Dissociation between behavioural and biochemical measures of mu and delta opioid receptors in rat central nervous system

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ABSTRACT

The opioid receptor family is comprised of three members: μ, δ and κ, all of which are G protein coupled receptors, primarily acting through $G\alpha_{i/o}$ subunits. Clinically, μ opioid receptor (MOR) agonists are used in the treatment of moderate to severe pain. δ opioid receptor (DOR) agonists are being developed as alternative analgesics, since stimulation of this receptor results in fewer adverse side effects. Characterization of behaviourally relevant μ and δ opioid receptors, as well as interactions between them, will provide a better understanding of opioid agonist-induced analgesia.

Although the behavioural knockdown after antisense targeting of MOR has been well characterized, few studies have examined the corresponding in vitro changes. Thus, the first aim of this thesis was to determine the neuroanatomical extent of MOR knockdown after pretreatment with peptide nucleic acid antisense in rats. Antisense pretreatment completely inhibited antinociception by the μ agonist DAMGO, but produced no detectable ex vivo changes in brain or spinal MOR labelling or functional responses. This study suggests that there may be a small, critical population of MORs that mediate antinociceptive responses to agonist.

The second aim of this thesis was to compare the CNS distribution of functional DOR with radioligand binding. DOR labelling was determined autoradiographically using an agonist, ([^{125}I]deltorphin II) and an antagonist ([^{125}I]AR-M100613) radioligand. In adjacent tissue sections, functional DORs were detected using deltorphin II-induced [^{35}S]GTPγS binding. Overall, radioligand binding did not strongly predict the magnitude

of [^{35}S]GTPγS responses, and this weak association is possibly explained by a paucity of DORs on the cell surface and/or heterogeneity in G protein receptor coupling. The highest [^{35}S]GTPγS responses were found in the basal ganglia, while areas involved with pain perception (spinal cord, brain stem, and periaqueductal grey) possessed low [^{35}S]GTPγS responses.

The low deltorphin II-induced [^{35}S]GTPγS binding in pain-related areas could explain the moderate degree of antinociception produced by δ agonists relative to their μ counterparts. Thus, the third aim of this thesis was to investigate two pharmacological treatments (short- and long-term morphine pretreatment) that are reported to enhance behavioural responses to δ agonists. As previously observed by others, short-term exposure to morphine resulted in sensitization to spinally administered δ agonists. In contrast, long-term morphine pretreatment resulted in profound tolerance to the antinociceptive and locomotor stimulant effects of deltorphin II. After chronic morphine pretreatment, there was no detectable change in DOR labelling or [^{35}S]GTPγS responses in the brain or spinal cord, suggesting that changes in downstream regulators may be responsible for this tolerance.

RÉSUMÉ

La famille des récepteurs opioïdes est composée de trois sous-types: μ, δ et κ. Ces trois récepteurs sont couplés aux protéines G et produisent leurs effets à travers les sous-unités Gαi/o. Les agonistes du récepteur μ opioïde (MOR) sont utilisés cliniquement pour combattre la douleur modérée et sévère. Les agonistes du récepteur δ opioïde (DOR) sont en phase de développement comme analgésique car ils produisent moins d'effets secondaires que les agonistes du MOR. Il est donc important de caractériser les effets comportementaux des agonistes MOR et DOR et des interactions entre ces deux sous-types de récepteurs opioïdes.

Malgré le fait que les changements comportementaux après l'injection d'antisense dirigé contre le MOR sont bien connus, les conséquences *in vitro* ont été caractérisé dans très peu d'études. Donc, le premier objectif de cette thèse était de déterminer l'expression du MOR au niveau neuroanatomique après l'injection d'antisense (acide nucléique peptidique) à des rats. Un traitement avec ces antisenses a complètement aboli l'effet anti-nociceptif normalement observé en présence de l'agoniste MOR DAMGO, mais n'a produit dans le cerveau ou la moelle aucun changement discernable sur la liaison d'agoniste MOR ou sur la fonction de ce récepteur. Cette étude suggère qu'une petite population de MOR est impliquée dans la production des effets anti-nociceptifs des agonistes MOR.

Le deuxième but de cette thèse était de comparer la distribution dans le système nerveux central (SNC) des DORs fonctionnels avec les sites de liaisons du DOR. Pour évaluer

ces sites de liaisons, un agoniste ([^{125}I]deltorphin II) et un antagoniste ([^{125}I]AR-M100613) radioactifs DOR ont été utilisé. Dans des sections de tissus adjacentes, les DORs fonctionnels ont été évalué en quantifiant la liaison de [^{35}S]GTPγS après stimulation avec l'agoniste DOR deltorphin II. En général, l'intensité du marquage radioactif produite par la liaison des ligands radioactifs dans les différentes régions du SNC ne correspond pas avec l'intensité du marquage produite par la liaison du [^{35}S]GTPγS en présence de deltorphin II dans ces mêmes régions. La pauvre association entre ces deux paramètres est possiblement expliquée par la faible densité des DOR à la surface cellulaire et/ou pourrait être causée par une hétérogenéité du couplage aux protéines G dans les différentes régions du SNC. Les plus hauts niveaux de liaison du [^{35}S]GTPγS ont été observé dans le ganglion basal tandis que les régions impliquées dans la perception de la douleur (telle que la moelle, le PAG et le tronc cérébral) ont produit de faibles niveaux de liaison du [^{35}S]GTPγS.

Les faibles niveaux de DOR fonctionnels (évalués en utilisant la liaison du [^{35}S]GTPγS en présence de l'agoniste deltorphin II) dans les régions impliquées dans la perception de la douleur pourraient être la cause des modestes effets anti-nociceptifs des agonistes DOR relatifs aux agonistes MOR. Donc, le troisième objectif de cette thèse était d'investiguer deux traitements pharmacologiques (traitement a la morphine de courte et de longue durée) qui augmentent les réponses comportementales des agonistes DOR. En accord avec des données publiées, un court pre-traitement avec la morphine (un agoniste du MOR) entraîne une sensibilisation aux agonistes DORs injectés dans la moelle. Par contre, un long traitement avec la morphine produit une tolérance importante aux effets

anti-nociceptif et locomoteur de la deltorphin II. Le traitement chronique avec la morphine ne produit aucun changement dans la moelle ou le cerveau sur les sites de liaison d'un ligand du DOR ou les sites de liaison du [^{35}S]GTPγS engendré par un agoniste DOR. Ces résultats suggèrent que des changements aux effecteurs intracellulaires pourraient être la cause de cette tolérance.

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GUIDELINES FOR THESIS PREPARATION

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

1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.)

2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges preceding and following each manuscript are mandatory.

3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include the following: (1) a table of contents; (2) a brief abstract in both English and French; (3) an introduction which clearly states the rational and objectives of the research; (4) a comprehensive review of the literature (in addition to that covered in the introduction to each paper); (5) a final conclusion and summary; (6) a thorough bibliography; (7)Appendix containing an ethics certificate in the case of research involving human or animal subjects, microorganisms, living cells, other biohazards and/or radioactive material.

4. As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

5. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.

CONTRIBUTION OF AUTHORS

This thesis is written in manuscript format as permitted by the McGill Faculty of Graduate Studies and is composed of three manuscripts. The contribution of each author is as follows.

Chapter 2: A.A.A. Pradhan & P.B.S. Clarke (2005). Pharmacologically selective block of mu opioid antinociception by peptide nucleic acid antisense in absence of detectable ex vivo knockdown. European Journal of Pharmacology 506:229-236.

I performed all of the experiments in this manuscript. The techniques included stereotaxic surgeries for chronic indwelling cannulae, antisense administration, antinociceptive testing (paw pressure), cryosectioning, autoradiography for [^{125}I]FK 33824 and DAMGO-induced [^{35}S]GTPγS binding, immunoautoradiography, quantitative autoradiographic analysis on the MCID M4 imaging system, and statistical analysis.

The manuscript was written and prepared by Amynah Pradhan and Paul Clarke.

Chapter 3: A.A.A. Pradhan & P.B.S. Clarke (2005). Comparison between δ opioid receptor functional response and autoradiographic labelling in rat brain and spinal cord. Journal of Comparative Neurology 481:416-426.

I performed all of the experiments in this manuscript. The techniques included cryosectioning, autroradiography for [^{125}I]deltorphin II and [^{125}I]AR-M100613, and

deltorphin II-induced [^{35}S]GTPγS binding, quantitative autoradiographic analysis on the MCID M4 imaging system, and statistical analysis.

The manuscript was written and prepared by Amynah Pradhan and Paul Clarke.

Chapter 4: A.A.A. Pradhan, C. Siau, A. Constantin, and P.B.S. Clarke (2005). Chronic morphine administration and withdrawal results in tolerance to delta opioid receptor mediated antinociception. *Submitted.*

I performed the majority of the experiments in this manuscript. My contributions were the following: stereotaxic surgeries for chronic indwelling cannulae, morphine administration, antinociceptive testing (formalin test), locomotor testing, cryosectioning, autoradiography for [^{125}I]deltorphin II and deltorphin II-induced [^{35}S]GTPγS binding, quantitative autoradiographic analysis on the MCID M4 imaging system, and statistical analysis.

Dr. Chiang Siau performed all acute intrathecal injections.

Annie Constantin assisted with the stereotaxic surgery, and the formalin testing.

The manuscript was written and prepared by Amynah Pradhan and Paul Clarke.

CLAIMS OF ORIGINALITY

In this thesis I presented the following original results:

Chapter 2

Initially, the goal of this study was to characterize the neuroanatomical extent of μ opioid receptor knockdown in central nervous system (CNS) following intracerebroventricular (i.c.v.) administration of peptide nucleic acid antisense (a novel antisense chemistry). The anti-μ opioid receptor antisense sequence abolished μ agonist-induced antinociception. Surprisingly, post mortem receptor autoradiographic analysis of CNS areas revealed no change in μ opioid receptor functional response ([^{35}S]GTPγS assay) or receptor labelling ([^{125}I]FK-33824 and μ opioid receptor immunoautoradiography). The antisense literature is rife with examples of small biochemical changes accounting for complete elimination of behavioural affects. These results provided the clearest example of antisense-induced knockdown at the behavioural level, in the absence of clear changes at the tissue level. This study suggests that there may be a small but critical population of μ opioid receptors that are responsible for the behavioural effects of μ agonists.

Chapter 3

The distribution of δ opioid receptors (DORs) in the rat CNS has been previously characterized by radioligand binding and immunohistochemistry. However, the functional neuroanatomy of DORs has not been mapped in any detail; this is potentially important, since these receptors appear to be primarily cytosolic. Opioid receptors can couple to $G_{i/o}$ G proteins, a process which is detected by agonist-stimulated [^{35}S]GTPγS

binding. The purpose of this study was to compare the functional population of DORs to agonist and antagonist radioligand binding. This study illustrated that for the DOR, radioligand binding only partially predicted the functional receptor population. This is an important finding as labelling of the receptor is not equivalent to the receptor having functional relevance. The divergence between the static and functional measures may possibly reflect regional heterogeneity in G protein receptor coupling, or in the subcellular localization of DOR.

Chapter 4

The literature suggests that after short term morphine pretreatment, rats are sensitized to spinal antinociception by deltorphin II (δ opioid receptor agonist). Clinically, this finding may be important as it suggests that switching patients from μ to δ agonists would prevent morphine tolerance, and the δ agonists would be better analgesics due to the pre-exposure to morphine. The aim of our study was to determine if chronic pretreatment with morphine would change δ opioid receptor responses. Surprisingly, we found that after chronic morphine animals became tolerant to the effects of the δ agonist, deltorphin II. More importantly, this tolerance lasted for 2 weeks after morphine withdrawal. Although initial studies suggested that short term morphine pretreatment primes the δ opioid receptor, our studies show that long term use of morphine results in a desensitization of the δ opioid receptors. Our results indicate that δ agonists may be limited in their use as analgesics if they are to be given to patients who have already been treated with morphine for their pain.

LIST OF ABBREVIATIONS

[^{35}S]GTPγS: guanosine 5'(γ-^{35}S-thio) triphosphate

5'-NTII: naltrindole 5'-isothiocyanate

ACTH: adrenocorticotropic hormone

AS: antisense

β-FNA: β-funaltrexamine

BNTX: 7-benzylidenenaltrexone

C5: cervical segment 5

cAMP: cyclic adenosine monophosphate

CNS: central nervous system

CP: caudate putamen

CTAP: D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2

CTOP: D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2

DADLE: [D-Ala^2,D-Leu^5]enkephalin

DALCE: [D-Ala^2,Leu^5, Cys^6]enkephalin

DAMGO: [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin

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

Deltorphin I: [D-Ala^2,Asn^4]-deltorphin

DH: dorsal horn

DOR: delta opioid receptor

DPDPE: D-Pen^2, D-Pen^5-enkaphalin

DSLET: [D-Ser^2, Leu^5, Thr^6]enkephalin

FrCtx: frontal cortex

GABA: glutamate and γ-amino-butyric acid

GIRK: G protein coupled inwardly rectifying potassium channel

GPCR: G protein coupled receptor

GRK: G protein coupled receptor kinase

ICI 174 864: N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH, where Aib is α-aminoisobutyric acid

i.c.v.: intracerebroventricular

i.p.: intraperitoneal

i.t.: intrathecal

L4: lumbar segment 4-5

LUM: lumbar spinal cord

M6G: morphine 6 glucoronide

MM: mismatch sequence

MOR: mu opioid receptor

MSH: melanocyte stimulating hormone

NMDA: N-methyl-D-aspartate

NSB: nonspecific binding

NTI: naltrindole

OcCtx: occipital cortex

PAG: periaqueductal grey

PNA: peptide nucleic acid

RGS: regulators of G protein signalling

RVM: rostroventral medulla (medial aspect)

SAL: saline

s.c.: subcutaneous

SEM: standard error of the mean

SNC80: (±)-4-[(α-R)-α-{2S,5R]-4-allyl-2,5-dimethyl-1-piperazinyl]-3-methoxybenzyl]-N,N-diethyl-benzamide

THAL: thalamus

TIPPψ: H-Tyr-Ticψ[CH_2-NH]Phe-Phe-OH

VEH: vehicle

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INTRODUCTION

1.1 History of Opioids

Opium has been used for centuries for its medicinal and euphoric properties, during which time it has been hailed both as a panacea for man's ills, and cursed as a scourge of civilization. Medical documentation of the use of opium soaked sponges to relieve pain during surgery can be found as early as 1500 BC (for review see Brownstein, 1993). Numerous literary works have portrayed opium as having near-mystical qualities. For instance, in Homer's Odyssey, Telemachus' grief over the loss of his father is stopped by a drug which will "lull all pain and anger and bring forgetfulness of every sorrow"; and the famous addict Samuel Taylor Coleridge described opium as "the milk of Paradise" in his laudanum-induced "Kubla Khan". The abuse liability of opium is so great, that in the 1830s China banned its use upon seeing how detrimental opium dens had become to Chinese society. The subsequent Opium War between China and Britain illustrated how the drug trade was as financially addictive to the merchants of the latter country as the drug itself was to the citizens of the former. Despite humanity's long familiarity with the derivatives of the poppy plant, there was almost total ignorance of how it worked.

One of the first breakthroughs in understanding the unique pharmacology of opium occurred in 1806, when Friedrich Wilhelm Serturner isolated the primary active ingredient in opium and called it Morphine, after Morpheus, the god of dreams. Serturner did not hesitate to experiment on himself, and after morphine administration he experienced a euphoric dream-like state, followed by depression and nausea; "I consider it my duty to attract attention to the terrible effects of this new substance in order that

calamity may be averted" (Scott, 1969). Morphine proved to be as addictive as opium, and so the hunt for opioid analgesics with low abuse liability began. The elucidation of the alkaloid structure of morphine led to the development of the synthetic opioid heroin, which was found to be more potent than morphine. Ironically, heroin was initially hailed as a non-addictive morphine substitute. Many other opioid agonists have since been characterized, but to date there are still no commercially available opioid drugs that are both analgesic and free from abuse liability.

The search turned from the drug's chemistry to mammalian anatomy, specifically the receptors in the central nervous system (CNS) that regulate opioid responses. The first opioid receptor was discovered in 1973, by three separate groups, all using radiolabelled opioid agonist binding in brain homogenates (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973). Evidence for multiple opioid receptor types was demonstrated by the different pharmacological profiles of morphine (μ opioid receptor, MOR), ketazocine (κ opioid receptor, KOR), and N-allylnormetazocine (SKF-10047, σ opioid receptors) in chronic spinal dog (Martin et al., 1976). Sigma receptors have since been shown to not be members of the opioid receptor family (Mannalack et al., 1986). The increased potency of the endogenous opioid peptide enkephalin to inhibit contractions in the mouse vas deferens relative to morphine led Kosterlitz and colleagues to propose the existence of δ opioid receptors (DOR)(Lord et al., 1977). The presence of DOR was later confirmed in rodent brain (Chang and Cuatrecasas, 1979).

Opioids are now defined as agonists that are displaced by naloxone (Dhawan et al, 1996). The opioid receptor family includes three members: the μ, δ, and κ opioid receptors (MOR, DOR and KOR respectively). All three are G protein coupled receptors, primarily acting through $G\alpha_{i/o}$ subunits (Dhawan et al., 1996). These receptors are found throughout the body, but two of the most important behavioural effects of opioids, analgesia and addiction, are mediated by opioid receptors in the brain and spinal cord.

1.2 Endogenous Opioid Peptides

It seemed unlikely that organisms would develop opioid receptors to respond to a plant-derived drug (morphine), with which they might never come in contact. The more likely explanation was that organisms produced an endogenous ligand for these receptors, and shortly after the discovery of opioid receptors, the hunt for their natural ligand began.

The earliest physiological evidence in support of endogenous opioids was that analgesia induced by electrical stimulation of certain brain areas was reversed by the pan-opioid antagonist naloxone (Akil et al., 1976). The first naturally occurring opioid peptides were discovered in pig brain, and named enkephalin meaning "in the head" (Hughes et al., 1975). The first two enkephalins discovered were chemically-related pentapeptides with the sequences Tyr-Gly-Gly-Phe-Leu (leu-enkephalin) and Tyr-Gly-Gly-Phe-Met (met-enkephalin). Of the opioid receptors, enkephalins had the greatest selectivity for DORs (Hughes et al., 1975). The subsequent discovery of β-endorphin, (equally selective for MOR and DOR (Loh et al., 1976)), and the KOR preferring dynorphins

(Goldstein et al., 1979) revealed that these three classes of endogenous opioid peptides shared the core sequence of Tyr-Gly-Gly-Phe-Leu/Met.

The majority of these endogenous opioids are derived from three precursors, which undergo peptidase cleavage to produce smaller functional opioid peptides. Thus, proopiomelanocortin produces β-endorphin, as well as a number of other biologically important peptides such as adrenocorticotropic hormone (ACTH), and melanocyte stimulating hormone (MSH)(Nakanishi et al., 1979). Preproenkephalin encodes one copy of leu-enkephalin, and four copies of met-enkephalin, including an octa- and a heptapeptide analogue (Table 1.2)(Noda et al., 1982a; Noda et al., 1982b; Khachaturian et al., 1985). Lastly, prodynorphin contains three leu-enkephalin core opioid sequences with C-terminal differences encoding dynorphin A, dynorphin B, and neo-endorphin (Kakidani et al., 1982). Although these endogenous peptides are in general not highly selective for any particular opioid receptor and are quickly degraded, their structure has lead to the development of numerous peptide agonists with greater selectivity and stability. For example, the highly selective MOR agonist D-Ala^2, MePhe^4, Gly^5-ol-enkephalin (DAMGO)(Handa et al., 1981) and the selective DOR peptide D-Pen^2, D-Pen^5-enkephalin (DPDPE)(Mosberg et al., 1983) are both based on the structure of endogenous enkephalin. Furthermore, endogenous opioid peptide derived from amphibian skin have yielded some of the most selective ligands for MOR (dermorphin) (Montecucchi et al., 1981) and DOR (deltorphin I and II)(Erspamer et al., 1989; see Table 1.1 for review).

Although the majority of clinically relevant opioid agonists act at MOR, none of the three above classes of endogenous opioids are highly selective for this receptor. It was not until 1997 that endogenous ligands with high affinity and selectivity for MOR were discovered (Zadina et al., 1997). These were termed endomorphin 1 and 2 and unlike other endogenous opioids they do not share the opioid peptide core, instead they are tetrapeptides with the sequence Tyr-Pro-Trp/Phe-Phe (Zadina et al., 1997). To date, the precursor peptide from which endomorphins are derived from is unknown (for review see Zadina et al., 1999).

Opioid receptors are located throughout the brain and spinal cord. As the activation of the κ opioid receptor results in dysphoria and hallucinations (reviewed in Martin and Eisenach, 2001), and upregulation of this receptor is associated with hyperalgesia (Wang et al., 2001), it does not pose a promising target for clinical use. The rest of this introduction will therefore focus on characteristics of μ and δ opioid receptors only.

Table 1.1: Commonly used ligands that act at μ and δ opioid receptors

Receptor	Endogenous Ligand	Peptide Agonist	Peptide Antagonist	Agonist	Antagonist
MOR	Endomorphin 1 Endomorphin 2 β-endorphin	DAMGO Dermorphin	CTOP CTAP	Morphine Fentanyl Sufentanyl	β-FNA Naloxone Naloxonazine
DOR	Leu^5-enkephalin Met^5-enkephalin Met^5-enkephalin-Arg^6-Phe^7 Met^5-enkephalin-$Arg^6Gly^7Leu^8$	DADLE DPDPE DSLET Deltorphin I Deltorphin II	DALCE ICI 174 864 TIPP TIPPψ	BW373U86 SNC80 TAN 67	Naltrindole (NTI) Benzylidenenaltrexone (BNTX) Naltriben (NTB) NTI 5'isothiocyanate (5'-NTII) AR-M100613

1.3 Neuroanatomical distribution of opioid receptors

1.3.1 Distribution of opioid receptors in the brain

The neuroanatomical localization of MOR and DOR has been extensively characterized using radioligand binding, immunohistochemistry and in situ hybridization. In general, these three methods are in agreement as to the location of these receptors in rodent brain (Table 1.2). Direct comparison of MOR vs. DOR indicates that MOR has a broader neuroanatomical distribution (Mansour et al., 1995a). Both are expressed in pain-related areas such as the periaqueductal grey and rostroventral medulla, although MOR is more consistently detected in these regions (Goodman et al., 1980; Mansour et al., 1994a; Mansour et al., 1994b; Mansour et al., 1995a; Arvidsson et al., 1995a; Mansour et al., 1995b; Cahill et al., 2001a). There is also an abundance of both receptors in the dopamine rich caudate putamen and nucleus accumbens, which play a role in reinforcing and locomotor stimulant effects of MOR and DOR agonists (Narita et al., 2001).

Although the different methods used to localize DOR are generally in agreement, several apparent discrepancies have been noted. For example, the majority of autoradiographic studies have been performed with [^{3}H]DPDPE or with [^{3}H]deltorphin analogs, and generally they produce overlapping distributions. However, in the nucleus accumbens DPDPE binding is consistently shown throughout this structure (Mansour et al., 1987; Tempel and Zukin, 1987; Blackburn et al., 1988; Sharif and Hughes, 1989), but binding by deltorphin analogs is limited (Dupin et al., 1991; Renda et al., 1993; Kitchen et al., 1995). This cannot be explained by the existence of DOR subtypes, as both DPDPE and deltorphin I preferentially binds to the putative δ1 subtype (see Section 1.8.1). There also appears to be an inconsistency between radioligand binding and immunohistochemical localization of DOR in the hypothalamus, periaqueductal grey and brain stem. In these structures DORs are poorly detected autoradiographically, but are moderately labelled by antibodies (Arvidsson et al., 1995a; Cahill et al., 2001a). This can be explained by the fact that these antibodies detect intracellular receptors (Svingos et al., 1995; Svingos et al., 1998; Svingos et al., 1999; Wang and Pickel, 2001; Cahill et al., 2001a) to which the radioligand may be insensitive.

Table1.2 μ and δ opioid receptors in the rodent brain

Brain Regions	MOR	DOR
Limbic System		
Hippocampus	Moderate	Low
Amygdala	High	Moderate
Hypothalamus	Moderate-low	Low
Cingulate Cortex	High	Moderate-low
Extended Striatum		
Caudate Putamen	High	High
Nucleus accumbens	High	High
Olfactory Tubercle	Low	High
Pain related areas		
Thalamus	High	Low
Periaqueductal grey	Moderate-low	Low
Raphe	Moderate	Low

References: Goodman et al., 1980; Mansour et al., 1987; Tempel and Zukin, 1987; Blackburn et al., 1988; Sharif and Hughes, 1989; Dupin et al., 1991; Renda et al., 1993; Gouarderes et al., 1993b; Mansour et al., 1994a; Mansour et al., 1994b; Kitchen et al., 1995; Arvidsson et al., 1995a; Mansour et al., 1995b; Arvidsson et al., 1995b; Hiller et al., 1996; Ding et al., 1996; Bakota et al., 1998; Unterwald et al., 1998; Cahill et al., 2001a; Abeyta et al., 2002

1.3.2 Evidence for MOR and DOR in the dorsal root ganglia

MOR and DOR are located on primary afferents which terminate in the spinal cord. Evidence for this is found after dorsal rhizotomy which results in a decrease in MOR and DOR binding and immunoreactivity in the superficial dorsal horn (Fields et al., 1980; Zajac et al., 1989; Besse et al., 1990; Gouarderes et al., 1991; Dado et al., 1993; Stevens and Seybold, 1995; Ding et al., 1996). Radioligand binding (Fields et al., 1980; Mennicken et al., 2003) and immunohistochemical (Dado et al., 1993; Mansour et al., 1995b; Arvidsson et al., 1995b; Ding et al., 1996) studies have also confirmed MOR and DOR labelling on cell bodies of dorsal root ganglion. However, it is unclear on which type of primary afferent fibre these opioid receptors are located, since evidence of every combination of C, Aδ and Aβ fibres has been reported (Dado et al., 1993; Mansour et al., 1994a; Arvidsson et al., 1995b; Ding et al., 1996; Wang and Wessendorf, 2001; Mennicken et al., 2003).

1.3.3 MOR in spinal cord

Consistent findings using radioligand binding, immunohistochemistry and in situ hybridization indicate that MOR is preferentially found in the superficial dorsal horn (lamina I and II) (Goodman et al., 1980; Sharif and Hughes, 1989; Zajac et al., 1989; Besse et al., 1990; Gouarderes et al., 1991; Hiller et al., 1994; Mansour et al., 1994b; Stevens and Seybold, 1995; Arvidsson et al., 1995b; Ding et al., 1996; Abbadie et al., 2001). Although primary afferent terminals account for some of the MOR found in the spinal cord, dorsal rhizotomy results in only a partial loss of MOR binding in lamina I and II (Zajac et al., 1989; Besse et al., 1990; Gouarderes et al., 1991; Stevens and

Seybold, 1995). Thus, it would appear that MOR is also expressed on intrinsic neurons within the spinal cord, and this has been confirmed by immunohistochemical detection of MOR on cell bodies in lamina II (Arvidsson et al., 1995b). Evidence from in situ hybridization (Mansour et al., 1994a; Mansour et al., 1994b; Wang and Wessendorf, 2001) and binding studies (Goodman et al., 1980; Gouarderes et al., 1991; Mansour et al., 1994b; Stevens and Seybold, 1995) indicate that MOR mRNA and protein may also be present in deeper laminae, but this expression is less abundant than that found in laminae I and II.

1.3.4 DOR in the spinal cord

There are conflicting reports of the distribution of DOR in the rodent spinal cord. Localization of DOR throughout the grey matter of the spinal cord has been shown using autoradiography with deltorphin analogs (Gouarderes et al., 1993b; Mennicken et al., 2003), DOR immunolabelling (Arvidsson et al., 1995a; Cahill et al., 2001a) and DOR in situ hybridization (Mansour et al., 1994a). However, several DOR radioligands (i.e. [^{125}I]DADLE, [^{3}H]DPDPE, [^{3}H]DTLET) have been found to label only the superficial dorsal horn (Goodman et al., 1980; Sharif and Hughes, 1989; Besse et al., 1990; Gouarderes et al., 1993b). To date, two DOR subtypes have been proposed, partly on the basis of pharmacological comparisons between δ1 (DPDPE, deltorphin I) and δ2 (deltorphin II, DTLET) selective agonists (Zaki et al., 1996)(see Section 1.8.1). Clearly, the differences in the above-mentioned DOR localization cannot be explained by these proposed subtypes, as ligands for both δ1 and δ2 can cause either profile.

