Delta Opioid Receptor (δOR) Trafficking in the Central Nervous System

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Abstract

Studies from our laboratory have demonstrated that delta opioid receptors (δORs) are primarily localized intracellularly and may be recruited to the plasma membrane upon prolonged treatment with morphine. Our first objective was to establish a model in vitro system to study the mechanism of morphine-induced δOR trafficking. We discovered that while prolonged morphine treatment decreased the density of δORs at the plasma membrane in GH3 pituitary cells, the same treatment induced a translocation of δORs from intracellular to plasma membrane-bound compartments in cultured dorsal root ganglion cells, suggesting that the baseline intracellular localization and morphine-induced trafficking of δORs is a phenomenon specific to neurons.

A second objective of this thesis was to address whether the phenomenon of morphine-induced δOR trafficking occurred in regions of the central nervous system other than the spinal cord. To resolve this issue, we analyzed by immunogold histochemistry the subcellular distribution of δORs in the nucleus accumbens, dorsal neostriatum, and frontal cortex in mice treated or not with morphine (48h). We observed that prolonged treatment with morphine induced a translocation of δORs from intracellular to sub-plasmalemmal and membrane compartments in dendrites from both the nucleus accumbens and the dorsal neostriatum, but not from the frontal cortex. These results demonstrate that prolonged morphine treatment can not only regulate the trafficking of δORs in the brain but does so in a region-specific manner.

In summary, results from the present work support previous findings that δORs expressed in the central nervous system are poised to participate in physiological processes by recruitment to neuronal plasma membranes following an appropriate

cellular stimulus. This phenomenon could have implications for a number of physiological processes in which opioid receptors are known to play a role.

Résumé

Dans notre laboratoire, nous avons démontré que les récepteurs delta opioïde (δORs), ayant une localisation principalement intracellulaire, peuvent être recrutés à la membrane plasmique suite à une stimulation prolongée à la morphine. Le premier objectif de ce travail était d'établir un système in vitro pour l'étude des mécanismes de régulation du trafic de δOR induit par la morphine. Dans les cellules hypophysaires GH3, nous avons trouvé que l'application prolongée de morphine diminue la densité de δORs à la membrane, alors qu'elle induit plutôt leur recrutement vers la membrane plasmique dans les neurones de ganglions spinaux en culture. Ces observations suggèrent donc que les niveaux de localisation intracellulaire ainsi que la régulation du trafic des δORs induit par la morphine est un phénomène spécifique aux cellules de type neuronal.

Le deuxième objectif de ce mémoire était de vérifier si le phénomène de modulation du trafic des δORs induit par la morphine est aussi présent dans des régions du système nerveux central autre que la moelle épinière. À l'aide d'une technique d'immunohistochimie adaptée à la microscopie électronique, nous avons donc étudié la distribution subcellulaire des δORs dans le noyau accumbens, le neostriatum dorsal, et le cortex frontal de souris traitées ou non avec la morphine (48 h). Nous avons trouvé qu'un tel traitement prolongé avec la morphine induit la translocation des δORs à partir de compartiments intracellulaires vers les espaces sous-membranaires et membranaires des dendrites du noyau accumbens et du neostriatum dorsal, mais pas de ceux du cortex frontal. Ces résultats démontrent qu'un

traitement à la morphine ne fait pas que réguler le trafic des δORs dans le cerveau mais le contrôle de façon spécifique selon les régions.

En conclusion, les résultats de ce travail supportent nos observations précédentes et montrent que l'expression de δOR dans le système nerveux central pourrait participer de façon importante à différents processus physiologiques suite à son recrutement à la membrane plasmique, un phénomène étroitement contrôlé par des stimuli appropriés.

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List of Abbreviations

7TMR: 7-transmembrane domain receptor

α-MSH: α-melanocyte stimulating hormone

β-MSH: β-melanocyte stimulating hormone

γ-MSH: γ-melanocyte stimulating hormone

AC: adenylyl (adenylate) cyclase

ACTH: adrenocorticotropic hormone

AP2: adaptor protein 2

cAMP: cyclic adenosine monophosphate

CFA: Complete Freund's Adjuvant

CGRP: calcitonin gene related peptide

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

CNS: central nervous system

DLT: refers to deltorphin II

Deltorphin: generic term referring to either deltorphin I or deltorphin II

Deltorphin I: [D-Ala^2, Asn^4]deltorphin I, Tyr-D-Ala-Phe-Asp-Val-Val-Gly

Deltorphin II: [D-Ala^2, Glu^4]deltorphin II, Tyr-D-Ala-Phe-Glu-Val-Val-Gly

DPDPE: [D-Pen2, Pen5]enkephalin, when pen is penicillamine

DRG: dorsal root ganglion

ERK-1: extracellular signal-regulated kinase 1

ERK-2: extracellular signal-regulated kinase 2

Fluo-DRM: ω-Bodipy-576/589 dermorphin 5-APA

Fluo-DLT: ω-Bodipy 576/589 deltorphin-I 5-APA

GABA: γ-amino-butyric acid

GASP: G protein-coupled receptor-associated protein

GDP: guanosine diphosphate

GIRK: G protein coupled inwardly rectifying potassium channel

GRK: G-protein coupled receptor kinase

GTP: guanosine triphosphate

GTPase: guanosine triphosphatase

ICV: intracerebroventricular

IT: intrathecal

KCl: Potassium chloride

KO: knock-out

LAMP-1: lysosomal-associated membrane protein 1

LAMP-2: lysosomal-associated membrane protein 2

LDCV: large dense core vesicle

mRNA: messenger ribonucleic acid

NAc: nucleus accumbens

NMDA: N-Methyl-D-Aspartate

OR: opioid receptor

PAG: periaqueductal grey

PB: 0.1 M phosphate buffer

PBS: 0.1 M phosphate-buffered saline

PKC: Protein kinase C

POMC: Pro-opiomelanocortin

RGS: regulators of G-protein signaling

s.c.: subcutaneous

SP: substance P

WT: wild-type

VTA: ventral tegmental area

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Preface to the Introduction

As the focus of this Master's thesis is the interaction between μ and δ opioid receptors, much of the text will be devoted to the description of these two subtypes. However, the third remaining opioid receptor subtype, the κOR, is not without physiological and historical relevance. Therefore, a portion of the introduction will be devoted to the description of this receptor, specifically, in sections of the historical review of the opioid field, the distribution of the three opioid receptor subtypes, and in functional studies where appropriate (i.e. knockout studies).

Introduction

I: Historical Review of Opioids

Ii: Discovery of opiates

The analgesic and euphoric properties of opioids have been exploited since time immemorial. The first opioid, commonly known as opium, was derived from the milky extracts of the *Papaver somniferum* seedpods, and historically was most often consumed in either dried form for smoking, or as laudanum, a beverage of wine, spices and crude opium[1,2]. The immediate effects following consumption of these preparations are well-described in the words of the famous 17th century physician, Thomas Sydenham[2,3]:

"*It causes a most agreeable, pleasant and charming sensation...like a most delicious and extraordinary refreshment of the spirits...*"

"*Among the remedies which it has pleased Almighty God to give man to relieve his sufferings, none is so universal and so efficacious as opium*".

Although used since ancient times, no one knew how opium produced its desirable effects until 1806, when German chemist Friedrich Sertürner isolated one of the forty alkaloids which crude opium contains. In light of the sleep-inducing properties of this pure substance following administration to- among others- stray animals wandering his laboratory as well as himself[4], he named this substance *morphine*, after Morpheus, the Greek god of dreams[1-4]. The discovery of other opium-containing alkaloids followed soon after, including codeine, thebaine, and papaverine[2,3]; however, the elucidation of the chemical structure[5] and the synthetic synthesis[6] of morphine did not occur until the 1920's and 1950's, respectively. By then, opium and its derivatives were banned substances in England and the United States, due to the increasingly

apparent liability for abuse when injection using hypodermic needles became widespread[3,7].

Iii: Discovery of opioid receptors

By the 1970's, it became clear that the actions of opiates were elicited through endogenous opioid receptors. This line of reasoning was derived from the high potency of opioid agonists, the discovery of selective antagonists, and the stereospecificity of opiate actions[8-10]. Goldstein was the first to report radioligand binding to opioid receptors using ^{3}H-levorphanol, but the crude protocol used and low specific activity of this agonist resulted in high nonspecific binding. However, Goldstein's paper did emphasize the importance of stereospecificity of opioid ligands, as the binding of ^{3}H-levorphanol could be displaced only by the pharmacologically active (-)-isomer and not the (+)-isomer[8,10]. Just two years later, the existence of specific, saturable binding sites for opioids in the central nervous system (CNS) was confirmed by three independent groups[11-13], who refined the radioligand binding technique by using highly specific radioligands and filtration manifolds which permitted extensive washing of samples to remove nonspecific labeling. In fact, this binding paradigm became the gold standard and was employed for the subsequent discovery of other important neurotransmitter receptors, including serotonin and dopamine receptors[8].

Even before the biochemical identification of opioid binding sites, it had been postulated that the effects of opioids were elicited through multiple receptors. This hypothesis followed observations in both humans and animal models that at high doses,

nalorphine (a mixed opioid agonist-antagonist) produced analgesia, but at low doses blocked morphine-induced analgesia[8,14,15]. Evidence supporting the notion of multiple receptors was reported by Martin and colleagues. Based on the pharmacological actions of various opioid ligands in dogs, these authors proposed the existence of three opioid receptors: *kappa*, which mediated the actions of ketocyclazocine, *mu*, which was activated by morphine, and *sigma*, which mediated the psychotomimetic actions of certain opiates[16]. However, definitive demonstration of the opioid receptor subtypes we know today was described largely through the work of Kosterlitz and colleagues. Using radioligand binding and isolated nerve-smooth muscle techniques, they discovered a high-affinity opioid receptor in the mouse vas deferens that was pharmacologically distinct from the high affinity 'morphine' receptor present in the guinea pig ileum; this receptor expressed in the mouse vas deferens came to be known as *delta*[17]. In subsequent work, they confirmed the existence of kappa opioid receptors using radiolabeled ketocyclazocine[18]. We know now that the sigma receptor as described by Martin is in fact the phencyclidine binding site[19] on the NMDA receptor. Therefore, the prevailing hypothesis of the early 1980's was that the three remaining opioid receptor (OR) subtypes- mu (μ), delta (δ) and kappa (κ)- were responsible for transducing the effects of all opioid ligands.

A major breakthrough in the study of opioid receptors occurred in the early 1990's following molecular cloning of the three receptor subtypes. Within a year, the genetic sequences encoding δOR[20,21,28], μOR[22-24,28] and κOR[25-27] were isolated, expressed in heterologous cell expression systems, and characterized using molecular and pharmacological techniques including radioligand binding, cyclic AMP production,

and electrophysiology[20-27]. Isolation of the genetic sequences for opioid receptors thereby allowed for distribution analysis by highly sensitive techniques including in situ hybridization and immunocytochemistry (discussion to follow). Although debate continues as to whether OR genes give rise to alternatively spliced isoforms, it is generally accepted now that all opioid receptors derive from the three currently identified genes. This has been unambiguously confirmed by Clarke and colleagues[29], who observed a complete loss of ^{3}H-naloxone binding in triple OR knockout mice.

Iiii: Our very own painkillers: Discovery of the endogenous opioids

Following the discovery of opioid receptor binding sites in the early 1970s, the search for their endogenous ligands hastened. The existence of such endogenous ligands was not just derived from philosophical reasoning, as evolution generally does not conserve proteins incapable of activation by endogenous means, but was proposed following a number of supporting observations. In 1974, it was reported that focal electrical stimulation of the periaqueductal grey induced a pronounced analgesia[30] that could be blocked by naloxone, an opioid receptor antagonist[31]. In addition, biochemical studies demonstrated that incubating tissue in brain extracts alone could inhibit opioid receptor binding to radioligands[32-34].

The first class of endogenous opioids to be discovered was the enkephalins, Greek for 'in the head'. Isolated by Hughes and colleagues[35,36] from porcine brain, the enkephalins are naloxone-displaceable pentapeptides possessing the canonical opioid sequence NH_2-Tyr-Gly-Gly-Phe-X-COOH, where X is either leucine or methionine. In

radioligand binding studies, these pentapeptides exhibited appreciable selectivity for the δOR, which was often referred to as the "enkephalin" receptor[39,40]. Shortly thereafter, a peptide with opiate activity was isolated from camel pituitary, which corresponded to the C-terminal amino acids 61-91 of the peptide β-lipotropin. In it's entirety, β-lipotropin had no opiate activity, but the C-terminal untriakontapeptide displayed ^{3}H-etorphine-displaceable binding to opiate receptors in radioligand binding assays. Coined β-endorphin, this peptide binds to both μ- and δORs, with moderate selectivity for μORs[37,38,41]. Finally, a series of κ-selective peptides containing the leucine-enkephalin motif were extracted from porcine pituitary and named the dynorphins[42,43]. Interestingly, among the most highly selective μ- and δOR peptides discovered have been derived from amphibian skin; these include the μOR-selective dermorphins[44] and the δOR-selective deltorphins[45]. Finally, a class of highly μ-selective peptides, known as the endomorphins, was recently isolated from mammalian brain and these peptides are postulated to be the primary endogenous ligands for the μOR[46].

The enkephalins, dynorphins and β-endorphin are each produced by cleavage from a distinct precursor protein. Peptides derived from the pro-opiomelanocortin (POMC) precursor protein include β-endorphin, along with several other peptides lacking opioid activity, including β-lipotropin, adrenocorticotropic hormone (ACTH), and α-, β- and γ-melanocyte stimulating hormone (MSH)[41,47]. Proteolytic cleavage of proenkephalin yields multiple opioid peptides, including 4 copies of met-enkephalin, one leu-enkephalin, one met-enkephalin-Arg^{6}-Phe^{7} and one met-enkephalin-Arg^{6}-Gly^{7}-Leu^{8} [41,48]. Finally, the κ-selective opioid peptides dynorphin A and B, as well as α- and β-neoendorphin, are derived from cleavage of the precursor molecule prodynorphin[41,49].

II: Insights into the biology of opioid receptors

IIi: Molecular biology and signaling of opioid receptors

Following the discovery of opiate binding sites, early studies of intracellular signaling suggested that opiate receptors belonged to the G-protein coupled superfamily of seven transmembrane domain receptors. Sharma and colleagues were the first to demonstrate that acute morphine treatment led to naloxone-reversible inhibition of adenylate cyclase activity in NG-108/15 cells, a neuroblastoma x glioma cell line[50-51]. Using this cell line, opioid receptors were also shown to be sensitive to Na^+ ion and GTP concentration[40,52,53], findings consistent with previous autoradiographic and ligand binding studies in rat brain[54,55]. In later work, opioid receptors were discovered to induce GTP hydrolysis and to couple directly to the inhibitory G proteins Gi/Go[56] in a pertussis toxin-sensitive manner[57,58], both of which are characteristic of G protein-coupled receptors (GPCRs).

Molecular cloning[20-28] has now firmly established all opioid receptors as members of the GPCR superfamily, structurally characterized by seven transmembrane domains, an extracellular N-terminus, a cytosolic C-terminus and alternating intra- and extracellular loops. Like many GPCRs, opioid receptors contain multiple sites for post-translational modifications such as glycosylation and palmitoylation[59]. Overall, opioid receptors share high sequence homology with one another, owing to the high homology within the transmembrane domains (75%) and intracellular loops (86-100%). The areas of greatest divergence are found in regions of the protein important in ligand recognition and binding as well as in those subject to post-translational modifications, including the N-terminus (9-10%), extracellular loops (14-72%), and C-terminus (20%)[60].

The intracellular signaling mechanisms of opioid receptors have been extensively characterized (reviewed in ref. 61). Following activation, opioid receptors regulate a spectrum of effectors; in addition to adenylyl cyclase[50,51,61], these include G protein-coupled inward rectifying K^+ channels[62], Ca^{++} channels[63,64], phospholipase C[65,66], and the mitogen-activated protein kinases ERK1 and ERK2[67,68]. Activation of opioid receptors is ultimately inhibitory at the cellular level, as activation of these signaling cascades leads to hyperpolarization of the plasma membrane.

An intensely studied area of opioid receptor pharmacology is receptor subtype interaction, particularly between μORs and δORs. A model of allosteric coupling between μORs and δORs was first proposed by Rothman and Westfall, following observations in rat brain preparations that μ-selective ligands could inhibit the binding of δ-selective ligands[99-101]. Around the same time, Pert and colleagues reported on the pharmacological "interconvertibility" of μOR and δOR, particularly in the rat neostriatum, whereby receptor subtype selectivity for μ and δ ligands was dependent on experimental conditions[102,103]. In support of these data, several subsequent studies demonstrated that sustained morphine treatment altered the level of δOR binding sites[104-107]. Therefore, even prior to molecular cloning, the evidence suggested that these distinct receptor entities could engage in pharmacological 'cross-talk'.

A novel and intensely studied area of GPCR pharmacology is receptor oligomerization, or the physical association of monomeric receptors to form unique pharmacological complexes. The advent of biochemical techniques to measure the physical association of proteins in vitro, namely immunoblotting and fluorescence resonance energy transfer, have been applied to opioid receptors and have

demonstrated that μORs and δORs form both homo[108,109] and hetero-oligomeric complexes[110, 111]. These in vitro studies have been recently corroborated by Gomes and colleagues who demonstrate the existence of μOR-δOR heterodimers in vivo[112]. However, this is the sole study to date demonstrating the in vivo existence of such complexes. In any event, physical association of these receptors likely represents just one level of interaction between these two receptor subtypes.

Iiii: Review: Desensitization and internalization of GPCRs

As with other receptor classes, agonist binding to a GPCR induces a conformational change, which acts as the molecular 'switch' that turns on subsequent intracellular processes. First, GDP is exchanged for GTP on the α subunit of the trimeric G protein. This leads to dissociation of the α subunit from the βγ subunit, which each separately activate or inhibit their target effectors. Termination of signaling occurs when GTP is hydrolyzed back to GDP on the α subunit, which occurs either through the intrinsic GTPase activity of the G-protein or aided externally by regulators of G-protein signalling (RGS) proteins (reviewed in refs. 69,70).

Along with their G protein partners, the receptors themselves undergo their own series of regulatory events. Following agonist-induced receptor stimulation, GPCRs are uncoupled from their heterotrimeric G-proteins as a consequence of phosphorylation by intracellular kinases, including G protein-coupled receptor kinases (GRKs; reviewed in ref. 71). GRK-phosphorylated receptors are the targets of another family of proteins known as the non-visual or β-arrestins, which bind GRK-phosphorylated receptors and

thereby sterically inhibit further G protein activation. This process, known as desensitization, is the first of several mechanisms designed to control cellular activation during periods of agonist exposure.

In addition to its role in receptor desensitization, β-arrestins serve to promote the internalization of inactivated receptors. By recruiting the clathrin adaptor protein AP2 along with clathrin itself, β-arrestins facilitate receptor endocytosis into clathrin-coated pits. In the final step, the GTPase dynamin pinches off the clathrin-coated endocytic vesicle, separating vesicle from plasma membrane. From there, post-endocytotic sorting of internalized receptors can follow many routes depending on receptor subtype; these include recycling back to the plasma membrane via recycling endosomes, detainment within intracellular compartments, or targeting to lysosomes for degradation (reviewed in ref. 71).

Iiiii: Desensitization, internalization and downregulation of opioid receptors

It is well established that following agonist stimulation, all opioid receptor subtypes undergo the processes of desensitization and internalization (reviewed in refs. 61, 72, 73). Opioid receptor phosphorylation was first demonstrated for the δOR[74], followed by the μOR[75], and appeared to be mediated by GRKs[74,75] and not other protein kinases which have been demonstrated to phosphorylate GPCRs, such as protein kinase C. Work by Chavkin and colleagues demonstrated that phosphorylation of μORs and δORs proceeds at serine and/or threonine residues within the second intracellular loop and/or C-terminal tail[76,77]. Studies on the μOR suggest that phosphorylation,

predictably, is positively correlated to agonist efficacy[78]. However, Zhang and colleagues have demonstrated evidence that morphine, while efficacious in inducing intracellular signaling cascades, does not induce receptor phosphorylation in vitro unless GRKs are overexpressed[79], suggesting that the morphine-μOR complex is a poor substrate for GRK-dependent phosphorylation.

