacological evidence for δ subtypes reflects the existence of subtypes derived from distinct genes, splice variants of a common gene, μ/δ receptor complexes, receptor homodimers or heterodimers, or the nonselective interaction of δ ligands with alternate receptors in the brain and spinal cord.

1.2.2 Evidence for μ Receptor Subtypes

Subtypes of the μ opioid receptor were first postulated on the basis of detailed radioligand binding experiments. [^{3}H]-Labeled μ, δ and κ-ligands displayed biphasic binding characteristics where each radioligand appeared to bind to a common, very high affinity site, classified as μ_1, and a second site (μ, δ or κ) dependent upon the nature of radioligand used (Wolozin & Pasternak, 1981). The μ_2 subtype was classified as the low affinity binding site revealed by μ ligands and this site appeared to correspond to the pharmacological activity of μ-agonists in bioassays or behavioural experiments (Pasternak & Wood, 1986). However, upon review of the binding data, correlation analysis between the binding potencies of twenty-one opioid ligands towards μ_1 and μ_2 receptors in calf thalamus membranes suggested that both binding assays were labeling a common receptor (Fowler & Fraser, 1994).

Subsequently, naloxozone (a hydrazone derivative of naloxone), and its active metabolite, naloxonazine were presented as selective, irreversible antagonists of the μ_1 receptor. Thus, these agents were reported to block μ_1 binding and inhibit only certain components of morphine activity; morphine antinociception was blocked, but not respiratory depression, dependence or gastric motility (Ling *et al.*, 1985; Ling *et al.*,

1984; Pick *et al.*, 1991). However, subsequent evaluation in other laboratories could not confirm the selectivity or irreversibility of naloxonazine binding at μ_1 sites (Nock *et al.*, 1993; Cruciani *et al.*, 1987).

Morphine analogues with substitutions at the 6 position, such as morphine-6β-gluconiride (M6G) and heroin, are μ agonists and their antinociceptive activity is not blocked by selective δ or κ antagonists (Brown *et al.*, 1997). However, these agents are pharmacologically quite different from morphine. Thus, they do not produce cross-tolerance with morphine, they are potently antinociceptive in CXBX morphine-insensitive mice (Rossi *et al.*, 1996), they differ from morphine with respect to their sensitivity to antisense treatment directed towards different regions of the cloned μ opioid receptor (MOR) (Rossi *et al.*, 1997), and they produce antinociception in MOR knockout mice where the disruption was introduced in exon 1, but not exon 2 (Schuller *et al.*, 1999). Remarkably, reverse-transcriptase polymerase chain reaction (RT-PCR) analysis demonstrated the continued expression of exons 2 and 3, despite the abolition of exon 1, in the M6G-sensitive knockout strain (Pasternak, 2001). Based on these findings, it was concluded that the antinociceptive actions of morphine-6β-gluconiride are mediated through a splice variant of the MOR gene, yielding a gene product differing from MOR in the exon 1 region (Schuller *et al.*, 1999; Pasternak, 2001).

The recent identification of a total of six MOR splice variants (Pan *et al.*, 1999; Zimprich *et al.*, 1995; Bare *et al.*, 1994) suggests that alternative splicing of the MOR gene may yield the μ receptor subtypes predicted by the pharmacology. However, pharmacological differences between splice variants have not yet been determined (Pan *et al.*, 1999). Furthermore, the abolition of a range of responses (antinociception, respiratory depression, dependence, gastric motility) to morphine and other common μ agonists (i.e. DAMGO) in MOR knockout mice suggests that all of these effects are mediated by a single common receptor (Kieffer, 1999).

1.2.3 Evidence for κ Receptor subtypes

The κ opioid receptor was first identified on the basis of the unique physiological responses elicited by ketocyclazocine in chronically spinalised dogs (Martin *et al.*, 1976). The dynorphins are presumed to be the endogenous ligands for κ receptors on the basis of binding selectivity data (Chavkin *et al.*, 1982; Gillan *et al.*, 1985). The proposed

existence of κ subtypes is based almost entirely upon radioligand binding assays using non-selective ligands to define putative sites. Thus, characterization of the κ opioid receptors in binding assays was initially attempted with the benzomorphan radioligands [^{3}H]ethylketocyclazocine ([^{3}H]EKC) and [^{3}H]bremazocine (Kosterlitz *et al.*, 1981) assayed in the presence of additional opioid ligands to minimize non-selective labeling of μ and δ sites (Weyhenmeyer & Mack, 1985). Under these assay conditions, selective κ agonists such as U69593 (Lahti *et al.*, 1985) appear to bind to a subset of the sites labeled by [^{3}H]EKC in rat (Nock *et al.*, 1988) or monkey brain (Butelman *et al.*, 1998). In addition, U69593-sensitive and insensitive sites have different neuroanatomical distributions in rat brain (Zukin *et al.*, 1988). The U69593-sensitive sites have been classified as κ_1 (Zukin *et al.*, 1988; Devlin & Shoemaker, 1990), and have been further subdivided into κ_{1A} and κ_{1B} sites on the basis of biphasic displacement curves of [^{3}H]U69593 binding by the endogenous opioids dynorphin B and α-neoendorphin (Clark *et al.*, 1989; Kinouchi & Pasternak, 1991; Rothman *et al.*, 1990). The cloned κ opioid receptor (KOR) appears to correspond pharmacologically with the κ_{1B} site based on its sensitivity to U69593 binding and the high affinity of α-neoendorphin binding (Lai *et al.*, 1994).

In contrast, [^{3}H]EKC-labeled sites remaining after blockade of μ, δ and κ_1 sites were classified as κ_2 (Zukin *et al.*, 1988) or ε opioid receptors (Nock *et al.*, 1990; Nock *et al.*, 1993). The κ_2 site appears to have similar pharmacology to the recently identified δ/κ heterodimer (Jordan & Devi, 1999). Rothman et al. have suggested further heterogeneity of κ_2 sites based on complicated radioligand binding paradigms in guinea pig brain and spinal cord using non-reversible ligands (Rothman *et al.*, 1990; Ni *et al.*, 1995).

The existence of a κ_3 opioid receptor subtype has also been suggested on the basis of studies with [^{3}H]naloxone benzoylhydrazone (NalBzOH), a compound that inhibits binding to μ, κ and δ receptors with nanomolar potency, demonstrates little selectivity between μ and κ receptors, and has been described as binding in a partly reversible and partly "pseudoirreversible" manner (Price *et al.*, 1989). The reversible portion of NalBzOH binding is postulated to be selective for the pharmacologically distinct κ_3 receptor (Clark *et al.*, 1989). It has been reported that κ_3 analgesia is not readily reversed by μ, δ or κ antagonists and shows no cross-tolerance with μ or κ_1 analgesics (Gistrak *et al.*, 1989; Paul *et al.*, 1990). However, similarities between the pharmacological

characteristics of the proposed κ_3 site and the traditional μ opioid receptor (μ_2) have been noted by numerous independent investigators (Wollemann *et al.*, 1993; Nock *et al.*, 1993; Fowler & Fraser, 1994).

Although it is clear from the above discussion that [^{3}H]EKC and [^{3}H]U69593 label different sites in rat brain, definitive pharmacological evidence supporting the existence of κ subtypes is lacking because of the absence of subtype-specific antagonists. The biphasic inhibition of [^{3}H]bremazocine binding (upon which the notion of κ subtypes is largely based) may, in actuality, correspond to different affinity states of the same receptor contingent upon the status of G-protein coupling (Richardson *et al.*, 1992). This hypothesis would correlate with the lack of functional evidence for κ subtypes in pharmacological assays performed on KOR knockout mice (Simonin *et al.*, 1998). Alternatively, the [^{3}H]U69593-insensitive binding sites labeled by [^{3}H]EKC and [^{3}H]bremazocine may represent either non-selective binding to MOR and DOR, or to combinations of MOR, DOR and KOR receptor complexes. The latter hypothesis is supported by the finding that [^{3}H]bremazocine binding is abolished in triple MOR-DOR-KOR knockout mice (Simonin *et al.*, 2001).

1.2.4 The ORL_1 Receptor

The 'opioid receptor-like receptor', ORL_1, was first identified in 1994 (Mollereau *et al.*, 1994; Bunzow *et al.*, 1994; Nishi *et al.*, 1994; Wang *et al.*, 1994; Fukuda *et al.*, 1994). This novel receptor is highly homologous to the classical opioid receptors (60% homology), however it has very distinct pharmacology. Non-selective opioid ligands such as naloxone, etorphine or diprenorphine have very low affinity for the ORL_1 receptor in comparison to their affinity for the other opioid receptors (Mollereau *et al.*, 1994). Conversely, only the endogenous peptide, nociceptin (a.k.a. orphanin FQ; Table 1.1), and synthetic derivatives thereof, interact with ORL_1 with high selectivity and affinity (Dooley *et al.*, 1997). However, these peptides are highly susceptible to degradation and thus are of limited use as pharmacological agents for the characterization of ORL_1. The recent synthesis of non-peptidic ORL_1 agonists (Wichmann *et al.*, 1999) and antagonists (Ozaki *et al.*, 1998) should support the further characterization of this receptor. Splice variants of the ORL_1 receptor have been reported (Wang *et al.*, 1994) (Xie *et al.*, 2000), however their physiological relevance requires further elucidation.

The pharmacology of nociceptin and its receptor, ORL_1 has been summarized in a recent review (Calo *et al.*, 2000).

1.2.5 Heterodimerization of Opioid Receptors

Receptor dimerization of G-protein coupled receptors is a potential mechanism for modulation of receptor function (Salahpour *et al.*, 2000). Delta (δ) receptors can exist as homodimers that are expressed on the surface of intact cells (McVey *et al.*, 2001). Delta (δ) homodimers appear to undergo agonist-mediated monomerization and subsequent receptor internalization (Cvejic & Devi, 1997). Kappa (κ) opioid receptors can also exist as homodimers; these complexes are resistant to agonist-induced monomerization and are more stable than their δ counterpart (Jordan & Devi, 1999). Also, δ and κ receptors can co-assemble to form functional heterodimers that exhibit distinct ligand binding and receptor trafficking properties (Jordan & Devi, 1999). The δ/κ dimer appears to correspond to the pharmacologically-defined κ_2 subtype based on its insensitivity to both κ-selective (i.e. U69593) and δ selective ligands (i.e. DPDPE) and its strong affinity for partially selective ligands such as EKC and bremazocine (Jordan & Devi, 1999; Jordan *et al.*, 2000).

Most recently, δ/μ heterodimers have also been identified by selective immunoprecipitation techniques performed on cells co-expressing both receptors (George *et al.*, 2000; Gomes *et al.*, 2000). The δ/μ dimer has similar (Gomes *et al.*, 2000) or decreased (George *et al.*, 2000) affinity for both μ (i.e. DAMGO) and δ (i.e. DPDPE) agonists. However, ligand binding to the δ/μ dimer and its consequent activation is enhanced when assayed in the presence of both μ and δ ligands suggesting the possible occurrence of allosteric binding interactions between μ and δ ligands in the heterodimer (Gomes *et al.*, 2000). In contrast to the independently expressed μ and δ receptors, the δ/μ dimer appears to interact with a PTX-insensitive G-protein as agonist affinity and opioid-induced inhibition of cAMP production are resistant to treatment with pertussis toxin (George *et al.*, 2000). Finally, the δ/μ dimer may be more resistant to receptor desensitization and internalization than each native receptor upon exposure to μ and δ selective agonists (George *et al.*, 2000). In total, these initial studies have demonstrated that the δ/μ dimer has a distinct pharmacological profile from both that of the

independently expressed cloned δ or μ receptors and the predicted δ and μ receptor subtypes (Gomes *et al.*, 2000).

1.3 Opioid Signal Transduction

1.3.1 Opioid receptor-coupled G-proteins

The cloning of the opioid receptors confirmed the extensive biochemical evidence that these receptors are members of the G-protein coupled receptor superfamily. All four cloned receptors (μ, δ, κ, ORL_1) appear to couple preferentially via the pertussis toxin (PTX) sensitive G_i/G_o protein families (Connor & Christie, 1999; Calo *et al.*, 2000). Initial studies in rat brain membranes demonstrated that G_i and G_o were often co-purified with opioid receptors (Wong *et al.*, 1989). Additional studies where activated G-proteins were irreversibly labeled (Offermanns *et al.*, 1991; Roerig *et al.*, 1992; Carter & Medzihradsky, 1993) or various combinations of opioid receptors and G-protein α-subunits were reconstituted in cultured cells have further confirmed that opioid receptors couple with a range of PTX-sensitive G-proteins including G_{i1-3} and G_{o1-2} (Burford *et al.*, 1998; Chan *et al.*, 1995; Chakrabati *et al.*, 1995; Prather *et al.*, 1995). Of the two most highly expressed G proteins in the mammalian CNS, the human δ opioid receptor (hDOR) activates $G_{i1\alpha}$ more efficiently than $G_{o1\alpha}$ (Moon *et al.*, 2001).

Reconstitution experiments have also demonstrated that all four opioid receptors can couple with the PTX-insensitive G-proteins, G_z (Tsu *et al.*, 1995; Chan *et al.*, 1995; Lai *et al.*, 1995; Chan *et al.*, 1998) and G_{16} (Chan *et al.*, 1998; Offermanns & Simon, 1995; Lee *et al.*, 1998). G_z is closely related to G_o and can inhibit adenylate cyclase (Wong *et al.*, 1992). G_{16} is closely related to G_q and can activate phospholipase C. Notably, δ and ORL_1 receptors appear to couple more efficiently to G_{16} than μ or κ receptors; G_{16} is the only effector mechanism identified thus far that demonstrates significant differences in the coupling efficiencies of different opioid receptors (Chan *et al.*, 1998; Offermanns & Simon, 1995; Lee *et al.*, 1998).

Antisense studies targeting G_s and G_q α subunits suggest that these proteins may have a role in modulating opioid receptor activity *in vivo* (Sanchez-Blazquez & Garzon, 1998; Standifer *et al.*, 1996). However, these results should be interpreted with caution for two reasons. Firstly, these findings oppose extensive biochemical data, including negative data from reconstitution experiments (Tsu *et al.*, 1995; Chan *et al.*, 1995), that opioid

receptors do not couple with PTX-insensitive G-proteins with the exceptions of G_z and G_{16}. Secondly, it is uncertain to what extent the antisense targeting of G-protein α subunits alters the general, ongoing activity of neurons by disrupting the G-protein coupling of other, non-opioid receptors.

The finding that the cloned opioid receptors couple to a common range of G-protein α subunits has two important implications. Firstly, the various responses evoked by either different opioid receptors or the same opioid receptor expressed in different cell types is more likely dependent upon the profile and stoichiometry of G-proteins and effectors expressed by a given cell than on the type of opioid receptor (Connor & Christie, 1999). Secondly, all opioid receptors activate the same second messenger systems, which principally include the inhibition of cAMP, the inhibition of voltage-operated calcium conductance and the activation of inwardly rectifying potassium conductance (Connor & Christie, 1999).

1.3.2 Inhibition of cAMP

Delta (δ) opioid receptors inhibit cAMP production via two distinct mechanisms. Firstly, δ receptor activation inhibits adenylate cyclase activity. This effect has been demonstrated in cultured cells expressing native receptor (Blume *et al.*, 1979), brain tissue (Law *et al.*, 1981; Izenwasser *et al.*, 1993) and cultured cells transfected with the cloned human δ opioid receptor (Knapp *et al.*, 1995). This response is likely transduced by one or more of the G-protein subunits (G_{i2}, G_{o2}, G_{i3}) shown to be coupled to δ receptors in the NG108-15 mouse neuroblastoma x rat glioma hybrid cell line (McKensie & Milligan, 1990; Roerig *et al.*, 1992). Secondly, the δ receptor has been shown to indirectly decrease cAMP levels via a PTX-insensitive G-protein that modulates the release of intracellular Ca^{2+} and the consequent promotion of Ca^{2+}/calmodulin phosphodiesterase activity (Law & Loh, 1993).

Opioid-mediated decreases in intracellular cAMP levels may have diverse implications resulting from the decreased activation of various target proteins by cAMP-dependent protein kinase (Fleming *et al.*, 1992). For example, opioid inhibition of cAMP levels may mediate antinociceptive pathways (Wang *et al.*, 1993) and respiratory depression (Ballanyi *et al.*, 1997). Alternatively, the high rebound cAMP levels observed after chronic opioid agonist pretreatment and subsequent exposure to forskolin in cultured

cells (Malatynska *et al.*, 1996) appears to correlate with the elevated cAMP levels involved in opioid dependence and withdrawal syndromes (Nestler & Aghajanian, 1997).

1.3.3 Ion Channels

Numerous studies have demonstrated the role of the δ opioid receptor in modulating the function of Ca^{2+} and K^+ channels. Delta (δ) opioid receptor inhibition of calcium (Ca^{2+}) channel currents was first demonstrated in the NG108-15 cultured cell line (Tsunoo *et al.*, 1986). This effect was inhibited by PTX-pretreatment and restored by intracellular administration of G_o or, to a less potent extent, G_i (Hescheler *et al.*, 1987). Additional studies demonstrated that the effects of δ agonists on Ca^{2+} currents were independent of cAMP regulation and modulated primarily by changes in N-type Ca^{2+} channel function (Taussig *et al.*, 1992; Sher *et al.*, 1996). Similarly, in cultured rat dorsal root ganglion neurons, the δ_2 selective agonists DADLE and deltorphin II, but not the δ_1 agonist DPDPE, inhibited L-, N-, P- and Q-type voltage-activated Ca^{2+} currents where the N-type currents contributed most to the overall current sensitive to δ_2 agonists (Acosta & López, 1999). The inhibition of Ca^{2+} channels may reduce neurotransmitter release and account for the presynaptic inhibitory effects of δ agonists on the conduction of nervous impulses in nociceptive pathways (Collin *et al.*, 1991; Wang *et al.*, 1996; Zachariou & Goldstein, 1996; Glaum *et al.*, 1994). A similar process has been demonstrated for μ opioid receptors on the unmyelinated, small nociceptive neurons that conduct dull, persistent pain (Taddese *et al.*, 1995). Alternatively, the modulation of intracellular Ca^{2+} levels may contribute to the regulation of various protein kinases as discussed in the final paragraph of this section.

Delta (δ) opioid receptors also appear to increase the conductance of an inwardly rectifying potassium (K^+) channel in the guinea pig submucous plexus leading to the subsequent hyperpolarization of the cell membrane (North *et al.*, 1987). Experiments with the non-hydrolyzable GTP analogue, guanosine 5'-[γ-thio]triphosphate (GTPγS), indicated that the δ opioid receptor is directly coupled to the K^+ channel via a G-protein. There was no evidence that a PKC or cAMP-dependent protein kinase is involved in the opioid mediated modulation of K^+ conductance (North *et al.*, 1987). Furthermore, in neuroblastoma X DRG hybrid F11 cells, DPDPE increases K^+ conductance at concentrations greater than 1 nM in a PTX-sensitive manner (Fan & Crain, 1995). Thus, apart from the effects on Ca^{2+} conductance described above, opioids may also inhibit

antinociceptive neurotransmission by increasing K^+ conductance, thereby hindering the movement of action potentials into the presynaptic terminal and lessening neurotransmitter release (North, 1993). This hypothesis is supported by the recent demonstration of a μ opioid receptor mediated increase in K^+ conductance and subsequent inhibition of GABAergic neurotransmission in the periaqueductal grey region of rat brain (Vaughan *et al.*, 1997b). Alternatively, opioid-mediated increases in K^+ conductance may also cause hyperpolarization of the postsynaptic membrane and consequently attenuate the transmission of nociceptive impulses (Grudt & Williams, 1994).

1.3.4 Protein Kinases

There is increasing evidence that δ opioid receptors modulate the activity of a number of kinases in cultured cells. Thus, the δ agonist DPDPE stimulates proteinase kinase C (PKC) activity and, following prolonged (24-hour) exposure, protein kinase A (PKA) activity in NG108-15 cells. This response is PTX-sensitive implicating signal transduction by the G_i/G_o protein families (Lou & Pei, 1997). The elevated PKA activity is consistent with a previous report demonstrating protein kinase A regulation of DOR mRNA levels following chronic exposure to δ agonist (Búzás *et al.*, 1997). Mitogen-activated protein kinase (MAP kinase) is also stimulated by δ agonists in a βγ and *Ras*-dependent, PTX and PKC-sensitive manner in cultured cells (Burt *et al.*, 1996; Fukuda *et al.*, 1996; Belchva *et al.*, 1998). The activation of receptor kinases may play a role in δ opioid receptor phosphorylation and subsequent receptor downregulation (Pei *et al.*, 1995).

1.4 Tissue Distribution of δ Opioid Receptors

The tissue distribution of the opioid receptors has been extensively covered in various review articles (Mansour *et al.*, 1987; Dhawan *et al.*, 1996; Mansour *et al.*, 1995). Here, the tissue distribution of the δ opioid receptor is discussed in detail. Emphasis will be placed on the distribution of the δ opioid receptor in the central nervous system, particularly at supraspinal sites, in keeping with the focus of this thesis.

1.4.1 Distribution in Brain

Delta (δ) opioid receptors have a more restricted distribution in the central nervous system than the other opioid receptors (Mansour *et al.*, 1987). Receptor autoradiography studies indicate that the highest density of δ opioid receptors are found in the olfactory bulb, neocortex, caudate putamen and nucleus accumbens, whereas a moderate to poor δ opioid receptor density is found in the thalamus, hypothalamus and brainstem in rat (Dupin *et al.*, 1991; Renda *et al.*, 1993; Gouardères *et al.*, 1993). These findings have been confirmed by immunohistochemical labeling of the cloned δ opioid receptor (DOR) (Arvidsson *et al.*, 1995). In situ hybridization studies demonstrate that there is a good correlation between DOR mRNA expression and δ opioid receptor autoradiography (Mansour *et al.*, 1987), suggesting local receptor synthesis in these δ receptor-rich brain regions. Ultrastructural localization studies in rat striatal patches indicate that DOR has a preferential presynaptic distribution in small axon terminals where DOR is predominantly associated with cytoplasmic organelles involved in the delivery of receptor proteins or neurotransmitters to the cell surface (Wang & Pickel, 2001).

1.4.2 Distribution in Spinal Cord

In the spinal cord, autoradiographic and immunohistochemical studies demonstrated labeling of δ opioid receptors predominantly in the superficial dorsal horn but also in the deeper lamina and the ventral horn (Gouardères *et al.*, 1993). In contrast, in situ hybridization studies detect cells expressing DOR mRNA in the dorsal and ventral horns of the spinal cord but not in the superficial layers (Wang & Wessendorf, 2001). However, DOR mRNA expression is observed in cells of the dorsal root ganglia (DRG) (Mansour *et al.*, 1994; Schafer *et al.*, 1994; Maekawa *et al.*, 1994; Wang & Wessendorf, 2001). Thus, it is likely that the δ opioid receptor sites in the superficial layers of the spinal cord are on presynaptic fibers projecting from the DRG. This hypothesis is consistent with the decreased expression of δ opioid receptors in laminae I and II of the spinal cord following dorsal rhizotomy (Besse *et al.*, 1992; Dado *et al.*, 1993). Ultrastructural immunohistochemistry studies have provided convincing, additional evidence for the presynaptic localization of δ opioid receptors on axon terminals projecting into the superficial layers of the dorsal horn (Cheng *et al.*, 1995; Zhang *et al.*, 1998). Presynaptic δ opioid receptors on primary afferents appear to modulate the inhibitory effects of opiates on the release of nociceptive neurotransmitters such as

substance P and calcitonin gene-related peptide (CGRP) in the dorsal horn of the spinal cord (Bourgoin *et al.*, 1994; Zhang *et al.*, 1998).

1.4.3 Distribution in Sensory Ganglia

There is also evidence that opioid receptors synthesized in DRG are transported into the peripheral terminals of primary afferent sensory neurons (Hassan *et al.*, 1993; Zhou *et al.*, 1998). Thus, immunohistochemistry studies of the upper dermal region of glabrous rat (Wenk & Honda, 1999) and monkey (Coggeshall *et al.*, 1997; Wenk & Honda, 1999) skin demonstrated δ opioid receptor labeling in sensory afferent fibers and terminals. Similarly, subcutaneous nerves innervating the lip, eyelid, cornea and papillary dermis also were positive for δ receptor-like immunoreactivity. Delta (δ) opioid receptor labeling was also found in subcutaneous nerves innervating tissues without any known nociceptive function such as hair follicles, glandular apparatus and blood vessels (Wenk & Honda, 1999). In all peripheral tissues, δ opioid labeling was confined to small diameter, unmyelinated neurons, a description consistent with the class of nerve fibers labeled positively for μ opioid receptors in rat tooth pulp (Taddese *et al.*, 1995). The role of δ opioid receptors in the non-nociceptive fibres is unknown. However, the presence of δ opioid receptors in subcutaneous nerve bundles in tissues such as skin is consistent with the predicted role of opioids in inhibiting peripheral nociception associated with tissue inflammation (Stein *et al.*, 1989; Zhou *et al.*, 1998).

1.5 Actions of δ Opioid Receptors *in vivo*

The opioid receptors modulate a wide variety of responses in nervous, cardiovascular, immunological, gastrointestinal and a host of other biological systems. In the current section, supraspinal opioid receptor function in pain transmission and the modulation of psychostimulant activity is discussed in detail in keeping with the focus of this thesis. Alternate biological actions of δ opioid receptors, and the other members of the opioid receptor family, are summarized in Table 1.3 at the end of this section.

1.5.1 Ascending Pain Pathways

Opiates produce analgesic effects by modulating the ascending and descending pain pathways (Basbaum & Fields, 1984; Figure 1.1). The primary function of the ascending pathway is to transmit nociceptive information from the skin, viscera and other peripheral organs to the brain, where the conscious appreciation of pain is realized (Millan, 1999).

As described above, δ opioid receptors are found in key tissues in the ascending pain pathway including the peripheral and afferent terminals of sensory neurons, dorsal root ganglia, spinal cord and the spinal trigeminal nucleus, although not in higher centers within the thalamus (Mansour *et al.*, 1987). Delta (δ) opioid receptors in the peripheral terminals of primary afferents appear to attenuate inflammatory pain transmission to the spinal cord based on the antinociceptive effects of δ agonists administered at peripheral sites of injury (Zhou *et al.*, 1998; Bilsky *et al.*, 1996). At the level of the spinal cord, δ opioid receptors also play a role in inhibiting ascending nociceptive transmission. Thus, electrophysiology studies have demonstrated that activation of presynaptic δ opioid receptors inhibit excitatory post-synaptic potentials in dorsal horn neurons (Dickenson *et al.*, 1987; Glaum *et al.*, 1994; Acosta & López, 1999). In addition, δ receptors inhibit the spinal release of nociceptive neurotransmitters including substance P, calcitonin gene-related peptide and glutamate (Collin *et al.*, 1991; Bourgoin *et al.*, 1994; Ueda *et al.*, 1995; Zachariou & Goldstein, 1996). Accordingly, intrathecal administration of δ agonists effectively inhibit nociceptive, hyperalgesic and allodynic pain states in animal models (Stewart & Hammond, 1994; Hylden *et al.*, 1991; Ho *et al.*, 1997; Hao *et al.*, 1998).

A secondary function of the ascending pathway may be to tonically depress antinociception mediated by endogenous supraspinal opioids acting to inhibit the descending pathway. Thus, suppression of the ascending pathway by spinal opioids may act to disinhibit antinociception produced by supraspinal opioids (Gear & Levine, 1995). It has been demonstrated that the spinal administration of δ agonists positively modulates supraspinal δ opioid antinociceptive effects (Miaskowski *et al.*, 1993; Kovelowski *et al.*, 1999). However, it is unclear to what extent this response is due to disinhibition of the descending pathway in comparison to the independent modulation of supraspinal and spinal structures involved in nociceptive transmission.

1.5.2 Descending Pain Pathways

Descending pathways originating at supraspinal sites function to modulate nociceptive transmission in the dorsal horn, generally by reducing the release of neurotransmitters from the terminals of peripheral afferents (Millan, 1999; Figure 1.1). It has been proposed that opioid-induced disinhibition of neurons in the periaqueductal gray (PAG) activates spinally projecting neurons in the rostroventral medulla (RVM) to attenuate

nociceptive signals originating from sites in the dorsal horn (Basbaum & Fields, 1984). The expression of μ and δ opioid receptors in the PAG and RVM support this model (Mansour *et al.*, 1995; Kalyuzhny *et al.*, 1996; Figure 1.1). Correspondingly, whole-cell patch clamp techniques demonstrate opioid disinhibition of ventrolateral PAG neurons projecting to the RVM in rat brain slices in response to [Met]enkephalin (δ-selective agonist) (Osborne *et al.*, 1996) likely via the presynaptic inhibition of GABAergic inhibitory postsynaptic currents (Vaughan & Christie, 1997a). Furthermore, in the RVM, immunocytochemistry studies demonstrate that about one-half of all spinally projecting neurons in the nucleus raphe magnus are apposed by presynaptic δ-immunoreactive varicosities (Kalyuzhny *et al.*, 1996). This finding is consistent with the hypothesis that the activation of presynaptic δ opioid receptors in the RVM causes disinhibition of neurons that form part of a descending antinociceptive pathway (Harasawa *et al.*, 2000; Thorat & Hammond, 1997). In turn, δ receptor activation in the RVM activates descending pain pathways projecting through the dorsolateral funiculus (DLF) to attenuate acute and tonic spinal nociceptive input (Kovelowski *et al.*, 1999).

1.5.2.1 GABAergic pathways

Delta (δ) opioid receptors have been implicated in the modulation of GABA, noradrenaline, serotonin and glutamate, all of which are key neurotransmitters in the modulation of descending inhibitory pain pathways (Millan, 1999; Figure 1.1). GABAergic transmission is perceived to tonically inhibit a descending, antinociceptive pathway. Whole-cell patch-clamp recordings in the rat PAG suggest that opioids such as [Met]enkephalin elicit analgesic responses by suppressing the inhibitory GABAergic synaptic transmission on neurons of the descending antinociceptive pathway (Vaughan *et al.*, 1997b). Additional studies are required to resolve whether this effect results from the direct activation of δ opioid receptors on GABAergic terminals (Bausch *et al.*, 1995; Commons & Milner, 1997) or the modulation of afferent input to GABAergic neurons (Kalyuzhny & Wessendorf, 1998), or both.

1.5.2.2 Noradrenergic pathways

Noradrenergic neurons projecting from the RVM to the spinal cord may comprise an antinociceptive pathway under modulation by GABAergic inputs. Direct administration of the δ agonist, deltorphin II, into the RVM appears to disinhibit bulbospinal noradrenergic neurons and elicit antinociception by the release of noradrenaline at spinal

sites and the consequent activation of α_2-adrenergic receptors in the dorsal horn (Grabow *et al.*, 1999).

1.5.2.3 Serotonergic pathways

Serotonergic neurons projecting from the RVM to the spinal cord appear to correspond to a nociceptive pathway (Wang & Wessendorf, 1999). The majority of spinally projecting serotonergic neurons projecting from the RVM to the spinal cord also express the DOR mRNA suggesting that δ opioid receptors may also play a significant role in the modulation of a descending serotonergic nociceptive pathway (Wang & Wessendorf, 1999). The appearance of δ receptors on serotonergic neurons is consistent with the findings of a recent study where the application of δ agonists inhibited serotonin release in the rat ventral spinal cord (Franck *et al.*, 1996).

1.5.2.4 Glutamatergic pathways

Glutamatergic neurotransmission activates excitatory postsynaptic currents in the brain and both ionotropic and metabotropic glutamate receptor agonists are associated with nociception (Vaughan & Christie, 1997a; Suzuki *et al.*, 2000). Electrophysiology studies performed in striatal, neocortical or PAG neurons indicate that δ selective agonists inhibit glutamatergic neurotransmission (Jiang & North, 1992; Vaughan & Christie, 1997a; Ostermeier *et al.*, 2000). The finding that both AMPA and NMDA post-synaptic excitatory currents were inhibited to a similar extent suggests that δ agonists decrease glutamatergic transmission by presynaptic inhibition of glutamate release (Ostermeier *et al.*, 2000). A similar mechanism has been proposed at the level of the spinal cord where δ agonists appear to inhibit glutamate release from primary afferents at synaptic junctions (Zhang *et al.*, 1998).

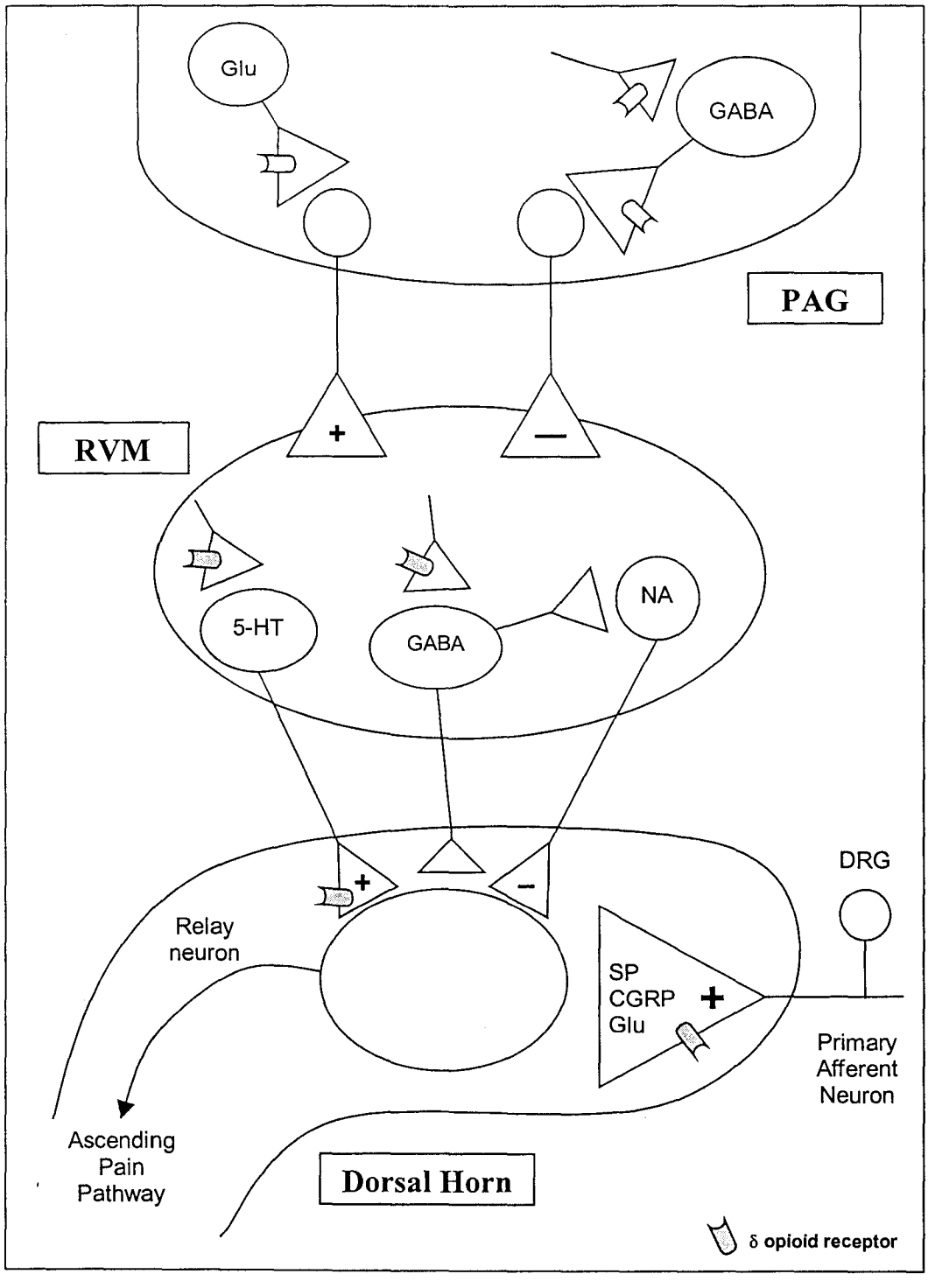

Figure 1.1 Mechanisms for δ opioid receptor-mediated pain modulation.
Delta (δ) opioid receptors attenuate pain transmission by disinhibition of a descending antinociceptive pathway tonically inhibited by GABA and presynaptic inhibition of a descending, serotonergic nociceptive pathway. Also, δ opioid receptors attenuate ascending pain transmission by presynaptic inhibition of the release of substance P (SP), calcitonin gene-related peptide (CGRP) and glutamate (Glu) from primary afferent terminals in the dorsal horn. *Abbreviations:* periaqueductal gray (PAG), rostral ventromedial medulla (RVM), noradrenergic pathway (NA), serotonergic pathway (5-HT), dorsal root ganglion (DRG). *Symbols:* (+) Nociceptive transmission, (-) Antinociceptive transmission.

1.5.3 Role of δ Opioid Receptors in Supraspinal Antinociception

In behavioural studies, the role of δ opioid receptors in modulating supraspinal antinociception has been demonstrated in mice, rats and primates (Jiang *et al.*, 1990; Ossipov *et al.*, 1995a; Negus *et al.*, 1998). However, there are discrepant reports of the antinociceptive efficacy of δ agonists that appear to be contingent upon the δ agonist tested, the nociceptive stimulus used and/or the supraspinal site of injection (Negri *et al.*, 1991a; Adams *et al.*, 1993; Ossipov *et al.*, 1995a). For example, in the hot plate and tail flick assays of thermal nociception, the δ_2 agonist, deltorphin II, elicited an antinociceptive response whereas the δ_1 agonist, DPDPE, was inactive following direct injection into the rat PAG or RVM (Rossi *et al.*, 1994; Ossipov *et al.*, 1995a). In comparison, deltorphin II and DPDPE were antinociceptive in the tail flick assay, although only deltorphin II was effective in the hot plate assay, following direct injection into the rat ventromedial medulla (Thorat & Hammond, 1997). In contrast, intracerebroventricular (i.c.v.) administration of DPDPE produced significant antinociception in assays of mechanical nociception (Miaskowski *et al.*, 1991). Similarly, in assays of mechanical or thermal allodynia following nerve injury, administration of DPDPE into the ventral (but not dorsal) PAG attenuated neuropathic pain symptoms in rats (Sohn *et al.*, 2000). The latter finding may suggest a unique therapeutic role for δ agonists, as μ agonists are ineffective in animal models of nerve injury causing allodynia (Bian *et al.*, 1995; Yaksh, 1999) and in the clinical treatment of neuropathic pain (Arner & Meyerson, 1988).

Supraspinal administration of the δ_2 agonist, deltorphin II, is more potent in inflammatory pain models associated with tissue injury and thermal hyperalgesia than in assays of acute thermal nociception (Hurley & Hammond, 2000; Fraser *et al.*, 2000a). The enhanced potency of deltorphin II during persistent inflammation may arise from an additive or synergistic interaction with increased levels of endogenous opioids (i.e. [Met]enkephalin) in the PAG, RVM or other sites in the descending pain pathways (Williams *et al.*, 1995; Ossipov *et al.*, 1995b; Hurley & Hammond, 2001). Alternatively, the potent response to deltorphin II is consistent with a more prominent role for δ_2 receptors in supraspinal pain processing as a consequence of the enhanced neuronal activity in descending pain pathways following peripheral inflammation (Ren & Dubner, 1996).