1.3.5 Subcellular distribution of MOR and DOR

Electron microscopy studies of MOR have found this receptor preferentially expressed on the cell surface (Svingos et al., 1996; Wang and Pickel, 2001; Aicher et al., 2001; Garzon and Pickel, 2002). In contrast, DOR are predominantly associated with intracellular organelles, as has been seen in the cortex, nucleus accumbens, caudate putamen and the lumbar spinal cord (Svingos et al., 1995; Svingos et al., 1998; Svingos et al., 1999; Wang and Pickel, 2001; Cahill et al., 2001a). This intracellular distribution may also explain why immunohistochemical staining tends to reveal DORs in more areas than radioligand binding. For example, the periaqueductal grey is labelled by antibodies targeted to DOR (Arvidsson et al., 1995a; Cahill et al., 2001a), yet this structure is rarely detected by radioligand binding (Mansour et al., 1987; Tempel and Zukin, 1987; Blackburn et al., 1988; Sharif and Hughes, 1989; Renda et al., 1993). Moreover, this region does not appear to possess functional DOR in terms of antinociception (Bodnar et al., 1988; Ossipov et al., 1995) and electrophysiological responses to DOR agonists (Vaughan and Christie, 1997). Antibodies which bind to intracellular DOR may also detect receptors that are at different stages of post-translational modification or breakdown (Cahill et al., 2001a). A further problem with DOR localization studies is that the majority of autoradiographic studies have used DPDPE, which appears to have a MOR component (Sora et al., 1997; Hosohata et al., 2000; Park et al., 2000; Fraser et al., 2000b). These different types of binding may not reflect the functional distribution of DOR, which may be better detected using the [^{35}S]GTPγS assay, a measure of G protein receptor coupling. Using DOR selective ligands, a direct autoradiographic comparison between DOR-mediated [^{35}S]GTPγS responses and radioligand binding would determine if functional

DORs are a subpopulation of labelled DORs. This idea will be further developed in Chapter 3.

1.4 Pain Pathways

Painful stimuli are transmitted from primary afferents to the spinal cord and subsequently to the brain via ascending pain pathways, and descending pain pathways send inhibitory or facilitatory information through parallel pathways back to the spinal cord and primary afferent fibres (Millan, 2002). There are three different types of primary afferents; small calibre, unmyelinated C fibres and medium calibre, thinly myelinated Aδ fibres transmit nociceptive stimuli, while large calibre myelinated Aβ fibres convey innocuous and mechanical stimuli to the spinal cord. In general, the threshold stimuli needed to activate nociceptors are stronger than those needed to activate Aβ fibres, although they need not be so strong as to cause tissue damage (Willis and Westlund, 1997).

C and Aδ fibres primarily terminate in the superficial dorsal horn (lamina I and II), while Aβ fibres terminate in deeper lamina III-VI (Millan, 2002). Primary afferents terminate on projection neurons, or excitatory and inhibitory interneurons within the spinal cord. The interneurons serve to modulate the projection neurons, and inhibitory interneurons can also inhibit excitatory interneurons and primary afferents (Millan, 2002). Projection neurons transmit information from the spinal cord to numerous regions in the brainstem and midbrain, including the thalamus, periaqueductal grey, parabrachial region, and bulbar reticular formation. Pain-related information is subsequently passed on to the cortex and limbic structures such as the hypothalamus, amygdaloid nucleus, septal

nucleus, and extended striatum (nucleus accumbens and olfactory tubercle in particular)(Willis and Westlund, 1997).

In response to painful stimuli, brain regions then send descending projection neurons either directly to the spinal cord, or to other structures that have a direct projection to the spinal cord. Direct descending projections from the cortex, hypothalamus, nucleus tractus solitarius, dorsal reticular nucleus, parabrachial area and rostroventral medulla (medial aspect) (Basbaum and Fields, 1984; Millan, 2002) carry inhibitory or facilitatory information back to the spinal cord and primary afferents. These descending projection neurons can terminate on ascending projections, interneurons (excitatory or inhibitory), primary axon terminals, and other descending projections (Millan, 2002), thereby completing the pain circuit.

Opioid agonists produce antinociceptive effects by modulated ascending and descending pain pathways (Basbaum and Fields, 1984). Consistent with its role in antinociception, MOR expression is found in dorsal root ganglia, superficial dorsal horn (lamina I and II), parabrachial nucleus, rostroventral medulla, nucleus of the tractus solitarius, periaqueductal grey, thalamus, limibic structures and cortex. DORs are also expressed in these brain regions, but at lower levels than MOR. Nevertheless, substantial DOR expression is detected in primary afferents, dorsal horn, amygdala, and cortex (see Table 1.2, Section 1.3 and references therein). Endogenous opioid peptides are also found in numerous structures involved with antinociception. Enkephalin immunoreactivity has been reported in the superficial horn, rostroventral medulla, periaqueductal grey,

thalamus and limbic structures (Hokfelt et al., 1977; Basbaum and Fields, 1984; Khachaturian et al., 1985). β-endorphin has a more limited CNS distribution, and immunohistochemical detection in the periaqueductal grey, thalamus and hypothalamus has been observed (Khachaturian et al., 1985). Endomorphins are also distributed in numerous pain related areas such as the dorsal horn, thalamus, frontal cortex, hypothalamus and amygdala (Martin-Schild 1999, Horvarth 2000). Overall, μ and δ opioid receptors along with their endogenous ligands are well placed to mediate antinociception.

1.5 Antinociceptive actions of MOR

Systemic administration of μ agonists, such as morphine, have long been known to be analgesic. The anatomical sites mediating these antinociceptive responses has been determined using intracerebroventricular (i.c.v.), intrathecal (i.t.), and intraparenchymal microinjections directly into brain sites.

Intracerebroventricular injection of MOR agonists results in antinociception (Yaksh and Rudy, 1978; Tseng and Fujimoto, 1985), which is blocked by naloxone (Tseng and Fujimoto, 1985). Further evidence for the role of brain MOR in antinociception is that direct infusion into the ventricle of MOR-targeting antisense blocks the systemic (Chen et al., 1995a; Tyler et al., 1998) i.c.v or intra-PAG effects of morphine (Chen et al., 1995a; Rossi et al., 1997). In addition, MOR knockout mice are unresponsive to the antinociceptive effects of i.c.v μ agonists (Mizoguchi et al., 1999; Hosohata et al., 2000).

The periaqueductal grey (PAG) is an important supraspinal site of MOR agonist-induced antinociception. For example, microinjection of morphine or DAMGO into the PAG results in an antinociceptive response (Yeung et al., 1977; Llewelyn et al., 1983; Jensen and Yaksh, 1986; Jones and Gebhart, 1988; Fang et al., 1989), and this is blocked by systemic and local injections of naloxone (Jensen and Yaksh, 1986). The PAG regulates responses to pain through its projection to the rostroventral medulla, a structure which provides a direct descending projection to the spinal cord (Basbaum and Fields, 1984). Evidence for this connection was seen by changes in neuronal firing in the RVM after morphine injections into the PAG (Heinricher et al., 1987). Microinjection of DAMGO and morphine directly into the RVM also results in antinociception in acute pain tests (Llewelyn et al., 1983; Jensen and Yaksh, 1986; Jones and Gebhart, 1988; Rossi et al., 1994). Within the RVM, microinjection of naloxone into, or lesions of, the nucleus raphe magnus blocks antinociception induced by systemic morphine (Chance et al., 1978; Azami et al., 1982). Other sites of MOR agonist action include the thalamus (Cohen and Melzack, 1985; Carr and Bak, 1988), habenula (Cohen and Melzack, 1985), hypothalamus (Manning and Franklin, 1998) and the ventral tegmental area (Altier and Stewart, 1998).

Spinal antinociceptive effects of MOR stimulation are also well established. In rats, intrathecal administration of the μ selective drugs; morphine, codeine, meperidene, methadone, fentanyl, and DAMGO lead to increased latencies in the hotplate and tail flick tests, and were blocked by systemic MOR antagonists (Yaksh and Rudy, 1976; Yaksh and Rudy, 1977; Pick et al., 1991). In addition, direct administration of antisense

targeting MOR into the spinal cord inhibited endomorphin-induced antinociception (Wu et al., 2002). Furthermore, evidence for the spinal antinociception by MOR agonists comes from knockout studies, where the deletion of MOR resulted in a loss of antinociceptive responses produced by systemic or intrathecal μ agonists (Hosohata et al., 2000).

1.6 Antinociceptive actions of DOR

Antinociception resulting from DOR stimulation is more complicated than its MOR counterpart. DOR agonists have been reported to be less antinociceptive than MOR agonists (Audigier et al., 1980; Porreca et al., 1984; Chaillet et al., 1984; Galligan et al., 1984). There is no doubt that DOR agonists can be antinociceptive when given i.c.v. (Porreca et al., 1987; Qi et al., 1990; Ossipov et al., 1995; Kovelowski et al., 1999a; Kovelowski et al., 1999b; Hosohata et al., 2000; Fraser et al., 2000b), and knockdown of brain DOR by antisense results in complete knockdown of DOR agonist induced antinociception (Lai et al., 1995; Fraser et al., 2000a; Fraser et al., 2000b). However, supraspinal sites of action are not as clear as those for MOR. For example, unlike MOR, DOR infusion into the PAG does not appear to produce supraspinal antinociception. Although one group did report that microinjections of deltorphin II directly into the PAG was antinociceptive in the tail flick test (Rossi et al., 1994), no other group has found this site to be effective (Bodnar et al., 1988; Ossipov et al., 1995). Furthermore, electrophysiological studies suggest that DORs in the PAG are not functional (Vaughan and Christie, 1997).

The thalamus may also be a supraspinal site of action for DOR agonist induced antinociception. For example, DADLE has been shown to produce increased latencies in the hot plate and tail flick tests when injected into the lateral thalamus (Walker and Yaksh, 1986), and DTLET injected into the ventrobasal thalamus decreases neuronal firing induced by noxious stimuli (Benoist et al., 1986). Characterization of these effects using more selective DOR ligands has not been done. DORs in the nucleus accumbens may also be important in antinociception to noxious peripheral stimuli (Schmidt et al., 2002).

The rostroventral medulla (RVM) has also been tested as a possible initiation site for DOR antinociception. Thus, injections of deltorphin II directly into the RVM results in antinociception in acute and chronic pain tests (Kiefel et al., 1993; Rossi et al., 1994; Ossipov et al., 1995; Thorat and Hammond, 1997; Kovelowski et al., 1999a; Kovelowski et al., 1999b; Hurley and Hammond, 2000). However, DOR agonist injection into this brain area was less potent than i.c.v. administration of the drug (Kovelowski et al., 1999b), suggesting that the RVM is not the most important site for supraspinal DOR antinociception. Perhaps supraspinal antinociception produced by DOR agonists could be explained by synergy of DORs in numerous brain regions.

Understanding of the antinociceptive effects of supraspinal DOR agonist action is further complicated by studies done in opioid receptor knockout mice. Animals that lacked DOR retained supraspinal antinociception following i.c.v. DPDPE and deltorphin II, and this effect was only partially antagonized by the DOR antagonist naltrindole (Zhu et al.,

1999). This antinociception was insensitive to MOR and KOR antagonists, but was completely blocked by naltrexone (Zhu et al., 1999). To explain these results, the authors proposed the existence of a δ-like opioid receptor that is distinct from DOR (Zhu et al., 1999). There is also the possibility that retention of supraspinal DOR response is due to some compensatory effect of genetic manipulation. Pharmacological specificity of deltorphin II and DPDPE may also be in question, and this will be further discussed in section 1.8.2.

DOR agonists can also produce antinociception via a direct action in the spinal cord, and the pharmacological nature of this response is better understood than those in the brain. Initial reports found that intrathecal administration of the DOR agonist DADLE could block acutely painful stimuli (Tung and Yaksh, 1982; Hylden and Wilcox, 1982). Although DADLE has affinity for both DOR and MOR, this antinociceptive effect was not blocked by the μ antagonist β-FNA (Hylden and Wilcox, 1982), and did not show cross tolerance to morphine (Tung and Yaksh, 1982) suggesting a wholly DOR action. Later studies with DPDPE and the highly DOR-selective agonist deltorphin II confirmed that intrathecal DOR agonists were antinociceptive (Porreca et al., 1984; Porreca et al., 1987). Responses to these agonists were blocked by DOR antagonists ICI 174 864, naltrindole, 5'-NTII and naltriben (Heyman et al., 1987; Mattia et al., 1991; Sofuoglu et al., 1991; Mattia et al., 1992; Stewart and Hammond, 1993) but not by MOR antagonists (Heyman et al., 1987; Jiang et al., 1991). Antisense knockdown of spinal DOR, and deletion of DOR also resulted in a lack of response to intrathecal DPDPE and deltorphin

II (Standifer et al., 1994; Tseng et al., 1994; Bilsky et al., 1996; Zhu et al., 1999). In summary, DOR agonists can clearly induce antinociception by acting at spinal DOR.

1.7 Opioid Receptor Signalling

Long before the cloning of the opioid receptors there were many indications that this receptor family was coupled to G proteins. For example, agonist binding was reduced in the presence of Na^+ (Pert and Snyder, 1973; Kosterlitz et al., 1988), and addition of GTP synergistically enhanced this effect (Childers and Snyder, 1980). Activation of opioid receptors resulted in inhibition of adenylate cyclase (Sharma et al., 1975), and this inhibitory effect was pertussis toxin sensitive (Hsia et al., 1984), dependent on GTP and Na^+ (Blume et al., 1979), and ultimately resulting in GTP hydrolysis (Koski and Klee, 1981). Further evidence that opioid receptors were G protein coupled came from reconstitution experiments where the addition of G_i or G_o Gα subunits to rat brain purified MORs increased the displacement of [^{3}H]naloxone by DAMGO (Ueda et al., 1988). In addition, these two Gα subunits were co-purified with opioid receptors from brain homogenates (Wong et al., 1989).

Cloning studies confirmed that opioid receptors belonged to the G protein coupled receptor (GPCR) super-family. The δ opioid receptor was the first to be cloned from the NG108-15 cell line (Kieffer et al., 1992; Evans et al., 1992). Oligonucleotide primers based on the DOR sequence were then used to clone KOR (Meng et al., 1993; Minami et al., 1993), and MOR (Chen et al., 1993; Fukuda et al., 1993; Thompson et al., 1993). The putative structure of these receptors is typical of the GPCR super-family. These

serpentine receptors have seven transmembrane alpha helices, three intracellular loops, three extracellular loops, an intracellular carboxy terminus and an extracellular amino terminus. The opioid receptors are ~60% homologous to one another, and the greatest homology is in the transmembrane domains (73-76%) and the intracellular loops (86-100%), which suggests that these receptors have similar intracellular interactions (Law et al., 2000b)(See Section 1.7). The greatest diversity, on the other hand, occurs at the extracellular face of the receptor, at the N terminus (9-10%) and extracellular loops (14-72%), thereby conferring ligand selectivity (Xie et al., 1990; for review see Akil et al., 1998 and Law et al., 2000) .

1.7.1 Identification of Gα subunits coupling to MOR and DOR

MOR and DOR primarily signal through $G\alpha_{i/o}$ subunits, and they appear to share many of the same Gα subunits. Both in cultured cells and in vivo, it appears that MORs and DORs primarily couple to G_{o1-2}, and G_{i1-3} (Connor and Christie, 1999). This has been determined by irreversibly labelling activated G proteins (Offermanns et al., 1991; Roerig et al., 1992; Laugwitz et al., 1993), by blocking the function of different Gα subunits using antibodies (Sanchez-Blazquez et al., 1993; Carter and Medzihradsky, 1993; Garzon et al., 1997b) or by antisense knockdown (Sanchez-Blazquez et al., 1995; Standifer et al., 1996; Sanchez-Blazquez and Garzon, 1998). This overlap in G protein coupling is supported by work done in SH-SY5Y cells where either SNC80 (δ agonist) or DAMGO (μ agonist) can promote dissociation of $[^{35}S]GTP\gamma S$ prebound to the other ligand (Alt et al., 2002). However, there are subtle differences between MOR and DOR coupling. For example, in human neuroblastoma cells, it appears that MOR preferentially couples to G_o

(Carter and Medzihradsky, 1993) and G_{i3} (Laugwitz et al., 1993), while DOR more efficiently couples to G_{i1} (Carter and Medzihradsky, 1993; Laugwitz et al., 1993).

MORs and DORs can also couple to non-$G_{i/o}$ Gα subunits. For example there is in vitro and in vivo evidence to suggest that opioid receptors can act through G_z, a subunit related to G_o but insensitive to pertussis toxin (Wong et al., 1992). In support of this coupling, in HEK 293 cells which express G_i subunits, coexpression of cloned MOR and G_z resulted in inhibition of adenylate cyclase that was only partially blocked by pertussis toxin (Chan et al., 1995). In addition, antibodies to G_z blocked MOR stimulated GTPase activity in rat periaqueductal grey membranes (Garzon et al., 1997b), and antisense knockdown of G_z blocked antinociception by MOR agonists (Sanchez-Blazquez et al., 1993; Sanchez-Blazquez et al., 1995). Unlike MOR, the evidence for G_z coupling to DOR is equivocal. Two separate groups have found that in vitro (Tsu et al., 1995), and in vivo (Standifer et al., 1996) DOR can efficiently couple to G_z. However, another group found no change in DOR responses after antibody blockade (Sanchez-Blazquez et al., 1993; Garzon et al., 1997a; Garzon et al., 1997b) or antisense targeting of G_z (Sanchez-Blazquez et al., 1995). Thus, the ability of DOR to couple to G_z is unclear.

Other non-$G_{i/o}$ Gα subunits that can couple MOR and DOR are the G_q related, G_{15} and G_{16}, respectively (Offermanns and Simon, 1995; Lee et al., 1998). This coupling can lead to activated phospholipase C and subsequently to inositol phosphate production. However, opioid receptor stimulation may also activate this pathway by Gβγ subunits (Yoon et al., 1999).

The Gα subunit selectivity profile for the μ and δ opioid receptors has primarily been determined using reconstitution studies in cell membranes or by in vivo antisense knockdown. There are limitations to both of these techniques. An important limitation of cell culture is that within these systems cellular distribution of opioid receptors is not representative of in vivo expression. For example, in cell culture DORs are expressed on the cell surface (Ko et al., 1999; Alt et al., 2002), while ultrastructual localization of DOR in brain and spinal cord indicates that these receptors are primarily intracellular (Svingos et al., 1995; Svingos et al., 1998; Svingos et al., 1999; Wang and Pickel, 2001; Cahill et al., 2001a). In vivo antisense studies are also limited, especially since knockdown of Gα subunits would be expected to disturb G protein coupling not only to opioid receptors but to other GPCRs that contribute to behavioural responses. In this regard, it is noteworthy that G_s and G_q opioid receptor coupling has only been detected in in vivo antisense studies (Standifer et al., 1996; Sanchez-Blazquez and Garzon, 1998), and not in in vitro studies (Connor and Christie, 1999). One important thing to note about G protein coupling of opioid receptors is the diversity in Gα subunits through which these receptors signal. The signalling response to opioid agonists may vary greatly depending on the population of Gα subunits expressed within a given cell and the abundance of other GPCRs that may be competing for these Gα subunits.

1.7.2 Opioid activation of Gβγ subunits

The characterization of opioid receptor coupling has primarily focused on Gα subunits. However, Gβγ subunits appear to regulate many of the downstream effects of opioid

receptor activation. One such example is the MOR- and DOR-mediated inhibition of voltage dependent Ca^{++} channels seen in cell culture (Seward et al., 1991; Schroeder et al., 1991; Morikawa et al., 1995) and in the brain (Stefani et al., 1994; Connor and Christie, 1998; Connor et al., 1999). This inhibitory effect is believed to be responsible for the attenuation of neurotransmitter release seen after opioid receptor activation (Bhoola and Pay, 1986; Schoffelmeer et al., 1986). Inhibition of Ca^{++} conductance for other $G_{i/o}$ GPCRs is produced by Gβγ subunits (Ikeda, 1996; Herlitze et al., 1996). However, opioid inhibition of Ca^{++} channels is blocked by pertussis toxin, and is restored upon addition of G_o and to a lesser extent G_i subunits (Hescheler et al., 1987). This suggests that Gα subunits may be necessary to couple the opioid receptors to this Gβγ effect.

Opioid receptor activation also results in an increased K^+ conductance, the most commonly observed being the G protein-activated inwardly rectifying conductance (GIRK)(North et al., 1987; Vaughan and Christie, 1997; for review see Williams et al., 2001). GIRK channel opening is due to a direct action of Gβγ released from pertussis toxin sensitive G proteins (Reuveny et al., 1994; Jan and Jan, 1997; Yamada et al., 1998). The inhibitory effect of increased K^+ conductance has two main consequences. First, it can result in a decrease in neurotransmitter release. For example, stimulation of MOR in the periaqueductal grey results in increased K^+ conductance followed by an inhibition of GABAergic synaptic transmission (Vaughan and Christie, 1997). Second, increased K^+ conductance results in hyperpolarization of the postsynaptic membrane, which reduces neuronal excitability (Grudt and Williams, 1994).

The Gβγ subunit may also be responsible for the stimulation of phospholipase Cβ observed after opioid receptor activation. Activation of this enzyme results in the generation of inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG), which in turn leads to Ca^{++} mobilization from intracellular stores and activation of protein kinase C (PKC). This signalling pathway has been detected in MOR (Smart et al., 1994; Smart et al., 1997) and DOR (Jin et al., 1994; Smart and Lambert, 1996). Although induction of phospholipase Cβ may also be mediated by opioid receptors coupling to G_q, this is unlikely as blockade of Gβγ and not G_q was necessary to inhibit the release of intracellular Ca^{++} following DOR stimulation (Yoon et al., 1999). However, it appears that $G_{i/o}$ coupling is necessary for this Gβγ effect to occur, since opioid-induced Ca^{++} release is pertussis toxin sensitive (Smart et al., 1994) and blocked by antisense knockdown of G_{i2} or G_o (Murthy and Makhlouf, 1996).

A further result of opioid-induced activation of Gβγ is stimulation of phosphoinositide 3-kinase (PI3K) which in turn stimulates mitogen activated protein (MAP) kinase (Hawes et al., 1996; Polakiewicz et al., 1998b). Activation of MAP kinase is also pertussis toxin sensitive, suggesting that it is mediated by coupling through $G_{i/o}$ (Burt et al., 1996). MAP kinase activation is important for opioid receptor desensitization (Polakiewicz et al., 1998a), and appears to play a role in synaptic plasticity induced by chronic morphine exposure (Eitan et al., 2003)

1.7.3 Opioid receptor coupling and desensitization

The general sequence for GPCR activation and desensitization is as follows: The inactive α subunit is bound to GDP and to βγ, and this trimer is bound to the receptor. Upon receptor activation by an agonist, the complex undergoes a change in conformation, resulting in the exchange of GDP for GTP at the α subunit. This guanine nucleotide exchange causes uncoupling of the heterotrimeric complex from the receptor, and α and βγ subunits dissociate, and activate or inhibit several down stream effectors. The signal is terminated by hydrolysis of GTP to GDP on the α subunit either by intrinsic GTPase activity or by GTPase Activating Proteins (GAPs) of the Regulators of G protein Signalling (RGS) family. The heterotrimeric complex is reformed following the hydrolysis of GTP to GDP.

After agonist-induced uncoupling from the heterotrimeric complex, the receptor also undergoes significant changes. In most cases, the activated receptor is desensitized by phosphorylation by GPCR kinases (GRKs), which in turn recruit β-arrestin to the cell surface. Arrestins recognize both the activated receptor confirmation as well as phosphorylated sites on the receptor (Luttrell and Lefkowitz, 2002; Perry and Lefkowitz, 2002), and thus prevent the agonist-bound receptor from further signalling. Arrestins also promote receptor internalization by binding to the clathrin adaptor protein, AP2, and to clathrin itself. This allows the desensitized receptor to be engulfed into clathrin coated pits (Goodman, Jr. et al., 1997; Laporte et al., 1999), and dynamin-dependent budding and fission delivers the ligand-bound receptors to early endosomes (Chu et al., 1997; Bohm et al., 1997). At this point the receptor is either sorted to the lysosome for

degradation, or resensitized and recycled to the cell surface (for review see Gainetdinov et al., 2004). Although agonist activation of MOR and DOR results in phosphorylation of the receptor by GRKs (Arden et al., 1995; Pei et al., 1995; Zhang et al., 1996; El Kouhen et al., 1999), subsequent internalization of MOR and DOR follow divergent pathways.

1.7.4 DORs are degraded after agonist activation

DOR have been shown to undergo degradation after agonist-induced internalization. This has been demonstrated in recombinant neuronal and non-neuronal cell lines transfected with DOR (Malatynska et al., 1996; Trapaidze et al., 1996; Afify et al., 1998; Ko et al., 1999), as well as in intact brain tissue (Tao et al., 1998). Internalization of DOR is dependent on clathrin-coated pits, since DOR is colocalized with transferring (a marker for this type of endocytosis), and is inhibited by sucrose (which blocks formation of clatherin pits) (Ko et al., 1999). In neuronal cells stably transfected with DOR, short term treatment with DADLE resulted in ~10% of internalized receptors being recycled to the cell surface, while a small portion was retained in the lysosome (Ko et al., 1999). In contrast, prolonged agonist treatment (4-24 h) results in profound DOR degradation by targeting to the lysosome. This degradation was blocked by chloroquine (an inhibitor of lysosomal acidification) (Ko et al., 1999), and DOR was colocalized with lysosome-associated membrane protein-1 and 2 (LAMP-1/2) (Ko et al., 1999; Whistler et al., 2002). The recently discovered GPCR associated sorting protein (GASP) was necessary to target DOR to the lysosome, and disruption of the interaction between DOR and GASP resulted in DOR recycling (Whistler et al., 2002).

Desensitization of DOR is not dependent on coupling to $G_{i/o}$ proteins, since it is insensitive to pertussis toxin, which suggests that agonist binding is sufficient to cause receptor downregulation (Chakrabarti et al., 1997; Remmers et al., 1998; Zaki et al., 2001). It also appears that phosphorylation of DOR by GRKs may be essential to induce internalization, but is not necessary to produce DOR trafficking to the lysosome (Whistler et al., 2001).

1.7.5 MOR are recycled after agonist activation

MOR ligands also produce endocytosis (Sternini et al., 1996; Keith et al., 1998; Garrido et al., 1999; Trapaidze et al., 2000), but unlike DOR, MOR is predominantly resensitized and recycled to the cell surface as seen in cell culture and CNS tissue (Koch et al., 1998; Wolf et al., 1999; Trafton et al., 2000; Law et al., 2000a; Koch et al., 2001; Whistler et al., 2002; Wang et al., 2003). Also unlike DOR, MOR internalization is sensitive to pertussis toxin, indicating that G protein coupling is necessary for endocytosis (Chakrabarti et al., 1997; Zaki et al., 2001).

A direct correlation between MOR phosphorylation and receptor desensitization has been demonstrated (Zhang et al., 1996), further establishing a relationship between GRK binding and uncoupling of the receptor. In addition, the ability of a μ agonist to induce receptor phosphorylation is also correlated to its efficacy (Yu et al., 1997), and agonists such as DAMGO and etorphine are reported to induce rapid receptor phosphorylation and internalization (Yu et al., 1997). However, morphine is exceptional as unlike many other

μ agonists it is a poor inducer of receptor phosphorylation and subsequent internalization (Arden et al., 1995; Keith et al., 1996; Sternini et al., 1996; Keith et al., 1998; Zhang et al., 1998). This effect may be due to agonist-induced conformational changes of MOR which make phosphorylation sites on the receptor more or less accessible by GRK. Morphine can induce receptor internalization in the presence of over-expressed GRK-2 (El Kouhen et al., 1999; Zhang et al., 1998), or over-expressed β-arrestin (Zhang et al., 1998; Whistler and Von Zastrow 1998).

The inability of morphine to cause MOR internalization may have implications for tolerance. Compared to other μ agonists, morphine causes rapid tolerance (Finn and Whistler, 2001) and this occurs mainly through adaptations downstream of MOR (Nestler and Aghajanian, 1997). It has been proposed that morphine causes accelerated tolerance development by providing continuous MOR signalling which is uninterrupted by the receptor endocytosis and resensitization that most μ agonists produce (He et al., 2002). In support of this idea, a low dose of DAMGO can facilitate the ability of morphine to internalize MOR, and this increased endocytosis results in a reduction in antinociceptive tolerance (He et al., 2002). This is a highly controversial hypothesis, as it runs contrary to the more conventional idea that tolerance to chronic morphine is due to downregulation of MOR.

In support of the hypothesis that internalization of MOR is necessary to induce tolerance, Bohn and colleagues found that internalization of MOR by β-arrestin 2 was critical for the development of morphine tolerance. In this study, β-arrestin 2 knockout mice did not

develop tolerance to the supraspinal antinociceptive effects of morphine (Bohn et al., 2000), and had a delayed and attenuated tolerance to spinal morphine antinociception (Bohn et al., 2002). β-arrestin 2 knockout mice also showed an enhanced antinociceptive response to morphine (Bohn et al., 1999; Bohn et al., 2002). These findings were explained by increased ability of MOR to couple to G proteins in pain-related areas (PAG, brain stem, spinal cord) in knockout vs. wild type animals (Bohn et al., 1999; Bohn et al., 2002). In addition, rewarding properties of morphine were also enhanced in β-arrestin 2 knockout animals, as seen by an increased conditioned place preference relative to wild type mice (Bohn et al., 2003). However, there was no difference in the physical withdrawal symptoms produced by naloxone between knockout and wild type animals (Bohn et al., 2000). Overall, these results suggest that MOR internalization is important for morphine induced antinociception and tolerance.

Although these studies suggest that morphine-induced endocytosis of MOR is important in the development of tolerance, one must consider that knockout of β-arrestin 2 affects internalization of numerous other types of receptors which may explain these results. For example, DORs are internalized after agonist stimulation, and these receptors have been shown to modulate tolerance to morphine (as discussed in Section 1.10.2), and may be sensitive to manipulations of β-arrestin 2. Furthermore, long term exposure to all MOR agonists eventually results in tolerance, suggesting that the ability of an agonist to induce internalization may not be important.