Following phosphorylation, μORs and δORs undergo ligand-induced internalization. This was initially demonstrated in clonal cell lines endogenously expressing δORs[80,81]; since then this phenomenon has been demonstrated for both μORs and δORs in transfected cell lines[75,82-86], in primary neuronal cultures[86-89], and in vivo[90-92]. Opioid receptor internalization appears to proceed via a β-arrestin and clathrin-dependent mechanism. This conclusion is based on observations demonstrating that internalized opioid receptors co-localize with the transferrin receptor[83,85] as well as the sensitivity of this phenomenon to disrupters of β-arrestin function and clathrin assembly[79,84,87,93].

Unlike the largely identical desensitization and internalization processes of μORs and δORs, their subsequent postendocytotic fates appear to be quite different. Following short-term agonist exposure, μORs are largely recycled back to the plasma membrane, as shown by Law and colleagues[94]. The recycling fate of μORs is also supported by studies demonstrating a lack of co-localization of μORs with the late endosome markers LAMP-1 and LAMP-2 as well as the absence of proteolysis of internalized μORs[95].

In contrast to the μOR, studies of the postendocytotic sorting of δORs indicate that these receptors are targeted to lysosomes. This was first demonstrated in early studies of the δOR, in which incubation of NG-108/15 cells with chloroquine, which disrupts

lysosomal function by elevating lysosomal pH, promoted the accumulation of radiolabeled enkephalin in lysosomes[81]. Chloroquine-induced inhibition of δOR degradation, and colocalization of internalized δORs with LAMP-2 after prolonged agonist stimulation, supported these early findings[83]. Using immunocytochemical approaches, von Zastrow and colleagues demonstrated δOR sorting to lysosomes[96,97], which appeared to be independent of ubiquitination[98]. Recently, a protein was discovered using yeast two-hybrid approach that interacts with the C-terminus of δORs. Known as GASP, for G protein coupled receptor-associated protein, this protein was shown to promote the targeting of endocytosed δORs to lysosomes[95]. Taken together, the presently available evidence suggests that unlike μORs, δORs do not appreciably recycle but are degraded following agonist exposure.

During periods of prolonged agonist exposure, it is well established that receptor internalization can lead to a decrease in total receptor density, a process known as downregulation. This phenomenon has been demonstrated to occur for both μORs and δORs in clonal and recombinant cell lines[83,85,113,114], and is thought to be an important cellular mechanism underlying opiate tolerance and dependence.

IIiv: Distribution of opioid receptors in the central nervous system

In the earliest reports of opiate receptor binding, it was immediately revealed that the distribution of opioid receptors in the CNS was not homogeneous. Indeed, Pert and colleagues showed using homogenized membrane fractions that the levels of opiate receptor binding were highest in preparations from the striatum, intermediate in the

cortex and midbrain, and low in the brainstem and cerebellum[11]. These studies were extended to human and monkey brain, where opiate receptor binding was highest in the amygdala, thalamus, hypothalamus, caudate and periaqueductal grey, and lowest in the brainstem and cerebellum[115]. However, these 'grind and bind' techniques were crude and did not permit fine elucidation of regional differences in opioid receptor distribution. Subsequent autoradiographic studies, following injection of intact animals with radiolabeled opiate ligands, were an improvement, and confirmed the previous regional distributions observed by membrane binding[116]. However, fine resolution was still lacking, notwithstanding other challenges of this method, including high diffusion of radioligand and high nonspecific binding.

Young and Kuhar refined the autoradiographic technique by developing a binding assay using thawed fresh-frozen tissue sections[117], which permitted more detailed anatomical distribution studies[118,119]. As the development of more subtype-selective ligands became available, the distribution maps for the three opioid receptor subtypes became more refined[120-122]. However, the molecular cloning of the three opioid receptor subtypes allowed for highly sensitive localization techniques, namely in situ hybridization and immunohistochemistry, to be employed in the regional distribution analysis of these receptors. Therefore, the distribution of opioid receptors has been extensively characterized using all of these techniques, and will be summarized below.

Taken together, the large body of data demonstrates distinct but overlapping distribution patterns for μOR, δOR and κORs in the rodent (mouse and rat) CNS[123-36]. High levels of μOR binding sites, mRNA and/or protein are found in the caudate putamen (striatum), nucleus accumbens (NAc), amygdala, thalamus, hypothalamus,

globus pallidus, ventral striatum, periaqueductal gray (PAG), colliculi, raphe nuclei, parabrachial nucleus, locus coeruleus, spinal cord and dorsal root ganglia (DRGs). Lower expression levels of μORs are found in the cerebral cortex, ventral tegmental area (VTA) and substantia nigra. High expression levels of δOR have been found in the cerebral cortex, caudate putamen, NAc, amygdala, thalamic nuclei, colliculi, pontine nuclei, spinal cord and DRGs, whereas low levels of δOR have been observed in the PAG, VTA, and the basal ganglia (substantia nigra, globus pallidus & ventral striatum). Finally, high κOR expression is found in the cerebral cortex, entorhinal cortex, caudate putamen, amygdala, NAc, basal ganglia, thalamus, hypothalamus, colliculi, VTA, locus coeruleus, raphe nuclei, spinal cord and DRGs. For a more detailed description, please refer to table 1.

IIv: Function of opioid receptors in the central nervous system

The viability of triple knockout OR mice[29] belies the crucial role of these receptors in a number of physiological processes in the central nervous system. In light of their widespread distribution, the physiological effects of opioid receptor activation are similarly diverse and include endogenous pain modulation, addiction, locomotion, autonomic function, and immunomodulation[137-141]. Given that the focus of this thesis is the expression and trafficking of δORs in CNS centers involved in pain modulation and reward, the following discussion will elaborate on the role of opioid receptors in these physiological processes.

I: Opioid receptors and pain modulation

It is perhaps counterintuitive that the ability to feel pain is one of the most adaptive endowments we could possibly ask for, as pain is a sensory experience that most do not enjoy. However, pain serves a fundamental physiological role by alerting us to avoid situations of imminent tissue injury, and when tissue damage has occurred, reminds us to leave our affected area alone to heal. To cope with this unpleasant experience, evolution has also conserved extensive circuitry in the CNS designed to modulate the perception of pain, the best understood of which is the endogenous opioid system.

Opioid receptors, by nature of their hyperpolarizing cellular effects, reduce neuronal excitability and inhibit neuronal firing. Following a noxious stimulus, a number of pro-inflammatory and pro-nociceptive compounds are released, both in the affected area and into the spinal cord. In the CNS, primary sensory afferent neurons release the pronociceptive peptides substance P (SP) and calcitonin gene-related peptide (CGRP) into the spinal cord dorsal horn, which in turn stimulate dorsal horn projection neurons to inform higher brain centers of the painful threat. In response, local interneurons in this region release enkephalins, which stimulate opioid receptors, particularly μORs, located pre- and post-synaptically on sensory afferent and projection neurons respectively. Activation of opioid receptors expressed on the terminals of sensory afferent neurons prevents the further release of these pronociceptive peptides by inhibiting the activation of voltage-gated calcium channels, while stimulation of postsynaptic opioid receptors located on target projection neurons leads to a dampening of neuronal transmission to higher brain centers. This 'two-pronged' approach is a well-established and effective

mechanism for the modulation of pain transmission at the level of the spinal cord[137,142,143].

Opioid receptors are also localized in many of the higher centers of the central nervous system (CNS) involved in pain perception and modulation. For example, neurons of the periaqueductal gray (PAG) are a critical origin for descending pain modulation. These neurons, by way of the rostroventral medulla and pontine reticular formation, modulate the excitability of spinal cord dorsal horn neurons, thereby affecting the ascending transmission of pain stimuli[137].

The link between the PAG and pain modulation was first demonstrated in a seminal study by Reynolds (1969), who reported that electrical stimulation in this region produced such profound analgesia in rats that surgery could be performed without the use of anesthetics[144]. The involvement of opiates in PAG-mediated pain inhibition was first demonstrated by Yaksh and Rudy[145], who showed that injection of morphine into the PAG produced a long-lasting analgesia that was blocked by naloxone. It is now believed that the role of the opioid system in the PAG is that of opioid-mediated inhibition of inhibitory GABAergic interneurons. These neurons, at steady state, exert a tonic inhibitory influence on the PAG projection neurons. Relief of this tonic inhibition potentiates the firing of these projection neurons, thereby augmenting analgesia[146].

The cerebral cortex is an important afferent target of pain processing. In primates, the primary somatosensory cortex is involved in processing the topography and intensity of painful stimuli, whereas neurons of the secondary somatosensory cortex have been reported to code temporal aspects of painful stimuli[143,147]. A third important locus of pain processing in the cortex is the anterior cingulate, which plays a

pivotal role in the attentional and emotional processing of pain[143,147]. In the rat, both the frontal and cingulate cortices express μOR[123-128] and δORs[126,129-134]. Therefore, these receptors are ideally poised to participate in pain processing in this region. However, the exact role of the opioid system in cortical pain processing is unknown.

Unfortunately, there are situations where the experience of pain is not useful, as when it is felt following stimuli which pose no threat of tissue damage, or where no tissue damage remains. Far from conferring survival advantages, these latter forms of pain tend to be chronic, debilitating and can significantly impact the quality of life of those afflicted. Chronic pain can take several forms, including post-herpetic neuralgia, diabetic neuropathy, cancer-induced neuropathy, rheumatoid arthritis, fibromyalgia, and low back pain[148].

The efficacy of opiate therapy for the treatment of chronic pain has been evaluated extensively, and several studies have demonstrated that μOR-selective opiates, the prototype of which is morphine, can be useful in treating many of these types of pain[149,150]. However, traditional opiate treatment is not without side effects, including constipation, nausea, sedation, and immunosuppression[150]. The most feared side effects of all are the development of tolerance and dependence with repeated or prolonged administration. The fear of tolerance and eventually addiction has resulted in the severely restricted administration of opiates in the clinical setting, to the detriment of many pain sufferers[149-151]. In contrast, studies where δOR agonists have been administered to patients with chronic pain demonstrate that δOR agonists can provide significant pain relief without many of the adverse effects of their μOR-selective counterparts[152,153]. Moreover, experimental studies corroborate these clinical

observations and have demonstrated that δOR agonists are effective in a number of chronic pain paradigms, including neuropathic[154-157] and inflammatory[156-159] pain. Therefore, it appears that the δOR is a potentially useful target for the treatment of chronic pain[160].

II: Opioid receptors and reward

Opioid receptors also play an important role in brain reward circuitry, the best described of which are the mesolimbic and mesocortical dopaminergic systems. In these circuits, dopamine-containing neurons originating in the VTA send axons to the nucleus accumbens, striatum and frontal cortex. The release of dopamine within these circuits enhances motivational states and positive reinforcement, a highly important evolutionary endowment considering the importance of motivational stimuli such as food and sex on survival. Opioid receptors, particularly μOR, promote mesolimbic/cortical activity by inhibition of inhibitory GABAergic neurons, thereby prolonging dopaminergic signaling. Under endogenous control, the opioid system plays an adaptive role in motivation and reward. However, the system becomes maladaptive upon prolonged exposure to exogenous opiates, due to overstimulation of these circuits and compensatory reactions by the brain which often leads to tolerance, dependence and eventually addiction. Opiate drugs that produce these effects include morphine, the prototypical prescription analgesic, and the illicit drug heroin. Finally, a common cause of death in opiate abuse is respiratory depression, owing to high μOR expression in the respiratory centers of the brainstem. Considering the adverse effects of exogenous opiates, there is great clinical concern for prescribing opiate drugs such as morphine even in severe pain conditions, underscoring the need for better targets.

III: Opioid receptors, pain and reward: Lessons from knockout mice

Molecular cloning of the opioid receptors has made it possible to study opioid receptor function using genetic approaches. Single and combinatorial mutant mouse strains lacking the genes for μOR, δOR and κOR have all been generated and extensively characterized, and not only have provided valuable insight into the role of these receptors in analgesia and reward, but highlight the complexity of this system in modulating these processes[138,164].

Studies in mice lacking the μOR gene unequivocally demonstrate that the μOR is the mandatory molecular target for morphine. As demonstrated by Matthes *et al*[161], acute subcutaneous administration of morphine had no analgesic effects in μOR-knockout (KO) mice as compared to wild-type controls. In contrast, morphine-induced analgesia was preserved in δOR-[162] and κOR-KO[163] mice, demonstrating that the presence of μOR is required to mediate the actions of morphine in nociceptive processing. In addition, the adverse effects of morphine treatment, including respiratory depression, tolerance, and withdrawal were also abolished in μOR-KO mice[161]. As the main biological actions of morphine, both therapeutic and beneficial, are clearly mediated through the μOR, it is clear from a therapeutic standpoint that any drugs selective for this receptor subtype are liable to produce the same undesired effects[138,164].

Studies of delta-mediated analgesia have been performed in both δOR and μOR KO mice. Ablation of the δOR gene (-/-) resulted in a significant decrease in the ED_{50} value for the δOR-selective agonist DPDPE when administered intrathecally. Though DPDPE-mediated analgesia was preserved in δOR-KO mice when given

intracerebroventricularly, this effect was abolished in experiments using wild type mice treated with δOR antisense oligonucleotides[163]. In addition, the preservation of supraspinal DPDPE analgesia in δOR-KO mice could be explained by the expression of alternate δOR binding sites, as low levels of ^{3}H-naltrindole binding were detected in these mice[163]. Nevertheless, this study clearly demonstrates that δORs are able to produce analgesia independently of μORs.

The effects of delta agonists in μOR-KO mice are equally interesting. Several studies have demonstrated that in the absence of μOR, deltorphin and DPDPE-mediated analgesia is attenuated[165,166,170], a finding which does not extend to mu and kappa agonists whose activities are maintained in the absence of δOR/κOR or δOR/μOR respectively (reviewed in ref. 138). In contrast, Hosohata *et al* found that in μOR-deficient mice, deltorphin analgesia is maintained while only DPDPE analgesia is attenuated[167], a finding supported by Sora and colleagues[168]; these results suggest that at least part of the analgesic effect of DPDPE is mediated by μOR activation. In contrast, deltorphin is the most highly selective δOR compound described to date, therefore, lack of selectivity cannot explain the reduction of deltorphin analgesia in μOR-KOs observed by Matthes and colleagues[165,170]. Taken together, these studies suggest the effects of mu and delta agonists are at least in part attributed to interactions between these two receptor subtypes.

III: Preceeding work of our laboratory

The work of the Beaudet lab has focused extensively on the localization and trafficking properties of δOR in the rodent central nervous system. Prior to Cahill *et al* (2001)[133], there were some disconcerting discrepancies in the literature with regard to the distribution of δORs in the CNS, particularly in the spinal cord. Hence, there was an extensive re-evaluation of the cellular and subcellular distribution of δORs in the rodent central nervous system, with an emphasis on the spinal cord, using 2 well characterized δOR antibodies[133]. Though the regional expression of δOR was largely consistent with that surmised by earlier autoradiographic and in situ hybridization studies, electron microscopic analysis of this receptor in the spinal cord revealed that at steady state, the δOR was primarily localized intracellularly and not at the neuronal plasma membrane[133]. Furthermore, unlike previous studies in which a significant proportion of intracellular δ ORs had been co-localized with large dense core vesicles (LDCVs)[171-173], the bulk of δ OR immunoreactivity observed by Cahill and colleagues was found associated with microtubules and/or small vesicles in the cytoplasm of labeled dendrites. Furthermore, only a small proportion of δORs was found to be associated with synaptic terminals, and within those terminals, was not localized to LDCVs[133].

This finding was confirmed and extended in later studies, when it was discovered that prolonged pretreatment of cultured cortical neurons with morphine (10μM, 48h) markedly enhanced the concentration of δORs at the cell surface[89]. This enhancement was observed as an increase in the density of plasma membrane-associated δORs as detected by electron microscopy, and was also seen by confocal microscopy as an increase in the internalization of a fluorescent δOR-selective peptide agonist, ω-bodipy-

deltorphin (fluoDLT)[89]. In vivo, prolonged treatment of rats with morphine over the same time period similarly resulted in an increase in plasma membrane-associated δORs in dendrites of the dorsal horn of the spinal cord, as measured by immunogold electron microscopy, as well as in an enhanced internalization of fluoDLT, as visualized by confocal microscopy[89,92]. Furthermore, this increased trafficking of δORs to the plasma membrane translated behaviourally into an augmentation of the antinociceptive potency of δOR-selective agonists[89]. Finally, the translocation of δORs induced by prolonged morphine treatment was shown to be dependent on stimulation through the μOR, as co-incubation of morphine with CTOP, a highly μOR-selective antagonist, abolished the increase in plasma membrane-bound δORs[89]. This result was confirmed by experiments with μOR-knockout mice, who did not demonstrate any increase in plasma membrane-bound δORs following the morphine treatment paradigm[174].

The translocation of δORs to neuronal plasma membranes in the spinal cord dorsal horn has also been demonstrated in our lab using a well-validated model of chronic inflammatory pain. Injection of complete Freund's adjuvant (CFA; 72 hrs. prior to experimentation), resulted in upregulation of δOR protein levels, recruitment of δOR from intracellular stores to the plasma membrane, and was correlated with an improved antinociceptive potency of the δOR-selective agonist deltorphin[175]. As observed in morphine-treated animals, this effect was abolished in μOR-KO mice, suggesting that the effects observed were due to stimulation of μOR by endogenous opioids in response to chronic inflammatory pain[176]. In recent work, Morinville and colleagues reported that the regulation of δOR trafficking by μOR activation is linked to somatosensory processing, following observations that the increase in plasma

membrane-bound δORs induced by prolonged morphine treatment was abolished in animals lacking primary afferent input[92].

Taken together, these results provide novel insight into the function of δORs in the CNS, as outlined in the following hypothesis. It appears that under basal conditions, there is minimal stimulation of δORs due to their predominantly intracellular location. However, these receptors may be 'called to action' to the plasma membrane by an appropriate cellular stimulus. In both paradigms studied, the induction of δOR trafficking towards neuronal plasma membranes was dependent on previous stimulation of μORs. Therefore, this phenomenon could represent a homeostatic mechanism to maintain sensitivity to opiate drugs in the absence (or preoccupation) of μORs. Alternatively, in situations of sustained release of endogenous opioid peptides, the desensitization and downregulation of μORs could also be compensated by the plasma membrane expression of δORs. A 'functional switch' in the bioavailable expression of opioid receptors suggests a second mechanism whereby sensitivity to opiates, in this case to endogenous opioids, can be preserved. In either scenario, δORs are surrogate transducers of opioid ligands; nevertheless this function may be crucial for the modulation of a number of physiological processes, including pain.

IV: Rationale of the present Master's thesis

The discovery of the unique trafficking properties of δORs has raised many important questions which we have attempted to address in the present thesis. Although these earlier studies had clearly uncovered a novel pharmacological cross talk between these two receptors, with important functional implications, the mechanism by which this phenomenon occurred remained unknown. Therefore, a primary task in the present thesis was to elucidate the mechanism of μOR-mediated δOR trafficking using an in vitro model. It was shown in our laboratory that this phenomenon could be observed in cultured cortical neurons[89]. However, these cultures, because of their heterogeneity, low neuronal cell number, and difficult preparation and maintenance, would have been a challenging model at best. Instead, we decided to use heterologous cell lines singly expressing δORs or dually expressing μORs and δORs, with the hypothesis that μOR-mediated δOR trafficking required the co-expression of both receptors in the same cell.

A second objective of this thesis was to determine whether cellular stimuli other than activation of μORs through prolonged morphine treatment could induce the translocation of δORs to neuronal plasma membranes. It has been shown recently by Bao and colleagues[177] that both selective and nonselective stimuli, including acute treatment with δOR-selective agonists and membrane depolarization by KCl, can induce the translocation of δORs to the plasma membrane of DRG cells. These results follow their previous finding that δORs are associated with LDCVs in these cells[173], and suggest that δORs are recruited to the plasma membrane by exocytosis through the regulated secretory pathway. However, this interpretation suggests that δORs might not be attractive targets for novel analgesic drugs, since stimuli that promote their

translocation to the cell surface would also cause the release of inflammatory neuropeptides such as substance P and CGRP. As studies from our laboratory have never observed the association of δORs with LDCVs in neurons of the spinal cord[133], we wished to reinvestigate this issue at the subcellular level in DRG cells.

