INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

ProQuest Information and Learning 300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA 800-521-0600

IMI[®]

.

INTERNALIZATION AND TRAFFICKING OF MU AND DELTA OPIOID RECEPTORS IN RAT PRIMARY CORTICAL CULTURE AND THEIR RESPONSE TO CHRONIC NALOXONE AND MORPHINE TREATMENT

By

Mao-Cheng Lee

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for The degree of Master of Science

> Department of Neurology and Neurosurgery McGill University Montréal, Québec, Canada December 20, 2000

> > © Mao-Cheng Lee, 2000



National Library of Canada

Acquisitions and Bibliographic Services

395 Wellington Street Ottawe ON K1A 0N4 Canada Bibliothèque nationale du Canada

Acquisitions et services bibliographiques

395, rue Wellington Ottawa ON K1A 0N4 Canada

Your file Votre référence

Our file Notre rélérance

The author has granted a nonexclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission. L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-70451-3

Canadä

LIST OF ABBREVIATIONS

MOR:	μ opioid receptor
DOR:	δ opioid receptor
KOR:	к opioid receptor
POMC:	Pro-opioimelanocortin
PENK:	Pro-enkephalin
PDYN:	Pro-dynorphin
ACTH:	Adrenocorticotropic hormone
LPH:	lipotropin
MSH:	Melanocyte stimulating hormone
CLIP:	Corticotropin-like peptide
ENK:	Enkephalin
Fluo-DRM:	Fluo-dcrmorphin (&Bodipy 576/589 [K7]DRM-I 5APA)
Fluo-DLT-I:	Fluo-deltorphin (ω-Bodipy 576/589 DLT-I 5APA)
CNS:	Central nervous system
DAMGO:	D-Ala ² -MePhc ⁴ -Glyol-enkephalin
DPDPE:	D-[Pen ^{2,5}] enkephalin
U69593:	5α,7α,8β-(-)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec- 8-yl]-benzene acetamide
GPCR:	G protein-coupled receptor
GRK:	G protein-coupled receptor kinase
AchR:	Acetylcholine receptor
SPR:	Substance P receptor

ABSTRACT

The Mu and Delta opioid receptors (MOR & DOR) have been shown to undergo complex regulatory changes in response to ligand exposure in transfected cells. However, little is known regarding receptor regulation in neurons. In this study, we have investigated the internalization and trafficking of the MOR and DOR agonists, fluodemorphin (fluo-DRM) and fluo-deltorphin-I (fluo-DLT-I), respectively, and the fate of these receptors following ligand binding. Experiments were performed on primary rat cortical neuronal cultures using a ligand binding internalization assay and immunocytochemistry. Incubation of neurons with fluo-DRM and fluo-DLT-I resulted in the specific internalization of the fluo-ligands. The internalization was blocked by phenylarsine oxide, indicating that it is clathrin-mediated. Both fluo-ligands accumulated in the soma while no change was evident in the neuronal processes. Exposure to the microtubule disruptor nocadazole resulted in the accumulation of fluo-ligands in the neuronal processes and decrease in their accumulation in soma, suggesting that fluoligands are targeted to the cell soma following internalization. Immunocytochemistry of MOR and DOR revealed that receptor compartmentalization did not change after exposure to their respective opioid agonists suggesting that in contrast to internalized ligands, internalized receptors are recycled locally. The internalization and trafficking of opioid ligands and receptors were also determined after chronic treatment of neuronal cultures with morphine. Chronic morphine treatment led to a profound decrease in fluo-DRM internalization along the processes, but had no effect on the intensity of MOR immunolabeling. However, DOR immunolabeling as well as fluo-DLT-I internalization were greatly increased. This study is the first to demonstrate the internalization and targeting of opioid receptor ligands in neurons. After ligand exposure, MOR and DOR internalize, dissociate from their bound ligands and recycle locally following internalization. Finally, chronic morphine treatment increases the availability of cell surface DOR available for internalization.

RÉSUMÉ

Plusieurs études faites sur des cellules transfectées ont demontré que l'expression des récepteurs aux opiacés Mu et Delta (MOR & DOR) était modulée en présence de leurs ligands respectifs. Toutefois, nous ne savons toujours pas si ces observations sont applicables aux neurones. L'objectif de notre étude consistait donc à étudier l'internalisation et le trafic intracellulaire des agonistes des récepteurs MOR et DOR, la fluo-demorphine (fluo-DRM) et la fluo-deltorphine-I (fluo-DLT-I), respectivement. Nous nous sommes également intéressés au sort des récepteurs MOR et DOR dans les mêmes conditions. Suite à l'incubations des neurones corticaux en culture primaire en présence des ligands fluorescents fluo-DRM et fluo-DLT-I, nous avons observé par microscopie confocale que ces deux ligands internalisent de façon spécifique. Cette internalisation est dépendante de la clathrine puisqu'elle est bloquée par l'oxide de phénylarsine. Les deux ligands fluorescents s'accumulent au niveau des corps cellulaires neuronaux alors qu'aucun changement n'est détecté dans les extensions neuronales. Les ligands demeurent concentrés au niveau des processus neuronaux, et leur accumulation est réduite au niveau du soma, lorsque les neurones sont incubés en présence de l'agent antitubulaire nocadazole, ce qui suggère que les ligands internalisés sont normalement acheminés de la périphérie vers le corps cellulaire. Des expériences immunocytochimiques ont démontré que la distribution des récepteurs MOR et DOR demeure inchangée en présence des agonistes aux opiacés, ce qui suggère que contrairement à leurs ligands, les récepteurs internalisés sont recyclés localement. Nous nous sommes également intéressé à l'internalisation et au trafic des ligands et récepteurs des opioïdes en présence de morphine, un agoniste sélectif du MOR. Suite à une stimulation prolongée par la morphine, nous avons détecté une diminution marquée de l'internalisation de la fluo-DRM au niveau des processus neuronaux, alors qu'aucun changement n'a été noté dans l'intensité du marquage immunocytochimique du DOR. Cependant, nous avons détecté une augmentation marquée du marquage immunocytochimique DOR ainsi que de l'internalisation du fluo-DLT-I dans les mêmes Cette étude est la première à démontrer et à décrire le phénomène conditions. d'internalisation des ligands opioïdes dans des neurones. De plus, nous avons démontré

une régulation de l'expression du DOR à la membrane plasmique suite à une exposition chronique à la morphine.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS	ii
ABSTRACT	iii
RÉSUMÉ	iv
ACKNOWLEDGEMENTS	ix
INTRODUCTION	1
Opioids and Opiates	1
Classification of opioid receptor subtypes	6
Structural characteristics of opioid receptors	7
Table 1 Table 2	8 9
CNS localization of the opioid receptors	11
Table 3 Table 4 Table 5	12 15 16
Functional implications of opioid receptors based on their localization in the rat CNS	20
G protein-coupled receptor activation and internalization	23
Table 6 Table 7	24 25
μ and δ opioid receptor internalization and trafficking	31
Opioid receptor interaction	36
AIM OF THE STUDY	39
MATERIALS AND METHODS	40
Preparation of receptor-encoding plasmids	40

	Culture and transfection of COS-7 cells	40
	Cortical primary cultures	41
	Binding and internalization of ω -Bodipy DRM-I 5APA and ω -Bodipy DLT-I 5APA in transfected COS-7 cells	42
	Binding and internalization of fluo-DRM and fluo-DLT-I in primary cortical cultures	43
	Immunocytochemistry	44
	Chronic Naloxone or Morphine pre-treatment experiments on primary cortical neurons	46
	Confocal microscopy, image processing, image analysis and statistical calculations	46
RESUL	TS	49
	Antibody Specificity	49
	Fluorescent Ligand Specificity	50
	Characterization of cells expressing MOR and DOR in Rat Cortical Primary Cultures by Immunocytochemistry in Steady State (prior to exogenous ligand exposure) conditions.	51
	Characterization of cells expressing MOR and DOR in P1 Rat Cortical Primary Cultures by fluo-ligand binding experiments.	52
	Assessment of Internalization of fluo-DRM and fluo-DLT-I in Rat Cortical Neurons in Culture.	53
	Distribution of internalized fluo-ligands over time	55
	Effect of ligand exposure on the distribution of Opioid receptor immunoreactivity in cortical neurons in culture	58
	Effect of 48 hours naloxone pre-treatment on the distribution of internalized fluo-DRM in rat cortical neurons in culture	60
	Effect of 48 hours naloxone pre-treatment on the distribution of internalized fluo-DLT-I in rat cortical neurons in culture	62

	Effect of 48 hours naloxone pre-treatment on the distribution of opioid receptor immunoreactivity in cortical neurons in culture upon exposure to their ligands	64
	Effect of 48 hours morphine pre-treatment on the distribution of internalized fluo-DRM in rat cortical neurons in culture	64
	Effect of 48 hours morphine pre-treatment on the distribution of internalized fluo-DLT-I in rat cortical neurons in culture	67
	Effect of 48 hours morphine pre-treatment on the distribution of opioid receptor immunoreactivity in cortical neurons in culture upon exposure to their ligands	68
	TABLES, FIGURES AND FIGURE LEGENDS	70
DISCU	SSION	105
BIBLIC	OGRAPHY	123

ACKNOWLEDGEMENTS

Glory to God in the highest, and on earth peace to men on whom his favor rests. Luke 2:14

It is with immense feelings of gratitude and respect that I wish to thank the following people for their invaluable help in making the present thesis become a reality.

My supervisor, Dr. Alain Beaudet who has given me the unique opportunity to learn and to work in an exceptional environment. His great patience, wisdom, understading as well as his constant encouragements and challenges are exemplary and truly inspiring to me.

Dr. Catherine Cahill for her friendship as well as her constant support and encouragements through both words and action. I owe her a great part of this thesis. Without her invaluable help, this thesis would hardly have seen the light.

Dr. Thomas Stroh for his friendship, his teachings and his constant help that allowed me to master the scientific methods.

My friend and colleague Pierre Villeneuve. His friendship, exemplary character, invaluable help, constant support and unrelenting encouragements inspire deep respect and speechless gratefulness within me. A friend in need is a friend indeed.

Anne Morinville, who has helped me so much in the latter part of my journey towards the completion of my experimental work. She has been a blessing to our lab!

Drs. Edith Hamel and Philippe Séguéla for accepting to be in my thesis committee.

Special thanks to the following friends and colleagues for making my research experience in the neuroanatomy laboratory a memorable one: Cheryl Savdie, Minh-ky ha Nguyen, Alexander Choi Jackson, paula Amaral Garrido, James Esdaile, Kelly Mclellan as well as Drs. Dominique Nouel, Hélène Boudin, Franck Vandenbulcke, Jose Domingues Fontencle Neto, Philippe Sarret, Mario Alonso-Vanegas.

Our exceptional and crucial team members Mariette Houle and Naomi Takeda for their friendship and for always being there to clear the way of all troubles.

The program coordinator, Monique Lederman, who has so often bent the deadlines and made the completion of this thesis possible. Fortunate is the one who has her as program coordinator!

My dearest parents and sister for their love and care, and for being there for me in my most difficult times. They are a special blessing in my life.

INTRODUCTION

The discovery of opioid receptors over twenty-five years ago led to a plethora of research that, through the years, brought a whole new dimension of insights and understanding of the neurobiology underlying the experience of pain (Pert and S.H., 1973; Terenius, 1973). Using different drugs and animal models, researchers proposed the existence of multiple opioid receptors (Gilbert and Martin, 1976; Martin et al., 1976). The existence of three genetically distinct subtypes of opioid receptors, referred to as μ , δ , and κ opioid receptors (MOR, DOR, and KOR), has since been firmly established (see below). So has the fact that these receptors were able to modulate pain at every level of the CNS. Opioid receptors mediate the effects of exogenous opiates such as morphine, as well as the effects elicited by endogenous opioids, which play a role in the regulation of pain transmission. Since the discovery of opioid receptors, much effort has been invested in their characterization, as well as in the investigation of their various physiological functions, including how they modulate and suppress pain. Although activation of opioid receptors is not the only mechanism whereby pain can be modulated, it is, notwithstanding, the most prominent effector in this process. Further knowledge regarding the cellular mechanisms through which these receptors and their ligands interact will prove vital to the understanding of their role in the process of modulating or altering the perception of pain.

OPIOIDS AND OPIATES

The mid 1970's brought with it the discovery of the enkephalins, the first of a class of neuroactive peptides referred to as the opioid peptides. Previously, the existence of endogenous opiate receptor agonists had only been inferred. Kosterlitz and Waterfield found that some component of brain extracts could inhibit acetylcholine release in guinea pig ileum (Kosterlitz and Waterfield, 1972; Waterfield and Kosterlitz, 1975); naloxone, which is an opioid receptor antagonist, was found to block this inhibition (Waterfield and Kosterlitz, 1975). The active components were isolated from swine brain, and found to be

pentapeptides with the sequences Tyr-Gly-Gly-Phe-Met, and Tyr-Gly-Gly-Phe-Leu; these molecules were named Met-enkephalin and Leu-enkephalin, respectively, and demonstrated to bind to opioid receptors (Hughes et al., 1975).

Nearly all currently identified opioid peptides present in mammals belong to one of four families; each family arises from the proteolytic cleavage of a distinct precursor polypeptide. The three precursors are proopiomelanocortin (POMC), proenkephalin (PENK), prodynorphin (PDYN) and pro-nociceptin/orphanin FQ (Nakanishi et al., 1979; Kakidani et al., 1982; Noda et al., 1982; Meunier et al., 1995). An exception are the endomorphins, a new family of high affinity MOR selective endogenous opioids discovered in recent years for which the precursor is still unknown.

A number of neuropeptides and peptide hormones derive their existence from their protein precursor POMC. POMC is proteolytically cleaved to different extents in various tissues and its products include β -endorphin (the principal POMC derived opioid), melanocyte stimulating hormones (α , β , and γ MSH) and adrenocorticotropic hormone (ACTH) (reviewed in Bertagna, 1994). The expression of POMC gene occurs mainly in the pituitary, but its expression in the arcuate nucleus of the hypothalamus, placenta, gonads, macrophages, adrenal medulla, and spleen has also been documented (Hollt, 1991). The POMC gene has been isolated and sequenced in human, bovine, rat, and mouse. The human gene is 7665 base pair long and localized on chromosome 2. Translated POMC mRNA, as well as newly translated PENK and PDYN mRNAs, contains a cysteine rich N-terminal signal sequence which is important for the intracellular transport of the resulting precursor proteins (Shields, 1991; Simon and Hiller, 1994). POMC is cleaved to yield a 16 kiloDalton N-terminal peptide, ACTH, and β -lipotropin (β -LPH). These are the dominant products in the anterior pituitary (reviewed in Dickerson and Nocl, 1991). In the cells of the intermediate lobe of the pituitary (melanotrophes), POMC cleavage products are more fully processed. For instance, β -LPH is cleaved into γ -LPH and β -endorphin, while ACTH is cleaved into α -MSH and CLIP (corticotropin-like peptide). In melanotrophes, α -MSH is acetylated, and the 31

residue β -endorphin is processed into two carboxy shortened and N-acetylated (and therefore inactive) products Ac- β -endorphin (1-27) and Ac- β -endorphin (1-26) (reviewed in Dickerson and Noel, 1991). Extremely low level of the protein precursor POMC is produced in the arcuate nucleus when compared to that in the pituitary. In addition, the POMC in the arcuate nucleus is mainly processed to β -endorphin (1-31) and α -MSH, only a small fraction of which become acetylated (reviewed in Dickerson and Noel, 1991).

Six copies of the Met-enkephalin sequence, and one of Leu-enkephalin are present in the Proenkephalin precursor protein (proenkephalin A). Additional opioid active peptides that also derive from proenkephalin are: Met-ENK Arg-Phe, Met-ENK Arg-Gly-Leu, BAM 22, peptide E, and peptide F (Schafer, 1991). The unacetylated N-terminal tyrosine of these peptides is essential for their interactions with opioid receptors (reviewed in Dickerson and Noel, 1991). Proenkephalin is very widely expressed in the brain (striatum, hypothalamus, cortex, hippocampus, brainstem) and spinal cord; it is also found peripherally in the chromaffin cells of the adrenal medulla (Hollt, 1991). The proenkephalin gene has been studied in humans and in rats. The human PENK gene is about 5,200 nucleotides long and contains four exons and three introns (Hollt, 1991). The typical size of the proenkephalin mRNA, as it is normally found in the brain and adrenal medulla is about 1450 nucleotides. Levels of PENK generally correlate with levels of PENK derived peptides.

Prodynorphin (also known as proenkephalin B) is processed to generate the opioid peptides dynorphin A, dynorphin B, and α and β -neo-endorphin (reviewed in Dickerson and Noel, 1991). Prodynorphin expression is largely confined to the brain; it is most concentrated in regions of the hypothalamus and hippocampus, pituitary, globus pallidus, substantia nigra, spinal cord, brain stem, heart, testis, gut, and adrenal gland. (Hollt, 1991; Schafer,). The prodynorphin gene has been sequenced in human, pig, and rat. The human gene is on chromosome 22 and, like PENK gene, it contains four exons and three introns (Hollt, 1991). Prodynorphin mRNA is typically about 3 kilobases, approximately half of which is in the 3' untranslated region (Hollt, 1991).

Nociceptin/orphanin FQ is processed from pro-nociceptin/orphanin FQ and is the endogenous ligand for the ORL1-receptor; it has little affinity for MOR, DOR, or KOR (Meunier et al., 1995; Reinscheid et al., 1995). The amino acid sequence of nociceptin/orphanin FQ has homology with other opioid peptides especially the prodynorphin fragment dynorphin A, suggesting a close evolutionary relationship between the precursors. Nociceptin/orphanin FQ, however, has a C-terminal phenylalanine (F) whereas peptides derived from the other precursors all have the pentapeptide sequence Tyr-Gly-Gly-Phe-Met/Leu (YGGFM/L) at their N-termini. Orphanin FQ/nociceptin act at the supraspinal CNS sites to reverse the opioid-mediated antinociception effects (Grisel et al., 1996; reviewed in Henderson and McKnight, 1997; Meunier et al., 1995; Reinscheid et al., 1995; Rossi et al., 1997). Many studies are being conducted currently to characterize this newly discovered opioid peptides.

Recently, endomorphins have been discovered that act as MOR agonists (McConalogue et al., 1999; Mentlein, 1999; Przewlocki et al., 1999; Zadina et al., 1999). In fact, of all the known endogenous opioids isolated from the brain, these peptides show the highest affinity and selectivity for MOR (Zadina et al., 1999). So far, 2 subtypes have been identified, namely endomorphin-1 with an amino sequence of Tyr-Pro-Trp-Phe-NH₂ and endomorphin-2 with an amino acid sequence of Tyr-Pro-Phe-Phe-NH2. The endomorphins are amidated tetrapeptides and are structurally unrelated to the other endogenous opioid peptides, most of which contain the sequence Tyr-Gly-Gly-Phe (Akil et al., 1976). Although the study of the cellular localization of these peptides is at an early stage, endomorphin-2 is found in discrete regions of rat brain, some of which are known to contain high concentrations of MORs (Schreff et al., 1998). Endomorphin-2 is also present in primary sensory neurones and the dorsal horn of the spinal cord where it could function to modulate nociceptive input (Martin-Schild et al., 1997). The isolation of relatively large amounts of endomorphin-1 and endomorphin-2 from human brain cortex has also reported (Hackler et al., 1997).