In total, these findings raise further questions about the role of δ subtypes in pain transmission, the anatomical distribution of δ subtypes in the brain, and the comparative role of subtype-specific δ agonists in the treatment of acute, neuropathic and inflammatory pain.

1.5.4 Role of δ Opioid Receptors in Locomotor Activity & Reward

The mesolimbic dopaminergic pathway extending from the ventral tegmental area to the nucleus accumbens has been characterized as important in the modulation of psychostimulant behaviour including increased locomotor activity and reward-seeking (Pennartz *et al.*, 1994; Koob, 2000). Substantial biochemical and behavioural evidence indicates that δ opioid receptors modulate mesolimbic dopaminergic pathways. Autoradiographic and *in situ* hybridization and immunocytochemical localization studies indicate the presence of δ opioid receptors within the mesocorticolimbic dopamine system (Dilts & Kalivas, 1990; Mansour *et al.*, 1995; Svingos *et al.*, 1998). Moreover, δ opioid receptor immunolabeling in axon terminals within the shell compartment of the nucleus accumbens provides ultrastructural evidence that δ receptor activation is primarily involved in inhibiting the presynaptic release of inhibitory neurotransmitters such as GABA (Svingos *et al.*, 1998; Svingos *et al.*, 1999). This observation is consistent with the finding that δ agonists decrease the tonic, inhibitory synaptic potentials mediated by GABA in striatal neurons (Jiang & North, 1992). Accordingly, brain microdialysis studies have demonstrated that δ agonists cause increased extracellular dopamine release in the nucleus accumbens (Spanagel *et al.*, 1990; Longoni *et al.*, 1991; Yoshida *et al.*, 1999). In total, these studies suggest that δ receptor activation directly inhibits tonic, inhibitory GABAergic transmission and, in turn, leads to disinhibition of dopaminergic striatal pathways.

In behavioural assays, the majority of reports suggest that δ agonists stimulate locomotor activity in rodents (Negri *et al.*, 1991a; Longoni *et al.*, 1991; Meyer & McLaurin, 1995). Recent antisense studies in mice and rats indicate that the enhanced locomotor activity in response to δ agonists is mediated by the cloned δ opioid receptor (Mizoguchi *et al.*, 1996; Negri *et al.*, 1999; Fraser *et al.*, 2000b). Studies correlating changes in δ receptor-mediated locomotor activity with the lesion of mesolimbic dopamine neurons (Calenco-Choukroun *et al.*, 1991b) or the stimulation of dopamine release in the nucleus accumbens (Longoni *et al.*, 1991) provide additional evidence for the role of δ receptors

in the modulation of dopaminergic activity in the brain. Nonetheless, aspects of the locomotor response to δ agonists remain to be resolved. For example, some published reports suggest that δ agonists have a depressant effect on locomotor activity that may be dose, time or agonist-dependent (Negri *et al.*, 1996; Meyer & McLaurin, 1995; Pohorecky *et al.*, 1999). Also, a recent antisense study suggests that there may be δ subtype-specific pharmacology underlying the hyperlocomotor response to δ agonists. Thus, treatment with an antisense sequence targeted against exon 2 of the cloned δ opioid receptor selectively inhibited the hyperlocomotor response to deltorphin II, but not DPDPE, whereas an antisense sequence targeted against exon 3 inhibited the response to both agonists (Negri *et al.*, 1999). The latter finding is consistent with the proposed existence of δ receptor subtypes arising from alternative splicing of the DOR gene (Rossi *et al.*, 1997). In total, additional studies are required to clarify the role of DOR in the modulation of locomotor activity particularly with regard to the effects of the non-peptidic δ agonists (i.e. SNC80) that are currently under consideration for clinical development.

Mesolimbic dopamine pathways terminating in the nucleus accumbens also play an important role in the modulation of reward-seeking behaviour (Koob, 2000). Delta (δ) agonists appear to have positive motivational properties based on conditioned place-preference studies in rodents (Shippenberg *et al.*, 1987; Longoni *et al.*, 1998; Suzuki *et al.*, 1997). Also, in rats, high doses of DPDPE were effective in establishing and maintaining lever-pressing associated with self-administration of drug directly into the VTA (Devine & Wise, 1994). Similarly, microinjections of DPDPE into the rat caudate putamen nucleus caused increased motor behaviours related to reward in a self-stimulation paradigm (Johnson & Stellar, 1994). In monkeys, SNC80 produced cocaine-like discriminative stimulus effects, but did not maintain responding in monkeys trained to self-administer cocaine, suggesting that this δ agonist has low abuse potential (Negus *et al.*, 1998). However, a recent study suggests that the weak rewarding effects observed for δ agonists may be due to non-specific interactions at the μ opioid receptor, as a lack of dependence and rewarding effects were observed for deltorphin II in μ opioid receptor (MOR) knockout mice (Hutcheson *et al.*, 2001).

Chronic exposure to peptidic δ agonists followed by administration of the general opioid antagonist, naloxone, has been reported to exacerbate a range of physical withdrawal

symptoms in rats including wet dog shakes, tremors, teeth chattering and ptosis (Cowan *et al.*, 1988; Maldonado *et al.*, 1990). However, δ agonists produce a mild degree of physical dependence in comparison to μ agonists where major signs of withdrawal related to a severe degree of abstinence, such as jumping, body weight loss and hypothermia, are routinely observed (Cowan *et al.*, 1988). Thus, it has also been suggested that the mild withdrawal effects observed for δ agonists reflect a weak, non-selective interaction of these compounds at μ receptors. This hypothesis is supported by the recent finding that the physical dependence induced by chronic treatment with deltorphin II is not observed in MOR knockout mice (Hutcheson *et al.*, 2001).

Behaviour	δ	μ	κ	ORL_1
Pain modulation	Spinal/supraspinal antinociception (Zhu *et al.*, 1999; Bilsky *et al.*, 1996); δ antinociception may be co-dependent on MOR expression (Sora *et al.*, 1997; Matthes *et al.*, 1998)	Spinal/supraspinal and peripheral antinocieption (Sora *et al.*, 1997; Loh *et al.*, 1998; Stein, 1993; Tian *et al.*, 1997; Matthes *et al.*, 1996)	Spinal/supraspinal antinociception; inhibition of visceral chemical nociception (Simonin *et al.*, 1998; France *et al.*, 1994)	Spinal antinociception; supraspinal pronociceptive or anti-opioid effects (Nishi *et al.*, 1997; Mogil *et al.*, 1996)
Spontaneous locomotor activity	Hyperlocomotion (Longoni *et al.*, 1991)	Hyperlocomotion (Tian *et al.*, 1997)	Hypolocomotion (Simonin *et al.*, 1998)	Hypolocomotion (Nishi *et al.*, 1997; Calo *et al.*, 2000)
Anxiety	Anxiolytic (Filliol *et al.*, 2000)	Anxiogenic (Filliol *et al.*, 2000)	No effect (Filliol *et al.*, 2000) or mild anxiolytic (Privette & Terrian, 1995)	Anxiolytic (Jenck *et al.*, 2000)
Dependence/ Reward	Reinforcing properties (Shippenberg *et al.*, 1987), but less potent that μ (Devine & Wise, 1994)	Euphoria, reward, withdrawal (Matthes *et al.*, 1996; Devine & Wise, 1994)	Dysphoria, aversion (Pfeiffer *et al.*, 1986; Simonin *et al.*, 1998)	No effect (Devine *et al.*, 1996)
Gastric Motility	Decrease (Broccardo & Improta, 1992; Negri *et al.*, 1999)	Decrease (Roy *et al.*, 1998b; Roy *et al.*, 1998a)	Decrease (Shukla *et al.*, 1995)	Decrease (Osinski *et al.*, 1999)
Respiratory	No effect (Takita *et al.*, 1997; Matthes *et al.*, 1998)	Decrease respiratory frequency (Takita *et al.*, 1997; Matthes *et al.*, 1998)	Decreased respiratory frequency, increased inspiration time (Takita *et al.*, 1997; Matthes *et al.*, 1998)	Inhibition of tachykinergic contraction of the bronchus (Fischer *et al.*, 1998)
Cardiovascular	No effect (Bachelard & Pitre, 1995; Shen & Ingenito, 1999)	Increase blood pressure, tachycardia, vasoconstriction (Bachelard & Pitre, 1995)	Decrease blood pressure and heart rate (Shen & Ingenito, 1999) anti-arrhythmic (Yu *et al.*, 1999)	Transient hypotension and bradycardia (Giuliani *et al.*, 1997; Madeddu *et al.*, 1999)
Renal	Diuretic, natriuretic (Sezen *et al.*, 1998)	Diuretic, antinatriuretic (Cabral *et al.*, 1997)	Diuretic, antinatriuretic (Brooks *et al.*, 1997; Cabral *et al.*, 1997)	Diuretic, antinatriuretic (Kapusta *et al.*, 1997)
Immunomodulation	Immunosuppression (Cheido *et al.*, 1996; Sharp *et al.*, 1998)	Immunosuppression (Gaveriaux-Ruff *et al.*, 1998; Roy *et al.*, 1998a)	Immunosuppression (Radulovic *et al.*, 1995; Sharp *et al.*, 1998)	To be determined

Table 1.3 Biological Effects of Opioid Receptors

2 Tools for the Determination of Gene Function

Genes are being cloned and identified at an accelerated pace as a consequence of recent advances in the field of molecular biology. The human genome comprises about 30,000 genes, 3-10% of which are estimated to be viable targets for drug development (Drews, 1996). Although the sequencing of the human genome was recently completed, the impact of this achievement cannot be fully realized until the function of each gene is determined *in vivo* (Table 2.1). At present, gene function is primarily studied via two distinct approaches: antisense 'knockdown' and homologous recombination 'knockout'. These methods share a common premise that gene function can be determined *in vivo* by preventing target gene expression and monitoring the impact of this manipulation on phenotype. This section aims to provide an overview of antisense technology with emphasis on the utility of this approach for the determination of gene function for targets expressed in the CNS. In addition, antisense technology will be briefly compared to homologous recombination knockout techniques for the determination of gene function *in vivo*.

2.1 Antisense Technology

Antisense is a naturally occurring phenomenon utilized by cells to repress gene function. Thus, cells are able to transcribe RNA complementary to an endogenous target mRNA such that hybridization of these nucleotide sequences prevents translation of the target sequence (Weintraub, 1990). Zamecnik and Stephenson (Zamecnik & Stephenson, 1978) were the first to demonstrate that synthetic oligonucleotides (ODN) designed complementary to an RNA target sequence of *Rous sarcoma* virus could prevent viral replication.

2.1.1 Antisense Mechanisms

Antisense technology is founded on the premise that any cloned gene can be specifically targeted as a consequence of Watson-Crick base pairing (Crooke, 1993). Antisense oligonucleotides (ODNs) suppress gene expression by hybridizing with a complementary target sequence to inhibit processes required for the flow of information from gene to protein via two main mechanisms: (1) steric inhibition of mRNA processing and (2) RNase H mediated cleavage of target mRNA (Figure 2.1).

Advantages	Disadvantages
Antisense Knockdown	
• Applicable to any stage of development • Amenable to studying gene function in any species • Exclusive targeting of central or peripheral gene expression • Knockdown of protein expression is reversible • Direct therapeutic application • Rapid, inexpensive paradigm	• Incomplete knockdown • Sequence-dependent and sequence-independent non-specific effects (especially with phosphorothioates) • Continuous or repeated administration is necessary to maintain effect
Homologous Recombination Knockout	
• Complete disappearance of gene product • Genetic manipulation is generally highly specific for the target gene • Permanent source of knockout animals • Technical advances now permit increased control over the manipulation (i.e. spatial and temporal knockouts)	• Limited to studies in mice • Compensatory developmental mechanisms might be operative • Possibility of lethal phenotype • Possible cross-over effect of knockout on adjacent genes • Genetic background may confound phenotype • Expensive, laborious and time-consuming

Table 2.1 Comparison of antisense knockdown and homologous recombination knockout approaches for determining gene function *in vivo*

2.1.1.1 Steric inhibition of mRNA processing

The hybridization of ODN to mRNA sequences can inhibit the interaction between the mRNA and various proteins or other factors required for mRNA processing leading to translation (Crooke, 1999). For example, key steps in the intermediary metabolism of mRNA are 5'-capping, 3'-polyadenylation, and the excision of introns (i.e. splicing). 5'-Capping plays a key role in both stabilizing the mRNA construct and directing the transport of mRNA out of the nucleus. Thus, antisense ODN targeted against the 5'-capping region effectively inhibited the binding of the translation initiation factor eIF-4a (Baker *et al.*, 1992). Polyadenylation occurring in the 3'-untranslated region also plays a key role in stabilizing mRNA. Although antisense ODNs directed against the 3'-untranslated region effectively block protein synthesis, it is not clear whether this effect is specifically due to the disruption of polyadenylation (Chiang *et al.*, 1991). Splicing reactions arise from sequence-specific spliceosome activity to produce mature mRNA for translation. Antisense ODN directed towards splice sites effectively inhibit gene

expression (Hodges & Crooke, 1995). The ability of antisense ODNs to selectively inhibit splicing at targeted sites has application in the restoration of correct splicing of mutant disease genes (Sierakowska *et al.*, 1996) and the regulation of expression of certain splice variants for research or therapeutic purposes (Taylor *et al.*, 1999).

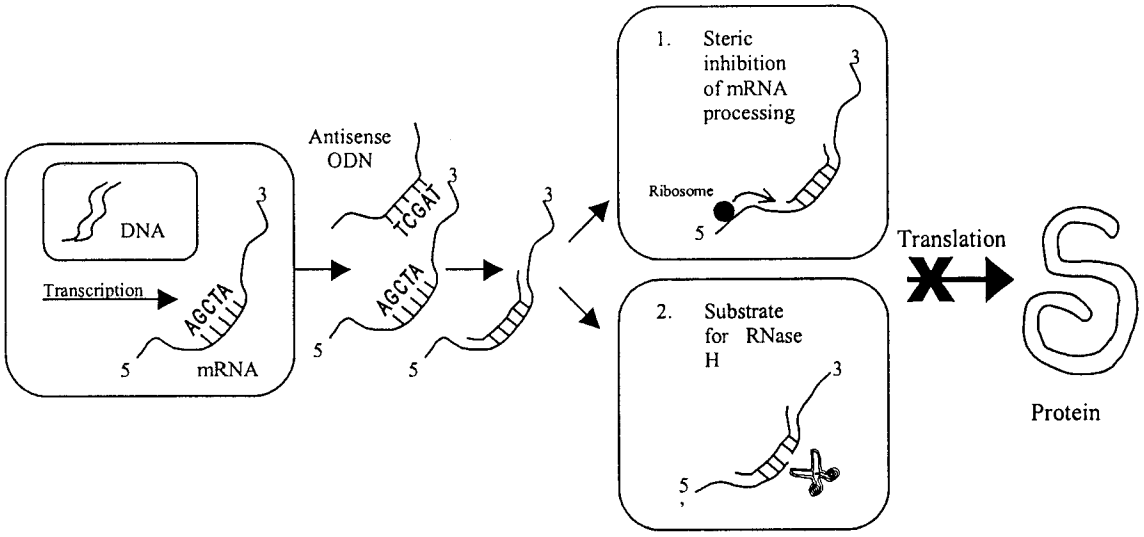

Figure 2.1 Antisense ODNs selectively hybridize to complementary target mRNA sequences. (Figure modified from Fraser & Wahlestedt, 1997a.)
Phosphodiester and phosphorothioate ODNs are able to inhibit protein expression by facilitating RNase H mediated cleavage of target mRNA. Alternatively, ODNs have the capacity to inhibit protein expression by sterically blocking the interaction of the target mRNA with cellular proteins which facilitate translation.

The efficient processing of mRNA can also be inhibited by ODN hybridization that causes a disruption of tertiary RNA structure. RNA spontaneously folds into certain structures to provide additional stability to the molecule and to expose recognition motifs for a variety of proteins, nucleic acids and ribonucleoproteins that play a role in mRNA processing. The tertiary structure of mRNA is induced by intramolecular hybridization and is therefore susceptible to disruption by antisense ODNs. For example, ODNs targeted against the stem-loop structure of the TAR element of HIV were shown to disrupt the structure of the mRNA molecule and inhibit the TAR-mediated production of a reporter gene (Vickers *et al.*, 1991).

Translational arrest is thought to be the most common mechanism by which antisense ODN block mRNA processing to inhibit protein synthesis. In this case, ODN complementary to the translation initiation codon hybridizes with the target mRNA and blocks the movement of the ribosome along the transcript, thus preventing protein synthesis (Boiziau *et al.*, 1991). Antisense

inhibition via translational arrest has been clearly demonstrated for various ODN analogues targeted against several different biological targets (Mirabelli *et al.*, 1991; Baker *et al.*, 1997; Cooper *et al.*, 1999). Certain ODN analogues (i.e. methylphosphonate, 2'-O-allyl diester and peptide nucleic acid; Figure 2.2) are presumed to inhibit protein synthesis exclusively by steric hindrance, resulting in translational arrest or some other change in mRNA processing as discussed above (Bonham *et al.*, 1995; Johansson *et al.*, 1994).

2.1.1.2 Activation of RNase H

RNase H is a cellular endonuclease that recognizes DNA:RNA heteroduplexes and cleaves the RNA portion of the duplex (Lima & Crooke, 1997b). The resulting cleavage products lack a 5'-cap and 3'-polyadenylation, respectively, and are thus susceptible to rapid degradation by 5'- and 3'exonucelases. It appears that phosphodiester (PDE) and phosphorothioate (PS) ODNs have the capacity to mimic the DNA strand and are substrates for RNase H when they are bound to their complementary mRNA targets (Dash *et al.*, 1987; Boiziau *et al.*, 1992). Thus, in cell-based assays, selective reduction of target mRNA and the formation of mRNA cleavage products have been demonstrated following treatment with PDE or PS ODN (Giles *et al.*, 1995; Condon & Bennett, 1996). In addition, there is a positive correlation between the ability of oligonucleotides to support RNase H activity *in vitro* and their antisense activity in cells (Monia *et al.*, 1993). RNase H-mediated cleavage of target mRNA is effectively catalytic, as the antisense ODN is resistant to degradation by RNase H and thus survives to mediate the RNase H-mediated cleavage of many additional target mRNA molecules (Neckers *et al.*, 1992; Flanagan *et al.*, 1996). A disadvantage of RNase H activation is that this enzyme can also be activated by unstable complexes arising from the transient hybridization of ODN to mismatched, non-target mRNA (Lima & Crooke, 1997b). Non-specific mRNA cleavage by PS-induced RNase H activity is considered to be an important contributor to the side effect profile observed with the use of PS ODN (Stein, 2000).

2.1.2 Methodological Considerations

Antisense techniques have been used to evaluate the function of a variety of targets including G-protein coupled receptors, ion channels, immediate-early genes, neurotransmitters and other non-receptor proteins (Weiss *et al.*, 1997). Antisense techniques can be used in any species at any stage of development. The acute nature of antisense knockdown allows gene function to be determined in the absence of compensatory developmental changes arising in response to the manipulation. The reversibility of antisense treatment permits subjects to be used as their own

controls following recovery of the target protein. Antisense technology is the quickest, most inexpensive method for determining gene function *in vivo*.

Recent technological advances have overcome many of the initial barriers limiting the use of antisense in biological systems. Chemical improvements in ODN structure have yielded molecules with increased stability, target specificity, hybridization affinity and potency (Cook, 1998). In addition, ODN permeability of cell membranes or the blood-brain barrier has been improved by using carrier-mediated transport systems (Boado, 1995).

Incomplete knockdown and poor target specificity are key limitations of existing antisense technology. Incomplete knockdown complicates the interpretation of antisense effects because changes in phenotype (if any) must be explained in the context of partial decreases in target protein expression. Thus, the level of expression and redundancy of the target protein may influence the efficacy of antisense treatment to change phenotype (Rosolen *et al.*, 1993; Chen *et al.*, 1995). Similarly, the possibility of non-specific effects to ODN treatment necessitates the use of appropriate controls to demonstrate that phenotypic changes arise from an antisense mechanism (Eckstein *et al.*, 1996). In this section, methodological considerations pertaining to the use of antisense technology are discussed with emphasis on techniques to minimize or control for the appearance of incomplete knockdown and poor target specificity.

2.1.2.1 ODN & PNA Chemistry

The current limitations of antisense technology, including incomplete knockdown, poor target specificity, and poor ODN stability and permeability, are to some extent characteristic of the ODN chemistry and thus may be circumvented by using alternate antisense molecules (Figure 2.2). The variety of ODN analogues that are currently available has been previously reviewed (Cook, 1998). They can be summarized briefly as follows.

Phosphodiester (PDE) ODN can be effective antisense agents when administered directly into the CNS (Wahlestedt *et al.*, 1993a; Wahlestedt *et al.*, 1993b). However, these ODNs are rapidly degraded by nucleases and proteases in the circulation and thus are not appropriate for targeting proteins expressed in the periphery (Wickstrom, 1986; Thierry & Dritschilo, 1992).

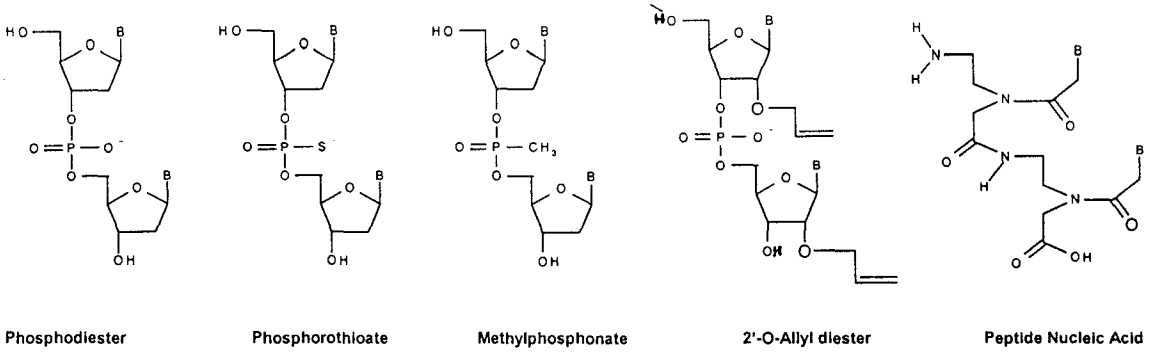

Figure 2.2 Structures of oligonucleotide analogues derived from synthetic modifications to the natural phosphodiester backbone.
B indicates the position of the nucleotide bases.

Phosphorothioate (PS) ODNs are less susceptible to nuclease and protease activity (Wickstrom, 1986; Campbell *et al.*, 1990) and effectively inhibit the expression of both central and peripheral targets (Akhtar & Agrawal, 1997). Moreover, the highly charged nature of PS ODN molecules leads to potent activation of RNase H and can produce substantial decreases in target mRNA expression (Stein *et al.*, 1988). However, the charged nature of these molecules also reduces their hybridization affinity for target mRNA due to electrostatic repulsion with the target sequence (Cooper *et al.*, 1999). Thus, the melting temperature of hybridization (T_m) of a PS ODN for RNA is approximately 0.5°C and 2.2°C less per nucleotide than the corresponding PDE ODN and RNA sequences, respectively (Crooke, 1999). Accordingly, PS ODN must be at least 17-20 nucleotides in length to have sufficient hybridization affinity to produce biological activity (Monia *et al.*, 1992). In addition, the charged PS backbone also promotes non-specific interactions with a variety of endogenous proteins and non-target mRNA sequences (Stein, 1996). Consequently, non-specific effects of phosphorothioate ODNs are often observed *in vivo* (LeCorre *et al.*, 1997; Abraham *et al.*, 1997; Stein, 1996). Finally, PS ODN synthesis typically yields a mixture of enantiomeric products, with corresponding differences in antisense activity, due to the chiral nature of the PS backbone.

Methylphosphonate (Jayaraman *et al.*, 1981), 2'-O-allyl diester ODNs (Johansson *et al.*, 1994) and other 2'-modifications to the phosphodiester backbone (Cook, 1998) have also been introduced. These ODNs have superior hybridization affinity in comparison to PDE or PS ODN. In addition, these ODNs are resistant to nuclease degradation and thus are appropriate for targeting proteins expressed in the periphery. However, these ODN analogues do not support

RNase H activity (Johansson *et al.*, 1994) and thus are generally less potent than PS ODN in preventing the expression of target gene products in cell-based assays.

Sugar-modified oligonucleotides such as locked nucleic acids (LNA; oligonucleotides containing 2'-C, 4'-C-oxy-methylene-linked bicyclic ribonucleotide monomers) have recently been reported in the literature. These antisense oligonucleotides have been shown to recruit and activate RNase H *in vitro* (Wahlestedt *et al.*, 2000).

Chimeric ODNs were constructed in an attempt to capitalize on the most advantageous traits of the different ODN types (Shibahara *et al.*, 1987). ODN constructs comprised of an internal PDE region and PS end regions were designed to minimize ODN degradation by exonucleases and provide a low-toxicity alternative to fully modified PS ODN (Hebb & Robertson, 1997). Alternatively, second generation, end-capped ODNs are characterized by a short region of phosphorothioate backbone (typically 6-8 nucleotides in length) in order to support RNase H activity for potent inhibition of protein expression. This short region is inserted between two stretches of steric inhibitor class ODNs (typically 2'-O-methyl modified ODNs) to promote ODN affinity for target mRNA, support the stability of the heteroduplex, and reduce the net polyanionic charge of the ODN (Altmann *et al.*, 1996; Giles & Tidd, 1992). Studies in cell-based assays indicate that chimeric ODNs show some promise as specific inhibitors of protein expression (Monia *et al.*, 1993; Monia *et al.*, 1996b). However, further studies are required to validate the potential of these chimeric ODNs *in vivo*.

Peptide Nucleic Acids

Peptide nucleic acids (PNA) are synthetic analogues of deoxynucleotide bases with an achiral, charge-neutral, pseudopeptide backbone formed from N-(2-amino-ethyl)-glycine units (Nielsen *et al.*, 1991). Although PNA is chemically more analogous to peptides than nucleotides, these sequences are capable of hybridizing with complementary DNA or RNA sequences according to Watson-Crick base pairing and helix formation (Egholm *et al.*, 1993; Wittung *et al.*, 1994; Brown *et al.*, 1994). The uncharged nature of PNA sequences eliminates electrostatic repulsion between the PNA oligomer and the target sequence to accommodate the rapid formation of a highly stable and specific hybridization complex (Egholm *et al.*, 1993; Smulevitch *et al.*, 1996). Thus, the stability of a PNA-DNA duplex under physiological conditions is ~1.5°C (T_m; melting temperature) per base pair higher than that of the equivalent DNA-DNA hybrid (Egholm *et al.*, 1993). Moreover, T_m values for PNA-RNA duplexes are on average 4°C higher than for PNA-DNA duplexes (Jensen *et al.*, 1997). In addition, PNA-DNA or PNA-RNA hybridization is very

sensitive to base mismatches in comparison to DNA-DNA complexes; a single mismatch within a 16*mer* PNA-DNA duplex can reduce the T_m by up to 15°C (Egholm *et al.*, 1993; Jensen *et al.*, 1997; Ray & Norden, 2000; Doyle *et al.*, 2001).

The superior hybridization affinity and mismatch discrimination shown by PNA permits the use of sequences of 12-18 bases in length to achieve selective and potent antisense effects (Tyler *et al.*, 1998; Fraser *et al.*, 2000b; Doyle *et al.*, 2001). In comparison, phosphorothioate ODN of similar lengths have been shown to be ineffective (Monia *et al.*, 1992). Moreover, PNA oligomers are more potent than their phosphorothioate analogues in *in vitro* assays measuring antisense efficacy (Norton *et al.*, 1996; Taylor *et al.*, 1997) although a direct comparison *in vivo* has not been performed. Nonetheless, the ability to reduce oligomer length and dose when using PNA sequences may improve the efficiency of cellular uptake (Loke *et al.*, 1989) and reduce the prevalence of sequence-dependent non-specific effects (Flanagan *et al.*, 1996; Woolf *et al.*, 1992), respectively. Furthermore, PNA demonstrate a poor affinity for proteins that normally bind nucleic acids, thus minimizing the appearance of sequence-independent side effects (Hamilton *et al.*, 1996). Also, the inability of PNA to activate RNase H eliminates the likelihood of unintended degradation of nontarget mRNA, a potential cause of the unintended effects of PS ODN (Stein, 2000).

Finally, PNA sequences have superior stability in a variety of biological fluids in comparison to traditional ODN analogues. The synthetic amide bonds in the PNA backbone are highly resistant to degradation by nucleases, proteases and peptidases. Thus, PNA incubated in human serum (37°C, 120 min) is completely resistant to degradation (Demidov *et al.*, 1994) unlike phosphodiester or phosphorothioate ODN incubated under similar conditions (Wickstrom, 1986).

Initially, *in vitro* studies suggested that PNA sequences were impermeable to cellular membranes (Wittung *et al.*, 1995; Bonham *et al.*, 1995; Gray *et al.*, 1997). This finding fostered research into conjugating PNA sequences to molecules known to facilitate physical or receptor-mediated cell uptake (Ray & Norden, 2000). However, the relevance of the initial findings of poor PNA uptake is in dispute. Firstly, it is now generally acknowledged that the uptake and efficacy of antisense ODN *in vivo* may not be adequately modeled by cell culture experiments (Myers & Dean, 2000). Secondly, recent studies have demonstrated the uptake of unmodified PNA sequences in rat cortical neurons (Aldrian-Herrada *et al.*, 1998), human myoblasts (Taylor *et al.*, 1997), lymphoma cells (Cutrona *et al.*, 2000) and prokaryotic cells (Good & Nielsen, 1998). Moreover, unmodified PNA sequences have been shown to have antisense effects *in vivo* following direct

administration into the brain (Tyler *et al.*, 1998; Fraser *et al.*, 2000b) and, in some cases, may even penetrate the blood-brain barrier (Tyler *et al.*, 1999, but see Pardridge *et al.*, 1995). In a direct comparison in the rat spinal cord, PNA coupled to a cellular transporter protein had similar antisense efficacy to the unmodified PNA sequence suggesting that modifications to improve cellular uptake may not be required for PNA applications *in vivo* (Pooga *et al.*, 1998; Rezaei *et al.*, 2001).

Unmodified PNA sequences inhibit protein expression by steric inhibition of mRNA processing rather than by RNase H mediated catalysis of target mRNA (Knudsen & Nielsen, 1996; Bonham *et al.*, 1995). However, various PNA analogues are currently in development that may support RNase H activity and thus potentially deliver improved antisense efficacy and potency. Modified PNA analogues such as 2', 5'-oligoadenylate-PNA conjugates are able to recruit RNase L (an endonuclease that degrades the targeted mRNA in the single-stranded region adjacent to the polyadenylate region; Torrence *et al.*, 1993) and appear to have potent antisense effects *in vitro* (Verheijen *et al.*, 1999). Alternatively, PNA-PDE chimeras have been developed that appear to maintain the high stability and affinity of PNA constructs while supporting RNase H-dependent cleavage of target RNA in *in vitro* assays (Uhlmann, 1998; Malchere *et al.*, 2000).

In contrast to other antisense reagents, PNA is based on peptide chemistry rather than nucleotide chemistry (Figure 2.2). This offers significant advantage for the continued development of PNA molecules. Firstly, PNA molecules can by synthesized efficiently and economically by conventional Boc or Fmoc-type solid-phase peptide synthesis. Secondly, unlike other antisense reagents, medicinal chemistry can be performed on PNA oligomers that may permit the optimization of their pharmacokinetic and pharmacodynamic properties (Nielsen *et al.*, 1998).

2.1.2.2 ODN & PNA design

Antisense ODNs and PNA capitalize on the principle of Watson-Crick base pairing to specifically hybridize to target mRNA sequences. Complementary nucleotide base pairs (adenine-thymine, guanine-cytosine) have a natural affinity for selective hybridization as a consequence of hydrogen bonding and the reduction in entropy resulting from coplanar base stacking in the double helix formed from strand hybridization (Crooke, 1993). Antisense ODNs and PNA bind to target mRNA in a competitive and reversible manner consistent with traditional receptor theory. However, target complementarity is merely one of many components that require consideration in the optimal design of antisense oligomers (Agrawal & Kandimalla, 2000). Other features of antisense sequence design are discussed below.

ODN & PNA Length

ODN length is an important determinant of the affinity and specificity of antisense ODNs. Statistical analysis suggests that antisense ODNs must be longer than 12-14 nucleotides in order to be uniquely complementary to a target mRNA within the vertebrate RNA pool (Woolf *et al.*, 1992). However, overall hybridization affinity also increases as a function of ODN length and, as a consequence, long ODNs are more inclined to hybridize with mismatched mRNA sequences (Herschlag, 1991; Flanagan *et al.*, 1996). Thus, the choice of ODN length is a compromise between optimizing hybridization affinity and sequence-specificity. Accordingly, phosphodiester and phosphorothioate ODNs are generally used at between 18-20 nucleotides (Dean *et al.*, 1996), whereas peptide nucleic acid (PNA) sequences can be used at shorter lengths, 12-18 nucleotides, because of their higher hybridization affinity (Tyler *et al.*, 1998; Fraser *et al.*, 2000b; Doyle *et al.*, 2001).

Target Accessibility

Hybridization affinity and specificity are a function of the accessibility of the target mRNA sequences to ODN binding. Messenger RNA (mRNA) possess a complex secondary structure that makes it difficult to accurately predict which target sites will be most accessible for hybridization. Secondary and tertiary structures within the flanking and distal regions of the target mRNA significantly influence ODN hybridization (Vickers *et al.*, 2000; Lima *et al.*, 1992; Rittner *et al.*, 1993). Thus, it has been proposed that specificity can be optimized by targeting short ODNs (i.e. ODNs less than 14 nucleotides in length) to regions of target mRNA particularly susceptible to ODN hybridization (Wagner *et al.*, 1996). However, an understanding of which RNA structures optimally bind ODNs has not yet been established. Cell-based assays suggest that ODN targeted to the AUG initiation codon demonstrate superior efficacy in inhibiting protein expression (Crooke, 1999; Knudsen & Nielsen, 1996). The secondary and tertiary structure of this site permits the interaction between the ribosome and mRNA for translation and thus may facilitate the hybridization of the antisense ODN as well. Thus, the initial antisense experiments *in vivo* were performed with ODNs designed to hybridize with a region of target mRNA either flanking or in close proximity to the AUG initiation codon (Wahlestedt *et al.*, 1993b; Wahlestedt *et al.*, 1993a). In particular, ODN analogues such as PNA that exert antisense effects by translational arrest (Figure 2.1) appear most effective when targeted to sites in close proximity to the initiation codon (Doyle *et al.*, 2001; Mologni *et al.*, 1998). However, it has been recently demonstrated that PNA sequences, but not the corresponding sequences of ODN analogues that do not support RnaseH activity (i.e. 2'-O-methyl and phosphoramidate ODN), can hybridize at

sites downstream and distant from the initiation codon and cause translational arrest (Dias *et al.*, 1999).

Recent hybridization studies performed with phosphorothioate (PS) analogues suggest that regions of mRNA throughout the transcript are accessible to ODN binding (Wagner *et al.*, 1993; Laptev *et al.*, 1994; Vickers *et al.*, 2000). The antisense activity of PS ODNs at sites downstream and distant from the initiation codon appears to occur by RNase H-mediated degradation of the target mRNA (Monia *et al.*, 1998).

ODNs targeted to different regions of the same mRNA transcript will have a range of activities (Dean *et al.*, 1996; Vickers *et al.*, 2000). Generally, only 10-15% of phosphorothioate ODN sequences are effective antisense agents in cell-based assays, depending on the nature of the target mRNA (Cooper *et al.*, 1999). A computational approach using thermodynamic indices suggests that the duplex formation energy for the ODN and mRNA target region is the most consistent predictor of ODN efficacy (Stull *et al.*, 1992). However, a general rule for selecting the most effective ODN sequences has not yet emerged. Thus, other ODN selection methods have recently been proposed. These are based on the screening of large pools of combinatorial ODN constructs that direct RNase H cleavage of mRNA accessible regions (Lima *et al.*, 1997a; Monia *et al.*, 1998), the mapping of RNA-accessible sites with ODN libraries (Ho *et al.*, 1998), or the binding of radiolabeled mRNA transcripts to ODN arrays immobilized on a solid support (Milner *et al.*, 1997). However, many of these ODN screening assays do not use full-length target mRNA sequences (which could affect secondary structure) nor do they account for interactions between target mRNA and cellular proteins that may occur in vivo. Thus, the predictive value of in vitro screening methods requires further clarification (Cooper *et al.*, 1999; Crooke, 1999) despite some examples of excellent correlation between ODN effects *in vitro* and *in vivo* (Monia *et al.*, 1996a; Ho *et al.*, 1998).

2.1.2.3 Treatment Paradigm

Antisense inhibition of gene expression often leads to phenotypic changes that persist for the duration of the antisense treatment. The time required to observe the antisense effects appears to be dependent upon the nature of the target protein. For example, antisense effects *in vivo* have been measured on the order of a few hours following ODN treatment directed towards G-protein α-subunits (Stone *et al.*, 1995) and immediate-early gene products such as *c-fos* or *c-jun* (Chiasson *et al.*, 1992; Heilig *et al.*, 1993). In comparison, antisense treatment of three to seven days is typically required to effectively decrease G-protein coupled receptor function (for review,

see Weiss *et al.*, 1997). These differences presumably reflect the turnover rate of the target protein.

ODN & PNA Pharmacokinetics

Nuclease resistant antisense agents such as phosphorothioates, methylphosphonates, 2'-O-allyl ODNs and peptide nucleic acids can be used to inhibit gene expression at peripheral sites (Crooke, 1999). A pharmacokinetic profile has been established for phosphorothioate ODNs and PNA. The terminal half-lives of phosphorothioate ODNs have been measured to be between 24-60 hours in mice, rats, monkeys and humans (Agrawal, 1996; Crooke *et al.*, 1994). There are numerous examples of phosphorothioate ODNs effectively inhibiting the expression of peripheral target proteins *in vivo* (Dean *et al.*, 1994; Hijiya *et al.*, 1994; Skorski *et al.*, 1994; Akhtar & Agrawal, 1997). Radiolabelling techniques indicate that these compounds have almost complete systemic bioavailability with the exception of very low distribution to the brain and other sites in the central nervous system. Similarly, initial pharmacokinetic data for PNA following intravenous administration to mice suggest that these molecules have good systemic bioavailability and a half-life of 2-3 hours (Nielsen, 2001).

Generally, ODN analogues are prevented from permeating the blood-brain barrier by their size and charge, unless they are conjugated to a vector-mediated drug delivery system (Pardridge *et al.*, 1995; Wu *et al.*, 1996; Penichet *et al.*, 1999). The only reported exception is for unmodified PNA oligomers having effects in the brain following intra-peritoneal administration (Tyler *et al.*, 1999). Thus, antisense ODNs are generally administered directly into the brain or spinal cord to evaluate the function of proteins expressed in the central nervous system. Local administration of antisense ODNs permits the study of gene function in specific brain regions (Wahlestedt, 1994) although investigators should be aware that ODN uptake might not be equivalent in all target tissues or cell types (Yee *et al.*, 1994; Yaida & Nowak, 1995; Szklarczyk & Kaczmarek, 1997).