In all previous studies of μOR-mediated δOR trafficking in vivo, only the dorsal horn of the rodent spinal cord was extensively studied at the subcellular level. However, given the wide expression of both μORs and δORs throughout the CNS, the question remained as to whether prolonged treatment with μOR agonists similarly induced an up-regulation of cell surface δORs in regions other than the spinal cord. To address this question, we performed δOR immunogold histochemistry followed by transmission electron microscopy on C57BL/6 mice treated or not with morphine for 48h. We analyzed the subcellular distribution and compartmentalization of δORs in three regions recognized as containing high concentrations of both μOR and δOR in the rodent brain[123-134]: the dorsal neostriatum, an area vital for the integration and regulation of motor and cognitive function, the nucleus accumbens, a limbic structure involved in the neural processing of addiction and reward, and the frontal cortex, involved in sensory processing. In carrying out these studies, we wished to provide further anatomical insight into the trafficking and expression properties of the δOR in the CNS. Furthermore, we were curious as to whether δORs are broadly poised to be 'called to action' to neuronal plasma membranes following previous μOR stimulation. If the case, this phenomenon could have implications for a number of physiological processes, such as pain modulation and addiction, in which opioid receptors are known to play a role. By understanding how to manipulate the expression of this potentially useful therapeutic

target, we may thereby improve strategies to manage these diseases in the clinical setting.

Table 1: Distribution of mu, delta and kappa opioid receptors in the rodent CNS[123-36].

Mu (μ)[123-28]	Delta (δ)[126,129-134]	Kappa (κ)[126,135,136]
Telencephalon		
Olfactory bulb	Olfactory bulb	
	Olfactory tubercle	Olfactory tubercle
	Claustrum	Claustrum
Cerebral cortex	Cerebral cortex	Cerebral cortex
		Entorhinal cortex
Hippocampus (Dorsal hippocampus and dentate gyrus, low)	Hippocampus (Dorsal hippocampus and dentate gyrus, high)	Hippocampus (not dorsal hippocampus nor dentate gyrus)
Caudate putamen (Striatal patches)	Caudate putamen (Homogeneous)	Caudate putamen
Nucleus Accumbens	Nucleus Accumbens	Nucleus Accumbens
Amygdala	Amygdala	Amygdala
Bed nucleus stria terminalis	Bed nucleus stria terminalis	Bed nucleus stria terminalis
Globus pallidus	Globus pallidus	Globus pallidus
Ventral striatum	Ventral striatum	Ventral striatum
Diencephalon		
Most thalamic nuclei	Thalamus	Medial thalamic nuclei
Hypothalamus	Ventromedial hypothalamus	Most hypothalamic nuclei
		Infundibulum
		Median eminence
Mesencephalon		
Superior & inferior colliculus	Superior & inferior colliculus	Superior & inferior colliculus
Central gray (high)	Central gray (low)	Central gray (moderate)
Substantia nigra (low)	Substantia nigra (low)	Substantia nigra (high)
VTA (low)	VTA (low)	VTA (high)
		Endopiriform nucleus
Oculomotor nucleus	Oculomotor nucleus	
	Red nucleus	
Met- and Myelencephalon		
	Pontine nuclei (v. high)	
	Reticulotegmental nucleus	
Medial Raphe nuclei		Raphe nuclei
Parabrachial nucleus		Parabrachial nucleus
Locus coeruleus		Locus coeruleus
Nucleus gracilis & cuneatus	Motor, spinal trigeminal nucleus	Paratrigeminal, spinal trigeminal
Dorsal motor nucleus of the vagus	Brainstem nuclei: Linear nucleus of	Nucleus solitary tract
Nucleus solitary tract	medulla, Lateral reticular nucleus,	
Nucleus ambiguus	hypoglossal nucleus	
	Cerebellum	
Spinal Cord & Peripheral Nervous System		
Superficial spinal cord	Superficial spinal cord	Superficial spinal cord
Dorsal root ganglia	Dorsal root ganglia	Dorsal root ganglia

Materials and Methods

Part A: *In vitro* Studies

I) GH3 Cells

Ii- Culture

GH3 cells were grown at 37°C in a humidified chamber (5% CO_2/ 95% O_2). Wild-type GH3 cells (GH3-WT) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 1% penicillin/streptomycin and 10% FBS (complete DMEM). GH3-DOR and GH3-MOR/DOR stably transfected cell lines were established previously as described[178,179]. GH3-DOR cells were grown in the above described medium supplemented with 250μg/mL hygromycin, while GH3-MOR/DOR co-transfected cells were maintained in complete DMEM containing 250μg/mL hygromycin plus 1mg/mL neomycin (G418).

For fluorescent ligand binding studies, cells were plated in complete DMEM at approximately 60% confluency on sterile 4-well plates containing poly-L-lysine-coated glass coverslips. Cells were plated at least 8 hours prior to the initiation of treatments or binding to ensure adherence to the coverslips. For radioligand binding studies, cells were plated at approximately 60% confluency on poly-L-lysine-coated 24-well plates in complete DMEM at least 8 hours prior to treatment or binding.

Iii- FluoDLT and fluoDRM binding studies

To determine the effects of prolonged morphine treatment on the density of cell-surface DORs, GH3-WT, GH3-DOR and GH3-MOR/DOR cells were first treated with

morphine sulfate (Sabex) for 48 hours. Following treatment, we performed ligand binding studies using the fluorescent ligands ω-bodipy-fluo-deltorphin 576/589 (fluoDLT) and ω-bodipy-fluo-dermorphin 576/589 (fluoDRM). Deltorphin and dermorphin, respectively, are highly DOR- and MOR-selective peptides first isolated from amphibian skin[44,45]. The specificity and selectivity of their fluorescently-labeled conjugates have been described previously[84].

For experimentation, growth medium was removed and cells were pre-incubated for 10min at 37°C in warmed Earle's buffer (140mM NaCl, 5mM KCl, 1.8mM $CaCl_2$, 0.9mM $MgCl_2$, and 25mM HEPES, pH 7.4) supplemented with 0.8mM phenathroline, 0.09% D-glucose (Gibco), and 0.2% bovine serum albumin (supplemented Earle's buffer). Following pre-incubation, cells were then treated with either fluoDLT or fluoDRM at a concentration of 10nM in warmed supplemented Earle's buffer and incubated at 37°C for 30min. To ensure binding specificity, some wells were co-incubated with 10μM naloxone (Sigma), a nonspecific opioid antagonist, or 10μM CTOP (Bachem), a MOR-selective antagonist. Following this incubation, cells were washed in a hypertonic acid solution (2.92g NaCl/100mL Earle's buffer, adjusted to a pH of 4.0 using glacial acetic acid) for 2 minutes. This step ensures that any remaining surface-bound ligand is removed, revealing only fluorescent ligand that has been internalized. Following this acid wash, cells were washed in cold Earle's buffer, fixed in 4% paraformaldehyde (PFA; in 0.1M phosphate buffer, pH 7.4), and mounted on glass slides using Aquamount. Slides were stored in the dark at 4°C until ready for imaging.

Imaging was performed within 48h following experimentation using a Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss Canada Ltd., Toronto ON)

equipped with a Leica Diaplan inverted microscope, Argon and Helium/Neon lasers (excitation wavelengths of 488nm and 543nm, respectively). This microscope was used for all subsequent confocal imaging studies. For fluoDLT binding studies, only the Helium/Neon laser was used. Confocal images were acquired through the nuclear plane, and processed using Zeiss LSM image browser version 5 and Photoshop version 6.0 (Adobe Systems Inc., San Jose, CA).

iii- Radioligand binding studies

To quantitatively assess the density of cell-surface DORs, whole-cell radioligand binding was performed on GH3 cells treated or not with morphine, etorphine hydrochloride (Wildlife Pharmaceuticals), or methadone hydrochloride (Royal Victoria Hospital, Montreal, QC) for 48h. For experimentation, cells were washed once with warmed Earle's supplemented buffer and incubated with radiolabeled deltorphin (^{3}H-DLT; specific activity 38.5 Ci/mmol, Perkin-Elmer) in supplemented Earle's buffer for 30min at 37°C. Following incubation with radioligand, cells were washed twice with Earle's buffer and lysed with 1mL/well of 0.1M NaOH for 10min at room temperature. The total contents of each well were then pipetted into individual scintillation vials and counted for 60 seconds/vial using a Wallac 1409 liquid scintillation counter. Graphs and statistical analyses were performed using GraphPad Prism software (San Diego, CA).

II) Dorsal Root Ganglia

IIi- Dissection and culture

All experiments using animals in this thesis have been approved by the animal care committee at McGill University in compliance with the policies of the Canadian Council on Animal Care. Under halothane anesthesia, adult male Sprague-Dawley rats were sacrificed by decapitation. The spinal canal was isolated and placed in warmed sterile HBSS (131mM NaCl, 5.4mM KCl, 0.4mM KH_2PO_4, 4.2mM $NaHCO_3$, 0.3mM Na_2HPO_4, 1mM HEPES, 1% w/v D-glucose, pH 7.4) and transported to a laminar flow hood for dissection. Under sterile conditions, DRGs were isolated following laminectomy and placed in DRG medium (Ham's F-12 medium supplemented with 3mM L-glutamine, 1% penicillin/streptomycin, 40mM D-glucose and 0.1% fungizone; all Gibco). The DRGs were then transferred to a Falcon® tube containing filter-sterilized Collagenase A (0.3% w/v; Roche) in DRG medium, and incubated for 90min at 37°C with moderate shaking. Following collagenase incubation, the DRGs were centrifuged, resuspended in trypsin (0.25% in HBSS no Ca^{++}/Mg^{++}; Gibco), and vigorously triturated with flame-polished Pasteur pipettes of decreasing bore diameter. Once dissociated, the DRG cells were passed through a 70μm cell strainer, centrifuged, resuspended in DRG medium containing 10% FBS and 40ng/mL NGF, and plated on a poly-L-lysine and laminin-coated substrate. DRG cells were allowed to differentiate in a humidified atmosphere with routine changing of medium every 2-3 days (50% conditioned medium, 50% fresh medium with FBS, NGF).

IIii- Immunocytochemistry

To verify the expression of δOR in DRG neurons, we performed immunofluorescence cytochemistry on DRG neurons in culture. DRG cells were plated on poly-L-lysine and laminin-treated glass coverslips at a density of 1 DRG/well and allowed to differentiate for 7 days as described above. For experimentation, cells were rinsed twice in 0.1M phosphate buffer pH 7.4 (PB), fixed in 4% PFA in PB for 20min at room temperature, and pre-incubated in 0.1M tris-buffered saline, pH 7.4, (TBS) containing 5% normal goat serum (NGS; Jackson Immunoresearch Laboratories) and 0.1% Triton X-100 for 15min at room temperature. Following pre-incubation, DRG cells were incubated in primary antibodies, diluted in TBS containing 0.5% NGS, overnight at 4°C with gentle shaking. To positively identify neurons, we used a mouse monoclonal anti-microtubule-associated protein 2 antibody (MAP-2, Chemicon). MAP-2 is a protein selectively expressed in the dendrites of differentiated neurons[180,181] and is therefore well-suited as a neuronal marker. For DOR immunolabeling, we used a polyclonal rabbit antiserum which recognizes amino acid residues 3-17 of the mouse DOR (Chemicon, catalog number AB1560). This antibody has been extensively characterized in our laboratory and was used for all DOR immunolabeling experiments in this thesis. Control conditions consisted of treatment of cells in the absence of primary antibody.

Following overnight incubation with primary antibodies, the cells were rinsed three times with TBS and treated for secondary immunolabeling. For MAP2, we used a goat anti-mouse IgG conjugated to Alexa 488 (Molecular Probes), while for secondary immunolabeling of DOR we used goat anti-rabbit IgG coupled to Texas Red (Jackson Immunoresearch Laboratories). Both were used at a concentration of 1:800 in TBS

containing 0.5% NGS for 40min at room temperature with gentle shaking. Cells were then rinsed three times with TBS, mounted on glass slides with aquamount, and stored at 4°C until confocal imaging. Dual fluorescence imaging was performed using the Argon and Helium/Neon lasers.

IIiii- FluoDLT binding studies

The protocol for fluoDLT binding on cultured DRG cells is identical to that described for GH3 cells above, with the exception that cells were incubated in fluoDLT at a concentration of 15nM.

IIiv- δOR Immunogold EM

DRG cells were plated on poly-L-lysine and laminin-treated 4-well plates and cultured as described above. To determine the effect of morphine treatment on the density of δORs at the plasma membrane, a subset of wells at 7-8 days in vitro (DIV) received a single dose of morphine (10μM final concentration) and were incubated in a humidified atmosphere for 48h. To ascertain whether nonspecific membrane depolarization could also increase the density of δORs at the plasma membrane, DRG cells at 8-9 DIV were incubated in 40mM KCl diluted in warmed unsupplemented Earle's buffer for 30sec or 5 min.

All treatments were followed immediately by fixation first in 2% acrolein in 2% PFA, then 2% PFA alone for 20min each. Cells were then rinsed with TBS, blocked in

TBS containing 3% NGS and 0.02% Triton-X, and incubated with our primary δOR antiserum, at a dilution of 1:4000 in TBS containing 0.5% NGS, for 40-48hr at 4°C with gentle shaking. Specificity controls were performed on a subset of wells in TBS-0.5% NGS in the absence of primary antibody.

Following primary antibody incubation, cells were rinsed first in 0.01M PBS, pH 7.4 (PBS; 3X5min) followed by washing incubation buffer (PBS containing 2% gelatin and 8% BSA; 1X5 min). The DRG cells were then incubated with a 1:50 dilution of colloidal gold-conjugated goat anti-rabbit IgG (1nm, BBI International or 0.6nm, Aurion) in washing incubation buffer for 2h at room temperature. After thorough washing in PBS, cells were fixed with 2% glutaraldehyde in PBS for 10min followed by rinsing with PBS and 0.2M citrate buffer. Immunogold grains were then intensified by ionic silver (IntenSE M silver enhancement kit, Amersham Biosciences), rinsed again with citrate buffer, postfixed in 2% OsO_4 in PB for 10min, and dehydrated in graded alcohols. Following dehydration, the wells were embedded in Epon, ultrathin-sectioned (80nm thick), counterstained with uranyl acetate and lead citrate, and examined using a JEOL 100CX or a Phillips 410 transmission electron microscope.

IIv- Data Analysis (δOR Immunogold EM)

DRG neurons were photographed from two to three grids from at least 1 well per condition per experiment. Only neurons containing 50 gold particles or more were included for analysis. For immunogold particle quantification, DRG cells were pooled from 2 (10μM morphine) and 1 (40mM KCl) independent experiments. Gold particles were considered membrane-associated if directly contacting or overlaying the plasma

membrane. Using computer-assisted morphometry (NeuroLucida and NeuroExplorer software; MicroBrightField Inc, Williston, VT), we first analyzed the density of immunogold particles per unit length of membrane (grains/μm). We then classified all immunolabeled grains into two bins (0-99nm and 100nm-) according to the segmental distance from their nearest plasma membrane. Statistical analyses were performed using GraphPad Prism software.

Part B: Animal Studies

I) Immunogold EM on wild-type C57BL6 Mice

Animals and Treatments

Male and female wild-type C57BL6 mice (6-11 months of age; Strasbourg, France) were housed in groups of 1 to 5 per cage and maintained on a 12hr light/dark cycle with experiments being performed during the light phase (10am-2pm). All experiments have been approved by the animal care committee at McGill University in compliance with the policies of the Canadian Council on Animal Care.

To determine the effect of prolonged morphine treatment on the subcellular distribution of δORs, a subset of mice were treated with morphine sulfate (Abbott Laboratories, Toronto, Canada) at 12hr intervals (5, 8, 10, 15mg/kg, subcutaneous) and processed for δOR immunogold histochemistry 12hr following the last morphine injection, for a total treatment duration of 48h. Non-treated and morphine-treated mice were perfusion-fixed under pentobarbital anaesthesia (2.9mg/mouse), first with 35mL of a mixture of 3.75% acrolein and 2% paraformaldehyde (PFA) in 0.1M phosphate buffer, pH 7.4 (PB), followed by 250mL of 2% PFA in PB. The brains were then post-fixed in 2% PFA in PB for 30min, and coronal sections (40-50μm in thickness) containing the nucleus accumbens, dorsal neostriatum and frontal cortex were collected in ice-cold PB using a vibratome.

Sections were first incubated in 1% sodium borohydride in PB followed by several rinses in PB, and then cryoprotected for 30min in a solution of 25% sucrose/3% glycerol in PB before snap-freezing with isopentane (-70°C), liquid nitrogen and thawing in PB. Sections were rinsed in 0.1M tris-buffered saline, pH 7.4 (TBS; 2X10min),

blocked in TBS containing 3% normal goat serum (NGS) for 30min, and incubated for 36-48h at 4°C with our primary δOR antiserum at a concentration of 1:4000 in TBS containing 0.5% NGS. As specificity controls, sections were processed in the absence of primary antibody.

Sections were subsequently rinsed in phosphate-buffered saline (PBS; 3X10min) followed by a rinse in PBS containing 2% gelatin and 8% BSA for 10min. Sections were then incubated for 2h at room temperature with 1nm colloidal gold-conjugated goat anti-rabbit IgG (1nm, BBI International) at a dilution of 1:50 in the same buffer. After thorough rinsing, sections were fixed with 2% glutaraldehyde in PBS for 10min and rinsed twice with 0.2M citrate buffer, pH 7.4. Immunogold particles were then intensified by incubation with ionic silver (IntenSEM silver enhancement kit, Amersham Biosciences), rinsed again with citrate buffer, postfixed in 4% OsO_4 in PB for 40min, and dehydrated in graded alcohols. Following dehydration, sections were embedded in Epon, ultrathin-sectioned (80nm thick), counterstained with uranyl acetate and lead citrate, and examined using a JEOL 100CX or a Phillips 410 transmission electron microscope. Negatives were scanned using an AGFA Duoscan T1200 Scanner, and the images processed using Photoshop version 6.0 software.

Data Analysis

As immunoreactive δORs were found to be predominantly associated with dendrites in all three regions (i.e., the frontal cortex, dorsal neostriatum, and nucleus accumbens), we focused our analysis on the association of gold particles with these

neuronal elements. Dendrites were identified by the following structural features: cylindrical shape, electron-light cytoplasm, presence of microtubules and rough endoplasmic reticulum and of post-synaptic connections with axon terminals[182]. Fifteen labeled dendritic profiles were photographed in two to three grids from each brain region per animal, for a total of 45 dendrites per region per condition. Profiles were considered immunolabeled if they contained 3 silver-gold particles or more.

First, we measured labeling density by counting the total number of gold particles per surface area of labeled profile (number particles/μm^2). Second, we analyzed the segmental distance of each immunogold particle from the nearest plasma membrane, using computer-assisted morphometry (NeuroLucida and NeuroExplorer software). Dendrite-associated immunogold particles were then classified into 100nm bins according to their distance from the plasma membrane. Graphs and statistical analyses were performed using GraphPad Prism software.

Results

Part 1- Studies with GH3 Cells

A primary task in this thesis project was to develop a model system to study μOR-mediated δOR trafficking. We used GH3 cells, a rat pituitary-derived cell line heterologously expressing δORs or co-expressing μORs and δORs. Both of these cell lines were established previously and have been used to study μOR-δOR interactions; specifically, the effect of single or co-expression of these receptors on ion channel activity[178,179].

To characterize the expression of μOR and δOR in these cell lines, we used our ligand internalization assay. In this assay, cells are incubated with fluorescent ligand then washed with a hypertonic acid solution to remove any remaining surface-bound ligand, thereby enabling the selective visualization of internalized ligand molecules. The ligands used for these experiments are both peptides derived from amphibian skin: deltorphin[45] (DLT), selective for δOR, and dermorphin[44] (DRM), selective for μOR. Fluorescent conjugates of these ligands using the red fluorophore ω-bodipy 576/589 were prepared by reverse-phase HPLC as described[84].