Opiates, on the other hand, refer to exogenous opioids that include both natural opiates - that is, drugs from the opium poppy – and opiate-related synthetic drugs, such as meperidine and methadone (reviewed in Lukoff, 1977). The opiates are found in a gummy substance extracted from the seed pod of the Asian poppy, Papaver somniferum. Opium is produced from this substance, and codeine and morphine are derived from opium (reviewed in Lukoff, 1977 & Way, 1979). Other drugs, such as heroin, are processed from morphine or codeine (Lukoff, 1977; Way, 1979). Opiates have been used both medically and non-medically for centuries. A tincture of opium called laudanum has been widely used since the 16th century as a remedy for "nerves" or to stop coughing and diarrhea (Way, 1979). By the early 19th century, morphine had been extracted in a pure form suitable for solution and, since then, it has been widely used clinically as a potent analgesic drug (Way, 1979). Physiologically, its effects are mediated via activation of the MOR.

Heroin (diacetylmorphine) was introduced in 1898 and was heralded as a remedy for morphine addiction. Although heroin proved to be a more potent analgesic and cough suppressant than morphine, it was also more likely to produce dependence and addiction (reviewed in Bewley, 1975).

Of the 20 alkaloids contained in opium, only codeine and morphine are still in widespread clinical use today. In this century, many synthetic drugs have been developed with essentially the same effects as the natural opium alkaloids. Opiate-related synthetic drugs, such as meperidine (Demerol) and methadone (Bewley, 1975; Lukoff, 1977), were first developed to provide an analgesic that would not produce drug dependence. Unfortunately, all opioids (including naturally occurring opiate derivatives and synthetic opiate-related drugs), while effective as analgesics, can also produce dependence (Bewley, 1975; Lukoff, 1977).

CLASSIFICATION OF OPIOID RECEPTOR SUBTYPES

The first evidence for the existence of specific opioid binding sites was originally suggested by behavioral and clinical studies and confirmation ensued with biochemical identification (Pert and Snyder., 1973). Several types of opioid receptors were postulated based on pharmacological evidence whereby opiate alkaloids from different families possess different profiles of pharmacological activity in vivo (Gilbert and Martin, 1976; Martin et al., 1976). Through animal behavioral paradigms, these studies demonstrated that different opioid ligands elicited various behaviors, which were interpreted to result from activation of different opioid receptors. Some opioid ligands induced overlapping behavioral effects, denoting that varying degrees of receptor cross-affinity existed for these ligands. Multiple opioid receptors were also postulated based on various opioid agonists displaying different rank orders of potency in different tissues. For instance, in bioassay experiments, enkephalins were noted to be more potent than morphine in mouse vas deferens but less potent than morphine in guinea-pig ileum. In similar experiments, it was also shown that β -endorphin was much more potent than morphine in rat vas deferens but not in guinea-pig ilcum (Hughes et al., 1975). In experiments using radiolabeled naloxone, an opioid receptor antagonist, Pert and Snyder demonstrated high affinity and stereospecific binding showing regional variation; in addition, the binding affinity of opiates was correlated with physiological potency (Pert and S.H., 1973). Their data effectively proved that there were specific opiate receptors. It was further shown that the dissociation constant (Ke) of naloxone varied within the same tissue, according to the agonists against which it is competing. In guinea-pig ileum, for example, the Ke for naloxone was 2-3 nM against normorphine (a mu opioid receptor agonist), but about 20-30 nM against dynorphin A, dynorphin B (both are kappa opioid receptor agonists), or Bncoendorphin (delta opioid receptor agonist) (Lord et al., 1977). This again supported the notion of multiple opioid receptor subtypes. Finally, it was shown that there was no cross-tolerance between selective opioid receptor agonists. For example, isolated preparations or whole animals can be made tolerant to one opioid agonist without any change in sensitivity to another (reviewed in Goldstein and James, 1984).

Despite the convincing pharmacological data supporting the existence of different opioid receptor subtypes, the criteria for differentiating between these have been unsatisfactory mostly due to the lack of ligand specificity. It was only after the successful cloning of, first, the δ -opioid receptor and, subsequently, other opioid receptors that the concept of multiple opioid receptor subtypes was firmly established (Chen et al., 1993; Evans et al., 1992). Since then, a torrent of information on the structure, anatomical distribution and pharmacological properties of these receptors has appeared. Cloning of the three types in different species was subsequently achieved (Table 1 and references).

To date, three main subtypes of opioid receptors exist, termed the μ , δ , and κ opioid receptors (MOR, DOR, and KOR), all of which have a relative preference for the endogenous agonist ligands endomorphin, enkephalin and dynorphin families, respectively. In the past few years, a novel orphan receptor, termed ORL-1, has been discovered and added into the family of opioid receptors based on its structural homology with the other opioid receptors. There is, however, no corresponding pharmacological homology. Even non-selective ligands that exhibit uniformly high affinity towards μ -, δ and κ -opioid receptors, have very low affinity for the ORL1 receptor, and for this reason as much as for the initial absence of an endogenous ligand, the receptor was called an "orphan opioid receptor". It was only recently that its endogenous opioid ligand has been identified, namely Nociceptin/orphanin FQ. Other opioid receptor subtypes have been proposed, namely the sigma (σ), epsilon (ϵ), iota (ι), and zeta (ζ), however, these receptors have not been classified as opioid receptors as they are not antagonized by naloxone. Albeit somewhat higher affinity for MOR, it is able to bind to the different types of opioid receptors with high affinity and prevent their activation by opioid receptor specific ligands. The key characteristics of MOR, DOR and KOR are summarized in Table 2.

STRUCTURAL CHARACTERISTICS OF OPIOID RECEPTORS

The amino acid sequences of MOR, DOR and KOR are extremely homologous among one another, bearing about 60 - 70% identical sequences, and are highly conserved

Opioid Receptor	Species
μ	Human (1, 2)
	Rat (3, 4, 5, 6, 26, 27)
	Mouse (7)
δ	Human (8, 9)
	Rat (10, 11)
	Mouse (12, 13, 14)
κ	Human (15, 16, 17)
	Guinea pig (18)
	Rat (19, 20, 21, 22, 23)
	Mouse (24, 25)

TABLE 1. The cloned opioid receptors

Modified from Quock, 1999 #97.

Sources: **1.** (Raynor, 1995 #116); **2.** (Wang, 1994 #121); **3.** (Bunzow, 1995 #100); **4.** (Chen, 1993 #101); **5.** (Fukuda, 1993 #104); **6.** (Minami, 1994 #112); **7.** (Min, 1994 #110); **8.** (Knapp, 1994 #106); **9.** (Simonin, 1994 #117); **10.** (Abood, 1994 #98); **11.** (Chen, 1993 #102);**12.** (Augustin, 1995 #99); **13.** (Evans, 1992 #103); **14.** (Kieffer, 1992 #105); **15.** (Mansson, 1994 #108); **16.** (Simonin, 1995 #118); **17.** (Zhu, 1995 #124); **18.** (Xie, 1994 #122); **19.** (Chen, 1993 #102); **20.** (Li, 1993 #107); **21.** (Meng, 1993 #109); **22.** (Minami, 1993 #111); **23.** (Nishi, 1993 #113); **24.** (Nishi, 1994 #115); **25.** (Yasuda, 1993 #123); **26.** (Thompson, 1993 #119); **27.** (Wang, 1993 #120).

	Mu (μ)	Delta (δ)	Карра (к)
Gene family	7TM GPC	7TM GPC	7TM GPC
Gene organization	Intronic	Intronic	Intronic
mRNA Size	10-16 kb	11.0 kb	5.2 kb
Amino acid length	398	372	380
Selective ligands	DAMGO Morphine CTOP Dermorphin	DPDPE DSLET Naltrindolc Deltorphin	U50488 DYNA(1-17) nBNI
Signal transduction	Coupled to G _i protein ↓ cAMP ↑ K' ↓ Ca ² '	Coupled to G_i protein \downarrow cAMP \uparrow K' \downarrow Ca ² '	Coupled to G _i protein ↓ cAMP ↑ K' ↓ Ca ² '
Number of			
glycosylation sites	5	2	2
Predominant mRNA distribution	Thalamus Cortex Striatum Locus coeruleus NST	Cortex Striatum Lateral reticular nucleus	Hypothalamus Nucleus accumbens Substantia nigra VTA NST

TABLE 2. Characteristics of cloned opioid receptors

Abbreviations (GPC: G-protein-coupled; G_i : inhibitory G; TM: Transmembrane; VTA: Ventral tegmental area; NST: Nucleus of the solitary tract). Table modified from Mansour, 1995 #96.

across species (Befort et al., 1996; Knapp et al., 1994). The deduced amino acid sequences of the opioid receptors predict seven transmembrane domains, with conserved proline and aromatic acid residues, which are characteristic of G-protein-coupled receptors (GPCRs) (*Fig. 4*; Probst, 1992 #155). A pair of cysteine residues exists that is proposed to form a disulfide bond in the first two extracellular loops (Evans et al., 1992). The opioid receptor family is strikingly homologous to that of the somatostatin receptors, the angiotensin receptors, and the receptors for the chemotactic factors, N-formyl peptide and interleukin-8 (Evans et al., 1992; Kieffer et al., 1992).

The highest homology between the opioid receptor subtype proteins is found in the putative transmembrane domains, the intracellular loops, and a portion of the Cterminal tail adjacent to the seventh transmembrane domain. The third intracellular loop of each receptor subtype has been implicated in the binding of G-proteins. It has been proposed that all three opioid receptors interact with the same G-protein complexes due to the high homology between different opioid receptor subtypes at this portion of the receptor (Zaki et al., 1996). This third intracellular loop also possesses sites for phosphorylation, which might mediate regulatory processes such as G-protein docking (Zaki et al., 1996). The most pronounced differences between the receptors are found in the second and third extracellular loops as well as in the N- and C-termini (Zaki et al., 1996). At the N-terminus, consensus sites are present for N-linked glycosylation, though MOR, DOR and KOR possess different numbers of glycosylation sites. Based upon prior studies of GPCRs, it is probable that the differences in the extracellular loops might account for the signature ligand-binding profiles of the three receptors (Probst et al., 1992).

Extending the screening of genomic and cDNA libraries in an effort to identify putative subtypes of the classical opioid receptors resulted in the identification of a novel receptor ORL-1 that bore as high a degree of homology towards the classical opioid receptor types, as they shared among each other. The receptor was identified in three species: rat, mouse and man, with the degree of homology among the species variants of more than 90% (reviewed in Henderson and McKnight, 1997). Close comparison of the deduced amino-acid sequences of the four receptors highlights structural differences that may explain the pharmacological anomaly. Thus there are sites near the top of each of the transmembrane regions, that are conserved in the μ -, δ - and κ -opioid receptors, but are altered in ORL1. Work with site-directed mutants of ORL1 (rat) has shown that it is possible to confer appreciable affinity on the non-selective benzomorphan bremazocine by changing Ala213 in TM5 to the conserved Lys of m, k and d, or by changing the Val-Gln-Val276-278 sequence of TM6 to the conserved Ile-His-Ile motif (Meng et al., 1996). A splice variant of the ORL1 receptor from rat has been reported ("XOR") (Wang et al., 1994) with a long form (XOR1L) containing an additional 28 amino acids in the third extracellular loop. In the homologous receptor from mouse (also sometimes referred to as "KOR-3"), five splice variants have been reported to date (Pan et al., 1998).

CNS LOCALIZATION OF THE OPIOID RECEPTORS

Opioid receptor localization was first characterized by autoradiographic techniques using selective radiolabeled ligands. The classical exogenous ligands used in most studies were [³H] D-Ala²-MePhe⁴-Glyol-enkephalin ([³H] DAMGO), [³H] D-[Pen^{2,5}] enkephalin ([H³] DPDPE) and [³H] 5α , 7α , 8β -(-)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1- oxaspiro(4,5)dec-8-yl]-benzene acetamide ([³H] U69593) to label the μ , δ , and κ - binding sites, respectively.

Using these radioligands, receptor autoradiographic studies showed that the MOR was distributed ubiquitously in the fore-, mid-, and hind-brain of the rat, a pattern that was similar to that found in the guinea pig and mouse brain (Mansour et al., 1988; Quirion et al., 1983; Sharif and Hughes, 1989). In the rodent CNS, MOR exhibited a high density labeling in the olfactory bulb, nucleus accumbens, amygdaloid complex, striatal regions, hippocampal pyramidal layer and dentate gyrus, thalamic nuclei, medial cortical laminae, inferior colliculi, superior colliculi, central grey, geniculate bodies, substantia nigra and interpeduncular nucleus (Table 3) (Sharif and Hughes, 1989). On the other hand,

ain region	Specifi	c binding
	(amol/	/mm²) ^Ψ
	Rat	Guinea pig
Olfactory tubercle	121±38	ND
Accessory olfactory bulb	547±123	620±160
Olfactory bulb		
Giomerular layer	361±98	393±112
External piexform layer	340±79	421±109
Granular layer	109±39	113±79
Amygdala Deceleteral evolution	206127	210145
Basolaicrai hucicus	296±27	210±45
Medial nucleus	240±21	201±39
	287±30	289±30
Lateral nucleus	267±59	253±40
Central nucleus	50±21	/1±36
Septonippocampal N.	113±19	99±31
Subiomical organ	195±18	ND
Stria terminalis	250±51	181±02
Striatum (non patch)	239±64	261±80
(non patch) head	106±23	81±25
(non patch) body	188±69	110±30
(non patch) tall	227±35	139±12
Striatum patches	628±/4	723±95
Streaks	422±155	616±86
N. accumbens	398±71	501±139
Hippocampus	100.10	1.50.05
Molecular layer	179±19	150±87
Pyramidal layer	211±25	49±13
Dentate gyrus (vent)	265±13	169±38
Inalamus	420.100	
Paraventricular N.	430±120	141±23
Centromedial N.	688±61	381±59
Intermedial lateral N.	448±95	284±59
Lateral post. N.	375±82	199±89
Habcnular N.	495±41	291±72
Ventroposterior N. (med)	635±51	188±48
Ventroposterior N. (vl)	551±50	302±75
Anterior N.	425±51	578±81
Posterior N.	745±89	489±69
Hypothalamic area	208±63	130±38
Supraoptic N.	189±33	ND
Cerebral cortex		
Layers I-II	163±54	100±13
Layers III-IV	325±41	176±16
Layers V-VI	149±31	109±18
Interpeduncular N.	323±59	ND
Superior collic. (sup)	496±125	493±49
Superior collic. (int)	273±70	149±81
Inferior collic.	440±130	615±185
Central grey	302±88	332±55
Substantia nigra	501±99	332±54
Lateral geniculate body	428±130	335±40
Spinal trigeminal N.	310±82	274±40
Solitary N.	470±189	295±89
Pontine N.	450±120	389±75
Spinal cord		
Substantia gelatinosa	ND	119±10

TABLE 3. Quantitative antoradiographic localization of MOR in rat and guinea pig brain

	Dorsal/ventral grey	ND	30±7
Ccrebellum		12±4	18±3

_

^Ψthe data (amol/sq.mm) are means±SEM from 3-4 animals of each species, using 3.0±0.2 nM (³H)DAGO and 1 μM DAGO for defining nonspecific binding. ND= not determined (from Sharif and Hughes, 1989).

DOR was generally less abundant than MOR binding and was primarily localized in the forebrain (Sharif and Hughes, 1989). Brain regions with high DOR binding included layer), striatum, nucleus accumbens, the olfactory bulb (external plexiform amygdala and cerebral cortex (layers I-II and V-VI) (Table 4) (Sharif and Hughes, 1989). With the exception of a higher concentration of DOR sites in the hindbrain, δ sites in the guinea pig brain displayed a similar distribution as in the rat. In contrast to both MOR and DOR, KOR showed a markedly distinct binding site distribution compared to that of MOR and DOR, re-emphasizing the different physiological roles of the different opioid receptor subtypes (Table 5) (Mansour et al., 1995a). In order of decreasing amounts, [³H] ctorphine showed KOR binding sites predominantly in the cerebellum (molecular layer), cerebral cortex (layers V-VI), nucleus accumbens, striatum, globus pallidus, cerebral cortex (layers I-II) and substantia nigra. KOR was found to be more heavily expressed in guinca pigs and humans than in rats (Mansour et al., 1995a).

The advent of molecular cloning has generated precise techniques for receptor mapping. Accordingly, it was made possible to visualize opioid receptor mRNA with *in situ* hybridization (Delfs et al., 1994; Mansour et al., 1994a; Mansour et al., 1994b; Mansour et al., 1994c; Mansour et al., 1993; Schafer et al., 1994) and opioid receptor proteins with immunocytochemical techniques employing antibodies raised against specific peptide sequences of the various opioid receptor subtypes (Arvidsson et al., 1995a; Dado et al., 1993; Ji et al., 1995; Mackawa et al., 1994; Minami et al., 1995). The development of both techniques has greatly overcome the limitations encountered with receptor autoradiography, proving useful for identification of receptor localization.

The distribution of the MOR, DOR and KOR receptor mRNA compared to that of their respective autoradiographically labeled binding sites demonstrated both similarities and differences (Mansour et al., 1994a; Mansour et al., 1994b; Mansour et al., 1994c; Mansour et al., 1993). Regions of positive correlation between MOR mRNA and MOR binding sites include the striatal clusters and patches of the nucleus accumbens and caudate-putamen, diagonal band of Broca, globus pallidus and ventral pallidum, bed TABLE 4. Quantitative antoradiographic localization of (³H)-DPDPE-labeled DOR in rat

and guinca pig CNS

rain region	Specifi	c binding
	(amol	/ mm²) ^Ψ
	Rat	Guinca pig
Olfactory tubercle	592±22	275±11
Accessory olfactory bulb	40±10	38±12
Olfactory bulb	49±13	30±13
Glomerular layer	625±112	396±131
External plexiform layer	181±90	172±62
Amygdala		
Basolateral nucleus	298±81	112±39
Medial nucleus	310±69	131±43
Cortical nucleus	272±89	132±61
Lateral nucleus	245±72	129±74
Central nucleus	59±21	40±11
Stria terminalis	33±4	84±7
Striatum	306±30	152±24
N. accumbens	597±22	327±17
Globus pallidus	50±13	111±12
Hippocampus		
Pyramidal layer	83±3	65±5
Thalamus	31±5	162±13
Habenula	50±8	90±10
Hypothalamus	49±6	82±21
Cerebral cortex		
Layers I-II	265±89	250±16
Layers III-IV	204±22	1 2 1±16
Layers V-VI	313±60	280±12
Superior collic.	33±4	197±18
Inferior collic.	68±8	94±8
Central grey	33±4	78±9
Substantia nigra	32±6	18±2
Lateral geniculate body	30±4	116±13
Spinal cord		
Substantia gelatinosa	36±6	58±7
Ventral grey	7±1	19±5

 Ψ Digital subtraction autoradiography of [³H]DPDPE-labeled delta receptors was performed. The data are mean±SEM from 3-5 sections from 3 animals of each species. The concentration of the radioligand was 8 nM, and the nonspecific binding was defined with 1 µM unlabeled DADLE. Specific [3H]DPDPE binding represented 80-90% of the total binding. ND= not determined (from Sharif and Hughes, 1989).