The poor stability of phosphodiester ODN following systemic administration precludes the use of these agents for inhibiting the expression of peripheral targets (Sands *et al.*, 1994). However, phosphodiester ODNs are sufficiently stable in cerebrospinal fluid to be effective following direct administration into the CNS (Whitesell *et al.*, 1993; Yee *et al.*, 1994; Szklarczyk & Kaczmarek, 1997). The expression of a number of central target proteins were significantly reduced and phenotype significantly altered in rodents following daily central administration of antisense phosphodiester ODNs (Wahlestedt *et al.*, 1993b; Wahlestedt *et al.*, 1993a; Weiss *et al.*, 1997).

Cell Uptake

ODN uptake has been studied extensively in cell culture-based assays. Uncharged ODNs such as methylphosphonates or PNA are internalized mainly by fluid-phase endocytosis (Shoji *et al.*, 1991). In comparison, negatively charged phosphodiester and phosphorothiate ODNs are internalized by both fluid-phase endocytosis and receptor-mediated endocytosis (Yakubov *et al.*, 1989; Gao *et al.*, 1993; Beltinger *et al.*, 1995). Receptor-mediated endocytosis is the more efficient mechanism and, in consequence, charged ODNs demonstrate higher levels of uptake than uncharged ODNs (Gray *et al.*, 1997). Uptake efficiency is influenced by a number of factors including ODN concentration, sequence, chemical class and the phase of the cell cycle (Temsamani *et al.*, 1994; Gray *et al.*, 1997). In addition, the efficiency of ODN uptake appears to be inversely correlated with ODN length (Loke *et al.*, 1989). However, there is poor correlation between cell uptake *in vivo* and in cell culture-based assays suggesting that ODN uptake *in vivo* may invoke different mechanisms (Myers & Dean, 2000).

2.1.2.4 Identification of target-specific effects

The range of non-specific effects inherent to ODNs makes it imperative that certain controls are implemented in order to determine whether an antisense mechanism is responsible for the observed response (Stein & Krieg, 1994; Eckstein *et al.*, 1996). The fast kinetics of RNase H cleavage imply that the transient hybridization of phosphodiester or phosphorothioate ODNs to non-target mRNAs may produce non-specific inhibition of protein expression (Stein, 2000). To exemplify this point, RNase H has equal affinity and similar rates of cleavage for single-mismatch and fully complementary sequences (Lima & Crooke, 1997b). Accordingly, antisense phosphorothioate ODNs reduce target expression only 3-5 fold more potently than mismatch controls in cell-based assays (Bennett *et al.*, 1994). In comparison, ODN analogues that inhibit target protein expression by RNase H-*independent* mechanisms are less susceptible to sequence-dependent non-specific effects. This is because target protein inhibition is dependent upon the stability of the ODN/mRNA heteroduplex and ODN hybridization to mismatched mRNA sequences typically results in complexes with short half-lives (Johansson *et al.*, 1994).

Sequence-independent non-specific effects are also encountered with phosphorothioate and other highly-charged ODN analogues. The polyanionic backbone of these ODNs promotes their binding to a variety of DNA-binding proteins such as other polyanions (e.g. heparin), transcription factors, growth factors, cellular enzymes and extra-cellular proteins (Stein, 1996). Additionally, the intrinsic activity of nucleoside and nucleotide degradation products from

phosphorothioate ODNs may affect cell proliferation and differentiation (Rathbone *et al.*, 1992; Kamano *et al.*, 1992).

Relating phenotypic changes to inhibition of protein expression

Control studies should focus initially on confirming that changes in phenotype are the result of an antisense-mediated effect. A key measure in this regard is to correlate phenotypic changes with decreases in target protein expression (Weiss *et al.*, 1997). Western blots or ligand binding techniques are often useful assays in this regard. Nonetheless, antisense treatment may be functionally silent in biological systems that have a great deal of spare capacity or redundancy in spite of significant changes in protein expression (Adams *et al.*, 1996).

Conversely, it is commonly found that a profound change in phenotype is associated with a small (i.e. <20%) or undetectable decrease in receptor B_{max} (Neumann *et al.*, 2000; Adams *et al.*, 1996; Bilsky *et al.*, 1996; Shah *et al.*, 1997). In this case, phenotypic changes may not correlate with *in vitro* measures of ODN activity because protein assays performed on whole tissue samples may dilute any highly restricted decreases in protein expression due to antisense treatment (Grzanna *et al.*, 1998). Alternatively, it has been proposed that only newly synthesized receptors are active, and thus the inhibition of new receptor synthesis by antisense treatment causes significant changes to phenotype in spite of the small decrease in total receptor B_{max} (Qin *et al.*, 1995; Kalra *et al.*, 1995; Hua *et al.*, 1998). In this regard, bioassays measuring receptor activation may be more relevant than binding assays in corroborating antisense effects *in vivo* (Fraser *et al.*, 2000b).

Sequence- and target-specificity

It is also important to demonstrate that ODN effects are highly selective for the target mRNA sequence. Sequence-specificity should be evaluated by testing mismatch, sense or scrambled (i.e. random) ODNs in parallel with the antisense sequence. Mismatch ODNs are constructed by reversing the order of a few pairs of nucleotide bases within the antisense sequence thereby maintaining the base composition and structural features of the antisense ODN as much as possible. In this regard, the fewer the mismatches, the more rigorous the control. Mismatch ODNs share the closest identity with the antisense ODN and thus provide more stringent controls than sense or scrambled sequences. Each mismatched base pair can correspond to an approximate 500-fold decrease in hybridization affinity for the target mRNA based on the change in Gibbs free energy for hybridization (Freier *et al.*, 1992).

Target-specificity can be evaluated by assaying the expression of related proteins with comparable half-lives, such as alternate subytpes of the same receptor family. The inability of the active ODN to reduce the expression of similar gene products supports two important conclusions; ODN treatment does not cause a non-specific inhibition of protein expression in the target tissue, and phenotypic changes can be directly correlated with a change in target protein expression.

2.1.3 Concluding Remarks

Antisense technology is a useful technique for determining gene function, as long as the limitations imposed by incomplete knockdown and target specificity are recognized and appropriate control studies are carried out. The power of antisense technology lies in the fact that antisense techniques can be performed in any species or accessible target tissue during any stage of development. The acute nature of antisense knockdown, in comparison to gene knockout techniques, minimizes the development of any compensatory mechanisms in response to the manipulation. The reversibility of antisense treatment allows animals to be used as their own controls following recovery of the target protein. Antisense technology is the quickest, most inexpensive method for determining gene function *in vivo*.

2.2 Homologous Recombination "Knockout" Techniques

Knockout techniques are becoming increasingly popular as they provide for a complete and specific elimination of target gene expression (Silva *et al.*, 1992a; Silva *et al.*, 1992b). Briefly, gene knockout technology refers to the irreversible disruption of target genes by homologous recombination in mouse embryonic stem cells (Capecchi, 1989; see Figure 2.3). These manipulated stem cells are then injected into blastocysts and implanted into foster mothers in order to establish a strain of mice deficient in the targeted gene. Within the opioid field, knockouts have been performed against preproenkephalin (König *et al.*, 1996) and the μ, κ and δ opioid receptors (for review, see Kieffer, 1999).

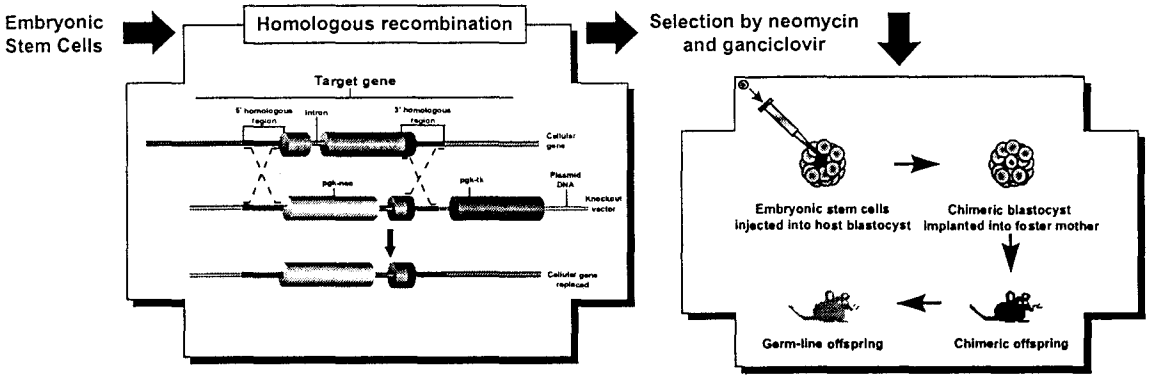

Figure 2.3 A schematic representation of the homologous recombination knockout technique. (Figure represented from (Fraser & Wahlestedt, 1997b).)

It is necessary to manipulate the genome of knockout mice at the earliest embryonic stages of development for all cells to inherit the knockout of the target gene. However, elimination of the target gene at this stage of development may alter the expression of other genes or affect various developmental programs (Routtenberg, 1995; Plomin *et al.*, 1994). Additionally, changes may occur throughout the course of development to compensate for the loss of the target gene (Giros *et al.*, 1996). As a consequence of these processes, some knockout mice strains may be inappropriate for study because changes in phenotype may not be directly related to target gene function (Routtenberg, 1995). Additionally, if manipulation of a target gene seriously impacts development, the appearance of a lethal phenotype precludes the study of adult mice (Copp, 1995). Finally, the gross disruption of the target gene in all cells of the mouse makes it difficult to determine tissue-specific changes in phenotype and thus establish tissue-specific gene function (Gu *et al.*, 1994; Tsien *et al.*, 1996).

There are examples of surprising inconsistencies in mice knockouts for a common gene. For instance, epidermal growth factor receptor knockout in three different strains of mice gave three unique phenotypes (Threadgill *et al.*, 1995). This and similar findings imply that the phenotypic effect of a knockout is contingent upon the genetic background of the strain and consequently suggests that it is invalid to use strain-specific knockout mice as a model to describe the general function of a target gene across species. This is an issue in transgenic studies of pain where the 129 mouse strain used as a source of embryonic stem cells and the recipient C57BL/6 strain have significantly different sensitivities in antinociceptive, hyperalgesic and allodynic pain models as well as different sensitivities to various analgesic compounds (Lariviere *et al.*, 2001). Thus, any differential phenotypes may not be directly related to the knocked-out gene, but rather arise from

the expression of polymorphism as a consequence of the hybrid genetic background of transgenic mice (Gerlai, 1996). Furthermore, inbreeding may cause a genetic shift between the knockout and wild-type strains that further limits the use of wild-type mice as controls.

A second example of knockout inconsistencies is the finding that three independent knockouts of the myogenic basic-helix-loop-helix (bHLH) gene *MRF4* also gave three unique phenotypes (Olson *et al.*, 1996). In this case the disparity could not be due to genetic background as all knockout mice were derived from a common strain. Instead, the source of phenotypic variation may be that the genetic manipulation was targeted to slightly different sites within the *MRF4* gene and that there was some crossover effect on adjacent genes. Perhaps these inconsistencies can be overcome as the knockout technique matures.

Recent developments give hope that some of these concerns may be circumvented. For example, a strategy for conditional, cell-type specific knockout of target genes has been introduced (Gu *et al.*, 1994). This technique makes use of the *Cre/loxP* recombination system of bacteriophage P_1 where *Cre* recombinase catalyzes recombination between *loxP* recognition sequences (Sauer & Hendersen, 1988). Briefly, *loxP* sites are inserted to flank the target gene in embryonic stem cells and a strain of mice is established. It is imperative that the insertions of the *loxP* sites do not have any impact on phenotype. The *loxP* strain is then crossed with a second transgenic strain of mice expressing *Cre* recombinase under the control of a tissue-type or cell-type specific promoter. Progeny homozygous for the *loxP* manipulation and expressing the *Cre* transgene are susceptible to target gene deletion in tissues expressing the *Cre* recombinase promoter. This technique has been used to study DNA polymerase β gene function in T cells (Gu *et al.*, 1994) and NMDA receptor 1 gene function in mouse forebrain (Tsien *et al.*, 1996; Wilson & Tonegawa, 1997).

The *Cre/loxP* system can also be used to establish inducible knockouts (Kühn *et al.*, 1995; Sauer, 1998). In this case, *Cre* recombinase is expressed in mice under the control of an inducible promoter that can be activated at the discretion of the investigator. Cell-type specific and temporal controls have been combined by expression of a chimeric protein consisting of a fusion between Cre and a mutated receptor binding domain under the control of tissue-specific promoters and activated by exposure to agonist (Schwenk *et al.*, 1995; Kellendonk *et al.*, 1999). Similarly, spatial and temporal control of DNA recombination can be achieved by local administration of adenovirus expressing Cre (Wang *et al.*, 1996; Burcin *et al.*, 1999). The evolution of knockout techniques now allows researchers to induce gene knockouts in post-

developmental stages in specific tissues to reduce the probability of lethal phenotypes and limit the appearance of compensatory mechanisms.

2.3 Closing Remarks

Antisense knockdown and homologous recombination knockout techniques offer two excellent choices for studying gene function *in vivo* (Table 2.1). Advances in ODN chemistry provide hope that increased target specificity and more complete knockdown can be achieved with antisense techniques. Antisense technology provides an assay for gene function with significantly higher throughput than knockout techniques and thus may be the more useful choice for screening the volume of novel gene clones that will be sequenced over the coming decade.

Knockout techniques are also in the process of being refined. Temporal, spatial and cell type-specific knockouts offer the most exact method for studying gene function although the difficulty of the method and the necessary investment of time and labor limit their mainstream use. The precision of knockout techniques supports their use in situations where the incomplete knockdown by antisense treatment in combination with a lack of selective ligands does not provide enough information to positively describe gene function.

3 Aim of the Thesis

The recent cloning of the δ opioid receptor and the coincident identification of SNC80 as its first selective, non-peptide agonist has spurred interest in the δ receptor as a biological target for the development of novel analgesics. However, at the time that this thesis work was initiated, δ opioid receptor pharmacology was largely uncharacterized due, in part, to a lack of selective pharmacological agents. Thus, the primary objective of this doctoral thesis is to characterize the function of the cloned δ opioid receptor in the rat brain using pharmacological and antisense techniques. The purpose of this research is to investigate the cloned δ opioid receptor, as opposed to an alternate δ receptor subtype, as a viable target for the development of novel analgesics. A secondary objective of this thesis is to establish a pharmacological profile for SNC80. SNC80 is a template for the design of non-peptidic δ agonists and, as such, it is key that the pharmacological profile of SNC80 is compared to that of the prototypical, peptidic δ agonists, deltorphin II and DPDPE. A final objective of this thesis is to demonstrate the use of peptide nucleic acids (PNA) as antisense agents for the determination of gene function *in vivo*. PNA have distinct chemical properties that may provide significant advantages over alternate DNA analogues with regard to their potential either as a tool for functional genomics or as a novel platform for antisense drug development.

Original Research Articles

4 Characterization of [^{125}I]AR-M100613, a high affinity radioligand for δ opioid receptors

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4.1 Preface

As described in section 2.1.2.4, behavioural changes associated with antisense treatment should be correlated with a demonstration of decreased target protein expression or function *in vitro*. This can be achieved by saturation binding and the demonstration of a lower target protein B_{max} in the tissue of interest. However, antisense treatment often causes only a modest decrease in target receptor B_{max}. Thus, a saturation binding assay able to detect small changes in δ opioid receptor B_{max} is required to support the antisense studies described in this thesis.

The expression of δ opioid receptors in rat brain is well established. However, an accurate determination of δ opioid receptor B_{max} in rat brain membranes is compromised by the poor selectivity (Table 1.2), high non-specific binding, low specific activity and/or agonist nature of the currently available radioligands. This study presents the pharmacological characterization of [^{125}I]AR-M100613 and an evaluation of its potential as a radiochemical probe for labeling δ opioid receptors in rat brain membranes. This study also demonstrates that all δ selective ligands tested (SNC80, deltorphin II, DPDPE) potently displaced [^{125}I]AR-M100613 binding in a manner that did not reveal differential radioligand binding to putative δ receptor subtypes.

4.2 Abstract

AR-M100613 ([I]-Dmt-c[-D-Orn-2-Nal-D-Pro-D-Ala-]) is the iodinated analogue of a cyclic casomorphin previously shown to be a potent antagonist at the δ opioid receptor. Specific [^{125}I]AR-M100613 binding to rat whole brain membranes was saturable, reversible and best fit to a one-site model (K_d = 0.080 ± 0.008 nM, B_{max} = 45.2 ± 4.4 fmol/mg protein). [^{125}I]AR-M100613 binding was displaced with high affinity by the δ opioid receptor ligands SNC-80, Deltorphin II and DPDPE but not the μ or κ-selective receptor ligands DAMGO and U69593. Residual non-selective binding of [^{125}I]AR-M100613 to μ opioid receptors is blocked by the addition of CTOP to the assay buffer. [^{35}S]GTPγS binding assays indicate that AR-M100613 is a potent, selective and reversible antagonist for δ opioid receptors in rat brain membranes. The high affinity, high specific activity, low non-specific binding and antagonist profile of [^{125}I]AR-M100613 favor its use as a radiochemical probe for δ opioid receptors.

4.3 Introduction

Radioligand binding studies have played an essential role in advancing opiate receptor pharmacology since the first demonstration of a specific binding site for opiate drugs in brain membranes (Pert & Snyder, 1973; Simon *et al.*, 1973; Terenius, 1973). Subsequent development of both binding and bioassays complemented the initial *in vivo* studies (Martin *et al.*, 1976) and led to the postulation of three major opioid receptor subtypes: μ, κ and δ (Lord *et al.*, 1977). This initial classification has been confirmed by the recent cloning of distinct genes encoding each receptor (Chen *et al.*, 1993; Evans *et al.*, 1992; Kieffer, 1999; Yasuda *et al.*, 1993). Further receptor heterogeneity has been proposed within each major class of opiate receptor partly on the basis of the binding profiles of putatively selective ligands (for review see Fowler & Fraser, 1994).

A number of radioligands have been used to characterize δ opioid receptors in binding assays (Knapp & Yamamura, 1992). These include ^{3}H or ^{125}I-labeled analogues of the peptide agonists DPDPE (Knapp & Yamamura, 1990; Knapp *et al.*, 1991) and Deltorphin (Búzás *et al.*, 1992; Dupin *et al.*, 1991; Fang *et al.*, 1992; Nevin *et al.*, 1994). However, the binding of opioid agonist radioligands is sensitive to the G-protein coupled state of the receptor and thus susceptible to modulation by the presence of cations or guanine nucleotides in the binding buffer. Also, saturation binding with agonist radioligands may underestimate receptor B_{max} due to the low affinity of agonists for uncoupled receptors (Richardson *et al.*, 1992). Although the binding of antagonist radioligands is unaffected by changes in G-protein coupling, the low specific activity of the presently available tritiated δ antagonists [^{3}H]Naltrindole, [^{3}H]TIPP and [^{3}H]TIPPψ (Contreras *et al.*, 1993; Nevin *et al.*, 1993; Nevin *et al.*, 1995; Yamamura *et al.*, 1992) limits their use to tissues with high receptor expression.

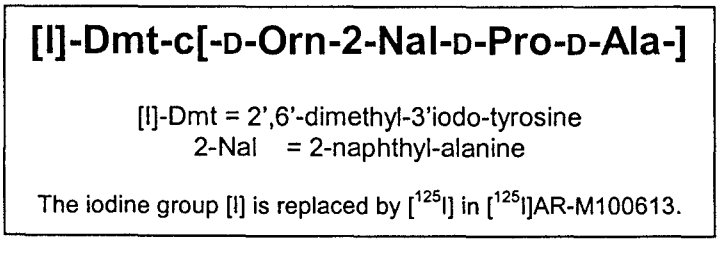


Figure 4.1 Structure of AR-M100613.

Recently, we have reported the synthesis of a number of cyclic casomorphin analogues with sufficient δ selectivity and antagonist potency *in vitro* (Schmidt *et al.*, 1994; Schmidt *et al.*, 1998) to warrant further investigation as ^{125}I-labeled radioligands for the δ opioid receptor. Here we present the characterization of [^{125}I]AR-M100613 (see Figure 4.1) binding to opioid receptors in rat brain membranes and demonstrate the utility of this potent antagonist as a radiochemical probe for δ opioid receptors.

4.4 Materials and Methods

4.4.1 Opioid Peptides and Drugs

AR-M100071 (Dmt-c[-D-Orn-2-Nal-D-Pro-D-Ala-]) and non-radioactive AR-M100613 were synthesized following published methods (Schmidt *et al.*, 1994). The opioid peptides used (i.e. DAMGO, CTOP, FK33824, Deltorphin II, and DPDPE) were purchased from BACHEM Bioscience (King of Prussia, PA). Diprenorphine, naloxone, naltrindole and U69593 were purchased from Research Biochemicals Inc. (Natick, MA). SNC-80 was purchased from Tocris Cookson Inc. (Ballwin, MO).

4.4.2 Chemicals

[^{35}S]GTPγS (specific activity, >1000 Ci/mmol), Na[^{125}I] (specific activity, 2200Ci/mmol) and [^{3}H]Naltrindole (specific activity, 34.7 Ci/mmol) were purchased from Dupont/New England Nuclear (Boston, MA). Tris, Hepes, bovine serum albumin (BSA), trifluoroacetic acid (TFA), ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), polyethylenimine (PEI), magnesium chloride, sucrose, dithiothreitol (DTT) and guanosine diphosphate (GDP) were purchased from the Sigma Chemical Co. (St. Louis, MO). Chloramine T and sodium metabisulfite were purchased from BDH Inc. (Toronto, ON). All other chemicals were obtained from commercial sources and were of analytical grade or better.

4.4.3 Preparation of Iodinated Radioligands

The peptides AR-M100071 (the uniodinated pre-cursor of AR-M100613), Deltorphin II, and FK33824 were dissolved in distilled water at a concentration of 10 mM. A volume of 1-2 µl of each peptide solution was transferred to a polypropylene centrifuge tube and diluted with 9 µl of 0.5 M phosphate buffer (pH 7.4). 1-2 mCi of Na[^{125}I] (i.e. 0.5-1nmole at 2200 Ci/mmole) was added to each peptide solution. The iodination reaction was initiated by the addition of 5 µl Chloramine T (2 mg/ml freshly prepared in 0.5 M phosphate buffer, pH 7.4) and the subsequent mixing of each peptide solution in the pipette tip for 1-2 minutes. The reaction was terminated by the addition of 20 µl sodium metabisulfite dissolved at 1 mg/ml in phosphate buffer or distilled water (in the case of FK33824). Each reaction mixture was diluted further by the addition of 100 µl distilled water and than purified by reverse-phase high performance liquid chromatography (BioRad Model 2800 HPLC) using a C18 analytical column (BioRad) eluted with a 20-minute linear gradient at 1 ml/min using an acetonitrile/TFA solvent system. Ultraviolet absorbance (214

nm - Pharmacia UVM-II) and radioactivity (Beckman Model 170 gamma monitor) were simultaneously recorded. The ^{125}I-labeled AR-M100613, Deltorphin II and FK33824 were purified to apparent homogeneity (2200 Ci/mmole) based on their elution 3-4 minutes after their non-iodinated precursors and prior to the di-iodinated product. The radioactive peaks corresponding to these mono-iodinated derivatives were positively identified by co-elution with their respective nonradioactive mono-iodopeptides. Aliquots of peptide radioligands were stored in solution at -20°C prior to use.

4.4.4 Membrane Preparation

Brain membrane binding studies were performed using tissue from male Sprague-Dawley rats (250-350g) supplied by Charles River Canada (St. Constant, QC). Rats were killed by decapitation and the whole brain (minus cerebellum) was rapidly removed on ice and stored at -70°C prior to preparation of tissue homogenates. On the day of homogenate preparation, brains were thawed and washed in 0.25 mM EDTA/0.5 M phosphate buffer solution (pH 7.4, 4°C). Brains were individually homogenized in a 20 ml solution of 50mM Tris buffer, 2.5 mM EDTA and 0.1 mM PMSF (pH 7.0). P_2 homogenate fractions were prepared following two consecutive low speed (1,200 x *g*) centrifugation steps and the collection and pooling of the subsequent supernatants. The supernatant was than centrifuged twice at 48,000 x *g* (20 minute for each spin) at 4°C. The P_2 pellet was resuspended in 50 mM Tris buffer (pH 7.4) and incubated at 37°C for 15 minutes to dissociate any receptor-bound endogenous opioid peptides. Membranes were centrifuged a third time at 48,000 x *g* as before and the final pellet was resuspended in 5 ml of 50 mM Tris buffer/0.32 M sucrose solution (pH 7.0). Protein content was determined by modified Lowry assay with sodium dodecyl sulfate (SDS) (3). Membrane aliquots were rapidly frozen in dry ice/ethanol and stored at -70°C until the day of the binding assay.

4.4.5 Measurement of [^{125}I]AR-M100613 stability

[^{125}I]AR-M100613 (0.68 nM) was incubated (1, 2, 4 and 6h, 22°C) in the presence or absence of rat brain membranes (80 µg/tube) in a solution of 50 mM Tris buffer, 3 mM $MgCl_2$ and 1 mg/ml bovine serum albumin (pH 7.4). The incubation was terminated by the addition of acetonitrile (300 µl, 4°C). Samples were then centrifuged at 4°C and the supernatants were collected for HPLC purification (performed as described above). [^{125}I]AR-M100613 stability was assessed by comparison of the radioactive peaks corresponding to [^{125}I]AR-M100613 incubated in the presence or absence of rat brain membranes.

4.4.6 Receptor Binding Assays

Saturation, competition and kinetic binding experiments were performed in a solution of 50 mM Tris buffer, 3 mM $MgCl_2$ and 1 mg/ml bovine serum albumin (pH 7.4). Radioligand, test compounds and membranes (final protein concentration of 60-80μg/sample) were combined in a final assay volume of 300 μl. Total and non-specific binding values were measured for the saturation, competition and kinetic binding experiments. Total binding was measured in the absence of any inhibitor whereas non-specific binding was defined as residual binding in the presence of 10 μM naloxone. The time course for association was measured by the addition of [^{125}I]AR-M100613 (0.13nM) to membranes at different times (i.e. 0-240 minutes) prior to filtration. The time course of dissociation was determined from the addition of 30 nM diprenorphine at different times (i.e. 0-120 minutes) before filtration of membranes previously incubated for 2 hours with [^{125}I]AR-M100613. For saturation binding experiments, 10 different concentrations of [^{125}I]AR-M100613 in the range of 0.004-0.4 nM were added to membranes and incubated at room temperature (25°C) 4 hours prior to filtration. For competition binding experiments, [^{125}I]AR-M100613 (0.04-0.06 nM), [^{125}I]Deltorphin II (0.15-0.2 nM) and [^{125}I]FK33824 (0.09-0.12 nM) were incubated for not less than 2 hours in the presence of different concentrations of various test compounds. All samples were filtered through 0.1% PEI-treated GF/B glass fiber filter strips (Xymotech Bioscience, Montreal QC) on a 24-well Brandel Cell Harvester (Gaithersburg MD). The filtrates were washed three times with ice cold wash buffer (50 mM Tris, 3 mM $MgCl_2$, pH 7.0) before transfer of filter disks into 12 x 75 mm polypropylene tubes for γ counting (Packard Cobra II Auto-gamma Counter, Meridien CT.).

4.4.7 [^{35}S]GTPγS Binding Assay

The assay was adapted from published procedures (Lorenzen *et al.*, 1993; Traynor & Nahorski, 1995). Rat brain membranes were thawed at 37°C, cooled on ice, passed 3 times through a 25-gauge needle, and diluted to 50-150 μg/ml in GTP assay buffer: 50 mM Hepes (pH 7.4), 20 mM NaOH, 5mM $MgCl_2$, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% BSA, 120 μM guanosine diphosphate (GDP). Test substances, [^{35}S]GTPγS (final concentration of 0.14 - 0.17 nM) and rat brain membranes (30 μg protein/well) were combined in a 96 deep well microtitre plate in a final assay volume of 300 μl. In the experiment showing competitive antagonism of SNC-80 by AR-M100613, the various concentrations of SNC-80 assayed were pre-incubated (25°C) with AR-M100613 (10 nM) for 30 minutes prior to the addition of [^{35}S]GTPγS. IC_{50} values were determined for various opioid antagonists based on the inhibition of maximally effective (E_{max})

concentrations of SNC-80 (3 μM) or DAMGO (10 μM). In all experiments, basal [^{35}S]GTPγS binding to rat brain membranes was determined in the absence of any test substances. All samples were incubated for 1 hour at 25°C prior to vacuum-filtration (TOMTEC Harvester 96, Orange CT) through GF/B Unifilter plates (Packard, Meridien CT) pre-soaked for 1 hour in water. After filtration, Unifilter plates were washed three times with ice cold wash buffer (50 mM Tris, 5 mM $MgCl_2$, 50 mM NaCl, pH 7.0) and than dried in an oven (55°C) for 2 hours. MICROSCINT 20 (Packard, Meridien CT) scintillation fluid (50 μl) was added to each Unifilter plate well and [^{35}S]GTPγS binding was measured in the 2.9-100 KeV window of the TopCount Microplate Scintillation Counter (Packard, Meridien CT).

4.4.8 Data Analysis

All analyses were performed using Prism (version 2.01) from GraphPad Software (San Diego, CA) unless otherwise stated. Receptor binding data was interpreted using non-linear regression analyses appropriate either for saturation, association, dissociation or competition binding paradigms. Saturation binding experiments were analyzed with regard to ligand depletion as described in the GraphPad Prism Manual (Swillens, 1995). Binding profiles were best-fit to either a one-site or a two-site model and the increase of goodness of fit was assessed by an F ratio based on the extra sum of squares. E_{max} and IC_{50} values for ligands affecting [^{35}S]GTPγS binding were obtained from non-linear curve fitting based on a 4-parameter sigmoidal dose-response curve model.

4.5 Results

4.5.1 [^{125}I]AR-M100613 stability in the presence of rat brain membranes

[^{125}I]AR-M100613 was stable following incubation with rat brain membranes. HPLC analysis revealed less than a 1 % decrease of intact radioligand following a 1, 2, 4 and 6h incubation at room temperature (data not shown).

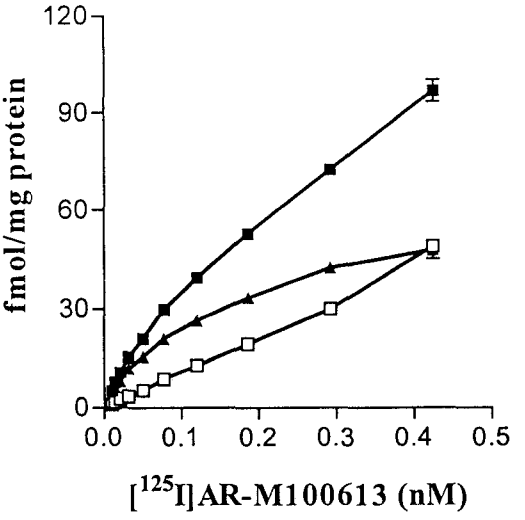
4.5.2 Saturation analysis of [^{125}I]AR-M100613 binding to rat brain membranes

Specific binding to rat brain membranes was saturable (Figure 4.2). Nonlinear regression analysis indicated a single class of binding sites with an apparent K_d value of 0.080 ± 0.008 nM and B_{max} of 45.2 ± 4.4 fmol/mg protein.

4.5.3 Kinetics of [^{125}I]AR-M100613 binding to rat brain membranes

Specific [^{125}I]AR-M100613 binding to rat brain membranes was time-dependent and reached steady-state at approximately 2 h (Figure 4.3A). [^{125}I]AR-M100613 binding was best fit to a one-phase model of association by non-linear regression analysis. The calculated association rate constant (k_1) was 0.126 min^{-1} nM^{-1}.

A] Saturation Isotherm



B] Scatchard Plot

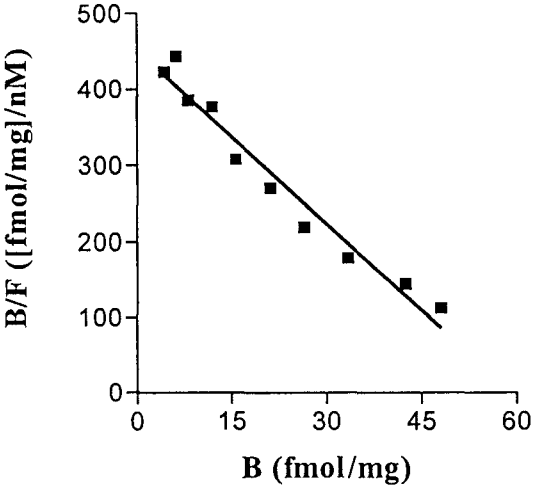
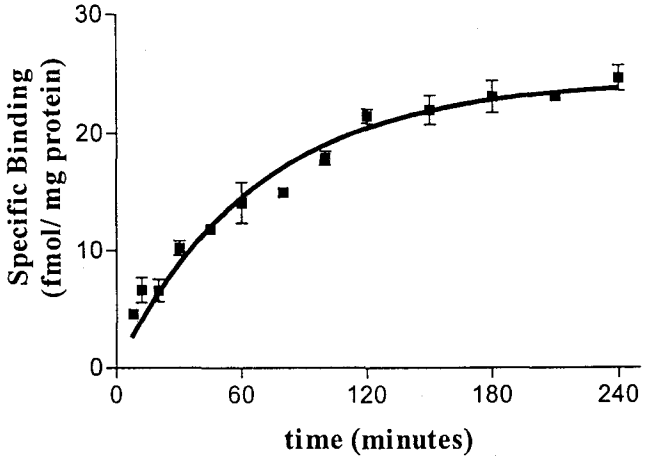


Figure 4.2 Saturation isotherm and Scatchard plot of [^{125}I]AR-M100613
Saturation isotherm [A] of [^{125}I]AR-M100613 binding to rat whole brain membranes. Specific binding (filled triangles) was obtained by subtracting, at each point, the nonspecific binding (open squares) from the total binding (filled squares). Each point represents the mean ± s.e.m. of triplet determinations from a single experiment. [B] Scatchard plot. The experiment was performed three times with a mean K_d = 0.080 ± 0.008 nM and B_{max} = 45.2 ± 4.4 fmol/mg protein.

A] Association Curve



B] Dissociation Curve

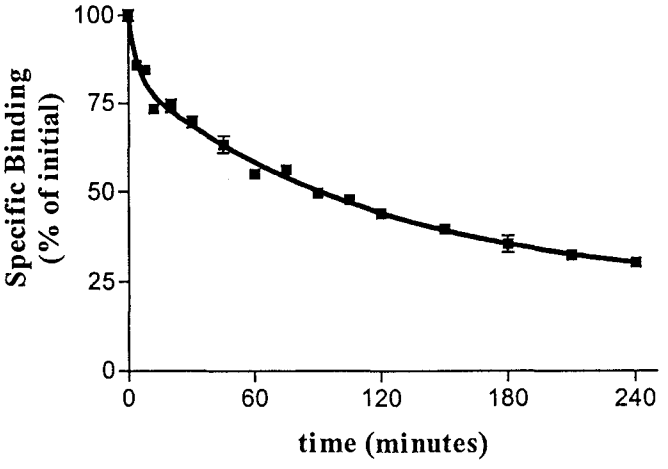


Figure 4.3 Time course of association and dissociation of 0.053 nM [^{125}I]AR-M100613 to rat whole brain membranes.

[A] Association was initiated by the addition of [^{125}I]AR-M100613 to membranes at different times before filtration. Each point represents the mean ± s.e.m. of triplicate samples from a single experiment. The experiment was replicated three times with similar results. [B] For dissociation studies, [^{125}I]AR-M100613 was allowed to associate as described in panel A for 3 hours prior to the addition of diprenorphine (60 nM) to prevent the rebinding of dissociated [^{125}I]AR-M100613. Dissociation was determined at various times after the addition of diprenorphine. Each point represents the mean ± s.e.m. of triplicate samples from a single experiment. The experiment was replicated three times with similar results. Data are presented as a percentage of the initial specific binding at equilibrium.

Specific [^{125}I]AR-M100613 binding dissociated in the presence of diprenorphine (Figure 4.3B). The kinetics of the dissociation of specific [^{125}I]AR-M100613 binding by excess diprenorphine was best fit to a two-phase model. The majority of the initial specific binding (~60%) was displaced in the initial phase of dissociation with a dissociation rate constant of k_{-1A} = 0.00839 min^{-1}. The slower phase of dissociation was determined to have a dissociation rate constant of k_{-1B} = 0.229 min^{-1}. The dissociation constant (K_d) determined from the ratio of k_{-1A}:k_1 was 0.067 nM which compares to an apparent K_d of 0.080 nM as determined from the saturation binding studies.

4.5.4 Comparison of the specific binding of [^{125}I]AR-M100613, [^{3}H]Naltrindole and [^{125}I]Deltorphin II to rat brain membranes

In order to compare the resolution of binding signal between [^{125}I]AR-M100613, [^{3}H]Naltrindole and [^{125}I]Deltorphin II, single point binding to rat brain membranes was performed with each radioligand at a concentration approximating its K_d value (Figure 4.4). Under these conditions, [^{125}I]AR-M100613 (0.78 nM) gave 6800 ± 170 dpm of specific binding where %specific binding/total binding (%SB/TB) was 70%. In comparison, [^{3}H]Naltrindole (0.15 nM) gave 160 ± 2 dpm of specific binding (%SB/TB = 63%) and [^{125}I]Deltorphin II (0.92 nM) gave 4300 ± 54 dpm of specific binding (%SB/TB = 55%).

The effect of cations and guanine nucleotides on specific [^{125}I]AR-M100613 and [^{125}I]Deltorphin II binding to rat brain membranes was also measured. Receptor binding performed in the presence of 100 mM NaCl and 120 μM GDP (i.e. the [^{35}S]GTPγS binding buffer) decreased specific [^{125}I]AR-M100613 (0.05 nM) and [^{125}I]Deltorphin II (0.46 nM) binding by 30% and 75% respectively in comparison to control binding performed in the opioid receptor binding buffer (data not shown).