By confocal imaging, we observed that following binding of fluorescent DLT (fluoDLT), there is a punctate intracellular distribution of fluorescence indicative of internalized ligand-receptor complexes. This punctate intracellular labeling was observed in both GH3-δOR- (Fig. 1A) and GH3-μOR/δOR- (Fig. 1C) expressing cells but not in wild-type GH3 cells (data not shown). Furthermore, this labeling was abolished in δOR-expressing cells co-incubated with naloxone, a nonselective opioid receptor antagonist, confirming the specificity of this ligand for δOR (Fig 1B, D). To

ensure that the fluoDLT labeling in dually transfected GH3-μOR/δOR cells was not due to nonspecific binding to μOR, we co-incubated a subset of wells with CTOP, a highly selective μOR antagonist. As shown in Figure 1E, we observed no change in the intensity of fluorescence, suggesting that fluoDLT was binding selectively to δOR in the co-transfected cells. Finally, to verify the expression of μOR in GH3-μOR/δOR cells, we performed fluorescent ligand binding using fluorescent dermorphin (fluoDRM) and observed punctate intracellular labeling that was abolished by co-incubation of fluoDRM with naloxone (Figs. 1F, G respectively).

We next wished to assess whether prolonged morphine treatment could increase the cell surface density of δORs in these cells. Using our internalization assay, we observed no increase in the internalization of fluoDLT following treatment of cells with 10μM morphine for 48h, as was observed previously in primary cortical neurons in culture[89]. In fact, by visual inspection it appeared that prolonged morphine treatment caused a decrease in fluoDLT internalization in both GH3-δOR and GH3-μOR/δOR cells, suggesting that morphine decreased the cell surface density of δORs in both cell lines (Fig 2A). To confirm this result quantitatively, we performed whole-cell radioligand binding using tritiated deltorphin (^{3}H-DLT). As shown in figure 2B, prolonged treatment with morphine led to a decrease in the specific binding of ^{3}H-DLT to both GH3-δOR and GH3-μOR/δOR cells by approximately 25% (** $p<0.01$, unpaired t-test). These experiments confirmed that morphine treatment significantly decreased the cell-surface density of δORs in both GH3-δOR and GH3-μOR/δOR cell lines.

To determine whether other μOR-selective agonists could similarly decrease the cell surface density of δORs, we performed whole-cell radioligand binding following

prolonged treatment with etorphine and methadone in addition to morphine. All treatments followed the same dose and time course as in morphine treated cells (10μM, 48h). We observed that etorphine and methadone were even more efficacious than morphine at decreasing the cell surface binding of δORs (rank order efficacy etorphine>>>methadone>morphine). Furthermore, this rank order and decrease of binding was identical in both the singly transfected GH3-δOR cells (Fig 3A) and the dually transfected GH3-μOR/δOR cells (Fig. 3B).

The dose of morphine and other μOR agonists for 48h treatment was 10μM, the same used for primary neurons in culture where an increase in plasma membrane-bound δORs was observed[89]. However, it is well known that cells heterologously expressing receptors contain a much higher density of receptors than does primary tissue and that these receptors tend to be well-expressed at the plasma membrane at steady state. Furthermore, given the restricted in vitro selectivity of morphine for μOR[138], it was conceivable that the dose of morphine used (10μM) could be directly activating δORs. It was therefore possible that cellular mechanisms which target δOR to the plasma membrane via μOR stimulation were being superseded or masked by direct activation and subsequent down-regulation of pre-existing plasma membrane-associated δORs. To test this hypothesis, we performed radioligand binding on GH3-δOR and GH3-μOR/δOR cells that had been treated for 48h with escalating doses of morphine (1nM to 10μM). As shown in figure 4, we observed that prolonged morphine treatment induced a dose-dependent decrease in ^{3}H-DLT binding in both GH3-δOR and GH3-μOR/δOR cell lines, with the lowest doses having no significant effect on ^{3}H-DLT binding. Taken together, these results suggest that pretreatment with μOR agonists do

not enhance the cell-surface expression of δORs in these cells. Furthermore, the fact that the effects observed were identical in both GH3-δOR and GH3-μOR/δOR cell lines suggests that the changes in cell-surface density of δORs directly result from δOR stimulation and are independent of μOR stimulation.

Part 2- Studies on Dorsal Root Ganglion Cells

To further study the trafficking of δORs at the cellular level, we developed a protocol for the culture of dorsal root ganglion (DRG) cells. DRGs are primary sensory neurons that convey all somatosensory information from the limbs and trunk to the central nervous system. Morphologically, they are pseudounipolar in shape, with a large ganglion cell body and one long afferent fibre tract, which projects from the periphery to the ganglion cell body (peripheral afferent) and then from the ganglion cell body to the spinal cord (central afferent). When dissociated and cultured, DRG cells retain not only their morphological characteristics, but also their endogenous expression of both μ- and δORs[183].

In MAP-2/δOR co-immunofluorescence experiments, we detected δOR immunoreactivity throughout the soma and afferent processes in a significant proportion of MAP-2 immunolabeled cells (Figure 5), confirming that cultured DRG cells express detectable levels of δORs. We next attempted fluoDLT internalization experiments on cultured DRG cells, but we observed that in the absence of any treatment the DRG cells exhibited a high level of autofluorescence which obfuscated the ability to detect specific fluoDLT internalization (Figure 6). Adjusting imaging parameters or experimental conditions could not reduce this autofluorescence in these cultures, and we thereby aborted using fluoDLT internalization to study δOR trafficking in favour of δOR immunogold cytochemistry.

By electron microscopy, we observed that a significant proportion of DRG cells was δOR-immunopositive (defined as >50 immunogold particles/DRG cell), consistent with our observations from the co-immunofluorescence experiments. At steady state,

the majority of immunogold-labeled δORs were intracellular, found either loosely distributed throughout the cytoplasm or associated with small clear intracellular vesicles (Figures 7A (arrowhead), 7B). This intracellular pool represented 97.4% of the total pool of immunolabeled δORs expressed in DRG cells (data not shown), with approximately 9% localized to cytoplasmic regions within 100nm of the plasma membrane, defined as the sub-plasmalemmal cytoplasmic compartment (Figure 8B). Large dense core vesicles (LDCVs) were distributed sparsely within the DRG cells independent of treatment condition. However, when observed, the vast majority of LDCVs were unlabeled (Figure 7A).

Following prolonged morphine treatment (10μM, 48h), we observed an increase in the density of immunogold particles at the plasma membrane of DRG cells (Figure 7C). Quantitative analysis revealed that this increase in immunolabeled δORs associated with the plasma membrane was highly significant (figure 8A; ** $p<0.01$, unpaired t-test). Within the cytoplasm, there was also a significant increase in the density of grains found within the sub-plasmalemmal cytoplasmic compartment (8.8% vs. 14.5%; * $p<0.05$, unpaired t-test), suggesting that morphine treatment also induced the trafficking of δORs towards the plasma membrane of DRG cells (figure 8B).

To assess whether nonspecific membrane depolarization induces the trafficking of δORs towards the plasma membrane, we performed experiments in which DRG cells were depolarized by acute incubation (30 sec and 5min) with 40mM KCl. Following KCl treatment, preliminary results (n=1) showed no increase in the density of immunolabeled δORs per unit length of membrane in either treatment condition (Figure 9C). In addition, acute KCl treatment did not induce any significant increase in

proportion of gold-silver particles within the sub-plasmalemmal cytoplasmic compartment (Figure 9D). There was, however, a unique observation in the KCl-treated DRG cells. Unlike untreated or morphine-treated DRG cells, the vast majority of which displayed smooth plasma membranes and large, electron-sparse nuclei, treatment with 40mM KCl appeared to induce morphological changes in DRG cells, including membrane blebbing (Figures 9A,B; arrows) and nuclear condensation (Figure 9B).

Part 3- Studies with Wild-type C57BL6 Mice

We used electron microscopy to determine the subcellular distribution of immunolabeled δORs in non-treated and morphine-treated mice in three brain regions, namely the nucleus accumbens, dorsal neostriatum and frontal cortex, each of which is known to express both μOR and δORs[126,132]. In all three regions, and in both groups of animals, the bulk of δOR immunoreactivity was localized to neuronal somata and dendrites, with low to moderate labeling of synaptic terminals (data not shown). Within these somatodendritic profiles, and again irrespective of the region or presence or absence of morphine treatment, the vast majority of immunolabeled δORs was intracellular (Figure 10A-F).

In control conditions, the overall density of δOR immunolabeling over sampled dendritic profiles was significantly lower in the nucleus accumbens as compared to the neostriatum and frontal cortex ($p<0.05$, one-way ANOVA with Tukey's post-hoc test; Fig. 11). By contrast, there was no statistically significant difference between dendritic labeling densities in frontal cortex and dorsal neostriatum (Fig. 11). Following morphine treatment, there was a significant increase in the density of δOR immunolabeling in the neostriatum (7.9±0.1 vs. 5.6±0.5, $p<0.05$, Mann-Whitney U test), but neither in frontal cortex nor in nucleus accumbens, as compared to controls. By way of consequence, the density of δOR immunolabeling in dendrites of the neostriatum was significantly higher than in both the nucleus accumbens (4.8±0.5; $p<0.01$, one-way ANOVA with Tukey's post-hoc test) and the frontal cortex (5.0±0.4; $p<0.01$, one-way ANOVA with Tukey's post-hoc test) (Fig. 11).

Quantitative analysis of immunogold particle proximity to the plasma membrane revealed that sustained exposure to morphine significantly increased the proportion of silver gold particles located directly over or immediately subjacent to the membrane in both the nucleus accumbens (18.5±2.2% vs. 36.5±4.5%; $p<0.05$, unpaired t-test) and dorsal neostriatum (18.1±1.7% vs. 35.7±1.7%, $p<0.01$) (Fig. 10A-B, D-E, G-H). This increase was accompanied by an overall diminution of the distance of intracellular particles from the plasma membrane and a corresponding decrease in the proportion of immunolabeled δORs in the dendritic core (Fig 10G-H). By contrast, we observed no redistribution of δORs following morphine treatment in the frontal cortex (Figure 10C, F, I).

Figure 1

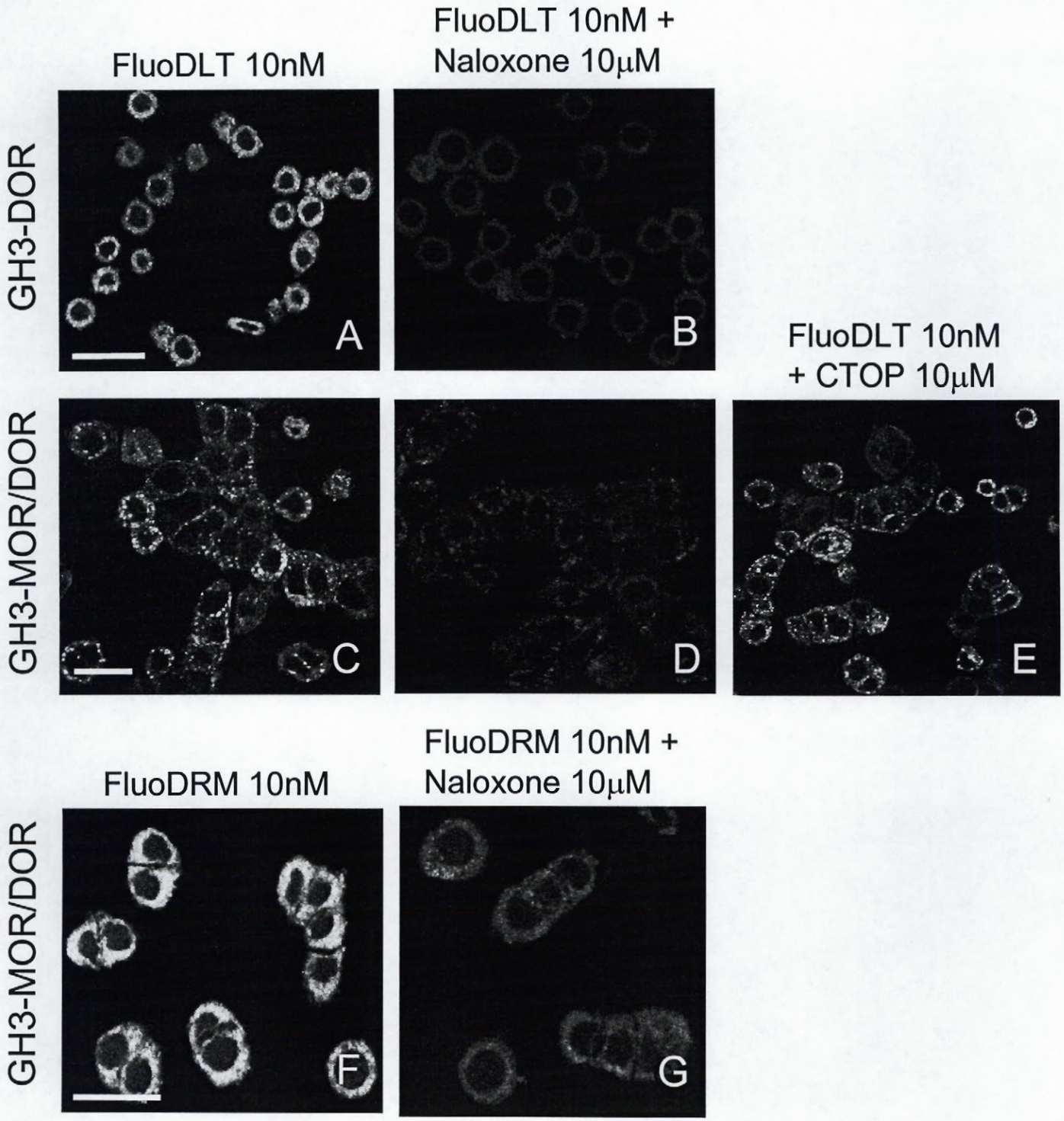
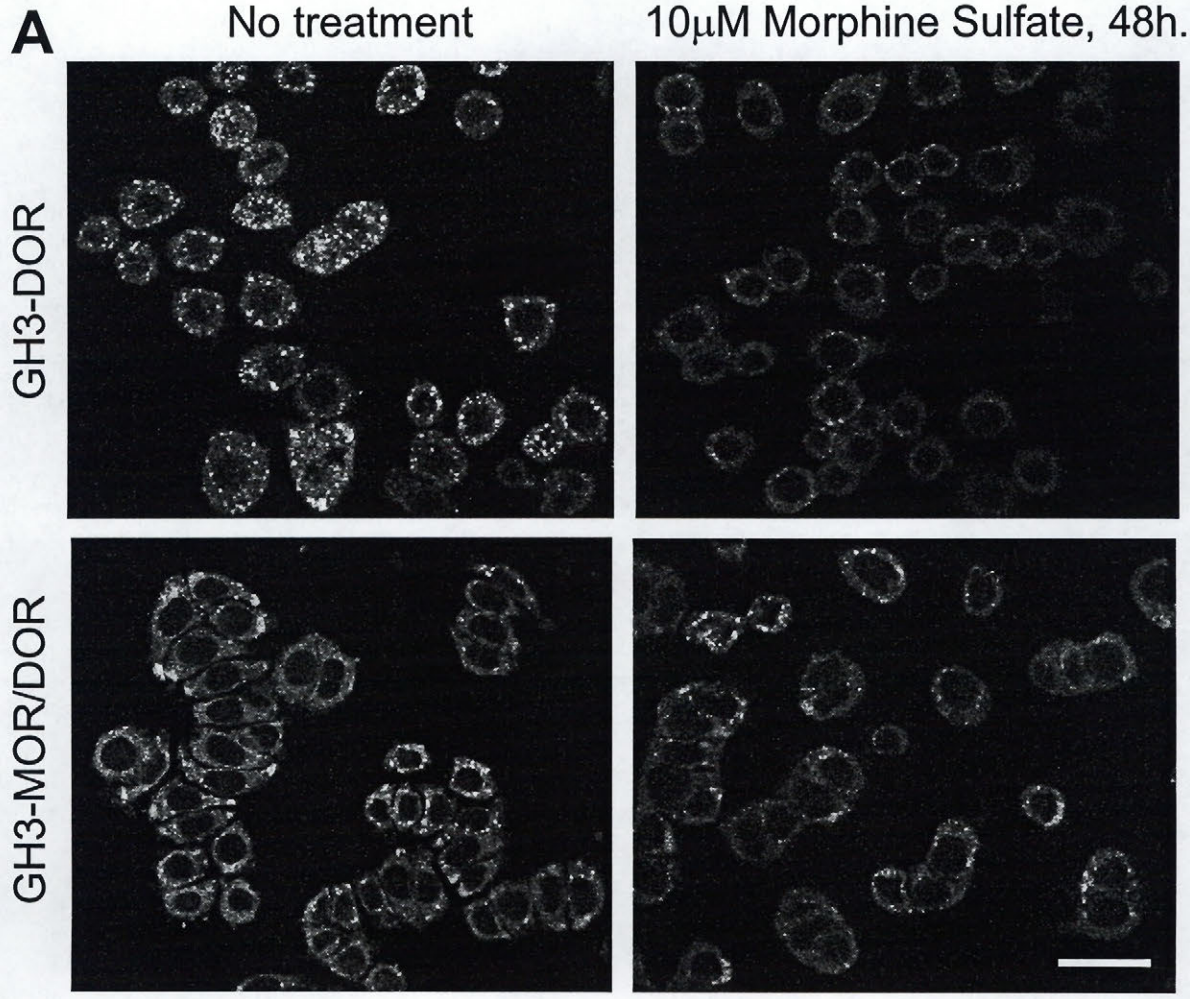

FluoDLT 10nM
FluoDLT 10nM +
Naloxone 10μM
GH3-DOR
A
B
FluoDLT 10nM
+ CTOP 10μM
GH3-MOR/DOR
C
D
E
FluoDRM 10nM
FluoDRM 10nM +
Naloxone 10μM
GH3-MOR/DOR
F
G

Figure 2


A
No treatment
10μM Morphine Sulfate, 48h.
GH3-DOR
GH3-MOR/DOR

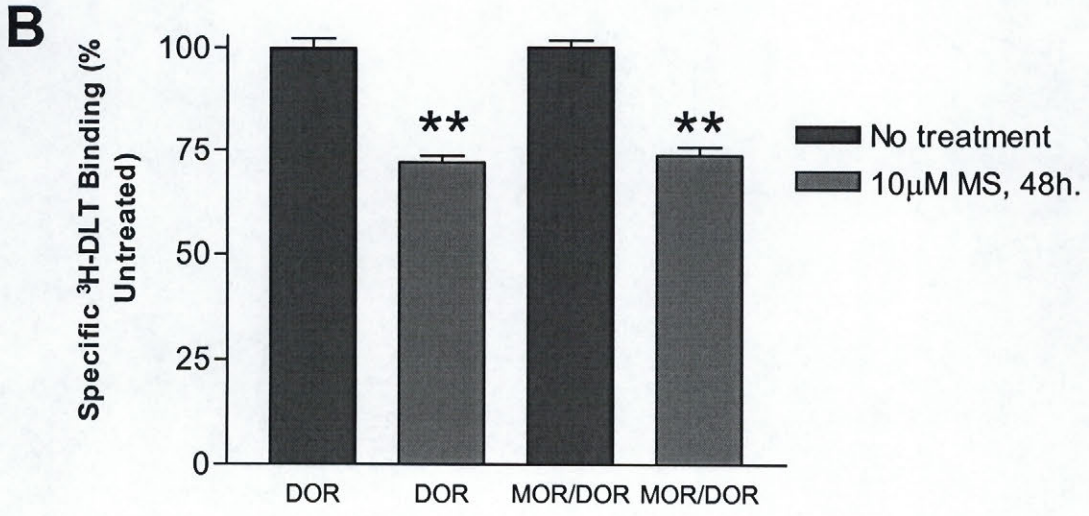

B
Specific ³H-DLT Binding (% Untreated)
100
75
50
25
0
**
**
DOR
DOR
MOR/DOR
MOR/DOR
No treatment
10μM MS, 48h.