Brain region	Specific binding (amol/mm ²) Ψ			
	[³ H]ET	[³ H]PD	[³ H]DYN	[125]]DYN
Amygdala	120	30	35	0.7
Striatum	630	115	274	4.2
Globus pallidus	580	84	240	4.8
N. accumbens	690	99	290	4.2
Hippocampus				
Molecular layer	331	94	118	2.0
Granular layer	221	53	41	0.6
Thalamus	180	72	57	0.5
Hypothalamic area	100	88	67	1.0
Cerebral cortex				
Layers I-IV	320	55	124	1.4
Layers V-VI	960	147	486	4.6
Superior collic.	240	78	65	0.9
Inferior collic.	430	91	108	1.5
Central grey	101	75	50	1.3
Substantia nigra	530	107	183	2.1
Spinal cord				
Substantia gelatinosa	400	ND	95	ND
Dorsal/ventral grey	270	ND	62	ND
Cerebellum				
Molecular layer	1050	143	ND	2.2
Granular layer	320	77	ND	1.0

TABLE 5. Quantitative antoradiographic localization of KOR in guinea pig CNS

^Ψthe data represent means from 3-6 sections from 2-3 animals for each radioligand. [³H]ET=etorphine (3 nM); [³H]PD117302 (5 nM); [³H] DYN = [³H] dynorphin (1-8) (6 nM). [¹²⁵I]DYN = [¹²⁵I]dynorphin (1-8) (0.2 nM). ND = not determined (from Sharif and Hughes, 1989). nucleus of the stria terminalis, most thalamic nuclei, medial and cortical amygdala, mammillary nuclei, presubiculum, interpeduncular nucleus, median raphé, raphé magnus, parabrachial nucleus, locus coeruleus, nucleus ambiguus and nucleus of the solitary tract (Mansour et al., 1994a; Mansour et al., 1994b; Mansour et al., 1994c; Mansour et al., 1993). Differences between MOR mRNA and MOR binding distributions were noted in the neocortex, superior colliculus, olfactory bulb, spinal trigeminal nucleus and spinal cord, which suggest that in these regions, the receptor had been transported away from the site of synthesis in the perikarya to the dendrites and/or axon terminals (Mansour et al., 1994a; Mansour et al., 1994b; Mansour et al., 1994c; Mansour et al., 1993).

In the case of DOR, a high correlation between its mRNA and its binding distributions were observed in the anterior olfactory nucleus, neocortex, caudateputamen, nucleus accumbens, olfactory tubercle, hippocampus, diagonal band of Broca, globus pallidus and ventral pallidum, septal nuclei, amygdala, and pontine nuclei. These findings suggest synthesis of DORs by local neuronal populations as per detected. Differences in DOR mRNA and binding distributions were, however, found in the following regions: substantia gelatinosa of the spinal cord, external plexiform layer of the olfactory bulb, the superficial layer of the superior colliculus, midbrain and brainstem (Mansour et al., 1994a; Mansour et al., 1994b; Mansour et al., 1994c; Mansour et al., 1993), indicating again that such receptor distribution pattern is the result of receptor transport away from the original site of synthesis in the perikarya to distal neuronal processes.

Lastly, a high correlation between KOR mRNA and KOR binding distributions were observed in the following regions: nucleus accumbens, caudate-putamen, olfactory tubercle, bed nucleus of the stria terminalis, medial pre-optic area, paraventricular, supraoptic, dorsomedial and ventromedial hypothalamic nuclei , amygdala, midline thalamic nuclei, periaqueductal grey, raphé nuclei, parabrachial nucleus, locus coeruleus, spinal trigeminal nucleus, and the nucleus of the solitary tract. Thus, these are regions of both KOR expression and synthesis. Regions showing discrepancies in mRNA

17

distribution compared to autoradiographical binding were: substantia nigra, pars compacta, ventral tegmental area and the neural lobe of the pituitary. KORs detected in these regions were localized either in dendrites, axons or synaptic terminals.

In recent years, immunohistochemical studies using antibodies against the C- or N-terminus of opioid receptors have provided additional knowledge regarding opioid receptor distribution at cellular and subcellular levels.

Many immunohistochemical studies, using anti-MOR antibodies against several different C-terminal regions of the receptor, report that MORs are widely distributed in the rat brain and spinal cord. Abundant perikarya-, fiber- and terminal-like patterns of immunoreactivity were localized to superficial layers of the spinal cord (lamina I and II), nucleus of the solitary tract, nucleus ambiguus, locus cocruleus, interpeduncular nucleus, and medial aspect of the lateral habenular nucleus (Ding et al., 1996; Moriwaki et al., 1996). Other regions showing prominent MOR immunoreactivity are the "patch" (striosomes) area and subcallosal streak in the striatum, medial terminal nucleus of the accessory optic tract, median raphe nucleus and parabrachial nucleus (Ding et al., 1996; Mansour et al., 1995b). Moderate perikarya-, fiber- and/or terminal densities were found in selected areas of the thalamus, hypothalamus, middle layer of the cerebral cortex and amygdala (Ding et al., 1996; Hiller et al., 1994; Mansour et al., 1995b; Moriwaki et al., 1996). Regions such as striosomes of the caudate-putamen, trigeminal spinal nucleus, nucleus accumbens, ventral tegmental area, preoptic area, spinal cord substantia gelatinosa, parabrachial nucleus, lateral and medial aspects of the septum, endopiriform nucleus, presubiculum, hippocampus, superior and inferior colliculus, central grey, dorsal motor nucleus of the vagus and stria terminalis were also reported to display fiber and/or perikaryal MOR immunolabeling (Mansour et al., 1995b). Most of these studies report a good concordance of their results with previously determined distributions of MOR ligand binding using autoradiography and MOR mRNA expression using in situ hybridization techniques. There is also a good correlation of MOR immunoreactivity among these studies despite the use of differing epitope-specific antibodies.

Striking discrepancies in regional distribution of DOR exist between immunocytochemical data and results derived from autoradiography and in situ hybridization. Thus, DOR immunolabeling was poorly correlated with that of DOR binding sites derived from autoradiography, particularly in cerebral cortex, caudate putamen and hippocampal formation in which binding sites are dense but immunohistochemical labeling was sparse (Bausch et al., 1995). Discrepancies between mRNA expression and immunoreactive distribution for DOR in the brain were also present in other regions. For instance, high DOR immunoreactivity was seen in the thalamus of the mouse, but low level of mRNA expression was observed in this region. Recently, Cahill et al. from our laboratory reported the first comprehensive immunocytochemical topographical map of DOR in CNS of the rat using antibodies raised against two different epitopes (Cahill et al., 2000). Similar to MORs, DORs show a widespread distribution in the rat brain. Densely immunolabeled DOR was localized to layer V of the neocortex, olfactory tubercle, basal ganglia (caudate-putamen, subthalamic nucleus and subthalamic nucleus), mesencephalon (pars compacta and pars reticulara of the substancia nigra), basal forebrain (the diagonal of Broca, islands of Calleja, magnocellular preoptic areas and ventral pallidum), hypothalamus (medial/lateral preoptic area, ventromedial hypothalamic nucleus and ventral tuberomammillary nucleus), pons (mesencephalic trigeminal nucleus, anterior tegmental nucleus and trapezoid nucleus) and throughout the grey matter of the spinal cord (Cahill et al., 2000). Moderate labeling was found in the hippocampal formation (Cahill et al., 2000). The patterns generated with the two anti-DOR antibodies correlated strikingly with each other throughout the rat CNS and closely matched those previously obtained using either autoradiography or in situ hybridization (Delay-Goyet et al., 1990; Mansour et al., 1987; Quirion et al., 1983; Temple and Zukin, 1987). Remaining regions of continued discrepancies between receptor protein and mRNA distributions are structures within the limbic system, namely the nucleus accumbens and the basolateral and medial amygdaloid nuclei. It is not yet clear why such discrepancies exist.

Immunohistochemical studies of the distribution of KOR in rat CNS demonstrated

immunoreactive perikarya and/or fibers in regions such as the deep layers of the temporal, parietal and the occipital cortex, central and medial amygdala, parasumbiculum, nucleus accumbens, bed nucleus of the stria terminalis, endopiriform nucleus, olfactory tubercle, claustrum, median eminence, hypothalamic nuclei, zona incerta, caudal linear and dorsal raphe, central grey, substantia nigra, pars reticulata, ventral tegmental area, parabrachial nucleus, spinal trigeminal nucleus, nucleus of the solitary tract, spinal cord and dorsal root ganglia (Lin et al., 1995; Mansour et al., 1996). Immunoreactive perikarya and/or fibers were also observed in the neural and intermediate lobes of the pituitary (Mansour et al., 1996). This immunohistochemical localization shows good correspondence with previously described kappa receptor mRNA and binding distributions.

FUNCTIONAL IMPLICATIONS OF OPIOID RECEPTORS BASED ON THEIR LOCALIZATION IN THE RAT CNS

Opioid receptors are known to be involved in a variety of physiological functions and activation of these receptors is dependent upon interaction with their specific ligands that include endogenous peptides as well as exogenous compounds. The CNS distribution of these receptors imparts a great amount of insight into their possible regional-specific roles.. For example, MORs have been shown to be present pre-synaptically in laminae I and II of the trigeminal nucleus caudalis of the medulla and dorsal horn of the spinal cord (Arvidsson et al., 1995b; Dado et al., 1993; Ding et al., 1996; Mansour et al., 1995b). MORs in these areas have been implicated in mediating analgesia by inhibiting capsaicinevoked, and noxious stimuli-induced, release of substance P (an important neuropeptide for central transmission of nociceptive stimuli) (Aimone and Yaksh, 1989; Hirota et al., 1985). MOR-immunoreactive neurons were also reported to be present in the periaqueductal grey (including the dorsal raphe nucleus) as well as in the nucleus raphe magnus and the gigantocellular reticular nucleus, two regions in which microinjections of mu agonists were reported to induce a profound naloxone-sensitive analgesia (reviewed in Pasternak, 1993).. However, since the periaqueductal grey has been shown to also contain DORs and KORs and since microinjections of δ and κ opioid peptides also induce profound analgesia, mediation of antinociceptive effects may possibly be shared among activation of all these opioid receptors. Observation of intense MOR immunostaining in locus coeruleus (Ding et al., 1996; Moriwaki et al., 1996) supports a role for MOR in analgesia induced by opioid microinjection in this region (Bodnar et al., 1988) and implicates MOR in opioid withdrawal effects known to partly be mediated by noradrenergic pathways originating from the locus coeruleus (Nestler, 1992). Lastly, MORs have also been implicated, at least in part, in controlling respiratory, cardiovascular and gastrointestinal functions via their localization in the medial parabrachial nucleus, n. of solitary tract and the ambiguus nucleus (Ding et al., 1996; reviewed in Olson et al., 1995 and Vaccarino, 1999 #29).

It has been proposed that DOR may also be implicated in mediating spinal and supraspinal analgesia (reviewed in Ferrante, 1996; Pasternak et al., 1995). The observation of dense DOR immunolabeling in the dorsal root ganglion, primary afferent fibers as well as in substantia gelatinosa and laminae layers I and II of the nucleus caudalis of the medulla and dorsal horn of the spinal cord supports this hypothesis and supports the view that DOR agonists may prove useful adjuncts in the treatment of pain. Advantages of DOR- over conventional MOR-targeted analgesics include: greater relief of neuropathic pain (Dickenson, 1997), reduced respiratory depression (Cheng, 1993; reviewed in Olson et al., 1995 and Vaccarino, 1999 #29) and constipation (Sheldon et al., 1990), as well as minimal risk for the development of physical dependence (Cowan et al., 1988; reviewed in Olson et al., 1995 and Vaccarino, 1999 #29). Despite these findings and advantages, no DOR agonists have been yet made available for clinical use. On the other hand, the widespread dense immunostaining of DOR observed in the olfactory system that encompasses the olfactory tubercle, diagonal band of Broca, bed nucleus of the stria terminalis and the amygdala further implicates this opioid receptor in the mediation of the central processing and integration of olfactory information known to take place in these regions (Abood et al., 1994; Cahill et al., 2000). Central processing of the auditory information has also been thought to involve DOR due to the substantial

immunohistochemical localization of this receptor in the neural pathways and nuclei known to be involved in the primary processing of auditory information (Cahill et al., 2000). These regions include the vestibular nuclei, the cochelear and trapezoid nuclei, the superior olivary complex and the inferior colliculi. DOR may also be involved in the processes of the visual system as DOR immunolabeling was found in the superior colliculi, suprachiasmatic nucleus, medial and lateral preoptic areas and the visual cortex (Cahill et al., 2000).

KORs have been implicated in various physiological processes that include hormonal regulation, mesolimbic and nigrostriatal function, modulation of pain pathways, and control of visceral responses. Among the better known effects is the ability of ligand-activated KOR to inhibit vassopressin and oxytoxin release, both of which are synthesized by neurons in the paraventricular and supraoptic nuclei, respectively, and transported across the internal layer of the median eminence to the neural lobe of the pituitary (Douglas et al., 1993; Hamon and Jouquey, 1990). This has been postulated to occur pre-synaptically since dense KOR immunostaining has been observed in the perikarya of neurons in the paraventricular and supraoptic as well as along the fibers in the internal layer of the median eminence and neural lobe (Lin et al., 1995; Mansour et al., 1996). In addition, widespread distribution of KOR fiber immunoreactivity in several hypothalamic nuclei supports a role for KOR in the broad range of neuroendocrine effects scen with KOR agonists microinjection in the hypothalamus, namely that of an increase in the release of prolactin, growth hormone, corticosteroids and propiomelanocortin peptides and a decrease in the release of luteinizing hormones (see references in Mansour, 1996 #232). KORs have also been implicated in mediating antinociceptive and gustatory effects (Fox and Burks, 1988; Millan, 1990). KOR agonists are potent analgesics and are particularly effective in modulating visceral and low intensity thermal and mechanical nociceptive responses (Millan, 1990). These effects are mediated by ascending pathways (perykarya of dorsal root ganglia, primary afferent fibers, substantia gelatinosa, superficial laminae of the spinal cord, spinal trigeminal nucleus, centrolateral nucleus of the thalamus, and deep layers of the parietal cortex) as well as descending pathways

(especially involving the central grey) in which KORs are densely localized (Fox and Burks, 1988; Lin et al., 1995; Mansour et al., 1996; Millan, 1990).

Opioid receptors possess other modulatory functions, for instance on the regulation of behavior, motor systems, or levels of consciousness. Many of these functions appear to be exerted through noradrenergic, cholinergic, dopaminergic and serotonergic systems (Mulder et al., 1991). Thus, electrophysiological studies (North et al., 1987) demonstrated that MOR agonists were able to inhibit the firing of noradrenergic neurons and the subsequent release of noradrenaline in the cortex (Mulder et al., 1987). A sub-population of the cells expressing DOR in the neostriatum are large diameter neurons bearing a similar distribution pattern to those of ACh-containing neurons within this same region, consistent with the observed inhibitory effects of DOR agonists on the release of Ach in the caudoputamen (Mulder et al., 1984). The high KOR mRNA labeling densities detected in the substantia nigra, pars compacta and ventral tegmental area suggest co-localization with dopamine and may implicate KOR in the modulation of the release of this neuropeptide (Defagot and Antonelli, 1997). Additional associations and possible co-localization with other neurotransmitters are listed in Table 6.

Lastly, opiate ligands and their receptors have been implicated in modulating many other physiological functions besides those mentioned above including stress, tolerance, dependence, cating, drinking, alcohol consumption, depression, learning memory, epilepsy, general activity and locomotion, mental illness, aggression, sex, and immunology (reviewed in Olson, 1995 #30 and Vaccarino, 1999 #29). Refer to Table 7 for a summary of some of the most important roles of opioid receptors in the mediation and/or modulation of bodily functions.

G-PROTEIN-COUPLED RECEPTOR ACTIVATION AND INTERNALIZATION

An increasing amount of evidence supports the notion that interaction between

	Receptor type	Anatomical site
Inhibition of NA	μ	Locus coeruleus
Inhibition of DA	κ	Nigrostriatal and mesolimbic dopamine systems (presynaptic) Tuberhypophysial (AN) Tuberoinfundibular (AN)
Stimulation of DA	μ	Nigrostriatal and mesolimbic dopamine systems (inhibition of inhibitory interneurons)
	δ	Nigrostriatal and mesolimbic dopamine systems (inhibition of
Inhibition of Ach	δ	Striatal Ach neurons
Stimulation of 5-HT	μ, κ	Median, dorsal, caudal linear raphé, raphé magnus
Inhibition of dynorphin	κ	Magnocellular PVN and SON or fibers in neural lobe

TABLE 6. Opioid receptor and regulation of neurotransmitter release

Table adapted from Mansour, 1995 #96.

Effects of μ , δ and κ receptors on neurotransmitter and peptide release and their likely anatomical site of action. Abbreviations: NA, noradrenaline; DA, dopamine, PVN, paraventricular nucleus; and SON, supraoptic nucleus.
Receptor	Prototypic ligands		Actions
(subtypes')	Endogenous	Exogenous	
Ми	β-endorphin endomorphin-1 endomorphine-2	Morphine methadone heroin codeine	Guinea-pig ilcum bioassay
(Mu ₁)			Supraspinal analgesia Prolactin release Feeding Acetylcholine release in the
(Mu ₂)			brain Spinal analgesia Respiratory depression Gastrointestinal transit Dopamine turnover in the brain Feeding Most cardiovascular effects
Delta	leu-enkephalin met-enkephalin		Mouse vas deferens bioassay
Delta ₁		DPDPE	Supraspinal analgesia
Delta ₂		D-Ala ² -deltorphin	Spinal and supraspinal analgesia
Карра	dynorphin	Ketocyclazocine U69593	
Kappa		0304880	Spinal analgesia
Kappa ₂			Diuresis ?analgesia
Kappa ₃			recomg Supraspinal analgesia

TABLE 7. Tentative Classification of Opioid Receptor Subtypes and Their Actions

Table adapted and modified from Ferrante, 1996 #160 and Pasternak, 1995 #31.

'evidences based on pharmacological studies.

receptor subtypes constitutes a critical step for the regulation of their biological activity (i.e. initiating molecular events that lead to receptor activation) as well as for the mediation of new physiological processes (reviewed in Hebert and Bouvier, 1998). It has recently been proposed that GPCRs may exist under mono-or heterodimeric forms. Such dimerization could affect both high-affinity ligand binding and subsequent intracellular signaling events. The dimerization of GPCRs and the role of dimerization in the function of these receptors are, however, still not well understood. Initially, the evidence that suggested the existence of GPCR-dimers came from pharmacological studies (Kobilka, 1988 #251; Maggio, 1993 #41; Monnot, 1996 #42). Accordingly, studies of α_2 , β_2 -adrenergic receptors, muscarinic receptors, and type I angiotensin II receptors showed that the co-expression of two mutant receptors, which on their own did not bind or mediate signal tranduction, resulted in receptors that bound and transduced signals (Kobilka, 1988 #251; Maggio, 1993 #41; Monnot, 1996 #42). Furthermore, there have also been studies reporting that two non-functional receptor subtypes, such as those of the GABA family of receptors, can heterodimerize to form a functional receptor (Maggio, 1993 #41; Monnot, 1996 #42; Jones, 1998 #43; Kaupmann, 1998 #44; White, 1998 #45; White, 1998 #45). For example, it was observed in yeast that heterodimerization of GABA(B) receptor-1 and GABA(B) receptor-2 is required for the formation of a functional GABA(B) receptor that possesses similar pharmacological characteristics as the endogenous mammalian brain GABA(B) receptors (White et al., 1998). Studies such as these using chimeric and mutant receptor constructs in functional reconstitution assay has in recent years brought revival on GPCR dimerization, a theme generally and previously believed to be unrelated to signaling and most likely representing technical artifacts.