1.8 Opioid receptor subtypes

1.8.1 DOR subtypes

DOR heterogeneity was first proposed based on radioligand binding studies in guinea pig cortical membrane and the DOR-rich NG108-15 cell line. In both tissues the non-selective opioid agonist [^{3}H]diprenorphine was inhibited biphasically by the DOR agonist DSELT (Werling et al., 1988). Further evidence to support the existence of DOR subtypes was provided by [^{3}H]DSLET and [^{3}H]DPDPE radioligand binding. In rat brain homogenates [^{3}H]DSLET was found to label 40% more sites than [^{3}H]DPDPE (Sofuoglu et al., 1992), and BNTX inhibited [^{3}H]DPDPE binding 100 times more potently than [^{3}H]DSLET (Portoghese et al., 1992). In addition, autoradiographic comparison of these two radioligands in the rat brain found that although they produced similar distributions, there were some regional differences, particularly in the hypothalamus, amygdala, cortex and periaqueductal grey (Hiller et al., 1996).

Behavioural studies also support the existence of DOR subtypes. For example, in acute pain tests supraspinal antinociception by DPDPE was blocked selectively by DALCE and BNTX. Conversely, antinociception by i.c.v. deltorphin II and DSLET was blocked by naltrindole 5'-isothiocyanate (5'-NTII) and naltriben (Calcagnetti et al., 1989; Sofuoglu et al., 1991; Jiang et al., 1991; Portoghese et al., 1992; Sofuoglu et al., 1993; Vanderah et al., 1994). Further evidence for the existence of DOR subtypes is provided by the finding that mice do not become cross tolerant after repeated administration of either DPDPE or deltorphin II (Mattia et al., 1991)(Table 1.3). On the basis of these behavioural findings two opioid receptor subtypes have been proposed: δ1 which is stimulated by

DPDPE and blocked by DALCE and BNTX, and δ2 opioid receptors were activated by deltorphin II and blocked by 5'-NTII and naltriben (for review see Zaki et al., 1996).

Table 1.3 Putative DOR subtype specific ligands

Receptor Subtype	Agonist	Antagonist
δ_1	DPDPE	BNTX DALCE
δ_2	Deltorphin II DSLET	Naltriben 5'-NTII
δ (combined)	SNC80	Naltrindole ICI 174 864

Biochemical studies in the brain also support the δ1/δ2 distinction. Basal (Buzas et al., 1994) and forskolin (Noble and Cox, 1996) stimulated adenylyl cyclase activity was inhibited by DPDPE and deltorphin II, and this inhibition was blocked by BNTX and naltriben respectively. In addition, G protein activation induced by δ agonists also adheres to the δ1/δ2 classification. A limited autoradiographic study in the mouse and rat forebrain and midbrain found that DPDPE- and deltorphin II-induced [^{35}S]GTPγS binding was inhibited by BNTX and naltriben, respectively (Tsuji et al., 1999).

The existence of δ1/δ2 subtypes has also been proposed in the spinal cord, but experimental evidence in support of this is equivocal. First, the δ2 antagonist naltriben was found to selectively antagonize antinociception by intrathecal deltorphin II and not

DPDPE in rat (Stewart and Hammond, 1993). Second, in a neuropathic pain model, both i.t. DPDPE and deltorphin II significantly reduced allodynia and were antagonized by BNTX and 5'-NTII, respectively (Mika et al., 2001). Third, electrophysiological studies of DOR in the spinal cord also suggest that DPDPE and deltorphin II differentially reduce excitatory postsynaptic currents and only the latter is inhibited by naltriben (Glaum et al., 1994). Other findings do not appear consistent with the δ1/δ2 distinction in the spinal cord. For example, 5'-NTII blocked both i.t. DPDPE and deltorphin II antinociception, where DALCE had no effect (Mattia et al., 1992). Additionally, i.t. antisense-targeting the DOR inhibited both i.t. DPDPE and deltorphin II induced antinociception in mice (Standifer et al., 1994). Based on the above evidence it is not clear if δ1/δ2 subtypes exist in the spinal cord.

1.8.2 Arguments against DOR subtypes

A major weakness in the case for DOR subtypes is that DPDPE, the classic δ1 agonist, has questionable selectivity for DOR. Evidence that DPDPE responses may have a MOR component is that in the brain the antinociceptive effects of DPDPE are blocked by CTOP (Fraser et al., 2000b), and in the spinal cord i.t. DPDPE is blocked by i.t. CTAP (He et al., 2002). Furthermore, MOR knockout mice have a significantly reduced response to i.c.v. and i.t. DPDPE (Sora et al., 1997; Hosohata et al., 2000), and antisense knockdown of DOR results in complete loss of deltorphin II-induced antinociception, but no concurrent inhibition of DPDPE (Bilsky et al., 1996; Fraser et al., 2000 but see Standifer et al., 1994; Tseng et al., 1994).

Biochemical analysis also supports the notion that DPDPE requires functional MOR to produce its effects. For example, a fraction of DPDPE binding was displaced by DAMGO and morphine (Cotton et al., 1985), and DPDPE-induced [^{35}S]GTPγS binding is reduced in MOR knockout animals (Hosohata et al., 2000; Park et al., 2000 but see Matthes et al., 1996 and Narita et al., 1996). These studies suggest that DPDPE is either not selective for DOR, or that DPDPE requires interaction between both DOR and MOR in order to produce its effects.

A further argument against the existence of DOR subtypes comes from molecular biology studies. In particular, only one clone has been identified for DOR with no polymorphisms or viable splice variants (Gaveriaux-Ruff et al., 1997; Wei and Loh, 2002). The evidence for DOR subtypes may only be an accident of pharmacology and could reflect the interplay between MOR and DOR (See Section 1.10).

1.8.3 MOR subtypes

MOR subtypes were first classified on the basis of binding studies in brain homogenates. Two subtypes (μ1 and μ2) were proposed based on the differential binding affinities of [^{3}H]morphine, [^{3}H]enkephalin, and [^{3}H]dihydromorphine (Wolozin and Pasternak, 1981). In animal studies all behavioural effects of morphine were blocked by β-funaltrexamine, but only a few were blocked by the μ1 selective antagonist naloxonazine. The μ1 subtype is suggested to mediate the following behavioural effects of morphine: supraspinal antinociception, prolactin release, catalepsy, feeding and hypothermia (Pasternak and Wood, 1986). Interestingly, the unwanted side effects of morphine administration -

physical dependence, respiratory depression, sedation, bradykardia and gastric motility were not regulated by this subtype, but by μ2 (Gintzler and Pasternak, 1983; Ling et al., 1984; Pasternak and Wood, 1986). Contrary to this classification, one study has found that morphine antinociception and respiratory depression was blocked by naloxonazine (Rourke and Shaw, 1984). Nevertheless, if the μ1/μ2 distinction is true it would suggest that a selective μ1 agonist would be an ideal clinical analgesic. To date, no such agonist has been found.

Clinically, all analgesics targeted to MOR ultimately result in tolerance after prolonged exposure. However, cross tolerance to other opioid analgesics is incomplete, which argues for heterogeneity in MOR populations. The discovery that the morphine metabolite, morphine 6β glucuronide (M6G) acted distinctly from morphine further complicated the nature and characterization of MOR subtypes. Unlike morphine, M6G is antagonized by low concentration of 3-O-methylnaltrexone (Walker et al., 1999), and is antinociceptive in CXBK mice (which are insensitive to morphine)(Rossi et al., 1996). In addition, M6G does not develop cross tolerance to morphine (Pasternak, 2001).

Antisense targeting different sequences of the MOR transcript suggests that morphine and M6G exert their antinociceptive effects via different splice variants. For example, exons 2 and 3 appear critical for M6G antinociception but not that of morphine, exon 1 is necessary for supraspinal morphine analgesia, and exon 4 is important for both supraspinal and spinal effects of morphine (Rossi et al., 1995a; Rossi et al., 1995b). Knockout studies support the distinction between morphine and M6G antinociception.

MOR knockout mice generated by deletion of exon 1 (Sora et al., 1997; Schuller et al., 1999) or of exon 2 and 3 (Matthes et al., 1996; Loh et al., 1998) lost their antinociceptive response to morphine. However, knockout mice with an exon 1 deletion continued to respond to M6G, and this response was blocked by naloxonazine or subsequent administration of antisense to exon 2 (Schuller et al., 1999).

These studies resulted in the search for splice variants of MOR, and to date seven have been found (MOP1, MOP1A-F). All identified splice variants possess exon 1-3, only MOP1 has exon 4, and the rest vary in their 3' terminus (Pan et al., 1999; Pan et al., 2000). However, whether these splice variants have any real meaning is debatable. It is difficult to reconcile these splice variants with the M6G studies, since all of these splice variants contain exons 1-3. Thus, it is difficult to understand how morphine-induced antinociception is retained after knockdown of exon 2 and 3. Equally, it is unclear how M6G antinociception is retained after knockdown or knockout of exon 1. Furthermore, autoradiographic analysis of triple opioid receptor knockout mice, where exon 1 of the MOR gene was deleted, reveal that naloxone binding was completely abolished (Clarke et al., 2002). Since opioid agonists are defined as naloxone displaceable (Dhawan et al., 1996), M6G could be acting at a non-opioid receptor.

1.9 Antisense approaches to characterize opioid receptors

The opioid receptors have been extensively targeted by antisense. In particular, much of the evidence for MOR heterogeneity comes from antisense studies. There are several advantages to the antisense approach. First, unlike knock-out models one need not worry

about unwanted alterations that might occur during genetic development. For example, the confusing supraspinal results reported for DOR knockout mice (see Section 1.6), may be explained by a compensatory response to gene deletion. Second, antisense approaches, unlike chronic antagonist exposure, do not directly bind to biologically active receptors which can result in unexpected responses. For instance, chronic administration of the opioid antagonist naltrexone results in upregulation of DOR in rat brain (Belcheva et al., 1994). Third, since antisense only targets a short sequence of mRNA it can be used to identify alternative splice variants, as has been determined for MOR subtypes (Pasternak, 2001).

However, commonly used antisense chemistries (phospodiester and phosphorothioate) are limited. The biggest problem is that first generation antisense compounds often lack efficacy and specificity. In addition, the most frequently used antisense reagent, negatively charged phosphorothioates, can have nonspecific interactions with proteins resulting in toxicity. Furthermore, these antisense chemistries activate RNase H, which recognizes DNA/RNA duplexes and cleaves the RNA portion (Lima and Crooke, 1997). This enzyme can also recognize unstable complexes formed by transient hybridization of the sequence to non-target mRNA (Stein, 2000).

The advent of the chemically novel peptide nucleic acid (PNA) oligomers may solve some of these problems. Relative to first generation antisense compounds, PNAs have a superior hybridization affinity and specificity (Nielsen, 2000), therefore relative to phosphorothioates shorter sequences can be use to target mRNA. In addition, PNA do

not activate RNase H, thereby foregoing irrelevant cleavage of non-target mRNA (for review see Larsen et al., 1999). A further advantage of PNAs is that systemic administration of these oligomers can have antisense effects in the brain (Tyler et al., 1999; McMahon et al., 2001; Tyler-McMahon et al., 2001; McMahon et al., 2002; Boules et al., 2004).

PNA antisense has been used successfully to target both MOR and DOR. Our group found that i.c.v. administration of PNA antisense to DOR, inhibited antinociceptive and locomotor stimulant effects of deltorphin II (Fraser et al., 2000a). Furthermore, relative to control animals, antisense pretreated rats had a 25% decrease in DOR [^{35}S]GTPγS responses in whole brain homogenates (Fraser et al., 2000a), with no detectable change in radioligand binding. From this study it can be concluded that the [^{35}S]GTPγS assay is a more sensitive measure to detect receptor changes after antisense pretreatment.

Rat MOR has also been successfully targeted with PNA antisense given i.c.v. (Tyler et al., 1998) or intraperitoneally (McMahon et al., 2001). Antisense pretreatment resulted in a significant (~70%) decrease in antinociceptive responses to systemic morphine in the tail flick test. In vitro analysis was limited to the periaqueductal grey, where Western blot showed a ~ 50% reduction of MOR protein in antisense treated rats (Tyler et al., 1998; McMahon et al., 2001). With regards to these studies, one must consider that injections of antisense directly into the PAG may result in nonspecific damage that would affect pain perception in general. Further, systemic administration of PNA may produce antinociception by blocking peripheral MOR. To date, a thorough examination of PNA

antisense effects in the brain has not been completed. We attempted to characterize the neuroanatomical extent of PNA antisense knockdown after i.c.v. administration with the [^{35}S]GTPγS in Chapter 2.

1.10 Interactions between MOR and DOR

1.10.1 Modulation of MOR by DOR

There are many reports of acute DOR agonists potentiating MOR antinociception. For instance, in mice a low dose of leu-enkephalin caused a leftward shift in the dose response curve to systemic morphine, nearly halving the ED50 (Vaught and Takemori, 1979; Lee et al., 1980; Barrett and Vaught, 1982). In addition, studies where the more DOR selective drug DPDPE was used, subantinociceptive doses given i.c.v. were able to potentiate the effects of i.c.v morphine, and this potentiation was blocked by the administration of the DOR antagonist ICI 174 864 (Heyman et al., 1989). In the spinal cord, the MOR agonist DAMGO and a low doses of DPDPE had synergistic responses (Riba et al., 2002), although DPDPE was unable to modulate DAMGO or sulfentanil antinociception in the mouse brain (Heyman et al., 1989). Additional evidence of MOR modulation by DOR was that the DOR antagonist DALCE could block DOR mediated antinociception in the mouse tail flick test, but could not block DPDPE potentiation of morphine-induced antinociception (Jiang et al., 1990; Porreca et al., 1992). These studies led to a proposed distinction between DORs which modulate morphine antinociception, and those that are responsible for DOR antinociception.

Further interactions between MOR and DOR are seen with respect to the rewarding and physical dependence induced by δ agonists. MOR knockout mice do not show a conditioned place preference to deltorphin II, nor do they show somatic signs of withdrawal after chronic exposure to the same δ agonist (Hutcheson et al., 2001).

1.10.2 The role of DOR in morphine tolerance

Convergent evidence suggests that activation of DOR is necessary for the induction of tolerance to antinociceptive effects of morphine. Thus, MOR tolerance can be inhibited by ablating DOR expression or function by knockout (Zhu et al., 1999), antisense (Kest et al., 1996), and pharmacological approaches (Adelhamid et al., 1991; Hepburn et al., 1997 but see Fundytus et al., 1995). The additional observation that preproenkephalin knockout mice do not show morphine tolerance (Nitsche et al., 2002), suggests that stimulation of DOR by enkephalin may be necessary to induce morphine tolerance. Regulation of morphine tolerance by DOR may be explained by the increased number of DOR sites seen after continuous morphine infusion (Gouarderes et al., 1993a).

It is controversial whether DOR activation plays an important role in morphine withdrawal. Physical withdrawal symptoms are reported to be attenuated, but not abolished, by coadministration of naltrindole with morphine (Fundytus et al., 1995; Suzuki et al., 1997), and after antisense knockdown of DOR (Sanchez-Blazquez 1997). However, DOR and preproenkephalin knockout mice did not show attenuated responses to naloxone-precipitated withdrawal (Zhu et al., 1999; Nitsche et al., 2002). Unlike tolerance to the antinociceptive effects of morphine, which is completely lost after

inhibition of DOR function, it is unclear if physical withdrawal is regulated by this receptor.

1.10.3 μ and δ opioid receptor complexes

Interactions between μ and δ agonists may be better understood by close physical interactions between MOR and DOR. Biochemical evidence for complexed MOR and DOR came from binding studies using the opioid agonist DADLE, which was believed to have high and low affinity binding sites for DOR and MOR respectively. It was later proposed that the high affinity site was a DOR noncomplexed site (δncx), and the low affinity site was DOR complexed to MOR (δcx) (Rothman et al., 1984). Therefore, under assay conditions which favoured the low affinity site (high Na^+ and Mn^{++}), [^{3}H]DADLE would detect the complexed opioid receptors (Rothman et al., 1984; Bowen et al., 1988).

Cloning of the μ and δ opioid receptors allowed further investigation into the nature of opioid receptor interactions. Two separate groups obtained evidence for MOR/DOR oligomerization (George et al., 2000) or dimerization (Gomes et al., 2000) using co-immunopercipitation in transfected cell systems. MOR/DOR oligomers may use alternate Gα subunits relative to either receptor alone, since inhibition by DAMGO or DPDPE of forskolin-stimulated cAMP production possessed altered sensitivity to pertussis toxin in those cells that expressed the oligomers (George et al., 2000). However, this altered G protein coupling was not detected in another cell expression

system (Law et al., 2005). Heterodimerization may also result in the complexed receptors sharing the same G protein heterotrimer (Law et al., 2005).

In vivo, heterodimerization of μ and δ opioid receptor has also been detected in mouse spinal cord membranes, and this interaction is proposed to be responsible for the potentiation of intrathecal morphine by the DOR antagonist TIPPψ (Gomes et al., 2004). The physical interaction between MOR and DOR is thought to stabilize either receptor in the active receptor conformation when the other is occupied, thus explaining how a DOR antagonist may potentiate morphine (Gomes et al., 2004).

In assessing the evidence for MOR/DOR heterodimers, one should keep in mind that in order for these two opioid receptors to directly interact, they must be coexpressed on the same cells and in the same subcellular compartment. Neuroanatomically MOR and DOR are expressed in similar but not identical CNS regions (Mansour et al., 1995b). Even when the two receptors are anatomically in the same CNS structure, they may not be coexpressed on the same cells. For example, an electron microscopic examination of DOR and MOR in the superficial layers of the cervical spinal cord, found that 21% of MOR labelled soma and dendrites also expressed DOR, and only 6% of DOR labelled cells coexpressed MOR (Cheng et al., 1997). Furthermore, even if the two receptors are expressed by the same neurons, their subcellular distributions may prevent any physical interactions. As discussed in section 1.3.5, ultrastructural localization of DOR is primarily on intracellular vesicles, while MOR is preferentially expressed on the cell surface (Svingos et al., 1995; Svingos et al., 1996; Svingos et al., 1998; Svingos et al.,

1999; Wang and Pickel, 2001; Aicher et al., 2001; Cahill et al., 2001a; Garzon and Pickel, 2002). However, there is evidently enough DOR on the cell surface to be behaviourally relevant, and perhaps a small number of MOR/DOR interactions would be enough to have a functional effect. The in vivo synergy seen between MOR and DOR agonists may also be explained by synaptic interactions between neurons expressing each receptor, or by a less direct interaction at the systems level.

1.10.4 The effects of morphine on DOR

The clinical use of δ agonists in pain management is hindered because these agonists are not as efficacious as their μ counterparts. The sensitization of DOR by morphine pretreatment may make DOR agonists a more viable analgesic target. The effects of morphine pretreatment were thoroughly characterized by one group that found that a 48 hour exposure to morphine resulted in behavioural sensitization of spinal DOR (Cahill et al., 2001b; Morinville et al., 2003; Morinville et al., 2004). Morphine pretreated animals possessed an enhanced response to intrathecal deltorphin II in the hot plate and formalin tests. The effects of deltorphin II were mediated by DOR, since they were blocked by naltrindole. Numerous lines of evidence indicate that MOR stimulation by morphine is probably responsible for this DOR enhancement. First, the concurrent administration of CTOP with morphine abolished this DOR effect. Second, no sensitization to deltorphin II was observed in MOR knockout mice pretreated with morphine (Morinville et al., 2003). Third, many μ agonists caused this enhancement of DOR (Morinville et al., 2003). This form of sensitization appears to be the result of increased trafficking of DOR to the cell surface. There is no concurrent increase in DOR protein abundance or mRNA

expression after morphine pretreatment (Cahill et al., 2001b), which could account for the transient nature of this sensitization (Morinville et al., 2003). Systemic morphine pretreatment does not appear to change DOR expression throughout the CNS, since increases have been detected in the lumbar spinal cord, nucleus accumbens and neostriatum, but not in the frontal cortex (Lucido et al., 2005). To date, only changes in DOR spinal antinociception have been characterized using this morphine regimen, and it has yet to be determined if the upregulation of DOR in the extended striatum translates to enhanced responses to the locomotor stimulant or rewarding effects of δ agonists.

Additionally, electrophysiological measures have detected sensitization of DOR after a longer morphine dosing regimen that results in morphine tolerance. Unlike the 48 hour dosing regimen used in the previous studies, in this study rodents had a five day exposure to a slow release morphine preparation, which was previously reported to induce physical dependence to morphine (Chieng and Christie, 1996; Ingram et al., 1998). This morphine pretreatment resulted in an induction of functional DORs in the periaqueductal grey, an area where DOR agonists do not normally induce presynaptic inhibition of GABA currents (Hack et al., 2005). This gain of function was also dependent on MOR and β-arrestin, as observed with knockout mice (Hack et al., 2005).

Sensitization of DOR-mediated effects after morphine exposure does not appear to be exclusive to antinociception. In rats, the locomotor stimulation induced by intracerebroventricular deltorphin II was greatly enhanced after chronic morphine pretreatment (Melchiorri et al., 1992). Importantly, this study found that enhancement of

DOR was greatest with chronic morphine regimens, either with daily injections of morphine or continuous infusion by minipump. Interestingly, sensitization to the locomotor stimulant effect of deltorphin II not only continued, but was further enhanced several weeks after morphine cessation. To date, the effects of chronic morphine pretreatment and withdrawal have not been assessed in antinociceptive assays. This issue is addressed in Chapter 4.

STATEMENT OF PURPOSE

The overall aim of this thesis was to characterize functional responses of μ and δ opioid receptors in rat central nervous system. Behaviourally relevant and G protein coupled MOR and DOR receptor populations were examined using a number of different approaches.

This thesis grew out of two related interests: peptide nucleic acid antisense and opioid receptors. As discussed in section 1.9, when I started my thesis work, the antisense approach was thought to have considerable potential as a tool for elucidating the physiological roles of proteins. Among the different antisense chemistries available, PNA appeared particularly promising but had been little characterized in vivo. We had demonstrated profound PNA-induced knockdown of DOR-mediated behavioural responses with little evidence of altered DOR protein in vitro (Fraser et al., 2000a). The first aim of my thesis was to extend this approach to MOR. Although behavioural knockdown of MOR by phosphorothioate antisenses has been previously reported (Rossi et al., 1994; Rossi et al., 1995a; Rossi et al., 1995b; Chen et al., 1995b; Shah et al., 1997; Leventhal et al., 1997; Tyler et al., 1998; McMahon et al., 2001), very few studies have characterized the in vitro changes associated with this knockdown (Shah et al., 1997; Tyler et al., 1998; McMahon et al., 2001). Behavioural and biochemical knockdown of MOR after intraparenchymal or systemic administration of peptide nucleic acid (PNA) had been determined, but only one brain area was examined (Tyler et al., 1998; McMahon et al., 2001). Therefore, the first aim of the thesis was to characterize the neuroanatomical extent of knockdown of PNA antisense targeting MOR.

The second aim of this thesis was to compare in vitro DOR functional responses with radioligand binding. DOR activation can produce numerous behavioural effects, including antinociception. However, ultrastructural localization of this receptor indicates that it is primarily located on intracellular membranes (See Section 1.3.5). Anatomical distribution of the DOR has been well characterized using radioligand binding and immunohistochemistry. However, neither of these methods measure any functional DORs, i.e. those that are coupled to their G proteins. Thus, the aim of Chapter 3 was to autoradiographically compare DOR agonist and antagonist radioligand binding with a measure of DOR function, namely deltorphin II-induced [^{35}S]GTPγS binding.

The final aim of this thesis was to observe the effects of chronic morphine pretreatment on behavioural responses mediated by DOR. Numerous interactions have been reported between the μ and δ opioid receptors (see Section 1.10). Recent studies have determined that short term morphine pretreatment can result in a sensitization of brain and spinal DORs (Cahill et al., 2001b; Morinville et al., 2003; Hack et al., 2005). Moreover, according to one report (Melchiorri et al., 1992) chronic morphine pretreatment results in a dramatic cross sensitization to the locomotor stimulant effects of deltorphin II, and this sensitization increases after morphine withdrawal. Therefore, the primary aim of Chapter 4 was to examine the effects of chronic morphine pretreatment and withdrawal on DOR mediated antinociception.

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INTERVENING SECTION 1

The initial aim of this thesis had been to characterize the effects of peptide nucleic acid (PNA) antisense in rat CNS. MOR was used as a model system since a published PNA antisense sequence targeting MOR was already known, and both in vivo and in vitro knockdown of this receptor could be determined. The aim of this chapter was therefore to survey the neuroanatomical extent of knockdown after antisense pretreatment. Although the behavioural knockdown of MORs using antisense oligodeoxynucleotides has been well characterized, very few of these studies have examined the corresponding in vitro knockdown after antisense pretreatment.

What this study actually succeeded in showing was that PNA antisense targeting MOR caused a complete inhibition of DAMGO-induced antinociceptive responses in the paw pressure test, without causing any change in DOR-mediated responses. However, there was no detectable change in MOR in vitro responses as measured by DAMGO-induced [^{35}S]GTPγS autoradiography, [^{125}I]FK 33824 labelling or immunoautoradiography. Overall, these results suggest that there may be a small population of MORs which are responsible for the behavioural effects of μ agonists but which are too small to detect with any of the assays used.

CHAPTER 2

PHARMACOLOGICALLY SELECTIVE BLOCK OF MU OPIOID ANTINOCICEPTION BY PEPTIDE NUCLEIC ACID ANTISENSE IN ABSENCE OF DETECTABLE EX VIVO KNOCKDOWN

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ABSTRACT

The goal of this study was to determine the neuroanatomical extent of mu opioid receptor knockdown in central nervous system (CNS) following intracerebroventricular (i.c.v.) administration of peptide nucleic acid antisense. Rats received subchronic i.c.v. injections of anti-mu opioid receptor antisense, mismatch or vehicle and were tested for paw pressure latency following i.c.v. mu opioid receptor agonist ([D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin; DAMGO) or delta opioid receptor agonist ((+)-4-[(*aR*)-*a*-((2*S*,5*R*)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide; SNC80). The anti-mu opioid receptor antisense (but not mismatch) sequence abolished DAMGO-induced antinociception with no reduction in the delta opioid receptor-mediated response. In contrast, post mortem receptor autoradiographic analysis of CNS areas revealed no change in mu opioid receptor functional response ([^{35}S]GTPγS assay) or receptor labelling ([^{125}I]FK-33824 and mu opioid receptor immunoautoradiography). These results provide further evidence for antisense-induced knockdown at the behavioural level in the absence of clear changes at the tissue level.

1. INTRODUCTION

Peptide nucleic acids are synthetic deoxynucleotide analogs based on a pseudo-peptide backbone (Nielsen et al., 1991). Their chemical properties confer several potential advantages for antisense applications. For example, peptide nucleic acid antisenses have high affinity for mRNA and poor tolerance for base mismatches (Dias et al., 1999; Larsen et al., 1999; Ray and Norden, 2000). Peptide nucleic acids are also highly resistant to nucleases and proteases (Demidov et al., 1994). The polyamide backbone of peptide nucleic acids is not only achiral but also charge-neutral, minimizing interactions with proteins (Larsen et al., 1999), and at effective doses, peptide nucleic acids have not been associated with toxicity (Fraser et al., 2000a; Turner et al., 2003). An additional advantage is that peptide nucleic acids act independently of ribonuclease H, thereby avoiding nonspecific effects resulting from cleavage of non-target mRNA (Stein, 2000).

As antisense agents, peptide nucleic acids have proven to be efficacious and target-selective both *in vitro* (Aldrian-Herrada et al., 1998; Pooga et al., 1998; Cutrona et al., 2000; Turner et al., 2003) and *in vivo* (Tyler et al., 1998; Pooga et al., 1998; Tyler et al., 1999; Fraser et al., 2000a; McMahon et al., 2001; Tyler-McMahon et al., 2001; Rezaei et al., 2001; Turner et al., 2003). Antisense effects have been reported in rodent brain and spinal cord, with evidence of CNS efficacy not only after central injection but even after systemic administration (Tyler et al., 1999; McMahon et al., 2001; Tyler-McMahon et al., 2001; McMahon et al., 2002; Turner et al., 2003).

The anatomical extent of protein knockdown following central peptide nucleic acid antisense administration is largely unknown (Tyler et al., 1998). To address this question in the present study, we used a peptide nucleic acid sequence that was previously shown to produce profound behavioural effects, with a concomitant reduction in brain mu opioid receptor (~55%) in the PAG and hypothalamus (Tyler et al., 1998; McMahon et al., 2001). In the present study, mu opioid receptor protein knockdown was assessed not only by radioligand binding and immunohistochemistry, but also by [^{35}S]GTPγS binding which is a potentially more sensitive measure (Fraser et al., 2000a). For greater anatomical resolution, mu opioid receptor abundance and function were assessed using tissue autoradiography.

2. MATERIALS AND METHODS

2.1 Animals

Male Sprague-Dawley rats (325-350g; Charles River, St Constant, QC, Canada) were housed in groups of two in a temperature- and humidity-controlled animal colony, lit from 7 a.m. to 7 p.m. Food and water were available ad libitum. All experiments were approved by the McGill University Animal Care Committee, in accordance with Canadian Council on Animal Care guidelines.