Figure 3

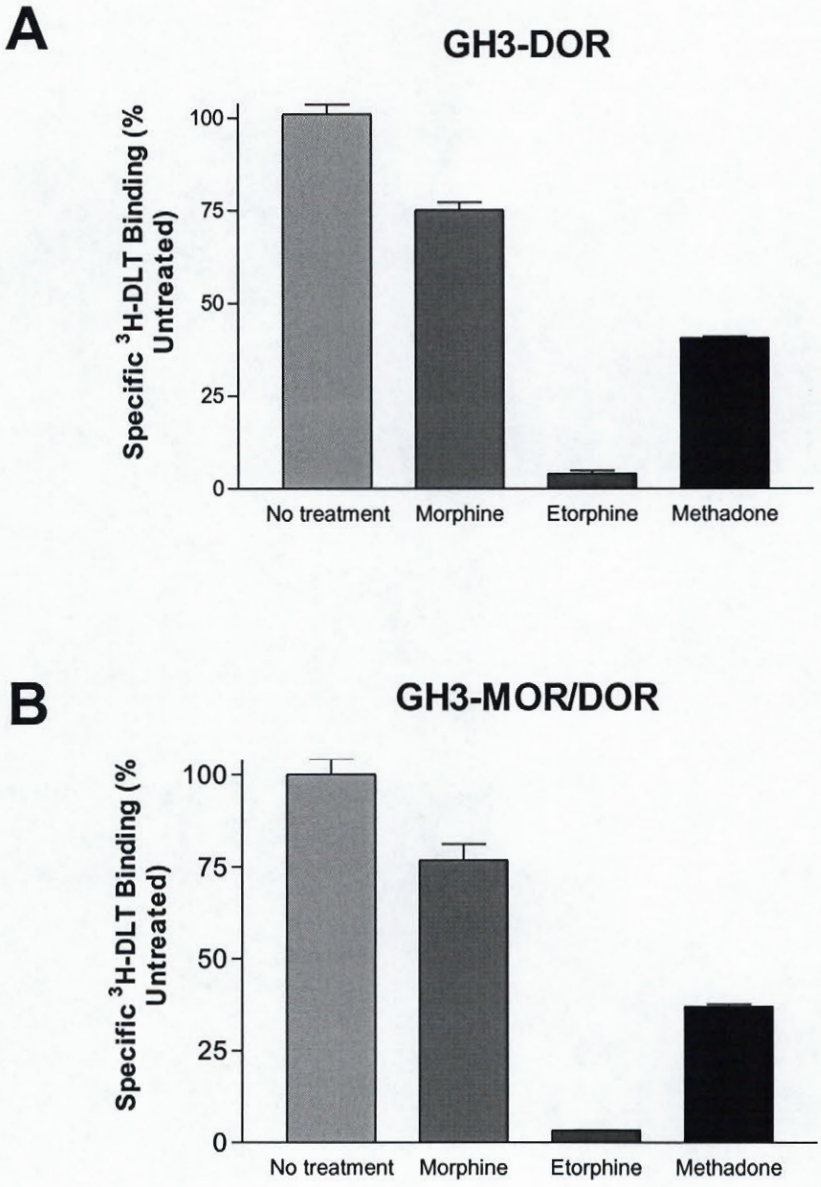

A
GH3-DOR
Specific ^{3}H-DLT Binding (% Untreated)
100
75
50
25
0
No treatment
Morphine
Etorphine
Methadone
B
GH3-MOR/DOR
Specific ^{3}H-DLT Binding (% Untreated)
100
75
50
25
0
No treatment
Morphine
Etorphine
Methadone

Figure 4

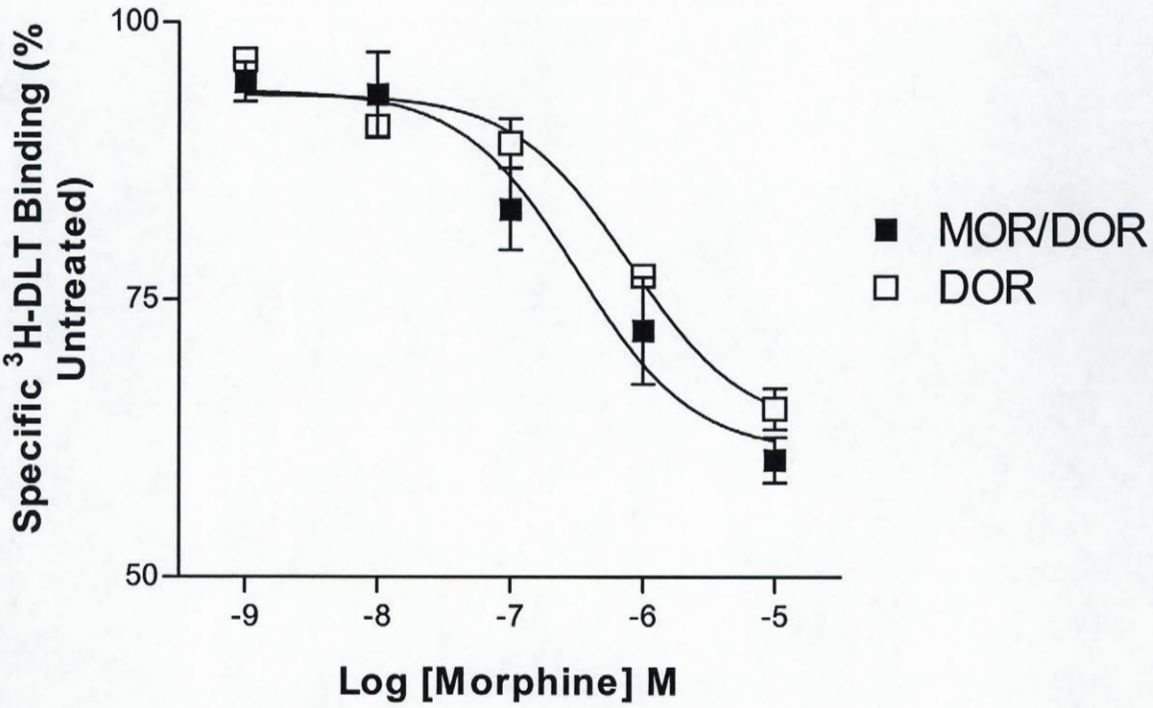

Specific ^{3}H-DLT Binding (% Untreated)
100
75
50
-9
-8
-7
-6
-5
Log [Morphine] M
MOR/DOR
DOR

Figure 5

Figure 6

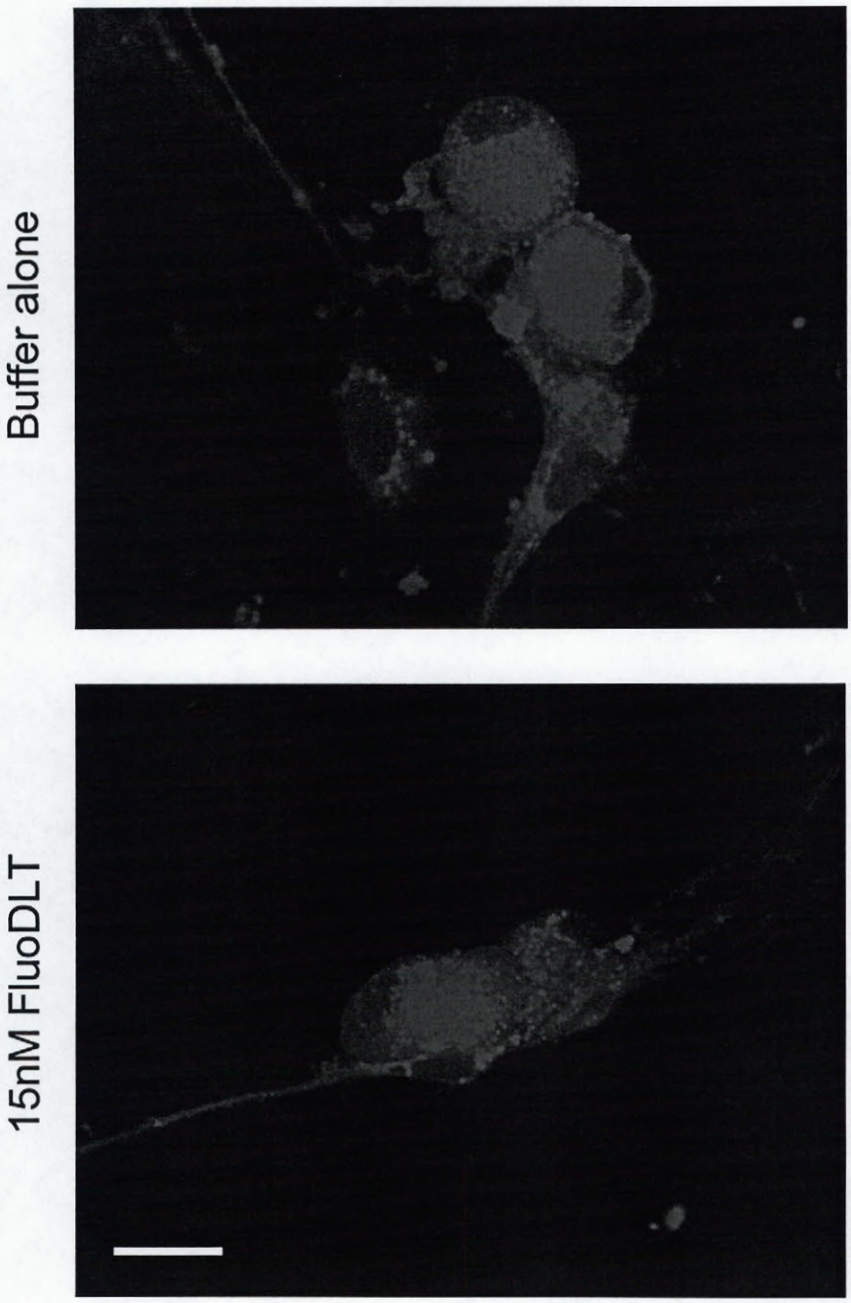
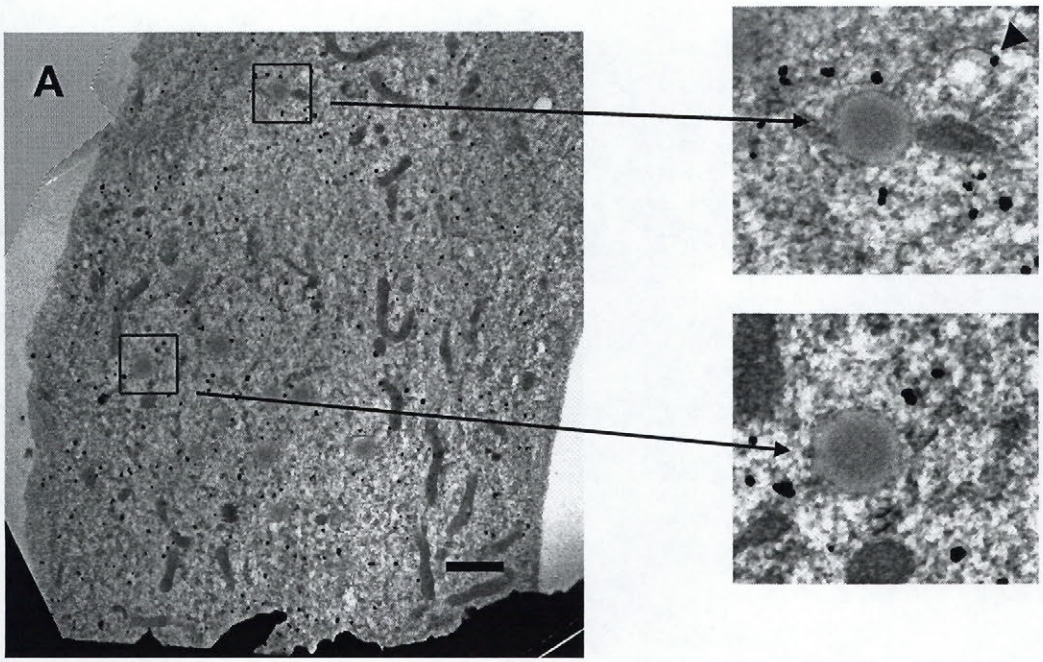


Figure 7



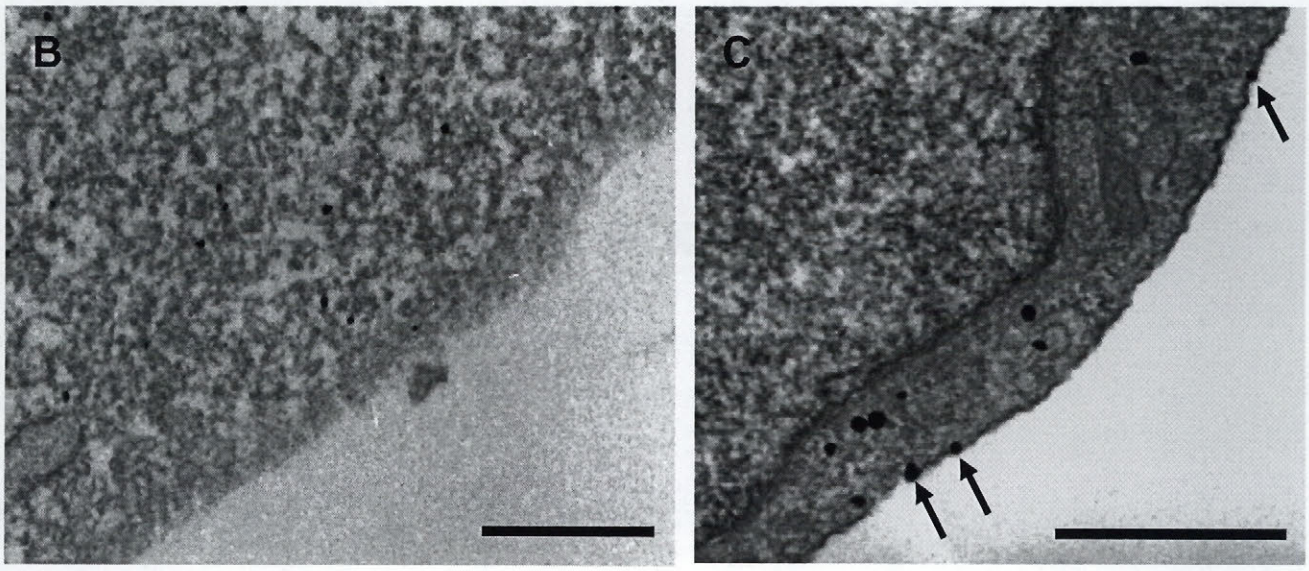


Figure 8

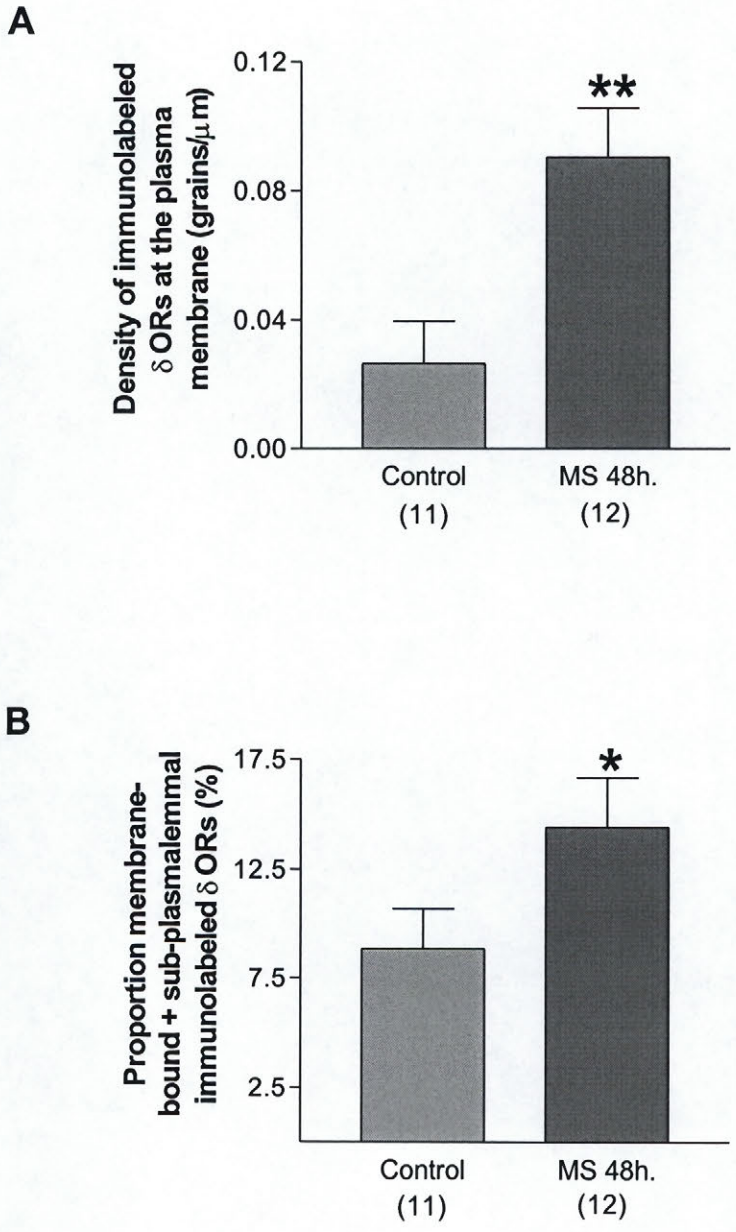

A
Density of immunolabeled δORs at the plasma membrane (grains/μm)
0.12
0.08
0.04
0.00
**
Control
(11)
MS 48h.
(12)
B
Proportion membrane-bound + sub-plasmalemmal immunolabeled δORs (%)
17.5
12.5
7.5
2.5
*
Control
(11)
MS 48h.
(12)

Figure 9

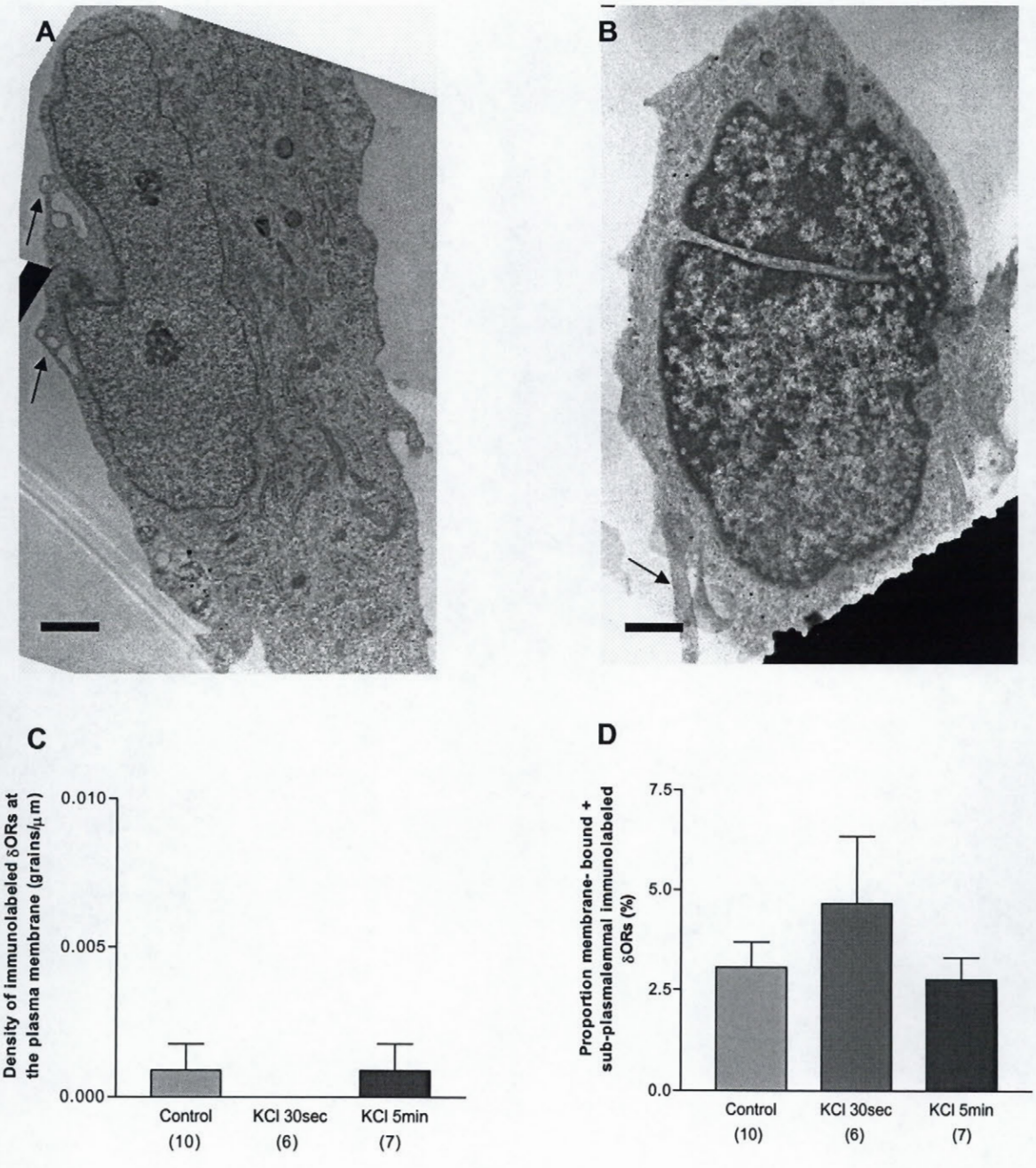

A
B
C
Density of immunolabeled δORs at the plasma membrane (grains/μm)
0.010
0.005
0.000
Control (10)
KCl 30sec (6)
KCl 5min (7)
D
Proportion membrane- bound + sub-plasmalemmal immunolabeled δORs (%)
7.5
5.0
2.5
0.0
Control (10)
KCl 30sec (6)
KCl 5min (7)