It is now evident that functional complementation can be achieved by intermolecular interaction between receptor molecules. It has been shown in previous studies that GPCRs, such as β_2 -adrenergic receptor, can interact to form functional homodimers (Gouldson et al., 1998; reviewed in Hebert and Bouvier, 1998). Moreover, Hebert et al. showed that a functionally inactive mutant of the β_2 -adrenergic receptor

(C341G) unable to form stable dimers by itself was able to dimerize with a wild-type β_2 adrenergic receptor when both were co-expressed in the same cells, and to affect downstream signaling in the same way as in cells expressing the wild-type β_2 -adrenergic receptor alone (reviewed in Hebert and Bouvier, 1998). To date, many receptors have been found to form dimers or even oligomers. These include: opioid receptors, β adrenergic receptor, α_1 and α_2 adrenergic receptor, D1 and D2 dopamine receptor, GnRH receptors, A₁ adenosine receptor, angiotensin II receptor, glutamate receptor, substance P receptor, neurokinin-2 receptor, glucagon receptor, $5HT_{1B}$ receptor, M₂ and M₃ muscarinic receptor, luteinizing hormone receptor, gonadotropin hormone releasing receptor and luteinizing hormone-releasing hormone receptor (reviewed in Hebert and Bouvier, 1998). Future studies should help determine the mechanisms underlying the formation of receptor dimers and the role of dimerization in the transduction process.

Irrespective of the form (monomer or oligomer) in which the receptor exists, its activation by an agonist, in addition to initiating signaling events, will usually result in its internalization. The process of internalization refers to the translocation of a receptor and/or ligand from the cell surface to an intracellular compartment. The terms sequestration or endocytosis also refers to the same process. Biochemically, internalization can be determined by assessing the amount of ligand that is sequestered into a cell following a hypertonic acid wash treatment, which strips off surface bound ligand, or by assessing the proportion of surface-bound receptors to intracellular receptors after ligand binding by using receptor constructs that include a fluorescent tag, thus enabling one to visualize the compartment to which the receptor is localized.

Internalization of cell surface receptors is a critically important cellular process which has been shown to be critical for both receptor desensitization and resensitization (reviewed in Tsao and Zastrow, 2000a) and may even play a role in G proteinindependent signaling (Lefkowitz and Caron, 1986; Sarret et al., 1999). Agonist-induced GPCR signaling typically requires the isomerization of the receptor to a high affinity agonist-binding conformation necessary to mediate the exchange of GDP for GTP on the heterotrimeric guanine nucleotide binding protein (G-protein) α -subunit (reviewed in Weiss and Schlessinger, 1998). Freely dissociated α - and β y-subunits then modulate the activity of numerous effector systems in the cells such as adenylyl cyclase and ion channels, among others (Gagnon et al., 1998). Besides facilitating receptor-G protein coupling, agonist binding also induces a change in GPCR conformation that is necessary for the interaction of the receptor with G-protein coupled receptor kinases (GRKs). GRKs specifically phosphorylate GPCRs at serine/threonine residues found in their carboxylterminal tails and/or third intracellular loops (reviewed in Ferguson et al., 1996a; Premont et al., 1995). GRK-mediated phosphorylation serves to promote the binding of arrestin proteins (i.e. *β*-arrestins), which when bound uncouple the receptor by preventing receptor-G protein interactions (Carman and Benovic, 1998). To date, the GRK protein family consists of six members (i.e. GRK, through GRK,), which can be further classified into subgroups according to sequence homology and functional similarities (reviewed in Ferguson et al., 1996a). The arrestin protein family also comprises six members, which arc sub-grouped on the basis of sequence homology and tissue distribution (reviewed in Ferguson et al., 1996a). Following binding of arrestin proteins and by some as yet unidentified mechanism, receptors cluster at specific sites along the plasma membrane, a process which is initiated by the recruitment of the cytosolic clathrin adaptor molecule, AP-2, to the plasma membrane, and aided by arrestins (Goodman et al., 1997; Laporte et al., 2000). The sites into which receptors cluster subsequently become sites of clathrincoated pits (reviewed in Hirst and Robinson, 1998; Larkin et al., 1986; Willingham et al., 1983). Following AP-2 binding to the plasma membrane, clathrin triskelia are moved to the plasma membrane and polymerize to form a curved polygonal lattice that provides the mechanical scaffold for the coated pit (reviewed in Hirst and Robinson, 1998; Larkin et al., 1986; Pearse et al., 2000). Shortly after the clathrin-coat is created, dynamin, which is a 100 kDa GTPase, self assembles into ring-like structures around the neck of the invaginating coated vesicle, undergoes a conformation change requiring the hydrolysis of GTP, and pinches off the coated vesicle from the plasma membrane to form a clathrincoated vesicle inside the cell (reviewed in Hinshaw, 2000; reviewed in Hirst and Robinson, 1998; Sever et al., 2000). Dynamin, AP-2, and clathrin are then released from

the vesicle and recycled to undergo another round of endocytosis (reviewed in Tsao and Zastrow, 2000a). Upon internalization, these receptors enter a common endocytic compartment via the clathrin-coated pit pathway. Subsequently, the ligands and receptors are translocated into more acidic endosomal compartments from where they dissociate and are routed to several destinations, including lysosomes, the cytosol, or the plasma membrane. (Krueger et al., 1997; Pippig et al., 1995; Roth et al., 1995; Vandenbulcke et al., 2000; Yamashiro and Maxfield, 1984).

Recent evidence suggest that GRKs and β -arrestins are required to both initiate and mediate receptor internalization (reviewed in both Ferguson et al., 1996a & Ferguson et al., 1996b). Co-expression studies revealed involvement of GRK₂ in the sequestration of muscarinic AchRs in COS-7 and BHK-21 cells whereas a mutated form of GRK₂ resulted in decreased endocytosis (Tsuga et al., 1994). Additionally, overexpression of GRK_{4.5.6} resulted in the rescue of both phosphorylation and sequestration of β_2 AR-Y326A, which is a mutant β_2 AR lacking a phosphorylation site, suggesting a general involvement of GRK-mediated phosphorylation in facilitating sequestration (Menard et al., 1996). It has also been shown that overexpression of β -arrestins can rescue sequestration of β_2 AR-Y326A, while expression of dominant-negative forms of β -arrestin result in a wild-type β_2 AR that is unable to internalize (reviewed in Ferguson et al., 1996b). Furthermore, co-expression of GRK₂ and β -arrestin led to the enhanced sequestration of β_2 AR and muscarinic AchR (reviewed in Ferguson et al., 1996b; Menard et al., 1997; Schlador and Nathanson, 1997).

Receptors such as β_2 -AR, m₁, m₃, m₄ muscarinic receptors, opioid receptors, dopamine D₁ receptor, LH/HCG receptor, GnRHR, gastrin-releasing peptide receptor, thyrotropin releasing hormone receptor, thrombin receptor, somatostatin 2a receptor, angiotensin II 1a receptor, adenosine 1A receptor, platelet activating factor receptor, olfactory receptor, cholecystokinin receptor, and parathyroid hormone receptor have all been shown to internalize employing a clathrin-coated pit mediated pathway (Anderson and Peach, 1994; Ashworth et al., 1995; Daniels and Amara, 1999; Ferrari et al., 1999; Gagnon et al., 1998; Gaudriault et al., 1997; Grady et al., 1995; Jennes et al., 1985; Koenig et al., 1997; Le Gouill et al., 1997; Luttrell et al., 1997; Rankin et al., 1999; Roettger et al., 1995b; reviewed in Tsao and Zastrow, 2000a; Vogler et al., 1999); the sequestration of all of these receptors was blocked when stimulated in the presence of a hypertonic sucrose solution which has been shown to selectively inhibit clathrindependent receptor endocytosis by disrupting the formation of coated pits and vesicles.

GPCR responsiveness to agonist stimulation wanes over time as the consequence of receptor phosphorylation by both second messenger-dependent protein kinases and GRKs. This process is termed receptor desensitization and represents a loss or dampening of receptor functional response despite the constant or persistent presence of agonist. While the molecular mechanisms contributing to GPCR desensitization are fairly well characterized, little is known about the mechanism(s) by which GPCR responsiveness is re-established, other than that receptor sequestration (internalization) might be involved. The same molecular intermediates that are required for receptor desensitization (i.e. GRKs and β -arrestins) (Benovic et al., 1987; Lohse et al., 1990) have recently been shown to be implicated also in mediating the resensitization of GPCR responsiveness. The term resensitization refers to a receptor's recovery of its functional response after desensitization. By entering the endocytic pathway, the activated receptor is able to get dephosphorylated in endosomes and recycled back to the plasma membrane where it is again responsive to agonist stimulation (reviewed in Ferguson et al., 1996a).

Although it appears that the majority of GPCRs studied to date do internalize via clathrin-coated pits, there are some receptors that have been shown to undergo a non-coated vesicle mediated internalization pathway (reviewed in Bishop, 1997). For instance, m_2 muscarinic receptor in cardiac myocytes, cholecystokinin-A receptor in CHO cells, the β -AR in human epidermoid carcinoma A-431 cells, bradykinin B₂ receptor and the platelet derived growth factor have all been shown to be targeted to caveola following agonist binding (Haasemann et al., 1998; Liu et al., 1996; Raposo et al., 1989; Roettger et al., 1995a; Roettger et al., 1995b). Caveola are non-coated plasma membrane

30

invaginations that contain the structural protein caveolin and serve as molecular scaffolds that provide binding sites for GPCRs and other signaling proteins. They can either internalize into the cell interior or to persist in the proximity of the plasma membrane (Parton et al., 1994).

Lastly, in pancreatic acinar cells, agonist stimulation induces migration of cholccystokinin-A receptors to a plasmalemma site which differs from caveolae or clathrin-coated pits where it becomes insulated from acid wash and does not respond further to agonist stimulation (Bishop, 1997; Roettger et al., 1995a). The functional significance of these alternative internalization pathways is unknown.

μ and δ opioid receptor internalization and trafficking

Binding of endogenous or exogenous opioid peptides causes MORs and DORs to promote guanine nucleotide exchange of heterotrimeric G-proteins of the Gi/Go class. Receptor-mediated activation of these G-proteins triggers the acute down-stream signaling of opioid receptors, including regulation of adenylate cyclase, activation of protein kinases, G-protein-gated inwardly rectifying K+ channels, and voltage-gated calcium channels (Dhawan et al., 1996; Fukuda et al., 1996). In the continued presence of agonist, the acute actions of receptor activation are followed by regulatory processes, such as desensitization and internalization that modulate the number and functional activity of opioid receptors present on the plasma membrane. The rapid process of receptor internalization, which occurs within several minutes after MOR or DOR activation, has been observed in transfected cells in vitro (Gaudriault et al., 1997; Keith et al., 1996; Trapaidze et al., 1996) and in myenteric neurons in vivo (Sternini et al., 1996). Down-regulation, a much slower process involving decreased receptor synthesis, can be observed after several hours of continuous exposure to agonists (Law et al., 1982; von Zastrow et al., 1993) Internalization of opioid receptors seems to depend on clathrincoated pits (Chu et al., 1997; Gaudriault et al., 1997; Keith et al., 1996) and a population

of endocytic vesicles similar or identical to those that mediate the endocytic trafficking of constitutively recycling transferrin receptor (Keith et al., 1996).

Opioid receptor internalization, whether in vivo (rat CNS) (Keith et al., 1998; Sternini et al., 1996) or in vitro (transfected cells) (Burford et al., 1998; Gaudriault et al., 1997; Keith et al., 1998; Trapaidze et al., 2000; Trapaidze et al., 1996), appears to depend on the type of opioid agonist to which it is exposed. For instance, morphine failed to induce internalization of MOR whereas etorphine triggered rapid internalization in heterologous transfection systems (Burford et al., 1998; Keith et al., 1998; Whistler et al., 1999). Even in the presence of saturating concentrations of morphine, which caused maximal receptor-mediated inhibition of adenylyl cyclase in stably transfected cells, MOR remained in the plasma membrane and were not internalized (Keith et al., 1996). These observation have been reproduced in vivo in myenteric neurons, which express native MOR (Sternini et al., 1996). Other drugs that also activate MOR such as DAMGO and methadone cause internalization of MORs (Burford et al., 1998; Whistler et al., 1999). MOR endogenous opioid ligand endomorphin-1 was also shown to cause MOR internalization (Burford et al., 1998). To determine if the differential effects of opioid agonist on internalization were due to differential receptor activation, Whistler et al. measured receptor-mediated activation of inwardly rectifying potassium channels (K_{IR} channels) in HEK 293 cells (Whistler et al., 1999). Using this system, they showed that morphine is substantially more effective than methadone and only marginally less effective than DAMGO in activating K_{IR} channels (Whistler et al., 1999). This observation led to the conclusion that the ability of these drugs to induce internalization could not be due to differences in agonist efficacy (Whistler et al., 1999). In follow up studies, these investigators constructed a chimeric μ/δ opioid receptor in which the cytoplasmic C terminus of MOR was replaced with the C terminus of the DOR (Whistler et al., 1999). Unlike for the native MOR, phosphorylation, recruitment of arrestins to the plasma membrane, and subsequent internalization of this chimeric receptor occurred in response to morphine (Whistler et al., 1999). These observations were further confirmed in primary hippocampal neuronal cultures that were transfected with native and chimeric

MORs using adenovirus-mediated gene tranfer (Whistler et al., 1999). It was proposed recently that the ability of distinct opioid agonists to differentially induce MOR internalization may be related to their ability to promote G protein-coupled receptor kinase (GRK)-dependent phosphorylation of the receptor. Zhang et al. have recently reported that overexpression of GRK2 resulted in the enhancement of MOR sequestration and in the rescue of MOR-mediated β -arrestin translocation (Zhang et al., 1999). Although these observations have enhanced our understanding of the possible molecular mechanisms underlying MOR differential response to different agonists, clear explanation of this phenomenon is still pending. These studies suggest the possibility that endocytic regulatory mechanism may play an important role in distinguishing the physiological actions of individual opiate analgesic drugs in the CNS and be the key determinants of the signals that underlie the cognitive and behavioral components of opiate tolerance and behavior.

As mentioned previously, DORs are known to undergo rapid, agonist-induced endocytosis by a conserved, β -arrestin and dynamin dependent mechanism mediated by clathrin-coated pits (Chu et al., 1997; Keith et al., 1996; Trapaidze et al., 1996; von Zastrow et al., 1994; Zhang et al., 1999). By generating various mutants of DORs lacking different portions of their C-terminal tail, especially point mutations of any of the Ser/Thr between Ser³⁴⁴ and Ser³⁶³, it was observed that these receptors exhibited a significant reduction in the rate of internalization (Trapaidze et al., 1996). A further study carried out by the same investigators after fluorescently labeling surface DORs with a specific anti-DOR antibody showed that in the absence of agonists, the mutant DORs exhibited a significantly higher level of constitutive internalization as compared to the wild type receptors (Trapaidze and Devi, 1999). This agonist-independent DOR internalization was also found to be blocked by co-expression of a dominant negative mutant of β -arrestin. Taken together, these two studies suggest that, in addition to agonist-dependent internalization, DORs undergo agonist-independent constitutive internalization via the clathrin-coated pit mediated pathway and that the receptor Cterminal tail plays an important role in the process of DOR internalization.

Investigation on HEK 293 cells by Zhang et al. has further shed light on the cellular regulation of DOR and MOR (Zhang et al., 1999). This group of investigators have already reported that differences in the ability of distinct agonists to promote receptor sequestration are related to their ability to induce GRK-mediated phosphorylation of the MOR. While both etorphine and morphine effectively activate DOR, only etorphine was observed to trigger robust DOR phosphorylation followed by plasma membrane translocation of beta-arrestin and receptor internalization. In contrast, morphine is unable to either elicit DOR phosphorylation or stimulate beta-arrestin translocation, correlating with its inability to cause DOR internalization. Unlike for the MOR, overexpression of GRK2 results in neither the enhancement of DOR sequestration nor the rescue of DOR-mediated beta-arrestin translocation. Therefore, this study not only suggests the existence of marked differences in the ability of different opioid agonists to promote DOR phosphorylation by GRK and binding to beta-arrestin, but also demonstrate differences in the regulation of two opioid receptor subtypes. These observation may have important implications for our understanding of how opioid tolerance and addiction can arise from activation by various opioids.

Recent observations in our laboratory, using biochemical and confocal microscopy studies, have provided a greater insight for the intracellular routing of MOR and DOR before and after agonist binding (Gaudriault et al., 1997). Using fluorescent ligand binding assays, MOR and DOR co-expressed in the same cells were found to internalize through partly distinct endocytic pathways as labeling of both receptors did not overlap completely with time. This suggested the possibility that opioid receptor subtypes may be sorted via different endocytic vesicles, suggesting also that each of these receptors could interact with distinct proteins mediating intracellular sorting and trafficking. In a recent report, Chu et al. had, in fact, confirmed our observations and further provided added understanding to the cellular mechanism underlying differential opioid receptor sorting after endocytosis (Chu et al., 1997). Using the alkaloid drug etorphine, a potent agonist of mu, delta, and kappa opioid receptors, it was observed that DORs were internalized from the plasma membrane within 10 min while KORs

expressed in the same cells remained in the plasma membrane and were not internalized for 60 min, even when cells are exposed to saturating concentrations of etorphine. It was further observed that the rapid internalization of DORs could be specifically inhibited in cells expressing K44E mutant dynamin I, suggesting that type-specific internalization of opioid receptors was mediated by clathrin-coated pits. Examination of a series of chimeric mutant kappa/delta receptors indicated that at least two receptor domains, including the highly divergent carboxyl-terminal cytoplasmic tail, determined the type specificity of this endocytic mechanism. Therefore, structurally homologous opioid receptors have been shown here to be differentially sorted by clathrin-mediated endocytosis following activation by a same agonist ligand.

Biochemical studies have suggested that DOR homodimerization might be critical for agonist-induced internalization (Cvejic and Devi, 1997). It was observed that a mutant DOR monomer lacking a C-terminal tail was unable to dimerize and to undergo agonistinduced internalization. It was further proposed that monomerization of DOR preceded internalization and was a prerequisite for the latter process to occur. It can be envisioned therefore that homodimerization of DOR upon ligand exposure, followed by subsequent monomerization of the receptor, play both crucial regulatory roles in allowing internalization of the receptor to occur (Cvejic and Devi, 1997).

A recent study on stably transfected HEK293 cells furthered our understanding of the fate of DOR following agonist-induced internalization (Tsao and von Zastrow, 2000b). Using radioligand binding to detect functional receptors and immunoblotting to detect total flag-tagged receptor proteins, these investigators reported that DOR exhibited substantial (>50%) agonist-induced down-regulation after being continuously exposed for 3 hours to DOR-specific agonists. Disappearance of DORs was sensitive to inhibitors of lysosomal proteolysis. Using fluorescent flow cytometric and surface biotinylation assays, they further noticed that differential sorting of DOR into non-recycling pathways, unlike a parallel observation on β_2 -adrenergic receptor which was sorted into the recycling pathways, could be detected within 10 min. after internalization, significantly before the onset of detectable proteolytic degradation of receptors (~60 min. postinternalization). Through additional pulsatile application of agonist, it was found that continued presence of agonist was not needed for subsequent steps of membrane transport leading to lysosomal degradation of DORs following internalization and specific sorting of the receptor. With the knowledge that both DOR and β_2 -adrenergic receptor employ the classic clathrin-mediated pathway, these findings suggests that distinct GPCRs differ significantly in endocytic membrane trafficking after internalization by the same membrane mechanism and that brief application of agonist can induce substantial downregulation of receptors, such as that seen with DORs.