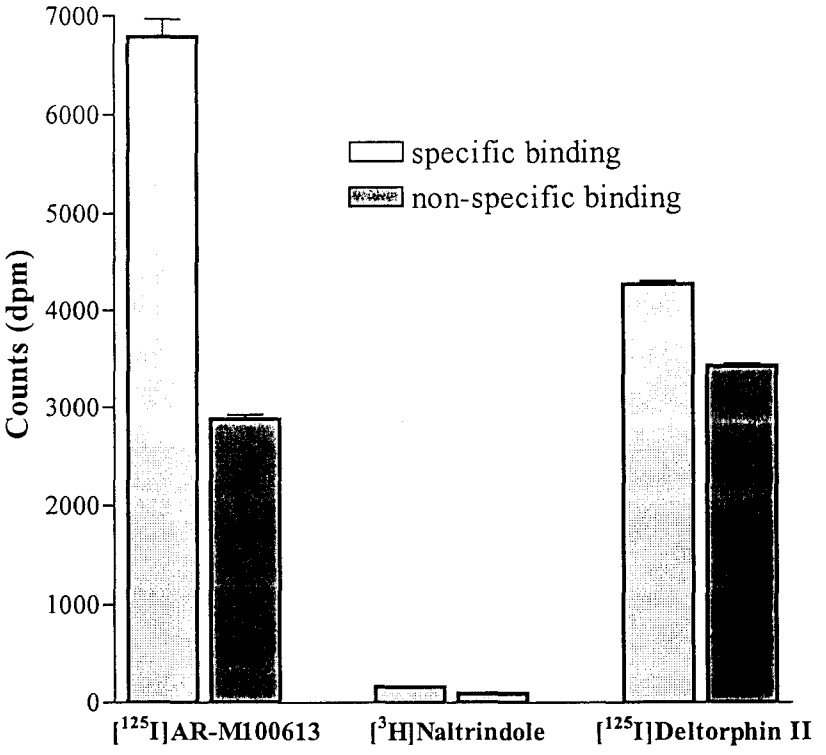


Figure 4.4 Resolution of signal from the binding of [^{125}I]AR-M100613 (0.078 nM), [^{3}H]Naltrindole (0.15 nM) and [^{125}I]Deltorphin II (0.92 nM) to rat whole brain membranes (70-80 μg protein/sample).

For comparison, each radioligand was used at a concentration approximating its K_d value. Specific binding (open bars) was determined by subtracting non-specific binding (shaded bars) from total binding (not shown). Each bar represents the mean ± s.e.m. of triplet determinations from three independent experiments.

4.5.5 Selectivity of AR-M100613 for μ and δ opioid receptor subtypes in rat brain membranes

The competition of specific [^{125}I]Deltorphin II (δ-subtype selective) and [^{125}I]FK33824 (μ-subtype selective) binding by AR-M100613 and a set of standard opioid ligands was studied in rat brain membranes. The K_i values and Hill coefficients (n_H) from these experiments are shown in Table 4.1. All competing ligands gave Hill coefficients with a value close to unity consistent with competition for a single class of binding sites. AR-M100613 was about 10-fold selective for the δ opioid receptor under these binding conditions ($K_{i\delta}$ = 0.160 ± 0.016 nM; $K_{i\mu}$ = 1.46 ± 0.11 nM). This is less δ-selective than naltrindole (70-fold) and SNC-80 (1100-fold). As expected, the μ agonist DAMGO demonstrated 650-fold selectivity for the μ opioid receptor in these assays.

Ligand	δ (*vs.* [^{125}I]Deltorphin II) $K_{i\delta}$ (nM)	n_H	μ (*vs.* [^{125}I]FK33824) $K_{i\mu}$ (nM)	n_H
AR-M100613	0.160 ± 0.016	1.03 ± 0.05	1.46 ± 0.11	0.88 ± 0.10
Naltrindole	0.081 ± 0.004	1.27 ± 0.14	5.66 ± 0.79	1.00 ± 0.03
SNC-80	0.429 ± 0.100	1.22 ± 0.13	471 ± 120	0.94 ± 0.03
DAMGO	177 ± 22	1.03 ± 0.08	0.270 ± 0.042	0.99 ± 0.04

Table 4.1 Selectivity of AR-M100613 for δ and μ opioid receptor binding sites in rat brain.

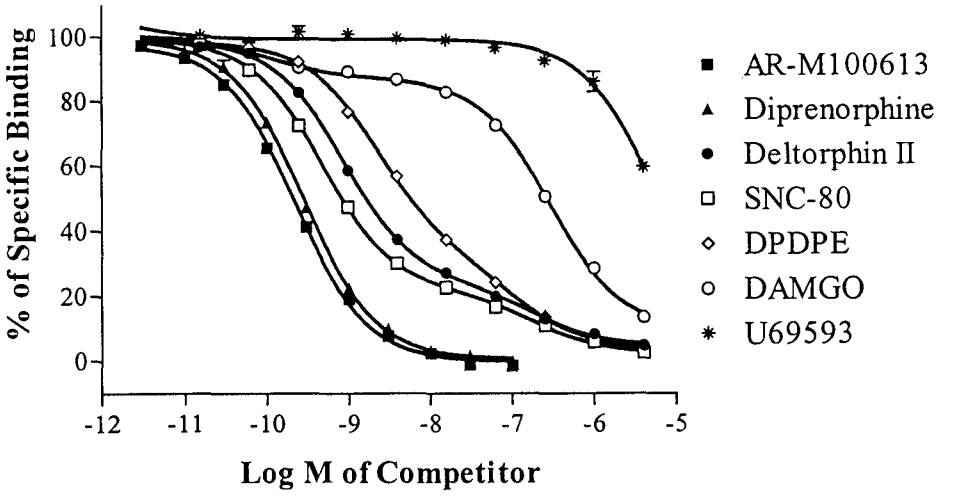
Competition binding assays were performed on membrane preparations. δ Opioid receptors were selectively labeled by [^{125}I]Deltorphin II (0.13-0.17 nM) and μ opioid receptors were selectively labeled by [^{125}I]FK33824 (0.10-0.16 nM). Individual binding experiments were performed using quadruplet samples. The data presented are the mean ± s.e.m. values determined from three experiments.

4.5.6 Inhibition of [^{125}I]AR-M100613 binding to rat brain membranes by various opioid receptor ligands

Specific [^{125}I]AR-M100613 binding to rat brain membranes was displaced by non-radioactive AR-M100613 and the non-selective opioid antagonist diprenorphine in a monophasic manner (Figure 4.5A & Table 4.2). Conversely, the opioid agonists Deltorphin II, SNC-80, DPDPE and DAMGO inhibited [^{125}I]AR-M100613 binding in a biphasic manner consistent with displacement of [^{125}I]AR-M100613 from multiple classes of receptor sites. However, when assayed in the presence of 50 nM CTOP, these agonists inhibited [^{125}I]AR-M100613 binding in a monophasic manner with the δ selective ligands Deltorphin II, SNC-80 and DPDPE inhibiting [^{125}I]AR-M100613 binding on the order of 200-fold more potently than that observed for the μ selective ligand DAMGO (Figure 4.5B & Table 4.2).

The κ opioid selective agonist U69593 did not displace [^{125}I]AR-M100613 at concentrations relevant to its affinity for the κ opioid receptor indicating undetectable levels of [^{125}I]AR-M100613 binding to κ receptors in rat brain membranes. With the exception of U69593 (for which only a partial curve was obtained over the concentration range tested), all other competing ligands inhibited [^{125}I]AR-M100613 binding to the same maximal level defined by 10 μM naloxone (non-specific binding) (Figure 4.5A).

A] Competition Curves



B] Competition Binding of [^{125}I]AR-M100613 with 50nm CTOP

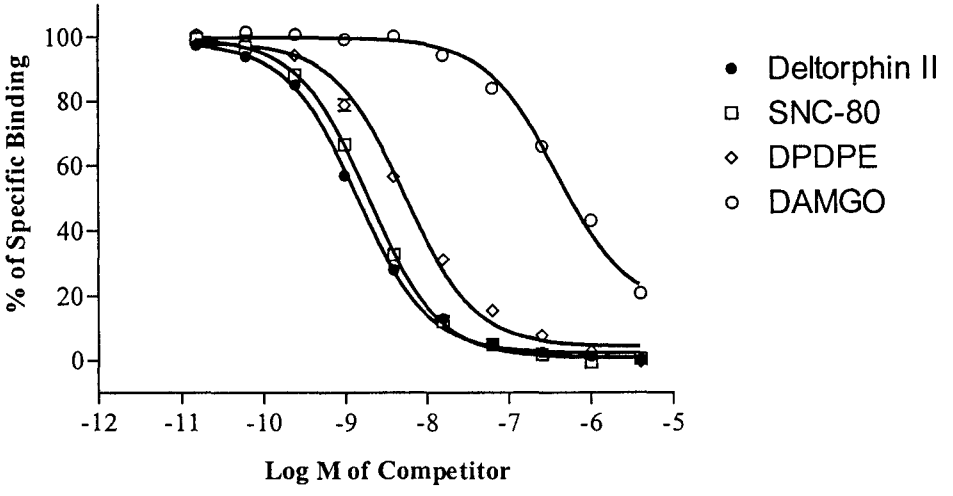


Figure 4.5 Inhibition of [^{125}I]AR-M100613 (0.04 - 0.06 nM) binding to rat whole brain membranes by varying concentrations of opioid agonists and antagonists.
Each point represents the mean ± s.e.m. of quadruplet determinations from a single experiment. [A] Competition curves for Deltorphin II, SNC-80, DPDPE and DAMGO were fit to a two-site model significantly better ($p<0.05$) than to a one-site model. The experiment was performed 5 or more times with similar results for each competing ligand. [B] Competition binding in the presence of 50 nM CTOP. Under these assay conditions, Deltorphin II, SNC-80, DPDPE and DAMGO inhibited [^{125}I]AR-M100613 binding in a monophasic manner. This experiment was performed three times with similar results for each competing ligand.

	K_{i1} (nM ± s.e.m.)	K_{i2} (nM ± s.e.m.)	%binding to high affinity site (% ± s.e.m.)	n
AR-M100613	0.140 ± 0.013	---	100	6
Diprenorphine	0.178 ± 0.009	---	100	5
Deltorphin II	0.538 ± 0.071	104 ± 38.6	76 ± 4	6
+CTOP	0.910 ± 0.086	---	100	3
SNC-80	0.312 ± 0.019	104 ± 36.5	74 ± 5	5
+CTOP	1.24 ± 0.16	---	100	3
DPDPE	1.62 ± 0.29	94.1 ± 28.2	61 ± 5	5
+CTOP	3.40 ± 0.32	---	100	3
DAMGO	0.243 ± 0.096	161 ± 17	14 ± 1	5
+CTOP	247 ± 20	---	100	3
U69593	>10000	n.m.	n.m.	5

Table 4.2. Inhibition of [^{125}I]AR-M100613 Binding to Rat Whole Brain Membranes by Opioid Receptor Ligands

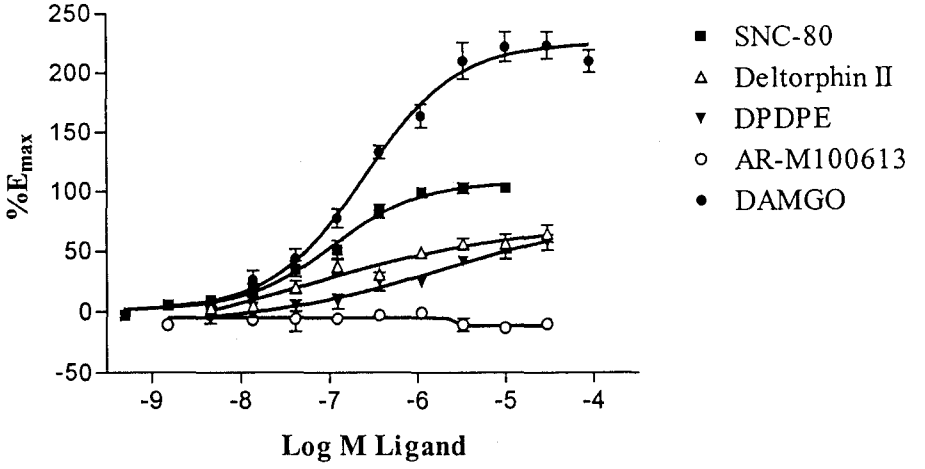
Note: Competitive inhibition of [^{125}I]AR-M100613 binding by AR-M100613 and diprenorphine was best fit to a one-site model. In addition, the competitive inhibition of [^{125}I]AR-M100613 binding by Deltorphin II, SNC-80, DPDPE and DAMGO was best fit to a one-site model when the experiment was conducted in the presence of 50 nM CTOP. n.m. denotes data values that were not measured.

4.5.7 Modulation of [^{35}S]GTPγS binding to rat brain membranes by AR-M100613

In this assay, $\%E_{max}$ values were determined relative to the maximal stimulation of [^{35}S]GTPγS binding in response to 10 μM SNC-80 and EC_{50} values were determined relative to the maximal effect of each particular ligand (Figure 4.6A). [^{35}S]GTPγS binding in rat brain membranes was stimulated by the δ agonists SNC-80 (E_{max} = 100%, EC_{50} = 107 nM), Deltorphin II (E_{max} = 64.3%, EC_{50} = 191 nM) and DPDPE (E_{max} = 64.6%, EC_{50} = 1480 nM) as well as the μ agonist DAMGO (E_{max} = 225%, EC_{50} = 252 nM). AR-M100613 did not stimulate [^{35}S]GTPγS binding within the dose range tested (Figure 4.6A). However, AR-M100613 (10 nM) shifted the agonist dose response curve of SNC-80 25-fold to the right without affecting $\%E_{max}$ suggesting that AR-M100613 is a competitive, reversible antagonist of SNC-80 mediated responses in rat brain

membranes (Figure 4.6B). Stimulation of [^{35}S]GTPγS binding by approximate E_{max} concentrations of the δ selective agonist SNC-80 (3 μM) and the μ selective agonist DAMGO (10 μM) were inhibited by a range of concentrations of the opioid antagonists AR-M100613, naltrindole, naloxone and CTOP. The K_e values from these inhibition curves are presented in Table 4.3. AR-M100613 was 72-fold more potent in inhibiting δ rather than μ mediated effects (based upon comparison of the relative K_e values). This compares with a δ:μ selectivity ratio of 48-fold for the δ selective antagonist naltrindole. Conversely, the standard opioid antagonist naloxone was about 6.4-fold selective for the μ receptor in this assay. The μ selective antagonist CTOP did not show any effective inhibition of SNC-80 mediated stimulation of [^{35}S]GTPγS binding within the dose range tested and is thus more than 850-fold selective for the μ receptor over the δ receptor.

A] Concentration-dependent stimulation of [^{35}S]GTPγS binding



B] Competitive antagonism of SNC-80 stimulated [^{35}S]GTPγS binding

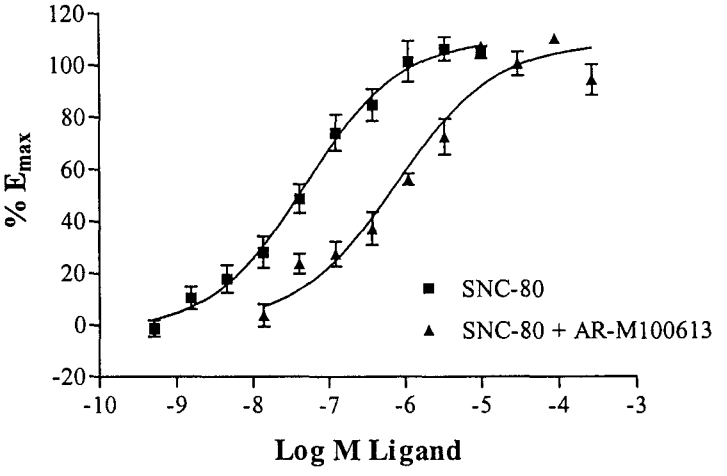


Figure 4.6 Modulation of [^{35}S]GTPγS (0.14 - 0.17 nM) binding to rat brain membranes in response to various opioid ligands.

[A] Concentration-dependent stimulation of [^{35}S]GTPγS binding by opioid agonists. %E$_{max}$ values were determined relative to the maximal stimulation of [^{35}S]GTPγS binding in response to SNC-80. In these experiments, average basal and SNC-80 (E$_{max}$) stimulated [^{35}S]GTPγS binding were 2140 cpm and 2900 cpm respectively. Each data point represents the mean ± s.e.m. from three individual experiments performed in duplicate. [B] Competitive antagonism of SNC-80 stimulated [^{35}S]GTPγS binding by AR-M100613 (10 nM). SNC-80 and AR-M100613 were pre-incubated (25°C) with membranes in the presence of 120 μM GDP for 30 minutes prior to the addition of [^{35}S]GTPγS. Average basal and SNC-80 (E$_{max}$) stimulated [^{35}S]GTPγS binding were 4840 cpm and 6530 cpm respectively in these experiments. Each data point represents mean ± s.e.m. from three individual experiments performed in duplicate.

	δ K_e (nM) (inhibition of SNC-80)	μ K_e (nM) (inhibition of DAMGO)
AR-M100613	0.861 ± 0.083	62.4 ± 9.1
Naltrindole	0.118 ± 0.012	5.75 ± 0.71
Naloxone	23.0 ± 4.0	3.59 ± 0.39
CTOP	>3,440	3.96 ± 0.40

Table 4.3 Antagonism of [^{35}S]GTPγS binding to rat brain membranes induced by δ and μ opioid receptor agonists.

Various opioid antagonists effectively inhibited [^{35}S]GTPγS (0.14 - 0.17 nM) binding to rat brain membranes induced by either SNC-80 (3 μM) or DAMGO (10 μM). CTOP did not significantly inhibit SNC-80 induced [^{35}S]GTPγS binding within the dose range tested. In all other cases, these antagonists maximally inhibited agonist-stimulated [^{35}S]GTPγS binding by approximately 100%. Data presented are the K_e values (mean ± s.e.m.) determined from analysis of the data from three individual experiments performed in duplicate.

4.6 Discussion

[^{125}I]AR-M100613 is a cyclic pentapeptide analog of β-casomorphin-5. Uniodinated analogues of [^{125}I]AR-M100613 have previously been shown to be potent and high affinity antagonists at the δ opioid receptor (Schmidt *et al.*, 1998). Similarly, the data presented here indicate that [^{125}I]AR-M100613 is a high affinity radioligand for δ opioid receptors with high specific activity and antagonist potency.

[^{125}I]AR-M100613 binding to rat brain membranes was reversible and saturable. The B_{max} determined for [^{125}I]AR-M100613 binding (45.2 ± 4.4 fmol/mg tissue) is consistent with previously reported B_{max} values in rat brain membranes determined using other δ selective radioligands (Yamamura *et al.*, 1992; Knapp *et al.*, 1991). The association kinetics of [^{125}I]AR-M100613 binding to rat brain membranes was best fit to a one-phase model. The dissociation kinetics of [^{125}I]AR-M100613 binding was best fit to a two-phase model. The initial phase of dissociation accounted for the majority of bound [^{125}I]AR-M100613 and occurred at a rate consistent with the apparent K_d measured in the saturation studies. The second, slower phase of dissociation likely reflects displacement of low affinity [^{125}I]AR-M100613 binding from μ opioid receptor sites.

[^{125}I]AR-M100613 gave a superior binding signal in comparison to other δ radioligands as a result of its high specific activity, low non-specific binding and antagonist profile. These properties favor its use in tissue preparations with low receptor expression. [^{125}I]AR-M100613 may have an additional application in anatomical studies performed by receptor autoradiography. ^{125}I-labeled radioligands are preferred for autoradiography studies because shorter film exposures can be used and differential quenching by tissue is not a concern, as it is with tritiated radioligands.

The large specific binding signal of [^{125}I]AR-M100613 permits its use at concentrations much lower than its K_d value. Although [^{125}I]AR-M100613 is only 10-fold selective for δ over μ opioid receptors, this radioligand predominantly labels δ receptors in rat brain membranes when used at concentrations less than its K_d. Thus, [^{125}I]AR-M100613 (0.04 - 0.06 nM) binding was inhibited about 200-fold more potently by the δ-selective ligands Deltorphin II, SNC-80 and DPDPE than the μ ligand DAMGO (Figure 4.5A). The selective labeling of δ sites is further improved by using [^{125}I]AR-M100613 in the presence of a selective μ antagonist such as CTOP (50 nM). Under these assay conditions, SNC-80, Deltorphin II and DPDPE inhibited [^{125}I]AR-M100613

binding in a manner consistent with displacement from a single binding site. The low affinity site revealed by the DAMGO inhibition of [^{125}I]AR-M100613 binding under these conditions is consistent with the concentration at which DAMGO begins to bind non-selectively to δ opioid receptors (refer to the DAMGO inhibition of [^{125}I]Deltorphin II binding presented in Table 4.1).

The existence of distinct δ opioid receptor subtypes has been postulated based, in part, on the pharmacology of the putative δ_2-subtype selective agonist Deltorphin II and the putative δ_1-subtype selective agonist DPDPE (Traynor & Elliot, 1993). However, the existence of distinct δ opioid receptor subtypes awaits confirmation as only a single δ opioid receptor (DOR) has been cloned to date (Evans *et al.*, 1992; Kieffer, 1999). Moreover, a recent study has demonstrated that [^{3}H]Deltorphin II, [^{3}H]DPDPE and [^{3}H]Naltrindole binding is not detectable in DOR-knockout mice (Zhu *et al.*, 1997). This finding precludes the existence of δ opioid receptor subtypes encoded by distinct genes but does not exclude the possibility that subtypes could exist arising from the common DOR gene. In any case, there is no indication that [^{125}I]AR-M100613 is δ opioid receptor subtype-preferring based on the monophasic nature of the saturation and competition (versus non-radioactive AR-M100613) binding curves in addition to the potent and complete inhibition of [^{125}I]AR-M100613 binding by both putative subtype-selective ligands, Deltorphin II and DPDPE.

The [^{35}S]GTPγS binding assay permits the measurement of the efficacy of ligands for G-protein coupled receptors (Traynor & Nahorski, 1995). The δ opioid agonists SNC-80, Deltorphin II and DPDPE as well as the μ agonist DAMGO all significantly increased [^{35}S]GTPγS binding in rat brain membranes. The higher maximal effect of SNC-80 in comparison to the peptide agonists Deltorphin II and DPDPE is consistent with previous reports (Clark *et al.*, 1997; Payza *et al.*, 1996).

In the absence of agonist, AR-M100613 did not alter [^{35}S]GTPγS binding in rat brain membranes. However, AR-M100613 did shift the SNC-80 concentration-response curve to the right without changing the $\%E_{max}$. This finding is consistent with AR-M100613 being a reversible antagonist at the δ opioid receptor.

In summary, the results of this study indicate that [^{125}I]AR-M100613 is a high affinity radioligand which can be used to label δ opioid receptors. The limitation imposed by the low selectivity of [^{125}I]AR-M100613 for δ over μ opioid receptors (which can be alleviated by the addition of a

selective μ opioid receptor antagonist, such as CTOP, to the assay buffer) should be considered together with the advantages that this radioligand offers. The high specific activity, low non-specific binding and antagonist profile of [^{125}I]AR-M100613 give it a significant advantage over other radioligands as a probe to label tissues with low δ opioid receptor expression. The antagonist properties of [^{125}I]AR-M100613 result in monophasic saturation curves that simplify the quantitation of δ opioid receptor B_{max}. Moreover, the binding potencies of agonists can be determined with [^{125}I]AR-M100613 even when the δ opioid receptor is in the low affinity state as is the case in the [^{35}S]GTPγS assay. Thus, [^{125}I]AR-M100613 can be used to correlate agonist binding affinity with agonist potency in [^{35}S]GTPγS dose-response curves in order to determine the intrinsic activity of δ opioid ligands. In comparison, the use of tritiated δ antagonists (i.e. [^{3}H]Naltrindole) in saturation or binding experiments is limited by the poor resolution of signal as a consequence of the low specific activity of these radioligands. Thus, the high specific activity, binding characteristics and antagonist profile of [^{125}I]AR-M100613 support its use as a radiochemical probe for δ opioid receptors.

5 The effects of δ agonists on locomotor activity in habituated and non-habituated rats

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5.1 Preface

Supraspinal δ opioid receptors appear to have a role in the modulation of psychostimulant activity, as discussed in section 1.5.4 of this thesis. However, the effects of δ agonists appear to vary based on the prior habituation of the test subjects, the interval of behaviour measured and the dose used. Also, the non-peptide, SNC80, appears to differ from δ agonist peptides such as deltorphin II with regard to the stimulation of dopamine release in striatal pathways modulating psychostimulant activity. Thus, the objectives of the current study were to characterize the locomotor response to δ agonists in various test paradigms and to compare the effects of SNC80 with those of the prototypical δ agonist, deltorphin II. Apart from the scientific findings reported in the current chapter, another important outcome of this study was the optimization of assay conditions to measure the role of the cloned δ opioid receptor in modulating hyperlocomotor activity using antisense technology as presented in Chapter 8.

5.2 Summary

The effects of the δ agonists SNC80 and deltorphin II on ambulation and rearing activity were measured in habituated and non-habituated rats. SNC80 (30, 100, 200, 400 nmol, i.c.v.) and deltorphin II (3, 15, 30, 60 nmol, i.c.v.) induced similar, dose-dependent biphasic locomotor effects in non-habituated subjects. An initial decrease in exploratory activity was associated with anxiogenic signs such as pilo-erection, freezing behaviour and pupil dilation for each drug. Pre-treatment with the δ antagonist naltrindole (10 nmol, i.c.v.) inhibited the depressant effect, but not the subsequent stimulant effect, on locomotor activity in response to 30 nmol deltorphin II in this assay ($P<0.05$). In habituated rats, deltorphin II (0.03, 0.1, 0.3, 3 nmol, i.c.v.) caused significant, naltrindole-reversible increases in locomotor activity ($P<0.05$ for all doses) at 1000-fold lower doses than those required for a similar response to SNC80 (10, 30, 100, 300 nmol, i.c.v.). Pharmacokinetic studies suggest that these compounds penetrate the brain to similar extents following i.c.v. injection. The substantial potency difference between deltorphin II and SNC80 in stimulating locomotor activity in habituated rats suggests pharmacological heterogeneity for these δ opioid receptor agonists.

5.3 Introduction

Most research on supra-spinal δ opioid receptors concerns their role in modulating pain transmission (Ossipov *et al.*, 1995a). However, δ agonists also modulate locomotor activity and reward-related behaviours in rodents (Shippenberg *et al.*, 1987; Devine & Wise, 1994; Meyer & McLaurin, 1995; Negri *et al.*, 1996; Longoni *et al.*, 1991). To date, these additional behavioural effects have been identified largely in tests using peptidic compounds such as deltorphin II and DPDPE. Less attention has been given to the non-peptide δ agonist SNC80 (Calderon *et al.*, 1994), which exerts antinociceptive actions in animal tests (Negus *et al.*, 1998; Bilsky *et al.*, 1995) and represents a prototype for the development of δ agonists with therapeutic potential as analgesics (Dondio *et al.*, 1997).

Several observations suggest that the pharmacological profile of SNC80 may differ from that of peptidic δ agonists. In vitro, SNC80 has significantly higher efficacy in the [^{35}S]GTPγS binding assay of receptor activation performed on rat brain homogenates (Fraser *et al.*, 1999). In vivo, SNC80 appears to share the reinforcing properties of the peptidic δ agonists (Longoni *et al.*, 1998). However, microdialysis studies indicate that SNC80 (Longoni *et al.*, 1998), unlike deltorphin II (Longoni *et al.*, 1991), does not increase dopamine outflow in the medial nucleus accumbens of freely-moving rats. Lastly, it is unclear whether SNC80 has similar effects on spontaneous locomotor activity since published reports appear to provide conflicting findings (Spina *et al.*, 1998; Pohorecky *et al.*, 1999). Thus, in the former study, systemic administration of SNC80 to drug-naïve rats resulted in a locomotor stimulant effect, whereas in the latter the drug effect was uniformly depressant.

The aim of the present study was therefore to compare the locomotor effects of SNC80 with those of deltorphin II. For most direct comparison, both drugs were given by the same intracerebroventricular route. Since drug effects on locomotion can depend importantly upon prior habituation (Kelley, 1993), locomotor activity was tested in both habituated and non-habituated subjects.

5.4 Methods

5.4.1 Animals

Male, Sprague-Dawley rats (250-300 g; Charles River, St. Constant, Qc.) were housed in groups of three and provided with food and water *ad libitum* under an artificial 12h light-dark cycle (lights on at 7:00 h) with a constant temperature (23°C) and relative humidity (60%). Animals were used in compliance with the guidelines established by the Canadian Council for Animal Care.

5.4.2 Surgery

Rats were anaesthetised by intraperitoneal injection of 80 mg/kg ketamine/12 mg/kg xylazine solution (Research Biochemicals Int., Natick, MA) and placed in a stereotaxic device. Each rat was implanted with a 23 gauge stainless steel cannula extending into the right lateral cerebral ventricle (i.c.v.; co-ordinates from bregma, AP: 0.8 mm, ML: 1.5 mm, DV: 3.5 mm). The guide cannula was fixed in place with dental cement applied to the surface of the skull. Animals were allowed three or more days to recover from surgery prior to random allocation into treatment groups and subsequent experimentation.

5.4.3 Drugs and Drug Administration

Deltorphin II was purchased from RBI (Natick, MA). SNC80 and naltrindole HCl were purchased from Tocris Cookson (Ballwin, MO). All drugs were dissolved in sterile 0.9% saline solution and administered (i.c.v.) via the guide cannula in volumes of 10 µl using a 50 µl Hamilton syringe attached via PE20 polyethylene tubing to a 30 gauge needle. Solution was injected over a period of 60 seconds and the needle was left within the guide cannula for an additional 30 seconds to prevent reflux. Saline solution was administered for all control injections.

5.4.4 Activity Testing

Activity was measured using the AM1051 Activity Monitor (Benwick Electronics, UK). The plastic cage within the monitor measured approximately 30 x 18 x 18 cm. The monitor was equipped with a 12 x 7 infra-red beam matrix (ie. 2.54 cm grid) on both the lower level (set at a height of 3 cm) and the upper level (set at a height of 12 cm). The activity monitor operates by recording the number of times the infra-red beams change from broken to unbroken. Horizontal locomotion and rearing (vertical movement) were recorded at 10-minute intervals throughout

each experiment. In experiments measuring the effects of drugs on exploratory behaviour, rats were placed in the activity monitor cage immediately following administration of opiate agonist or saline. In a second series of experiments, rats were habituated in the activity monitor cage for 1 hour prior to drug administration and the subsequent measurement of locomotor stimulant drug effects. In all cases, data recording was started immediately following the injection of agonist. In addition to the recording of locomotor activity, the general appearance of the animals (including freezing behaviour, piloerection and pupil dilation) was observed intermittently for the first three 10-minute intervals after agonist administration. All activity experiments were conducted with counter-balanced treatment groups between 8h30 and 15h. Each rat was tested once.

5.4.5 Drug Concentrations in Whole Brain Homogenates

Brain penetration following i.c.v. injection was measured in previously untested subjects. Briefly, rats were decapitated at various time intervals after drug treatment. The brain (minus cerebellum) was collected, frozen in liquid nitrogen and stored at –80°C for determination of parent drug concentration. On the day of analysis, tissues homogenates were prepared in phosphate buffer (100 mM KH_2PO_4, pH 7.4; 2 ml/mg tissue), diluted in two volumes of ice-cold acetonitrile and centrifuged at 11000g for 10 minutes. Aliquots (250-500 µl) of the supernatant fraction were evaporated to dryness under a stream of nitrogen. Dry supernatant residues were dissolved in 100 µl of acetonitrile:0.04% formic acid mixture prepared according to the mobile phase for each compound (20:80, %v/v; mobile phase for deltorphin II and 40:60, %v/v; mobile phase for SNC80, respectively). Samples were analyzed following chromatographic separation using a C18 column (Phenomenex, Luna C-18 ODS2, 3 µm particle size). For deltorphin II, 20 µl sample volumes were passed through a 50 x 2.0 mm column at a flow rate of 0.25 ml/min. For SNC80, 30 µl sample volumes were passed through a 75 x 4.6 mm column at a flow rate of 1 ml/min. Drug concentrations were determined by LC/MS analysis (HP1100/Benchtop MS detector with API-ES source, Hewlett-Packard, Quebec, Canada) and comparison to calibration curves established using drug-free rat brain homogenates spiked with known amounts of deltorphin II or SNC80. The limit of quantitation for this procedure was 0.029 nmol drug/g of brain tissue.

5.4.6 Statistical Analyses

All data were analysed using GraphPad Prism™ (San Diego, CA). Data are presented as mean ± standard error of the mean (s.e.m.). Differences between treatment groups were analyzed by one-way or two-way analysis of variance (ANOVA) with DOSE and TIME as between-subject and

within-subject factors, respectively. Post-hoc analyses were performed using Dunnett's multiple comparison test on log-transformed data where appropriate. Values of $p < 0.05$ were judged to be statistically significant.

5.5 Results

SNC80 and deltorphin II elicited dose- and time-dependent changes in horizontal locomotion and rearing behaviour. Since the two behavioural measures were affected in a similar fashion in each experiment, only data describing the drug effects on horizontal locomotion are presented.

5.5.1 Drug Effects on Exploratory Behaviour

Saline-treated rats exhibited peak locomotion and rearing activity upon presentation of the novel environment. Ambulation and rearing rapidly subsided over time until a lower level of activity was established about 40 minutes after rats were placed in the activity cage. SNC80 and deltorphin II exerted biphasic dose-dependent effects on locomotor activity [dose x time interaction, respectively: $F(15,180) = 9.15$; $p < 0.0001$ and $F(33,348) = 3.69$; $p < 0.0001$; Figure 5.1 and Figure 5.2, respectively]. These δ opioid agonists significantly decreased locomotion during the initial exploratory phase of the test session (Figure 5.1B and Figure 5.2B). The initial dose-dependent decrease in locomotion was accompanied by freezing behaviour, pilo-erection and pupil dilation; these signs were most prominent in rats tested with SNC80. There was no indication of sedation or catalepsy in any treatment group.

SNC80 and deltorphin II elicited a second phase of behavioural stimulation that became apparent 20-30 minutes after drug administration (Figure 5.1C and Figure 5.2C, respectively). The increase in locomotion persisted for up to 60 and 100 minutes after drug administration for the highest doses of SNC80 and deltorphin II, respectively. During this phase, SNC80 treated rats frequently lost balance while rearing. This phenomenon was not observed for deltorphin II-treated rats.

Pre-treatment with the δ opioid antagonist naltrindole (10 nmol i.c.v., administered 10 minutes prior to agonist) completely blocked the initial decrease in exploratory activity induced by deltorphin II (30 nmol, i.c.v.) in the first 10-minute test interval ($p<0.05$; Figure 5.3). Naltrindole pretreatment also appeared to prevent deltorphin II-induced freezing behaviour, pilo-erection and pupil dilation. Naltrindole pretreatment did not significantly affect the subsequent locomotor stimulant response to deltorphin II at any other test interval. Furthermore, animals treated with naltrindole alone did not demonstrate any differences in exploratory behaviour in comparison to saline-treated controls.

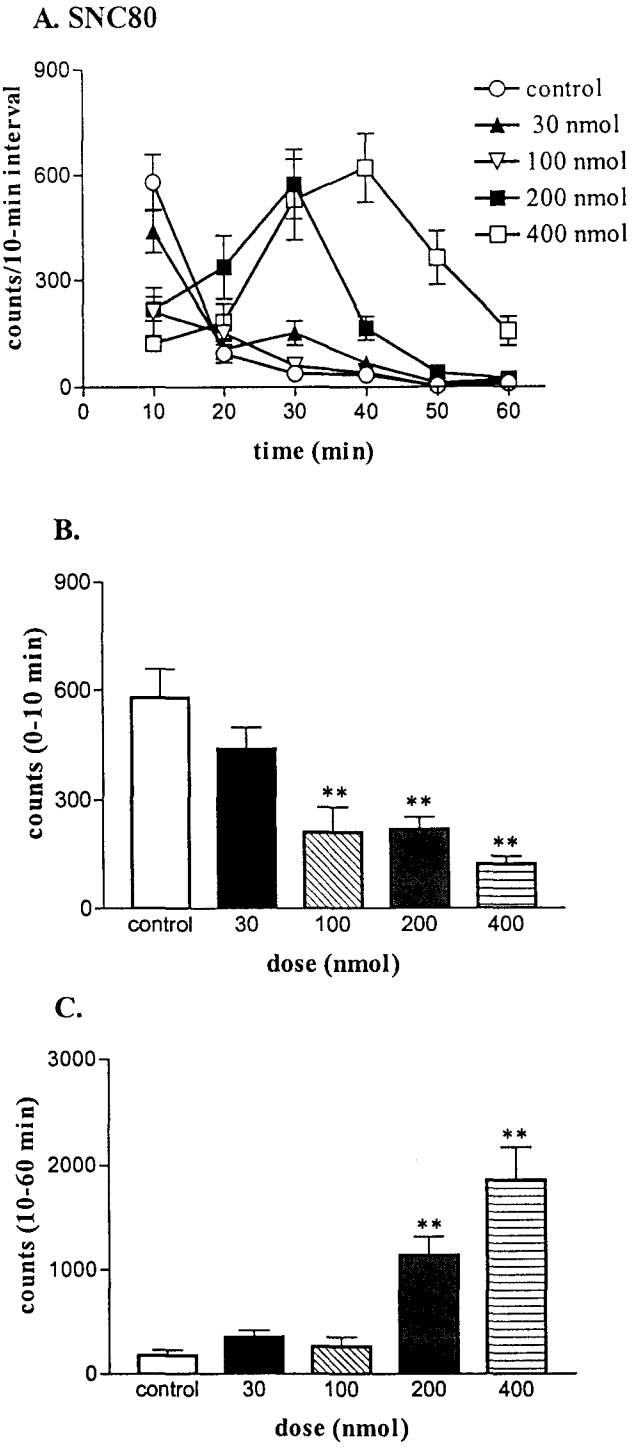

Figure 5.1 Effects of SNC80 (i.c.v.) on locomotion associated with exploration of a novel environment.

The complete time-course for the response to SNC80 is shown in panel A. The initial hypolocomotor and subsequent hyperlocomotor effects of SNC80 are presented in panels B and C respectively. Each data point represents the mean ± s.e.m activity of 8 - 9 rats. ** different from the control group, $p < 0.01$.

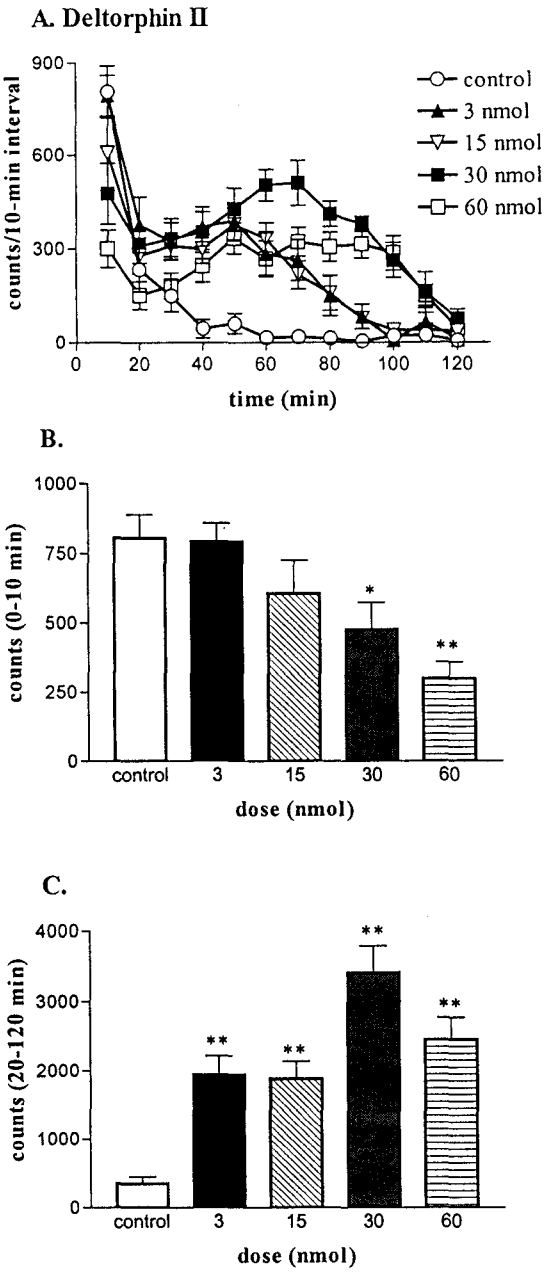

Figure 5.2 Effects of deltorphin II (i.c.v.) on locomotion associated with exploration of a novel environment.