Figure 10

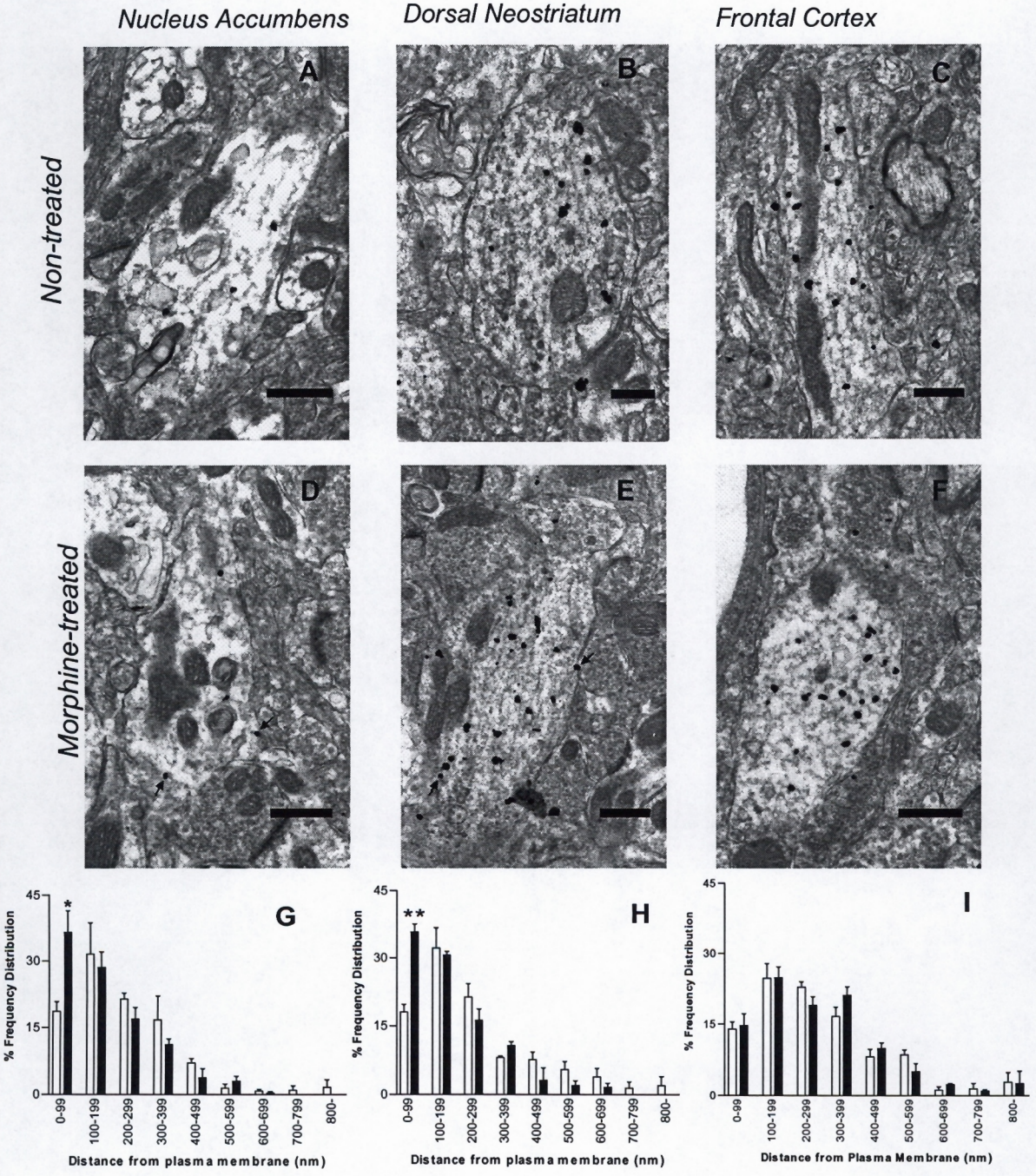

Nucleus Accumbens
Dorsal Neostriatum
Frontal Cortex
Non-treated
Morphine-treated
A
B
C
D
E
F
G
H
I
% Frequency Distribution
Distance from plasma membrane (nm)
Distance from Plasma Membrane (nm)
0-99
100-199
200-299
300-399
400-499
500-599
600-699
700-799
800-
*
**

Figure 11

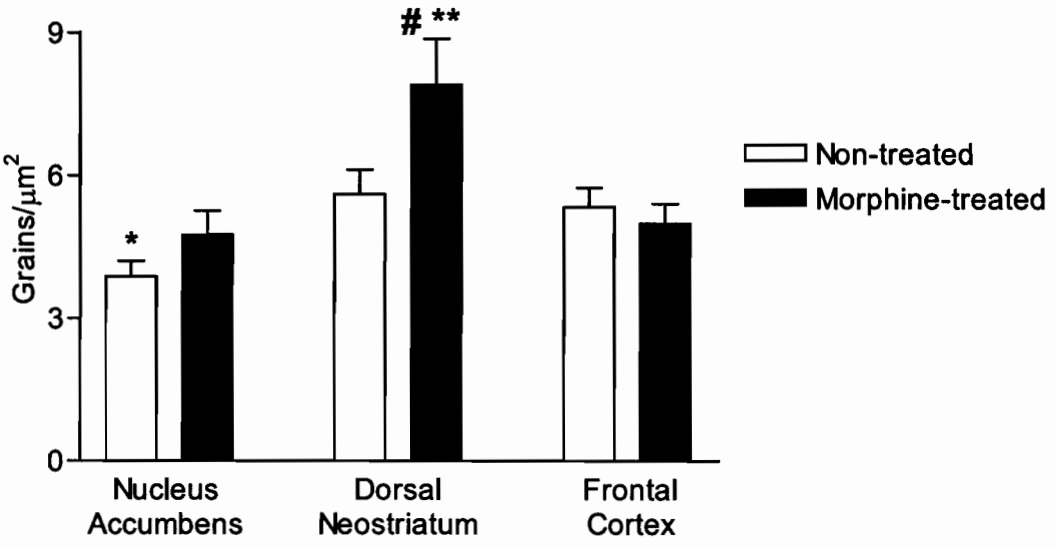

9
6
3
0
Grains/μm²
*
#**
Nucleus Accumbens
Dorsal Neostriatum
Frontal Cortex
Non-treated
Morphine-treated

Figure Legends

Figure 1. **Specific binding and internalization of fluoDLT and fluoDRM in GH3 cells.** GH3 cells transfected with δOR (A,B) or with both μOR and δOR (C-G) were incubated for 30min with 10nM of either fluoDLT (A-E) or fluoDRM (F,G), fixed, and imaged by confocal microscopy. Following treatment with fluoDLT, both GH3-δOR (A) and GH3-μOR/δOR (C) cells exhibit a punctate intracellular distribution of fluorescence that is abolished when the incubation is carried out in the presence of naloxone (B, D). In the presence of CTOP, fluoDLT labeling is retained in the co-transfected cells, confirming the selectivity of fluoDLT for δOR. Following treatment with fluoDRM, GH3-μOR/δOR cells exhibit punctate intracellular labeling (F) that is abolished when cells were co-incubated with naloxone (G). Images are representative of 2 (fluoDRM) or 3 (fluoDLT) independent experiments with samples prepared in triplicate.

Figure 2. **Effect of prolonged morphine treatment on GH3 cells.** GH3-δOR and -μOR/δOR cells were incubated for 48h with morphine sulfate (10μM) and assessed for changes in fluoDLT internalization (A) and receptor binding using ^{3}H-deltorphin (B). By confocal microscopic analysis, morphine treatment does not increase the intensity of fluoDLT labeling in neither the GH3-δOR nor the GH3-μOR/δOR cells (n=2) (A). Quantitative analysis of radioligand binding demonstrates a significant decrease in ^{3}H-deltorphin binding, indicative of a decrease in the density of δORs, in both GH3-δOR and GH3-μOR/δOR cell lines (n=3) (B).

Figure 3. **Effect of prolonged treatment with μOR-selective ligands on the density of δORs in GH3 cells.** GH3-δOR and GH3-μOR/δOR cells were incubated for 48h with morphine, etorphine, or methadone at a concentration of 10μM, and assessed for changes in δOR density by ^{3}H-DLT binding. Quantitative analysis reveals a significant decrease in the specific binding of ^{3}H-DLT compared to untreated cells, with an identical rank order of efficacy (etorphine>>>methadone>morphine) in both cell lines. Results are representative of 2 independent experiments prepared in triplicate.

Figure 4. **Dose-response analysis of prolonged morphine treatment on δOR binding in GH3 cells.** GH3-δOR (?) and GH3-μOR/δOR (¦) cells were treated with increasing doses of morphine (1nM to 10μM) for 48h, and assessed for changes in the density of plasma membrane-expressed δORs by ^{3}H-DLT binding. These studies demonstrate a dose-dependent decrease in ^{3}H-DLT binding as compared to untreated cells, in both GH3-δOR and GH3-μOR/δOR cells.

Figure 5. **Double immunocytochemical labeling of MAP-2 and δOR in cultured dorsal root ganglion cells.** Dissociated neurons were maintained in culture for 7 days prior to staining with MAP-2/Alexa 488-conjugated (A) and δOR/Texas Red-conjugated (B) antibodies. As detected by confocal microscopic imaging, δOR immunoreactivity is distributed throughout the soma and afferent processes in a significant proportion of

MAP-2 immunolabeled cells. Figure 5C represents the merged image of the dually immunolabeled MAP-2/δOR DRG cell shown in A (MAP-2) and B (δOR). Scale bar, 20μm.

Figure 6. **FluoDLT internalization in cultured dorsal root ganglion cells.** Cells were incubated with either buffer alone (A) or 15nM fluoDLT (B) for 30min. By confocal imaging, a high level of autofluorescence is evident in untreated DRG cells, with a comparatively low level of signal in fluoDLT-treated DRG cells. Scale bar, 20μm.

Figure 7. **Electron microscopic localization of immunogold-labeled δORs in cultured DRG cells.** For all conditions, the bulk of δOR immunoreactivity is distributed throughout the cytoplasm (A-C), often in association with small clear vesicles (A, top right panel, arrowhead). Note the absence of gold particles over LDCVs (fig 7A, top and bottom right panels). In morphine-pretreated DRGs, an increase of silver-gold particles indicative of immunolabeled δORs are evident along plasma membrane (C). Scale bars, 1μm.

Figure 8. **Frequency distribution analysis of immunogold-labeled δORs in non-treated and morphine-pretreated DRGs.** Following morphine pretreatment, there is a significant increase in the density of immunolabeled δORs over the plasma membrane

(A; ** $p<0.01$) as well as within cytoplasmic compartments located proximal to (>100nm), but excluding, the plasma membrane (B; * $p<0.05$). Results are pooled from 2 independent experiments, with 5-6 DRGs analyzed per condition per experiment.

Figure 9. **Electron microscopic (A,B) and frequency distribution (C,D) analysis of immunolabeled δORs following depolarization with 40mM KCl.** By visual inspection, KCl treatment did not induce the redistribution of silver-gold labeled δORs as compared to untreated DRG cells, but does induce morphological changes in a significant population of DRG cells, including membrane blebbing (A,B; arrows) and nuclear condensation (B). Following analysis by computer-assisted morphometry, there is no quantitative change in the density of immunolabeled δORs either at the plasma membrane (C) or within the sub-plasmalemmal cytoplasmic compartment (D). Scale bars, 1μm.

Figure 10.* **Electron microscopic localization of immunogold-labeled δORs in non-treated (A-C) and morphine-treated (D-F) C57BL/6 mice.** In both morphine-treated and non-treated mice, the vast majority of immunolabeled δORs are found inside dendrites. In dendrites from non-treated mice, gold particles are rarely seen in association with plasma membranes. Following morphine-treatment, there is an increase in the number of silver-gold particles visible over plasma membranes in the

nucleus accumbens and dorsal neostriatum (D, E; arrows), but not in frontal cortex (F). Scale bars, 0.5μm.

G-I, Frequency distribution analysis of immunogold particles in dendrites from non-treated (white columns) and morphine-treated (black columns) mice. Note the significant increase in the proportion of labeled δORs over the plasma membrane and sub-plasmalemmal zone (0-99nm) as compared to intracellular compartments in the nucleus accumbens (* $p<0.05$) and dorsal neostriatum (** $p<0.01$).

Figure 11. * **Analysis of the density of silver/gold particles in labeled dendrites.** In non-treated mice (white columns), the density of immunolabeled δORs is significantly lower (* $p<0.05$) in the nucleus accumbens compared to the dorsal neostriatum or the frontal cortex. Following morphine treatment (black columns), δOR density is significantly higher in dendrites from the dorsal neostriatum as compared to control dendrites (# $p<0.05$), as well as compared to dendrites from the nucleus accumbens and frontal cortex of the same animals (** $p<0.01$).

* These figures were reproduced, with permission, from the following manuscript:
Lucido A, Morinville A, Gendron L, Stroh T, Beaudet A. (2005) Prolonged Morphine Treatment Selectively Increases Membrane Recruitment of Delta Opioid Receptors in the Mouse Basal Ganglia. *J Mol Neurosci.* 25(3): 207-14.

Discussion

Studies with GH3 cells

In the present Master's thesis, we carried out studies directed at further understanding the subcellular localization and trafficking properties of δORs in the rodent CNS. We first wished to tease out the cellular mechanism underlying μOR-mediated δOR trafficking using a heterologous expression system. Unlike primary cortical neurons, prolonged morphine treatment did not increase the internalization of fluoDLT in GH3 cells heterologously expressing opioid receptors. Instead, we observed that sustained exposure to morphine decreased the cell-surface density of δORs in both singly transfected GH3-δOR and dually transfected GH3-μOR/δOR cells, as measured by ^{3}H-DLT binding. This result is consistent with those previously reported by Liu and Prather[184]. Using membrane preparations from GH3-δOR cells, these authors showed that 48h of morphine pretreatment decreased the specific binding of ^{3}H-diprenorphine, a nonselective opioid antagonist, by approximately 30%[184].

It is well known that morphine is highly selective for μORs in vivo[161]. However, several in vitro studies have reported that mu-selective agonists, including morphine, can also recognize endogenously[114] or heterologously[184,201-202] expressed δORs. Though it had been shown in primary cortical neurons that identical morphine conditions (10μM, 48h) were selectively stimulating μORs[89], the expression levels of δORs, particularly at the plasma membrane, are much higher in the transfected GH3 cells than in native neurons. Therefore, we hypothesized that in the present system morphine could be acting directly on plasma membrane-bound δORs, thereby promoting receptor downregulation rather than trafficking of δORs to the plasma membrane.

To test this hypothesis, we carried out two sets of experiments. First, we assessed whether other mu-preferring agonists could similarly downregulate the plasma membrane expression of δORs. We used etorphine, a highly efficacious opioid agonist with moderate selectivity for μORs, and methadone, a less efficacious but highly selective μOR agonist[3]. Using the same dose and treatment duration as was used for sustained morphine treatment, we observed that both methadone and etorphine decreased the cell surface binding of ^{3}H-DLT, with an even greater potency than morphine.

In the second set of experiments, we assessed whether the downregulation of cell surface δORs induced by sustained morphine treatment was dose-dependent. Specifically, we wanted to determine whether morphine, which did not alter the binding of δORs in singly transfected GH3-δOR cells, could enhance the plasma membrane expression of δORs when μORs were co-expressed (i.e., in the GH3-μOR/δOR cells). However, we observed identical dose-response phenomena in both the GH3-δOR and GH3-μOR/δOR cells, whereby low doses of morphine had no effect on ^{3}H-DLT binding, and high doses led to a decrease ^{3}H-DLT binding. Taken together, these results suggest that the μOR agonists tested directly bound and downregulated δORs, thereby decreasing the binding of ^{3}H-DLT to δORs, irrespective of μOR expression.

Previous radioligand binding and cAMP accumulation studies using δOR-expressing mammalian cell lines have demonstrated similar results following treatment with μOR agonists[184,201-202]. However, these previous studies, in addition to those reported in this thesis, should not be interpreted as the result of a lack of selectivity of these drugs, particularly morphine and methadone, for μORs over δORs. As reported by

Kristensen and colleagues, the affinity of methadone and morphine for μORs over δORs is greater than 1000-fold, with IC_{50}s in the nanomolar range for μORs[203]. The doses of methadone and etorphine used in this thesis were within the range for directly activating δORs; however, in light of the morphine dose-response experiments it is clear that dose was not a factor in the lack of μOR-induced δOR trafficking observed in the co-transfected GH3-μOR/δOR cells.

It is possible that the lack of δOR membrane trafficking observed in transfected cells is due to a differential regulation of δOR trafficking in heterologous cell lines versus neurons. Indeed, at the subcellular level, it was observed both in cultured cortical neurons[89] and in DRG cells (present thesis) that the vast majority of δORs were distributed intracellularly and not at the plasma membrane. Interestingly, a recent report by Kim and von Zastrow demonstrated using PC12 neurosecretory cells that in their undifferentiated state, recombinant δORs were expressed at the plasma membrane, and their differentiation into a neuronal phenotype with nerve growth factor (NGF) induced the targeting of newly synthesized δORs to intracellular stores[185]. In addition, these authors demonstrated in cultured hippocampal neurons that transfected δORs were largely localized intracellularly, while transfected μORs were expressed at the plasma membrane[185]. Taken together, it appears that the intracellular retention of δORs is not dependent on endogenous expression, but is specific to cells of a neuronal phenotype. If this is the case, reproducing the complex pharmacological interactions between μORs and δORs may require more than just co-expression of the two receptors within the same cell.

In the future, it would be informative to study the differential targeting mechanisms of δORs between heterologous cell lines and cultured neurons. In HEK293 cells, the synthesis and maturation of δORs has been extensively studied by Petäjä-Repo and colleagues, who showed that the rate-limiting step controlling expression of δORs at the plasma membrane was export from the endoplasmic reticulum[186]. A yeast two-hybrid screen using the c-terminal tail of the δOR recently uncovered a protein, GASP, which interacts with internalized δORs and targets them to lysosomes[95]. It is therefore possible that other proteins exist which interact directly with δORs, either to retain δORs within the cytoplasm or to induce their targeting to the plasma membrane. These molecular approaches are clearly needed to resolve how δORs are targeted to intracellular compartments in neurons. Furthermore, a better understanding of the synthesis and neuron-specific intracellular targeting of δORs could provide valuable insight into how mu stimulation induces δOR trafficking to the plasma membrane.

Studies in Dorsal Root Ganglion cells

In light of our findings in GH3 cells, we decided to continue our studies of δOR trafficking using a primary neuronal culture model. To this aim, we designed a protocol for the culture of dorsal root ganglion cells. DRG cells possess several advantages over other primary neuronal culture models: they display robust growth and differentiation in vitro, are clearly distinguishable from other cell types due to their unique morphology, and are well known to endogenously co-express μORs and δORs at high levels[183].