OPIOID RECEPTOR INTERACTION

Interactions between opioid receptor sub-types has been extensively studied pharmacologically. Antinociceptive synergy between MOR and DOR agonists has also been well documented in several studies (Hassan et al., 1993; Jiang et al., 1990; Malmberg and Yaksh, 1992; Ossipov et al., 1995).Recent studies using specific opioid receptor subtype agonists in MOR gene knockout mice suggest that MOR may play a role in δ -mediated analgesia (reviewed in Childers, 1997). As expected, it was observed that MOR- mediated analgesia was totally climinated in MOR knockout mice. However, in the same animals, δ -mediated analgesia was also partially reduced. Similarly, studies performed by He et al. suggested that the selective δ agonist DPDPE mediated antinociception in the spinal cord through MORs. In this study, they observed that DPDPE-mcdiated antinociception in the spinal cord could be antagonized by CTAP, a MOR-selective antagonist. This was not to say that DORs were not involved in the antinociceptive effects of the drug since they were also abolished by naltrindole, a DORselective antagonist. Subsequently, when DAMGO (MOR agonist) and DPDPE were administered intrathecally at ratios raging from 1:200 to 1:500, the antinociceptive dose (AD_{so}) of DAMGO was lowered as much as 10-fold relative to its AD_{so} when given alone. Thus DPDPE exhibited a potentiating effect on DAMGO. The reverse was not seen. Furthermore, it was also observed that such potentiation was lost in animals made

tolerant to systemic morphine. It was proposed that in morphine tolerant rats, DOR function was altered as the AD_{so} of DAMGO given intrathecally alone to tolerant animals was about the same as for naïve animals while the AD_{so} of DPDPE given alone increased by 4-fold. Moreover, the AD_{so} of DPDPE in tolerant animals was only slightly increased by naltrindole whereas CTAP still exhibited potent antagonism. Thus this study provided a strong evidence that δ -mediated analgesia, at least in the spinal cord, is mediated through MORs (He and Lee, 1998).

In another study, Jordan et Devi (1999), reported that heterodimerization of two fully functional DOR and KOR resulted in a new receptor that displayed ligand binding and functional properties clearly distinct from those of the individual receptors (Jordan and Devi, 1999). They further reported that this KOR-DOR heterodimerization (1) led to the development of a new binding site that is able to strongly bind highly selective ligands synergistically, (2) altered the trafficking properties of these receptors, and (3) was activated synergistically by selective ligands. These findings provide further evidence that heterodimerization represents a potent mechanism for modulating receptor function (see above). It was recently proposed that closely related receptors, such as DOR and MOR, may interact with each other directly and thus have the potential to create novel signaling units (George et al., 2000). Co-expressing both MOR and DOR receptors, thes authors observed a functional enhancement in the affinity of these receptors for endomorphin-1 and Leu-enkephalin, respectively, over that observed when MOR and DOR were expressed individually. This suggested the formation of a novel binding pocket. Moreover, hetero-oligomers were identified by co-immunoprecipitation and it was observed that these co-precipitating receptors showed insensitivity to pertussis toxin (unlike the individually expressed receptors) and continued signal transduction. This study, together with a more recently published one by Devi et al. (2000), thus suggests that MOR and DOR are able to form oligomers, with the generation of novel pharmacology, and to interact with different subtypes of G-protein.

Most of the knowledge accrued to date regarding opioid receptors- and more

specifically MORs and DORs- has been derived from studies done mostly on neuroblastoma cell lines or on non-neuronal systems. Thus questions of whether these findings in the heterologous cell systems would reflect what really goes on in the native neuronal systems remain. Studies have yet to be performed in *in vitro* and in *in vivo* neuronal systems to determine or confirm the mechanisms underlying MOR and DOR regulation, activation and signaling, internalization as well as their subsequent fate upon internalization. As mentioned previously, there is evidence that MOR does internalize in neurons, but there is no report in the literature to date in this regard about DOR. Furthermore, the fate of bound ligands in the course of MOR and DOR internalization needs to be answered. Do these ligands internalize with their receptors? Are they depleted at the site of internalization? Are they trafficked into their target cells? At the same time, what is the fate of internalized receptors? Where do they dissociate from their ligand? And are they recycled? The present study seeks to address some these questions.

AIM OF THE STUDY

In order to understand more in depth how opioid receptors effect their physiological functions, certain facts such as their regulation, ligand-receptor interaction, as well as their processing and trafficking within neuronal cells need to be first thoroughly established. Clinically, the knowledge of how exogenous drugs interact and modulate the regulation of MOR and DOR bears great implications with regards to better and more effective pain management. Therefore, the present investigation has the aim to describe the processes of internalization and trafficking of MOR and DOR in *in vitro* rat cortical neurons upon their timely exposure to the selective fluorescent derivatives fluodermorphin and fluo-deltorphin, respectively. Neurons derived from the rat cortices were used in this study because high amounts of both MOR and DOR are found in this region, thus facilitating the parallel study of both receptors. While DORs are moderately-to-highly concentrated in the outer (I-II) and inner laminae (V-VI), MOR sites are mainly found in the mid-cortical laminae (layers III-IV) (Sharif, 1989). The second reason for choosing this region in our investigation relates to the relative technical ease to culture this neuronal population.

We also set out to investigate the fate of MOR and DOR in the above processes. A final aim in our study is to determine the effect of chronic treatment with naloxone and morphine on the internalization and trafficking of both MOR and DOR and their respective ligands. In this report, we propose that whereas the internalized ligands are targeted to the cell body, their receptors appear to recycle locally following internalization. We also postulate that chronic naloxone treatment upregulates MOR but not DOR and that chronic morphine treatment decreases the number of surface MOR, but upregulates and increases the number of cell surface DOR available for internalization. Finally, although there is less internalization of fluo-dermorphin in morphine treated cultures, chronic treatment with naloxone or morphine modifies distribution of internalized ligand to some extent, but they do not generally result in changes to its kinetics of internalization.

MATERIALS AND METHODS

Preparation of Receptor-encoding Plasmids

Rat μ (MOR) (Thompson, 1993) and (DOR) (Abood, 1994) opioid receptor cDNAs were amplified from rat brain cDNAs by polymerase chain reaction with specific oligonucleotides. Polymerase chain reaction products were subcloned in pcDNAI. pcDNAI-MOR, and pcDNAI-DOR were transfected in COS cells by the DEAE-Dextran method (Perlman, 1992).

Culture and transfection of COS-7 cells

COS-7 cells were grown at 37°C in humidified conditions of 5% CO₂/ 95% oxygen in Dulbecco's modified Eagle's medium (DMEM; GibcoBRL, NY) supplemented with 10% Fetal Bovine Serum (FBS; Harlan Bioproducts Inc., Indianapolis, IN) and 1% penicillin/ streptomycin (GibcoBRL, NY). COS-7 cells were passaged every 3-4 days. Approximately 10⁶ cells/ 100 mm diameter culture dish were seeded the night before carrying out the transfection procedure. Employing DEAE-dextran/chloroquine method of transfection, 1 μ g/ml of pcDNAI-MOR or pcDNAI-DOR was used per dish. After 48-72 h, cells were harvested with a PBS solution containing 0.05% trypsin and 0.53 mM EDTA, plated onto poly-L-lysine-coated coverslips in four-well tissue culture plates and equilibrated with 10% FBS in DMEM for 2 h at 37°C prior to experimentation.

Cortical cell culture was prepared from P1 Sprague Dawley rats. In short, P1 litters were sacrificed by rapid decapitation and their brains were quickly removed from the skull and washed in icc-cold Hank's Balanced Salt Solution (HBSS without Ca⁺⁺ or Mg⁺⁺) (Gibco BRL, Burlington, Ont., Canada). Cortical hemispheres were dissected and transferred into 15 ml Falcon tubes containing cold HBSS. The tissue was then washed 3 times in HBSS, transferred into a sterile 15 ml Falcon tube and rinsed 3 more times with HBSS before exposure to Trypsin (Gibco BRL, Burlington, Ont., Canada) for 15 minutes at 37°C. Trypsin was thoroughly washed away by rinsing the tissue 3 times with HBSS, followed by three additional washes with culture medium (DMEM supplemented with 20 mM KCl, 110 mg/ml sodium pyruvate, 2 mM glutamine, 100 µg/ 100 ml penicillin/streptomycin, and 50 µg/ 100 ml fungizone). Corticies were immersed in culture medium containing 10% FBS and the cells were mechanically separated by gentle trituration through three fire-constricted pasteur pipette of decreasing tip diameter. The resulting cloudy cell suspension was filtered through a sterile 70 µm nylon screen into a 50 ml Falcon tube and diluted with 10% FBS-containing culture medium to a final concentration of 2 x 10^6 cells/ml. One hundred microliters of this cell suspension was then seeded onto poly-L-lysine-coated glass coverslips in the wells of 24-wells culture plates containing 900 µl of culture medium supplemented with 2% B27 growth factors (Gibco, Burlington, Ont., Canada). Neurons were grown an atmospheric condition of 5% CO₂/ 95% O₂ at 37°C. Cortical cells were then used for experiments after 7-9 days in culture at which point the neurons are fully differentiated. All chemicals used for cell culture were purchased from Life Technologies (Burlington, Ont., Canada).

Binding and internalization of ω-Bodipy DRM-I 5APA and ω-Bodipy DLT-I 5APA in transfected COS-7 cells

The specificity of fluorescent-labeled agonists for the μ opioid receptor, ω -Bodipy 576/589 [K7]DRM-I 5APA, and for the δ opioid receptor, ω -Bodipy 576/589 DLT-I 5APA (kindly provided by Dr. J.P. Vincent, University of Nice), was determined in COS-7 cells transiently transfected with either pcDNAI-MOR or pcDNAI-DOR. Cells were pre-incubated for 10 minutes at 37°C in Earle-HEPES binding buffer (140 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 0.9 mM MgCl₂, and 25 mM HEPES) supplemented with 0.8 mM of the peptidase inhibitor 1,10 Phenantroline (Sigma, St. Louis, MO), 0.01% Bacitracin, 0.09% Glucose and 0.2% Bovine Serum Albumin (BSA; Bochringer Mannheim, QC., Canada) followed by incubation for 30 minutes with either 10 nM fluo-DRM or fluo-DLT-I and rinsing in ice-cold Earle-HEPES buffer. To demonstrate fluoligand internalization, ligand-exposed cells were subjected to hypertonic acid buffer wash (2.92 g NaCl/ 100 ml Earle-HEPES buffer adjusted to a final pH of 4.0 with glacial acetic acid) for 2 minutes in order to strip off all surface bound ligand and thereby reveal only internalized fluo-ligands. Cells were then fixed with 4% paraformaldehyde (PFA; in 0.1 M phosphate buffer (PB), pH 7.4) for 20-30 minutes at 37°C, rinsed with ice-cold Earle-HEPES buffer and mounted onto slides with Aquamount (Polyscience, ON, Canada). Cells were viewed under a Zeiss laser scanning confocal microscope attached to an Axiovert 100 inverted microscope (Carl Zeiss Canada Ltd., Con Mills, Ont., Canada). To test for the selectivity and specificity of binding, experiments using each of these fluoligands were carried out in pcDNA-MOR- and pcDNA-DOR- transfected COS-7 cells as well as in control non-transfected COS-7 cells. Specificity of binding was further tested

by the addition of 10μ M naloxone, an opioid receptor antagonist. The displaceability of the fluo-ligands as assessed under confocal microscopy serves as an indicator of the specificity of binding.

Binding and internalization of fluo-DRM and fluo-DLT-I in primary cortical cultures

Binding procedures on primary cortical neurons were performed exactly as described above for COS-7 cells. The only alteration made to the protocol was the incubation times with fluorescent opioid receptor ligands. Binding of fluo-DRM and fluo-DLT-I was determined after both 5 and 30 minutes of ligand exposure in primary cortical neurons in order to study the changes in the distribution of internalized ligand with time.

In order to determine whether the internalization of the ligand-receptor complex was mediated through clathrin-coated pits, neurons were incubated for 30 minutes in equilibration buffer (supplemented Earles-HEPES buffer) containing the fluorescent ligand in the presence or absence of the endocytosis inhibitor phenylarsine oxide (PAO; Sigma, St. Louis, MO). The drug was at a concentration of 10 μ M at which protein internalization was reported to be optimal and cellular deleterious effects to be minimal (Gibson et al., 1989). For the neuronal cultures that were treated with PAO, a pre-incubation with the same concentration of PAO for 30 minutes was performed. Other experiments were performed in the presence or absence of Nocodazole (Sigma, St. Louis, MO), a microtubule assembly blocker, in order determine whether the distribution of internalized ligand was microtubule-dependent. For this experiment, cells were pre-incubated for 60 or 90 minutes in equilibration buffer containing 10 μ M Nocodazole (Yu

et al., 1993; Takatsuki et al., 1993) prior to the binding of fluorescent ligands. For both experiments, the binding of fluo-DRM or fluo-DLT-I was carried out in the presence of the same concentration of the respective drug above and treated as described in the binding procedure.

Immunocytochemistry

To determine the specificity and selectivity of both rabbit anti-MOR (1 μ g/ml; Incstar Corp., Stillwater, Minnesota), a polyclonal antibody that recognizes residues 384-398 from the predicted C-terminus of the rat MOR1 protein, and rabbit anti-DOR (1:10000, Chemicon, Temecula, CA), which is a polyclonal antibody that recognizes residues 3-17 (LVPSARAELQSSPLV) from the predicted amino acid sequence of the Nterminus of the mouse DOR protein (Keiffer et al., 1992), immunocytochemitry was carried out in pcDNAI-MOR transfected, pcDNAI-DOR transfected as well as nontransfected COS-7 cells. Immunocytochemistry was performed as follows. pcDNAI-MOR transfected, pcDNAI-DOR transfected and non-transfected COS-7 cells were equilibrated for 10 minutes at 37°C in pre-warmed Earle-HEPES binding buffer. The cells were then rinsed three times in 0.1 M PB, pH 7.4, and fixed with 4% PFA for 20-30 minutes at 37°C. Following fixation, these cells were rinsed twice with 0.1 M PB and twice with 0.1 M Tris-buffered saline (TBS), pH 7.4, and pre-incubated in TBS containing 10% normal goat serum (NGS) and 0.1% triton X-100 for 15 minutes at 37°C. They were then incubated overnight at 4°C with rabbit anti-MOR or rabbit anti-DOR in antibody dilution buffer (TBS supplemented with 0.5 NGS and 0.1 triton X-100). The following day, the cells were rinsed three times with TBS and incubated with goat antirabbit-texas red at a dilution of 1:200 (purchased from Jackson ImmunoResearch Laboratories Inc., West Grove, PA) in antibody dilution buffer for 45-60 minutes at room temperature. At the end of the incubation, cells were rinsed three times with TBS and mounted onto slides with Aquamount. The specificity of the secondary goat anti-rabbit antibody was tested on these cells by incubating it in the absence of the primary antibody.

To determine the phenotype and proportion of opioid receptor expressing neurons as well as to study the changes in the distribution of activated opioid receptor over time, simultaneous double immunocytochemistry with or without agonist pre-exposure for varying time-points (5 and 30 minutes) was performed on primary cortical neurons with a few modifications of the protocol used for COS-7 cells. Neurons were equilibrated for 10 minutes at 37°C in pre-warmed Earle-HEPES binding buffer followed by incubation with non-fluorescent ligand for 5 or 30 minutes. Neurons were then rinsed six times in 0.1 M PB, pH 7.4, and fixed with 4% PFA for 20-30 minutes at 37°C. The rest of the experimental procedure was identical to the protocol employed for COS-7 cells. Additionally, to correctly identify neurons in our culture from glial cells, a selective marker for neurons, namely a mouse antibody against microtubule associated protein-2 (anti-MAP-2, Bochringer Mannheim, QC, Canada), was incubated simultaneously with anti-MOR or anti-DOR at a dilution of 1:500 in antibody dilution buffer (TBS supplemented with 0.5% NGS and 0.1% triton X-100). Furthermore, to assess whether the distribution of the opioid receptor was microtubule-dependent, additional experiments were performed in which neurons were pre-incubated for 60 minutes with Nocodazole prior to carrying out the immunocytochemical procedure.

Chronic Naloxone or Morphine pre-treatment experiments on primary cortical neurons

Regulation of MOR and DOR was further studied in primary neurons by determining the effects of chronic treatment with either naloxone or morphine. For these experiments, neurons were grown in culture for 6-7 days or until fully differentiated and then subjected to 48 hours chronic exposure to 10 μ M of naloxone or 10 μ M Morphine. Cultures were then rinsed with and preincubated for 10 minutes in Earle-HEPES binding buffer before proceeding with the fluorescent ligand internalization assay. Both ligand internalization studies and immunocytochemistry experiments were performed on these cells in order to investigate the effects of these treatments on opioid ligand and receptor trafficking. All experiments were performed in parallel with and in exact similar fashion as those done for the untreated control cultures.

Confocal microscopy, image processing, image analysis and statistical calculations

Fluorescent ligand or immunocytochemically labeled cells were examined and images acquired on a Zeiss laser scanning confocal microscope attached to an Axiovert 100 inverted microscope (Carl Zeiss Canada Ltd., Con Mills, ON., Canada) and operated via a IBM-compatible computer. The acquired images were processed using Adobe Photoshop v.4.0.1 and Adobe Illustrator v.7.0 (Adobe Systems Inc., San Jose, CA) on an IBM-compatible computer.

Single optical sections through the center of the cells were used to acquire images. The corresponding resolution was 32 scans/frame for the immunolabeled neurons and 8 scans/frame for the bodipy fluo-ligand labeled neurons. Red dyes, including Texas Red, fluo-DRM and fluo-DLT-I labelings were imaged in the LUTS mode. For green dyes, such as in FITC-antibody immunostainings, the RGB mode was utilized. Images from immunocytochemical double-labeling of rat primary cultures were acquired sequentially in the red and in the green channel. The parameters used for image acquisition of the fluo-DRM and fluo-DLT-I labeled neurons were set constant across all experimental conditions. The same principle of preset constant parameters was applied to the image acquisition of MOR- and DOR-immunoreactive neurons. This allows for the comparison and evaluation of the changes that might occur across the different conditions. Furthermore, in order to ascertain neuronal identity in fluo-ligand binding experiments, concurrent phase-contrast images were acquired and used to discriminate cortical neurons from glial cells. Standard neuronal morphologies employed in this phase contrast discrimination approach were derived from anti-MAP2 immunostaining studies of the neurons in our system. On the other hand, cell phenotype was identified in immunocytochemical experiments on the basis of MAP-2 immunoreactivity.

Quantification of the acquired images from fluo-DRM and fluo-DLT-I binding experiments was performed using Biocom 200 Photometric System for Image Analysis software v.1.4 (Biocom Imagerie Instrumentation Biotechnologique, ZA COURTABOEUF, B.P. 53-91942, FRANCE) running on an IBM-compatible computer and connected to a Panasonic WV-CD50 camera for digitization of analogue images. Using autoradiography (RAG), a software component of BIOCOM 200, cell area, area of labeled fluorescent "hot spots" as well as number of fluorescent "hot spots" were obtained. Total surface of fluorescently labeled areas were then expressed as a ratio of the total cell surface area, the result of which is termed hereafter as *Cell Occupancy Ratio*. Data are mean of three experiments, each performed in duplicate. Statistical analyses for these binding experiments were done using Kruskal-Wallis One-Way Analysis of Variance.