2.2 Surgery

Rats were anesthetised by intraperitoneal injection of ketamine/xylazine (80/16 mg/kg) solution (Bioniche, Belleville, ON, Canada and Novopharm, Toronto, ON, Canada) and placed in a stereotaxic device. Each animal was implanted with a 24-gauge guide cannula (Plastics One, Roanoke, VA, USA) extending into the right lateral ventricle of the brain (coordinates from bregma: AP, -0.8 mm; ML, 1.5 mm; DV, 4.1 mm) and fixed with dental cement. Rats were given dipyrone analgesic (100 mg/kg, Vétoquinol, Lavaltrie, QC, Canada) immediately following surgery. To prevent occlusion, guide cannulae were kept patent by stainless steel inserts which extended 0.5 mm beyond the cannulae tip. Rats were allowed 5-7 days to recover from surgery before random allocation into treatment groups.

2.3 Peptide nucleic acid antisense constructs

All peptide nucleic acid sequences were donated by Applied Biosystems (Framingham, MA). Peptide nucleic acid sequences were HPLC purified as TFA salts then converted to HCl salts by freeze-drying from a dilute aqueous HCl solution. The completeness of the conversion was confirmed by ion exchange chromatography. The anti-mu opioid receptor peptide nucleic acid sequence (5'-CAG CCT CTT CCT CT-3') and the mismatch sequence (CCG CAT CCT CTT CT) were designed according to Tyler et al. (1998). Peptide nucleic acid sequences were reconstituted in a stock solution of sterile ddH_2O (1 mM) and stored at 4°C. On each antisense treatment day peptide nucleic acid antisense was diluted to 0.1 mM (1 nmol/10 µl) in Dulbecco's phosphate buffered saline (DPBS; 0.5 mM $MgCl_2$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 7.3 mM NaCl, 8.0 mM Na_2HPO_4), and the concentration was verified by determining the absorption of the solution at a wavelength of 260 nm. The following formula was used to quantify the peptide nucleic acid concentration: (A_{260}/extinction coefficient of the sequence) x dilution factor. The presence of soluble aggregates of peptide nucleic acid was also scanned for at 300 nm, and was found to be negligible for all sequences used.

2.4 Intracerebroventricular injections

Antisense or vehicle (DPBS) was administered i.c.v. in daily bolus injections for 5 days. Antisense and opioid drugs were administered by the i.c.v. route to conscious rats through an indwelling 30 gauge injection cannula (Plastics One) connected via PE50

polyethylene tubing to a 100 µl Hamilton syringe. Solutions (10 µl) were injected over 1 minute, and the injection cannula was left within the guide cannula for an additional 30 seconds.

2.5 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 was gently restrained by hand and an increasing force was gradually applied to the right hind paw until the threshold force causing the rat to withdraw its paw was determined. A maximal cut-off force of 510 g was implemented for this study. Data are presented as percentage maximum possible effect (%MPE), calculated as follows: %MPE = [(response-baseline)/(cut-off-baseline)] x 100%.

Animals were tested 18-20 hours after the last antisense injection. 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. ED80 doses of DAMGO (0.2 nmol), and SNC80 (400 nmol) were determined by Fraser et al. (2000b).

2.6 Preparation of tissue

Rats were decapitated 3 hours after the hour-long test session and the brains and spinal cords were rapidly removed, frozen in 2-methylbutane (–50 °C for 30 s) and stored at –40 °C. Brain and spinal cord sections were cryostat-cut at 20 μm. All sections were taken according to Paxinos and Watson (1997). Sections for the caudate putamen were cut between 10.7 and 7.7 mm above the interaural line. The thalamic and periaqueductal grey sections were taken between 6.44 and 4.2, and 3.2-1.2 mm above the interaural line, respectively. Brain stem sections were cut between 1.3 and 2.6 mm below the interaural line. Sections were thaw-mounted onto gelatin-coated slides, air dried at room temperature for 10-15 min and vacuum dried with desiccant at 4°C overnight. Slides were then stored at -40°C until further use.

2.7 [^{35}S]GTPγS autoradiography

[^{35}S]GTPγS autoradiography was performed using a protocol modified from Hyytia et al. (1999). Sections were thawed at room temperature and rehydrated for 20 minutes in assay buffer containing 50 mM Tris HCl, 5 mM $MgCl_2$, 100 mM NaCl, and 1 mM EDTA (pH 7.4). Sections were then preincubated for 1 hour with assay buffer plus 2 mM guanosine 5'-diphosphate sodium salt (GDP; Sigma Chemical Co., St. Louis, MO, USA) and 1 μM 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, adenosine A(1) receptor antagonist, Sigma Chemical Co., St. Louis, MO, USA). The sections were incubated in plastic slide mailers for 1.5 hours with assay buffer plus 2 mM GDP, 1 μM DPCPX, 1

mM dithiothreitol, 225 pM guanosine 5'(γ-^{35}S-thio) triphosphate ([^{35}S]GTPγS, 1250 Ci/mmol, Perkin Elmer Life Science Products, Woodbridge, ON, Canada). Slide mailers were allocated to three incubation conditions: basal (i.e. no agonist present), agonist EC50 (with added mu opioid receptor agonist DAMGO 0.3 μM (Sigma)), agonist EC100 (10 μM DAMGO) and non-specific (i.e. 10 μM unlabelled GTPγS (Sigma) with no agonist present). Sections were then rinsed in ice-cold buffer (50 mM Tris HCl and 5 mM $MgCl_2$, pH 7.4, 2 x 5 min), and distilled water (2s), then blow-dried. Sections were exposed to X-ray film for 24 hours in light-proof X-ray cassettes. Co-exposure with [^{14}C] microscale autoradiographic standards (American Radiolabeled Chemicals, Inc., St. Louis, MO, USA) permitted quantification of the [^{35}S] radioisotope (Miller, 1991). The films were processed with D19 developer and GBX fixer (Kodak).

2.8 [^{125}I]FK-33824 autoradiography

[^{125}I]FK-33824 autoradiography was performed using a protocol modified from Fraser et al. (1999). [^{125}I]FK-33824 was donated by AstraZeneca R&D Montreal (specific activity 2200 Ci/mmol). Sections were thawed at room temperature and incubated at room temperature for 2 hours in assay buffer comprising 50 mM Tris HCl, 3 mM $MgCl_2$, 0.1% bovine serum albumin (pH 7.4) and a non-saturating concentration of 0.03 nM [^{125}I]FK-33824. Non-specific binding was defined by the addition of the highly selective mu opioid receptor antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP, 1 μM; Tocris, Ellisville, MO, USA). Following incubation, sections were rinsed in ice-cold wash buffer (50 mM Tris HCl, 3 mM $MgCl_2$; 3 x 5 min) and distilled water (2 s), then

blow-dried. Sections were exposed to Kodak X-OMAT AR X-ray film together with [^{125}I] microscale autoradiographic standards (Amersham Pharmacia Biotech, Piscataway, NY, USA) for 24 hours in light-proof X-ray cassettes. The films were processed with D19 developer and GBX fixer (Kodak).

2.9 Immunoautoradiography

Immunoautoradiography of the mu opioid receptor was performed using a protocol modified from Grant and Clarke (2002). Sections were post-fixed in an aqueous solution containing 6% paraformaldehyde, 20% absolute alcohol, 20% ethylene glycol, 10% glycerol, and 0.32 M sucrose for 1 hour at -20°C. After washing (2 x 5 min then 1 x 30 min) in buffer (0.1 M phosphate buffer in 0.1 M NaCl (PBS)/0.3% Tween-20), sections were incubated in a blocking solution containing 30% skim milk powder (Carnation), 3% goat serum (Vector) and 0.05% NaN_3 for 2 hours at room temperature. After washing with buffer (1 x 10 min), sections were incubated with rabbit polyclonal anti-mu opioid receptor antibody (1:5000; Neuromics Inc. Minneapolis, MN, USA) in 1.5% goat serum and 0.05% NaN_3 overnight at 4°C. As a control, non-specific binding was determined by incubating adjacent sections with 0.3 mM blocking peptide (NHQLENLEAETAPLP; Sheldon Biotech, McGill University, Montreal, QC, CANADA). After washing with buffer (1 x 5 min, 1 x 10 min, 1 x 30 min), the secondary antibody [^{125}I]-labelled goat anti-rabbit IgG (Perkin Elmer Life Science Products; specific activity 1200 Ci/mmol) was applied (8 pM) for 1 hour at room temperature. This antibody was added to a solution containing 10% skim milk powder, 5% goat serum, and 0.05% NaN_3. Sections

were rinsed in ice-cold buffer (2 x 30 min), dipped briefly in distilled water, then blow-dried. Sections were exposed to Kodak X-OMAT AR X-ray film together with [^{125}I] microscale autoradiographic standards (Amersham Pharmacia Biotech, Piscataway, NY, USA) for 3 days in light-proof X-ray cassettes. The films were processed with D19 developer and GBX fixer (Kodak).

2.10 Quantitative image analysis

Film autoradiographs were quantified using an M4 MCID computer-based system (Imaging Research, St. Catherines, ON, Canada). Specific binding was determined by subtraction of non-specific binding measured in adjacent sections. Agonist-stimulated [^{35}S]GTPγS binding was calculated by subtracting basal binding from agonist stimulated binding. Regions of interest were identified by reference to adjacent Nissl-stained sections.

2.11 Statistical analysis

Non-linear regression analysis of concentration-response data (sigmoidal curve fit) was performed by GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Multiple comparisons (i.e. t-tests with Bonferroni adjustment) and power analyses were performed using Systat v10.2 (SPSS Inc., Chicago, IL, USA).

2.12 Overview of experiments

Four separate experiments were performed using different sets of animals. In each experiment, animals were pretreated with antisense or vehicle, tested behaviourally, and sacrificed for *in vitro* analysis. Experiment 1 investigated target selectivity *in vivo* (i.e. delta opioid *vs.* mu opioid receptor mediated antinociception), following which evidence of functional knockdown *in vitro* ([^{35}S]GTPγS assay) was sought in the caudate-putamen and in brain areas that mediate mu opioid receptor antinociception. Experiment 2 tested whether behavioural knockdown was associated with changes in mu opioid receptor labelling ([^{125}I]FK-33824 and immunoautoradiography) in the brain. Experiment 3 investigated sequence selectivity *in vivo* (i.e. antisense *vs.* mismatch peptide nucleic acid). Experiment 4 tested for *in vitro* changes in the spinal cord that might account for the behavioural knockdown.

3. RESULTS

3.1 Antisense abolished mu opioid receptor-mediated antinociception.

In all four experiments, vehicle pretreated animals responded maximally or near maximally to the mu agonist DAMGO in the paw pressure assay. The peak drug effect occurred at 15 minutes post i.c.v. injection. In Experiments 1 and 3, pretreatment with anti-mu opioid receptor peptide nucleic acid antisense abolished this antinociceptive effect, as illustrated in Fig. 1. This effect was also seen in Experiments 2 and 4; thus no DAMGO response was observed after antisense pretreatment (mean ± SEM percent maximal possible effect, -1.6 ± 4.1, and 3.3 ± 5.8 respectively).

In order to test for target selectivity, antisense-pretreated rats were tested with the delta opioid receptor agonist SNC80 (Fig. 1A). The anti-mu opioid receptor antisense did not detectably reduce the response to this drug ($P>0.2$). In a test for sequence selectivity, pretreatment with a mismatch peptide nucleic acid sequence did not significantly alter the response to DAMGO ($P>0.3$, Fig. 1B). Lastly, pretreatment with antisense did not alter baseline antinociceptive responses (data not shown).

3.2 Anti-mu opioid receptor peptide nucleic acid antisense did not produce a detectable knock-down in CNS tissues

The functional response of the mu opioid receptor was determined *in vitro* using

[^{35}S]GTPγS autoradiography. Based on an initial characterization (Fig. 2), an approximate EC50 and maximal concentration of DAMGO (0.3 and 10 μM) were selected for further testing in brain and spinal cord. The mu opioid receptor agonist DAMGO increased [^{35}S]GTPγS binding in mu opioid receptor rich areas, consistent with the pattern of conventional radioligand binding ([^{125}I]FK-33824) and immunoautoradiography (Fig. 3).

In all four experiments, the caudate putamen and the periaqueductal grey were assessed for in vitro changes in mu opioid receptor function. Antisense pretreatment produced no detectable change in DAMGO-induced [^{35}S]GTPγS binding in these regions, as represented in Fig. 4A and C. Subsequent analysis of other pain-related areas revealed no antisense effect, i.e. in thalamus, rostroventral medulla (Fig. 4B and D), cervical segment 5 and lumbar segment 4 regions (Fig. 4E and F).

Possible knockdown of brain mu opioid receptor abundance was determined using a mu opioid receptor -specific radioligand ([^{125}I]FK-33824). No change was detected after antisense pretreatment in the three areas assayed (i.e. caudate putamen, thalamus, and periaqueductal grey, Table 1). Finally, no change in mu opioid receptor immunoautoradiographic labelling was detected (Table 1).

Power analyses were performed on the most sensitive measures in order to determine the smallest detectable antisense effects. To this end, the effects of 10 μM DAMGO on [^{35}S]GTPγS binding were normalized (i.e. mean DAMGO effect of vehicle-pretreated

group defined as 100%) and pooled across all four experiments. On this basis, we would have been able to detect a 23% or greater knock-down of the DAMGO response in the caudate putamen, whereas only a 3% reduction was actually observed. In the periaqueductal grey, a 17% knockdown in [^{125}I]FK-33824 binding would have been detectable, but instead a 2% increase was observed in the antisense group.

FIGURE 1

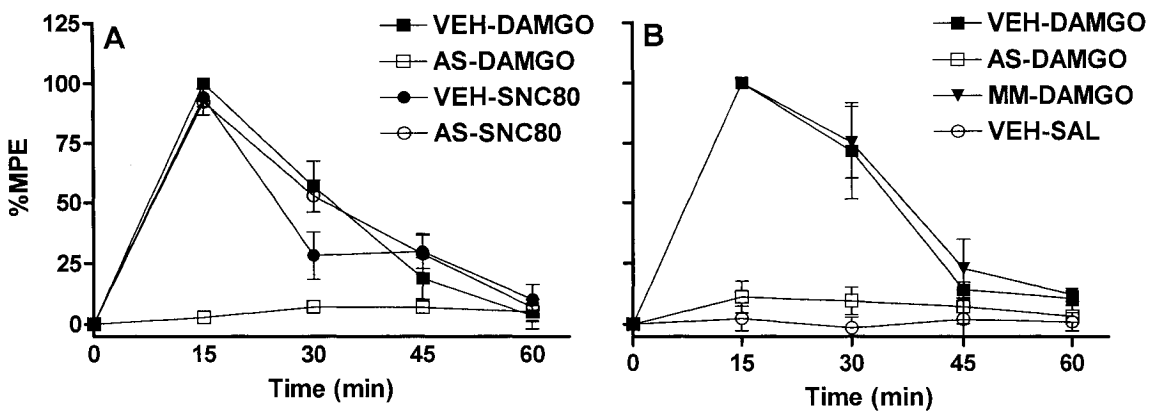

Figure 1. Effect of antisense pretreatment on mu opioid receptor-mediated antinociception. Experiments 1 and 3 are depicted in panels A and B respectively. Depending on the experiment, rats were pretreated with antisense, mismatch or vehicle for 5 days followed by acute challenge with DAMGO (0.2 nmol), SNC80 (400 nmol) or saline. Peptide nucleic acid antisense targeting mu opioid receptors abolished the antinociceptive response to DAMGO **(A and B)**. SNC80-induced antinociception was not affected by pretreatment with antisense **(A)**. A three base pair mismatch sequence did not alter DAMGO-induced antinociception **(B)**. The y axis represents the mean ± SEM response (n=4-8 rats/group), expressed as a percentage of the maximal possible antinociceptive effect. The x axis shows time relative to injection of challenge drug. Veh, vehicle; AS, peptide nucleic acid antisense; MM, peptide nucleic acid mismatch; sal, saline.

FIGURE 2

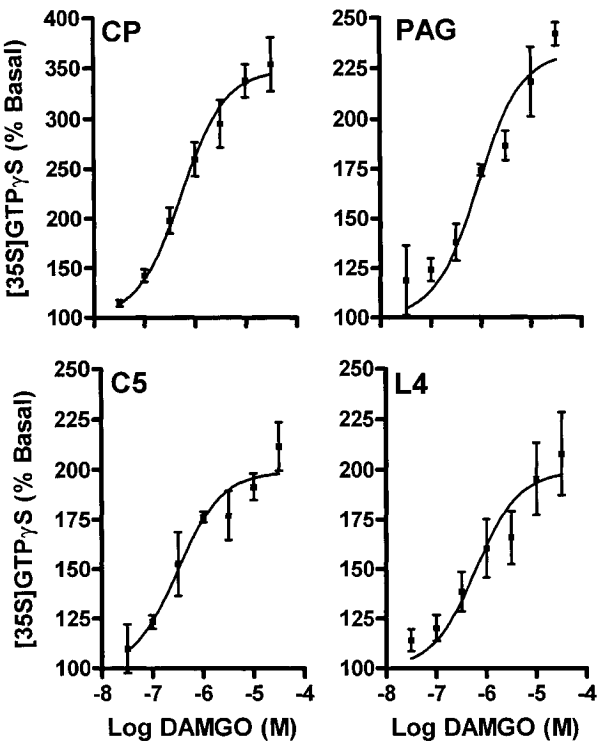

Figure 2. Effect of DAMGO on [^{35}S]GTPγS binding in selected rat brain and spinal regions. DAMGO stimulated binding in the caudate putamen (CP), periaqueductal grey (PAG), cervical segment 5 (C5), and lumbar segment 4 (L4). The y axis shows mean ± SEM specific [^{35}S]GTPγS binding expressed as a percentage of basal binding (i.e. absence of agonist) (n=6-8 sections).

FIGURE 3

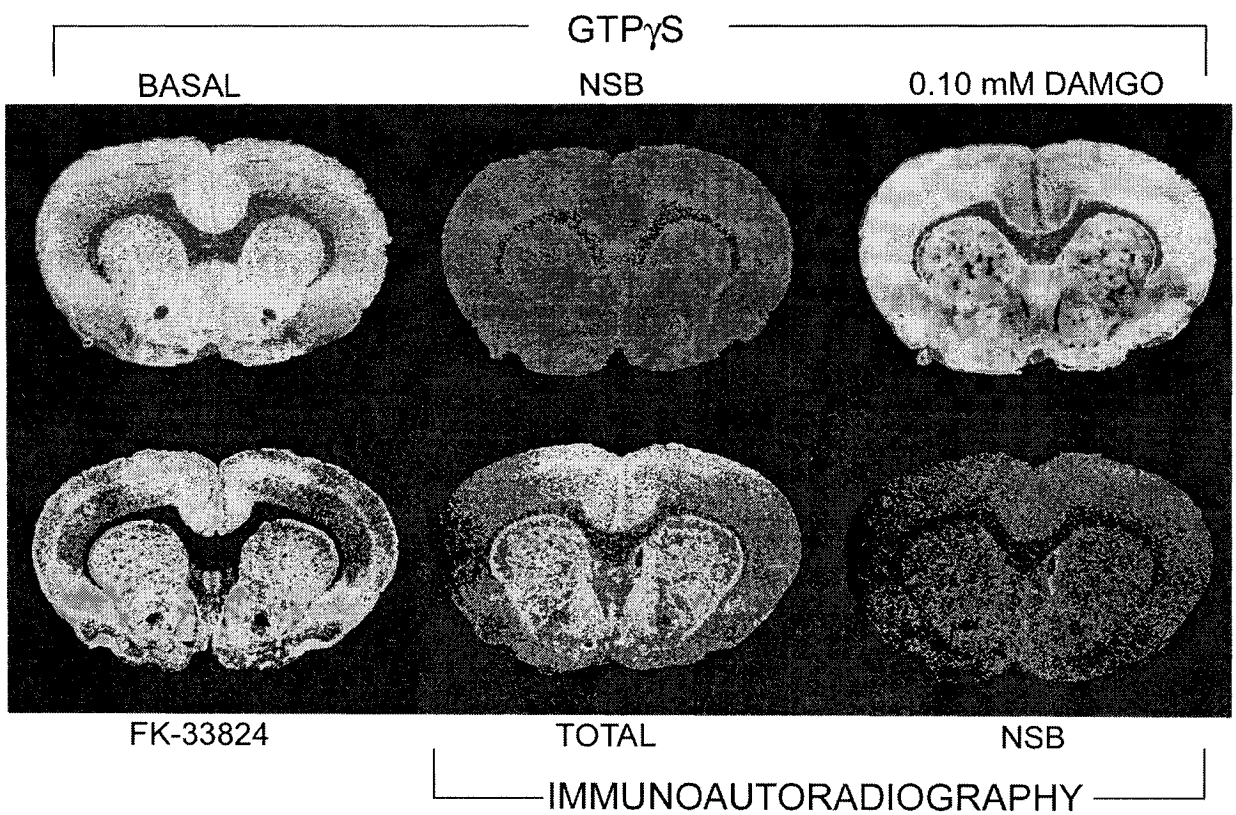

Figure 3. Representative autoradiograms of DAMGO-stimulated [^{35}S]GTPγS binding, [^{125}I]FK-33824, and mu opioid receptor immunoautoradiography. In the [^{35}S]GTPγS assay, basal binding was determined in the absence of agonist, and non-specific binding was determined in the presence of excess cold GTPγS. Non-specific binding for [^{125}I]FK-33824 was determined by addition of 1 μM CTOP, and was virtually undetectable. Non-specific binding for immunoautoradiography was determined by the addition of 0.3 mM of blocking peptide.

FIGURE 4

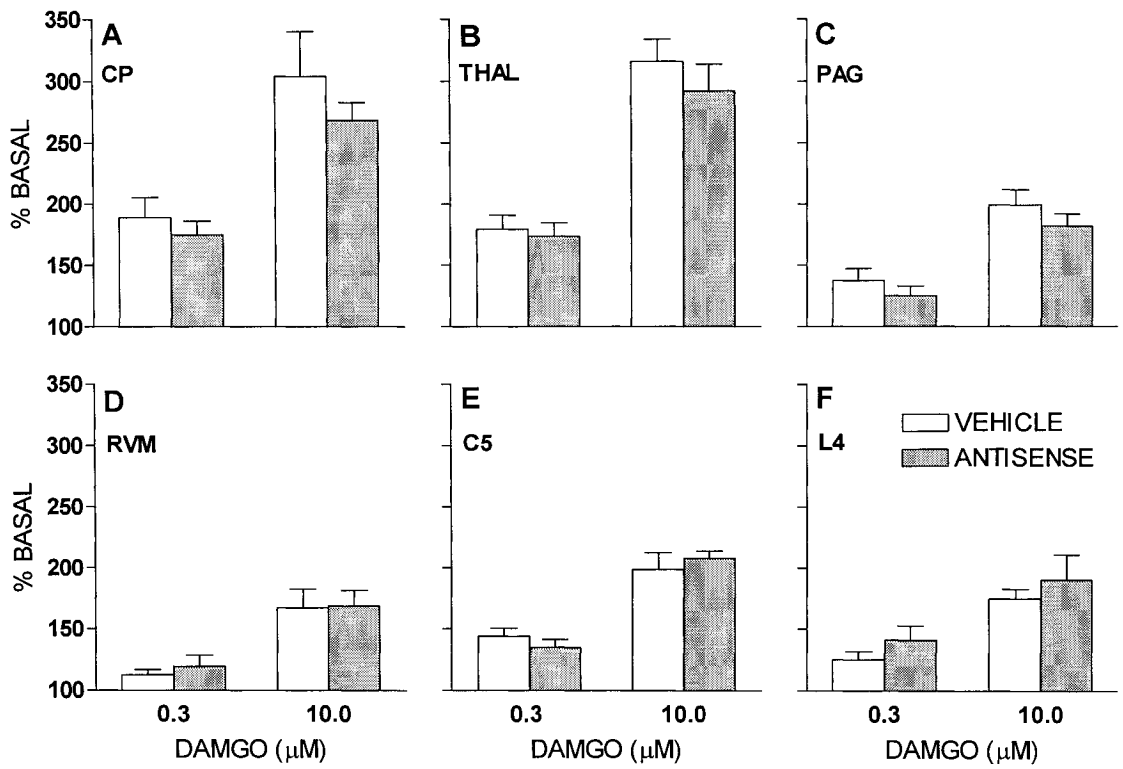

Figure 4. Lack of antisense induced knockdown of DAMGO-stimulated [^{35}S]GTPγS binding in rat CNS. Autoradiographic analysis of the response to EC50 (0.3 μM) and maximal (10 μM) concentrations of DAMGO revealed no difference between antisense *vs.* vehicle pretreated rats in any area examined: **(A)** caudate putamen (CP), **(B)** thalamus (Thal), **(C)** periaqueductal grey (PAG), **(D)** rostroventral medulla, **(E)** cervical segment 5 or **(F)** lumbar segment 4. Panels A-D are derived from Experiment 1; panels E-F are from Experiment 4. The y axis shows mean ± SEM specific [^{35}S]GTPγS binding expressed as a percentage of basal binding (i.e. in the absence of agonist) (n=4-8 rats/group).

TABLE 1: Autoradiographic labelling of mu opioid receptor with [^{125}I]FK-33824 and immunoautoradiography after vehicle or antisense pretreatment (mean ± SEM).

CNS Area	[^{125}I]FK-33824		Immunoautoradiography	
	Vehicle	**Antisense**	**Vehicle**	**Antisense**
CP	1.27 ± 0.11	1.44 ± 0.18	0.05 ± 0.02	0.08 ± 0.03
Thalamus	1.14 ± 0.12	1.07 ± 0.14		
PAG	0.44 ± 0.04	0.45 ± 0.06	0.13 ± 0.01	0.12 ± 0.01

In each case, non-specific binding (NSB) was subtracted from the total binding. Non-specific binding for [^{125}I]FK-33824 and immunoautoradiography was defined by addition of 1 μM CTOP and 0.3 mM blocking peptide, respectively (n=6-8 rats/group).

4. DISCUSSION

The main finding of the present study was the clear dissociation of behavioural and biochemical effects of peptide nucleic acid antisense targeted to mu opioid receptor. Thus, antisense treatment abolished DAMGO-induced antinociception with little or no detectable loss of mu opioid receptor protein or function *in vitro*.

It is likely that the behavioural knockdown represents a true antisense effect for the following reasons. First, the effect was sequence-dependent, as demonstrated by the mismatch control. Second, the peptide nucleic acid antisense effect appeared to be target selective in behavioural tests. Thus, we observed no knockdown of SNC80-induced antinociception, a response mediated by delta opioid receptor (Bilsky et al., 1995; Fraser et al., 2000b) and independent of mu opioid receptor (Fraser et al., 2000b). Sequence- and target-dependent effects have also been reported following intraparenchymal (Tyler et al., 1998) or systemic (McMahon et al., 2001) administration of the same sequence.

In trying to reconcile our negative *in vitro* findings with previous mu opioid receptor peptide nucleic acid studies, several procedural differences may be significant. For example, we administered antisense intracerebroventricularly, whereas in the earlier studies, it was administered either intraperitoneally or directly into brain tissue. We also gave the antisense daily, while in the previous studies it was given less frequently (Tyler et al., 1998; McMahon et al., 2001). It is important to note that our animals were sacrificed within hours of behavioural testing to insure that the antisense effect was still present. It is unlikely that our assays were less sensitive than those used previously.

Indeed, we assessed not only mu opioid receptor abundance (by radioligand binding and radioimmunohistochemistry) but also mu opioid receptor function using the [^{35}S]GTPγS assay which we previously found to be more sensitive to antisense treatment (Fraser et al., 2000a).

In view of the present mismatch between behavioural and biochemical responses it is important to note that all of the CNS regions mediating mu opioid receptor antinociception were assayed in the present study. The caudate putamen was also examined, because of its proximity to the site of antisense injection. All these areas are abundant in mu opioid receptor (Mansour et al., 1994; Mansour et al., 1995), and therefore the signals in these regions were large enough for changes to be detectable. Since both the peptide nucleic acid antisense and DAMGO were given intracerebroventricularly, changes in supraspinal mu opioid receptor were anticipated. However, no change was found in sites thought to mediate supraspinal antinociception by DAMGO (i.e. thalamus, PAG, and rostroventral medulla)(Carr and Bak, 1988; Fang et al., 1989; Rossi et al., 1994). It is not known whether a significant concentration of peptide nucleic acid antisense would accumulate in the spinal cord after i.c.v. administration. Therefore, as a final check, spinal regions that might contribute to DAMGO-induced antinociception were assayed, with the same negative result.

The peptide nucleic acid antisense sequence used in this study is also complementary to several other rat transcripts (i.e. metabotropic glutamate receptor 6, ephrin B1, and succinate semialdehyde dehydrogenase), raising the possibility that our behavioural

knockdown was not mu opioid receptor -mediated. This possibility is unlikely for the following reasons. First, metabotropic glutamate receptor 6 is located only in the eye (Nomura et al., 1994). Second, ephrin B1 is known to decrease chronic inflammatory pain, but is reported not to play a role in transmission of acute pain stimuli (Battaglia et al., 2003). Third, inhibition of succinate semialdhyde dehydrogenase expression would disrupt GABA metabolism and tend to produce a general behavioural disruption (Gupta et al., 2003). Importantly, the preservation of delta opioid receptor-mediated antinociception following peptide nucleic acid treatment renders all these possibilities unlikely.