By immunogold electron microscopy, we observed that δORs are largely expressed intracellularly in cultured DRG cells at steady state. When quantified, this intracellular pool represented almost the entire proportion of δORs expressed in these cells. The intracellular localization of δORs had been reported previously in primary sensory afferents; however, in these other studies a significant proportion of δOR-like immunoreactivity was localized to large dense core vesicles (LDCVs), as well as large electron-lucent vesicles, multivesicular bodies, and golgi apparatus[173,177]. While we frequently observed immunolabeled δORs on the membranes of small clear intracellular vesicles, or loosely distributed throughout the cytoplasm, we almost never observed δORs localized to the membranes of LDCVs.

Following prolonged treatment with morphine, we observed an increase in the density of δORs both at the plasma membrane, as well as the sub-plasmalemmal cytoplasmic compartment just beneath the plasma membrane, of cultured DRG cells. This result is consistent with recent studies from our laboratory, in which treatment of rats using our morphine treatment paradigm (5, 8, 10, 15mg/kg morphine sulfate, 48h) increased the in vivo internalization of fluoDLT in small, medium and large-size

DRGs[187]. Taken together, it appears that the phenomenon of morphine-mediated δOR trafficking observed in the spinal cord also occurs in primary sensory afferents. Considering that these cells transmit nociceptive information from the periphery to the central nervous system, it is possible that in addition to the spinal cord dorsal horn, the DRG neuron is a second anatomical site whereby functional δOR expression can be manipulated to enhance the potency and efficacy of δOR agonists.

Using this model, we also wished to study whether other cellular stimuli could induce the translocation of δORs towards the plasma membrane; specifically, neuronal depolarization. Activity-dependent trafficking of receptors in the central nervous system is an important mechanism for the regulation of neuronal signaling, a well-characterized example of which is the AMPA receptor family. AMPA receptors are highly mobile ion channel proteins which can be inserted into neuronal plasma membranes in an activity-dependent manner[204,205]; this phenomenon of receptor insertion is a key cellular step in the process of memory formation by long-term potentiation (reviewed in ref. 188).

Though δORs had not been traditionally considered to undergo activity-dependent neuronal trafficking, we wished to assess whether neuronal depolarization could affect the density of δORs at the plasma membrane. We were fuelled by a recent result using a similar model of cultured DRGs that acute KCl treatment increased the density of functional δORs at the plasma membrane, as measured by electrophysiology, immunoblotting and immunogold cytochemistry[177]. Furthermore, this finding was extended to δOR-transfected PC12 cells[177,185]. However, Bao and colleagues[177] reported that the insertion of δORs following membrane depolarization was coupled to the Ca^{++}-dependent release of excitatory neuropeptides, including CGRP. Combined

with observations that δORs co-localized with LDCVs, they interpreted that δOR insertion contributes to pronociceptive processing.

Although preliminary, our results are in clear contrast to this work. We first report in this thesis that there was virtually no colocalization of δORs with LDCVs, neither at steady state nor following any of the treatment conditions tested. This is consistent with previous findings from our laboratory in the rodent spinal cord as well as cultured cortical neurons[89,92,133], and is also consistent with our observations in the mouse brain as described in this thesis. It is possible that the discrepancy could be due to the antibodies recognizing distinct immunological forms of the δOR, assuming that different immunoreactive forms of the δOR are targeted to LDCVs as compared to other cellular compartments. However, considering that the epitope from which the δOR antiserum was raised[173,177,189] was identical to what we have used in our laboratory, this seems unlikely.

Following stimulation by acute treatment with KCl, we observed no redistribution of δORs in DRG cells by immunogold cytochemistry. Furthermore, we observed that even short treatment with KCl (30sec and 5min) induced morphological changes in a significant population of DRG cells, including membrane blebbing and nuclear condensation, which are characteristic of cells undergoing apoptosis[190]. Though we restricted our analysis to DRG cells which appeared healthy and did not exhibit these morphological changes, we did not adjust our treatment conditions to more closely resemble those of Kim and von Zastrow[185], who used KCl at a higher concentration (55mM) and for a much longer time period (30min) than was used in our own experiments. Therefore, our preliminary results support the hypothesis that the

membrane targeting of δORs occurs only following selective cellular stimuli. This hypothesis is supported by work by Christie and colleagues, who observed in periaqueductal grey neurons that while KCl-induced neuronal depolarization did not enhance the δOR-mediated inhibition of GABAergic synaptic currents[191], 48h of morphine pretreatment significantly increased the inhibition of these currents following acute treatment with deltorphin[192].

Studies *in vivo*: C57BL/6 mice

The principal aim of our in vivo study was to determine whether sustained morphine treatment would affect the expression and trafficking of δORs in CNS centers other than the spinal cord. We found that morphine was capable of modulating δOR trafficking in some areas, namely the nucleus accumbens and neostriatum, but not in others, namely the cerebral cortex, indicating that the effects of the drug were region-specific.

In all three regions examined, immunoreactive δORs predominated in the somatodendritic compartment of nerve cells. Furthermore, in all three areas, somatodendritic δORs were mainly intracellular, in conformity with earlier data on the sub-cellular distribution of δORs in either the same[89,193] or other[89,92] regions of the CNS. However, quantitative analysis revealed that in non-treated mice, the density of both membrane-bound and intracellular receptors was lower in dendrites from the nucleus accumbens than from either the neostriatum or the cerebral cortex, indicating that under baseline conditions, the expression, and hence the sensitivity, of target neurons to δOR agonists was variable between brain regions.

The salient finding of the present study was that sustained treatment with morphine induced a recruitment of δORs from intracellular compartments to the plasma membrane and sub-plasmalemmal zone in two of the three regions studied. This effect is likely to be μOR-mediated as earlier studies have demonstrated that in the spinal cord, the morphine-induced membrane recruitment of δORs could no longer be elicited in μOR KO mice[92]. The reason why this recruitment effect was observed in the basal ganglia but not in cerebral cortex therefore probably lies with a regional variability in the

availability of μOR for interacting with δOR. It is well established that the neostriatum, nucleus accumbens, and frontal cortex all contain both μOR and δOR, as demonstrated by immunohistochemistry, autoradiography, and *in situ* hybridization[121,124,126,131]. However, whereas in rat neostriatum, dual labeling electron microscopic immunocytochemistry has shown that a high proportion (39%) of δOR-expressing dendrites also expressed μOR[193], in rat cerebral cortex, single cell PCR studies have failed to find μOR and δOR co-expressed within the same neurons[194]. To date, there are no μOR-δOR dual-labeling studies in the nucleus accumbens, although dendrites of this region have been shown in separate electron microscopic studies to express both μOR and δOR[195,196]. Therefore, it is tempting to postulate that the induction of δOR trafficking by morphine requires co-localization of the two receptor sub-types within the same neurons.

It should be noted that prolonged μOR stimulation by morphine did increase the density of δORs at the plasma membrane of cortical neurons in culture[98]. However, this result was not corroborated by the present in vivo electron microscopic study and may reflect changes induced by culturing of these neurons. Alternatively, this discrepancy may also reflect the fact that the cultured neurons were derived from dissection of the entire cortex, thereby encompassing a number of anatomically and functionally distinct regions, whereas in the current study we sampled selectively the frontal cortex. It is therefore possible that the cultured neurons were derived from other regions of the cortex that were not analyzed here.

Of the two regions in which morphine induced a redistribution of dendrite-associated δORs, only the neostriatum exhibited a concomitant increase in δOR

receptor density. The lack of δOR up-regulation in the nucleus accumbens is consistent with our earlier observations in rodent spinal cord using the same morphine regimen[89,92], and suggests that the augmentation in δOR cell surface density is a true trafficking event and not merely the consequence of an overall increase in receptor levels within the whole cell. Whether the increase in the density of δOR observed in the neostriatum reflects a distinct compensatory feedback mechanism present in this region but not in the nucleus accumbens, or merely one that has a different time course in the neostriatum and nucleus accumbens remains to be determined.

Whether the increase in cell surface δOR observed in the present study translates into a functional increase in the activity of δOR in the basal ganglia also remains to be investigated. In the spinal cord, the increase in plasma membrane-associated δORs was found to improve the antinociceptive potency of intrathecally administered δOR agonists[89]. In neurons of the periaqueductal grey, electrophysiological studies by Christie and Hack[191] have revealed that 48h of morphine treatment enhanced δOR-mediated inhibition of GABAergic synaptic currents. We know that δOR stimulation can modulate motor and reward behaviours in the neostriatum and nucleus accumbens[197-199]. Considering that prolonged agonist exposure leads to the desensitization and down-regulation of many plasma membrane-bound receptors[72], including the predominantly surface-bound μORs[185,193,200], it is possible that the recruitment of δORs induced by prolonged stimulation of μOR represents an endogenous mechanism to maintain sensitivity to motor and reward stimuli by prolonging opioidergic transmission.

Final thoughts and conclusions

In this thesis, we describe complimentary yet novel findings about how δORs are expressed and trafficked in the CNS. First, we observed that the vast majority of δORs were found to be intracellular in all neuronal profiles analyzed, in vitro and in vivo. Second, by confirming that prolonged morphine treatment induces the trafficking of δORs towards the plasma membrane of DRG cells, we have identified a second anatomical site whereby an increase in the density of bioavailable δORs could modulate the antinociceptive potency of δOR agonists. Third, we have discovered that in addition to primary nociceptive targets, including the spinal cord dorsal horn and primary sensory afferent neurons, prolonged morphine treatment can regulate the trafficking of δORs in CNS centers with diverse physiological functions, suggesting that this phenomenon may help regulate opioidergic transmission throughout the CNS. Finally, it appears that the phenomenon of μOR-mediated δOR trafficking is restricted to neurons, as it was not observed in cells of a non-neuronal phenotype.

From these results, we can conclude that δORs in the CNS are not just classical receptor proteins that transduce cellular stimuli in a ligand-dependent manner. It appears that these receptors, when expressed in cellular compartments where they are inaccessible to their ligands, have an important purpose. Rather than being nonfunctional, these intracellular receptors await recruitment to the plasma membrane in conditions where their functional expression is required. In this way, δORs are important modulators of opioidergic transmission in the CNS.

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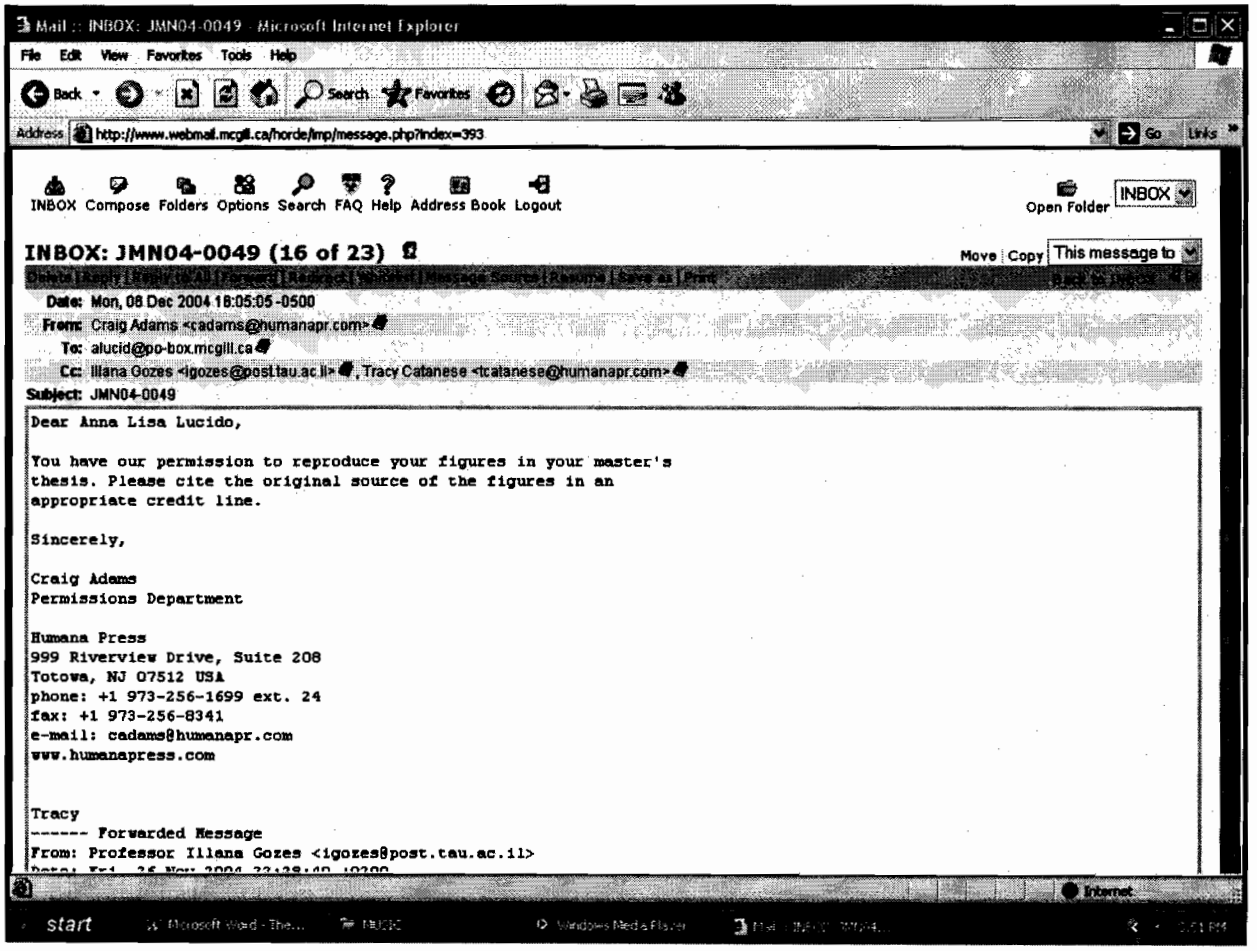
Appendix

I) Copyright waivers for:

Lucido AL, Morinville A, Gendron L, Stroh T, Beaudet A. (2005) Prolonged Morphine Treatment Selectively Increases Membrane Recruitment of Delta Opioid Receptors in the Mouse Basal Ganglia. *J Mol Neurosci.* 25(3): 207-14.



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Cc: Illana Gozes <igozes@post.tau.ac.il>, Tracy Catanese <tcatanese@humanapr.com>
Subject: JMN04-0049

Dear Anna Lisa Lucido,

You have our permission to reproduce your figures in your master's thesis. Please cite the original source of the figures in an appropriate credit line.

Sincerely,

Craig Adams
Permissions Department

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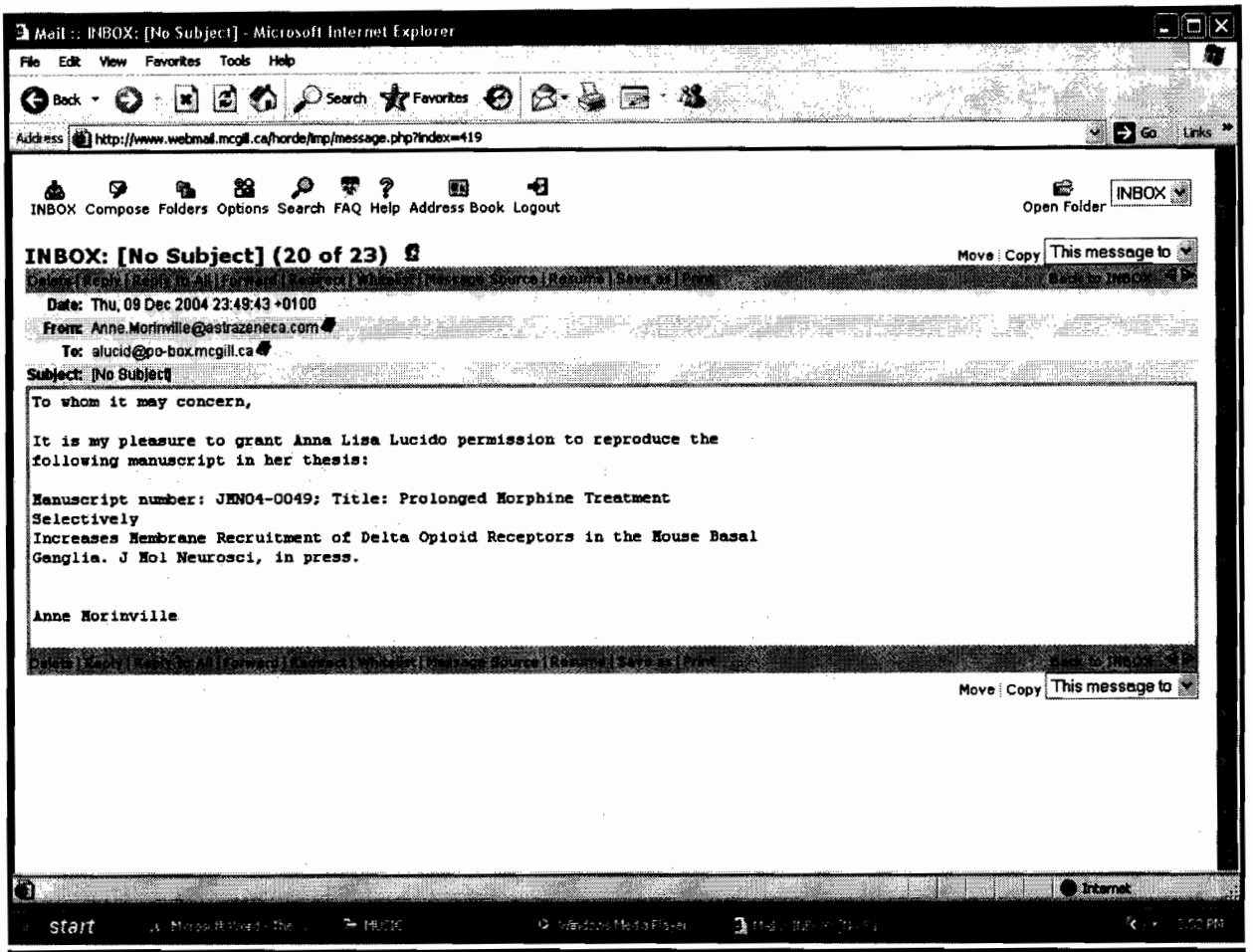
Tracy
------ Forwarded Message
From: Professor Illana Gozes <igozes@post.tau.ac.il>