Analysis of the images from the immunolabeling experiments was done using Scion Image Software (Scion Corporation, Frederick, Maryland 21703, USA). Surface area of neuronal soma and processes as well as integrated fluorescent densities were measured using this software. An average of the integrated density per unit area was calculated from measurements of neurons taken from the same experiment. When studying trafficking and distribution of MOR and DOR in the soma and along the processes, the integrated density per unit area measured in the soma and along the processes was expressed as a percentage of the total integrated density as measured in the whole cell. The parameter for all acquisitions were kept absolutely identical for all of these quantification experiments. Data are out of three experiments, each performed in duplicate. Statistical analyses for the immunolabeling studies were done using Twosample t-test combined with Dunn-Sidak and Bonferroni Adjusted Probabilities.

48

RESULTS

ANTIBODY SPECIFICITY

The specificity of the MOR antibody, directed against amino acids 384-398 in the C-terminus of the rat MOR, was determined by immunocytochemical experiments in pcDNA-MOR-transfected COS-7 cells. Rabbit anti-MOR-labeled pcDNA-MOR-transfected COS-7 cells showed strong, diffuse immunoreactivity throughout the cytoplasm of the cells (Figure 1-A). There was no obvious preferential distribution of the label at the level of the plasma membrane. Nuclei were entirely label-free. No labeling was observed in either non-transfected controls or in pcDNA-DOR-transfected COS-7 cells (Figure 1-B, C). Omission of the primary MOR antibody also resulted in the absence of immunolabeling in pcDNA-MOR transfected COS-7 cells (Figure 1-D).

DOR receptors expressed in pcDNA-DOR-transfected COS-7 cells showed specific immunostaining with a DOR antibody that recognizes residues 3-17 (LVPSARAELQSSPLV) from the predicted amino acid sequence of the N-terminus of the mouse DOR protein (Keiffer et al., 1992). Similar to that observed for MOR, immunolabeling with rabbit anti-DOR showed a strong, diffuse intracytoplasmic labeling of the cells (Figure 2-A). The cellular plasma membrane was not preferentially labeled. The nuclei were also entirely label-free. No immunoreactivity was observable in non-transfected controls nor in pcDNA-MOR-transfected COS-7 cells (Figure 2-B, C). Moreover, no immunolabeling was detectable when the primary DOR antibody was omitted (Figure 2-D).

FLUORESCENT LIGAND SPECIFICITY

Fluorescent agonists fluo-DRM and fluo-DLT-I, respective derivatives of [Lys7] dermorphin and deltorphin-I were used in the present investigation to study opioid peptide internalization. A previous study had demonstrated the specific binding and internalization of these ligands in transfected COS-7 cells (Gaudriault et al. 1997). Nonetheless, the specificity was confirmed in the present study in COS-7 cells transiently transfected with pcDNAI-MOR- or pcDNAI-DOR.

Confocal microscopic examination of pcDNAI-MOR -transfected COS-7 exposed to 10 nM fluo-DRM for 30 minutes and rinsed with either isotonic ice-cold Earles-HEPES binding buffer or with hypertonic acid buffer (to dissociate surface-bound ligand) revealed specific fluorescent labeling of nearly 15-20 % of the total number of cells present in the cultures (Figure 3A). This labeling yield is in keeping with the reported efficiency of transfection using the DEA-Dextran method in this cell line. At this 30 minutes time point, both in the acid and non acid-washed conditions (images for the latter not shown), labeling was observed to form multiple small fluorescent clusters in the cytoplasm of the cell. Binding to MOR was specific as labeling was no longer detected when the incubation was carried out in the presence of 10 µM naloxone, a non-specific opioid receptor antagonist (Figure 3B). No labeling was observed in either pcDNAI-DOR-transfected or non-transfected control COS-7 cells (Figure 3C, D).

Similarly, 15-20 % of pcDNA-DOR-transfected COS-7 cells incubated with fluo-DLT-I for 30 minutes followed by either isotonic or acid wash showed specific fluorescent labeling (Figure 4A). Again, labeling was displaceable with 10 μ M naloxone in both treatments and was completely absent in control non-transfected or pcDNA-MOR transfected COS-7 cells (Figure 4B, C, D).

CHARACTERIZATION OF CELLS EXPRESSING MOR AND DOR IN RAT CORTICAL PRIMARY CULTURES BY IMMUNOCYTOCHEMISTRY IN STEADY STATE (PRIOR TO EXOGENOUS LIGAND EXPOSURE) CONDITIONS.

Simultaneous incubation of the cortical neuronal cultures with neuron-specific mouse anti-MAP2 monoclonal antibody and glia-specific rabbit anti-GFAP polyclonal antibody revealed the presence of both neurons and glial cells within our primary cultures (result not shown). Omission of these primary antibodies resulted in no immunoreactivity (result not shown).

Immunolabeling of P1 rat cortical neuronal cells with MOR antibody revealed that $5.69 \pm 0.86\%$ (n=123) of neurons in culture stained positively for MOR (Figures 5A and 6-"MOR" *solid bar*). These neurons were distinguished from glial cells by their colabeling with anti-MAP2 monoclonal antibody (Figure 5A'). The average cross sectional surface area for these cells were $54.535 \pm 3.880 \ \mu\text{m}^2$ (n= 22). Morphologically, MORimmunopositive P1 rat cortical neurons were either polyhedral or bipolar in shape. Observations from confocal images of neurons immunolabeled for MOR in the absence of ligand stimulation (i.e. at 0 minutes) revealed a fairly homogeneous diffuse labeling in the soma and along segments of the processes (Figure 5A).

Immunolabeling of cortical neurons with anti-DOR antibody revealed that 11.60 \pm 0.66% (n= 250) of neurons in culture expressed DOR (Figure 5B and 6-"DOR" solid bar). These neurons were distinguished from glial cells by their co-labeling with neuron-specific anti-MAP2 monoclonal antibody (Figure 5B'). The average cross sectional surface area of DOR+ neurons was 60.986 \pm 2.287 μ m² (n=100). This value does not significantly differ from that obtained for MOR+ neurons (P=0.760; unpaired two-tailed t-test). As for MOR immunoreactive cells, one population of DOR+ neurons was

polyhedral while the other displayed a bipolar morphology. In the absence of ligand stimulation (i.e. at 0 minutes), anti-DOR immunocytochemistry showed intense DOR immunoreactivity diffusely and homogeneously distributed in the soma and in segments along the processes of these cortical neurons in culture (Figure 5B).

Some vesicular-like fluorescence was detected in the glial cells in our cultures following incubation with either anti-MOR or anti-DOR antibody. It is not known whether this labeling is specific as further investigation of this population subset of our culture was not carried out in the present study.

CHARACTERIZATION OF CELLS EXPRESSING MOR AND DOR IN P1 RAT CORTICAL PRIMARY CULTURES BY FLUO-LIGAND BINDING EXPERIMENTS.

Analysis of P1 rat cortical neurons in culture following 30 minutes incubation with 10 nM fluo-DRM and washing with isotonic non-acid solution revealed that $6.34 \pm$ 0.50% (n=142) of these neurons bound fluo-DRM at 37°C (Figure 7A; Figure 6-"MOR", *open bar*). This total binding was specific as it was no longer detectable when the incubation was carried out in the presence of 10 µM naloxone (Figure 7A'). The proportion of cells that bound to fluo-DRM within our cultures was not significantly different from that measured by MOR-immunolabeling (5.69± 0.86%; P= 0.696) (Figure 6-"MOR" *open bar* vs. *solid bar*). Confocal microscopic examination revealed a strong punctate fluorescent labeling that was contained within vesicular-like structures and distributed in the soma and along neuronal processes (Figure 7A). Nuclei were label-free.

Similarly, total fluo-DLT-I binding experiments showed that $11.49 \pm 0.55\%$ (n= 348) of the P1 rat cortical neurons in culture were selectively labeled following 30

minutes of incubation with 10 nM fluo-DLT-I at 37°C (Figure 6-"DOR" open bar and Figure 7B). Again, binding was specific as it was abolished by the addition of 10 μ M naloxone (Figure 7B'). This proportion of DOR+ cortical neurons was not statistically different from that determined by DOR immunostaining (11.60 ± 0.66%) (P=0.841) (Figure 6-"DOR" solid bar). Examination of fluorescent labeling under the confocal microscope revealed, in general, diffuse punctate labeling contained in vesicular-like structures and located prominently in the soma and, to a lesser extent, along the processes. No labeling was detected in the nuclei. The labeling was as intense as that observed in fluo-DRM-labeled neurons (Figure 7B).

ASSESSMENT OF INTERNALIZATION OF FLUO-DRM AND FLUO-DLT-I IN RAT CORTICAL NEURONS IN CULTURE.

In order to determine whether fluo-DRM and fluo-DLT-I were internalized in P1 rat cortical neurons in culture, the cultures were incubated with either of these fluoagonists for 30 minutes at a concentration of 10 nM and at 37°C. The internalization of fluorescent ligand was terminated by washing the cultures either with isotonic ice-cold Earle-HEPES binding buffer or with hypertonic acid buffer for a period of 2 minutes. Washing with isotonic buffer reveals both surface- bound and internalized ligand ("Total binding"), whereas washing with hypertonic acid buffer strips off all surface bound ligand, sparing only what has been internalized. Specificity of binding and internalization of either fluo-DRM or fluo-DLT-I in these cortical cultures was verified by co-incubation with 10 μ M naloxone in every experiment as a competitive antagonist of MOR and DOR. Hypertonic acid washing conditions were somewhat detrimental to the neuronal cell membrane integrity. The morphology of these neurons after such treatment was usually not as well preserved as in the controls. To preserve the morphology, cell fixation with paraformaldehyde was employed prior to mounting of the samples. The labeling intensity after both treatments was not affected significantly. Cortical neurons were distinguished from glial cells in culture by virtue of their characteristic morphology displayed under phase contrast microscopy. Reference to cortical neuronal morphology was derived from initial characterization studies of cultures using anti-MAP2 immunocytochemistry. Moreover, analyses were done to compare the proportion of neurons that bound either fluo-DRM or fluo-DLT-I and that of neurons which internalized the ligands. Results revealed no differences between these two.

Total binding results showed a strong fluorescent labeling confined in vesicularlike structures and distributed diffusely in the soma and along neuronal processes (Figure 8A). The nuclei of neurons were label-free. Following hypertonic acid wash, fluorescence hot spots were still present, albeit somewhat diminished in intensity, and their distribution was unchanged as compared to neurons subjected to total binding conditions alone (Figure 8A'). In both cases, the labeled hot spots were abolished with the concurrent addition of 10 μ M naloxone, indicating that binding and internalization of the fluorescent ligand were specific (not shown).

To determine whether fluo-DRM internalization was mediated via the classic clathrin-mediated pathway, neurons were pre-incubated for 30 minutes at 37°C in an equilibration buffer containing the endocytosis inhibitor PAO (10 μ M). Total binding in the presence of PAO and in the absence of a hypertonic wash showed positive fluorescent labeling of cortical neurons (Figure 8B). This labeling was still detected in the form of "hot spots", but these "hot spots" were larger than those observed in the non-PAO treated control (Figure 8B vs. A). The labeling was displaceable with 10 μ M naloxone indicating that the binding was specific (not shown). When neurons pre-treated with PAO were subjected to hypertonic acid wash, fluorescent-labeling was no longer detectable in these cortical neurons indicating that the ligand was confined to the cell surface (Figure 8B').

Similar assay was performed for fluo-DLT-I. Total binding on P1 rat cortical neurons showed a strong fluorescent labeling contained within vesicular structures, which were diffusely distributed prominently in the soma and more discretely along neuronal processes (Figure 9A). The nuclei of neurons were label-free. The fluorescence hot spots were still present following hypertonic acid wash, although their overall intensity was decreased compared to the neurons in total binding assays (Figure 9A'). Their distribution in the neurons was, however, unchanged by the acid wash treatment. Binding and internalization of fluo-DLT-I was completely displaceable with 10 μ M naloxone (not shown).

To determine whether fluo-DLT-I was internalized via clathrin-dependent pathway, a similar experiment using 10 μ M PAO was performed as described for fluo-DRM above. Total binding in the presence of PAO and in the absence of hypertonic wash showed fluorescent hot spots visible mainly in the soma of neurons (Figure 9B). This labeling was displaceable with 10 μ M naloxone indicating that the binding was specific (not shown). Treatment with 10 μ M PAO changed the somatic distribution of these fluorescent hot spots, which by and large were larger and less numerous than in the non-PAO-treated control (Figure 9B vs. A). Again, labeling along neuronal processes was less prominent (Figure 9B). When neurons pre-treated with PAO were washed with hypertonic acid solution, fluorescent labeling of the neurons was completely abolished, indicating that the label was confined to the cell surface (Figure 9B').

DISTRIBUTION OF INTERNALIZED FLUO-LIGANDS OVER TIME

Internalization of fluo-DRM or fluo-DLT-I in rat cortical neurons in culture was studied at 5 and 30 minutes after incubation with each fluo-ligand respectively. Quantification of the amount of fluo-ligand internalized at each of these time points was determined after acid-wash by measuring the proportion of the cell's cytoplasmic surface occupied by fluorescent clusters using the Biocom 200 Photometric System. As described in methods, this proportion is referred to as the "cell occupancy ratio".

After 5 minutes of fluo-DRM exposure, a number of hot spots was seen in the neurons that remained despite acid-wash and were displaceable with 10 μ M naloxone. These hot spots were in widely distributed throughout the neuron (Figure 11B). At 30 minutes, there was an observable increase in the number and intensity of hot spots detected over neuronal perikarya as compared with 5 minutes. This is reflected in the cell occupancy ratio which doubled from 0.025 ± 0.002 at 5 minutes to 0.050 ± 0.008 by 30 minutes (P<0.01) (Figure 10; Table 8). At the same time, the number of hot spots doubled from 5 minutes to 30 minutes, that is, from an average of 15 ± 4 vesicles per neuron at 5 minutes to an average of 28 ± 10 vesicles per neuron (P<0.001) (Table 8).

When further analysis was performed by dividing the cell into somatic and processes compartments, there was a significant increase in the labeling proportion of fluo-DRM from 5 to 30 minutes observed in the soma compartment while no observable changes occurred in the processes. Thus, the labeling proportion in the soma increased from 0.011 \pm 0.001 at 5 minutes to 0.031 \pm 0.005 at 30 minutes (P<0.01) (Figure 11A *solid bar*; Table 8). No significant changes were seen along the processes (i.e. 0.015 \pm 0.001 of fluo-DRM labeling proportion at 5 minutes vs. 0.016 \pm 0.003 at 30 minutes, P=0.566) (Figure 11A *open bar*; Table 8). There was, in addition, a significant increase in the number of hot spots in the somatic compartment from 5 to 30 minutes of fluo-DRM exposure, namely from 6 \pm 2 to 18 \pm 5 (P<0.001) (Table 8). No significant changes in hot spot number were seen in the processes compartment with time. The size of the hot spots did not vary significantly at any given time-point or compartment (Table 8).

Study of fluo-DLT-I internalization and trafficking in rat cortical neurons in

culture was performed similarly to that described for fluo-DRM above. Comparable observations were obtained for fluo-DLT-I regarding its labeling pattern at 5 and 30 minutes of exposure. Specifically, few hot spots were initially seen in the neurons at 5 minutes that were neither acid-washable nor displaceable with 10 μ M naloxone (Figure 13B). These hot spots were vesicular in form and widely distributed in the neuron. By 30 minutes, there was an observable increase in the intensity as well as in the number of hot spots without major changes in the labeling pattern (Figure 13B'). Quantification of internalized fluo-DLT-I in these neurons showed an average cell occupancy ratio of 0.034 ± 0.004 after 5 minutes of exposure to fluo-DLT-I (Figure 12). This labeling index increased to 0.060 ± 0.009 after incubation for 30 minutes (P<0.01)(Figure 12; Table 9). At the same time, the number of hot spots increased significantly from 12 ± 5 at 5 minutes to 21 ± 9 by 30 minutes during this interval (P<0.01) (Table 9).

Division of the neurons into soma and processes compartments revealed a significant increase of fluo-DLT-I labeling in the soma compartment of neurons between 5 and 30 minutes from a cell occupancy ratio of 0.021 ± 0.002 at 5 minutes to 0.049 ± 0.007 at 30 minutes (P<0.01) (Figure 13-A *solid bar*). No significant changes were seen along the processes between time points (i.e. 0.015 ± 0.003 for fluo-DLT-I's cell occupancy ratio at 5 minutes vs. 0.016 ± 0.003 at 30 minutes, P=0.08) (Figure 13-A *open bar*; Table 9). In addition, there was a significant increase in the number of hot spots from 5 to 30 minutes of fluo-DLT-I exposure in the somatic compartment, namely 7 ± 3 to 15 ± 6 (P<0.01) (Table 9). There were no other significant changes in the number or size of hot spots in between the two compartments during this interval.

To determine whether the increase in the fluo-DLT-I labeling observed in the soma over time could be due in part to retrograde transport of ligand internalized at the level of processes, additional experiments were performed in the presence of Nocodazole,

a microtubule assembly blocker. Only 30 minutes exposure to fluo-ligand was studied in this set of experiments. The result of the experiment showed that the subcellular distribution of hot spots was clearly different between the untreated and the Nocodazole treated population. Visually, in the untreated control population, internalized fluo-DLT-I accumulated prominently in the soma compartment, whereas, in the Nocodazole treated population, fluorescent hot spots were most numerous along neuronal processes, and only very few were visible in the somatic compartment (Figure 14B vs. B'). Quantitatively, analysis of the results confirmed our visual observations in that there was a much higher proportion of cell occupancy by fluo-DLT-I in the soma compartment of the untreated control population (0.049 \pm 0.007) as opposed to that in the Nocodazole treated neurons (0.019 ± 0.003) (P<0.001) (Figure 14A solid vs. open bar under "Soma"). On the other hand, cell occupancy by fluo-DLT-I was markedly lower in the processes of the untreated control population (0.016 \pm 0.003) as compared to that in the Nocodazole treated population (0.029 ± 0.006) (P<0.05) (Figure 14A solid vs. open bar under "Processes"). Similar experiments were carried for fluo-DRM and similar visual observations as that in fluo-DLT-I experiments were obtained. However, no quantitative analysis was performed as the sample size of the results was too small.

EFFECT OF LIGAND EXPOSURE ON THE DISTRIBUTION OF OPIOID RECEPTOR IMMUNOREACTIVITY IN CORTICAL NEURONS IN CULTURE

Cortical neurons were pre-incubated with non-fluorescent ("cold") DRM for 5 or 30 minutes and immunostained with anti-MOR antibody or were treated with nonfluorescent FLT-I for the same time periods and immunostained with anti-DOR antibody. Whole cell integrated labeling densities were measured from digitized confocal images using Scion Image Software and the resulting values were standardized to their respective total cell surface area. To evaluate changes in the distribution of MOR with different fluo-DRM exposure time (i.e. 5 vs. 30 minutes), measurements of the integrated labeling density per unit neuronal area were separately analysed for the soma and processes compartments. The resulting value for each compartment is expressed as a percentage (%) of the integrated density per unit area obtained for the whole cell, that is, percent of total MOR immunolabeling.

MOR-immunolabeled neurons showed no significant time-dependent changes in distribution of the immunoreactivity in either soma or processes compartments following exposure to DRM. Visually, the intensity of MOR immunolabeling in the soma and along the processes of cortical neurons at the 5 and 30 minutes time points were indistinguishable (Figure 15B and B'). The integrated density was determined to be 62.5 \pm 2.8% of total MOR immunolabeling in the soma at 5 minutes while 65.0 \pm 1.0% was the resulting measurement obtained in the same compartment at 30 minutes (P=0.420) (Figure 15A *solid bar*). Similarly, 37.5 \pm 2.8% of total MOR immunolabeling was located along the processes at 5 minutes as opposed to 35.0 \pm 1.0% at 30 minutes (P=0.420) (Figure 15A *open bar*).