The complete time-course for the response to deltorphin II is shown in panel A. The initial hypolocomotor and subsequent hyperlocomotor effects of deltorphin II are presented in panels B and C respectively. Each data point represents the mean ± s.e.m response of 8 - 11 rats. * and ** different from the control group, $p < 0.05$ and 0.01 respectively.

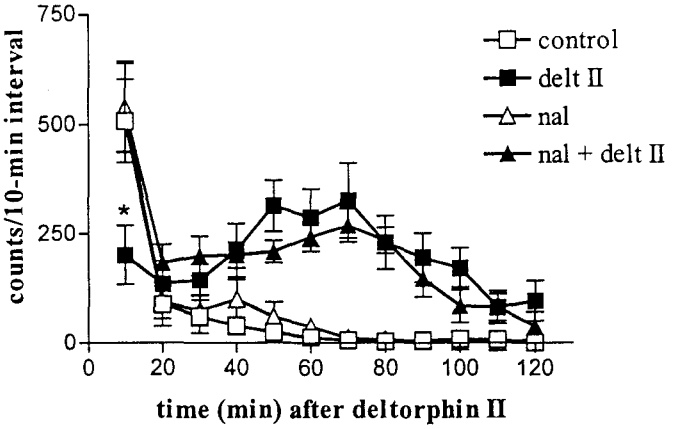

Figure 5.3 Deltorphin II (30 nmol, i.c.v.) mediated inhibition of exploratory locomotor activity is reversed by pretreatment with naltrindole (10 nmol, i.c.v.).
Each data point represents the mean ± s.e.m response of 7 - 8 rats. * represents a significant difference between the 'delt II' and 'nal + delt II'-treatment groups, $p < 0.05$.

5.5.2 Drug Effects on Locomotor Activity in Habituated Subjects

Saline-treated rats habituated to the activity cages exhibited low locomotor activity scores early in the session in comparison to non-habituated subjects. SNC80 and deltorphin II induced significant dose-dependent increases in locomotor activity [$F(3,162) = 9.55$, $p<0.001$ and $F(3,162) = 13.8$, $p< 0.0001$ respectively; Figure 5.4] in habituated subjects. Acute pilo-erection, freezing behaviour and pupil dilation were observed for rats treated with the highest doses of SNC 80 but not for any rats treated with deltorphin II.

In habituated subjects, pretreatment with naltrindole (10 nmol i.c.v., administered 10 minutes prior to agonist) significantly attenuated the hyperlocomotor response to deltorphin II (0.3 nmol, i.c.v.; $p < 0.05$ - Dunnett's test; Figure 5.5). Naltrindole alone did not affect the locomotion scores of habituated rats based on comparison of the naltrindole-treated group and the saline-treated control group ($p > 0.05$ – Dunnett's).

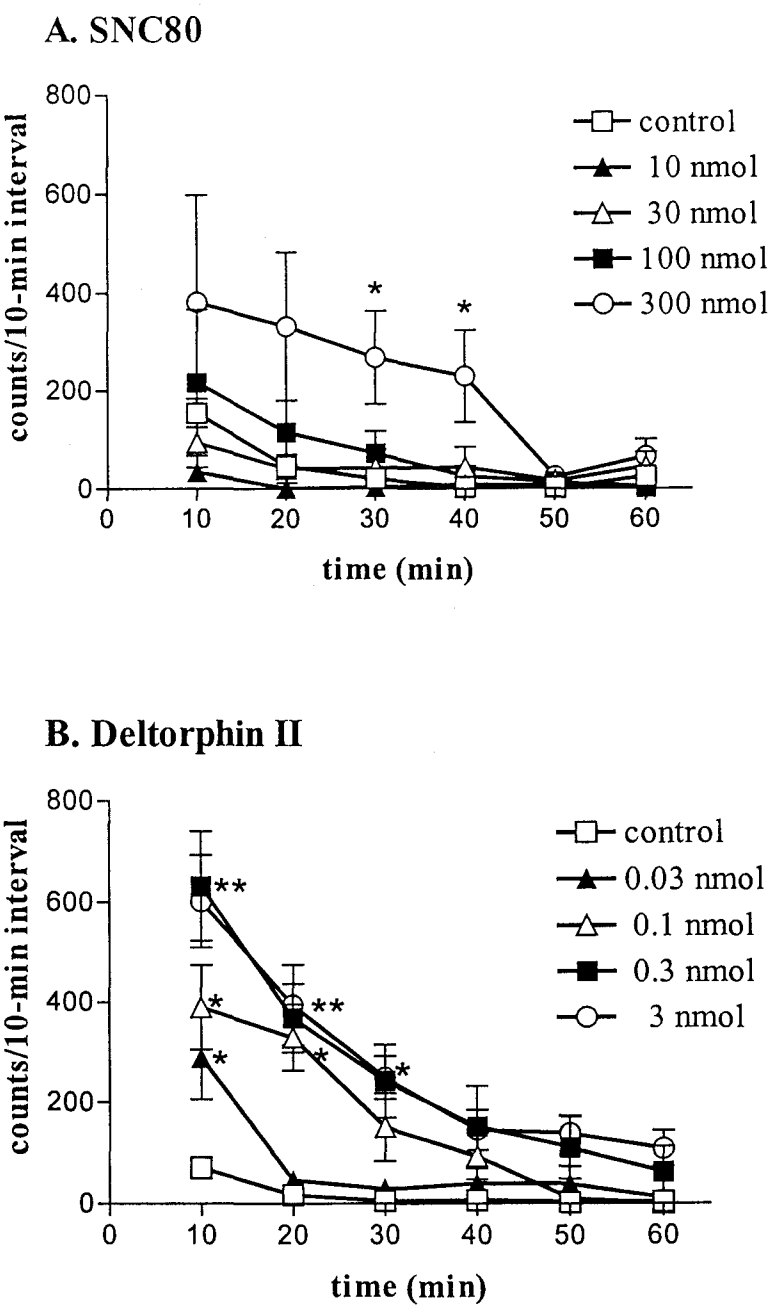

Figure 5.4 Effects of SNC80 and deltorphin II on locomotor activity in habituated subjects.

Each data point represents the mean ± s.e.m response of 7 - 10 rats. * and ** different from the control group where $p < 0.05$ and 0.01 respectively.

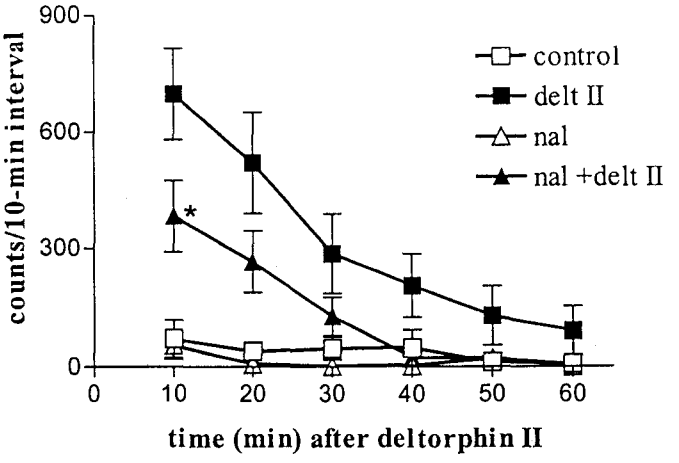

Figure 5.5 Deltorphin II (0.3 nmol, i.c.v.) mediated stimulation of locomotor activity in habituated rats is partially reversed by pretreatment with naltrindole (10 nmol, i.c.v.). Each data point represents the mean ± s.e.m response of 7 - 10 rats. * represents a significant difference between the 'delt II' and 'nal + delt II'-treatment groups, $p < 0.05$.

5.5.3 In Vivo Brain Penetration of SNC80 and Deltorphin II

The brain penetration profiles for SNC80 (8.8 nmol, i.c.v.) and deltorphin II (10 nmol, i.c.v.) did not differ significantly at any of the test intervals (Figure 5.6). In both cases, less than 20% of the administered dose was present in the brain 0.5 h after drug administration. Both compounds approached undetectable levels (i.e. < 30 pmol/g tissue) at 1.5 h after treatment.

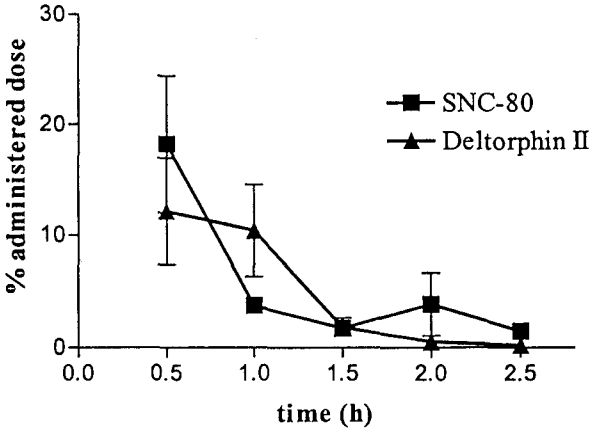

Figure 5.6 In vivo brain penetration of SNC80 (8.8 nmol, i.c.v.) and deltorphin II (10.0 nmol, i.c.v.). Each data point represents the mean % ± s.e.m. of the administered dose recovered from brain homogenates prepared from 3 rats.

5.6 Discussion

The present study compared the effects of the δ agonists, deltorphin II and SNC80, on locomotor activity in non-habituated and habituated rats. These drugs were found to exert broadly similar effects despite large differences in potency in habituated rats. Thus, in rats that were naive to the testing apparatus, locomotor stimulation was preceded by a clear depressant effect, whereas in habituated rats, only a stimulant effect was observed. Both drugs were given by the intracerebroventricular route in order to reduce possible differences in brain penetration, and direct measurement revealed no major difference in this regard.

Previous reports have suggested that δ agonists have both stimulant and depressant effects on locomotor activity. Thus, central administration of peptidic agonists increased locomotor activity (Longoni *et al.*, 1991; Negri *et al.*, 1991a; Calenco-Choukroun *et al.*, 1991a) although some investigators have noted a transient depressant effect at higher doses (Meyer & McLaurin, 1995; Negri *et al.*, 1996). Similarly, either depression or stimulation of locomotor activity was reported after systemic administration of the non-peptidic δ agonist SNC80 to drug-naïve rats (Pohorecky *et al.*, 1999; Spina *et al.*, 1998). The variable outcomes presented in published reports are likely related to differences in the experimental methods used. Thus, the present findings demonstrate that the locomotor effects of δ agonists depend importantly on time after injection and prior exposure to the testing apparatus. Depressant effects were noted only in non-habituated subjects within minutes of drug administration. This observation may help to account for the mixed results previously reported with peptidic and non-peptidic delta agonists. Thus, the previously reported depressant effects of DPDPE (a peptidic δ agonist) and SNC80 were observed in drug- and apparatus-naïve animals that were tested between 5 and 15 minutes after drug administration (Meyer & McLaurin, 1995; Pohorecky *et al.*, 1999). In contrast, a pure stimulant effect of these agonists was reported in rats that were habituated to the testing apparatus prior to injection (Spina *et al.*, 1998; Klitenick & Wirtshafter, 1995), unless high doses of drug were given (Pohorecky *et al.*, 1999). The depressant effects of δ agonists on locomotor activity are likely contingent upon the dose used. It has previously been reported that a high dose of deltorphin II transiently inhibits locomotor activity in habituated rats although lower doses of drug potently stimulated locomotor activity (Negri *et al.*, 1996).

In the present study, the early locomotor depression in response to δ agonist coincided with the transient phase of increased ambulation and rearing that characterizes exploration in non-habituated rats (Kelley, 1993). The animals treated with δ agonists did not appear motorically

impaired. Thus, the depressant locomotor effects of δ agonists are distinct from the previously reported cataleptic response to μ opioid receptor agonists (Negri *et al.*, 1996; Calenco-Choukroun *et al.*, 1991a). Rats treated with deltorphin II or SNC80 manifested clear signs of anxiety (i.e. pupil dilation, freezing behaviour and pilo-erection); inhibition of exploratory activity in open-field tests is a common response to anxiogenic substances (File, 1985; Otter *et al.*, 1997). The similarities in both the behavioural syndromes and the relative potencies for deltorphin II and SNC80 suggests that these drugs activate common pathways in the brain to depress exploratory activity in non-habituated rats.

The transient depressant effects of δ agonists in non-habituated subjects were immediately followed by increased locomotor activity. Natural exploratory activity (Kelley, 1993), delayed by the initial depressant effects of δ agonist, may comprise a component of this response. However, the duration of enhanced locomotor activity following treatment with deltorphin II was significantly longer than the duration of exploratory activity in control animals suggesting a specific response to drug. The finding that naltrindole inhibited the anxiogenic effects of deltorphin II but not the consequent hyperlocomotor activity suggests that the latter is not the direct result of the former. Thus, the failure of naltrindole pretreatment to block the subsequent phase of increased locomotor activity suggests that either this response is not due to the direct activation of δ opioid receptors or that there was insufficient naltrindole present in relevant brain regions at the time that this response was manifested. To the best of our knowledge, data pertaining to the time-course of naltrindole (i.c.v.) inhibition of δ opioid receptor mediated locomotor activity in non-habituated rats has not been previously reported. However, in other assays, naltrindole has been reported to act for between one and three hours after central administration of comparable doses (Yoshida *et al.*, 1999; Schad *et al.*, 1996). Thus, it is unlikely that the effects of naltrindole would have subsided prior to the hyperlocomotor phase. Consequently, it would appear that the hyperlocomotor phase does not arise from the direct activation of δ receptors.

Exploratory activity is minimised in habituated rats. Thus, this test paradigm is particularly appropriate for measuring the stimulant effects of drugs on locomotor activity (Kelley, 1993). Deltorphin II treatment potently increased locomotor activity in these experiments in agreement with published findings (Longoni *et al.*, 1991; Negri *et al.*, 1991a). The δ opioid nature of this response was confirmed by blockade with naltrindole. Deltorphin II caused significant increases in locomotion at doses 1000-fold lower than those found to depress exploratory activity in non-

habituated rats. In addition, deltorphin II had far more potent effects on locomotor activity than antinociception based on both our own recent findings (Fraser *et al.*, 2000a)) and previous studies on the role of δ agonists in modulating supraspinal pain transmission (Ossipov *et al.*, 1995a; Negri *et al.*, 1996).

The mesolimbic dopamine pathway extending from the ventral tegmental area to the nucleus accumbens has been characterised as important in the modulation of spontaneous and pharmacologically stimulated locomotion (Kelly *et al.*, 1975; Fink & Smith, 1980; Clarke *et al.*, 1988). Previous work with peptidic δ agonists suggests that δ opioid receptor activation reduces the firing of GABAergic interneurones resulting in decreased tonic inhibition of dopaminergic pathways (Dilts & Kalivas, 1990; Jiang & North, 1992). Accordingly, brain microdialysis studies have demonstrated that intracerebroventricular administration of DPDPE or deltorphin II cause increased extracellular dopamine release in the nucleus accumbens (Longoni *et al.*, 1991; Spanagel *et al.*, 1990). Substantial evidence suggests that heightened dopaminergic activity underlies the stimulation of locomotor activity in response to peptidic δ agonists (Longoni *et al.*, 1991; Kalivas *et al.*, 1983; Calenco-Choukroun *et al.*, 1991b).

SNC80 was demonstrated to be far less potent (i.e. ~1000-fold) than deltorphin II in stimulating locomotor activity in habituated rats. This outcome does not correlate with differences in brain penetration for these compounds, nor is it consistent with the superior binding affinity and efficacy of SNC80 at δ opioid receptors in rat brain homogenates (Fraser *et al.*, 1999). The weak locomotor stimulant response to SNC80 seen in the present study appears consistent with evidence that SNC80, unlike deltorphin II, does not appreciably increase extracellular dopamine concentrations in the nucleus accumbens of freely-moving rats (Longoni *et al.*, 1998).

In the present study, deltorphin II depressed locomotor activity in non-habituated rats at doses 1000-fold greater than those stimulating locomotor activity in habituated rats. In contrast, doses of SNC80 that depressed locomotor activity in non-habituated rats were similar to those that increased locomotor activity in habituated animals. The basis for the divergent potencies for deltorphin II and SNC80 on pharmacologically stimulated locomotor activity is not known. Differences in drug penetration into specific brain areas may be responsible, however this appears unlikely given that the whole brain penetration profiles for each drug were similar. Alternatively, it is possible that SNC80 and deltorphin II differentially act on pharmacologically distinct δ receptor populations (Traynor & Elliot, 1993) that arise either from different genes, from

alternative splicing (Rossi *et al.*, 1997) or receptor homo- (Cvejic & Devi, 1997) or heterodimerization (Jordan & Devi, 1999).

6 Antihyperalgesic Effects of δ Opioid Agonists in a Rat Model of Chronic Inflammation

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6.1 Preface

Animal models measuring nociceptive responses to an acute, noxious stimulus are useful for demonstrating the presence of a target receptor in pain pathways. However, these models are not clinically relevant as most patients present pain associated with a chronic, noxious stimulus originating from tissue injury or disease. Moreover, recent evidence suggests that tissue injury and chronic inflammation may alter the way nociceptive information is processed. Thus, the role of a target receptor in modulating nociceptive activity may differ depending upon the chronicity of the noxious stimulus. In pain models associated with persistent inflammation, δ agonists administered at the peripheral site of inflammation or into the spinal cord appear to be highly effective in attenuating hyperalgesic responses. The current study demonstrates that inflammation-based hyperalgesia is also attentuated by the administration of δ agonists (deltorphin II, SNC80) directly into the brain (*i.c.v.*). This finding supports the development of δ agonists for the treatment of pain associated with tissue injury. Also, this study demonstrates that δ opioid receptors in the brain are important sites for the treatment of hyperalgesia associated with peripheral inflammation or tissue injury.

6.2 Summary

- Opioid receptors in the brain activate descending pain pathways to inhibit the nociceptive response to acute noxious stimuli. The aim of the present study was to clarify the role of supraspinal opioid receptors in modulating the nociceptive response to persistent inflammation in rats.

- Subcutaneous administration of 50 μl of Freund's Adjuvant (CFA) into the plantar surface of the hindpaw induced a significant decrease in paw withdrawal latency to thermal stimuli ($P<0.01$) at 24 hours post-injection.

- Intracerebroventricular (i.c.v.) administration of the μ opioid receptor agonists, DAMGO and morphine, and the δ opioid receptor agonists, deltorphin II and SNC80, significantly reversed the hyperalgesic response associated with peripheral inflammation in a dose-dependent manner ($P<0.0001$).

- The μ and δ agonists also significantly attenuated the antinociceptive response to acute thermal stimulation in rats ($P<0.001$). However, deltorphin II and SNC80 were less potent, and in the case of SNC80 less efficacious, in modulating the response to acute thermal nociception in comparison to hyperalgesia associated with persistent inflammation.

- These results indicate that μ and δ opioid receptors in the brain modulate descending pain pathways to attenuate the nociceptive response to acute thermal stimuli in both normal and inflamed tissues. The heightened response to δ agonists in the hyperalgesia model suggests that δ opioid receptors in the brain are promising targets for the treatment of pain arising from chronic inflammation.

6.3 Introduction

It has been proposed that opioid-induced disinhibition of neurons in the periaqueductal gray (PAG) activates spinally projecting neurons in the rostroventral medulla (RVM) to attenuate nociceptive signals originating from sites in the dorsal horn (Basbaum & Fields, 1984). This model is supported by autoradiographic and immunocytochemical studies demonstrating the expression of μ and δ opioid receptors in the PAG and RVM (Mansour *et al.*, 1987; Kalyuzhny *et al.*, 1996). *In vivo*, the modulation of nociceptive transmission in the cord by descending inputs from the brainstem, and the effect of opiates in this paradigm, have been demonstrated using acute measures of nociception such as the tail flick assay (Rossi *et al.*, 1994). However, chronic pain following tissue damage leads to persistent functional changes in the nervous system (Dubner & Ruda, 1992). Accordingly, intra-plantar injection of inflammatory agents such as complete Freund's adjuvant (CFA) causes increased firing of peripheral afferents in the spinal cord leading to hyperexcitability of dorsal horn nociceptive neurons and consequent hyperalgesia in response to mechanical or thermal stimuli (Hargreaves *et al.*, 1988). In turn, this elevated nociceptive input in the spinal cord appears to trigger increased neuronal activity in descending pain pathways originating in the brain (Schaible *et al.*, 1991; Ren & Dubner, 1996). It is unclear to what extent the increased activity in descending pathways associated with peripheral hyperalgesia is susceptible to modulation by exogenous opioids.

The antinociceptive effects of μ opioid agonists such as morphine and DAMGO ([D-Ala^2, N-Me-Phe^4, Gly-ol^5]-enkephalin) in the brain have been well established in various acute pain assays. These compounds have also proven to be effective in models of chronic inflammatory pain, although it is unclear to what extent these antihyperalgesic effects were mediated at supraspinal sites (Joris *et al.*, 1990; Ho *et al.*, 1997; Zhou *et al.*, 1998). In contrast, studies of the supraspinal antinociceptive effects of δ agonists have produced conflicting results in rats, perhaps reflecting differences in the type of acute pain tests used (Negri *et al.*, 1991a; Ossipov *et al.*, 1995a). To the

best of our knowledge, the antihyperalgesic efficacy of δ agonists administered directly into the brain of conscious animals has not been previously demonstrated.

In the present study, thermal hyperalgesia associated with CFA-induced persistent inflammation of the rat hind paw was evaluated using the plantar test (Hargreaves *et al.*, 1988). This experimental paradigm appears to be highly predictive of thermal hyperalgesia in humans (Montagne-Clavel & Oliveras, 1996). The effects of μ and δ opioid agonists in the thermal hyperalgesia assay were compared to their effects in the tail flick assay of nociception.

6.4 Methods

6.4.1 Preparation of animals

Animals were handled in strict adherence to the guidelines established by the Canadian Council for Animal Care. Male Sprague-Dawley rats (250-300g) were anaesthetized with 80 mg kg^{-1} body weight ketamine-xylazine solution (*i.p.*; RBI, Natick MA) and placed in a stereotaxic device. Each animal was then implanted with a 23 gauge cannula extending into the right lateral ventricle (*i.c.v.*; coordinates from bregma, AP: 0.8 mm, ML: 1.5 mm, DV: 3.5 mm). The guide cannula was fixed in place with dental cement. Rats were allowed three or more days to recover from the surgery prior to random allocation into treatment groups. Pre-habituation to the *i.c.v.* injection procedure was effected by administering 10 µl of 0.9% saline solution via the indwelling cannula 24 hours prior to experimentation.

6.4.2 Inflammation

Rats were briefly anaesthetized by inhalation of isofluorane (5% saturation in O_2, flow rate of 800-900 ml min^{-1}). Inflammation was produced by the subcutaneous injection of 20, 50 or 100 µl of complete Freund's Adjuvant (CFA; Sigma, St.Louis, MO) into the plantar surface of the right hind paw. Only rats designated for testing in the thermal hyperalgesia assay were treated with CFA.

6.4.3 Plantar Test

Thermal hyperalgesia was assessed in unrestrained rats using a procedure adapted from published reports (Hargreaves *et al.*, 1988). Rats (n = 6-8 per group) were placed in opaque, plastic chambers (13 x 24 x 13 cm) positioned on a glass surface. Animals were allowed to habituate in this environment for 20 minutes prior to testing. Paw withdrawal latency in response to radiant heat was measured using the plantar test apparatus (Ugo Basile, Comerio, Italy). The heat source was positioned beneath the plantar surface of the affected hind paw and activated. The digital

timer connected to the heat source automatically recorded the response latency for paw withdrawal to the nearest tenth of a second. A cut-off time of 22 seconds was used to prevent tissue damage. The paw withdrawal latency of each rat was measured three times at each test interval and the median score recorded. The effects of opioid agonists on paw withdrawal latency were measured 24 hours after the injection of CFA. Control (saline-injected) and dose treatment groups were tested in parallel for each drug. Paw withdrawal latencies were converted to % anti-hyperalgesia using the following equation:

$$\% \text{ anti-hyperalgesia} = [(\text{drug} - \text{CFA}) \div (\text{baseline} - \text{CFA})] \times 100$$

where 'drug' represents the response latency for each treatment group in response to opioid agonist. 'CFA' represents the average paw withdrawal score for all groups of rats prior to opiate agonist treatment and 'baseline' represents the average of all baseline scores prior to CFA treatment.

6.4.4 Tail Flick Assay

The antinociceptive effects of opioid agonists were measured using the tail flick apparatus (IITC Inc., Woodland Hills, CA). Rats were positioned on a flat surface and held gently by the experimenter. Tail withdrawal latencies were recorded in response to heat from a light beam focused on the dorsal surface of the tail (approximately 2 cm from the tip). A digital timer automatically recorded response latencies to the nearest tenth of a second. The light beam intensity was adjusted to produce a baseline latency of 3-5 seconds. The recommended cut-off time of 12 seconds was used to prevent tissue damage. On the day of testing, two baseline responses were recorded 5 and 15 minutes prior to injection of drug to habituate the rats to the testing procedure. The antinociceptive effects of opioid agonists were measured 15, 30, 45 and 60 minutes after drug treatment. Control (saline-injected) and dose treatment groups were tested

in parallel for each drug. Tail flick response latencies were converted to percent of maximum possible effect (% MPE) according to the formula:

$$\% \text{ MPE} = [(\text{post-drug latency} - \text{control}) \div (\text{cut-off latency} - \text{control})] \times 100$$

6.4.5 Drug administration

The opioid agonists (DAMGO and deltorphin II supplied by RBI, Natick, MA; SNC-80 supplied by Tocris Cookson Inc., Ballwin, MO; morphine sulfate supplied by BDH, Toronto, ON) were dissolved in 0.9% saline solution and administered to rats via the guide cannula (*i.c.v.*) immediately prior to behavioural testing. All opioid drug solutions were injected in a volume of 10 µl using a 50 µl Hamilton syringe attached to a catheter (15 cm) constructed from PE20 polyethelene tubing and terminating in a 30 gauge needle. Solution was injected slowly over a period of 60 seconds and the needle was left within the guide cannula for an additional 30 seconds after the injection. In all cases, additional rats were treated concomitantly with 0.9% saline solution as a control for the drug treatment paradigm.

6.4.6 Statistical analyses

Data are presented as mean ± standard error of the mean (s.e.m.). Differences between treatment groups were analyzed by one-way or two-way analysis of variance (ANOVA) with DOSE and TIME as between-subject and within-subject factors, respectively. Post-hoc analyses were performed with Dunnett's multiple comparison test where appropriate. ED_{50} values were determined by linear regression analysis of the dose response curves. All analyses were performed using GraphPad Prism software (San Diego, CA).

6.5 Results

6.5.1 Thermal hyperalgesic response to CFA

Intra-plantar injection of 20, 50 or 100 µl volumes of CFA caused localized erythema and oedema in the affected hind paw. The degree of erythema and oedema appeared to increase in relation to the injection volume (data not shown). There were no obvious changes in weight gain, grooming or social interactions following CFA treatment over the duration of the 48-hour test period. The largest and most clear dose-dependent decrease in paw withdrawal latency occurred at 24 hours (Figure 6.1). Rats treated with the highest dose of CFA (100 µl) also exhibited spontaneous paw licking and decreased weight bearing for the affected paw at this test interval. Therefore, subsequent experiments measuring the effects of opioid agonists were performed on rats pre-treated with 50 µl CFA (*i.pl.*) 24 hours prior to drug testing.

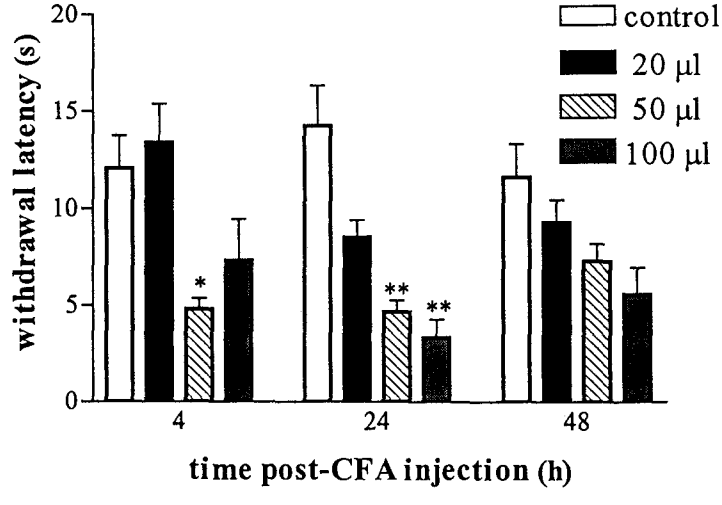

Figure 6.1 Dose-related effects of CFA (*i.pl.*) on paw withdrawal latency following exposure to radiant heat.
Only response latencies for the injected paw were measured. * and ** represent significant differences between the control (saline-injected) group and the CFA-treated groups ($P<0.05$ and $P<0.01$ respectively; Dunnett's test). Each bar represents the mean ± s.e.m. response of 6-9 rats.

6.5.2 Anti-hyperalgesic effects of opioid agonists

The effects of the δ agonists deltorphin II and SNC80 in the thermal hyperalgesia model are presented in Figure 6.2. Dose-response curves derived from these data are presented together with the corresponding data for the μ agonists DAMGO and morphine (Figure 6.4A). The peak antihyperalgesic effects for DAMGO, morphine and SNC80 occurred at the 20-minute test interval for all doses, whereas the peak effects for deltorphin II occurred at the 40-minute test interval. Each opioid agonist reversed thermal hyperalgesia by >90%. There was a significant effect of drug treatment (*i.c.v.*) for each compound (DAMGO – $F_{(3,60)} = 9.085$, $P<0.0001$; morphine – $F_{(3,26)} = 20.3$, $P<0.0001$; deltorphin II – $F_{(4,90)} = 48.4$, $P<0.0001$; SNC80 – $F_{(3,75)} = 25.77$, $P<0.0001$). There was no significant difference between groups for both the baseline scores and the pre-drug CFA scores in all four experiments. There were no clear decreases in locomotor activity or other signs of sedation for any of the compounds tested.

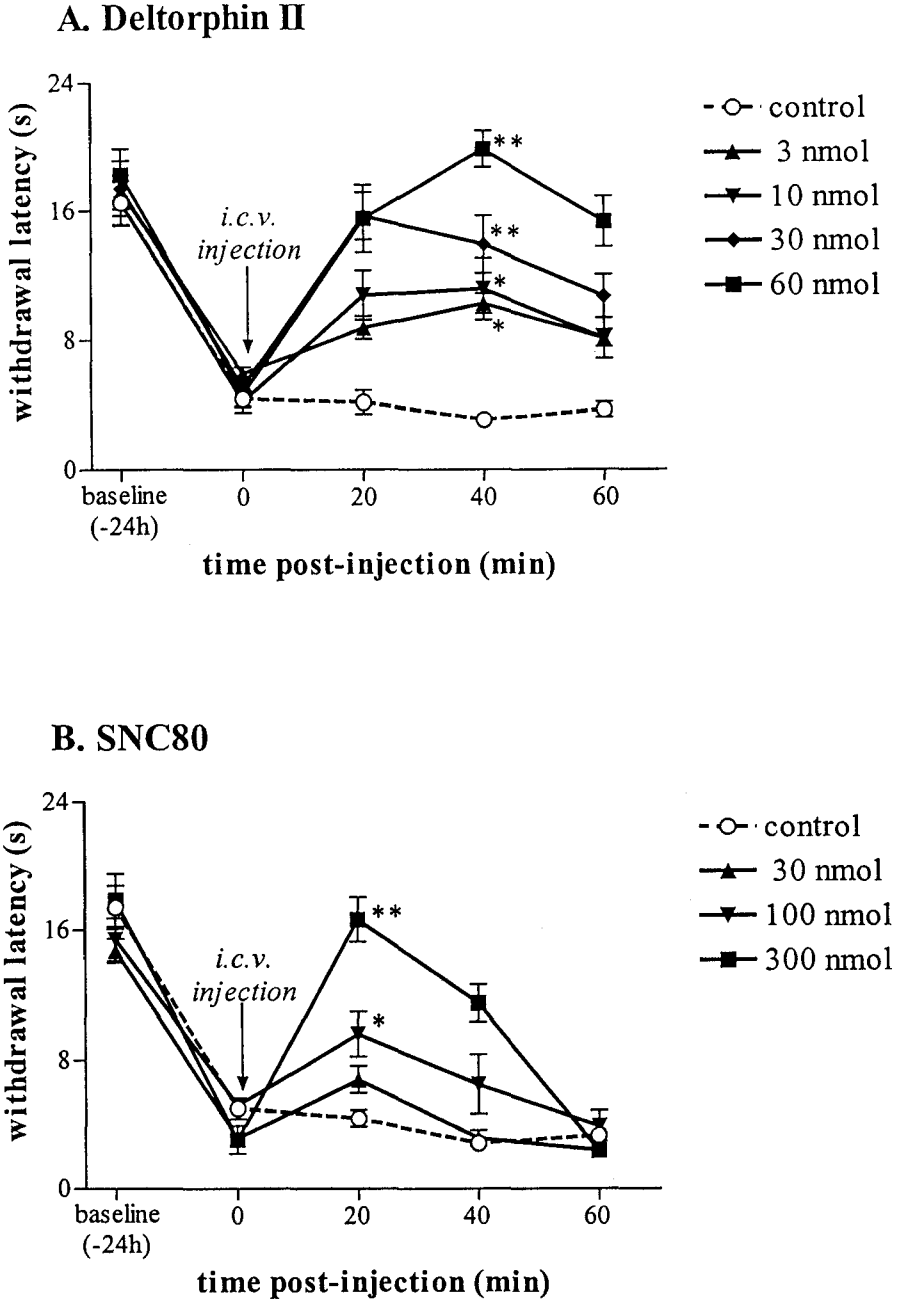

Figure 6.2 Anti-hyperalgesic effects of deltorphin II and SNC-80.
Baseline paw withdrawal latencies were measured for all rats prior to administration of CFA (50 µl *i.pl.*). The effects of δ agonists were measured 24h after CFA treatment. * and ** represent significant differences between the control group and the drug treatment groups ($P<0.05$ and $P<0.01$ respectively; Dunnett's test). Each curve represents the mean ± s.e.m. response of 6-8 rats.

6.5.3 Effects of opioid agonists in the tail flick assay

The peak antinociceptive effects for DAMGO, morphine and SNC80 occurred at the 15-minute test interval for all doses, whereas the peak effects for deltorphin II occurred at the 30-minute test interval. These data are presented in dose-response format (Figure 6.3B). Treatment (*i.c.v.*) with DAMGO, morphine, and deltorphin II significantly increased response latencies in the tail flick assay to >90% of MPE at the highest doses. In comparison, SNC80 significantly increased response latencies to a sub-maximal level in the tail flick assay over the dose range tested (E_{max} = 60% of MPE). There was a significant effect of drug treatment for each compound (DAMGO – $F_{(3,120)} = 21.2$, $P<0.0001$; morphine – $F_{(3,84)} = 30.6$, $P<0.0001$; deltorphin II – $F_{(4,144)} = 34.6$, $P<0.0001$; SNC80 – $F_{(4,136)} = 11.1$; $P<0.0001$). D_{50} and ED_{50} values for the opioid agonists in the plantar test and tail flick assay are presented and compared in Table 6.1.

	Plantar Test ***(D_{50}, nmoles)***	**Tail Flick** ***(ED_{50}, nmoles)***	**Potency** ***Ratio***
DAMGO	0.10	0.11	1.1
Morphine	5.6	9.8	1.7
Deltorphin II	11	37	3.4
SNC80	120	340	2.9

Table 6.1 Comparison of the antinociceptive potency of opioid agonists in the plantar test and tail flick assays.
D_{50} and ED_{50} values were determined by linear regression analysis of the dose-response curves presented in Figure 6.4.

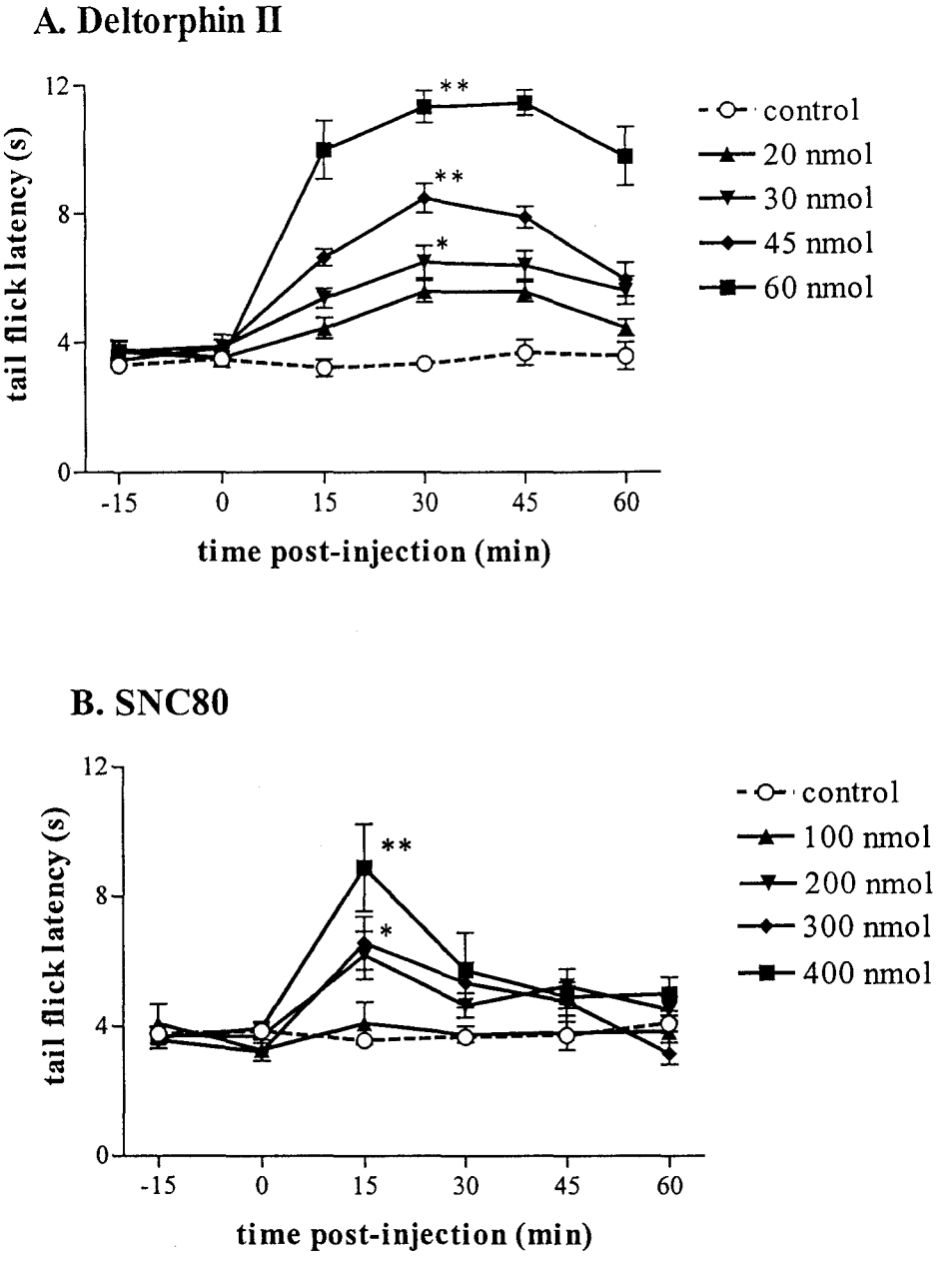

Figure 6.3 Antinociceptive effects of deltorphin II and SNC80.
Baseline tail flick latencies were measured for all rats prior to the administration of drug. * and ** represent significant differences between the control group and the drug treatment groups ($P<0.05$ and $P<0.01$ respectively; Dunnett's test). Each curve represents the mean ± s.e.m. response of 7-12 rats.