It therefore appears that peptide nucleic acid antisense treatment suppressed expression of a behaviourally relevant mu opioid receptor population which was not detected in our *in vitro* assays. One possibility is that our antisense treatment differentially targeted splice variants of mu opioid receptor (Pasternak, 2001), but this is unlikely since our sequence targeted the 5' non-coding region of the mu opioid receptor transcript. A second possibility is that DAMGO-induced antinociception occurred via G-proteins that are not readily detected by the [^{35}S]GTPγS assay. However, it remains to be explained why no knockdown of mu opioid receptor was observed in our receptor binding and immunohistochemical assays.

The present findings are reminiscent of our previous results using a peptide nucleic acid antisense sequence targeting delta opioid receptor (Fraser et al., 2000a). In the latter study, a marked inhibition of delta opioid receptor-mediated behavioural effects occurred,

with only a small (25%) inhibition of delta opioid receptor-mediated [^{35}S]GTPγS response and no significant reduction in [^{3}H]-naltrindole binding in whole brain homogenates (Fraser et al., 2000a). A large discrepancy between behavioural knockdown and *in vitro* G-protein coupled receptor (GPCR) expression has also been reported for phosphodiester and phosphorothioate oligodeoxynucleotides (Weiss et al., 1993; Qin et al., 1995; Shah et al., 1997).

Such discrepancies may be especially surprising given that GPCRs, including the mu opioid receptor (Sora et al., 2001), are commonly associated with a receptor reserve (i.e. "spare receptors"). Hence, it has been proposed that newly synthesized receptors are especially susceptible to antisense treatment, and contribute disproportionately to *in vivo* pharmacological responses (Qin et al., 1995; Hua et al., 1998; Van Oekelen et al., 2003). Consistent with this notion, our results show a clear and selective behavioural knockdown in the absence of readily detectable *in vitro* changes.

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INTERVENING SECTION 2

Although the results from Chapter 2 were intriguing, further PNA antisense studies could not be pursued for the following reasons. It became apparent that the antisense sequence used in Chapter 2 was not selective for MOR. Using a Basic Local Alignment Search Tool (BLAST), it was discovered that this published sequence, previously thought to be unique, also showed 100% homology for three other targets (succinate semialdehyde dehydrogenase, ephrin B1 and metabotropic glutamate receptor 6). Although two additional MOR-selective PNA sequences were screened, they did not prove to be effective antisense agents (Pradhan & Clarke, unpublished results). Therefore, in order to continue this research, a new MOR-specific and effective sequence would have had to be identified. The search for such a sequence became unfeasible when the company that was donating antisense sequences stopped its collaborations with all academic groups. In addition, at this time the siRNA method of protein knockdown was being developed for in vivo use, and it appeared that the days of antisense were numbered. For these reasons, opioid receptors became the sole focus of the thesis.

During the development of the [^{35}S]GTPγS autoradiographic assay used in Chapter 2, it was noted that the δ agonists (deltorphin II and SNC80) produced a much lower response relative to the μ agonist DAMGO. Furthermore, electron microscopy studies revealed that DORs were primarily associated with intracellular membranes. This raised the possibility that many DORs are non-functional, at least with respect to conventional pharmacology. Thus, the main objective of this study was to map functional DOR, and to compare this distribution with conventional radioligand binding.

CHAPTER 3

COMPARISON BETWEEN δ OPIOID RECEPTOR FUNCTIONAL RESPONSE AND AUTORADIOGRAPHIC LABELLING IN RAT BRAIN AND SPINAL CORD

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ABSTRACT

The distribution of delta opioid receptors (DORs) in the rat CNS has been previously characterized by radioligand binding and immunohistochemistry. However, the functional neuroanatomy of DORs has not been mapped in any detail; this is potentially important, since these receptors appear to be primarily cytosolic. Opioid receptors can couple to $G_{i/o}$ G proteins, a process which is detected by agonist-stimulated [^{35}S]GTPγS binding. The purpose of this study was therefore to determine the distribution of functional DORs, as assessed by [^{35}S]GTPγS autoradiographic labelling in response to the DOR agonist deltorphin II. For comparison, adjacent sections were labelled with [^{125}I]deltorphin II or the DOR antagonist [^{125}I]AR-M100613. In all three assays, mu opioid receptors were blocked pharmacologically. The distributions of [^{125}I]deltorphin II and [^{125}I]AR-M100613 were highly correlated but not identical. Deltorphin II increased [^{35}S]GTPγS binding in a concentration-dependent and naltrindole-sensitive manner. The regional [^{35}S]GTPγS response to deltorphin II was only moderately predicted by agonist or antagonist radioligand binding ($r = 0.67$ and 0.50 respectively). [^{35}S]GTPγS responses to deltorphin II were strongest in the extended striatum (caudate putamen, nucleus accumbens, olfactory tubercle) and cerebral cortex. In contrast, some areas reported to mediate DOR analgesia (brain stem, spinal cord) possessed a much lower [^{35}S]GTPγS response. These findings demonstrate the existence of a partial mismatch between DOR radioligand binding and [^{35}S]GTPγS response. This divergence possibly reflects regional heterogeneity in G protein receptor coupling, or in the subcellular localization of DOR.

INTRODUCTION

Delta opioid receptors (DORs) are widely distributed throughout the brain and spinal cord (Mansour et al., 1995). Activation of these G-protein coupled receptors mediate numerous behavioural effects, including locomotor stimulation (Negri et al., 1991), reward (Longoni et al., 1998), and analgesia (Improta and Broccardo, 1992). Clinically, the most effective opioid analgesics are mu opioid receptor (MOR) agonists, but treatment with these drugs often results in undesirable side effects. In contrast, DOR agonists are promising candidates for drug development since they produce analgesia without the respiratory depression (Takita et al., 1997) and physical dependence (Devine and Wise, 1994) associated with μ opioid analgesics.

The CNS distribution of the DOR has been well characterized in rodents using radioligand autoradiography and immunohistochemistry (Tempel and Zukin, 1987; Blackburn et al., 1988; Gouarderes et al., 1993; Mansour et al., 1995; Arvidsson et al., 1995; Hiller et al., 1996; Cahill et al., 2001). However, it is unclear whether these approaches reliably detect *functional* DORs, since most DORs appear to be intracellular rather than located on the cell membrane (Svingos et al., 1999; Wang and Pickel, 2001; Cahill et al., 2001). Like other opioid receptors, DORs act primarily through coupling to $G_{i/o}$ G-proteins (Reisine et al., 1996; Connor and Christie, 1999) which can be localized with [^{35}S]GTPγS autoradiography (Sim et al., 1995). Using this method, a thorough mapping of functional δ opioid receptors has been reported only in guinea pig brain (Sim

and Childers, 1997), and analogous studies in the rat have been limited to a few brain areas (Hyytia et al., 1999; Sim-Selley et al., 2002).

The primary aim of this study was therefore to compare the CNS distribution of DOR *function* ([^{35}S]GTPγS assay) with DOR radioligand binding. To date, only one report has provided a direct comparison between radioligand binding and [^{35}S]GTPγS response using the same DOR agonist (DPDPE) in both assays (Hyytia et al., 1999). However, the selectivity of this agonist is questionable since several of its actions appear to be dependent on MORs (Sora et al., 1997; Hosohata et al., 2000; Park et al., 2000; Fraser et al., 2000). In the present study, we instead employed deltorphin II (DELT), which appears more DOR selective (Hosohata et al., 2000; Fraser et al., 2000). As a further precaution, MORs were blocked by the addition of the μ selective antagonist CTOP. Thus, [^{125}I]DELT labelling was directly compared with DELT-stimulated [^{35}S]GTPγS binding. Further comparisons were made with the DOR antagonist radioligand [^{125}I]AR-M100613 since it reportedly offers several potential advantages for autoradiography (Fraser et al., 1999).

EXPERIMENTAL PROCEDURES

Subjects

Subjects were male Sprague Dawley rats, weighing 325-350g (Charles River, St Constant, Quebec, Canada). Rats were housed in groups of two in a temperature- and humidity-controlled animal colony, lit from 7 a.m. to 7 p.m. Food and water were available ad libitum. All experiments were approved by the McGill University Animal Care Committee, in accordance with Canadian Council on Animal Care guidelines.

Preparation of tissue

Rats were decapitated and the brains and spinal cords were rapidly removed and frozen in 2-methylbutane (–50 °C for 30 s) and stored at –40 °C. Brains were cryostat-cut (20 μm sections) throughout the rostro-caudal extent of the brain (Paxinos and Watson, 1997). At each rostro-caudal level, seven consecutive sections were collected for autoradiographic comparison of DOR antagonist ([^{125}I]AR-M100613), DOR agonist ([^{125}I]DELT), and [^{35}S]GTPγS binding. Sections were thaw-mounted onto gelatin-coated slides, air dried at room temperature for 10-15 min and vacuum dried with desiccant at 4°C overnight. Slides were then stored at -40°C until further use.

[^{125}I]AR-M100613 and [^{125}I]DELT autoradiography

Radioligand autoradiography was performed using a protocol modified from Mennicken et al. (2003). Sections were thawed and preincubated at room temperature for 30 min in assay buffer comprising 50 mM TrisHCl with added 1 mM $MgCl_2$ and 120 mM NaCl (pH 7.4). In the main experiment, the sections were then incubated in assay buffer in the presence of a non-saturating concentration of 0.4 nM [^{125}I]DELT (for 1 hour) or 0.03 nM [^{125}I]AR-M100613 (for 2 hours). In an additional experiment, an autoradiographic saturation binding analysis was performed with mid-striatal sections, using a range of 7-8 radioligand concentrations ([^{125}I]DELT 4.5 pM - 4.5 nM, [^{125}I]LMA 205 0.8 pM - 2.4 nM). Both radioligands (specific activity 2200 Ci/mmol) were gifts from AstraZeneca R&D Montreal. The incubation buffer (pH 7.4) comprised 50 mM TrisHCl, 1 mM $MgCl_2$, 120 mM NaCl, 0.5% bovine serum albumen (BSA), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and a saturating concentration of the highly selective MOR antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP 1 μM; Tocris, Ellisville, MO, USA). Non-specific binding was defined by the addition of the DOR selective antagonist naltrindole hydrochloride (0.1 μM, Tocris). Following incubation, sections were rinsed in ice-cold assay buffer (3 x 3 min) and distilled water (2 s), then blow-dried. Sections were exposed to Kodak X-OMAT AR X-ray film together with [^{125}I] microscale autoradiographic standards (Amersham Pharmacia Biotech, Piscataway, NY, USA) for 24 hours ([^{125}I]DELT) or 72 hours ([^{125}I]AR-M100613) in light-proof X-ray cassettes. The films were processed with D19 developer and GBX fixer (Kodak).

[^{35}S]GTPγS autoradiography

[^{35}S]GTPγS autoradiography was performed using a protocol modified from Hyytia et al. (1999). Sections were thawed at room temperature and rehydrated for 20 minutes in assay buffer containing 50 mM TrisHCl, 5 mM $MgCl_2$, 100 mM NaCl, and 1 mM EDTA (pH 7.4). Sections were then preincubated for 1 hour with assay buffer plus 2 mM guanosine 5'-diphosphate sodium salt (GDP; Sigma Chemical Co., St. Louis, MO, USA) and 1 μM 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, adenosine A(1) receptor antagonist, Sigma Chemical Co., St. Louis, MO, USA). The sections were incubated in plastic slide mailers for 1.5 hours with assay buffer plus 2 mM GDP, 1 μM DPCPX, 1 μM dithiothreitol (DTT), 1 μM CTOP, 225 pM guanosine 5'(γ-^{35}S-thio) triphosphate ([^{35}S]GTPγS, 1250 Ci/mmol, Perkin Elmer Life Science Products, Woodbridge, ON, CAN). Slide mailers were allocated to three incubation conditions: basal (i.e. no agonist present), 10 μM deltorphin II (DELT, Tocris, Ellisville, MO, USA), and non-specific (i.e. 10 μM unlabelled GTPγS (Sigma) with no agonist present). Sections were then rinsed in ice-cold buffer (50 mM TrisHCl and 5 mM $MgCl_2$, pH 7.4, 2 x 5 min), and distilled water (2s), then blow-dried. Sections were exposed to X-ray film for 24 hours in light-proof X-ray cassettes. Co-exposure with [^{14}C] microscale autoradiographic standards (American Radiolabeled Chemicals, Inc., St. Louis, MO, USA) permitted quantification of the [^{35}S] radioisotope (Miller, 1991). The films were processed with D19 developer and GBX fixer (Kodak).

Quantitative image analysis

Film autoradiographs were quantified using an M4 MCID computer-based system (Imaging Research, St. Catherines, ON, Canada). Binding was calculated based on autoradiographic standards and expressed as fmol/mg wet tissue equivalent. Non-specific binding was subtracted from the total binding of [^{125}I]DELT and [^{125}I]AR-M100613. Agonist-stimulated [^{35}S]GTPγS binding was calculated by subtracting basal binding. Regions of interest were identified by reference to adjacent Nissl-stained sections.

Statistical analysis

Non-linear regression analysis of saturation binding data (hyperbola) and concentration-response data (sigmoidal curve fit) was performed by GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). The Partial F test was used to compare one *vs.* two site binding models. Multiple comparisons were made using Bonferroni t-tests. Scatterplots were subjected to Deming linear regression, since variability occurred in both x and y dimensions (Motulsky and Christopoulos, 2003). Statistical comparison of r^2 values was performed according to Zar (1984).

RESULTS

Concentration response analysis of DELT-stimulated [^{35}S]GTPγS binding

The [^{35}S]GTPγS response to DELT varied across the eight CNS regions assayed (Fig. 1). A concentration-dependent DELT response was observed within the extended striatum (caudate putamen and olfactory tubercle) and cortex. Concentration response curves appeared sigmoidal, and EC50s were 0.14, 0.21, and 0.18 μM for the caudate putamen, olfactory tubercle and cingulate cortex respectively. Maximal responses to DELT were seen at 3 μM and above. Of all the brain areas tested, the caudate putamen showed the greatest maximal response (84% increase at 10 μM of DELT). No significant response was detected in the periaqueductal grey, rostroventral medulla, substantia nigra pars compacta, cervical or lumbar spinal cord in response to DELT at any concentration tested (Fig.1).

DOR mediation of DELT-stimulated [^{35}S]GTPγS binding

The [^{35}S]GTPγS response to a maximal concentration of DELT (10 μM) was assessed in the presence vs. absence of the DOR antagonist naltrindole (Fig. 2). Naltrindole did not significantly alter basal or non-specific binding. The antagonist completely inhibited 10 μM DELT-stimulated binding in all areas examined (caudate putamen, frontal and occipital cortices).

Comparison of DOR vs. MOR agonist stimulation of [^{35}S]GTPγS binding

We next compared the relative magnitude of delta vs. mu opioid receptor responses. For this purpose, maximal concentrations of DELT and of the MOR agonist DAMGO were tested in several areas possessing appreciable MOR and DOR binding (Mansour et al., 1995). In all areas tested, the DAMGO response was greater (Fig.3).

Comparison of antagonist and agonist radioligand binding

Saturation binding analysis of caudate-putamen yielded k_D and B_{max} values of 0.96±0.06 nM and 19.6±0.5 fmol/mg for [^{125}I]DELT and 0.42±0.04 nM and 29.1±0.9 fmol/mg for [^{125}I]AR-M100613. Corresponding values for frontal cortex were as follows: 0.91±0.06 nM and 12.8±0.3 fmol/mg ([^{125}I]DELT) and 0.18±0.02 nM and 16.4±0.4 fmol/mg ([^{125}I]AR-M100613). The data for all four binding isotherms closely conformed to a one-site model ($r^2 > 0.997$), and a two-site model did not yield a better fit (Partial F test, $F<0.3$ for all).

In the main autoradiographic study, non-saturating concentrations of [^{125}I]AR-M100613 (0.03 nM) and [^{125}I]DELT (0.4 nM) were used, with predicted receptor occupancies of approximately 10% and 45%, respectively, based on the above saturation analysis. Representative autoradiographs of [^{125}I]AR-M100613 (antagonist) and [^{125}I]DELT (agonist) binding are shown in Fig. 4. The radioligand [^{125}I]AR-M100613 bound with a poor signal to noise ratio, and the pattern of non-specific binding was not uniform across CNS areas. In contrast, non-specific binding of [^{125}I]DELT was virtually undetectable.

Binding of the two radioligands was highly correlated across CNS areas (r=0.87, P<0.0001). This close relationship was also evident when the regional data were divided into quartile ranges (Table 1). However, a significant component of [^{125}I]AR-M100613 binding remained in the absence of [^{125}I]DELT binding, as revealed by linear regression analysis (P<0.0001).

Receptor binding vs. DELT-stimulated [^{35}S]GTPγS response

DOR radioligand binding was then compared to the [^{35}S]GTPγS response to DELT in adjacent sections (Fig. 5, Table 1). For the functional assay, a maximal concentration of 10 μM DELT was chosen which was completely antagonized by naltrindole (see above). As predicted from the earlier concentration response analysis, DELT-stimulated [^{35}S]GTPγS binding was highest in the extended striatum and cortical regions. Significant responses were even detected in the periaqueductal grey and rostroventral medulla which were unresponsive in the concentration-response study shown in figure 1. This difference is possibly attributable to the greater number of sections used per condition in the main study.

Linear regression analysis was used in order to explore the relationship between radioligand binding and [^{35}S]GTPγS response. Both DOR antagonist and agonist binding were significantly but only moderately correlated with the functional response (AR-M100613 r=0.50, P<0.0001; DELT r=0.67, P<0.0001, Fig.5A and B). In this respect, [^{125}I]DELT and [^{125}I]AR-M100613 binding were not significantly different (comparison

of r^2 values: P=0.059, one-tailed). As shown in figure 5, many points appeared to form distinct clusters; some clustering appeared to have a functional and/or anatomical basis (e.g. pain-related areas, thalamic nuclei, dopaminergic areas, and cortical regions).

FIGURE 1

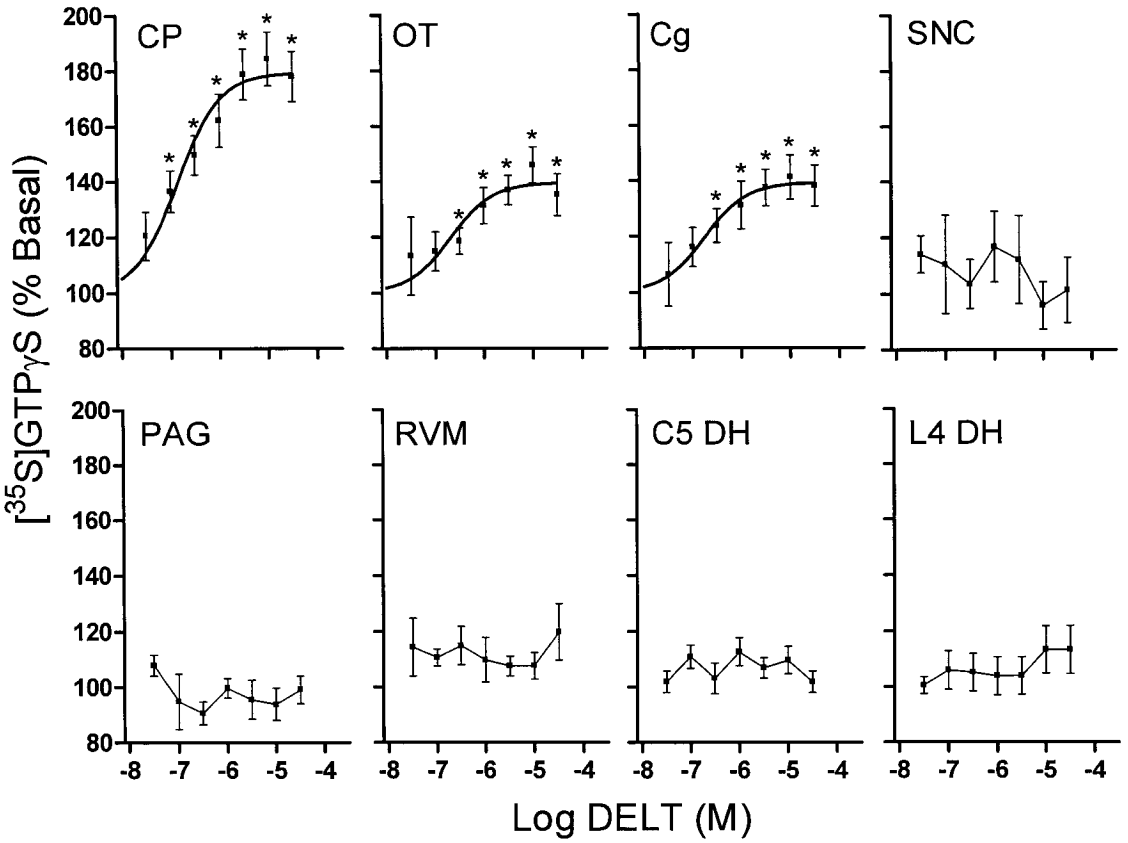

Figure. 1. Effect of DELT on [^{35}S]GTPγS binding in rat CNS. DELT stimulated [^{35}S]GTPγS binding in the caudate putamen (CP), olfactory tubercle (OT) and cingulated cortex (Cg) in a concentration-dependent manner. No significant increase in [^{35}S]GTPγS binding occurred in the substantia nigra pars compacta (SNC), periaqueductal grey (PAG), rostroventral medulla (RVM), cervical segment 5 dorsal horn (C5 DH) or lumbar segment 4 dorsal horn (L4 DH). The y axis shows mean ± SEM specific [^{35}S]GTPγS binding expressed as a percentage of basal binding (i.e. in absence of agonist). * $P<0.02$ *vs.* basal condition (Bonferroni t-tests, n=6-12 brain sections).

FIGURE 2

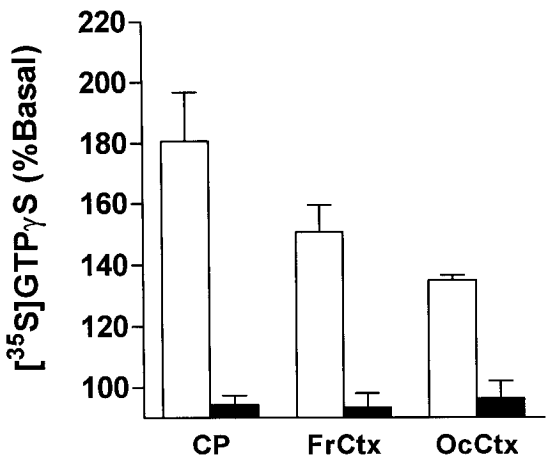

Figure 2. Antagonism of DELT-stimulated [^{35}S]GTPγS binding by naltrindole in caudate putamen (CP), frontal cortex (FrCtx) and occipital cortex (OcCtx). Responses to 10 μM DELT (open bars) were blocked by the addition of 1.0 μM naltrindole (closed bars). The y axis shows mean ± SEM specific [^{35}S]GTPγS binding expressed as a percentage of basal binding (i.e. absence of agonist), n=6 brain sections.

FIGURE 3

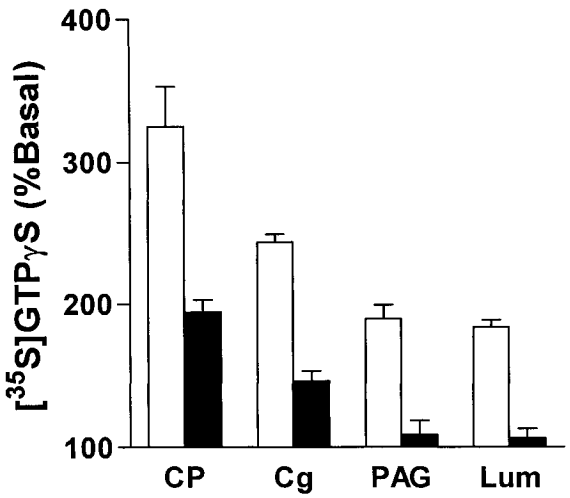

Figure 3. Stimulation of [^{35}S]GTPγS binding by μ vs. δ opioid agonists applied at maximal concentrations. Responses to DAMGO (10 μM, open bars) were consistently greater than to DELT (10 μM, dark bars). The y axis shows mean ± SEM specific [^{35}S]GTPγS binding expressed as a percentage of basal binding (i.e. absence of agonist), n=5 rats. Abbreviations: CP, caudate putamen; Cg, cingulate cortex; PAG, periaqueductal grey; Lum, lumbar segment 4.

FIGURE 4

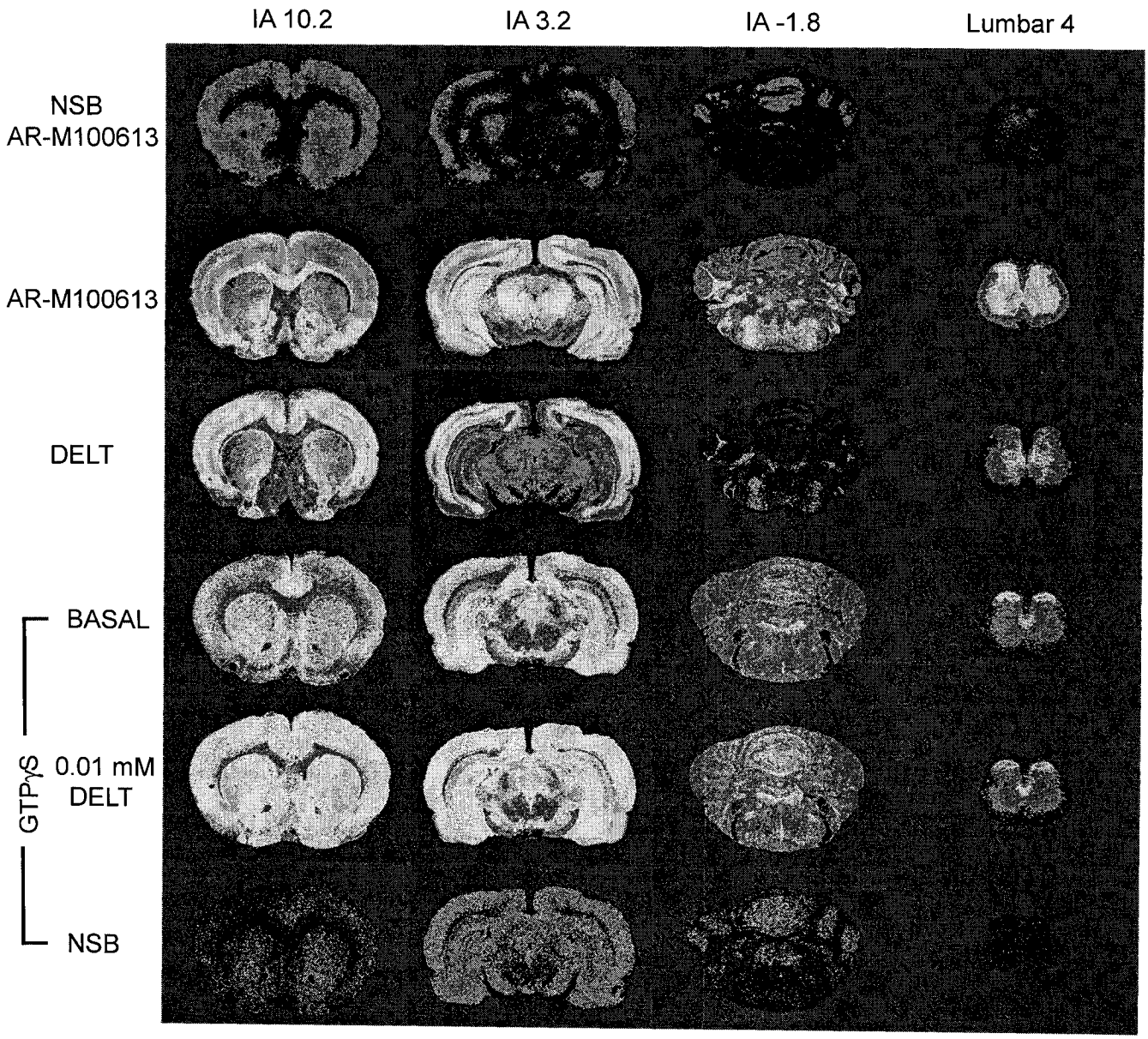
IA 10.2
IA 3.2
IA -1.8
Lumbar 4
NSB
AR-M100613
AR-M100613
DELT
BASAL
GTPγS
0.01 mM
DELT
NSB

Figure 4. Representative autoradiograms of [^{125}I]AR-M100613, [^{125}I]DELT and DELT-stimulated [^{35}S]GTPγS binding in rat forebrain, midbrain, brain stem, and spinal cord (lumbar 4). IA values in the figure refer to distance (mm) anterior to the interaural line (Paxinos and Watson, 1997). Non-specific binding (NSB) for agonist and antagonist radioligands was determined by addition of 0.1 μM naltrindole. [^{125}I]DELT produced virtually no non-specific binding. In the [^{35}S]GTPγS assay, basal binding was determined in the absence of agonist, and non-specific binding was determined in the presence of excess cold GTPγS.