Similarly, DOR-immunostained neurons showed no significant time-dependent changes in the cellular distribution of the immunoreactivity in either somatic or processes compartments following exposure to non-fluorescent DLT-I for 5 or 30 minutes (Figure 16B vs. B'). Thus, $76.2 \pm 1.8\%$ of total DOR immunoreactivity was associated with the soma at 5 minutes, while $71.8 \pm 1.9\%$ was found in the same compartment at 30 minutes (P=0.104) (Figure 16A *solid bar*). Similarly, $23.8 \pm 1.8\%$ of DOR immunoreactivity was located along processes at 5 minutes as opposed to $28.2 \pm 1.9\%$ at 30 minutes (P=0.104) (Figure 16A *open bar*).

EFFECT OF 48 HOURS NALOXONE PRE-TREATMENT ON THE DISTRIBUTION OF INTERNALIZED FLUO-DRM IN RAT CORTICAL NEURONS IN CULTURE.

In order to investigate the effects of chronic treatment with the non-selective opioid receptor antagonist naloxone on the internalization and trafficking of fluo-DRM and fluo-DLT-I in P1 rat cortical cultures, neurons were treated with 10 μ M naloxone for 48 hours prior to experimental manipulation. Subsequent procedures and analysis were carried out in the same way as described for the fluo-ligand binding experiments in the untreated control cultures.

Comparisons within the naloxone treated group

Upon visual inspection under the confocal microscope, fluorescent labeling of naloxone-treated cultures greatly increased between 5 and 30 minutes of exposure to fluo-DRM (Figure 17B and B'). Fluo-DRM labeling was resistant to acid-wash and displaceable with 10 μ M naloxone indicating that it corresponded to internalized ligand. Visual examination of cells exposed to fluo-DRM for 5 minutes revealed hot spots in both somatic and processes compartments. No gross differences in intensity, number, or size of these hot spots were noted between these two compartments (Figure 17B). At 30 minutes, however, the intensity, number and size of hot spots observed in the somatic compartment increased considerably as compared to that observed at 5 minutes (Figure 17B'). By contrast, the intensity, number and size of hot spots did not change in the processes compartment as compared visually to that at 5 minutes. Quantitatively, the proportion of cell surface occupied by internalized fluo-DRM in the chronically naloxone-treated cultures was 0.019 \pm 0.004 at 5 minutes. This value doubled to 0.040 \pm 0.006 after 30 minutes of incubation with the fluorescent ligand (P<0.01) (Figure 18).
The number of hot spots also doubled significantly from an average of 12 ± 6 to an average 23 ± 10 hot spots per whole neuron (P<0.001)(Table 8). Overall, there was a significant increase in the cell occupancy ratio, number and size of hot spots in the somatic compartment between 5 and 30 minutes of fluo-DRM exposure (Table 8). By contrast, there were no quantitative differences or changes in all three measurements in the processes compartment in either time point (Table 8). During this interval, the cell occupancy ratio in the somatic compartment doubled from 0.007 \pm 0.001 to 0.025 \pm 0.004 (P<0.05; Table 8). Moreover, there was a parallel increase in the number of hot spots in this compartment from an average of 5 ± 2 to 14 ± 7 (P<0.001) hot spots per neuronal soma. The size of hot spots also increased significantly, namely from 0.080 \pm 0.007 μ m² at 5 minutes to 0.117 \pm 0.008 μ m² at 30 minutes (P<0.001) (see Table 8).

Comparison between naloxone-treated and control groups

When measurements were visually compared between untreated and naloxonetreated cultures, there was no significant difference in the labeling pattern or intensity of fluo-DRM binding (Figure 17A, A' vs. 17B, B'). Quantitatively, labeling proportion in soma of untreated neurons measured 0.011 ± 0.001 as compared to 0.007 ± 0.001 in soma of chronically naloxone-treated cells at 5 minutes of fluo-DRM exposure (P=0.106). At 30 minutes, the ratio of cell occupancy by the label was 0.031 ± 0.005 in the soma of untreated control cells whereas that in the chronically naloxone-treated population was 0.025 ± 0.004 (see Figure 19A *solid* vs. *open bars*). Again no significant difference was evident between these two conditions at 30 minutes (P=0.776). Along the neuronal processes, there was a lower cell occupancy ratio for neurons chronically treated with naloxone (0.010 ± 0.002) compared to untreated ones (0.015 ± 0.001) (P<0.05) following 5 minutes of fluo-DRM exposure. At 30 minutes, the difference in labeling between untreated controls (0.016 ± 0.003) and naloxone-treated neuronal processes ($0.011 \pm$ 0.002) became insignificant (P=0.107) (Figure 19-B solid vs. open bars). Also, there was an increase in the average size of hot spots within the somatic comparment of the naloxone-treated neurons (0.117 \pm 0.008 μ m²) as compared to that in the untreated culture (0.083 \pm 0.008 μ m²) (P<0.001) (Table 8). No other differences in measurements were noted.

EFFECT OF 48 HOURS NALOXONE PRE-TREATMENT ON THE DISTRIBUTION OF INTERNALIZED FLUO-DLT-I IN RAT CORTICAL NEURONS IN CULTURE.

Comparison within the naloxone-treated group

Confocal microscopic visualization of the fluo-DLT-I labeled, naloxone-treated neurons revealed similar features as the ones observed within the untreated cultures, namely an increase in the intensity as well as in the quantity of fluo-DLT-I labeling of the somatic compartment with time without major changes along the processes (Figure 20B, B' vs. 20A, A'). Again, fluo-DLT-I labeling was resistant to acid-wash and displaceable with 10 μ M naloxone indicating that it was internalized and that the internalization was receptor-dependent. The average cell occupancy ratio in the chronically naloxone-treated whole neuron following binding with fluo-DLT-I measured 0.045 ± 0.010 at 5 minutes, and increased to 0.068 ± 0.005 at 30 minutes of incubation (P<0.05) (Figure 21; Table 9). There was no significant increase in total number of hot spot between these two time points (see Table 9, Figure 21 vs. 25). Further analysis revealed a significant increase in the proportion of cell surface occupied by the label and in the number of hot spots in the somatic compartment of the naloxone-treated neurons with time, from 9 ± 3 hot spots at 5 minutes with a cell occupancy ratio of 0.015 ± 0.002, to 16 ± 6 hot spots at 30 minutes with a cell occupancy ratio of 0.02 ± 0.003 (P values are <0.001 and <0.01 respectively)

(Table 9). There were, on the other hand, no observable changes in the cell occupancy or number of hot spots along the processes between 5 and 30 minutes time points. The size of hot spots also did not differ significantly in time or between compartments (Table 9).

Comparison between the naloxone-treated and control groups

Visual comparison between naloxone-treated vs. untreated cultures showed a higher amount of labeling in the processes compartment of the treated population (Figure 20-B, B' vs. 20-A, A'). No other visually discernable differences in terms of amount of surface labeling nor in terms of hot spot numbers or size. Quantitative analysis on the effect of chronic naloxone-treatment on the surface occupied by fluo-DLT-I hot spots showed a significant decrease in the somatic compartment of neurons chronically treated with naloxonc, namely 0.015 ± 0.002 at 5 minutes compared to that in the untreated control, 0.021 ± 0.002 (P<0.05) (Figure 22A). At 30 minutes, however, no significant differences were found between the surface of perikarya occupied by hot spots in the chronically naloxone-treated vs. control neuronal population (0.042 \pm 0.003 vs. 0.049 \pm 0.007; P=0.575). (Figure 22-A, solid vs. open bars; Table 9). The surface occupied by the label was, on the other hand, significantly higher in the "processes" compartment of the chronically naloxone-treated population after both 5 and 30 minutes of fluo-DLT-I exposure compared to the control group (Figure 22-B open vs. solid bars; Table 9). At 5 minutes, the ratio of occupied surface in the processes compartment of the control population measured 0.015 \pm 0.003, which was significantly lower than that of 0.038 \pm 0.007 measured in the chronically naloxone-treated culture (P<0.05). At 30 minutes, there were still significant differences in the surface occupied by the label between the untreated control population (0.016 \pm 0.003) and the naloxone-treated one (0.025 \pm (0.003) (P<0.05). The number of hot spots or the size of these did not differ significantly between the two populations at any time point (Table 9).

EFFECT OF 48 HOURS NALOXONE PRE-TREATMENT ON THE DISTRIBUTION OF OPIOID RECEPTOR IMMUNOREACTIVITY IN CORTICAL NEURONS IN CULTURE UPON EXPOSURE TO THEIR LIGANDS.

In order to investigate the effects chronic Naloxone treatment on MOR or DOR immunoreactivity in rat cortical neurons in culture, MOR or DOR immunolabeling experiments were performed after 48 hours treatment with 10 μ M naloxone. The quantitative analysis for these immunocytochemical studies was expressed as Integrated density per unit area (ID/A) using Scion Image software.

A significant increase in MOR immunoreactivity was observed following chronic naloxone treatment (Figure 23A, A') (naloxone-treated ID/A=148.06 \pm 3.44 vs. Control ID/A=126.04 \pm 4.68; P<0.001) (Figure 24-"MOR" open vs. solid bar). However, no detectable change in DOR immunoreactivity was evident between the control (Figure 23B, B') (ID/A=126.90 \pm 3.22) and the chronically naloxone-treated neuronal population (ID/A=117.71 \pm 4.09) (P=0.139) (Figure 24-"DOR" solid vs. open bar). When the intensity of MOR or DOR immunoreactivity was divided between the soma and the processes compartments no significant changes were observed between these two conditions for either time point or either compartment (results not shown).

EFFECT OF 48 HOURS MORPHINE PRE-TREATMENT ON THE DISTRIBUTION OF INTERNALIZED FLUO-DRM IN RAT CORTICAL NEURONS IN CULTURE.

In order to investigate the effects of chronic exposure to the MOR agonist morphine on the time-dependent internalization and distribution of fluo-DRM and fluo-DLT-I in rat cortical cultures, 10 μ M morphine was applied to these cultures 48 hours prior to experimental manipulation. Subsequent procedures and analysis were carried out in the same way as that described for the fluo-ligand binding experiments in the untreated control cultures.

Comparison within morphine-treated group

Within-group confocal microscopic observations revealed a similar tendency of fluo-DRM labeling in the morphine-treated group as seen within the untreated group (Figure 25-B, B' vs. 25-A, A'). A prominent accumulation of hot spots was seen to accumulate in the somatic compartment of these morphine-treated neurons from 5 to 30 minutes of fluo-DRM exposure. This labeling was resistant to acid wash and displaceable with naloxonc (Figure 25-B and B'). Almost no labeling was visible in the processes compartment at either time points. The average cell occupancy ratio was 0.014 ± 0.002 at 5 minutes of fluo-DRM exposure. This ratio incremented to 0.027 ± 0.003 at 30 minutes of fluo-DRM exposure (P<0.01) (Figure 26; Table 8). Additional analysis showed that this increase in the proportion of the cell surface occupied by fluo-DRM was attributable solely to an increase within the somatic compartment as opposed to the processes compartment (from 0.009 \pm 0.001 to 0.022 \pm 0.003, P<0.01)(refer to Table 9 under "Morphine treated"). The overall number of hot spots measured also increased significantly and proportionally from 11 ± 3 at 5 minutes to 22 ± 6 at 30 minutes (P<0.001). The size of hot spots did not differ significantly across compartments or time points within these chronically morphine-treated population.

Comparison between morphine-treated and control groups

Visually, compared to the confocal microscopic observations on untreated neurons, there was less fluo-DRM labeling along the processes of neurons treated with

morphine compared to control neurons. No other major differences were seen. These observations were confirmed quantitatively. Whole neuron fluo-DRM labeling proportion decreased significantly with chronic morphine treatment as compared to untreated groups (P<0.01). At 5 and 30 minutes of fluo-DRM exposure, fluo-DRM surface labeling was 0.014 ± 0.002 and 0.027 ± 0.003 , respectively, compared to those seen in the untreated group, namely 0.025 ± 0.002 and 0.050 ± 0.008 respectively (Table 8). Separation of the data between soma and processes demonstrated no significant difference in the occupancy of the soma of morphine-treated cells by fluo-DRM compared to that in the untreated control neurons at either 5 or 30 minutes. The average cell occupancy ratio for the somatic compartment in the morphine-treated population at 5 minutes measured 0.009 \pm 0.001, which did not differ significantly from 0.011 \pm 0.001 measured in the control group (P=0.378). At 30 minutes, the average cell occupancy ratio in the morphine-treated population measured 0.022 \pm 0.003, which again did not differ from 0.031 \pm 0.005 obtained for the control group (P=0.347) (Figure 27A open vs. solid bars). By contrast, in the processes compartment, there was a drastic difference between chronically morphinetreated and control populations. At 5 minutes, cell occupancy ratio in the chronically morphine-treated population was found to be 0.005 ± 0.001 , which was significantly lower than that observed in the control population, namely 0.015 ± 0.001 (P<0.01). At 30 minutes, there was still a significant difference in the cell occupancy ratio between the chronically morphine-treated (0.005 ± 0.001) and the control population (0.016 ± 0.003) (P<0.01) (Figure 27B open vs. solid bars). A drastic reduction in hot spot number, but not size, measured in the processes compartment of morphine-treated culture at 5 and 30 minutes of fluo-DRM exposure was also observed in comparison to that in the untreated cultures (P < 0.001) (Table 8).

EFFECT OF 48 HOURS MORPHINE PRE-TREATMENT ON THE DISTRIBUTION OF INTERNALIZED FLUO-DLT-I IN RAT CORTICAL NEURONS IN CULTURE.

Comparison within morphine-treated group

Fluo-DLT-I binding experiments performed on cultures treated with morphine for 48 h revealed a very striking increase in the intensity of labeling as well as in the overall number of hot spots by 30 minutes of exposure (Figure 28B'). The hot spots were resistant to acid wash and abolished when the incubation was carried out in the presence of 10 μ M naloxone. The average cell occupancy by the label measured 0.048 \pm 0.009 at 5 minutes, and increased dramatically to 0.119 \pm 0.014 at 30 minutes of incubation (P<0.001) (Figure 29; Table 9). There was a parallel augmentation in the number of hot spots from 22 \pm 11 at 5 minutes to 42 \pm 12 at 30 minutes that was attributable to both an accumulation of hot spots within the somatic compartment and an increment of these along the processes during this time interval (P<0.001). Moreover, there was a very significant increase in the size of hot spots seen at 30 minutes compared to 5 minutes of fluo-DLT-I exposure (P<0.001) (see Table 9). No such increase was documented along the processes.

Comparison between morphine-treated and control groups

Visually, a dramatic increase in fluo-DLT-I internalization was observed at both time points following 48 hours treatment with morphine. Many more hot spots, albeit smaller, were seen in the neuronal soma and along the processes. In the soma, hot spots were confluent and occupied a large part of the somatic surface. Division of the data between soma and processes showed a significant increase in the average fluo-DLT-I

labeling occupancy in the soma of neurons chronically treated with morphine (0.030 \pm 0.005) as compared to that seen in the untreated control (0.021 \pm 0.002) at 5 minutes (P<0.01). At 30 minutes, more striking significant differences were observed between the cell occupancy ratio of fluo-DLT-I in the morphine-treated (0.086 ± 0.008) vs. that in the control neurons (0.049 \pm 0.007)(P<0.001). (Figure 30-A open vs. solid bars). As regards to the processes compartment, the labeling occupancy was not significantly different between the chronically morphine-treated group (0.018 ± 0.004) and the control group at 5 minutes (0.015 \pm 0.003) (P=0.703), but became statistically different at 30 minutes of fluo-DLT-I exposure (Figure 30-B open vs solid bars). At 30 minutes, the chronically morphine-treated group measured 0.033 ± 0.005 in mean fluo-DLT-I surface labeling, which doubled in value over that of the control population $(0.016 \pm 0.003)(P<0.05)$ (Figure 30-B open vs. solid bars). Interestingly, in comparison to the control groups, the number of hot spots in these morphine-treated cultures were constantly and significantly more numerous in both compartments and in both time points (see Table 9). However, the size of these hot spots were consistently and significantly smaller than that seen in the untreated group (see Table 9).

EFFECT OF 48 HOURS MORPHINE PRE-TREATMENT ON THE DISTRIBUTION OF OPIOID RECEPTOR IMMUNOREACTIVITY IN CORTICAL NEURONS IN CULTURE UPON EXPOSURE TO THEIR LIGANDS.

In order to investigate the effects chronic Morphine treatment on the intensity of MOR or DOR immunoreactivity in rat cortical neurons in culture, MOR or DOR immunolabeling experiments were performed in a similar way as that described for the chronic naloxone experiments. Again, the quantification of immunolabeling was assessed as "Integrated density per unit area". In the case of MOR, chronic morphine treatment did not alter the density of MOR immunoreactivity. Compare MOR immunoreactivity of 122.83 ± 3.91 in the chronically morphine-treated to that in the control, namely 126.04 ± 4.68 (P<0.798) (Figure 31-A vs. A'; Figure 32-"MOR" *open* vs. *solid bar*). In the case of DOR, there was a drastic enhancement in the intensity of DOR immunoreactivity in the cultures after chronic morphine treatment. This value increased from 126.90 ± 3.22 in the control group to 209.31 ± 6.44 in the chronic morphine treated group (P<0.001) (Figure 31-B vs. B'; Figure 32-"DOR" *open* vs. *solid bar*). Both observations can be appreciated visually in Figure 14b. When the intensity of MOR and DOR immunoreactivity was divided between the somatic and the processes compartments, no significant changes were observed between these two conditions for either time point or either compartment (results no shown).



TABLES, FIGURES AND FIGURE LEGENDS

Hot Spots		5 minutes			30 minutes	
Untreated	Processes	Soma	Total	Processes	Soma	Total
Cultures						
Cell	0.015 ±	$0.011 \pm$	0.025 ±	0.016 ±	$0.031 \pm$	$0.050 \pm$
Occupancy	0.001	0.001	0.002	0.003	0.005	0.008
ratio						***
Number of	9 ± 3	6 ± 2	15 ± 4	13 ± 4	18 ± 5	28 ± 10
vesicles				0.004		
Vesicular	$0.103 \pm$	$0.091 \pm$	-	$0.084 \pm$	$0.083 \pm$	-
Size (µm ⁻)	0.007	0.007		0.004	0.008	l
Naloxone						
treated		0.007.		0.011		
Cell	$0.010 \pm$	$0.007 \pm$	$0.019 \pm$	$0.011 \pm$	$0.025 \pm$	$0.040 \pm$
occupancy	0.002*	0.001	0.004	0.002	0.004	0.000
Number of	7 + 4	5 + 7	12 + 6	8+4	$14 + 7^{***}$	$23 \pm 10^{***}$
vesicles	/ _ 4	512	12 - 0	0 - 4	14 ± /	25 ± 10
Vesicular	0.078 +	0.080 +	-	0.096 +	0117+	-
Size (um^2)	0.007	$0.000 \pm$		0.006	0.008	
5120 (74.17)	0.001	0.007			000	
Morphine			<u> </u>			
treated						
Cell	0.005 ±	0.009 ±	0.014 ±	0.005 ±	0.022 ±	0.027 ±
Occupancy	0.001000	0.001	0.002°°	0.001 ***	0.003**	0.003** ^{,00}
ratio						
Number of	4 ± 2°°°	7 ± 1	11 ± 3	4 ± 3°°°	17 ± 4***	22 ± 6***
vesicles						
Vesicular	0.084 ±	0.082 ±	-	0.081 ±	0.074 ±	-
Size (µm²)	0.006	0.004		0.006	0.006	

TABLE 8. Number and Size of Fluo-DRM Hot-Spots at Different Time-points.