start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Date: Thu, 09 Dec 2004 23:49:43 +0100
From: Anne.Morinville@astrazeneca.com
To: alucid@po-box.mcgill.ca
Subject: [No Subject]
To whom it may concern,
It is my pleasure to grant Anna Lisa Lucido permission to reproduce the
following manuscript in her thesis:
Manuscript number: JMN04-0049; Title: Prolonged Morphine Treatment
Selectively
Increases Membrane Recruitment of Delta Opioid Receptors in the Mouse Basal
Ganglia. J Mol Neurosci, in press.
Anne Morinville
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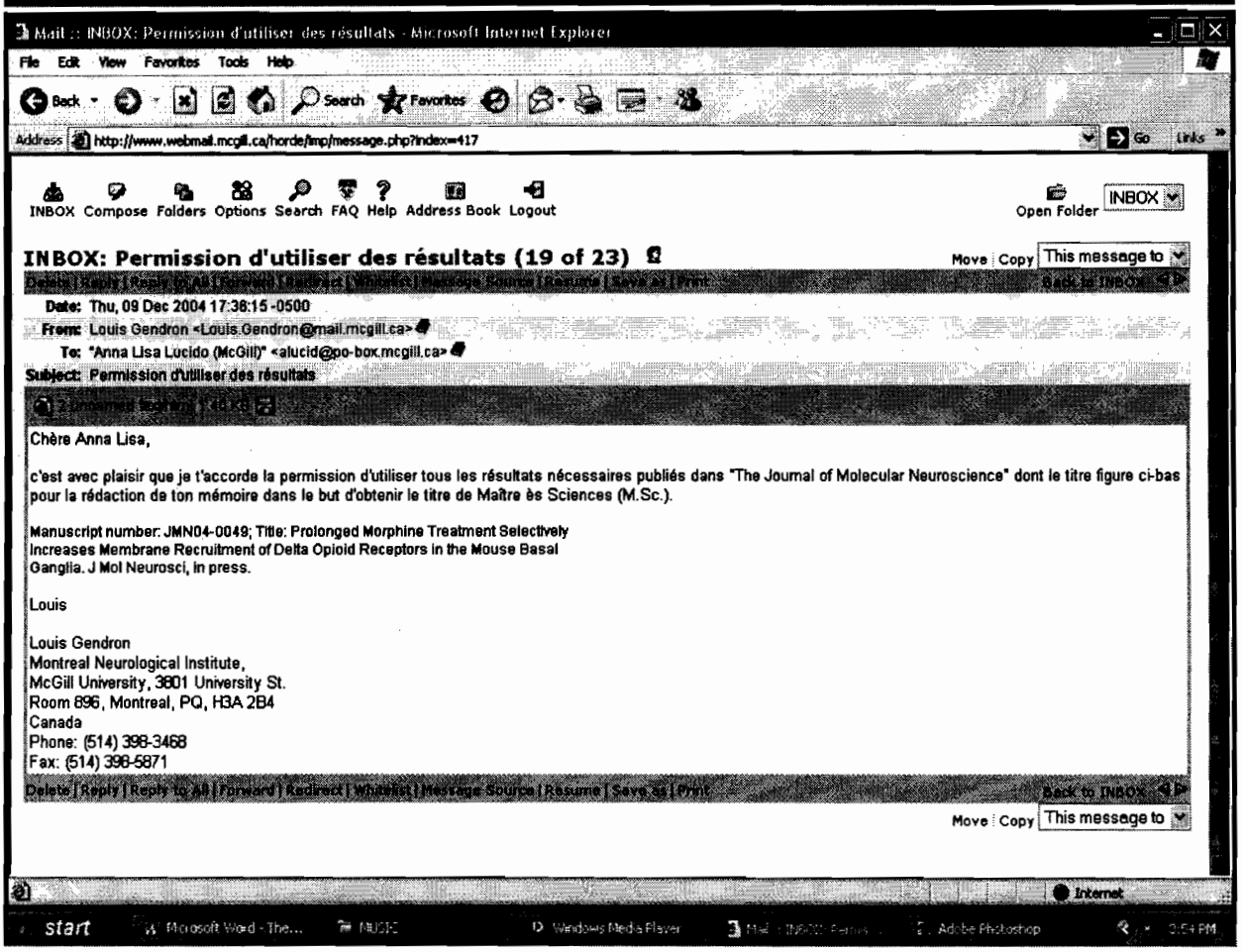
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Date: Thu, 09 Dec 2004 17:36:15 -0500
From: Louis Gendron <Louis.Gendron@mail.mcgill.ca>
To: "Anna Lisa Lucido (McGill)" <alucid@po-box.mcgill.ca>
Subject: Permission d'utiliser des résultats
Chère Anna Lisa,
c'est avec plaisir que je t'accorde la permission d'utiliser tous les résultats nécessaires publiés dans "The Journal of Molecular Neuroscience" dont le titre figure ci-bas pour la rédaction de ton mémoire dans le but d'obtenir le titre de Maître ès Sciences (M.Sc.).
Manuscript number: JMN04-0049; Title: Prolonged Morphine Treatment Selectively
Increases Membrane Recruitment of Delta Opioid Receptors in the Mouse Basal
Ganglia. J Mol Neurosci, in press.
Louis
Louis Gendron
Montreal Neurological Institute,
McGill University, 3801 University St.
Room 896, Montreal, PQ, H3A 2B4
Canada
Phone: (514) 398-3468
Fax: (514) 398-5871
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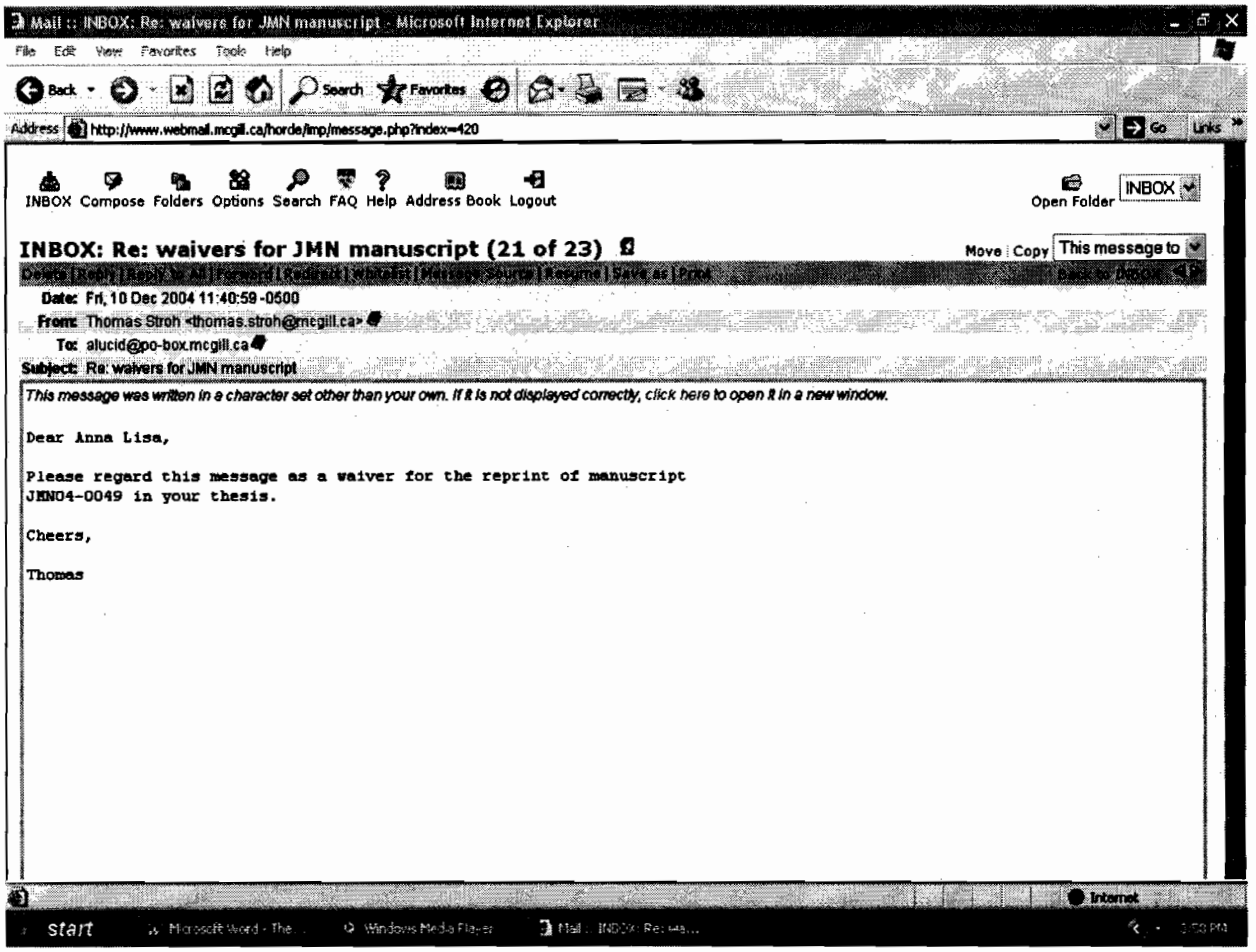
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Date: Fri, 10 Dec 2004 11:40:59 -0500
From: Thomas Stroh <thomas.stroh@mcgill.ca>
To: alucid@po-box.mcgill.ca
Subject: Re: waivers for JMN manuscript
This message was written in a character set other than your own. If it is not displayed correctly, click here to open it in a new window.
Dear Anna Lisa,
Please regard this message as a waiver for the reprint of manuscript
JMN04-0049 in your thesis.
Cheers,
Thomas
Internet
start

MONTREAL NEUROLOGICAL INSTITUTE AND HOSPITAL
McGill University

INSTITUT ET HÔPITAL NEUROLOGIQUES DE MONTRÉAL
Université McGill

Alain Beaudet, M.D., Ph.D.
Professor
Department of Neurology and Neurosurgery

December 13, 2004

Re: Manuscript JMN04-0049

To Whom It May Concern,

It is my pleasure to grant Anna Lisa LUCIDO permission to reproduce the following manuscript in her thesis:

Manuscript number: JMN04-0049; A.L Lucido et al., title: Prolonged Morphine Treatment Selectively Increases Membrane Recruitment of Delta Opioid Receptors in the Mouse Basal Ganglia. *J Mol Neurosci,* in press.

Sincerely,

Alain Beaudet, M.D., Ph.D.
Professor
McGill University

President and CEO
Fonds de la recherche en santé du Québec

AB/nt

3801, rue University
Montréal, Québec
Canada H3A 2B4
Téléphone : (514) 398 1913
Télécopieur/Fax : (514) 398 5871
Courriel/E-mail :
alain.beaudet@mcgill.ca

II) Role of candidate and co-authors in:

Lucido AL, Morinville A, Gendron L, Stroh T, Beaudet A. (2005) Prolonged Morphine Treatment Selectively Increases Membrane Recruitment of Delta Opioid Receptors in the Mouse Basal Ganglia. *J Mol Neurosci.* 25(3): 207-14.

I (Anna Lisa Lucido) performed all electron microscopic imaging and data analysis, and wrote the manuscript.

Anne Morinville treated the animals and processed all the tissue for immunogold electron microscopy.

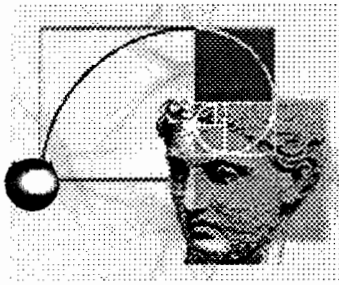
Louis Gendron assisted with the statistical analysis of the data.

Thomas Stroh provided technical expertise for the use of the electron microscope.

Alain Beaudet was the primary editor of this manuscript, and all the research for this manuscript was performed in his laboratory under his supervision.

Research Compliance Certificates (next 3 pages):

- Internal radioisotope user permit (2 pages)
- Animal use protocol (1 page)



Montreal Neurological Institute and Hospital

A Teaching and Research Institute at McGill University

Internal Radioisotope User Permit

Issued by:

The Radiation Safety Committee of the
Montreal Neurological Institute and Hospital
Authorized by the Canadian Nuclear Safety Commission
CNSC Radioisotope Licence Number: 01187-2-08.1 (and any revisions thereof)

1.	**Radioisotope User Permit Number**	:	**MNI_003 (Rev. 1)**
	Classification	:	**Basic**
	Date of Issue	:	**May 1, 2003**
	Revision Date	:	**January 30, 2004**
	Expiry Date	:	**April 30, 2008**
2.	**Name of Principal Investigator**	:	**Beaudet, Alain**
3.	**Department**	:	**Neurobiologie**
4.	**Location(s) approved by this permit**	:	**884, 894**
5.	**Radioisotopes approved by this permit**	:	**See non-shaded cells in table below.**

Note: The permit holder needs written authorization by the CNSC for projects requiring more than 10,000 exemption quantities (E. Q.) of a radioactive substance.

approved for use of:	^{3}H		^{35}S	^{33}P	^{55}Fe	^{125}I
10'000 exemption quantities (E. Q)	10 TBq or 270 Ci		1 TBq or 27 Ci	10 GBq or 270 mCi	10 GBq or 270 mCi	10 GBq or 270 mCi
your possession limit is:	1 GBq or 27mCi		370 MBq or 10 mCi	370 MBq or 10 mCi	37 MBq or 1 mCi	370 MBq or 10 mCi

6. **Personal Dosimeters Required** : **YES**

7. **Method of Disposal** : **All radioactive waste (solid and liquid) must be disposed of through the containers provided by the McIntyre Waste Management Facility (McGill)**

RSO Office: 398-8927
RSO Pager: 406-3069
Locating: 8888-53333

4. **Location(s) approved by this permit** : **884, 894**

5. **Radioisotopes approved by this permit** : **See non-shaded cells in table below.**

Note: The permit holder needs written authorization by the CNSC for projects requiring more than 10,000 exemption quantities (E. Q.) of a radioactive substance.

approved for use of:	^{3}H		^{35}S	^{33}P	^{55}Fe	^{125}I
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your possession limit is:	1 GBq or 27mCi		370 MBq or 10 mCi	370 MBq or 10 mCi	37 MBq or 1 mCi	370 MBq or 10 mCi

6. **Personal Dosimeters Required** : **YES**

7. **Method of Disposal** : **<u>All radioactive waste</u> (solid and liquid) must be disposed of through the containers provided by the McIntyre Waste Management Facility (McGill) and brought to the central waste storage cage in the basement of the MNH/I (room 045 B).**

RSO Office: 398-8927
RSO Pager: 406-3069
Locating: 8888-53333
Home: 525-0220

8. Special Conditions:

Gloves and lab coats mandatory.
Weekly wipe tests required in areas where radioisotopes are used.
Ring badges required for staff using > 50MBq (~1.5mCi) of ^{32}P.
Monitoring of all work surfaces where ^{32}P is used at the end of the work day.
Radio-iodinations: Must be carried out in a working fume hood. Schedule thyroid monitoring. Use of proper survey equipment during radio-iodine manipulations.

The Radiation Safety Officer, MNH/I: _____ **(E.Meyer, RSO, ext. 8927)**

McGill University
Animal Use Protocol – Research

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

Protocol #: 4158
Investigator #: 893
Approval End Date: June 30, 2005
Facility Committee: MNI

☐ Pilot ☐ New Application ☒ Renewal of Protocol # 4158-D

Title (must match the title of the funding source application): Interactions between mu and delta opioid receptors: implications for pain control.

D level

1. Investigator Data:

Principal Investigator:	Alain Beaudet	Office #:	1913
Department:	Neurology and neurosurgery	Fax#:	5871
Address:	MNI, 3801 University, Montreal, Quebec, H3A 2B4	Email:	alain.beaudet@mcgill.ca

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

Name:	Thomas Stroh	Work #:	1913	Emergency #:	(514) 287-7166
Name:	Louis Gendron	Work #:	1913	Emergency #:	(514) 934-1772

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: April 2003 - March 2006	Funding period:

ACTION | DATE | 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: Thomas Stroh (See attached letter) Date: JUN 22 2004

JUN 22 2004

Approval Signatures:

Chair, Facility Animal Care Committee:	Ken Hastings	Date: June 23, 2004
University Veterinarian:	[signature]	Date: July 26, 2004
Chair, Ethics Subcommittee(as per UACC policy):	[signature]	Date: July 27, 2004
Approved Period for Animal Use	Beginning: July 1, 2004	Ending: June 30, 2005

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

29 JUIN 2004

INBOX: JMN04-0049 (16 of 24)

Date: Mon, 06 Dec 2004 16:05:05 -0500
From: Craig Adams <cadams@humanapr.com>
To: alucid@po-box.mcgill.ca
Cc: Illana Gozes <igozes@post.tau.ac.il>, Tracy Catanese <tcatanese@humanapr.com>
Subject: JMN04-0049

Dear Anna Lisa Lucido,

You have our permission to reproduce your figures in your master's thesis. Please cite the original source of the figures in an appropriate credit line.

Sincerely,

Craig Adams
Permissions Department

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e-mail: cadams@humanapr.com
www.humanapress.com

Tracy
------ Forwarded Message
From: Professor Illana Gozes <igozes@post.tau.ac.il>
Date: Fri, 26 Nov 2004 22:28:49 +0200
To: Tracy Catanese <tcatanese@humanapr.com>
Cc: alucid@po-box.mcgill.ca
Subject: Fwd: JMN04-0049

Dear Tracy,

Could you please follow-up with Anna Lisa Lucido?

Thanks,

Illana

----- Forwarded message from alucid@po-box.mcgill.ca -----
Date: Fri, 26 Nov 2004 12:45:43 -0500
From: alucid@po-box.mcgill.ca
Reply-To: alucid@po-box.mcgill.ca
Subject: JMN04-0049
To: "igozes@post.tau.ac.il" <igozes@post.tau.ac.il>

Hello Dr. Gozes,

I am writing regarding the figures from the manuscript submitted a few weeks ago
from our laboratory (JMN04-0049). I would like to use these figures as part of
my Master's thesis. I have tried contacting Humana Press for copyright
permission, and I have received no response. Could you help me in this regard?
How do I go about obtaining copyright permission?

Thank you, Anna Lisa Lucido

----- End forwarded message -----

--
Illana Gozes, Ph.D.
Professor of Clinical Biochemistry
The Lily and Avraham Gildor Chair for the Investigation of Growth Factors
Sackler Faculty of Medicine
Tel Aviv University
Tel Aviv 69978
Israel

Chief Scientific Officer
Allon Therapeutics, Inc.

Editor-in-Chief
Journal of Molecular Neuroscience

------ End of Forwarded Message

INBOX: Re: waivers for JMN manuscript (21 of 24)

Date: Fri, 10 Dec 2004 11:40:59 -0500
From: Thomas Stroh <thomas.stroh@mcgill.ca>
To: alucid@po-box.mcgill.ca
Subject: Re: waivers for JMN manuscript

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Dear Anna Lisa,

Please regard this message as a waiver for the reprint of manuscript JMN04-0049 in your thesis.

Cheers,

Thomas

> Hi there,
>
> I've been checking over my 'Thesis Submission Checklist' and I will need
> from
> all of you a signed waiver for my thesis, allowing me permission to
> reprint the
> data from our manuscript. I imagine you know better than I do if a
> printed email
> is acceptable or whether I need something signed (Anne?). If an email is
> fine,
> if you could write back with your permission that would be great.
>
> Manuscript number: JMN04-0049; Title: Prolonged Morphine Treatment
> Selectively
> Increases Membrane Recruitment of Delta Opioid Receptors in the Mouse
> Basal
> Ganglia. J Mol Neurosci, in press.
>
> Thanks, Anna Lisa
>

--
Thomas Stroh, Ph.D.
Montreal Neurological Institute
McGill University
3801 University Street, Room 896
Montreal, PQ, H3A 2B4, CANADA
Phone: (514) 398-3468, Fax: (514) 398-5871

INBOX: [No Subject] (20 of 24)

Date: Thu, 09 Dec 2004 23:49:43 +0100
From: Anne.Morinville@astrazeneca.com
To: alucid@po-box.mcgill.ca
Subject: [No Subject]

To whom it may concern,

It is my pleasure to grant Anna Lisa Lucido permission to reproduce the following manuscript in her thesis:

Manuscript number: JMN04-0049; Title: Prolonged Morphine Treatment Selectively
Increases Membrane Recruitment of Delta Opioid Receptors in the Mouse Basal Ganglia. J Mol Neurosci, in press.

Anne Morinville

INBOX: Permission d'utiliser des résultats (19 of 24)

Date: Thu, 09 Dec 2004 17:36:15 -0500
From: Louis Gendron <Louis.Gendron@mail.mcgill.ca>
To: "Anna Lisa Lucido (McGill)" <alucid@po-box.mcgill.ca>
Subject: Permission d'utiliser des résultats

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Chère Anna Lisa,

c'est avec plaisir que je t'accorde la permission d'utiliser tous les résultats nécessaires publiés dans "The Journal of Molecular Neuroscience" dont le titre figure ci-bas pour la rédaction de ton mémoire dans le but d'obtenir le titre de Maître ès Sciences (M.Sc.).

Manuscript number: JMN04-0049; Title: Prolonged Morphine Treatment Selectively Increases Membrane Recruitment of Delta Opioid Receptors in the Mouse Basal Ganglia. J Mol Neurosci, in press.

Louis

Louis Gendron
Montreal Neurological Institute,
McGill University, 3801 University St.
Room 896, Montreal, PQ, H3A 2B4
Canada
Phone: (514) 398-3468
Fax: (514) 398-5871

MONTREAL NEUROLOGICAL INSTITUTE AND HOSPITAL
McGill University

INSTITUT ET HÔPITAL NEUROLOGIQUES DE MONTRÉAL
Université McGill

Alain Beaudet, M.D., Ph.D.
Professor
Department of Neurology and Neurosurgery

December 13, 2004

Re: Manuscript JMN04-0049

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Sincerely,

Alain Beaudet, M.D., Ph.D.
Professor
McGill University

President and CEO
Fonds de la recherche en santé du Québec

AB/nt

3801, rue University
Montréal, Québec
Canada H3A 2B4

Téléphone : (514) 398 1913
Télécopieur/Fax : (514) 398 5871
Courriel/E-mail :
alain.beaudet@mcgill.ca