FIGURE 5

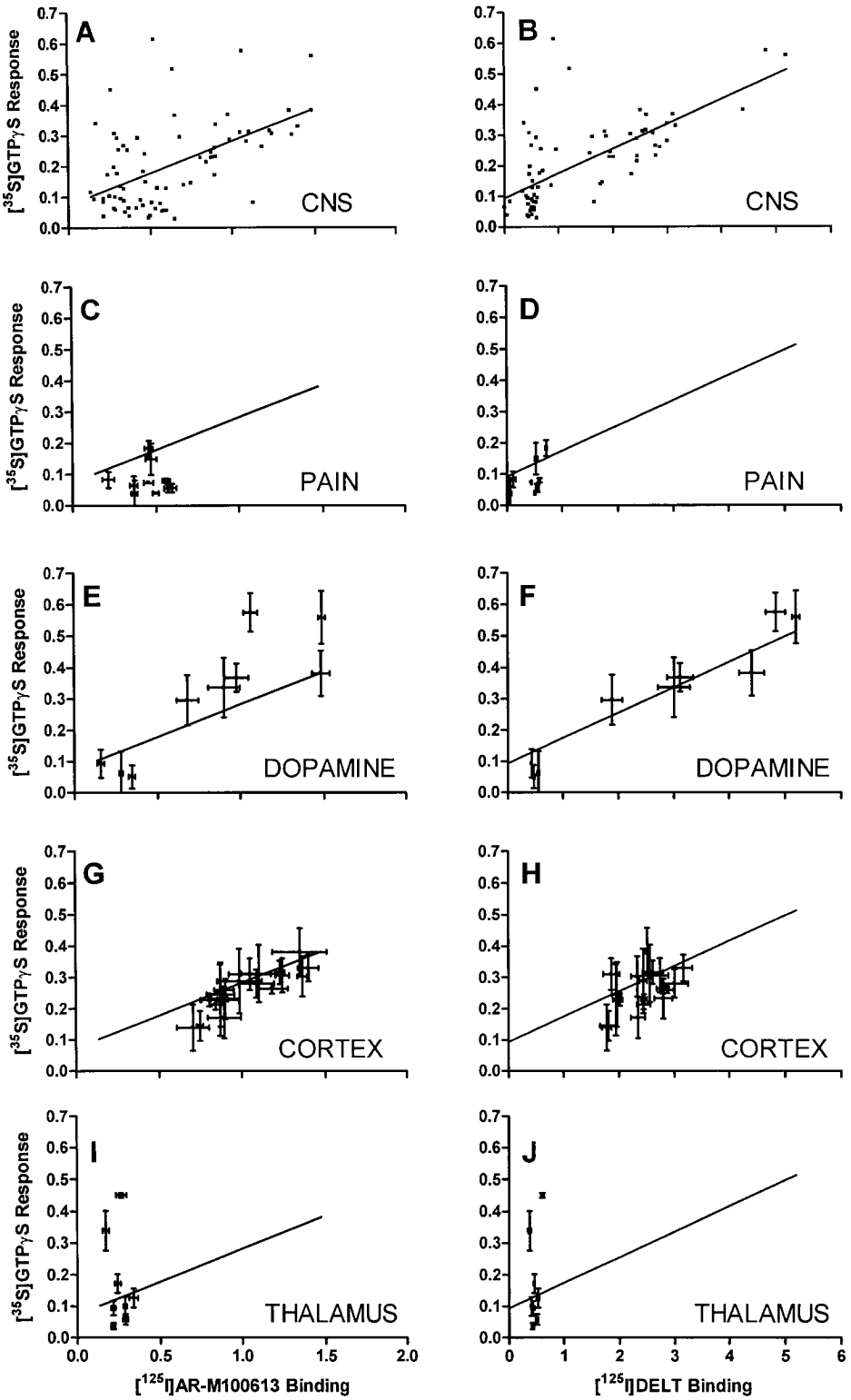
A
CNS
B
CNS
C
PAIN
D
PAIN
E
DOPAMINE
F
DOPAMINE
G
CORTEX
H
CORTEX
I
THALAMUS
J
THALAMUS
[35S]GTPγS Response
[125I]AR-M100613 Binding
[125I]DELT Binding

Figure 5. Poor prediction of DOR functional response by [^{125}I]AR-M100613 and [^{125}I]DELT binding. **A and B** All 74 CNS areas are shown. Each point represents the mean value (n=5 rats), and SEM bars are omitted for clarity. Specific radioligand binding was determined by subtraction of non-displaceable binding. Agonist-stimulated [^{35}S]GTPγS binding was calculated by subtracting basal binding. All measures are expressed as fmol/mg tissue. Correlational analysis revealed moderate associations between DELT-stimulated [^{35}S]GTPγS binding and labelling with [^{125}I]AR-M100613 (A, r=0.50; P<0.0001, n=74) or [^{125}I]DELT (B, r=0.67; P<0.0001, n=74). Panels C, E, and G contain a subset of CNS areas from panel A together with its regression line. Panels D, F, and H relate in the same way to panel B. **C and D** Areas associated with DOR-mediated analgesia possessed low radioligand binding and [^{35}S]GTPγS response (see text). **E and F** Mesolimbic and nigrostriatal dopaminergic terminal regions possessed much higher DOR binding and functional responses than the corresponding cell body regions. **G and H** Cortical regions had moderate to high DOR binding and [35S]GTPγS responses. **I and J** Thalamic regions mainly showed low binding in most measure.

TABLE 1.Comparison of Regional Distributions of DELT-Stimulated [^{35}S]GTPγS Binding with DOR Labeling by [^{125}I]DELT And [^{125}I]LMA205 in Rat CNS[1]

Region	[^{35}S]GTPγS Response	mean ± SEM	[^{125}I]DELT	mean ± SEM	[^{125}I]LMA205	mean ± SEM
Cerebral Cortex						
Cingulate Cortex	++++	0.31 ± 0.05	++++	2.70 ± 0.15	++++	1.24 ± 0.04
Frontal Cortex						
Lamina I	++++	0.31 ± 0.05	+++	1.90 ± 0.15	++++	1.05 ± 0.13
Lamina II III	++++	0.32 ± 0.03	++++	2.60 ± 0.18	++++	1.23 ± 0.03
Lamina V	++++	0.33 ± 0.04	++++	3.20 ± 0.16	++++	1.40 ± 0.06
Lamina VI	++++	0.30 ± 0.06	+++	2.30 ± 0.12	++++	1.36 ± 0.03
Parietal Cortex						
Lamina I	+++	0.23 ± 0.02	+++	2.00 ± 0.05	+++	0.81 ± 0.06
Lamina II III	+++	0.26 ± 0.01	++++	2.90 ± 0.12	+++	0.90 ± 0.06
Lamina IV	+++	0.21 ± 0.02	++++	2.40 ± 0.12	+++	0.84 ± 0.03
Lamina V	+++	0.23 ± 0.02	++++	2.50 ± 0.05	+++	0.89 ± 0.03
Lamina VI	+++	0.26 ± 0.02	++++	2.80 ± 0.11	++++	1.18 ± 0.09
Temporal Cortex						
Lamina I	+++	0.25 ± 0.10	+++	2.00 ± 0.08	+++	0.87 ± 0.08
Lamina II III	+++	0.29 ± 0.10	++++	2.40 ± 0.07	++++	0.99 ± 0.13
Lamina IV	NS	0.23 ± 0.11	+++	1.90 ± 0.05	+++	0.87 ± 0.11
Lamina V	++++	0.31 ± 0.09	++++	2.60 ± 0.06	++++	1.11 ± 0.11
Lamina VI	++++	0.38 ± 0.08	++++	2.50 ± 0.03	++++	1.35 ± 0.16
Occipital Cortex						
Lamina I	++	0.15 ± 0.05	+++	1.80 ± 0.15	+++	0.75 ± 0.02
Lamina II III	++	0.17 ± 0.07	+++	2.30 ± 0.13	++++	0.90 ± 0.10
Lamina IV	NS	0.14 ± 0.07	+++	1.80 ± 0.09	+++	0.71 ± 0.10
Lamina V	+++	0.23 ± 0.06	++++	2.80 ± 0.16	++++	0.90 ± 0.09
Lamina VI	+++	0.28 ± 0.05	++++	3.00 ± 0.24	++++	1.09 ± 0.11
Basal Ganglia						
Anterior Caudate Putamen	++++	0.56 ± 0.08	++++	5.20 ± 0.07	++++	1.49 ± 0.02
Posterior Caudate Putamen	++++	0.58 ± 0.06	++++	4.80 ± 0.17	++++	1.06 ± 0.04
[illegible]us accumbens - [illegible]	++++	0.30 ± 0.08	+++	1.90 ± 0.18	+++	0.68 ± 0.07
Nucleus accumbens - shell	++++	0.34 ± 0.10	++++	3.00 ± 0.29	++++	0.90 ± 0.10
Anterior Olfactory Tubercle	++++	0.38 ± 0.07	++++	4.40 ± 0.22	++++	1.48 ± 0.06
Posterior Olfactory Tubercle	++++	0.37 ± 0.05	++++	4.80 ± 0.17	++++	0.98 ± 0.07
Lateral Septum	NS	0.10 ± 0.07	++	0.56 ± 0.03	+	0.25 ± 0.01
Medial Septum	NS	0.09 ± 0.07	++	0.61 ± 0.02	+	0.32 ± 0.03
Vertical Band of Broca	++++	0.31 ± 0.10	+	0.47 ± 0.08	+	0.29 ± 0.05
Horizontal Band of Broca	+++	0.18 ± 0.06	++	0.65 ± 0.02	+	0.30 ± 0.04
Ventral Pallidum	++	0.14 ± 0.09	+++	0.88 ± 0.06	++	0.32 ± 0.04
Bed Nucleus of the Stria Terminalis	NS	0.29 ± 0.25	++	0.60 ± 0.03	+	0.30 ± 0.04
Globus Pallidus	+++	0.25 ± 0.05	+++	0.97 ± 0.09	++	0.36 ± 0.05
Magnocellular Preoptic Area	NS	0.09 ± 0.14	++	0.51 ± 0.04	++	0.34 ± 0.02
Thalamus						
Anterior Thalamic Group	++	0.13 ± 0.03	++	0.53 ± 0.02	++	0.34 ± 0.03
Reunions Nucleus	+++	0.17 ± 0.03	+	0.46 ± 0.02	+	0.24 ± 0.02
Paraventricular Thalamus	++++	0.34 ± 0.06	+	0.37 ± 0.03	+	0.17 ± 0.02
Reticular Thalamus	++	0.10 ± 0.03	+	0.41 ± 0.02	+	0.29 ± 0.01
Ventral Thalamic Group	+	0.06 ± 0.02	++	0.51 ± 0.02	+	0.29 ± 0.02
Laterodorsal Thalamus	+	0.04 ± 0.01	+	0.43 ± 0.02	+	0.22 ± 0.01
Mediodorsal Thalamus	++	0.09 ± 0.02	+	0.44 ± 0.03	+	0.22 ± 0.01
Zona Incerta	++++	0.45 ± 0.01	++	0.61 ± 0.02	+	0.27 ± 0.03

TABLE 1 Continued

Region	[^{35}S]GTPγS Response	mean ± SEM	[^{125}I]DELT	mean ± SEM	[^{125}I]LMA205	mean ± SEM
Hypothalamus						
Anterior Hypothalamus	+++	0.27 ± 0.05	+	0.51 ± 0.04	++	0.34 ± 0.02
Lateral Hypothalamus	+++	0.20 ± 0.01	+	0.47 ± 0.02	+	0.28 ± 0.02
Arcuate Hypothalamus	NS	0.01 ± 0.12	+	0.33 ± 0.01	++	0.40 ± 0.07
Ventromedial Hypothalamus	NS	0.61 ± 0.27	+++	0.40 ± 0.00	++	0.52 ± 0.06
Paraventricular Hypothalamus	NS	0.12 ± 0.06	+	0.34 ± 0.02	+	0.14 ± 0.02
Amygdala						
Medial Amygdala	++++	0.52 ± 0.12	+++	1.22 ± 0.08	+++	0.64 ± 0.06
Central Amygdala	NS	0.25 ± 0.16	+++	0.68 ± 0.06	+	0.31 ± 0.02
Basolateral Amygdala	++++	0.37 ± 0.06	++++	2.60 ± 0.12	+++	0.65 ± 0.04
Basomedial Amygdala	++++	0.29 ± 0.11	+++	1.60 ± 0.14	++	0.42 ± 0.02
Cortical Amygdala	+++	0.24 ± 0.05	+++	1.60 ± 0.08	++	0.47 ± 0.06
Mesencephalon						
Red Nucleus	+	0.06 ± 0.02	++	0.52 ± 0.01	++	0.41 ± 0.17
Ventral Tegmental Nucleus	NS	0.05 ± 0.04	+	0.47 ± 0.02	++	0.35 ± 0.02
Periaqueductal Grey	+++	0.18 ± 0.03	+++	0.71 ± 0.02	++	0.46 ± 0.03
Substantia Nigra pars compacta	NS	0.06 ± 0.07	++	0.55 ± 0.02	+	0.28 ± 0.01
Substantia Nigra pars reticulata	NS	0.09 ± 0.05	+	0.43 ± 0.01	+	0.16 ± 0.02
Pons						1.13 ± 0.10
Pontine	NS	0.08 ± 0.04	+++	1.70 ± 0.17	++++	
Raphe magnus and pallidus	++	0.08 ± 0.03	NS	0.11 ± 0.05	+	0.22 ± 0.04
Medulla						
[illegible]leus Reticularis Gigantocellularis	NS	0.04 ± 0.04	NS	0.05 ± 0.03	+	0.37 ± 0.02
Vestibular Nucleus	NS	0.06 ± 0.03	NS	0.00 ± 0.04	++	0.37 ± 0.02
Spinal Cord						
Cervical						
Lamina I II	++	0.13 ± 0.06	++	0.62 ± 0.02	+++	0.54 ± 0.03
Lamina III IV	NS	0.06 ± 0.09	+	0.50 ± 0.01	++	0.53 ± 0.01
Lamina V VI	NS	0.03 ± 0.02	++	0.60 ± 0.02	+++	0.65 ± 0.02
Lamina VII	+	0.06 ± 0.01	++	0.54 ± 0.02	+++	0.58 ± 0.02
Lamina VIII	++	0.13 ± 0.04	++	0.52 ± 0.02	+++	0.60 ± 0.01
Lamina IX	NS	0.03 ± 0.02	+	0.44 ± 0.02	++	0.49 ± 0.02
Lumbar						
Lamina I II	++	0.15 ± 0.05	++	0.53 ± 0.03	++	0.47 ± 0.03
Lamina III IV	+	0.04 ± 0.01	+	0.50 ± 0.01	++	0.50 ± 0.02
Lamina V VI	+	0.05 ± 0.01	++	0.55 ± 0.02	+++	0.57 ± 0.02
Lamina VII	+	0.08 ± 0.01	++	0.59 ± 0.01	+++	0.56 ± 0.02
Lamina VIII	+	0.06 ± 0.01	++	0.54 ± 0.01	+++	0.60 ± 0.03
Lamina IX	+	0.07 ± 0.00	+	0.44 ± 0.01	++	0.46 ± 0.03

[1]All measures were performed as described in the Materials and Methods, and are expressed as fmol/mg tissue. To aid comparison across measures, the mean values of DELT-stimulated [^{35}S]GTPγS, [^{125}I]DELT and [^{125}I]LMA205 in all CNS regions were divided into quartiles (+ first quartile, very low; ++ second quartile, low; ++ + third quartile, moderate; ++ ++ fourth quartile, high). NS indicates non-significant binding as determined by a one tailed, single-sample t-test (i.e. mean *vs.* zero). Non-specific binding was subtracted from the total binding of [^{125}I]LMA205 and [^{125}I]DELT. Agonist stimulated [^{35}S]GTPγS binding was determined by subtracting basal binding (absence of agonist) from agonist-stimulated binding.

DISCUSSION

This study provides the first extensive anatomical characterization of DOR function in both rat brain and spinal cord. The present study is also the first to compare [^{125}I]DELT and [^{125}I]AR-M100613 autoradiographically. These data provided the basis for a large-scale comparison of functional *vs.* conventional DOR radioligand measures. This comparison revealed that both [^{125}I]DELT and [^{125}I]AR-M100613 binding are only moderately predictive of DOR function, as determined by DELT-stimulated [^{35}S]GTPγS binding.

Neuroanatomical distribution of [^{125}I]DELT

In the brain, [^{125}I]DELT labelling was highest in the striatum and cerebral cortex and was lowest in thalamus, mesencephalon, and medulla. This general pattern has also been found in binding studies using structural analogs of deltorphin (i.e. [^{3}H]deltorphin I, [^{3}H]Ile 5,6-deltorphin II) (Gouarderes et al., 1993; Renda et al., 1993; Goody et al., 2002; Clarke et al., 2003; Ploj and Nylander, 2003), or using the agonist [^{3}H]DPDPE binding (Mansour et al., 1987; Blackburn et al., 1988; Sharif and Hughes, 1989). Immunohistochemistry has also revealed a high receptor abundance in the striatum and cerebral cortex (Cahill et al., 2001). However, immunolabelling and [^{125}I]DELT binding clearly diverge, particularly in mesencephalon, pons and medulla. In these regions, moderate DOR immunolabelling occurred in many nuclei, whereas [^{125}I]DELT binding was sparse. Thus, it appears that antibodies detect a broader population of DOR than

[^{125}I]DELT. This might be expected, since DOR antibodies can detect intracellular receptors (Svingos et al., 1999; Cahill et al., 2001; Wang and Pickel, 2001), may be insensitive to agonist affinity state, and may not discriminate between putative DOR subtypes.

In the spinal cord, the distribution of DOR has been mapped with several radioligands (Goodman et al., 1980; Sharif and Hughes, 1989; Besse et al., 1990; Gouarderes et al., 1993). Our quantitative analysis of [^{125}I]DELT binding demonstrated a homogeneous distribution across different laminae, in accordance with an earlier descriptive study using the same radioligand (Mennicken et al., 2003). The same pattern has been reported for [^{3}H]deltorphin I autoradiography (Gouarderes et al., 1993), DOR immunolabelling (Cahill et al., 2001) and DOR mRNA (Mansour et al., 1994). In contrast, several other DOR radioligands (i.e. [^{125}I]DADLE, [^{3}H]DPDPE, [^{3}H]DTLET) have been found to label the superficial dorsal horn preferentially (Goodman et al., 1980; Sharif and Hughes, 1989; Besse et al., 1990; Gouarderes et al., 1993). These two distinct patterns cannot be explained by the proposed δ1/δ2 receptor subtype classification (Zaki et al., 1996), since either of these two binding patterns can be produced by putative δ1 (DPDPE, deltorphin I) or δ2 (DELT, DTLET) ligands.

[^{125}I]AR-M100613 autoradiography

The DOR antagonist radioligand ^{125}I-AR-M100613 offers several theoretical advantages for autoradiography (Fraser et al., 1999). As an antagonist, its binding is not sensitive to

the affinity state of the receptor; in addition, it appears not to differentiate between DOR subtypes (Fraser et al., 1999). Furthermore, while other DOR antagonist radioligands are all tritiated, [^{125}I]AR-M100613 has high specific activity with negligible differential quenching in white vs. grey matter (Happe and Murrin, 1990). A high signal to noise ratio has also been predicted for [^{125}I]AR-M100613 on the basis of membrane binding studies (Fraser et al., 1999), but this was not borne out in our autoradiographic assay. In fact, the high non-specific binding was the major reason we chose to use a radioligand concentration that resulted in only ~10% receptor occupancy.

In general, the distribution of agonist ([^{125}I]DELT) and antagonist ([^{125}I]AR-M100613) binding was similar. In absolute terms, [^{125}I]DELT binding was greater than [^{125}I]AR-M100613 in most areas, likely reflecting the greater degree of receptor saturation produced by [^{125}I]DELT (i.e. 45% *vs.* 10%). However, two observations suggest that [^{125}I]AR-M100613 recognized a wider receptor pool than [^{125}I]DELT. First, saturation analysis revealed a higher B_{max} for the antagonist radioligand in the areas sampled. Second, regression analysis predicted a component of [^{125}I]AR-M100613 binding occurring in the absence of [^{125}I]DELT binding. This residual component, which was naltrindole-displaceable and hence likely to reflect DOR binding, was evident in a few areas that possessed virtually no [^{125}I]DELT binding. Perhaps in these areas, the DORs exist predominantly in a low affinity state for agonist and hence are not readily detected in the [^{125}I]DELT assay. Alternatively, [^{125}I]AR-M100613 may recognize one or more DOR subtypes that are not readily detected with [^{125}I]DELT.

DELT-stimulated [^{35}S]GTPγS binding

Previous studies have revealed only small stimulatory effects of DELT on [^{35}S]GTPγS binding in rodent brain, with maximal stimulation approximating 10-25% above basal binding (Fraser et al., 1999; Hosohata et al., 2000). However, whole membrane preparations were used, potentially masking strong responses in certain areas. The present autoradiographic study revealed considerable anatomical heterogeneity. Thus, [^{35}S]GTPγS responses ranged from near-zero in many areas to an 80% increase in caudate putamen. This result contrasts with previous findings using the DOR agonist pCl-DPDPE, which produced responses of similar magnitude across several forebrain areas (Sim-Selley et al., 2000). Hence, our results provide further evidence that pCl-DPDPE differs pharmacologically from DELT (Fraser et al., 2000).

In our [^{35}S]GTPγS assay, responses to the μ agonist DAMGO were considerably higher than responses to DELT in every area examined. This finding is consistent with published results using either brain membrane homogenates (Fraser et al., 1999; Alt et al., 2002; Sim-Selley et al., 2002) or autoradiography (Sim-Selley et al., 2002). There are several possible factors that may contribute to the greater functional response of the MOR compared to the DOR. First, the sampled areas possess similar densities of MOR and DOR (Mansour et al., 1988), and hence relative abundance is unlikely to play a major role. Second, there may be increased G protein coupling efficiency of MORs *vs.* DORs. Third, a greater number of MORs may be located on the plasma membrane. Finally,

DAMGO is a full agonist, whereas deltorphin II has been reported to be a partial agonist (Szekeres and Traynor, 1997; Clark et al., 1997).

DELT-stimulated [^{35}S]GTPγS binding vs. radioligand binding

A correspondence exists between patterns of radioligand binding and [^{35}S]GTPγS response for several G protein coupled receptors (Sim et al., 1995). In this respect, a particularly strong correlation has been reported for both μ (r=0.90) and κ (r=0.98) opioid receptors in rat brain (Hyytia et al., 1999). However, in an analogous study of DOR using DPDPE as both radioligand and agonist, only a tenuous correspondence was seen (r=0.61, nonsignificant) (Hyytia et al., 1999). In the present study, radioligand binding revealed a highly significant but moderate correlation with DELT-mediated function. A minor source of divergence was the residual [^{35}S]GTPγS response that occurred in areas that lacked significant radioligand binding; possibly, this small response is not mediated by DORs. A greater source of mismatch between radioligand binding and function could reflect one of at least four factors, as follows.

First, the affinity of [^{125}I]DELT or [^{125}I]AR-M100613 for DOR may vary across CNS regions, although we are unaware of any published evidence to support this. However, the close relationship between agonist and antagonist binding across CNS areas (r = 0.87) suggests that [^{125}I]DELT and [^{125}I]AR-M100613 affinity are either correlated or are relatively invariant.

Second, divergence in our assay conditions could potentially contribute to the differences between our agonist radioligand and functional measures. Both the [^{125}I]DELT and [^{35}S]GTPγS binding conditions (high Na^+ and GDP, respectively) favoured the low agonist affinity state of the receptor. However, it cannot be assumed that this agonist affinity shift occurred to the same extent in each assay across CNS regions.

Third, efficiency of coupling between DOR and G proteins may be regionally heterogeneous, as has been proposed for mouse DOR (Oakley et al., 2003) and for rat MOR (Sim-Selley et al., 2000). In this context, variations in [^{35}S]GTPγS response may reflect signalling through G proteins that are not readily detectable in this assay (Milligan, 2003).

A fourth potential factor is the extent to which DORs are intracellular, and whether these receptors would be detectable under our test conditions. In cryostat-cut sections, intracellular DORs are presumably accessible but whether any of our three assays is capable of detecting them is an open question. Electron microscopy studies suggest that in striatal patches (Wang and Pickel, 2001), nucleus accumbens shell (Svingos et al., 1999), and lumbar spinal cord (Cahill et al., 2001), the majority of DORs occur on intracellular organelles and not on the outer cell membrane. Whether this is the case in other CNS areas remains to be determined.

The existence of subtypes of DOR has been proposed, based partly on comparisons between putative δ1 (e.g. DPDPE) and δ2 (e.g. DELT) agonists (for review see Zaki et al.

(1996). Although there is considerable *in vivo* evidence for such a distinction, published *in vitro* data are equivocal (Sofuoglu et al., 1992; Buzas et al., 1994; Noble and Cox, 1995; Hiller et al., 1996; Zaki et al., 1996; Kim et al., 2001; Parkhill and Bidlack, 2002). In one receptor autoradiographic study, support for the δ1/ δ2 distinction has been claimed on the basis of regional differences between [^{3}H]DPDPE and [^{3}H]DSLET binding (Hiller et al., 1996). However, our own reanalysis of these data reveal a strong correlation between these markers (r=0.94). Since the CNS distribution of DOR subtypes is uncertain, it is not clear whether our [^{125}I]DELT labelling corresponds to either the putative δ1 or δ2 receptor subtype.

Behavioural implications

The highest DOR-mediated [^{35}S]GTPγS response and radioligand binding were found in the extended striatum (caudate putamen, olfactory tubercle, nucleus accumbens). DORs within this large forebrain structure have been implicated in several psychobiological effects, including arousal, stereotypy (Longoni et al., 1991; Spina et al., 1998), reinforcement (Longoni et al., 1998), and analgesia (Schmidt et al., 2002). Interestingly, DOR agonists exert anti-parkinsonian effects in several animal models (Pinna and Di Chiara, 1998; Hudzik et al., 2000; Hille et al., 2001), likely via DOR activation in the caudate putamen. The present results encourage the further research of DOR function in the extended striatum.

A prominent feature of the present study was the paucity of DELT-stimulated [^{35}S]GTPγS binding in some areas thought to mediate DOR antinociception (Ossipov et al., 1995; Thorat and Hammond, 1997; Kovelowski et al., 1999a; Kovelowski et al., 1999b; Hurley and Hammond, 2000). This was the case even at high concentrations of DELT, and despite clear evidence of stimulation in other CNS areas tested in parallel. The marginal response in the spinal cord was particularly surprising since we have previously observed an appreciable increase in this region (Morinville et al., 2004); one potential explanation for this discrepancy is that in the present study, μ/δ receptor interactions were rendered unlikely by the addition of a MOR antagonist.

How then can the present findings be reconciled with the numerous published reports of spinal and supraspinal DOR-mediated analgesia? One possible explanation is that DOR-mediated antinociception is produced by a critical but sparse population of $G_{i/o}$ coupled DORs that fall below the detection threshold of the assay. Alternatively, DOR signalling in these regions may occur mainly through non-$G\alpha_{i/o}$ subunits that are intrinsically difficult to detect in the [^{35}S]GTPγS assay (Milligan, 2003). In this context, DELT-induced spinal analgesia appears partially mediated by the phosphoinositol pathway (Sanchez-Blazquez and Garzon, 1998; Narita et al., 2000), and by $G\alpha_q$ subunits in particular (Sanchez-Blazquez and Garzon, 1998).

CONCLUSION

The partial mismatch between conventional radioligand binding and GTPγS responses indicates that to some extent, these markers detect different aspects of DOR expression

and function. The finding that some areas thought to mediate DOR analgesia possess little or no GTPγS response encourages the further characterization of alternate signalling mechanisms. In contrast, the strong GTPγS response in forebrain areas provides a potential focus for future behavioural studies.

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INTERVENING SECTION 3

In chapter 3 it was shown that DOR in pain related areas (brain stem, spinal cord, and periaqueductal grey) possess low in vitro functional responses to deltorphin II. This may help to explain why DOR agonists are not as potent as MOR agonists in antinociceptive tests. Recent studies had shown that short term pretreatment with μ agonists can result in an enhanced behavioural response to spinally administered deltorphin II (Cahill et al., 2001; Morinville et al., 2003), and increased electrophysiological responses to DOR agonists in the periaqueductal grey (Hack et al., 2005). In addition, profound sensitization to the locomotor stimulant effects of deltorphin II had been observed after long term morphine pretreatment, and withdrawal (Melchiorri et al., 1992). In the previous chapters of this thesis, in vivo and in vitro assays characterizing MOR and DOR effects were established, therefore allowing us to examine interactions that may occur by agonist stimulation of these two receptors. Thus, the aim of this study was to determine the effects of chronic morphine pretreatment on DOR-mediated behavioural and in vitro responses.

CHAPTER 4

CHRONIC MORPHINE ADMINISTRATION AND WITHDRAWAL RESULTS IN TOLERANCE TO DELTA OPIOID RECEPTOR MEDIATED ANTINOCICEPTION

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Submitted

ABSTRACT

Delta opioid receptor (DOR) agonists produce only a moderate degree of antinociception, possibly reflecting the predominantly intracellular location of DOR. Recent studies suggest that short term morphine pretreatment can increase DOR mediated antinociception by promoting the translocation of DOR to the cell surface. In the present study we examined the effects of longer term morphine pretreatment and withdrawal on DOR mediated antinociception in the formalin test. Male adult rats were pretreated daily with morphine (10 mg/kg s.c.) or saline for 10 days, and after 7 days of withdrawal were tested acutely with the DOR agonist [D-Ala^2,Glu^4]-deltorphin (DELT). Unexpectedly, chronic morphine pre-exposure resulted in tolerance to DELT- but not morphine-induced antinociception; cross-tolerance to DELT was lost at 14 days of withdrawal. Cross-tolerance was also observed to the locomotor stimulant effects of DELT. Thus, no evidence of DOR sensitization was found after chronic morphine pretreatment. However, consistent with previous reports, short term (48 hour) pretreatment with morphine did result in sensitization to DELT. Subsequent in vitro analysis, using [^{35}S]GTPγS and [^{125}I]DELT autoradiography, did not detect any changes in DOR resulting from chronic morphine pretreatment. In conclusion, short term exposure to morphine resulted in DOR sensitization, whereas chronic morphine administration caused profound tolerance to DOR-mediated behavioral effects with no clear change at the receptor level.