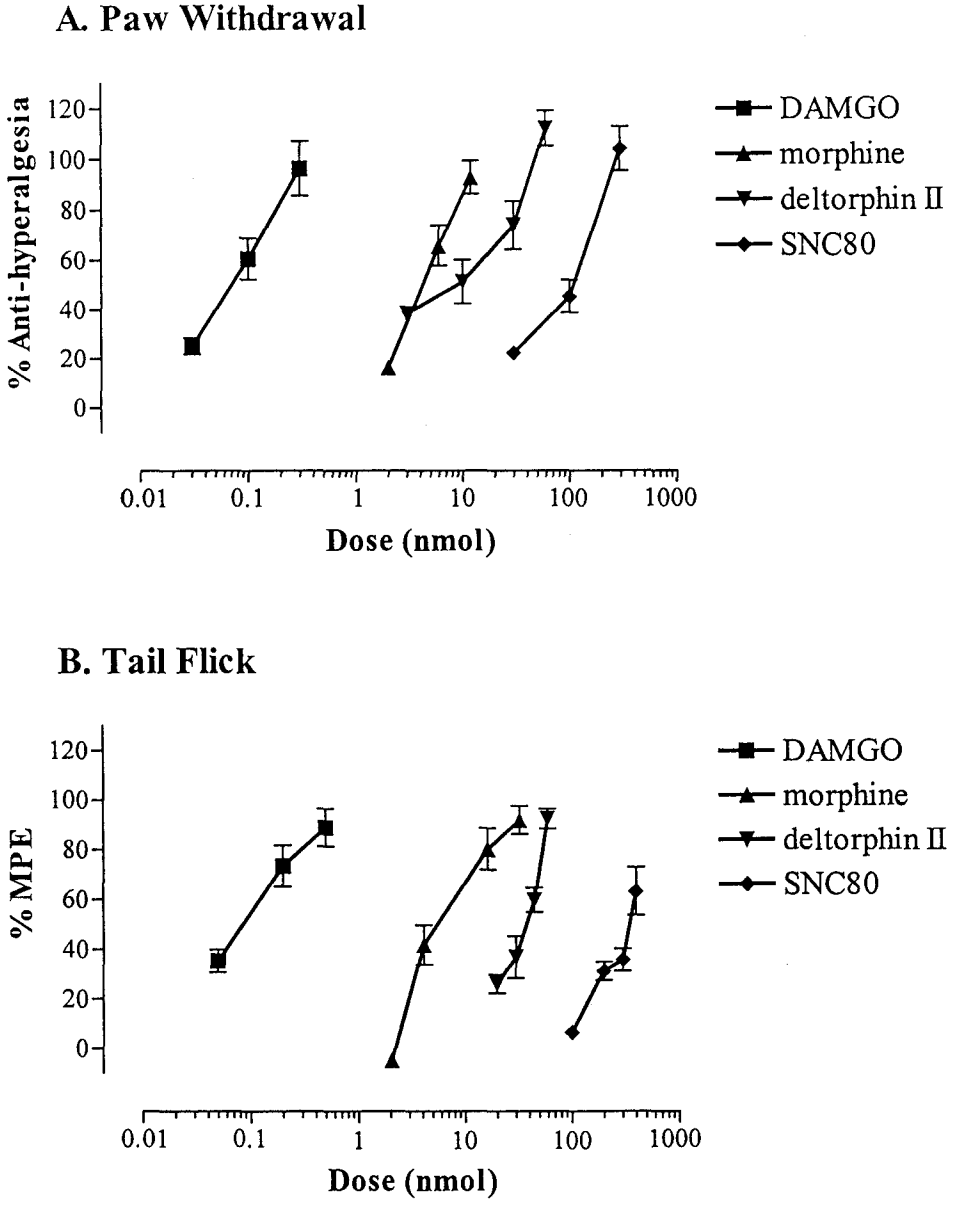

Figure 6.4 Opioid agonists (*i.c.v.*) have similar response profiles in antihyperalgesic and antinociceptive models.

[A] Thermal hyperalgesia was measured using the Hargreave's assay. % Anti-hyperalgesia was determined relative to the baseline paw withdrawal response to radiant heat prior to CFA treatment. Each data point represents the peak antihyperalgesic response to drug, which occurred at 20 minutes post-injection for DAMGO, morphine and SNC80 and 40 minutes post-injection for deltorphin II. [B] Antinociception was measured in the tail flick assay. % MPE was determined relative to the pre-determined cut-off for the test apparatus. The dose-response curves represent the peak antinociceptive response to drug at 15 minutes post-injection for DAMGO, morphine and SNC80, and 30 minutes post-injection for deltorphin II. Each data point represents the mean ± s.e.m. response of 6-12 rats.

6.6 Discussion

The main findings of this study are that δ agonists are effective antihyperalgesics when administered directly into the brain. Moreover, deltorphin II and SNC80 had improved potency in rats with persistent peripheral inflammation compared to normal rats tested in the tail flick assay. Our findings complement those of previous reports suggesting that δ agonists reverse peripheral hyperalgesia following administration directly into the inflamed tissue (Zhou *et al.*, 1998) or intrathecal space (*i.t.*; Ho *et al.*, 1997; Hylden *et al.*, 1991). Increased potency of δ agonists (*i.t.*) has been demonstrated in rats with unilateral hindpaw inflammation (Hylden *et al.*, 1991), but these findings were not corroborated in other published reports (Ho *et al.*, 1997). The lesser potency for δ agonists administered *i.c.v.* and tested in acute pain assays is consistent with the data presented in previous reports (Negri *et al.*, 1991a; Ossipov *et al.*, 1995a). Although δ opioid receptor subtypes have been postulated (e.g. Mattia *et al.*, 1991; Vanderah *et al.*, 1994, antisense studies suggest that supraspinal antinociception in response to deltorphin II and SNC80 is predominantly mediated by the cloned δ opioid receptor (DOR-1; Fraser *et al.*, 2000b). The potency difference for δ agonists (*i.c.v.*) in chronic versus acute pain models suggests a more prominent role for DOR-1 in supraspinal pain processing centers (Kalyuzhny *et al.*, 1996) as a consequence of the enhanced neuronal activity in descending pain pathways following peripheral inflammation (Ren & Dubner, 1996).

The antinociceptive effects of the μ agonists DAMGO and morphine in the tail flick assay are consistent with those described in previous reports (Rossi *et al.*, 1994). We are not aware of any previous reports demonstrating the supraspinal effects of μ agonists in models of chronic inflammation. However, μ agonists have been shown to have antihyperalgesic effects following peripheral (Joris *et al.*, 1990) or intrathecal administration (Hylden *et al.*, 1991). In general, the antihyperalgesic potency of μ agonists in rats with unilateral inflammation of the hindpaw is

much greater than the antinociceptive activity observed in normal animals. The enhanced potency of systemically active μ opioids in animals with peripheral inflammation has been ascribed to the activity of these drugs at sites proximal to the inflamed tissue or changes in spinal systems that would specifically affect the function of μ opioid receptors (Stanfa & Dickenson, 1995). However, the findings of the present study indicate that these reported changes at peripheral or spinal sites are not accompanied by μ-specific changes in supraspinal pain processing since μ agonists inhibited the nociceptive thresholds in chronic and acute pain models with similar potency.

In summary, supraspinal δ opioid receptors have an enhanced role in inhibiting nociceptive signals following chronic inflammation and thus represent promising targets for the treatment of clinical hyperalgesia. In contrast, supraspinal μ opioid receptors have a similar role in inhibiting nociceptive signals associated with both acute and chronic pain states.

7 Supraspinal antinociceptive response to [D-Pen^{2,5}]-Enkephalin (DPDPE) is pharmacologically distinct from that to other δ–agonists in the rat

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JPET **295**:1135-1141, 2000

7.1 Preface

Previous studies, including the work presented in the preceding chapter, demonstrate that δ agonists administered directly into the brain modulate antinociception. However, it is not clear whether supraspinal antinociception is modulated by the cloned δ opioid receptor, an alternate δ receptor subtype, a δ/μ receptor complex, or non-specific interactions at μ opioid receptors. The present study uses antisense technology to characterize the role of the cloned δ opioid receptor in this response. The major finding of this study is that the cloned δ opioid receptor modulates the antinociceptive response of all the δ agonists tested except for the prototypical δ_1-selective agonist, DPDPE. Additional experiments with the μ antagonist, CTOP, demonstrated a complete inhibition of DPDPE antinociception, but no effect on the response to deltorphin II or SNC80. These findings validate the cloned δ opioid receptor as a target for the development of novel analgesics. Also the data demonstrate that the supraspinal antinociceptive response to DPDPE requires either direct or indirect involvement of μ receptor activation, and that this response is pharmacologically distinct from that of the other δ agonists tested.

7.2 Abstract

The cloned δ–opioid receptor (DOR) is being investigated as a potential target for novel analgesics with an improved safety profile over μ–opioid receptor agonists such as morphine. The current study used antisense techniques to evaluate the role of DOR in mediating supraspinal antinociception in rats. All of the opioid agonists tested (δ-selective: deltorphin II, DPDPE, pCl-DPDPE, SNC80; μ-selective: DAMGO; i.c.v.) provided significant, dose-dependent antinociception in the paw pressure assay. Administration of a phosphodiester antisense oligonucleotide (i.c.v.) targeted against DOR inhibited antinociception in response to SNC80, deltorphin II and pCl-DPDPE compared with mismatch and saline-treated controls. However, antisense treatment did not inhibit the response to DPDPE or DAMGO. In contrast, the highly selective μ–antagonist CTOP blocked antinociception in response to ED_{80} concentrations of DAMGO and DPDPE, reduced the response to pCl-DPDPE, and did not alter the response to deltorphin II or SNC80. In total, these data suggest that DOR mediates the antinociceptive response to deltorphin II, SNC80 and pCl-DPDPE at supraspinal sites and further demonstrates that the DOR-mediated response to deltorphin II and SNC80 is independent of μ–receptor activation. Conversely, supraspinal antinociception in response to DPDPE is mediated by a receptor distinct from DOR; this response is directly or indirectly sensitive to μ-receptor blockade. The distinct pharmacological profile of DPDPE suggests that either this prototypical δ–agonist mediates antinociception by a direct, nonselective interaction at μ–receptors or DPDPE interacts with a novel δ-subtype that, in turn, indirectly activates μ–receptors in the brain.

7.3 Introduction

Opioid receptors are expressed throughout the central nervous system and are believed to modulate a variety of behavioral responses including antinociception, mood, dependence, motivation, and depression (Dhawan *et al.*, 1996). Three opioid receptor subtype genes (δ, μ, κ) have been cloned to date (Evans *et al.*, 1992; Kieffer *et al.*, 1992; Chen *et al.*, 1993; Yasuda *et al.*, 1993) and further receptor heterogeneity for all three classes of opioid receptors has been proposed (Dhawan *et al.*, 1996). Common analgesics such as morphine and related compounds preferentially interact with the μ–opioid receptor subtype (Pasternak, 1993). However, the therapeutic benefit of μ–opioid receptor agonists is diminished by the appearance of side effects including dependence, constipation and respiratory depression (Pasternak, 1993). Consequently, the therapeutic potential of agonists selective for other opioid receptors is under investigation. In this context, δ–agonists are of particular interest because they mediate antinociception in laboratory animals yet produce fewer adverse effects than μ–agonists (Quock *et al.*, 1999).

δ–Opioid receptors have been proposed to exist in two pharmacologically distinct subtypes, the evidence being based in large part on comparisons between the prototypical agonists deltorphin II and DPDPE. Thus, deltorphin II and DPDPE-mediated adenylyl cyclase stimulation in rat brain preparations (Búzás *et al.*, 1994; Olianas & Onali, 1995) as well as antinociception in both rats (Thorat & Hammond, 1997) and mice (Jiang *et al.*, 1991; Sofuoglu *et al.*, 1991b; Vanderah *et al.*, 1994) was differentially antagonized by various δ-antagonists. In addition, cross-tolerance in mice was not observed between the antinociceptive effects of DPDPE and deltorphin II, or with either of these peptides and the μ–agonist DAMGO (Mattia *et al.*, 1991). In total, these studies provide strong evidence that DPDPE and deltorphin II interact with distinct sites. However, the determination of the identity and function of these unique sites is complicated by the heterogeneous population of opioid receptors expressed in tissues such as brain (Mansour *et al.*, 1995) and the limited selectivity of the pharmacological tools used to resolve individual sites.

Antisense and genetic knockout approaches provide powerful alternative methods for the determination of receptor function (Fraser & Wahlestedt, 1997b). Antisense studies performed in mice support the existence of δ–receptor subtypes mediating antinociception in the brain and further suggest that these subtypes may arise from splice variants of the cloned δ–opioid receptor (DOR) gene (Rossi *et al.*, 1997). In contrast, supraspinal antinociception in response to δ–agonists, including DPDPE and deltorphin II, is reported to persist in DOR knockout mice

(Zhu *et al.*, 1999). The latter observation implies that certain δ–agonists interact with receptors other than DOR in the mouse brain, a finding that calls into question the role of DOR in mediating supraspinal antinociception.

The primary objective of the present study was to re-evaluate the role of DOR in the modulation of supraspinal antinociception in the rat. A second objective was to investigate discrepancies in the pharmacology of common δ–agonists with application to the possible existence of δ–opioid receptor subtypes.

7.4 Methods

7.4.1 Animals

Male Sprague-Dawley rats (250-300 g; Charles River, St-Constant, Québec, Canada) were housed in groups of three under an artificial 12 h light/dark cycle in a climate-controlled environment (23°C, relative humidity 60%). Food and water were provided ad libitum to animals throughout the housing period. Animals were used in compliance with the guidelines established by the Canadian Council for Animal Care.

7.4.2 Surgery

Rats were anesthetized by intra-peritoneal injection of ketamine (80 mg/kg) / xylazine (12 mg/kg) solution (Research Biochemicals International, Natick, MA) and placed in a stereotaxic device aligned with the interaural line. Each animal was implanted with a 23-gauge stainless steel cannula extending into the right lateral ventricle of the brain (i.c.v.; coordinates from bregma, AP, 0.8 mm; ML, 1.5 mm; DV, 3.5 mm). The guide cannula was fixed into place with dental cement applied to the surface of the skull. Rats were allowed 3 to 7 days to recover from surgery prior to random allocation into treatment groups.

7.4.3 Oligodeoxynucleotides

Phosphodiester antisense and mismatch oligodeoxynucleotides (ODN) were synthesized by Midland Certified Reagent Co. (Midland, TX). The 20-base antisense ODN (5'-GCA CGG GCA GAA GGC AGC GG-3') was designed complementary to nucleotides 112 to131 (exon 1) of the rat δ–opioid receptor, a region analogous to the 5' end of the coding sequence previously targeted in mouse (Bilsky *et al.*, 1996). A mismatch sequence (5'-GCA GCG GCA AGA GGA CGC GG-3') comprising the same base composition as the antisense sequence was designed to test the sequence-specificity of the antisense ODN. A search of the GenBank database confirmed that neither ODN sequence was homologous to any known nontarget genes in the rat. ODNs were

reconstituted in sterile 0.9% saline solution on the first treatment day and stored at 4°C for the duration of the treatment period. ODNs were administered i.c.v. in bolus injections of 20 μg/10 μl at 12 h intervals for 5 days. Vehicle-treated control subjects were dosed concurrently.

7.4.4 Chemicals

Naloxone and the opioid peptides [D-Ala^2,Glu^4]-deltorphin (deltorphin II), [D-Pen^{2,5}]-enkephalin (DPDPE), [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO) and D-Phe-c[-Cys-Tyr-D-Trp-Orn-Thr-Pen]-Thr-NH_2 (CTOP) were purchased from Research Biochemicals International. [D-Pen^2, pCl-Phe^4, D-Pen^5]-enkephalin (pCl-DPDPE) was purchased from Bachem (Basel, Switzerland). SNC80 was purchased from Tocris Cookson (Ballwin, MO). All drugs were weighed out and dissolved in 0.9% saline solution (or 10% dimethylsulfoxide (DMSO) for pCl-DPDPE) immediately before experimentation. The radioligand, [^{125}I]ARM-100613 ([^{125}I]-Dmt-c[-D-Orn-2-Nal-D-Pro-D-Ala-]), was synthesized in our laboratories as previously described (Fraser *et al.*, 1999).

7.4.5 Intracerebroventricular injections

ODNs and opioid drugs were administered via the intracerebroventricular (i.c.v.) route to conscious rats via the indwelling guide cannula. Injections were made using a 50-μl Hamilton syringe attached via PE20 polyethylene tubing to a 30-gauge injection cannula. Solution was injected over a period of 60 s. The injection cannula was left within the guide cannula for an additional 30 s to minimize reflux.

7.4.6 Antinociceptive testing

Each rat was tested on only one occasion. The same investigator performed all antinociceptive testing. Acute mechanonociception was measured using an analgesy meter (Ugo Basile, Varese, Italy). Briefly, a rat is gently restrained by hand and an increasing force is gradually applied to the right hind paw at a constant rate until the threshold force causing the rat to withdraw its paw

is determined. A maximal cut-off force of 510 g was implemented for this study. Data presented as % maximum possible effect (%M.P.E.) were determined using the following calculation:

$$\%MPE = [(response - baseline)/(cut\text{-}off - baseline)] \times 100\%$$

Animals were tested 12 h after the last ODN injection in experiments measuring antisense modulation of δ–opioid receptor function. In all experiments, baseline response thresholds were measured immediately before the administration of opioid agonist. The antinociceptive response to opioid agonists was measured at 15, 30, 45 and 60 minutes after drug treatment.

7.4.7 Radioligand binding studies

Antisense, mismatch and vehicle-treated control rats were decapitated immediately after the hour-long test session. The whole brain (minus cerebellum) was rapidly dissected and stored at -70°C before preparation of membrane homogenates. Brain homogenates were prepared from antisense, mismatch and saline-treated animals administered deltorphin II or DPDPE (n = 4 sets for each δ–agonist, respectively). On the day of homogenate preparation, brains were thawed and washed in 0.25 mM EDTA/0.5 M phosphate buffer solution (pH 7.4, 4°C) and then individually homogenized in a 20-ml solution of 50 mM Tris buffer, 2.5 mM EDTA and 0.1 mM phenylmethanesulfonyl fluoride (pH 7.0). P_2 homogenate fractions were prepared from two consecutive low speed centrifugation steps (1,200g). The resulting supernatant was then centrifuged twice at 48,000g (20 minutes for each spin) at 4°C. The P_2 pellet was resuspended in 5 ml of 50 mM Tris buffer (pH 7.4) and incubated at 37°C for 15 minutes to dissociate any receptor-bound endogenous opioid peptides. Membranes were centrifuged a final time at 48,000 x g and the pellet was resuspended in 5 ml of 50 mM Tris buffer/0.32 M sucrose solution (pH 7.0). Protein content was determined by modified Lowry assay with sodium dodecyl sulphate. Membrane aliquots were rapidly frozen in dry ice/ethanol and stored at -70°C until the day of the binding assay.

Saturation binding experiments were performed with the δ-selective radioligand [^{125}I]AR-M100613 (Fraser *et al.*, 1999) in the presence of 50 nM CTOP to minimize residual binding to μ-opioid receptors. Homogenates prepared from rats treated with vehicle, antisense or mismatch ODNs were assayed in parallel. Binding assays were performed in a solution of 50 mM Tris buffer, 3 mM $MgCl_2$ and 1 ml/mg bovine serum albumin (pH 7.4) on samples containing 60 to 80 μg protein in a total assay volume of 300 μl. Non-specific binding was determined by the addition of naloxone (10 μM). Samples were incubated for 3 h at room temperature before filtration (Brandel M-24 harvester) through Whatman GF/B filter strips previously soaked in 0.1% polyethyleneimine for 1 h. The filtrates were washed three times with 4 ml of ice-cold wash buffer (50 mM Tris (pH 7.0) with 3 mM $MgCl_2$) before transfer of filter disks into 12 x 75 mm polypropylene tubes for counting of γ-radiation (Packard Cobra II auto-gamma counter, Meridien, CT).

7.4.8 Data analysis

All analyses were performed using Prism (version 2.01) from GraphPad software (San Diego, CA). Dose-response effects were analyzed by two-way ANOVA with dose and time as between-subject and within-subject factors, respectively. ED_{50} and ED_{80} values were determined by linear regression analyses of the dose-response curves. Comparisons between the saline, antisense and mismatch-treated test groups were made by one-way ANOVA. Post hoc analyses were performed with Dunnett's multiple comparison test or Bonferroni *t* tests, as appropriate. Receptor binding data were analysed by nonlinear least-squares regression analysis.

7.5 Results

7.5.1 Opioid agonists modulate acute mechanonociception in the paw pressure assay

Dose-response curves were established for the μ–agonist, DAMGO, and the putative δ–agonists deltorphin II, DPDPE, pCl-DPDPE and SNC80. The different doses of each agonist were tested in parallel in comparison to vehicle-treated controls. Dose-response effects were normalized to the control baseline and data presented as %M.P.E. to facilitate comparison of dose-response curves for agonists tested on different days. All five test compounds gave a similar response profile; antinociception was maximal at the 15-min test interval, and also the 30-min test interval in the case of deltorphin II, but not significant at the 60-min test interval in comparison to saline-treated controls (data not shown). Treatment with each opioid significantly increased response thresholds in a dose-dependent manner (Figure 7.1): DAMGO, $ED_{50} = 0.096$ nmol; $F_{4,156} = 36$, $P < .001$; deltorphin II, $ED_{50} = 34$ nmol, $F_{4,184} = 39$, $P < .001$; DPDPE, $ED_{50} = 53$ nmol, $F_{3,124} = 22$, $P < .001$; pCl-DPDPE, $ED_{50} = 100$ nmol, $F_{4,152} = 32$, $P < .001$; SNC80, $ED_{50} = 240$ nmol, $F_{4,164} = 25$, $P < .001$.

7.5.2 Antisense inhibition of δ opioid receptor mediated antinociception

The antinociceptive response to ED_{80} concentrations of the opioid agonists (derived from the data presented in Figure 7.1) were measured in rats pretreated with antisense (or mismatch) oligonucleotides (i.c.v.) targeted against the δ–opioid receptor in comparison to vehicle-treated controls. As expected, the peak antinociceptive effects for each opioid agonist were observed at the 15- to 30-min test intervals in vehicle-treated subjects. Figure 7.2, A to E, shows the effects of antisense (and mismatch) treatment on rats administered opioid agonists. Antisense treatment significantly inhibited increases in nociceptive response thresholds in response to SNC80, deltorphin II and pCl-DPDPE (Figure 7.2, A-C, respectively) but not DPDPE or the μ–agonist DAMGO (Figure 7.2, D and E respectively). In comparison, treatment with the mismatch

sequence did not significantly alter the antinociceptive response to any of the opioid agonists at any test interval ($P > .05$). In addition, antisense or mismatch treatment did not significantly alter the baseline nociceptive responses measured for all treatment groups just prior to the administration of opioid agonists.

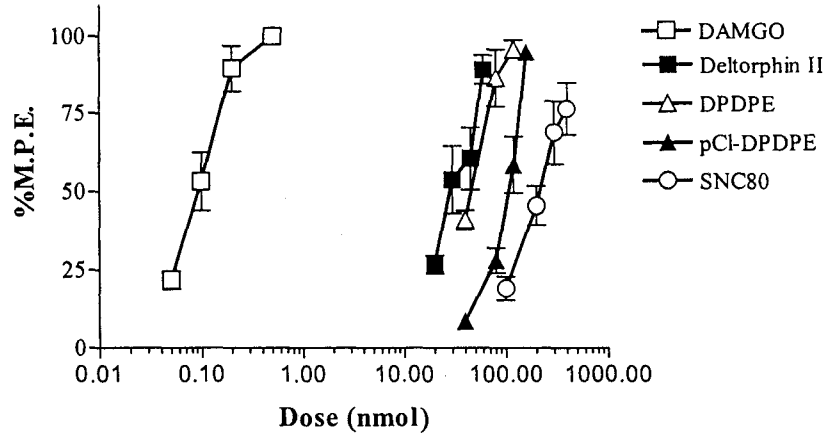

Figure 7.1: Antinociceptive dose-response curves for DAMGO, deltorphin II, DPDPE, pCl-DPDPE and SNC80 in the paw pressure assay.
The data represent the peak antinociceptive effects for each agonist measured at 15 min (or 30 min for deltorphin II) after injection (i.c.v.) for each drug. Data are presented as a percentage of the maximum possible effect (%M.P.E.) that can be measured using this test paradigm. Each data point represents the mean ± S.E.M. response of 8 to12 rats.

To determine whether the antisense inhibition of δ–agonist-induced antinociception was associated with changes in δ–opioid receptor density, saturation binding was performed in parallel on rat brain membrane homogenates prepared from vehicle, antisense and mismatch-treated subjects. [^{125}I]AR-M100613 binding (in the presence of 50 nM CTOP) was saturable and best fit to a one-site model in membranes prepared from all treatment groups (data not shown). Determination of receptor B_{max} values revealed a significant 25% decrease in δ–opioid receptor density in membranes prepared from antisense-treated rats in comparison to vehicle-treated controls (Dunnett's test: $P < .05$, Table 7.1). The degree of receptor knockdown was not significantly different in antisense-treated rats tested with either DPDPE or deltorphin II. In comparison, mismatch treatment did not significantly alter δ–opioid receptor density. Also, there were no significant differences in receptor binding affinity (K_d) between treatment groups.

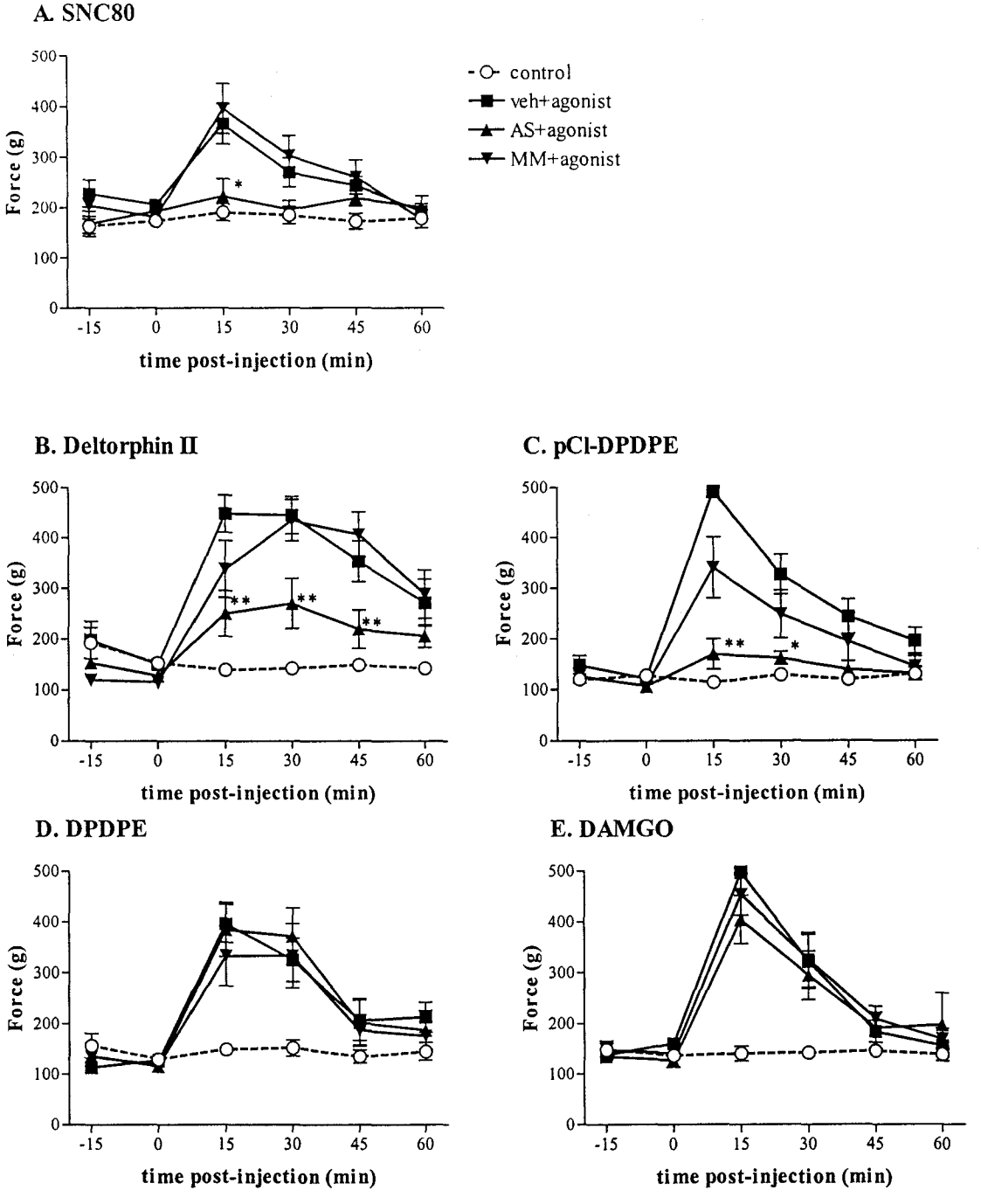

Figure 7.2: Administration of antisense oligonucleotides targeting δ–opioid receptors inhibited the antinociceptive response to SNC80 (400 nmol), deltorphin II (60 nmol) and pCl-DPDPE (160 nmol), but not DPDPE (100 nmol) or DAMGO (0.2 nmol).

* and ** represent significant differences in comparison to the vehicle + agonist group where $P < .05$ and .01, respectively (Dunnett's *t* test). Each data point represents the mean ± S.E.M. response of 7 to11 rats. Veh, vehicle; AS, antisense ODN; MM, mismatch ODN. Control rats were administered saline (i.c.v.) twice daily to simulate the antisense treatment regimen and also administered saline (i.c.v.) to control for drug treatment on the test day.

	B_{max}	K_d	N
	(fmol/mg protein)	*(nM)*	
Saline-treated	68.1 ± 5.4	0.090 ± 0.005	8
Antisense-treated	51.1 ± 4.6*	0.088 ± 0.006	8
Mismatch-treated	67.0 ± 4.2	0.081 ± 0.006	8

Table 7.1: Effect of antisense treatment on δ-opioid receptor density in whole brain homogenates

[^{125}I]AR-M100613 saturation binding was performed on sets of whole brain homogenates from saline-, antisense- and mismatch-treated rats administered either deltorphin II or DPDPE (n = 4 sets for each δ-agonist). Binding assays were performed in the presence of 50 nM CTOP to minimize residual binding of the radioligand to μ–opioid receptors. Each homogenate sample was assayed separately. Data are presented as mean ± S.E.M. *Significant difference from the saline-treated group ($P < .05$, Dunnett's test).

7.5.3 Inhibition of antinociception by the μ-opioid antagonist CTOP

Preliminary experiments indicated that 0.5 nmol CTOP (i.c.v., given 10 min before agonist) was the minimal dose required to completely block the antinociceptive effects of the μ–agonist DAMGO (0.2 nmol i.c.v.; data not shown). Figure 7.3 shows the effects of CTOP (0.5 nmol i.c.v., given 10 min before agonist) on the antinociceptive responses to ED_{80} concentrations of deltorphin II, SNC80, pCl-DPDPE, DPDPE and DAMGO (60, 400, 160, 100 and 0.2 nmol i.c.v., respectively; tested 15 min after dosing). This experiment was performed in two parts where deltorphin II, DPDPE and DAMGO, and then SNC80 and pCl-DPDPE, were tested in parallel alongside vehicle and CTOP-treated controls. The response thresholds from the vehicle and CTOP-treated control subjects did not differ between experiments; these data were pooled and are presented in Figure 7.3. Pretreatment with CTOP significantly inhibited the antinociceptive responses to DAMGO and DPDPE (Bonferroni t test: $t = 9.58$, df = 16, $P < .001$ and $t = 9.03$, df = 16, $P < .001$, respectively). Little, if any, residual agonist response occurred in the presence of the antagonist. In addition, CTOP inhibited the antinociceptive response to pCl-DPDPE (Bonferroni t test: $t = 3.49$, df = 12, $P < .005$), although a significant agonist response occurred in the presence of the antagonist in comparison to CTOP-treated controls (Bonferroni t test: $t = 3.69$, df = 8, $P < .01$). In contrast, CTOP did not inhibit the response to deltorphin II nor SNC80

(Bonferroni t test: $t = 0.89$, df = 16, $P = 0.39$ and $t = 0.92$, df = 15, $P = 0.37$, respectively), or alter the response threshold in saline-treated controls (Bonferroni t test: $t = 1.19$, df = 15, $P = 0.25$).

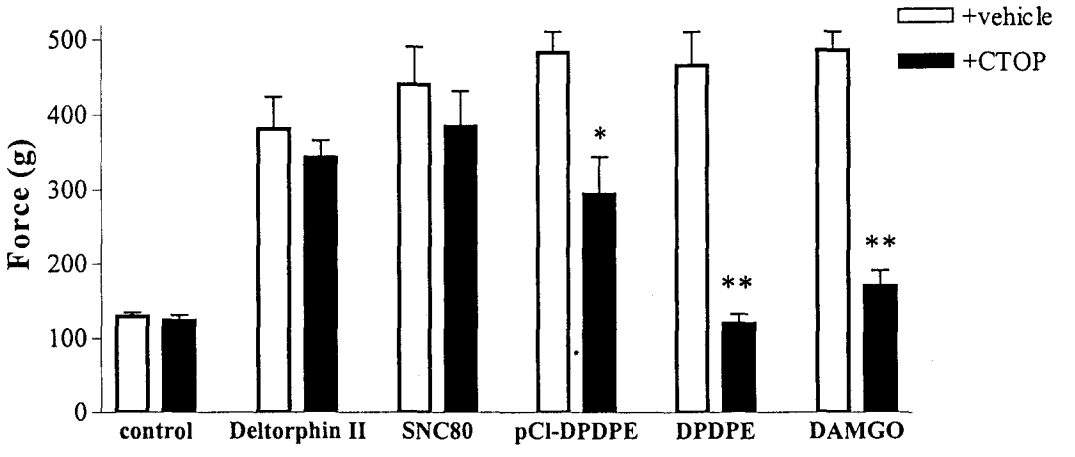

Figure 7.3: Pretreatment with CTOP (0.5 nmol, i.c.v., 10 min before agonist) antagonized the antinociceptive response to DAMGO (0.2 nmol), DPDPE (100 nmol), and pCl-DPDPE (160 nmol), but not deltorphin II (60 nmol) or SNC80 (400 nmol).

The figure depicts the antinociceptive response to opioid agonist (i.c.v.) at 15 min post injection. Each column (□, + vehicle; ■, + CTOP) represents the mean ± S.E.M of 6 to 9 rats. * and ** represent significant differences between the CTOP-treated and untreated groups for each agonist condition where $P < .005$ and $P < .001$, respectively (Bonferroni t test).

7.6 Discussion

The present study demonstrates that the antinociceptive effects of deltorphin II, SNC80 and pCl-DPDPE, but not DPDPE, were inhibited by antisense treatment targeted against the cloned DOR. Additional studies demonstrated that the antinociceptive response to DPDPE was completely blocked by pretreatment with the selective μ–antagonist CTOP. In total, these findings confirm the role of DOR in the modulation of antinociception at supraspinal sites and further suggest that the pharmacological actions of DPDPE are distinct from those of other δ–agonists.

Opioid receptors in the brain modulate descending pain pathways and consequently increase nociceptive response thresholds (Basbaum & Fields, 1984). The antinociceptive response to μ–agonists administered into the brain has been clearly demonstrated (Fang *et al.*, 1986). In comparison, in studies performed in rats, δ-opioid agonists (administered i.c.v.) have been reported to have discrepant effects on nociception that appear to be contingent upon the agonists and the nociceptive assays used (Negri *et al.*, 1991a; Adams *et al.*, 1993; Ossipov *et al.*, 1995a; Adams *et al.*, 1993). The paw pressure assay is more sensitive to the effects of opioids (i.c.v.) than tests measuring spinal reflex responses (Hayes *et al.*, 1987; Miaskowski *et al.*, 1991). The outcome of this nociceptive test, the paw withdrawal response, is an organized, unlearned behavior requiring supraspinal processing (Dubner, 1989). In the present study, all the compounds tested attained maximal efficacy in the paw pressure assay.

It has been suggested that the antinociceptive response to high concentrations of various δ–agonists may in fact be a consequence of a low affinity, non-selective direct activation of μ–receptors (Negri *et al.*, 1996). This hypothesis was tested in the present study using antisense and CTOP administration to assess possible DOR and μ–receptor involvement, respectively. Antisense treatment inhibited the antinociceptive response to deltorphin II, pCl-DPDPE and SNC80 in a sequence-specific and pharmacologically selective manner. The inhibition of

response to these agonists was associated with a reduction of δ–opioid binding sites in brain homogenates prepared from antisense-treated rats. These findings suggest that DOR plays an important role in the modulation of supraspinal pain pathways in the rat, a finding consistent with that of previous antisense studies performed in the mouse (Standifer *et al.*, 1994; Bilsky *et al.*, 1996; Rossi *et al.*, 1997). Moreover, DOR-mediated antinociception is independent of μ–receptor activation based on the inability of the selective μ–antagonist CTOP to inhibit deltorphin II or SNC80 mediated increases in paw withdrawal latency.