INTRODUCTION

Agonists at the delta opioid receptor (DOR) display analgesic activity in numerous animal models, and have fewer adverse effects than the widely-used mu agonists. Although promising, delta agonists are less efficacious than their mu counterparts. However, recent studies indicate that their efficacy can be enhanced by pretreatment with morphine and other mu agonists in rodents. For example, in antinociceptive tests, sensitization of spinal DOR occurred after short term exposure to morphine (Cahill et al., 2001; Morinville et al., 2003). At the cellular level, electrophysiological studies have shown that functional DORs are induced in the periaqueductal grey by morphine pretreatment (Hack et al., 2005). This gain of function is thought to be mediated by a MOR-dependent translocation of DOR from intracellular compartments to the cytosolic membrane (Cahill et al., 2001; Morinville et al., 2003).

The facilitatory effects of morphine pretreatment on DOR-related behavior are not restricted to antinociception. Thus, morphine pretreatment markedly increased the locomotor stimulant effect of DELT in rats (Melchiorri et al., 1992). Importantly, this study showed that cross-sensitization was greatest with *chronic* morphine regimens, and continued to increase up to several weeks after morphine cessation. These findings have yet to be applied to pain paradigms.

The aim of the present study was therefore to determine the effects of chronic morphine pretreatment and withdrawal on DOR-mediated antinociception.

MATERIALS AND METHODS

Subjects

Subjects were male Sprague Dawley rats, weighing 225-250g (Charles River, St Constant, Quebec, Canada). Rats were housed in groups of two in a temperature- and humidity-controlled animal colony, lit from 7 a.m. to 7 p.m. Food and water were available ad libitum. All experiments were approved by the McGill University Animal Care Committee, in accordance with Canadian Council on Animal Care guidelines.

Morphine Pretreatment

Rats were randomly allocated to pretreatment groups, receiving daily subcutaneous (s.c.) injections of either 10 mg/kg morphine sulphate (Sabex, Boucherville, Quebec, Canada) or vehicle (0.9% saline) for 10 days. Behavioral testing occurred on the 7^{th} day of withdrawal unless otherwise stated.

For short-term morphine pretreatment rats received escalating doses of morphine (5, 8, 10, 15 mg/kg, s.c.) every 12 hours for 48 hours. Behavioral testing occurred 10-18 hours after the last injection.

Formalin Pain Test

Animals were habituated to the test boxes for 10 minutes on the day before testing, and for 45 minutes immediately before the test session. Rats were given intrathecal [D-Ala^2,Glu^4]-deltorphin (DELT, Tocris, Ellisville, MO, USA) or saline under general anaesthesia (1.5-2.0% isoflurane in O_2), allowed 10 minutes to recover, and then injected

intraplantar with 2.5% formalin (50 µl). Observation to determine nociceptive responses began immediately after formalin injection and continued for the next 50 min. A nociceptive score was determined for each 5 min block by measuring the amount of time spent in each of the four behavioral categories as previously described (Dubuisson and Dennis, 1977): 0, the position and posture of the injected hind paw is indistinguishable from the contralateral paw; 1, the injected paw has little or no weight placed on it; 2, the injected paw is elevated and is not in contact with any surface; 3, the injected paw is licked, bitten or shaken. A time-weighted nociceptive score, ranging from 0 to 300 was then calculated.

Surgery

Rats were anesthetized with ketamine/xylazine (80/16 mg/kg, i.p.)(Bioniche, Belleville, ON, Canada and Novopharm, Toronto, ON, Canada) and placed in a stereotaxic device. Each animal was implanted with a 24-gauge guide cannula (Plastics One, Roanoke, VA, USA) extending into the right lateral ventricle of the brain (coordinates from bregma: AP, -0.8 mm; ML, 1.5 mm; DV, 4.1 mm) and fixed with dental cement. Rats were given dipyrone analgesic (100 mg/kg, Vétoquinol, Lavaltrie, QC, Canada) immediately following surgery. To prevent occlusion, guide cannulae were kept patent by stainless steel inserts which extended 0.5 mm beyond the cannulae tip. Rats were allowed 5-7 days to recover from surgery before random allocation to treatment groups.

Locomotor testing

Eight locomotor cages (58.1 cm long x 28.8 cm wide x 53.0 cm high) were used, each comprising four outer walls made of white plastic-coated particle board (Melamine) and an open top. Cages sat on linoleum flooring covered with a thin layer of Beta Chip bedding. The location and movements of rats during behavioral testing were monitored by a closed circuit television video camera (Panasonic) linked to a commercial tracking system (EthoVision v3.0, Noldus Information Technology, Leesburg, VA).

Animals were habituated to the locomotor test cage for 1 hour the day before testing, and again for 30 minutes immediately prior to testing. A repeated measures design was used, where each rat was tested on days 7 and 8 of withdrawal; once with intracerebroventricular (i.c.v.) DELT (0.4 μg) and once with i.c.v. vehicle (0.9% saline), in a counterbalanced order. Test solutions (10 μl) were injected over 1 minute, and the injection cannula was left within the guide cannula for an additional 30 seconds. Locomotor activity was measured as the total horizontal distance moved, and the effects of DELT were expressed as a difference between DELT and vehicle test scores.

Preparation of tissue

Rats were decapitated and the brains and spinal cords were rapidly removed, frozen in 2-methylbutane (–50 °C for 30 s) and stored at –40 °C. Tissue was cryostat-cut (20 μm thick) and sections were taken through the caudate putamen (10.7 to 8.7 IA), periaqueductal grey (3.2-1.0 IA), rostroventral medulla (-1.3 to -2.6 IA), and L4-L5 segments of the lumbar spinal cord (Paxinos and Watson, 1997). At each rostro-caudal

level, seven consecutive sections were collected for autoradiographic comparison of DOR and MOR agonist mediated [^{35}S]GTPγS binding, and [^{125}I]DELT labeling. Sections were thaw-mounted onto gelatin-coated slides, air dried at room temperature for 10-15 min and then stored at -40°C until further use.

[^{125}I]DELT autoradiography

Radioligand autoradiography was performed using a protocol modified from Mennicken et al. (2003). Sections were thawed and preincubated at room temperature for 30 min in assay buffer comprising 50 mM TrisHCl with added 1 mM $MgCl_2$ and 120 mM NaCl (pH 7.4). The sections were then incubated in assay buffer in the presence of a non-saturating concentration (0.4 nM) of [^{125}I]DELT for 1 hour. [^{125}I]DELT (specific activity 2200 Ci/mmol) was a gift from AstraZeneca R&D Montreal. The incubation buffer (pH 7.4) comprised 50 mM TrisHCl, 1 mM $MgCl_2$, 120 mM NaCl, 0.5% bovine serum albumen (BSA), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and a saturating concentration of the highly selective MOR antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP 1 μM; Tocris, Ellisville, MO, USA). Non-specific binding was defined by the addition of the DOR selective antagonist naltrindole hydrochloride (0.1 μM, Tocris). Following incubation, sections were rinsed in ice-cold assay buffer (3 x 3 min) and distilled water (2 s), then blow-dried. Sections were exposed to Kodak X-OMAT AR X-ray film together with [^{125}I] microscale autoradiographic standards (Amersham Pharmacia Biotech, Piscataway, NY, USA) for 24 hours in light-proof X-ray cassettes. The films were processed with D19 developer and GBX fixer (Kodak).

[^{35}S]GTPγS autoradiography

[^{35}S]GTPγS autoradiography was performed using a protocol modified from Hyytia et al. (1999) Sections were thawed at room temperature and rehydrated for 20 minutes in assay buffer containing 50 mM TrisHCl, 5 mM $MgCl_2$, 100 mM NaCl, and 1 mM EDTA (pH 7.4). Sections were then preincubated for 1 hour with assay buffer plus 2 mM guanosine 5'-diphosphate sodium salt (GDP; Sigma Chemical Co., St. Louis, MO, USA) and 1 μM 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, adenosine A(1) receptor antagonist, Sigma Chemical Co., St. Louis, MO, USA). The sections were incubated in plastic slide mailers for 1.5 hours with assay buffer plus 2 mM GDP, 1 μM DPCPX, 1 μM dithiothreitol (DTT), 225 pM guanosine 5'(γ-^{35}S-thio) triphosphate ([^{35}S]GTPγS, 1250 Ci/mmol, Perkin Elmer Life Science Products, Woodbridge, ON, CAN). Slide mailers were allocated to six incubation conditions: basal (i.e. no agonist present), 0.3 μM DELT, 10 μM DELT, 0.3 μM [D-Ala^2, NMe-Phe^4, Gly^5-ol]-enkephalin (DAMGO), 0.5 μM morphine sulphate, and non-specific (i.e. 10 μM unlabelled GTPγS (Sigma) with no agonist present). Sections were rinsed in ice-cold buffer (50 mM TrisHCl and 5 mM $MgCl_2$, pH 7.4, 2 x 5 min), distilled water (2s), and then blow-dried. Sections were exposed to X-ray film for 24 hours in light-proof X-ray cassettes. Co-exposure with [^{14}C] microscale autoradiographic standards (American Radiolabeled Chemicals, Inc., St. Louis, MO, USA) permitted quantification of the [^{35}S] radioisotope (Miller, 1991). The films were processed with D19 developer and GBX fixer (Kodak).

Quantitative image analysis

Film autoradiographs were quantified using an M4 MCID computer-based system (Imaging Research, St. Catherines, ON, Canada). Binding was calculated based on autoradiographic standards and expressed as fmol/mg wet tissue equivalent. Non-specific binding for [^{125}I]DELT was negligible. Agonist-stimulated [^{35}S]GTPγS binding was calculated by subtracting basal binding. Regions of interest were identified by reference to adjacent Nissl-stained sections.

Statistical analysis

Area under the curve values and linear regression analyses for the formalin test were generated using Prism 4.0 (GraphPad Software, San Diego California USA, www.graphpad.com). Multiple comparisons were performed using t-tests (Systat v10.2, SPSS, Chicago, IL, USA).

RESULTS

Except where noted, all rats were pretreated daily with morphine (10 mg/kg) or saline for 10 days, and all testing occurred on the seventh day of withdrawal.

In formalin pain tests (Fig. 1A and B), morphine pretreatment did not alter basal nociceptive scores obtained after acute saline challenge. As expected, intrathecal [D-Ala^2,Glu^4]-deltorphin (DELT) dose-dependently decreased phase 2 formalin-induced nociception in saline pretreated rats (linear trend $p<0.01$; Fig. 1A). However, chronic morphine pretreatment resulted in tolerance to this effect of DELT (linear trend $p>0.5$; Fig. 1B and 1C). Tolerance at the highest concentration of DELT (0.3 μg/μl) was particularly clear ($p<0.02$), and was confirmed in a subsequent experiment in a different group of rats ($p<0.001$; Fig. 2A). Tolerance to DELT was lost by 2 weeks of withdrawal from morphine pretreatment (Fig. 2B).

We next tested whether the same chronic morphine regimen would result in tolerance to acute morphine challenge given at the 7 day time interval (i.e. after withdrawal). For this purpose, a sub-maximal challenge dose of morphine was used (4 mg/kg; Abbott et al., 1982). Tolerance was clearly absent (chronic morphine *vs.* saline, $p=0.63$; Fig.3).

The effects of short term (48 hour) exposure to morphine were also tested. In contrast to the chronic dosing regimen, this short-term pretreatment resulted in sensitization to DELT-induced antinociception ($p<0.02$; Fig. 4).

The occurrence of tolerance to spinal DELT antinociception following *chronic* morphine was unexpected, since the same chronic regimen has been reported to increase locomotor stimulant responses to i.c.v. DELT (Melchiorri et al. 1992). Therefore, in a final behavioral study, the effects of this chronic pretreatment regimen were tested on locomotor responses to i.c.v. DELT. In the control (saline pretreated) group, a significant locomotor stimulant effect of DELT was observed in the first 30 minutes ($p<0.001$). Morphine pretreatment resulted in partial tolerance to this effect ($p<0.05$). The mean ± SEM DELT-vehicle difference scores for chronic morphine *vs.* saline were 3172 ± 824 and 6810 ± 1292 cm, respectively.

Possible changes in DOR function were assessed in vitro using the [^{35}S]GTPγS assay. For this purpose, parallel groups of rats were chronically treated with morphine or saline but were otherwise drug-naive. Assays were performed on three pain related CNS areas and on the DOR-rich caudate putamen (CP). Morphine pretreatment did not significantly alter GTPγS responses to DELT, except in the rostroventral medulla (RVM; $p<0.02$, Table 1). However, in this brain region tolerance occurred only at the higher concentration of DELT. To further investigate this result, the RVM and CP were assayed using a wider range of DELT concentrations in a new group of pretreated animals. Here, tolerance was not detected in either brain area (Fig. 5). In both the above experiments, mu agonist-induced GTPγS responses were not significantly affected by morphine pretreatment (Table 1 and Fig. 5). Finally, there were no significant differences in [^{125}I]DELT binding between morphine and saline pretreatment groups (Table 2).

FIGURE 1

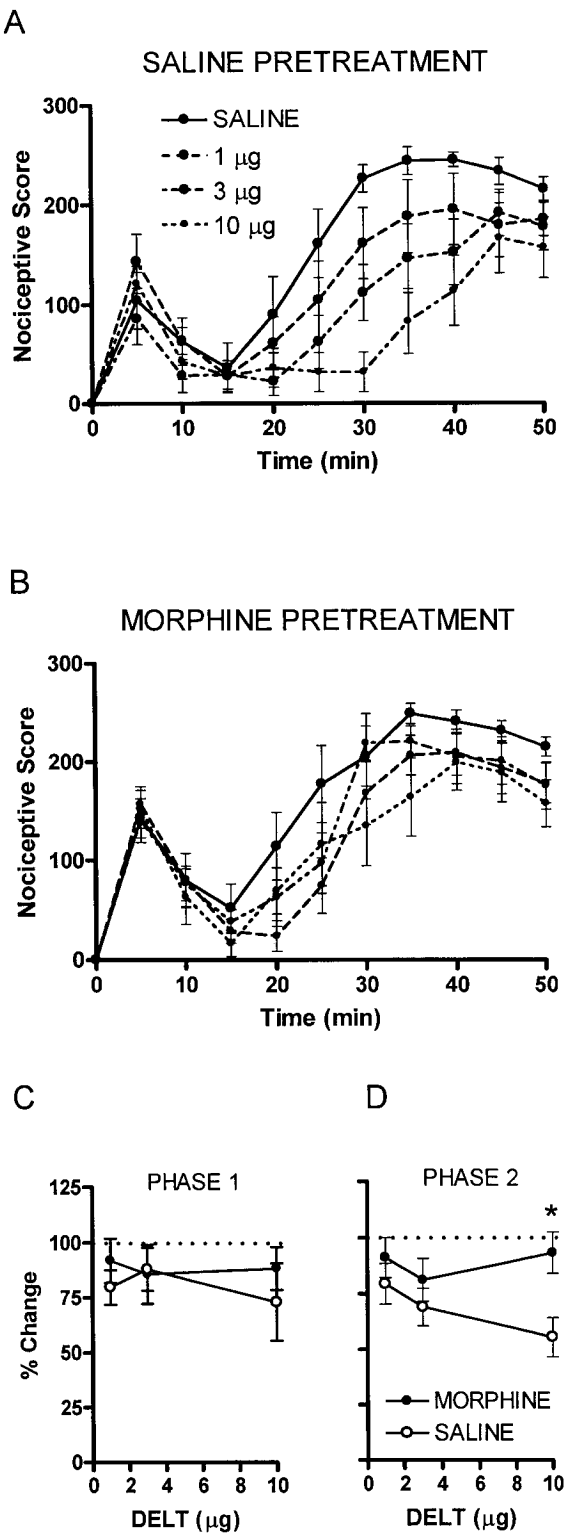
A
SALINE PRETREATMENT
SALINE
1 µg
3 µg
10 µg
Nociceptive Score
Time (min)
B
MORPHINE PRETREATMENT
Nociceptive Score
Time (min)
C
D
PHASE 1
PHASE 2
% Change
DELT (µg)
DELT (µg)
*
MORPHINE
SALINE

Figure1. The occurrence of tolerance to the antinociceptive effects of DELT after chronic morphine pretreatment. Intraplantar injection of 2.5% formalin produced a biphasic nociceptive response, which was dose-dependently decreased by DELT in saline pretreated rats (A), but not in the morphine pretreated group (B)(n=7-8 rats/group). The y axes for panels A and B show the mean ± SEM nociceptive score in response to formalin. Panels C and D show the antinociceptive dose response curves for phases 1 (0-10 min) and 2 (15-50 min), respectively. The y axes in panels C and D represent area under the curve values, expressed as percent change from control (i.e. acute saline challenge). *$p<0.02$ morphine *vs.* saline pretreatment.

FIGURE 2

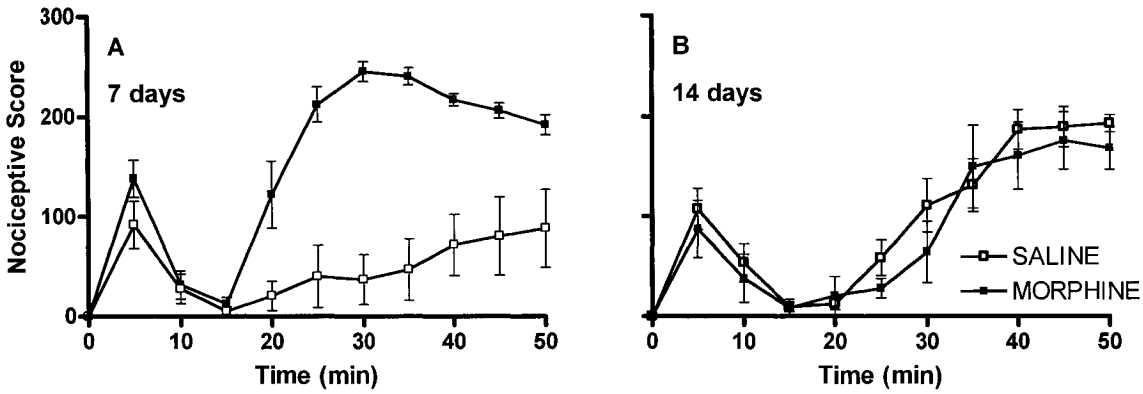

Figure 2. Antinociceptive response to DELT returns after 14 days of withdrawal. Rats were pretreated with chronic morphine or saline and challenged with acute DELT (10 μg, i.t.) on day 7 (A) or day 14 (B) of withdrawal. Peak nociception occurred between 25-35 min after intraplantar formalin injection, and these scores were averaged in order to perform a t-test between the two pretreatment groups. Morphine and saline pretreated groups differed significantly in their response to i.t. DELT on day 7 ($p<0.001$) but not on day 14 ($p=0.54$) of withdrawal. The y axes show the mean ± SEM pain score in response to formalin, n=7 rats/group.

FIGURE 3

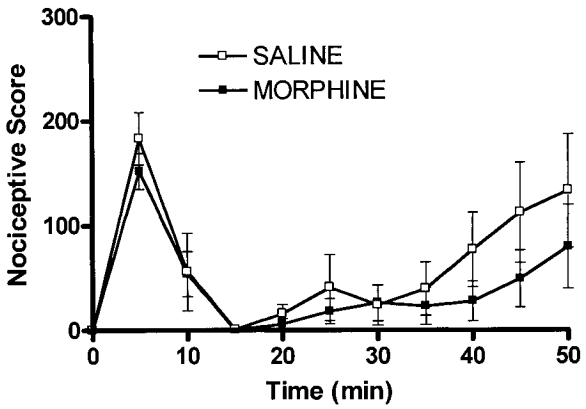

Figure 3. Morphine pretreatment did not result in tolerance to morphine-induced antinociception. Rats were pretreated for 10 days with morphine (10 mg/kg) or saline, and tested on day 7 of withdrawal with a challenge dose of 4 mg/kg morphine. Nociceptive scores were averaged between 25-35 min after formalin injection, and a t-test revealed no significant difference between the two pretreatment groups ($p=0.63$). The y axes show the mean ± SEM pain score in response to formalin, n=5-7 rats/group.

FIGURE 4

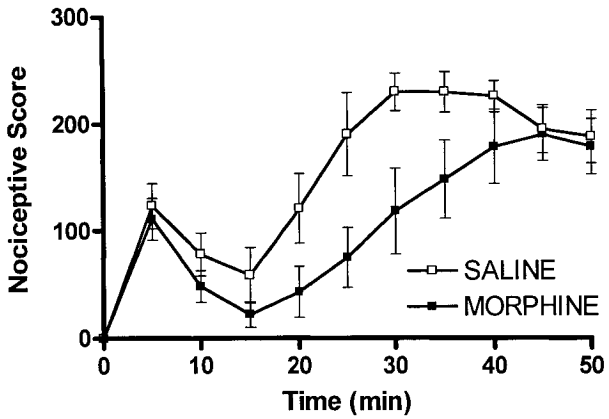

Figure 4. Sensitization induced by short term pretreatment with morphine. Rats were pretreated every 12 hours with increasing doses of morphine (5, 8, 10, and 15 mg/kg) for 48 hours, and challenged with DELT (3 μg, i.t.). Mean nociceptive scores at times 25, 30 and 35 min after formalin injection were compared by t-test and revealed a significant difference between the two pretreatment groups ($p<0.02$, n=7-9 rats/group).

FIGURE 5

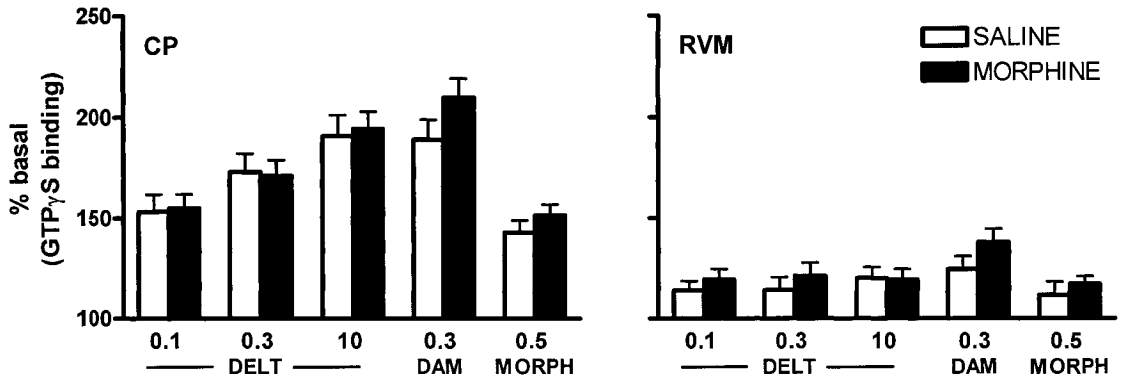

Figure 5. No change in MOR or DOR mediated [^{35}S]GTPγS responses after morphine pretreatment. Rats were pretreated with chronic morphine or saline, and on day 7 of withdrawal, in vitro responses to opioid agonists were assayed autoradiographically in the caudate putamen (CP) and rostroventral medulla (RVM). Analysis of the response to lower (0.1 and 0.3 μM) and maximal (10 μM) concentrations of DELT, and EC50 concentrations of DAMGO (0.3 μM) and morphine (0.5 μM) revealed no difference between morphine *vs.* saline pretreated animals. The y axis shows mean ± SEM specific [^{35}S]GTPγS binding expressed as a percentage of basal binding (i.e. in the absence of agonist; n=8-9 rats/group).

TABLE 1

Autoradiographic labeling of DOR and MOR mediated [^{35}S]GTPγS binding after chronic morphine or saline pretreatment

	CP		PAG		RVM		DH	
AGONIST	**SAL**	**MORPH**	**SAL**	**MORPH**	**SAL**	**MORPH**	**SAL**	**MORPH**
0.3 μM DELT	163 ± 9	162 ± 12	103 ± 3	102 ± 3	125 ± 13	119 ± 9	99 ± 4	109 ± 4
10 μM DELT	168 ± 6	165 ± 10	106 ± 3	100 ± 5	145 ± 18	99 ± 7*	111 ± 4	113 ± 3
0.3 μM DAMGO	173 ± 15	158 ± 12	141 ± 8	130 ± 5	147 ± 12	141 ± 10	132 ± 6	140 ± 13
0.5 μM MORPH	138 ± 6	129 ± 8	114 ± 4	106 ± 6	138 ± 10	117 ± 7	112 ± 12	123 ± 7

In each case, [^{35}S]GTPγS binding is expressed as a percentage of basal binding (i.e. in the absence of agonist; n=7-8 rats/group). Abbreviations: CP, caudate putamen; PAG, periaqueductal grey; RVM, rostroventral medulla; DH, L4-5 dorsal horn. * $p<0.02$ unprotected t-test, shows difference between morphine *vs.* saline pretreatment groups. A partial replication of this experiment is shown in Fig. 5.

TABLE 2

Autoradiographic labeling of DOR by [^{125}I]DELT after chronic morphine or saline pretreatment.

AREA	PRETREATMENT	
	SAL	MORPH
CP	4.62 ± 0.30	4.92 ± 0.15
PAG	0.48 ± 0.04	0.45 ± 0.02
RVM	0.32 ± 0.04	0.32 ± 0.01
L4-5 DH	0.43 ± 0.02	0.44 ± 0.01

Abbreviations: CP, caudate putamen; PAG, periaqueductal grey; RVM, rostroventral medulla; DH, L4-5 dorsal horn. There were no significant differences between pretreatment groups. Values are expressed as fmol/mg (mean ± SEM), n=7-8 rats/group.

DISCUSSION

Previously published reports suggest that both short and long term morphine pretreatment results in DOR sensitization. In contrast, we now report the novel observation that chronic morphine pretreatment can result in tolerance to DOR agonist-induced behavioral responses. This tolerance was observed one week after morphine withdrawal, a time at which animals were not tolerant to the effects of morphine. In vitro analysis in pain-related areas revealed no concomitant changes in DOR function or binding.

In the present study, the morphine dosing regimen proved critical for the induction of tolerance *vs.* sensitization to DELT. Thus, long term treatment (10 days and 7 days of withdrawal) resulted in near-total tolerance to DELT, whereas short term (48 hour) morphine exposure resulted in an enhanced response. The latter observation is consistent with previous findings based on the same dosing regimen (Cahill et al., 2001; Morinville et al., 2003). This form of sensitization appears transient, since it was lost 48 hours after the final morphine injection (Morinville et al., 2003).

The chronic morphine regimen employed in the present study has been reported to cause a dramatic sensitization to the locomotor stimulant effects of DELT (Melchiorri et al., 1992). This sensitization appeared robust, in that it was obtained with several short and long term morphine regimens, and increased with time up to several weeks of withdrawal. In contrast, we observed clear tolerance to DELT-induced locomotion. There is no obvious explanation for these divergent findings. Several factors cannot be

responsible as they were kept constant between studies (e.g. morphine dosing regimen, withdrawal interval, DELT challenge dose, rat strain, age and sex).

It is important to emphasize that the antinociceptive effects of DELT in this study were almost certainly mediated by a direct action on DOR. First, the antinociceptive effect of DELT given at the highest dose used here (10 μg, i.t.), is sensitive to blockade by the DOR antagonist naltrindole (Cahill et al., 2001). Second, antisense targeting rat spinal DOR inhibited the effects of an even higher dose of i.t. DELT (Bilsky et al., 1996). Third, in the present study, the antinociceptive effect of morphine, which is dependent on MOR but not DOR (Matthes et al., 1996; Sora et al., 1997) was still maintained concurrent with tolerance to DELT.

Chronic morphine has been reported to induce cross-tolerance to behavioral effects of delta agonists in some but not all previous studies (Yoburn et al., 1990; Adams and Holtzman, 1991; Stevens and Yaksh, 1992; Kalso et al., 1993; Catheline et al., 1996). However, the animals in all these reports were tested while still tolerant to morphine. In contrast, the present study included a one-week withdrawal period after which tolerance to morphine was not observed. Our findings appear to provide the first in vivo evidence for morphine-induced tolerance to a DOR agonist in the absence of residual tolerance to morphine. To our knowledge, the only analogous finding is provided by an in vitro study of adenylate cyclase activity (Noble and Cox, 1996). However, differential tolerance to DOR vs. MOR agonists was only seen in one of several brain regions examined, and the animals were tested directly after morphine pretreatment.