The pharmacology of DPDPE was distinct from that of the other δ–agonists used in this study, in two respects: insensitivity to antisense treatment and complete antagonism by CTOP. It is unlikely that these findings reflect differences in agonist efficacy between DPDPE and the other δ-agonists tested because all agonists were used at approximately ED_{80} concentrations in these experiments. The observed lack of inhibition by the antisense sequence suggests either that DPDPE does not modulate supraspinal nociception exclusively via the DOR receptor or that DPDPE activates an anatomically distinct receptor population that is differentially affected by the antisense treatment. Similar findings have been reported in antisense studies performed in mice (Bilsky *et al.*, 1996; Rossi *et al.*, 1997). In addition, a recent study demonstrates that the effects of DPDPE (i.c.v.), but not deltorphin II, on locomotor activity are resistant to antisense treatment in rats (Negri *et al.*, 1999). The results of these antisense studies may appear to contrast with published reports where δ-selective antagonists have been found to block the effects of DPDPE (Búzás *et al.*, 1994; Sofuoglu *et al.*, 1991b). However, the antisense techniques used in the present study specifically target DOR, whereas the antagonists previously used may inhibit the effects of DPDPE via interactions with a heterogeneous population of sites.

The second distinctive feature of DPDPE antinociception, in comparison to that of other δ–agonists, was its complete blockade by the μ-selective antagonist CTOP. This finding is in

agreement with data presented in previous studies in mice where the antinociceptive effects of DPDPE were blocked by pretreatment with the highly selective μ–antagonist CTAP (D-Phe-c[-Cys-Tyr-D-Trp-Arg-Thr-Pen]-Thr-NH_2) at the level of the brain (Kramer *et al.*, 1989) or spinal cord (He & Lee, 1998). Further support for a μ component to DPDPE-mediated antinociception has been provided by studies with μ-receptor knockout mice (Sora *et al.*, 1997; Fuchs *et al.*, 1999; Matthes *et al.*, 1998; Hosohata *et al.*, 2000; but see Loh *et al.*, 1998). The present study demonstrates that the μ-dependent effects of DPDPE can occur at doses that are submaximal with respect to antinociception. In comparison, the DPDPE analogue pCl-DPDPE appears to mediate supraspinal antinociception via both μ-dependent and -independent sites based on the inhibition of pCl-DPDPE effects by both DOR antisense and CTOP pretreatment.

Several findings suggest that the observed μ–receptor dependence of DPDPE antinociception may reflect, at least in part, a direct interaction of the agonist with supraspinal μ–opioid receptors. For example, DPDPE proved more potent than pCl-DPDPE in the present antinociceptive assay even though DPDPE has a lower binding affinity for δ–opioid receptors, and a much higher affinity for μ–receptors (Kramer *et al.*, 1993). Also, the greater sensitivity of DPDPE to CTOP inhibition is consistent with its inferior δ/μ receptor binding selectivity in comparison to pCl-DPDPE (Kramer *et al.*, 1993). Furthermore, binding studies performed on cell lines expressing recombinant human opioid receptors have revealed only moderate (approximately 100-fold) δ/μ selectivity for DPDPE and for the reversible δ-antagonists reported to block the effects of DPDPE [i.e. naltrindole, BNTX (9,7-ben-zylidene naltrexone), naltriben, ICI174,864, all less than 200-fold δ/μ selective (Payza *et al.*, 1996)]. Similarly, the irreversible antagonist DALCE, which blocks certain effects of DPDPE (Jiang *et al.*, 1991; Vanderah *et al.*, 1994) also appears to have some affinity for μ–receptors (Bowen *et al.*, 1987). Thus, in tissues such as brain where μ–receptors are predominant (Mansour *et al.*, 1995), it is conceivable that

even low levels of μ–receptor occupancy by DPDPE and by δ-selective antagonists may be behaviorally significant.

Alternatively, DPDPE may elicit supraspinal antinociception by acting on certain δ–sites that, in turn, potentiate μ–receptor activity (Traynor & Elliot, 1993). This hypothesis is supported by neuroanatomical studies demonstrating that δ– and μ–opioid receptors are coexpressed in certain brain regions (Mansour *et al.*, 1995). In addition, previous studies have shown that the coadministration of DPDPE with μ–agonists caused a synergistic increase in supraspinal antinociception (Miaskowski *et al.*, 1991; Negri *et al.*, 1995). Although the nature of this μ/δ receptor interaction is unclear at present, it likely does not occur at the level of signal transduction because δ–agonist-induced G-protein activation or adenylyl cyclase inhibition were not affected in μ–receptor knockout mice (Matthes *et al.*, 1998). Alternatively, pharmacological data supports the existence of a μ/δ receptor complex (Rothman *et al.*, 1988; Traynor & Elliot, 1993) such as the recently identified hetero-oligomer formed between DOR and the cloned μ–opioid receptor (George *et al.*, 2000). Nevertheless, the antisense experiments in the present study suggest that any indirect activation of μ–receptors by DPDPE was likely mediated by DOR-independent sites.

The existence of δ–opioid receptor subtypes has been postulated, in large part, on the basis of differences in the pharmacology of the prototypical δ–agonists, deltorphin II and DPDPE (Jiang *et al.*, 1991; Mattia *et al.*, 1991; Vanderah *et al.*, 1994). However, a second subtype arising from a gene distinct from DOR was not revealed by [^{3}H]DPDPE or [^{3}H]deltorphin II binding in brain homogenates prepared from DOR knockout mice (Zhu *et al.*, 1999). Alternatively, previous antisense studies in mice suggest that splice variants of the common DOR gene may give rise to receptor subtypes (Rossi *et al.*, 1997). The present study demonstrates that DPDPE interacts with a site that is distinct from that targeted by other δ–agonists; this site is directly or indirectly

associated with μ–opioid receptors. Further studies are required to determine whether the DPDPE site is a novel δ–opioid receptor (possibly arising from a different gene or DOR splice variant) or the μ–opioid receptor.

Acknowledgements

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8 Inhibition of δ Opioid Receptor Gene Function *in vivo* by Peptide Nucleic Acids

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8.1 Preface

The findings presented in the previous chapter demonstrated the power of antisense technology as a tool for the determination of gene function *in vivo*. However, the phosphodiester oligonucleotides used in Chapter 7, and the phosphorothioate oligonucleotides commonly used, have serious limitations as tools for functional genomics due, in part, to their nucleotide backbone. Peptide nucleic acids (PNA) are comprised of a peptide backbone and thus may have significant advantages as antisense agents over traditional oligonucleotides. The primary objective of this study was to demonstrate the effects of PNA as an antisense agent *in vivo*. The cloned δ opioid receptor was targeted using the same antisense treatment regimen used for the phosphodiester oligonucleotide in Chapter 7. This study demonstrates that repeated exposure to a PNA sequence complementary to a portion of the cloned δ opioid receptor inhibited δ agonist mediated antinociception and locomotor activity in a target-specific, sequence-specific and reversible manner consistent with an antisense mechanism. These findings confirm the role of the cloned δ opioid receptor in modulating antinociceptive and psychostimulant behaviour and also provide one of the first demonstrations that PNA molecules are effective antisense agents *in vivo*.

8.2 Abstract

Peptide nucleic acids (PNA) are synthetic analogues of DNA that hybridize to complementary oligonucleotide sequences with exceptional affinity and target specificity. The stability of PNA in biological fluids together with the unique hybridization characteristics of these structures suggests that PNA may have considerable potential as antisense agents for experimental use *in vivo*. To test this hypothesis, we attempted to modulate supraspinal δ opioid receptor function in rats using PNA sequences designed to be complementary to a region of the rat δ opioid receptor. Repeated intracerebroventricular administration of PNA over a period of five days significantly inhibited the antinociceptive response and locomotor response to selective δ opioid receptor agonists. PNA attenuated δ opioid receptor function in a sequence-specific, target-specific and reversible manner characteristic of the functional inhibition caused by an antisense mechanism. There were no apparent toxicities arising from the PNA treatment based on the behaviour of the animals and inspection of the treated tissues. Saturation binding studies on brain homogenates did not reveal any significant difference in receptor B_{max} between treatment groups. However, [^{35}S]GTPγS binding assays demonstrated a significant decrease in agonist efficacy in homogenates prepared from antisense treated rats. Taken together, these results demonstrate that peptide nucleic acids are effective antisense agents *in vivo* and suggest that PNA may be a useful alternative to phosphodiester or phosphorothioate oligonucleotides, or variants thereof, for determination of gene function *in vivo*.

8.3 Introduction

Antisense technology has already proven to be useful both as an experimental tool in functional genomics (Wahlestedt *et al.*, 1993b) and as a source of novel therapeutics. However, antisense studies performed with phosphodiester- or phosphorothioate-based oligonucleotides are often limited by the appearance of incomplete knockdown of the gene product and sequence-independent effects in brain and other tissues. These limitations are likely characteristic of the oligodeoxynucleotide chemistry and thus may be circumvented by using alternative antisense molecules (Fraser & Wahlestedt, 1997a).

Peptide nucleic acids (PNA) are synthetic analogues of deoxynucleotide bases (Nielsen *et al.*, 1991; Fraser & Wahlestedt, 1997b) capable of hybridizing with complementary DNA or RNA sequences via Watson-Crick base pairing and helix formation (Egholm *et al.*, 1993; Brown *et al.*, 1994). PNA oligomers have demonstrated sufficient uptake to support antisense activity in cultured cells (Good & Nielsen, 1998; Taylor *et al.*, 1997) and primary cultures of rat cortical neurones (Aldrian-Herrada *et al.*, 1998). In addition, it has been reported that naked (Tyler *et al.*, 1998) or modified PNA oligomers are effective antisense agents *in vivo* (Pooga *et al.*, 1998). PNA oligomers likely inhibit gene function by hybridizing with target mRNA to sterically obstruct translation and the consequent synthesis of target protein (Bonham *et al.*, 1995; Knudsen & Nielsen, 1996).

The achiral, charge-neutral polyamide backbone of the PNA molecule cannot contribute to the electrostatic interaction essential for protein binding. Thus, PNA oligomers can avoid the sequence-independent effects of traditional antisense oligonucleotides, which indiscriminately interact with a variety of endogenous proteins (Stein, 1996). PNA oligomers also do not induce ribonuclease H activity (Bonham *et al.*, 1995) and consequently are not prone to sequence-dependent side effects resulting from ribonuclease H-mediated cleavage of non-target mRNA (Weidner & Busch, 1994; Lima & Crooke, 1997b). In addition, PNA oligomers are not susceptible to degradation by endogenous nucleases or proteases and consequently demonstrate improved stability in biological fluids in comparison to the traditional antisense oligonucleotides (Demidov *et al.*, 1998). Finally, the charge-neutral backbone of PNA oligomers increases both the affinity and specificity of hybridization to complementary nucleotides (Egholm *et al.*, 1993). Together, these characteristics suggest that PNA oligomers may provide a more complete knockdown of the target gene product with an improved toxicity profile over traditional antisense oligonucleotides *in vivo*.

To investigate the potential of PNA as antisense agents in the living brain, PNA sequences were designed complementary to the rat δ opioid receptor gene. The δ opioid receptor was chosen as a target for PNA treatment based on its susceptibility to antisense treatment *in vivo* using conventional oligonucleotides (Bilsky *et al.*, 1996; Negri *et al.*, 1999). Receptor function was evaluated in antinociceptive and locomotor behavioural assays in keeping with the predicted role of supraspinal δ opioid receptors in the rat (Ossipov *et al.*, 1995a; Longoni *et al.*, 1991). In this report, we demonstrate sequence-specific and target-specific inhibition of δ opioid receptor gene function in the rat and suggest that PNA oligomers are a viable alternative to phosphodiester or phosphorothioate-based oligonucleotides for use in antisense studies *in vivo*.

8.4 Materials and Methods

8.4.1 PNA constructs.

PNA sequences inhibit functional gene expression by the steric hindrance of proteins involved in the process of translation. Antisense agents that inhibit protein function in this manner appear to be most effective when directed to areas close to the initiation codon where the secondary and tertiary structure of the mRNA facilitates protein interaction (Bonham *et al.*, 1995). Consequently, the antisense PNA sequence (5'-GTGTCCGAGACGTTG-3') was designed complementary to a region proximal to the start codon of the δ opioid receptor mRNA (Evans *et al.*, 1992; Kieffer *et al.*, 1992). A mismatch sequence (5'-GTTGCCGAGACTGTG-3') where two base pairs are reversed was designed as a measure of the sequence-specificity of the antisense oligomer. The mismatch sequence maintained the base composition and oligomer polarity of the antisense sequence and thus provided a stringent control. A search of the GenBank© database confirmed that the PNA sequences were not homologous to any known non-target genes in the rat. Unmodified PNA sequences were synthesized and HPLC purified by PerSeptive Biosystems (Framingham, MA). The 15*mer* PNA antisense oligomer presented in this report proved to be the most effective of three PNA sequences tested in preliminary assays (data not shown).

8.4.2 Preparation of animals for administration of PNA constructs and opioid agonists

Animals were handled in strict adherence to the guidelines established by the Canadian Council for Animal Care. Male Sprague-Dawley rats (250-300g) were anaesthetized with 80 mg/kg body weight ketamine/xylazine solution (RBI, Natick MA) and placed in a stereotaxic device. Each animal was then implanted with a 23 gauge canula extending into the right lateral ventricle (coordinates from bregma, AP: 0.8 mm, ML: 1.5 mm, DV: 3.5 mm) and fixed into place with dental cement. Correct canula placement was confirmed by histology performed on brains obtained from control rats. Rats were allowed three or more days to recover from the surgery prior to random allocation into treatment groups and subsequent administration of PNA. PNA constructs were diluted in sterile 0.9% saline solution (Astra Canada, Mississauga ON) and administered via the guide canula at a dose of 0.45 nmol twice daily for 5 days. Twelve hours after the final PNA treatment, the antinociceptive response to opioid agonists was measured in either the paw pressure assay or the locomotor activity assays. The opioid agonists (DAMGO and deltorphin II supplied by RBI, Natick, MA; SNC80 supplied by Tocris Cookson Inc., Ballwin, MO) were dissolved in 0.9% saline solution and administered to rats via the guide

canula immediately prior to testing. All PNA and drug treatments were injected via the guide cannula in a volume of 10 µl using a 50 µl Hamilton syringe attached to a catheter (15 cm) constructed from PE20 polyethelene tubing and terminating in a 30-gauge needle. Solution was injected slowly over a period of 60 seconds and the needle was left within the guide cannula for an additional 30 seconds after the injection. In all cases, rats were treated concomitantly with 0.9% saline solution as a control for the PNA/drug treatment paradigm.

8.4.3 Paw pressure assay.

The antinociceptive response to opioid agonists was measured using an analgesy-meter (Ugo Basile, Italy). Briefly, an increasing amount of force is applied to the right hind paw of each rat until a threshold force is determined (i.e. the amount of force causing the rat to attempt to withdraw its paw). A maximal cut-off force of 510 g was implemented for this study. Data presented as % maximal possible effect (%MPE) were determined using the following calculation:

$$\%MPE = [(response - baseline)/(cut\text{-}off - baseline)] \times 100\%$$

8.4.4 Locomotor activity testing.

Activity was measured using the AM1051 Activity Monitor (Benwick Electronics, UK). The plastic cage within the monitor measured approximately 30 x 18 x 18 cm. The monitor was equipped with a 12 x 7 infra-red beam matrix (ie. 2.54 cm grid) on both the lower level (set at a height of 3 cm) and the upper level (set at a height of 12 cm). The activity monitor operates by recording the number of times the infra-red beams change from broken to unbroken (or vice versa) and incrementing the relevant counters. Horizontal locomotion and rearing (vertical movement) were recorded for each 10 minute interval throughout the duration of the experiment. Rats were habituated in the activity monitor cages for approximately 1h before drug administration. In order to minimize disturbing these habituated animals, rats were injected with either deltorphin II (0.3 nmol) or 0.9% saline solution in the activity monitor cage with minimal handling. Data recording was started immediately following the injection. All activity experiments were conducted with parallel treatment groups between 8h30 and 15h.

8.4.5 Tissue preparation.

Immediately following the behavioural testing, rats were decapitated and brains (minus cerebellum) were rapidly removed and stored at -70°C. Previous studies with phosphorothioate oligodeoxynucleotides indicate that these structures have limited distribution proximal to the injection site following i.c.v. administration (Grzanna *et al.*, 1998). Based on these findings, the brain hemisphere ipsilateral to the injection site was used to prepare membrane homogenates in the present study. On the day of homogenate preparation, brain hemispheres were thawed and washed in 0.25 mM EDTA/0.5 M phosphate buffer solution (pH 7.4, 4°C). Tissues were individually homogenized in a 20 ml solution of 50 mM Tris buffer, 2.5 mM EDTA and 0.1 mM PMSF (pH 7.0). P_2 homogenate fractions were prepared following two consecutive low speed (1,200 x *g*) centrifugation steps and the collection and pooling of the subsequent supernatants. The supernatant was than centrifuged twice at 48,000 x *g* (20 minute for each spin) at 4°C. The P_2 pellet was resuspended in 50 mM Tris buffer (pH 7.4) and incubated at 37°C for 15 minutes to dissociate any receptor-bound endogenous opioid peptides. Membranes were centrifuged a third time at 48,000 x *g* as before and the final pellet was resuspended in 5 ml of 50 mM Tris buffer/0.32 M sucrose solution (pH 7.0). Protein content was determined by modified Lowry assay with sodium dodecyl sulphate (SDS). Membrane aliquots were rapidly frozen in dry ice/ethanol and stored at -70°C until the day of the binding assays. [^{3}H]Naltrindole and [^{35}S]GTPγS binding assays were assayed in parallel using a common membrane aliquot.

8.4.6 Saturation binding assay

Saturation binding curves were performed on rat brain homogenates with the selective δ opioid receptor radioligand [^{3}H]naltrindole (DuPont NEN, Wilmington, DE; specific activity 34.7 Ci/mmol). The incubation buffer was comprised of 50 mM Tris (pH 7.4) with 3 mM $MgCl_2$ and 1mg/ml bovine serum albumin, with the peptide CTOP (50 nM; RBI, Natick, MA) added to block residual binding of the radioligand to μ opioid receptors. The binding assay was performed on samples containing 70-90 μg tissue protein in a total assay volume of 300 μl. Non-specific binding was determined by the addition of diprenorphine (1 μM; RBI, Natick, MA). Samples were incubated for 2 hours at room temperature. The assay was terminated by filtration (Brandel M-24 harvester, Gaithersberg, MD) through Whatman GF/B filter strips previously soaked in 0.5% polyethlyeneimine for 1 hour. Filters were washed three times with 4 ml of ice-cold wash buffer (50 mM Tris (pH 7.0) with 3 mM $MgCl_2$). Radioactivity was measured using a liquid scintillation counter (Tri-carb 2100TR, Packard, Meridien, CT).

8.4.7 [^{35}S]GTPγS binding assay.

This assay was adapted from published procedures (Traynor & Nahorski, 1995). The incubation buffer was comprised of 50 mM HEPES (pH 7.4), 20 mM NaOH, 5 mM $MgCl_2$, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% BSA and 120 μM guanosine diphosphate (GDP). In addition, 2 μM CTOP was added to the incubation buffer to block any residual SNC80 mediated increases in [^{35}S]GTPγS binding due to activation of μ opioid receptors. SNC80 (0.1 – 10,000 nM), [^{35}S]GTPγS (final concentration of 0.14 - 0.17 nM) and rat brain membranes (32-34 μg tissue protein/sample) were combined in a final assay volume of 300 μl. Basal [^{35}S]GTPγS binding was determined in parallel in the absence of SNC80. All samples were incubated for 1 hr at room temperature prior to filtration (Brandel M-24 harvester, Meridien CT) through Whatman GF/B filters that were pre-soaked for 1 hour in water. Filters were washed three times with 4 ml of ice cold wash buffer (50 mM Tris, 5 mM $MgCl_2$, 50 mM NaCl, pH 7.0). [^{35}S]GTPγS binding was measured using a liquid scintillation counter (Tri-carb 2100TR, Packard, Meridien, CT).

8.4.8 Data analysis.

All analyses were performed using Prism (version 2.01) from GraphPad Software (San Diego, CA). The data from the behavioural assays were analyzed by one-way ANOVA and Dunnett's test (where applicable) for each time-point. Comparisons were made between the saline-treated (+drug) group and the antisense and mismatch-treated groups. Receptor binding data were subjected to non-linear least squares regression analysis appropriate for saturation binding to a single site. [^{35}S]GTPγS binding data were analyzed by non-linear regression analysis using a sigmoidal dose-response (variable slope) model. Maximal stimulation of SNC80 induced [^{35}S]GTPγS binding is defined as the peak increase over basal levels observed in brain homogenates prepared from saline-treated animals. The % maximal stimulation data presented in Table 1 was determined from the upper plateau of the dose-response curve determined from the non-linear regression analysis. EC_{50} values were determined relative to the maximal effect of SNC80 on [^{35}S]GTPγS binding for individual homogenate samples. Statistical analysis of these data was performed by one-way ANOVA followed by Dunnett's post-hoc test (comparison to the saline-treated group) where applicable.

8.5 Results

8.5.1 Antinociceptive response to opioid agonists in the paw pressure assay

Concentration-response curves were established for the opiate receptor agonists DAMGO, deltorphin II and SNC80 in the paw pressure assay of acute mechano-nociception (Figure 8.1). All three opioid agonists had a similar response profile; antinociception was maximal 15 minutes post injection and the duration of response lasted less than 1 hour for each dose. Each opiate agonist was able to reduce the nociception index by up to 80% within the dose ranges tested. Agonist concentrations giving 80% of maximal response (EC_{80}) were determined for each compound (i.e. 60 nmol, 400 nmol and 0.2 nmol for deltorphin II, SNC80 and DAMGO respectively). These agonist concentrations were used in subsequent studies investigating the capacity of PNA oligomers to inhibit agonist-induced antinociception.

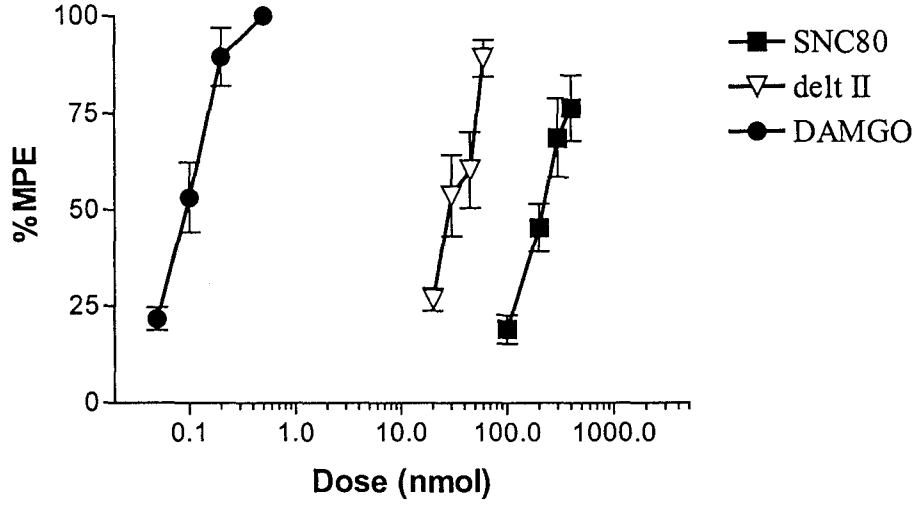

Figure 8.1 Antinociceptive effects of DAMGO, deltorphin II and SNC80 in the paw pressure assay.
The data represent the peak antinociceptive effects for each agonist measured at 15 minutes after injection (i.c.v.). %MPE is a measure of the antinociceptive effect of each opioid agonist (in comparison to saline-treated controls) as a percentage of the maximal possible effect that can be measured using this paradigm. Data is presented as mean $\pm$ s.e.m. (n = 8-12 rats)

8.5.2 Inhibition of δ opioid receptor mediated antinociception by PNA

The antinociceptive response to EC_{80} concentrations of the selective δ opioid receptor agonists deltorphin II and SNC80 are shown in Figure 8.2A and Figure 8.2B, respectively. As expected, the antinociceptive response to both compounds peaked at 15 minutes after injection and was barely detectable at 1 hour after injection. Treatment with the PNA antisense sequence significantly reduced the antinociceptive response to deltorphin II and SNC80 over the course of the test session ($p<0.001$ and $p<0.01$, respectively). By comparison, treatment with the PNA mismatch sequence did not significantly alter the antinociceptive response to either δ agonist at any time interval ($p>0.05$). In addition, neither PNA antisense nor PNA mismatch treatment were effective in inhibiting the antinociceptive response to an EC_{80} concentration of the μ agonist DAMGO (Figure 8.2C). Finally, treatment with PNA antisense or PNA mismatch did not alter the baseline nociceptive responses of animals in the paw pressure assay measured before the administration of the opiate agonists (Figure 8.2A-C).

The restoration of the antinociceptive response to deltorphin II was measured following the termination of PNA treatment (Figure 8.3). A recovery period of 5 days was chosen to accommodate the delay contingent upon the rate of δ opioid receptor turnover (Jiang *et al.*, 1991). Full recovery of deltorphin II mediated antinociception was observed in rats previously treated with PNA antisense.

8.5.3 Inhibition of δ opioid receptor mediated locomotor activity by PNA

PNA antisense treatment did not alter baseline exploratory activity in rats in comparison to saline treated controls (data not shown). However, PNA antisense treatment significantly attenuated deltorphin II mediated increases in horizontal locomotor activity (HLA) and rearing activity in comparison to saline and mismatch treated controls at the 10 and 20 minute intervals of the test session (Figure 8.4A-B). The mismatch-treated group did not vary significantly from the saline-treated group at any test interval in these locomotor assays ($p>0.05$).

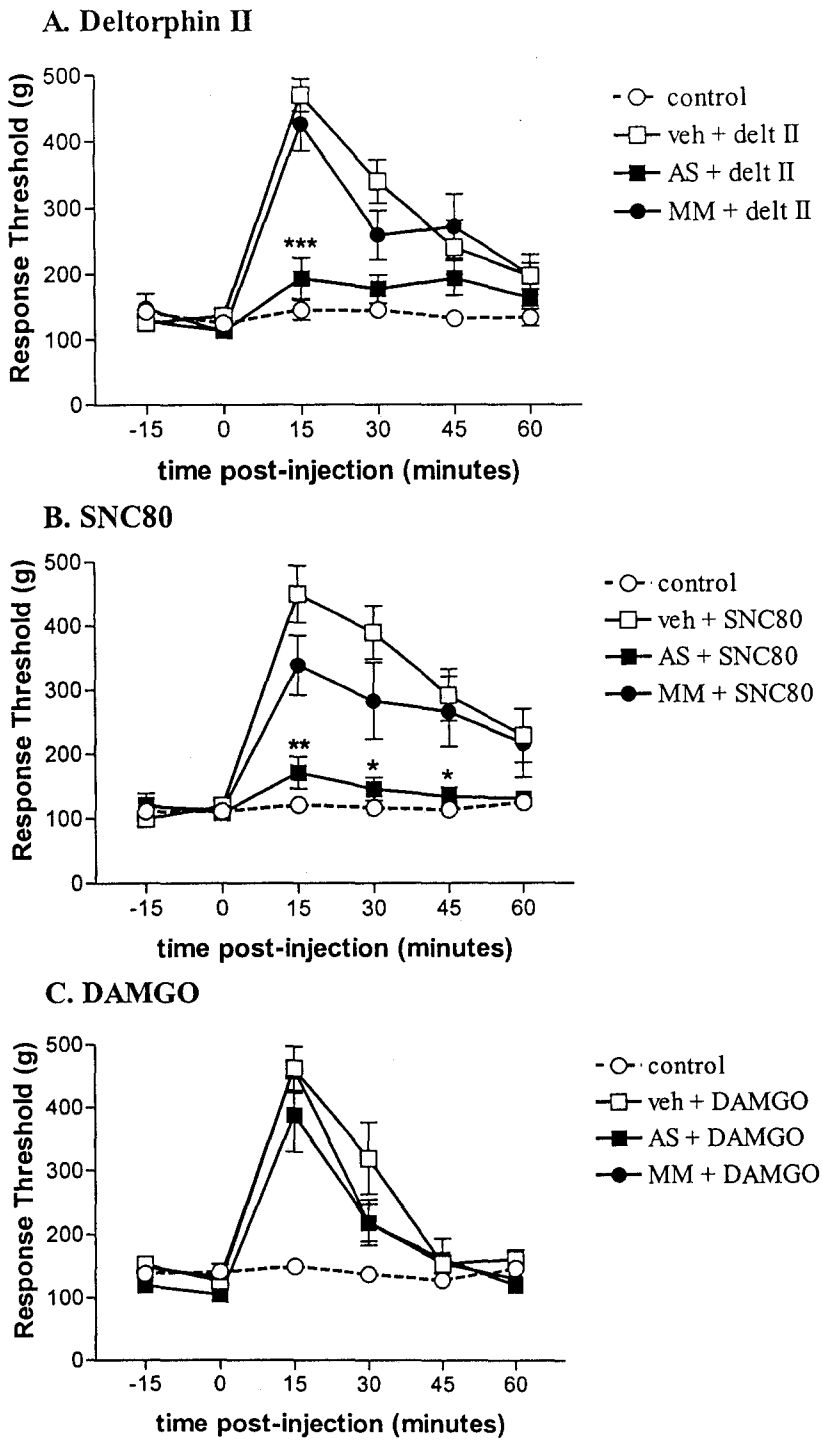

Figure 8.2. PNA antisense treatment inhibited the antinociceptive response to deltorphin II (60 nmol) and SNC80 (400 nmol) but not DAMGO (0.2 nmol).
Values represent saline-treated controls (○), saline-treated (vehicle) + agonist (□), antisense-treated + agonist (■) and mismatch-treated + agonist (●). *, **, *** represent significant differences between the antisense group and the saline (+ agonist) and mismatch groups where $p < 0.05$, 0.01 and 0.001, respectively. Each curve represents the mean ± s.e.m. response of 7-11 rats.

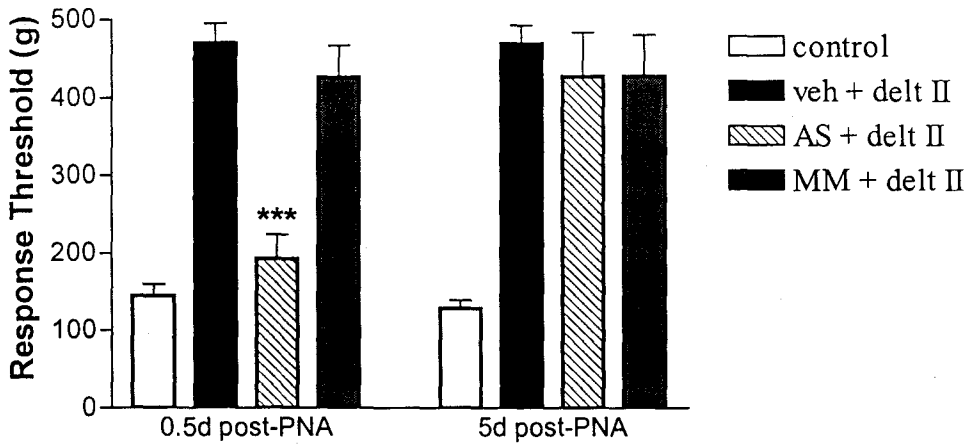

Figure 8.3. Recovery of δ opioid receptor function following PNA treatment.
Twice-daily i.c.v. injections of PNA antisense over a period of 5 days inhibited the antinociceptive response to deltorphin II (60 nmol) at 0.5 days but not 5 days after PNA treatment. Testing at 0.5 days and 5 days was performed on the same groups of rats. *** represents a significant difference between the antisense group and the saline (+ agonist) group where $p<0.001$. Each bar represents the mean ± s.e.m. antinociceptive response to Deltorphin II observed at 15 minutes after injection (n = 5-7 rats per group).

8.5.4 General observations pertaining to PNA toxicity

At no time during the course of the antisense (or mismatch) treatment did the animals display any behaviour indicating a toxic response to the PNA. Comparison of body weights before and after PNA treatment revealed no significant differences in comparison to saline-treated control rats ($p>0.05$, data not shown). Also, visual inspection of brain tissues did not show any gross signs of tissue necrosis in response to PNA treatment.

8.5.5 δ Opioid receptor density in brain homogenates

Binding of the δ opioid selective radioligand [^{3}H]naltrindole was saturable and best fit to a one-site model in brain membrane homogenates prepared from all treatment groups (data not shown). Analysis of [^{3}H]naltrindole saturation binding revealed an 11 to 13% decrease in whole brain δ opioid receptor density following antisense treatment compared with that of mismatch and saline-treated control groups as shown in Table 1. This difference in receptor B_{max} was not significant ($p>0.05$). In addition, there was no significant difference between the associated K_d values determined for each treatment group ($p>0.05$).

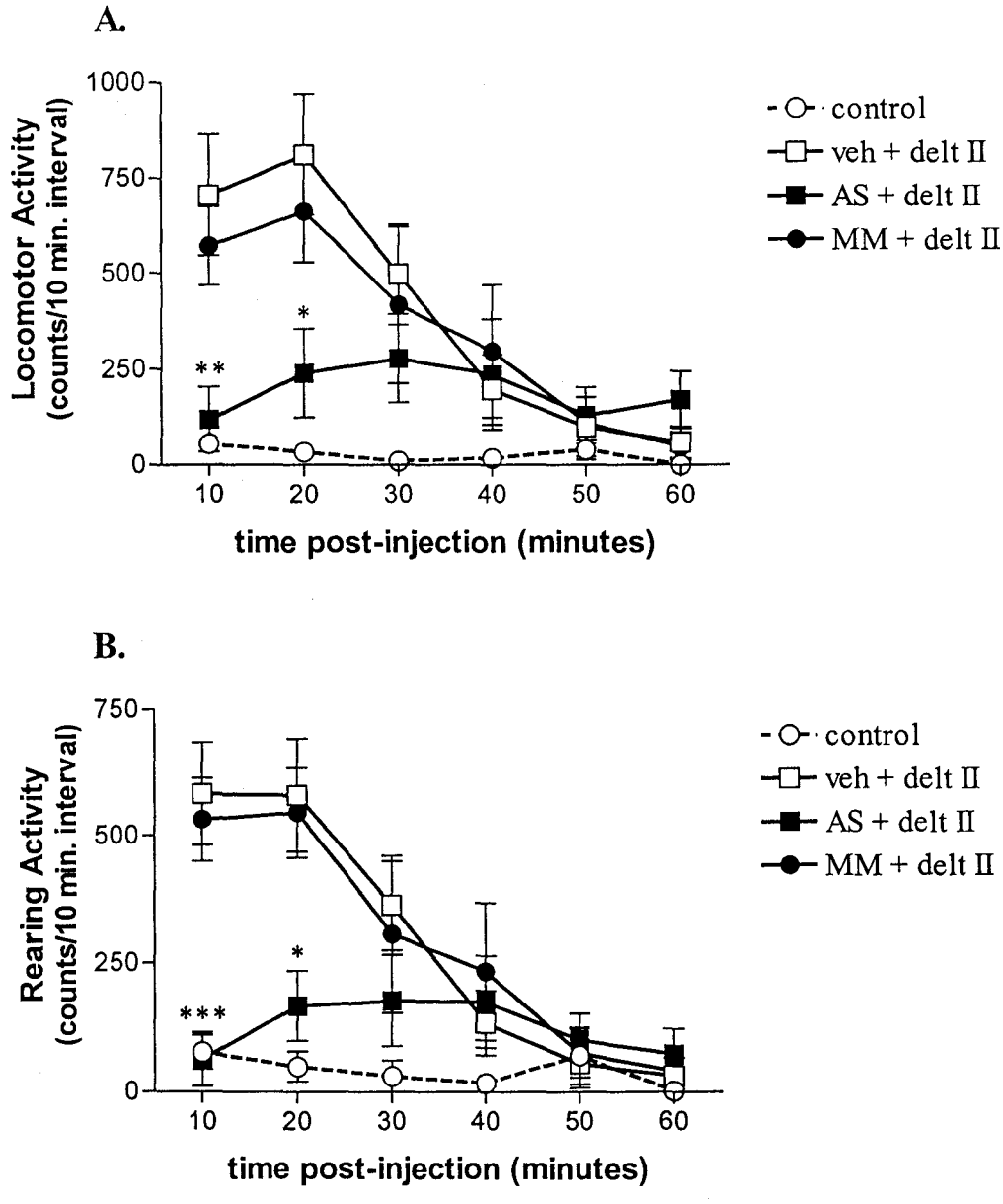

Figure 8.4. PNA inhibition of δ opioid receptor-mediated locomotor activity.
PNA antisense treatment inhibits [A] the increased horizontal locomotor activity (HLA) and [B] the increased rearing activity in response to the δ agonist deltorphin II (0.3 nmol, i.c.v.). Values represent saline-treated controls (○), saline-treated (vehicle) + deltorphin II (□), antisense-treated + deltorphin II (■) and mismatch-treated + deltorphin II (●). *, **, *** represent significant differences between the antisense group and the saline (+ agonist) and mismatch groups where $p < 0.05$, 0.01 and 0.001, respectively. Each curve represents the mean ± s.e.m. response of 7-10 rats.

8.5.6 SNC80 stimulated [^{35}S]GTPγS binding in brain homogenates

SNC80 (0.1 – 10,000 nM) induced [^{35}S]GTPγS binding in brain homogenates prepared from all treatment groups. Dose response relationships were best fit to a sigmoidal curve as shown in Figure 8.5. Basal [^{35}S]GTPγS binding did not differ significantly between treatment groups ($p>0.05$; data presented in caption for Table 8.1). SNC80 (10 μM) induced a maximal stimulation of 40.4 ± 2.4% above basal levels in brain homogenates prepared from saline-treated rats; maximal stimulated binding values for each treatment group were determined as a percentage of this value as shown in Table 1. EC_{50} values were determined relative to the maximal effects observed for each treatment group. The EC_{50} value for SNC80 stimulated [^{35}S]GTPγS binding was 20% higher in brain homogenates prepared from antisense-treated rats compared with the control group. However, one-way ANOVA comparison of the treatment groups just failed to indicate a significant difference ($p=0.084$). In contrast, maximal SNC80-stimulated [^{35}S]GTPγS binding was significantly lower in homogenates prepared from the antisense-treated group compared with those prepared from the control group (~25% lower, $p<0.05$). There was no significant difference in maximal SNC80-stimulated [^{35}S]GTPγS binding between the control group and the mismatch group ($p>0.05$).

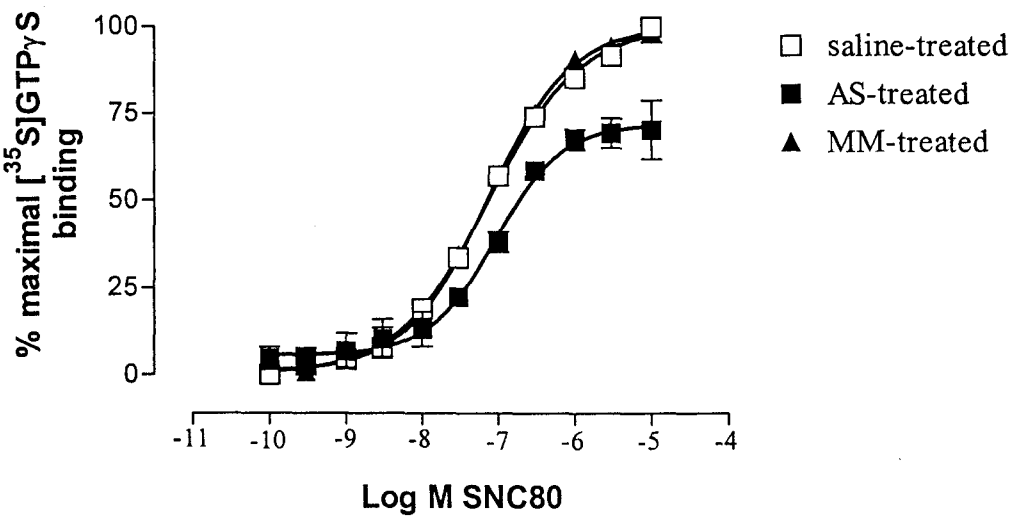

Figure 8.5. Representative dose-response curve for [^{35}S]GTPγS (0.14 – 0.17 nM) binding to rat brain membranes in response to SNC80 (0.1 – 10,000 nM).
Homogenates were prepared separately for each saline (□), antisense (■) and mismatch-treated (▲) rat. The data are from a single assay (i.e. one rat per group). Homogenates from each treatment group were assayed in parallel and each binding experiment was performed once with quadruplet samples.

	[^{3}H]Naltrindole Saturation Binding		[^{35}S]GTPγS Binding	
	K_d (nM)	B_{max} (fmol/mg protein)	EC_{50} (SNC80, nM)	% maximal stimulated binding
Saline-treated (n = 5)	0.059 ± 0.007	44.3 ± 2.8	71.5 ± 6.2	100.0 ± 5.8
Antisense-treated (n = 5)	0.055 ± 0.010	38.6 ± 2.2	85.5 ± 5.4	74.8 ± 5.0*
Mismatch-treated (n = 5)	0.069 ± 0.010	43.4 ± 0.9	65.8 ± 5.8	97.0 ± 8.1

Table 8.1 Effect of PNA Antisense Treatment on δ Opioid Receptor Density.
Saturation and [^{35}S]GTPγS binding were performed on homogenates of brain hemispheres from saline-, antisense- and mismatch-treated rats. The data from each rat brain homogenate was analyzed separately. Basal [^{35}S]GTPγS binding was 3240 ± 120, 3230 ± 150 and 3220 ± 120 cpm for the saline, antisense and mismatch-treated groups respectively; there was no significant difference between treatment groups ($p>0.05$). Maximal stimulated binding is defined as the peak increase over basal levels for SNC80-induced [^{35}S]GTPγS binding in brain homogenates prepared from saline-treated animals. Saturation binding and [^{35}S]GTPγS binding were assayed in parallel on the same brain homogenates. * represents a significant difference in comparison to the saline-treated group ($p<0.05$). Data are presented as mean ± s.e.m.

8.6 Discussion

This present study demonstrates that an unmodified PNA oligomer is an effective antisense agent *in vivo*. In addition, this study confirms that the cloned δ opioid receptor mediates both the antinociceptive and the locomotor effects of δ agonists administered directly into the brain of conscious rats. Finally, the findings presented in this report indicate that the [^{35}S]GTPγS binding assay is more sensitive than saturation binding experiments for evaluating the effects of antisense treatment on tissue samples *in vitro*.

Before antisense testing, the effects of δ (deltorphin II, SNC80) and μ (DAMGO) opiate receptor agonists were assessed in the paw pressure assay of antinociception. DAMGO was approximately 1000-fold more potent than the δ agonists consistent with the predominant expression of μ opioid receptors in supraspinal pain pathways (Mansour *et al.*, 1995).

Pretreatment with the PNA antisense sequence significantly inhibited the antinociceptive response to deltorphin II and SNC80. The sequence-specific nature of inhibition by the antisense but not the mismatch sequence implies that the PNA oligomer is effective via an antisense mechanism. In order to verify that the effect of the PNA antisense sequence was also target-specific (i.e. selective for δ opioid receptors), a separate group of rats were treated with PNA and than challenged with the μ opioid receptor agonist DAMGO. The μ opioid receptor was chosen as a control target based on its similarity to the δ opioid receptor in mediating antinociceptive responses and its supraspinal distribution. The δ antisense (and mismatch) PNA sequences were not complementary to any region of the μ opioid receptor mRNA (Chen *et al.*, 1993). The lack of effect of either PNA sequence on DAMGO mediated antinociception suggests that the inhibition of response to deltorphin II and SNC80 by PNA treatment in the paw pressure assay is due to an inhibition of δ opioid receptor function as opposed to a more general change in the functioning of supraspinal nociceptive pathways.

An advantage of antisense techniques as a method of determining gene function is that inhibition of target gene expression is transient in nature, thus mimimising the development of any compensatory changes as a consequence of the manipulation (Fraser & Wahlestedt, 1997a). To confirm that the behavioural effects of PNA antisense treatment in the paw pressure assay were due to a reversible inhibition of δ opioid receptor function, the antinociceptive effects of deltorphin II were remeasured in rats following the termination of PNA treatment. The allowed

recovery period is consistent with the expected rate of δ opioid receptor turnover (Jiang *et al.*, 1991). The complete recovery of deltorphin II efficacy in rats formerly treated with PNA antisense supports the proposed δ receptor-specific action of the PNA antisense sequence. In addition, this finding suggests that the inhibited response to δ agonists following PNA treatment was caused by neither a general neurotoxicity nor a long-term change in non-opioid receptor systems.

Distinct populations of δ opioid receptors in the pain pathways and striatal regions of the brain, respectively, mediate the antinociceptive and locomotor responses to δ agonists (Mansour *et al.*, 1995). PNA antisense treatment significantly inhibited deltorphin II-mediated increases in locomotor activity in a sequence-specific manner. This finding provides additional evidence that PNA sequences are effective antisense agents *in vivo*. In addition, it confirms that a common δ opioid receptor subtype mediates the locomotor and antinociceptive effects of deltorphin II. Finally, this observation implies that PNA oligomers are able to penetrate more than one region of the brain after i.c.v. injection.

Pretreatment with PNA antisense oligomers did not alter baseline response thresholds in the paw pressure assay. This observation is consistent with previous reports that the antagonism (Jiang *et al.*, 1991) or inhibition of expression (Kest *et al.*, 1996; Bilsky *et al.*, 1996) of δ opioid receptors does not alter the baseline response of animals in acute pain models. Similarly, PNA antisense treatment did not alter baseline exploratory locomotor activity in the present study. The finding that repeated i.c.v. injections of PNA did not alter baseline antinociceptive or locomotor responses suggests that there is no toxicity in response to the PNA treatment affecting either the motor response required for paw withdrawal, the cognition and processing of nociceptive signals, or the supraspinal processes that control basic exploratory activity. In addition, there were no obvious changes in the general behaviour or the body weight of the animals indicative of any untoward effects of the PNA. Also, there was no indication of tissue damage at the injection site, which compares favourably to the side effect profile after treatment with phosphorothioate oligonucleotides, where gross tissue necrosis proximal to the injection site is a common outcome (LeCorre *et al.*, 1997).

Saturation binding studies suggest that there may have been a small diminution (i.e. ~13%) in receptor B_{max} in brain homogenates prepared from antisense-treated rats in comparison to saline-treated controls. However, this difference in receptor B_{max} is not significant. This finding is consistent with a number of other reports of antisense studies directed against G-protein coupled

receptors *in vivo* where substantial changes in antisense-mediated behaviour were not accompanied by comparable decreases in receptor density. In studies where receptor B_{max} values were reported, examples of antisense modulation of supraspinal opioid or dopamine receptors coincided with either no change (Shah *et al.*, 1997) or a modest change (i.e. <20%) in receptor binding sites (Bilsky *et al.*, 1996; Niesbrand *et al.*, 1995; Qin *et al.*, 1995). Although such small changes in receptor population might seem insufficient to account for the changes in behaviour, receptor binding on whole tissue homogenates may dilute highly restricted decreases in protein expression (Grzanna *et al.*, 1998). However, this explanation appears to be insufficient to account for the present findings, in which the effects on both pain and locomotor activity imply that PNA oligomers effectively penetrate multiple brain regions. An alternate hypothesis is that only a small pool of newly synthesized G protein-coupled receptors are functional and that antisense treatment inhibits the replenishment of this receptor pool (Hua *et al.*, 1998; Qin *et al.*, 1995). This hypothesis was tested using the [^{35}S]GTPγS binding assay which measures the efficacy of ligands at G protein-coupled receptors (Traynor & Nahorski, 1995). Comparison of the EC_{50} values describing SNC80-induced stimulation of [^{35}S]GTPγS binding suggest a reduced agonist potency in brain homogenates prepared from antisense treated animals. Moreover, the efficacy of SNC80 was significantly reduced in homogenates prepared from the antisense treatment group. These changes in the SNC80 dose-response relationship are consistent with pharmacological models describing dose-response profiles generated in the presence of a non-competitive antagonist. The [^{35}S]GTPγS binding data provides an *in vitro* correlate for the behavioural differences observed in the antisense treatment groups *in vivo* and seems to be a more sensitive assay than saturation binding for measuring the efficacy of antisense treatment. Taken together, the saturation binding and [^{35}S]GTPγS binding data support the notion that antisense treatment preferentially inhibits the replenishment of a functional receptor pool.

The hybridization properties of PNA have made these synthetic oligomers very useful tools for a diverse number of scientific applications including hybridization techniques (Perry-O'Keefe *et al.*, 1996), high-throughput DNA or RNA screening (Webb & Hurskainen, 1996; Weiler *et al.*, 1997) and site-directed mutagenesis (Faruqi *et al.*, 1998). In addition, the superior hybridization affinity of PNA increases their versatility as antisense agents in comparison to phosphodiester or phosphorothioate oligonucleotides. Specifically, the high hybridization affinity of PNA-mRNA hybrids permits the use of short oligomer sequences to achieve antisense effects. Thus, a 15-base sequence was chosen for use in this study although it has been shown that phosphorothioate oligonucleotides of comparable length are ineffective antisense agents (Monia *et al.* 1992). Also,

the concentration of PNA required to achieve antisense effects *in vivo* (i.c.v.) in this study is about 10-fold less than the concentrations of oligonucleotide sequences used in previous reports of antisense knockdown of the δ opioid receptor in rats (Negri *et al.*, 1999; Fraser *et al.*, 2000). This is consistent with the improved *in vitro* antisense potency of PNA sequences in comparison to their phosphorothioate analogues (Norton *et al.*, 1996). The reduced dose of PNA required is probably a product of the high hybridization affinity and improved stability of these synthetic oligomers (Demidov *et al.*, 1998). The ability to reduce oligomer length and dose when using PNA sequences *in vivo* may be of benefit in improving the efficiency of cellular uptake and in reducing the prevalence of non-specific effects (Woolf *et al.*, 1992; Flanagan *et al.*, 1996).

In conclusion, the sequence-specific and target-specific inhibition of G protein-coupled receptor function in the living brain described previously (Tyler *et al.*, 1998) and in this report demonstrates that unmodified PNA oligomers are effective antisense agents *in vivo*. We anticipate continued advances in PNA chemistry to further improve the potency and toxicity profile of PNA oligomers over conventional oligonucleotides for application in the domain of functional genomics.

General Discussion

9 Summary

The main findings presented in this thesis are that the supraspinal administration of δ agonists caused antinociceptive and antihyperalgesic behaviour in various pain assays as well as heightened locomotor activity in rats. Also, antisense studies confirmed that these antinociceptive and locomotor responses were mediated by a specific activation of the cloned δ opioid receptor, DOR. Radioligand binding studies on rat brain membranes and behavioural assays using antisense techniques and selective antagonists did not provide evidence to support the hypothesized existence of multiple δ receptor subtypes. Although DPDPE had a differential pharmacology in comparison to the other δ agonists tested *in vivo*, its selective antagonism by CTOP suggested that these effects were due to an interaction with μ receptors rather than the activation of a novel δ receptor subtype. In total, these data advance the field of δ opioid receptor pharmacology, validate the pursuit of the cloned δ opioid receptor (DOR) as a target for novel analgesics, and demonstrate the application of peptide nucleic acids (PNA) as antisense agents for the determination of gene function.

9.1 δ Opioid Receptor Pharmacology

Previous studies have predicted the existence of δ opioid receptor subtypes based largely on the distinct pharmacological profiles of DPDPE (δ_1-selective) and deltorphin II (δ_2-selective) in radioligand binding, second messenger and behavioural assays as summarized in section 1.2.1. However, the work presented in this thesis, and that presented in the recent literature, does not support the postulated existence of δ receptor subtypes encoded by distinct genes. As communicated in Chapter 4 of this thesis, radioligand binding studies with [^{125}I]AR-M100613 yielded monophasic saturation and competition binding curves with complete inhibition of radioligand binding by both deltorphin II and DPDPE. These findings are in contrast to previous reports where biphasic saturation and competition binding curves were observed in mouse or rat brain membranes with [^{3}H]DPDPE, [^{3}H]deltorphin II and [^{3}H]DSLET (Negri *et al.*, 1991b; Sofuoglu *et al.*, 1992). The differences in the binding data may reflect important methodological improvements in our binding assay. Firstly, [^{125}I]AR-M100613 is an antagonist and its binding to the δ opioid receptor is insensitive to the state of G-protein coupling. In contrast, agonist radioligands at opioid receptors have previously been shown to yield biphasic displacement curves as a result of the different affinity states contingent upon the status of receptor-G protein coupling (Lutz & Pfister, 1992; Richardson *et al.*, 1992); these biphasic displacement curves may

have been previously misinterpreted as binding to distinct receptor subtypes. Secondly, the current radioligand binding studies were conducted in the presence of CTOP to block non-selective binding to μ receptors. Similar precautions should have been taken in previous studies with the peptide radioligands, but were not. The δ agonists DPDPE, DSLET and deltorphin II have all been shown to interact with low affinity at μ receptors (Payza *et al.*, 1996) (see Table 1.2 for selectivity ratios). Thus, low affinity displacement from μ sites, rather than from δ subtypes, may contribute to the appearance of biphasic displacement binding curves with these radioligands.

Numerous reports in the literature have described differences in the pharmacology of the prototypical δ agonists, DPDPE and deltorphin II, *in vivo*. For example, BNTX and DALCE selectively antagonized the supraspinal antinociceptive activity of DPDPE, whereas naltriben and NTII selectively antagonized that of deltorphin II (Jiang *et al.*, 1991; Vanderah *et al.*, 1994; Thorat & Hammond, 1997). Also, cross-tolerance between DPDPE and deltorphin II was not observed in mice despite the development of tolerance to their respective antinociceptive effects following repeated exposure to either agonist given individually (Mattia *et al.*, 1991). Finally, antisense studies, including the one presented in Chapter 7 of this thesis, have demonstrated that the antinociceptive response to supraspinal administration of deltorphin II, but not DPDPE, is blocked by antisense targeted against DOR (Bilsky *et al.*, 1994; Tseng *et al.*, 1994). Thus, it is well established that DPDPE and deltorphin II have distinct pharmacology *in vivo*. These data have been interpreted as evidence for the existence of δ subtypes (Zaki, 1996).

In contrast, the work presented in this thesis, as well as various reports in the literature, suggest an alternate explanation for the distinct pharmacology of DPDPE *in vivo*. As described in Chapter 7, pretreatment with the μ antagonist, CTOP, appeared to completely inhibit the antinociceptive response to DPDPE, but had no effect on the response to deltorphin II and SNC80. Similar findings of DPDPE antagonism by CTAP, an analogue of CTOP and a selective μ antagonist, have been reported in rodents at the level of the brain (Kramer *et al.*, 1989) and spinal cord (He & Lee, 1998). These antagonist studies are consistent with reports that DPDPE elicits μ-like behavioural effects in rodents (Cowan & Murray, 1989; Weinger *et al.*, 1996). Finally, in μ receptor knockout mice, DPDPE-mediated antinociception (Sora *et al.*, 1997; Fuchs *et al.*, 1999; Matthes *et al.*, 1998; Hosohata *et al.*, 2000) and DPDPE-stimulated GTPγS binding activity in brain membrane preparations (Hosohata *et al.*, 2000) were significantly reduced. In total, these data suggest that the differential pharmacology between DPDPE and the other δ agonists may

arise from an interaction of DPDPE at the μ opioid receptor rather than at an alternate δ receptor subtype.

Opioid receptor homo- and heteroligomers (δ/μ, δ/k) have recently been identified using immunoprecipitation techniques (Cvejic & Devi, 1997; Jordan & Devi, 1999; George *et al.*, 2000; Gomes *et al.*, 2000). Initial studies with DPDPE and deltorphin II indicate that these different receptor complexes display different ligand binding profiles, G protein coupling and receptor trafficking and desensitization activities (Cvejic & Devi, 1997; George *et al.*, 2000; Gomes *et al.*, 2000; McVey *et al.*, 2001). Thus, it is possible that the predicted δ receptor subtypes associated with the different pharmacology described for deltorphin II versus DPDPE may correspond to interactions at δ monomers, dimers and heterodimers rather than receptors derived from distinct genes (Jordan *et al.*, 2000).

There is anecdotal evidence that the δ/μ heterodimer may be an exciting target for novel analgesics based on the premise that it modulates the effects observed following dual exposure to δ and μ agonists (Jordan *et al.*, 2000). For example, synergistic effects have been reported between δ and μ agonists in assays of receptor binding (Martin & Prather, 2001), receptor activation (Martin & Prather, 2001; Chen *et al.*, 2001), adenosine release from spinal cord synaptosomes (Cahill *et al.*, 1996) and antinociception (Malmberg & Yaksh, 1992; Negri *et al.*, 1995). Also, δ antagonists attenuate the reinforcing properties, and block the development of antinociceptive tolerance and dependence, to morphine (Abdelhamid *et al.*, 1991; Suzuki *et al.*, 1994; Hepburn *et al.*, 2001). The latter findings have spurred interest in the development of mixed μ-agonist/δ-antagonist compounds (Wells *et al.*, 2001). Thus, the continued investigation of opioid receptor oligomers, such as the δ/μ heterodimer, is a potentially important area for the optimization and drug development of opiate analgesics.

9.2 Characterization of DOR function in rat brain

To date, a single gene has been cloned that appears to encode for a δ opioid receptor, DOR (Evans *et al.*, 1992; Kieffer *et al.*, 1992). The pharmacology of DOR is similar to that described for the predicted δ_2-receptor subtype in both radioligand binding and behavioural studies in rodents (Raynor *et al.*, 1994; Bilsky *et al.*, 1996). In this thesis, antisense studies were used to selectively inhibit DOR expression in order to characterize the role of DOR in modulating supraspinal pain pathways. The data presented in Chapters 7 and 8 of this thesis demonstrate that the selective, antisense inhibition of DOR expression in rat brain is correlated with a decreased

response to δ agonists (excepting DPDPE, as discussed above) in antinociceptive assays. These data implicate DOR as a biological target for the modulation of supraspinal pain pathways in the rat, a conclusion consistent with that of previous antisense studies performed in the mouse (Standifer *et al.*, 1994; Tseng *et al.*, 1994; Bilsky *et al.*, 1996; Rossi *et al.*, 1997).

The clinical manifestation of pain is typically associated with tissue damage, inflammation or nerve injury. Thus, an important consideration for the development of δ agonists as analgesics is the efficacy of these compounds in the treatment of hyperalgesia and allodynia arising from tissue damage and nerve injury, respectively. The data presented in Chapter 6 of this thesis demonstrate that the supraspinal administration of deltorphin II and SNC80 reversed hyperalgesia associated with persistent hindpaw inflammation in rats. Moreover, these δ agonists were approximately three-fold more potent in reversing hyperalgesia associated with paw inflammation than in an assay of acute thermal nociception. This finding is supported by another recently published report demonstrating that the antinociceptive effects of deltorphin II (i.c.v.) were potentiated following persistent hindpaw inflammation in rats (Hurley & Hammond, 2000). In total, these studies demonstrate that pain pathways in the brain are an important site of action for δ agonists in the treatment of hyperalgesia associated with peripheral tissue inflammation. Previous work has demonstrated that δ receptors in the spinal cord (Hylden *et al.*, 1991; Ho *et al.*, 1997; Cao *et al.*, 2001) and at the peripheral site of inflammation (Zhou *et al.*, 1998) also play a role in reversing hyperalgesia in rats. Thus, δ agonists with the capacity to access supraspinal, spinal and peripheral sites would be expected to provide the greatest clinical efficacy in the treatment of pain following inflammation associated with tissue injury. This hypothesis could be tested using non-peptidic δ agonists such as SNC80 that have bioavailability in the CNS following peripheral administration (Negus *et al.*, 1998). In view of that, a recent study has demonstrated that in monkeys, SNC80 (subcutaneous dosing) is more broadly effective than NSAIDs in the treatment of hyperalgesia associated with peripheral inflammation caused by capsaicin, prostaglandin E_2 and complete Freund's adjuvant (CFA) (Brandt *et al.*, 2001).

The physiological basis for the enhanced response to δ agonists in reversing hyperalgesia associated with inflammation (as discussed above) and allodynia associated with neuropathic pain (Sohn *et al.*, 2000; Mika *et al.*, 2001) is an area for further research. Three mechanisms have been proposed that may explain the heightened role of δ opioid receptors in chronic pain states; these mechanisms are not mutually exclusive. The first hypothesis stems from the finding that persistent pain transmission appears to cause increased release of endogenous [Met]- and

[Leu]enkephalin in descending pain pathways (Williams *et al.*, 1995; Ossipov *et al.*, 1995b; Hurley & Hammond, 2001). Thus, the increased release of endogenous enkephalins may have an additive or synergistic response in combination with exogenous δ agonists (Hurley & Hammond, 2001). The second hypothesis suggests that the elevated nociceptive input associated with persistent pain may trigger increased neuronal activity in descending pain pathways expressing δ opioid receptors (Ren & Dubner, 1996; MacArthur *et al.*, 1999), thereby providing for an enhanced antinociceptive response to δ agonists. The third hypothesis is based on the premise that persistent pain neurotransmission may cause increased δ opioid receptor expression on the axon terminals of primary afferents. Delta (δ) opioid receptors are expressed on large dense-core vesicles containing pain neurotransmitters such as substance P and CGRP in dorsal root ganglion neurons. Thus, it has been suggested that the exocytotic release of these pain neurotransmitters should correlate with increased exposure of the δ opioid receptor and, consequently, a heightened response to δ opioid receptor agonists (Zhang *et al.*, 1998).

Potential adverse effects of δ agonists include increased locomotor activity (in rodents) (Longoni *et al.*, 1991), reward-seeking behaviour (Shippenberg *et al.*, 1987) and physical dependence (Maldonado *et al.*, 1990). Dopaminergic pathways innervating the extended striatum modulate all of these behaviours and, correspondingly, show significant expression of δ opioid receptors (Mansour *et al.*, 1995). In Chapter 6 of this thesis, we demonstrate that deltorphin II and SNC80 administered directly into the brain cause increased locomotor activity in previously habituated rats. Antisense studies presented in Chapter 8 of this thesis indicate that this response to deltorphin II was mediated by activation of DOR. In general, the effects observed for deltorphin II (Negri *et al.*, 1991a; Longoni *et al.*, 1991; Negri *et al.*, 1999) and SNC80 (Spina *et al.*, 1998; Negri *et al.*, 1999) correlate well with those presented in the literature. However, our study was the first to directly compare these δ agonists and demonstrate that deltorphin II is greater than 1000-fold more potent than SNC80 on the stimulation of locomotor activity. The weak locomotor stimulant response observed for SNC80 in this study appears consistent with reports that SNC80, unlike deltorphin II (Longoni *et al.*, 1991), does not appreciably increase dopamine release in the nucleus accumbens of freely-moving rats (Longoni *et al.*, 1998). More importantly, antinociceptive and antihyperalgesic doses of SNC80, unlike deltorphin II, did not produce significant locomotor activity based on comparison of the work presented in Chapters 6 and 7 of this thesis. In total, these data suggest that SNC80 may have an improved side effect profile in comparison to other δ agonists. In addition, these data indicate that compounds in development as DOR analgesics should be screened for unwanted psychostimulant effects.

The data presented in this thesis, as well as recent data presented in the literature and cited herein, suggest that non-peptidic δ agonists selective for DOR could display therapeutic benefit as analgesics with minimal side effects (Dondio *et al.*, 1997). Accordingly, peptidomimetic and non-peptide ligands based on the structure of DPDPE are under investigation in academic laboratories (Hruby, 2001). Also, various pharmaceutical companies are currently supporting drug development programs for non-peptidic δ agonists. Toray Industries Inc. (Japan) and SmithKline Beecham plc (now GlaxoSmithKline, UK) have independently identified selective δ agonists derived from the structure of the δ antagonist, naltrindole (Knapp *et al.*, 1995; Dondio *et al.*, 1995). This series of compounds has been optimized to isolate structures with high brain penetration after oral administration and antihyperalgesic activity only three-fold less potent than morphine (Dondio, 2000). In comparison, AstraZeneca plc (UK) have developed a series of compounds derived from SNC80 (Calderon *et al.*, 1994). These δ agonists exhibit high selectivity over μ receptors (μ/δ binding affinity ratio = 4370) and oral bioavailability (Wei *et al.*, 2000). It is anticipated that the intense commercial interest in the development of non-peptidic δ agonists will champion the clinical testing of these agents as early as 2002.

9.3 Antisense Inhibition of Gene Function by Peptide Nucleic Acids

Antisense technology has broad application as a tool for the determination of gene function and target validation, and as a new class of therapeutic agents (Koller *et al.*, 2000; Agrawal & Kandimalla, 2000). However, the current workhorse antisense oligonucleotides, phosphorothioates (or variants thereof), are losing favour because of their propensity to cause non-specific effects related, in part, to their chiral, charged nucleotide backbone (Stein, 1996). Phosphodiester oligonucleotides can be effective antisense agents as demonstrated in the work presented in Chapter 7 of this thesis. However, their use is limited exclusively to central targets as these agents are rapidly degraded by proteases in the bloodstream (Wickstrom, 1986; Thierry & Dritschilo, 1992). Also, the charged phosphodiester backbone suggests that these agents could be prone to a similar side effect profile as phosphorothioates. Thus, alternate chemistries for antisense agents are required.

The peptide nucleic acids (PNA) are interesting candidate antisense agents, in part, because of their charge-neutral, achiral peptidic backbone (Ray & Norden, 2000). The physical properties of PNA and the possible application of these structures as antisense molecules were reviewed in section 2.1.2.1. PNA are effective antisense agents in *in vitro* or cell-based assays (Taylor *et al.*,

1997; Doyle *et al.*, 2001), however their use *in vivo* had not been demonstrated prior to the initiation of this thesis work. Thus, the data presented in Chapter 8 of this thesis is one of the first demonstrations that unconjugated PNA sequences can inhibit gene function by an antisense mechanism *in vivo*. In this study, PNA treatment inhibited DOR function in a sequence-selective, target-specific and reversible manner. PNA inhibition of gene function has similarly been demonstrated for the neurotensin (NTR-1), μ opioid and galanin (GalR1) receptors in the rat (Tyler *et al.*, 1998; Rezaei *et al.*, 2001). Additional studies are required to demonstrate the applicability of PNA antisense to both different target families (i.e. other than G-protein coupled receptors) and different target tissues (i.e. other than neurons). Progress in this area is anticipated shortly now that the antisense application of PNA *in vivo* has been realized. Recent *in vitro* studies have indicated that PNA antisense sequences are effective against a diverse range of targets including *c-myc* (Pardridge *et al.*, 1995), telomerase (Shammas *et al.*, 1999), the *gag-pol* domain of HIV type-1 (Sei *et al.*, 2000), inducible nitric oxide synthase (iNOS) (Scarfi *et al.*, 1999) and various antibacterial targets (Good & Nielsen, 1998; Good *et al.*, 2001).

Optimizing sequence design and increasing cellular permeability could further improve the antisense activity of PNA. One aspect of sequence design that is currently under investigation is determining the optimal length of PNA sequences. In a recent study where PNA was introduced to transfected cells by lipid-mediated transfection, it was demonstrated that longer sequences (i.e. up to 18 bases) provided the greatest inhibition of target gene expression (Doyle *et al.*, 2001). However, a second cell-based study that relied on PNA to permeate the cell membrane to achieve its antisense effects concluded that sequences between 9-12 bases in length provided optimal activity (Good *et al.*, 2001). In total, these studies indicate that the optimal PNA length is a balance between hybridization affinity (which increases with length) and uptake efficiency (which apparently decreases with length). The optimal PNA length has not been investigated *in vivo*, but it would be expected to rely on these same principles in addition to any effects of sequence length on bioavailability and pharmacokinetics. In this thesis and in another study (Tyler *et al.*, 1998), it was demonstrated that PNA sequences between 12-15 bases in length have significant antisense effects *in vivo*, even following peripheral administration (Tyler *et al.*, 1999). Thus, PNA sequences appear to have greater versatility than phosphorothioate oligonucleotides, which are generally ineffective at these lengths (Monia *et al.*, 1992).

The impact of PNA permeability (or lack thereof) on the efficacy of these molecules *in vivo* is another area of intense research. The data presented in this thesis, and that of another recent study (Rezaei *et al.*, 2001), indicate that PNA sequences are taken up by neurons in amounts

sufficient to inhibit target gene function following local administration *in vivo*. Also, it has recently been reported that unmodified, unconjugated PNA can permeate the blood-brain barrier to inhibit central targets following peripheral administration in rats (Tyler *et al.*, 1999). However, the latter finding is controversial, as other investigators have demonstrated that PNA must be conjugated to vector-delivery systems to pass through the blood-brain barrier (Pardridge *et al.*, 1995; Wu *et al.*, 1996; Penichet *et al.*, 1999). Although PNA can enter cells by passive diffusion (Ardhammar *et al.*, 1999), there is an emerging consensus that the conjugation of PNA to molecules that enhance physical or receptor-mediated cellular uptake can further improve cell membrane permeability and consequently lead to improved antisense efficacy (Aldrian-Herrada *et al.*, 1998; Cutrona *et al.*, 2000; Good *et al.*, 2001). Thus, the optimization of PNA delivery systems is an area of great scientific interest that is expected to further improve PNA antisense effects.

The current interest in PNA is spurred by two factors: the recent demonstration of their antisense effects (Tyler *et al.*, 1998; Fraser *et al.*, 2000b) and the realization that alternative deoxynucleotide mimics to phosphorothioates are required to develop antisense therapeutics (Monteith & Levin, 1999; Hollon, 2001). PNA are excellent alternatives to phosphorothioate oligonucleotides because of their high hybridization affinity to mRNA, improved sequence-selectivity, and stability in biological fluids (discussed in section 2.1.2.1). Also, in comparison to oligonucleotide compounds, PNA have the additional advantages of efficient and economical synthesis by Boc or Fmoc solid-phase techniques as well as the possibility of lead optimization by medicinal chemistry approaches (Nielsen, 2001). Biotechnology companies such as Pantheco A/S (Denmark) have been formed to exploit the antisense effects of PNA for the development of novel therapeutics, initially in the areas of anti-infectives, diabetes and cardiovascular disease. Pantheco A/S has presented a preliminary report describing PNA antisense compounds with antibiotic effects when targeted against multiresistant *E. coli* in a mouse model of peritonitis/sepsis (Schou *et al.*, 2000). Thus, lead PNA antisense compounds with antibiotic activity have been identified and pharmacokinetic and toxicology studies are currently underway in rodents. It is anticipated that the clinical development of PNA antisense compounds will begin by 2002.

9.4 Current and Future Applications of Antisense Technology

Antisense technology has application in three aspects of drug discovery and development: (1) a tool for functional genomics and target validation, (2) a tool for the modulation of gene splicing and (3) a novel class of therapeutics.

9.4.1 Functional Genomics and Target Validation

The advent of genomics is a boon for antisense technology. It is estimated that the number of biological targets that can be exploited for drug therapy is approximately ten-fold greater than the total number of targets for all currently approved drugs (Drews, 2000). Nonetheless, the pool of biological targets suitable for drug development represents a small fraction of the total number of genes in the human genome. Thus, there is a demand for quick, efficient methods to screen and select biological targets for drug development. A critical review of the suitability of antisense technology for the determination of gene function is provided in Chapter 2 of this thesis. Antisense technology compares favourably with other methods, including overexpression systems, small-molecule inhibitors, monoclonal antibodies and gene knockouts, for the determination of gene function. Principally, no other system consistently provides the desired combination of high versatility with respect to the classes of targets that can be inhibited, high target specificity and low technical and monetary resource requirements (Koller *et al.*, 2000). The process for determination of DOR gene function described in this thesis is not representative of the current pace of antisense technology in a dedicated, industrial setting. Thus, oligonucleotide synthesis and antisense inhibition assays (*in vitro* and cell-based) can be fully automated and integrated to accommodate the testing of antisense inhibitors for all the genes in a biological pathway in a matter of days. Hits from the *in vitro* screen can than be tested directly in appropriate *in vivo* models. One of the most advanced technology platforms of this type is GeneTrove™, a service-based subsidiary of ISIS Pharmaceuticals Inc. (San Diego, CA) dedicated to the rapid provision of functional genomics data. It is too early to evaluate the quality of hits yielded from the antisense, or from any of the other approaches in the field of functional genomics.

9.4.2 Modulation of Alternative Splicing

The pharmacological regulation of splice site selection is an application of antisense technology that is gaining recognition. In this case, antisense oligonucleotides are targeted to specific sequence elements that encode alternatively spliced protein variants of a given gene, block the

translation of the targeted exon and thereby inhibit the synthesis of unwanted protein variants and shift the splicing pattern of the target gene. Antisense molecules with high hybridization affinity for complementary mRNA, such as 2'-*O*-methoxyethyl ODN (Karras *et al.*, 2000) and PNA (Karras *et al.*, 2001), are effective modulators of alternative splicing. Antisense modulation of alternative splicing can be used to determine the function of splice variants of a common gene (Hodges & Crooke, 1995). In addition, this technique has various clinical applications. For example, genetic diseases such as β-thallassemia and muscular dystrophy are caused by mutations leading to the aberrant splicing of the *β-globin* gene and *dystrophin* gene, respectively. In both cases, *in vitro* studies have demonstrated that antisense oligonucleotides directed against the atypical splice sites can restore the function of the defective gene (Sierakowska *et al.*, 1996; Dunckley *et al.*, 1998). Alternatively, this approach can be used to decrease the expression of proteins associated with disease. For example, the *bcl-x* gene encodes two alternatively spliced proteins with antagonistic functions: Bcl-xL (antiapoptotic) and Bcl-xS (proapoptotic). Antisense oligonucleotides directed against a splice site in the Bcl-xL mRNA transcript leads to the predominant synthesis of Bcl-xS and the consequent modulation of cell survival in response to apoptotic stimuli (Taylor *et al.*, 1999; Mercatante *et al.*, 2001). Thus, control of the expression of the *bcl-x* gene by antisense technology may have clinical application in cancer therapy. Antisense techniques that control protein expression by the modulation of mRNA splicing will become increasingly popular with the interpretation of the human genome and the identification of splice variants of target proteins and related mutations thereof that are fundamental to genetic diseases.

9.4.3 Antisense Drugs

Antisense technology is extremely well positioned to accept the multitude of target genes revealed by functional genomics and to rapidly convert this information into highly selective drugs. However, the success rate of antisense drugs in clinical development has been poor for two main reasons – low efficacy (due, in part, to poor bioavailability at target sites) and toxicity. Only one antisense agent, Vitravene™ (fomivirsen; ISIS Pharmaceuticals Inc.), has been approved by the FDA out of the numerous oligonucleotides that have been entered into clinical trials over the last 10 to 15 years. Vitravene™ is a 21-base phosphorothioate oligonucleotide approved for the treatment of cytomegalovirus (CMV)-induced retinitis, which is prevalent in AIDS patients (Perry & Balfour, 1999). Its use is limited to a small market (12-month sales (2000): $157,000; Hollon, 2001) that is destined to become even smaller with the growing success of the HIV protease inhibitors in delaying the onset of AIDS.

The clinical success of the first-generation phosphorothioate antisense oligonucleotides is limited by their non-specific effects, poor accessibility to target sites *in vivo* and high manufacturing costs (Akhtar & Agrawal, 1997). The current approach for developing phosphorothioate oligonucleotides is to avoid these limitations by focusing on diseases that can be treated by local administration of drug (Hollon, 2001). For example, Vitravene™ is administered directly into the intravitreal space of the eye. However, local administration of drugs can be uncomfortable for the patient and expensive, particularly if administration requires clinical supervision as is the case for Vitravene™. Thus, the market potential of products that must be administered by local administration can be limited. Also, the scope of diseases that can be treated by local administration of drug is restricted.

A second, more constructive approach to developing antisense drugs is to move to an alternate chemistry platform as is reflected by the new, preclinical development pipelines presented by most antisense drug companies including ISIS Pharmaceuticals (www.ISIP.com) and Hybridon Inc. (www.hybridon.com). The toxicity and pharmacokinetic profile of the first-generation phosphorothioate oligonucleotides is largely a product of their chiral, polyanionic backbone chemistry (Stein, 1996, and previously discussed in Chapter 2). In comparison, second-generation antisense oligonucleotides now comprise 2'-O-(2-methoxy)ethyl, phosphoroamidate or morpholino oligonucleotide backbones (Nielsen, 2001), or combination mixed-backbone oligonucleotides (Agrawal & Kandimalla, 2000), with the overall goal being the minimization of polyanion-related effects and consequently decreased toxicity and increased protease resistance. In this regard, peptide nucleic acids (PNA) compare favourably with the second-generation oligonucleotides currently in preclinical development. PNA oligomers are devoid of polyanion-related effects leading to toxicity and poor stability. Also, the charge-neutral backbone of PNA increases both the affinity and specificity of hybridization to complementary nucleotides (Egholm *et al.*, 1993, and previously discussed in Chapter 2). Furthermore, PNA are easier to manufacture and chemically modify than the second-generation oligonucleotides listed above. Thus, PNA oligomers can be synthesized efficiently and economically by Boc or Fmoc solid-phase techniques. In addition, the peptidic structure of PNA can be modified by medicinal chemistry approaches for lead optimization (Nielsen, 2001).

The demonstration of PNA antisense effects *in vivo* presented in this thesis, and in other reports (Tyler *et al.*, 1998; Rezaei *et al.*, 2001) is a first step towards the commercialization of PNA oligomers as antisense agents. The preclinical development of PNA antisense oligomers has begun with Pantheco A/S (Denmark) presenting preliminary toxicological and pharmacokinetic

data for PNA sequences with antibacterial activity (Schou *et al.*, 2000). At the present time of writing, I anticipate continued advances in PNA chemistry to further improve the efficacy and toxicity profile of PNA oligomers over competing oligonucleotides and I look forward to the successful clinical development of PNA antisense oligomers in the next few years.

10 Reference List

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