In the present study, the mechanism underlying the behavioral tolerance to DELT was investigated using two in vitro assays: DELT-induced [^{35}S]GTPγS binding and [^{125}I]DELT autoradiography. Responses in this GTPγS assay reflect DOR function, but are only partially correlated with [^{125}I]DELT labeling (Pradhan and Clarke, 2005). Based on antisense experiments (Fraser et al., 2000), the GTPγS assay may be more sensitive to experimental manipulations. We observed a GTPγS response to DELT in all CNS regions tested, except in the PAG, consistent with previous findings (Pradhan and Clarke, 2005). This negative finding accords with electrophysiological evidence suggesting that DORs are non-functional in this brain area (Vaughan and Christie, 1997; Connor and Christie, 1998; Hack et al., 2005). No clear effect of chronic morphine was detected in either the [^{125}I]DELT or [^{35}S]GTPγS assay. The only possible indication of tolerance was a decrease in [^{35}S]GTPγS binding in the rostroventral medulla seen at a single (maximal) concentration of DELT (Table 1). However, this result was probably a false positive, since it would have been non-significant after Bonferroni correction and no such effect was detected at any DELT concentration in a subsequent experiment (Fig. 5).

These negative in vitro results suggest that the behavioral tolerance to DELT was not due to changes in DOR abundance or to reduced coupling between DOR and G proteins. Several alternative explanations may be offered. First, DOR function may have been reduced in other brain areas that were not assayed. However, the major CNS areas that mediate antinociception were investigated (rostroventral medulla, lumbar spinal cord, and periaqueductal grey) (Rossi et al., 1994; Ossipov et al., 1995; Thorat and Hammond, 1997; Kovelowski et al., 1999a; Kovelowski et al., 1999b). Second, changes in DOR

after morphine pretreatment possibly occur only in certain neuronal subcompartments. By way of analogy, morphine exposure appears to selectively change the distribution of dendritic, not somatic MOR (Haberstock-Debic et al., 2003; Hack et al., 2005). If a similar redistribution of DOR were to occur, it would not necessarily be detectable by our in vitro assays. Third, DORs primarily signal through $G\alpha_{i/o}$, whereas evidence in mice suggests that DELT-induced spinal analgesia may be partially mediated by $G\alpha_q$ subunits (Sanchez-Blazquez and Garzon, 1998); coupling through $G\alpha_q$ is not readily detected by the [^{35}S]GTPγS assay (Milligan, 2003).

There are at least two additional mechanisms that might explain behavioral tolerance to DELT following chronic morphine administration. Although DOR and MOR share downstream signaling cascades (Connor and Christie, 1999), differential tolerance could potentially occur if these two receptor pathways were segregated between cellular compartments or neuronal populations. In addition, we cannot rule out the possibility that DELT antinociception occurs through mu/delta heterodimers, which in turn may be downregulated by chronic morphine exposure. It is currently unknown whether [^{35}S]GTPγS responses and [^{125}I]DELT autoradiography would detect such changes.

DOR agonists, despite potential therapeutic advantages over mu agonists, have suffered from low antinociceptive efficacy in preclinical tests. Although several reports have indicated that morphine pretreatment can enhance DOR function, the present results show that DOR-mediated responses may be either sensitized or attenuated, depending on the dosing regimen.

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Chapter 5

GENERAL DISCUSSION

5.1 Summary

Several behavioural and biochemical aspects of μ and δ opioid receptors were presented in this thesis. It was shown that PNA antisense targeting of MOR lead to a complete inhibition of μ agonist-induced antinociception. This knockdown was only seen at the behavioural level, and there was no corresponding decrease in brain or spinal MOR labelling or functional responses. During the development of the antisense study it was noted that δ agonists possessed a much lower [^{35}S]GTPγS response relative to MOR stimulation. This observation lead to the next chapter of the thesis, in which the neuroanatomical distribution of functional DOR was determined and compared to DOR radioligand binding. Autoradiographic comparison of deltorphin II-induced [^{35}S]GTPγS binding vs. [^{125}I]deltorphin II and [^{125}I]AR-M100613 revealed that agonist or antagonist radioligand binding was a poor predictor of functional DOR. The CNS areas with the greatest [^{35}S]GTPγS responses were the extended striatum and cortex, while very low responses were detected in pain related areas. In the final experimental chapter, the μ and δ opioid receptors were examined together by observing the changes in DOR after chronic morphine pretreatment. Long term exposure to morphine followed by a one week withdrawal period resulted in tolerance to deltorphin II-induced antinociception and locomotor stimulation. This tolerance was not detected in vitro with either radioligand or [^{35}S]GTPγS binding. Taken together, the results presented in this thesis suggest that MOR/DOR agonist interactions should be further characterized if δ agonists are to be

used clinically, and that in vitro assays used in this thesis are not always predictive of behavioural responses.

5.2 Methodological Limitations

Throughout this thesis we were unable to detect significant biochemical changes in the central nervous system to explain significant loss of function in behavioural tests. Therefore, it is important to understand the limitations of our in vitro assays. A major limitation of the autoradiographic [^{35}S]GTPγS assay is that there is high basal binding (i.e. absence of agonist), which can be seen in Fig 4, chapter 3. This can be particularly problematic in areas such as the periaqueductal grey or dorsal horn, since small increases in agonist induced binding may not be detected due to the low signal to noise ratio. More importantly, further decreases in such areas brought about by experimental manipulations (e.g. morphine pretreatment) would be even more difficult to detect.

The [^{35}S]GTPγS autoradiographic assay provides a method for surveying large numbers of CNS areas, and detecting highly localized drug effects. However, use of tissue homogenates allows for more replicates to be assayed. Using whole brain homogenates we previously detected a significant decrease in δ agonist-induced responses after PNA antisense targeting DOR (Fraser et al., 2000a). Antisense pretreated rats had a 25% decrease in SNC80-induced [^{35}S]GTPγS binding relative to control animals. A further advantage of using brain homogenates in this assay, is that it would be possible to detect changes in non-$G_{i/o}$ G protein coupling. Relative to other Gα subunits, $G_{i/o}$ possess a substantially higher rate of basal guanine nucleotide exchange, and thus masks signals by

other Gα subunits (Milligan, 2003). In order to detect non-$G_{i/o}$ G coupling, antiserum against Gα subunits of interest can be used to bind to and isolate the Gα-[^{35}S]GTPγS complex (Milligan, 2003; Harrison and Traynor, 2003). Non-$G_{i/o}$ Gα coupling has been reported for both MOR and DOR (see Section 1.7.1), and it is possible that these alternate signalling mechanisms are important for the behavioural effects observed in this thesis.

There are certain general limitations that one must keep in mind with regard to autoradiography. First, several of the most commonly used radioligands used to label the opioid receptor are agonists ([^{125}I]DELT, [^{125}I]FK33824, [^{3}H]DPDPE, [^{3}H]DAMGO), which are sensitive to the affinity states of the receptor, and could be sensitive to putative receptor subtypes. Antagonist radioligands, in contrast, are not sensitive to affinity states of GPCRs, and are commercially available for MOR ([^{3}H]CTOP) and DOR ([^{3}H]naltrindole and [^{3}H]TIPPψ). However, these particular radioligands suffer from low specific activity provided by the tritium label, as well as differential quenching in white vs. grey matter (Happe and Murrin, 1990). DOR tritiated antagonists pose additional concerns as [^{3}H]naltrindole has a low DOR to MOR selectivity (~ 6 fold) (Payza et al., 1996), and TIPPψ was reported to be a partial agonist for DORs (Martin et al., 2001).

It was hoped that the novel DOR antagonist AR-M100613 would provide a viable alternative, as it has a high selectivity for DOR over MOR, and does not discriminate between DOR subtypes (Fraser et al., 1999). A further advantage is that AR-M100613 is iodinated, and therefore has high specific activity and is not quenched in tissue.

Unfortunately, this radioligand is unsuitable for autoradiographic labelling of DOR, since it possessed a low signal to noise ratio in autoradiographic sections. This high nonspecific binding suggests that [^{125}I]AR-M100613 also labels a non-DOR population. Thus, there continues to be an absence of antagonist radioligands specific for DOR autoradiography.

Alternative techniques that could be used to detect changes in MOR after PNA antisense treatment, and DOR after chronic morphine pretreatment are as follows. One potential strategy would be to use immunohistochemistry or immunoblotting. However, a major problem in the opioid field is that there is a lack of reliable antibodies which selectively detect MOR or DOR. Although many groups have reported immunhistochemical detection of both opioid receptors, the antibodies used are usually polyclonal, and highly susceptible to batch variation (Dr. Anne Morinville, personal communication). In fact a commonly used antibody to characterize the distribution of DOR (Dado et al., 1993; Arvidsson et al., 1995; Tao et al., 1998) was found to be blocked by substance P (Arvidsson et al., 1995). Furthermore, another group has found that all of the available opioid receptor antibodies continue to show immunostaining in their respective knock out animals, and sometimes even in triple knock out animals (Dr. Brigitte Kieffer, personal communication). Because of this poor antibody selectivity, a thorough study of the colocalization of MOR and DOR has not been done. Poorly selective opioid antibodies not only limit immunohistochemical localization, but also Western blot analysis. Thus, there is a real need for highly selective antibodies targeting the separate opioid receptors.

The advantage of immunolabelling of opioid receptors is that subcellular populations of the receptor can be visualized. This ability may prove to be crucial since morphine appears to selectively affect MOR in dendrites, but not axons (Haberstock-Debic et al., 2003). For example, if chronic morphine pretreatment was producing tolerance to deltorphin II by subtly changing DOR in selective neuronal subcompartments, autoradiography would not provide the high resolution needed to detect these changes.

A second approach to detect changes in MOR or DOR after antisense or chronic morphine pretreatment, would be to examine changes in down stream regulators of these receptors. There is some difficulty in this approach as all opioid receptors ultimately result in the same downstream signalling pathways. Thus, assays that do not depend on selective opioid receptor ligands (e.g. changes in MAP kinase phosphorylation) could not be used. However, monitoring changes in agonist-induced cAMP inhibition or GIRK or Ca^{++} currents may reveal differences in experimentally manipulated animals relative to controls. One shortcoming of these alternative assays is that they do not allow the anatomical survey that can be performed with receptor labelling.

Third, changes in MOR and DOR mRNA could be detected after the experimental manipulations used in this thesis. Changes in mRNA can be detected with in situ hybridization or RT PCR. In terms of antisense targeting the MOR, PNA binding to mRNA does not activate RNase H, thereby activating mRNA cleavage. In cell culture PNA has been reported to decrease (Aldrian-Herrada et al., 1998), or not change (Kilk et

al., 2004) mRNA levels. Only one study has characterized mRNA levels after PNA antisense pretreatment, and this group found an increase in target mRNA, which they propose to be a compensatory mechanism in response to the drop in protein that was detected (Boules et al., 2004). Thus, it is unclear if changes in mRNA levels would reflect changes at the protein level. Even if reductions in mRNA were detected after antisense or chronic morphine pretreatment it would still not explain the behavioural effects as changes at the protein level were not detected.

5.3 Behaviourally relevant populations of MOR and DOR

There are clear differences between MOR and DOR in vitro responses. In vitro results presented in Chapter 2, and a direct comparison of MOR vs. DOR [^{35}S]GTPγS binding in Chapter 3, confirms that MORs have a larger in vitro functional responses compared to DORs. This greater response of MOR vs. DOR has been previously reported in the literature using whole brain homogenates (Fraser et al., 1999; Alt et al., 2002; Sim-Selley et al., 2002) and autoradiography (Sim-Selley et al., 2002). A likely explanation for the greater MOR response is that this receptor is abundantly expressed on the cell surface, while DOR are primarily found intracellulary (see Section 1.3.5). In addition, MOR possibly couples to G proteins more efficiently than DOR. These in vitro findings indicate the existence of a large pool of functional MOR. However, the results from antisense targeting of MOR seen in Chapter 2, suggests that a small critical population of receptors is responsible for the behavioural effects of DAMGO.

Previous MOR antisense studies provide only a limited basis of comparison, as the majority have only demonstrated knockdown of behavioural effects, and have not quantified corresponding changes in CNS tissue (Rossi et al., 1994; Chen et al., 1995; Rossi et al., 1995a; Rossi et al., 1995b; Leventhal et al., 1997). However, one group did study changes in [^{3}H]DAMGO binding after phosphorothioate i.c.v. and i.t. antisense pretreatment in mice. Similar to our results, this group found that there was no change in [^{3}H]DAMGO binding in whole brain or spinal cord homogenates in the presence of a significant reduction in acute antinociceptive responses to systemic morphine (Shah et al., 1997). Two antisense studies have targeted rat MOR using the same PNA sequence as used in Chapter 2. This group found that antisense pretreatment resulted in a significant (~70%) decrease in antinociceptive responses to systemic morphine in the tail flick test, along with a ~ 55% reduction in MOR protein (as measured by Western blots) after either intra-PAG or systemic administration of antisense (Tyler et al., 1998; McMahon et al., 2001). These latter results suggest that not all MORs on the cell surface act equally, and that even with almost half of the receptors still intact, behavioural effects are not maintained. A possible explanation for these antisense results is that newly synthesized receptors are particularly important for μ agonist-induced antinociception in tests of acute pain. The extra receptors may play a modulatory role, and could be important in more complex behaviours such as inescapable or chronic pain, tolerance and dependence.

The low in vitro responses for DOR suggest that agonists at this receptor have low antinociceptive potential. Ultrastructural localization finds the majority of DORs on intracellular vesicles, raising the question of how they interact with exogenous ligand.

However, DOR agonists produce significant behavioural responses, including supraspinal (Chapter 2) and spinal (Chapter 4) antinociception (for review see Section 1.6). Apparently, the few receptors that are found on the cell surface are enough to mediate these behavioural effects. One possibility is that additive or synergistic responses from DORs in several brain and spinal structures are responsible for deltorphin II-induced antinociception. In support of this hypothesis, coadministration of deltorphin II into the periaqueductal grey and rostroventral medulla results in an additive antinociceptive effect (Rossi et al., 1994), and synergistic responses have been demonstrated when deltorphin II is concurrently injected into the rostroventral medulla and spinal cord (Kovelowski et al., 1999). Overall, the antinociceptive actions of DOR agonists were not predicted by our in vitro assays.

5.4 Non-antinociceptive uses for DOR agonists

To date, the literature on DOR has primarily focused on antinociception. However, in view of the finding that DOR labelling and [^{35}S]GTPγS responses were highest in the basal ganglia, cortex and amygdala, perhaps more emphasis should be placed on finding therapeutics that are relevant to these brain regions. For instance, δ agonists represent a potential therapeutic in the treatment of Parkinsonian symptoms. Animal studies have shown that DOR agonists such as SNC80 can induce ipsilateral turning at low doses, and contralateral turning at high doses, in rats that have sustained unilateral 6-OHDA lesions of the striatum (Pinna and Di Chiara, 1998; Hudzik et al., 2000). These effects were blocked by the DOR antagonist naltrindole (Pinna and Di Chiara, 1998; Hudzik et al., 2000). Thus, these findings suggest that SNC80 acts on DOR in intact and lesioned sides

of the striatum. SNC80 also exerts dose-dependent "restorative" effects in other animal models of Parkinson's disease. Thus, this drug increased locomotion in reserpine-treated rats, reversed akinesia induced by haloperidol or SCH23390, and reversed Parkinson-like symptoms in MPTP treated non-human primates (Hille et al., 2001). Systemic SNC80 appears to potentiate DA receptor transmission, but does not itself increase DA release (Longoni et al., 1998), and may serve as a concurrent therapy with L-DOPA. This pairing would allow a decrease in the amount of L-DOPA used, hence prolonging the time before onset of dyskinesias associated with long term L-DOPA therapy (for review see Olanow et al., 2004).

Further characterization of DOR in the forebrain may also be important in the development of antidepressants. Several findings indicate that DORs may be important in emotional regulation. First, mice lacking this receptor have shown an increase in anxiogenic-like and depressive-like behaviours (elevated plus maze, light-dark box; and forced swim test) (Filliol et al., 2000). Second, preproenkephalin knock out animals also show an increased response in tests of fear and anxiety (Ragnauth et al., 2001) along with increased aggression (Konig et al., 1996). Finally, DOR agonists (BW-372U86 and SNC80) can also decrease immobility in the forced swim test (a test which is predictive of effective antidepressants) (Broom et al., 2002). Our results showed that structures such as the amygdala and cortex which are involved in regulating emotional responses (Diamond 2004) had particularly high DOR binding and functional responses.

5.5 Tolerance to DOR agonists after morphine pretreatment

Tolerance to the behavioural effects of deltorphin II were seen after chronic morphine pretreatment and withdrawal (Chapter 4), in the absence of changes in DOR labelling and [^{35}S]GTPγS responses. This study raises numerous questions. Whether this cross tolerance can be induced by other μ agonists needs to be determined. This may be a very important point, particularly as morphine does not cause substantial internalization of MORs, unlike many other μ agonists (see Section 1.7.5). Previous studies examining the sensitization of DOR responses after short term pretreatment with morphine had found that this cross sensitization could be produced by several MOR agonists (fentanyl, methadone,etorphine), and that cross sensitization did not occur in MOR knockout mice (Morinville et al., 2003). These findings indicate that analogous studies adapted to the chronic use and withdrawal regimen used in Chapter 4 may provide further insights on the role of MOR in producing DOR tolerance.

In Chapter 4, tolerance to deltorphin II was detected one week after chronic morphine pretreatment. The experimental design did not distinguish whether chronic morphine pretreatment, withdrawal or an interaction between the two was responsible. This question may be addressed by challenging animals with deltorphin II on the final day of morphine pretreatment, and to test another group on day seven of withdrawal.

Several hypotheses may help to explain the tolerance seen at DOR after chronic morphine pretreatment and withdrawal. The first such hypothesis is that deltorphin II acts at MOR/DOR heterodimers to exert its antinociceptive effects, and that chronic morphine pretreatment downregulates these receptors and/or their associated signalling pathways. To date, there is very little in vivo evidence available to support the notion that deltorphin II exerts its behavioural effects by binding to MOR/DOR heterodimers. In fact, the existence of MOR/DOR heterodimers in animals was only demonstrated in 2004 (Gomes et al., 2004). In rats, the μ agonist CTOP does not block antinociception produced by an ED80 dose of i.c.v. deltorphin II in an acute pain test (paw pressure), suggesting a wholly DOR mediated behavioural response (Fraser et al., 2000b). However, in cells coexpressing MOR and DOR, CTOP has been found to reveal [^{3}H]deltorphin II binding sites possibly by stabilizing MOR/DOR heterodimers (Gomes et al., 2000). According to these results deltorphin II should be potentiated by CTOP, and perhaps repeating the above mentioned in vivo study with a lower concentration of deltorphin II would reveal this effect. It is also possible that in the more complicated and longer lasting formalin pain test (used in Chapter 4), deltorphin II relies on MOR and DOR interactions, but this has yet to be determined.

In our study, rats were tolerant to the antinociceptive effects of deltorphin II, but they were not concurrently tolerant to morphine. The antinociceptive effects of morphine are completely abolished in MOR knockout mice, indicating that this drug acts exclusively through MOR. If deltorphin II is acting through MOR/DOR heterodimers, then our results suggest that these heterodimers are downregulated for a longer time than MOR

alone. In cell culture and mouse spinal cord MOR/DOR heterodimers have been shown to have different signalling properties (George et al., 2000; Gomes et al., 2000; Gomes et al., 2004), and may even act through a different host of G proteins (pertussis toxin insensitive) (George et al., 2000), thus it is not inconceivable that this complex could also be differentially downregulated. It is unknown if the autoradiographic assays used in this thesis would detect MOR/DOR heterodimers, but given the methodological limitations presented earlier, it is unlikely.

A second possibility is that chronic morphine exposure results in an increase in enkephalin release which in turn downregulates DORs. In support of this idea, a microdialysis study showed a 340% increase in met-enkephalin release in rat PAG on the last day of chronic morphine pretreatment (Nieto et al., 2002). In addition, preproenkephalin mRNA in the PAG was increased from 40-70% during the first three days of morphine withdrawal (Fukunaga et al., 1996; Fukunaga et al., 1998). Increases in met-enkephalin after morphine pretreatment have also been detected in cat brain and spinal cord (Jhamandas et al., 1984). The putative downregulation of DOR clearly does not occur early during morphine pretreatment, since surface DORs have been shown to be upregulated after short term morphine exposure (Cahill et al., 2001). However, DOR downregulation might become the dominant process during longer-term morphine exposure or during withdrawal. Experiments designed to observe changes in brain and spinal enkephalin during the dosing regimen used in Chapter 4 may help to further characterize the cross tolerance seen at DOR after morphine pretreatment.

Lastly, a better understanding of DOR tolerance after morphine pretreatment may be obtained by using MOR tolerance as a heuristic. For example, tolerance at MOR and DOR is accompanied by cAMP superactivation (Nestler and Aghajanian, 1997; Varga et al., 2003). In contrast, NMDA antagonists appear only to prevent the development of tolerance to chronic morphine but not deltorphin II (Bilsky et al., 1996). Currently, nothing is known about the cellular mechanisms that mediate tolerance at DOR after morphine pretreatment

5.6 Concluding Remarks

A major reoccurring theme in this thesis was the presence of behavioural effects which were not predicted by in vitro assays. In Chapter 2, a complete and reliable knockdown of MOR antinociception was observed after PNA antisense pretreatment, and in Chapter 4 tolerance to both the antinociceptive and locomotor stimulant effects of deltorphin II were observed after chronic morphine pretreatment and withdrawal. However, in both cases there was no detectable change in brain or spinal receptor labelling or [^{35}S]GTPγS responses. Furthermore, a thorough anatomical characterization of deltorphin II-induced [^{35}S]GTPγS responses suggested that DOR in pain related areas (spinal cord, brain stem and periaqueductal grey) had very low functional activity, whereas DOR agonists produce reliable antinociception by acting at supraspinal and spinal sites (Chapter 2 and 4). The pharmaceutical industry continues to rely on in vitro screening of compounds

(and orphan receptors), yet from this thesis it is clear that not all biochemical assays reliably predict therapeutically-relevant activity in the whole animal.

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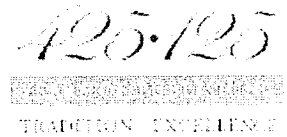

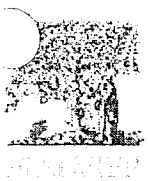

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Amynah A.A. Pradhan
McGill University
3655 Prom. Sir William Osler, Room 1320
Montreal
Canada H3G 1Y6

Dear Amynah A.A. Pradhan

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April 29, 2005

Amynah Pradhan
McGill University
Dept. of Pharmacology and Therapeutics
3655 Prom. Sir William Osler, Room 1320
Montreal, QC, Canada, H3G 1Y6
VIA FACSIMILE: 514-398-6690

Dear Amynah Pradham:

RE: Your recent request for permission to republish your material on pages 416-426 from Journal of Comparative Neurology (2005) Vol. 481. This material will appear in your forthcoming dissertation, to be published by McGill University in 2005.

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Montreal, QC, Canada H3G 1Y6

Unité de recherche en anesthésie
Université McGill
3655, Prom. Sir William Osler
Montréal, QC, Canada H3G 1Y6

05 May 2005

To Whom It May Concern:

I, Dr. Chiang Siau, am a co-author on the manuscript entitled "Chronic morphine administration and withdrawal results in tolerance to delta opioid receptor mediated antinociception."

I agree that this manuscript, which has been submitted for publication, can be included in the Ph.D. thesis written by Amynah A.A. Pradhan.

Thank you.

Sincerely,

Dr. Chiang Siau
Anesthesia Research Unit, Rm 1213
McIntyre Building, McGill University
3655 Prom. Sir William Osler
Montreal, QC, Canada H3G 1Y6
Tel: (514) 398 7562
Fax: (514) 398 8241

August 15, 2005

To Whom It May Concern:

I, Annie Constantin, am a co-author on the manuscript entitled "Chronic morphine administration and withdrawal results in tolerance to delta opioid receptor mediated antinociception."

I agree that this manuscript, which has been submitted for publication, can be included in the Ph.D. thesis written by Amynah A.A. Pradhan.

Thank you.

Sincerely,

Annie Constantin

McGill University
Animal Use Protocol – Research

Guidelines for completing the form are available at www.mcgill.ca/fgsr/rgo/animal/

Protocol #: 4368
Investigator #: 459
Approval End Date: MARCH 31, 2005
Facility Committee: MED

☐ Pilot ☐ New Application ☒ Renewal of Protocol # 4368

Title (must match the title of the funding source application): Antisense effects of peptide nucleic acid in brain

D level

1. Investigator Data:

Principal Investigator:	Paul Clarke	**Office #:**	398-3616
Department:	Pharmacology and Therapeutics	**Fax#:**	398-6690
Address:	McIntyre Building Room 1325	**Email:**	pclarke@pharma.mcgill.ca

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name:	Amynah Pradhan	**Work #:**	398-3616 or 00585	**Emergency #:**	276-8174 (H)
Name:	Paul Clarke	**Work #:**	398-3616 or 00585	**Emergency #:**	932-5852 (H)

3. Funding Source:

External ☒	**Internal** ☐
Source (s): CIHR	**Source (s):**
Peer Reviewed: ☒ YES ☐ NO**	**Peer Reviewed:** ☐ YES ☐ NO**
Status : ☒ Awarded ☐ Pending	**Status:** ☐ Awarded ☐ Pending
Funding period: 1/4/2001-30/3/2005	**Funding period:**

ACTION	✓	DATE
CCs		
OB	V	Apr 5 '04
APPROVED		

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed . e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/fgsr/rgo/animal/

Proposed Start Date of Animal Use (d/m/y): **or ongoing** ☒

Expected Date of Completion of Animal Use (d/m/y): **or ongoing** ☒

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator: **Date:** 23/2/2004

Approval Signatures:

Chair, Facility Animal Care Committee:		**Date:** 2/3/04
University Veterinarian:		**Date:** March 2, 2004
Chair, Ethics Subcommittee(as per UACC policy):		**Date:** 3/18/04
Approved Period for Animal Use	**Beginning:** April 1, 2004	**Ending:** MARCH 31, 2005

☐ This protocol has been approved with the modifications noted in Section 13.

03 MAR 2004

McGill University Animal Care Committee
AMENDMENT to Animal Use Protocol

www.mcgill.ca/rgo/animal/

rec'd 9/15/04
Conditional Approval 9/17/04

Principal Investigator: Paul CLARKE | Protocol # 4368

Protocol Title: Grant Title: Antisense Effects of Peptide Nucleic Acid in Brain | Phone: 398-3616

Unit, Dept. & Address: Pharmacology & Therapeutics, McIntyre Room 1320 | Fax: 398-6690

Email: paul.clarke@mcgill.ca | Level: D | Funding: CIHR

1. ADDITIONAL ANIMALS REQUESTED: *(justify additional animal numbers in box 5 below)*

Species	Strain	Supplier / Source	# Animals Purchased	# By Breeding	Age	Sex	Weight	# Needed at One Time	#/Cage	Total Per Year
NONE										

2. ADDITIONAL PERSONNEL: *If an undergraduate student is involved, the role of the student and the supervision received must be described. Training is mandatory for all personnel listed. Refer to www.animalcare.mcgill.ca for details. Each person listed in this section must sign to indicate that s/he has read the main protocol.*

Name	Classification	Animal Related Training	Occupational Health & Safety Program	Signature

3. CHANGE IN FUNDING SOURCE AND/OR TITLE *(title of grant must appear on the cover page of the animal protocol)*

4. OTHER: *(including housing, procedure, anaesthetic/analgesic, problems anticipated) If additional procedures, specify how many and which animals are to be used.*

PLEASE SEE ATTACHED

NOV 8 2004
APPROVED

5. WHY ARE THESE AMENDMENTS NECESSARY? *If requesting additional animals, justify the numbers*

PLEASE SEE ATTACHED

Approval Signatures:

Principal Investigator/ Course Director	Date	10 Sept 2004
Chair, Facility Animal Care Committee	Date	10/8/04
UACC Veterinarian	Date	Oct 18, 2004
Chairperson, Ethics Subcommittee (D level or Teaching Protocols Only)	Date	NOV 9, 2004

Form revised January 2004

Note: the above modifications are valid until the expiration date of the main protocol.

20 OCT. 2004

McGill University Animal Care Committee
AMENDMENT to Animal Use Protocol

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Principal Investigator: Paul CLARKE Protocol # 4368 (4368)

Protocol Title: Grant Title: Antisense Effects of Peptide Nucleic Acid in Brain Phone: 398-3618

Unit, Dept. & Address: Pharmacology & Therapeutics, McIntyre Room 1320 Fax: 398-6690

Email: paul.clarke@mcgill.ca Level: D Funding: CIHR

1. ADDITIONAL ANIMALS REQUESTED: *(justify additional animal numbers in box 5 below)*

Species	Strain	Supplier / Source	# Animals Purchased	# By Breeding	Age	Sex	Weight	# Needed at One Time	#/Cage	Total Per Year
Rat	SD	CR	132	0		M	275-350	30	3	132

2. ADDITIONAL PERSONNEL: *If an undergraduate student is involved, the role of the student and the supervision received must be described. Training is mandatory for all personnel listed. Refer to www.animalcare.mcgill.ca for details. Each person listed in this section must sign to indicate that s/he has read the main protocol.*

Name	Classification	Animal Related Training	Occupational Health & Safety Program	Signature

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PLEASE SEE ATTACHED

5. WHY ARE THESE AMENDMENTS NECESSARY? *If requesting additional animals, justify the numbers*

We wish to follow up recent interesting observations - see attached for more details.

Approval Signatures:

Principal Investigator/ Course Director		Date	8/6/2004
Chair, Facility Animal Care Committee		Date	07/7/04
UACC Veterinarian		Date	June 17, 2004
Chairperson, Ethics Subcommittee (D level or Teaching Protocols Only)		Date	17 04

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