P values from within group comparison between 5 and 30 minutes \rightarrow

• P<0.05 ; •• P<0.01 ; ••• P<0.001

P values from comparison with untreated group within same time point \rightarrow

° P<0.05 ; °° P<0.01 ; °°° P<0.001

With the exception of hot spot numbers (followed by S.D. value), all values in the table are followed by S.E.

Hot Spots		5 minutes			30 minutes	
Untreated cultures	Processes	Soma	Total	Processes	Soma	Total
Cell Occupancy ratio	0.015 ± 0.003	0.021 ± 0.002	0.034 ± 0.004	0.016 ± 0.003	0.049 ± 0.007**	0.060 ± 0.009**
Number of vesicles	5 ± 2	7 ± 3	12±5	6 ± 3	15±6	21 ± 7**
Vesicular	0.150 ±	0.154 ±	-	0.160 ±	0.173 ±	-
Size (µm²)	0.012	0.012		0.018	0.015	
Naloxone treated						
Cell	0.038 ±	0.015 ±	0.045 ±	0.025 ±	0.042 ±	0.068 ±
Occupancy ratio	0.007°	0.002°	0.010	0.003°	0.003	0.005
Number of vesicles	12 ± 8	9 ± 3	21 ± 11	9 ± 4	$16 \pm 6^{***}$	25 ± 10
Vesicular	0.140 ±	0.147 ±	-	0.126 ±	0.173 ±	-
Size (µm²)	0.026	0.033		0.020	0.023	
Morphine treated						
Cell	0.018 ±	$0.030 \pm$	0.048 ±	0.033 ±	0.086 ±	0.119 ±
Occupancy ratio	0.004	0.005°°	0.009°°	0.005*, °	0.008	0.014,
Number of vesicles	13 ± 7°°°	11±5°	22 ± 11°	17 ± 9°°°	25 ± 8****, °	42 ± 12 ^{***} .
Vesicular Size (µm²)	0.087 ± 0.007°°°	0.066 ± 0.009°°°	-	0.102 ± 0.012°	0.126 ± 0.014 ^{****}	-

TABLE 9. Number and Size of Fluo-DLT-I Hot-Spots at Different Time-points.

P values from within group comparison between 5 and 30 minutes →

• P<0.05 ; •• P<0.01 ; ••• P<0.001

P values from comparison with untreated group within same time point \rightarrow

° P<0.05 ; °° P<0.01 ; °°° P<0.001

With the exception of hot spot numbers (followed by S.D. value), all values in the table are followed by S.E.

Figure 1. Specific MOR immunostaining of μ receptor-transfected cells. MOR immunoreactivity is seen only in pcDNAI-MOR transfected COS-7 cells (A) and is absent in non-transfected or pcDNAI-DOR transfected COS-7 cells (B and C respectively). Omission of the primary antibody resulted in no immunoreactivity (D). Images were acquired on single confocal optical sections.

A	Β
MORir pcDNAI-MOR transfected	+ Anti-MOR Ab Non-transfected
C	D

Figure 2. Specific DOR immunostaining of δ receptor-transfected cells. FOR immunoreactivity is seen only in pcDNAI-MOR transfected COS-7 cells (A) and is absent in non-transfected or pcDNAI-DOR transfected COS-7 cells (B and C respectively). Omission of the primary antibody resulted in no immunoreactivity (D). Images were acquired on single confocal optical sections.

A	Β
DORir pcDNAI-DOR transfected	+ Anti-DOR Non-transfected
C	D
+ Anti-DOR pcDNAI-DOR transfected	- Anti-DOR + 2 Ab pcDNAI-DOR transfected

Figure 3. Specific fluo-DRM labeling of μ receptor-transfected cells. COS-7 cells transfected with pcDNAI-MOR (A, B), pcDNAI-DOR (C) and non-tranfected cells (D) were incubated for 30 min at 37 °C with fluo-DRM in the absence (A, C, D) or in the presence (B) of 10 μ M naloxone. Note the presence in A, but not in B, C, or D of intense fluorescent capping indicative of cell surface clustering of receptor-ligand complexes. Images were acquired on single confocal optical sections.

A	Β
fluo-DRM pcDNAI-MOR transfected	fluo-DRM + Naloxone pcDNA-MOR transfected
С	D
fluo-DRM	

Figure 4. Specific fluo-DLT-I labeling of δ receptor-transfected cells. COS-7 cells transfected with pcDNAI-DOR (A, B), pcDNAI-MOR (C) and non-tranfected cells (D) were incubated for 30 min at 37 °C with fluo-DLT-I in the absence (A, C, D) or in the presence (B) of 10 μ M naloxone. Note the presence in A, but not in B, C, or D of intense fluorescent capping indicative of cell surface clustering of receptor-ligand complexes. Images were acquired on single confocal optical sections.



Figure 5. Confocal microscopic images of MOR or DOR immunoreactive primary rat cortical neurons. Single confocal optical sections. Double immunocytochemical labeling of MOR (A) or DOR (B) and corresponding anti-MAP-2 immunoreactivity (A',B'). P1 rat cortical primary neurons cultured in vitro conditions stained positively for MOR (A) and DOR (B). Fluorescence labeling can be seen distributed in the soma as well as along the processes of these cortical neurons. MAP-2 staining provides a way to distinguish neurons from astrocytes in our culture.



Figure 6. Percentage of cortical neurons in rat primary culture that express the MOR and DOR. The proportion of neurons in our primary culture that expressed MOR and DOR, as determined by both immunocytochemical (*solid bars*) and ligand-labeling (*open bars*) methods, were comparable. Approximately 6% are MOR+ and 12% DOR+ neurons. Statistical analyses were done using Kruskal-Wallis One-Way Analysis of Variance.



Figure 7. Selective labeling of cortical neurons with fluo-DRM and fluo-DLT-I. Total ligand binding at 30 minutes exposure of fluo-DRM (A) or fluo-DLT-I (B) revealed strong punctate fluorescent labeling that are contained within vesicular-like structures and seen most prominently in the soma and, to a lesser extent, along the processes. Both are displaceable with 10 μ M naloxone (A'and B').



Figure 8. Clathrin-mediated internalization of fluo-DRM in rat cortical neurons in culture. Single confocal optical sections. *Upper two panels* show the results of total fluo-DRM binding (A) and that following hypertonic acid wash (A'). *Bottom two panels* show similar experiment as done for A and B, but in the additional presence of phenylarsine oxide (PAO). Panel C shows total fluo-DRM binding in the presence of PAO while panel D shows parallel treatment as for neuron in Panel C but with additional hypertonic acid wash.



Figure 9. Clathrin-mediated internalization of fluo-DLT-I in rat cortical neurons in culture. Single confocal optical sections. *Upper two panels* show the results of total fluo-DLT-I binding (A) and that following hypertonic acid wash (A'). *Bottom two panels* show similar experiment as conducted for A and B, but in the additional presence of phenylarsine oxide (PAO). Panel C shows total binding of fluo-DLT-I in PAO treated neurons while panel D shows parallel treatment as for neuron in Panel C but with additional hypertonic acid wash.



Figure 10. Fluo-DRM internalization in whole rat cortical neurons in culture. At 5 minutes of fluo-DRM exposure, the cell occupancy ratio of fluo-DRM measured 0.025 ± 0.002 . At 30 minutes, it increased to 0.050 ± 0.008 , which represents twice as many internalized fluo-DRM ligands as was seen at 5 minutes (**P<0.01). Quantification of internalized fluo-DRM was done by measuring the proportion of the cell's cytoplasmic surface occupied by fluorescent clusters using the Biocom 200 Photometric System on analogue images acquired by confocal microscopy. Data are mean of three experiments, each performed in duplicate. Statistical analyses were done using Kruskal-Wallis One-Way Analysis of Variance.



Figure 11. Distribution of internalized fluo-DRM in whole rat cortical neurons in culture. A significant increase in fluo-DRM's cell occupancy ratio was seen in the neuronal soma between 5 and 30 minutes of ligand exposure (**P<0.05) (A, *solid bars*). No changes were seen along the processes (A, *open bars*). Panel B shows internalization of fluo-DRM at 5 minutes while Panel B' shows that at 30 minutes. Significant increase in fluo-DRM could be observed in the somatic compartment while changes in the processes were minimal. Images were acquired as single confocal optical sections. Statistical analyses were done using Kruskal-Wallis One-Way Analysis of Variance.



Figure 12. Fluo-DLT-I internalization in whole rat cortical neurons in culture. The cell occupancy ratio of fluo-DLT-I increased significantly from 0.034 ± 0.004 at 5 minutes to 0.060 ± 0.009 at 30 minutes (**P<0.01). Quantification of internalized fluo-DRM was done by measuring the proportion of the cell's cytoplasmic surface occupied by fluorescent clusters using the Biocom 200 Photometric System on analogue images acquired by confocal microscopy. Data are mean of three experiments, each performed in duplicate. Statistical analyses were done using Kruskal-Wallis One-Way Analysis of Variance.


Figure 13. Distribution of internalized fluo-DLT-I in whole rat cortical neurons in culture. A significant increase in fluo-DLT-I's cell occupancy ratio was seen in the neuronal soma between 5 and 30 minutes of ligand exposure (**P<0.01) (A, *solid bars*). No significant changes were seen along the processes (A, *open bars*). Panel B shows internalization of fluo-DLT-I at 5 minutes while Panel B' shows that at 30 minutes. Significant increase in fluo-DLT-I could be observed in the somatic compartments while changes in the processes were non-apparent. Images were acquired as single confocal optical sections. . Statistical analyses were done using Kruskal-Wallis One-Way Analysis of Variance.



Figure 14. Effect of nocodazole on the distribution of internalized fluo-DLT-I in whole rat cortical neurons in culture. Nocodazole treatment resulted in impaired retrograde transport of fluo-DLT-I from the processes to the neuronal soma. Accordingly, significantly more fluo-DLT-I labeling was measured in the processes and much less in the soma of nocodazole-treated neurons than in the control cultures (**P<0.05; *P<0.01)(A, *open vs. solid bar*). Panel B shows internalization of fluo-DLT-I in the control culture at 30 minutes of ligand exposure that is prominently somatic in distribution. Panel B'shows internalization of fluo-DLT-I in the nocodazole-treated culture at 30 minutes of ligand exposure that is very prominently seen along the processes. Images were acquired as single confocal optical sections. Statistical analyses were done using Kruskal-Wallis One-Way Analysis of Variance.



Figure 15. Distribution of MOR in rat cortical neurons in culture following 5 and 30 minutes of non-fluorescent DRM exposure. No sub-compartmental changes in MOR distribution were detected within the cortical neurons between 5 and 30 minutes of ligand exposure (A). Panel B shows MOR immunoreactivity at 5 minutes following non-fluorescent DRM exposure while Panel B' shows that at 30 minutes. Note the similar intensity of MOR immunolabeling in the soma and along processes of the cortical neurons in these two panels. Images were acquired as single confocal optical sections. Statistical analyses were done using Two-sample t-test.



Figure 16. Distribution of DOR in rat cortical neurons in culture following 5 and 30 minutes of non-fluorescent DLT-I exposure. No sub-compartmental changes in DOR distribution were detected within the cortical neurons between 5 and 30 minutes of ligand exposure (A). Panel B shows DOR immunoreactivity at 5 minutes following non-fluorescent DLT-I exposure while Panel B' shows that at 30 minutes. Note the similar intensity of DOR immunoreactivity in the soma and along processes of the cortical neurons in these two panels. Images were acquired as single confocal optical sections. Statistical analyses were done using Two-sample t-test.



Figure 17. Effect of 48 hrs. naloxone treatment on the distribution of fluo-DRM in rat cortical neurons in culture. *Left panels* show internalized fluo-DRM in control cortical cultures at 5 and 30 minutes of fluo-DRM exposure (A and A'). *Right panels* show internalized fluo-DRM in naloxone-treated cortical cultures at 5 and 30 minutes of fluo-DRM cortical cultures at 5 and 30 minutes of fluo-DRM exposure (B and B'). No significant differences can be appreciated in the distribution of fluo-DRM in these two conditions. Note the dramatic increase of hot spots in the soma from 5 to 30 minutes of fluo-DRM exposure in the two conditions. Images were acquired on single confocal optical sections.



Control

Naloxone pre-treated

Figure 18. Effect of 48 hrs. naloxone treatment on the internalization dynamics of fluo-DRM in rat cortical neurons in culture. Fluo-DRM's cell occupancy ration increased significantly from 0.019 ± 0.004 at 5 minutes to 0.040 ± 0.006 at 30 minutes of ligand exposure (**P<0.01). Quantification of internalized fluo-DRM was done by measuring the proportion of the cell's cytoplasmic surface occupied by fluorescent clusters using the Biocom 200 Photometric System on analogue images acquired by confocal microscopy. Data are mean of three experiments, each performed in duplicate. Statistical analyses were done using Kruskal-Wallis One-Way Analysis of Variance.



Figure 19. Quantification of the effect of 48 hrs. naloxone treatment on the distribution of fluo-DRM in rat cortical neurons in culture. Panel A shows no differences in fluo-DRM's cell occupancy ratio of the soma between control and naloxone-treated cultures. Panel B shows only a significant decrease of fluo-DRM's cell occupancy ratio of the processes at 5 minutes between control and naloxone-treated cultures (*P<0.05), but otherwise no differences at 30 minutes. Data are mean of three experiments, each performed in duplicate. Statistical analyses were done using Kruskal-Wallis One-Way Analysis of Variance.



Figure 20. Effect of 48 hrs. naloxone treatment on the distribution of fluo-DLT-I in rat cortical neurons in culture. *Left panels* show internalized fluo-DLT-I in control cortical cultures at 5 and 30 minutes of fluo-DLT-I exposure (A and A'). *Right panels* show internalized fluo-DLT-I in naloxone-treated cortical cultures at 5 and 30 minutes of fluo-DLT-I exposure. More fluo-DLT-I can be seen internalized in the processes of the chronically naloxone-treated cultures at 5 and 30 minutes at 5 and 30 minutes timepoints as compared to those in the control population. Images were acquired on single confocal optical sections.



Figure 21. Effect of 48 hrs. naloxone treatment on the internalization dynamics of fluo-DLT-I in rat cortical neurons in culture. Following 5 minutes of fluo-DLT-I exposure, fluo-DLT-I' cell occupancy ratio was measured to at 0.045 ± 0.010 . At 30 minutes, it increased to 0.068 ± 0.005 (*P<0.05). Quantification of internalized fluo-DRM was done by measuring the proportion of the cell's cytoplasmic surface occupied by fluorescent clusters using the Biocom 200 Photometric System on analogue images acquired by confocal microscopy. Data are mean of three experiments, each performed in duplicate. Statistical analyses were done using Kruskal-Wallis One-Way Analysis of Variance.



Figure 22. Quantification of the effect of 48 hrs. naloxone treatment on the subcompartmental distribution of fluo-DLT-I in whole rat cortical neurons in culture. Panel A shows a significant decrease in fluo-DLT-I's cell occupancy ratio at 5 minutes but not at 30 minutes when control cultures were compared to naloxone-treated cultures. Naloxone treatment resulted in a significant increase in fluo-DLT-I's cell occupancy ratio along the neuronal processes over that in the control cultures (Panel B)(*P<0.05). Statistical analyses were done using Kruskal-Wallis One-Way Analysis of Variance.





Figure 24. Effect of 48 hrs. naloxone treatment on MOR and DOR immunoreactivity in rat cortical neurons in culture. Graph shows a significant increase in MOR immunoreactivity following chronic naloxone treatment (***P<0.001) (MOR, *open vs solid bar*), whereas there was no observable changes in DOR immunoreactivity between the control and the chronically naloxone treated neuronal population (DOR, *open vs. solid bar*). "Integrated density/Arca" (ID/A) refers to whole cell integrated densities measured from digitalized confocal images using Scion Image Software and standardized against their respective total cell surface area. Statistical analyses were done using Twosample t-test.



Figure 25. Effect of 48 hrs. morphine treatment on distribution of fluo-DRM in rat cortical neurons in culture. *Left panels* show internalized fluo-DRM in control cortical cultures at 5 (A) and 30 minutes (A') of fluo-DRM exposure. *Right panels* show internalized fluo-DRM in morphine-treated cortical cultures at 5 (B) and 30 minutes (B') of fluo-DRM exposure. Less overall amount of fluo-DRM was internalized in the latter condition. Note the increase of hot spots in the soma from 5 to 30 minutes of fluo-DRM exposure in the two conditions. Images were acquired on single confocal optical sections.



Figure 26. Effect of 48 hrs. morphine treatment on internalization dynamics of fluo-DRM in rat cortical neurons in culture. Fluo-DRM's cell occupancy ratio increased significantly from 0.014 \pm 0.002 at 5 minutes to 0.027 \pm 0.003 (**P<0.01). Data are mean of three experiments, each performed in duplicate. Statistical analyses were done using Kruskal-Wallis One-Way Analysis of Variance.



, #

Figure 27. Effect of 48 hrs. morphine treatment on the sub-compartmental distribution of fluo-DRM in rat cortical neurons in culture. No difference in fluo-DRM distribution can be observed in the somatic compartment of either control or morphine-treated cultures at 5 or 30 minutes of ligand exposure (Panel A). However, chronic morphine-treatment resulted in a significant decrease of fluo-DRM's cell occupancy ratio along the processes at all timepoints (***P<0.001)(Panel B, *open vs. solid bars*). Data are mean of three experiments, each performed in duplicate. Statistical analyses were done using Kruskal-Wallis One-Way Analysis of Variance.



Figure 28. Effect of 48 hrs. morphine treatment on distribution of fluo-DLT-I in rat cortical neurons in culture. *Left panels* show internalization of fluo-DLT-I in control cultures at 5 (A) and 30 minutes (A'). *Right panels* show internalized fluo-DLT-I in morphine-treated cortical cultures at 5 (B) and 30 minutes (B') of fluo-DLT-I exposure. Morphine treatment caused a dramatic increase in the amount of fluorescent hot spots at 30 minutes of fluo-DLT-I exposure (B' vs. A'). Images were acquired on single confocal optical sections.



Control

Figure 29. Effect of 48 hrs. morphine treatment on internalization of fluo-DLT-I in rat cortical neurons in culture. Following 5 minutes of fluo-DLT-I exposure, fluo-DLT-I's cell occupancy ratio was measured to be 0.048 ± 0.009 . At 30 minutes, this labeling index increased dramatically to 0.119 ± 0.014 (***P<0.001). Data are mean of three experiments, each performed in duplicate. Statistical analyses were done using Kruskal-Wallis One-Way Analysis of Variance.



Figure 30. Effects of 48 hrs. morphine treatment on the sub-compartmental distribution of fluo-DLT-I in rat cortical neurons in culture. Morphine treatment led to a dramatic increase in fluo-DLT-I's cell occupancy ratio in somatic compartment at 5 and 30 minutes (Panel A, *open bar*) and along processes at 30 minutes (Panel B, *open bar*) as compared to that in control cultures (Panel A and B, *solid bar*). Data are mean of three experiments, each performed in duplicate. Statistical analyses were done using Kruskal-Wallis One-Way Analysis of Variance. (*P<0.05; **P<0.01; ***P<0.001).



Figure 31. Effect of 48 hrs. morphine treatment on MOR and DOR immunoreactivity in rat cortical neurons in culture. Control and morphine-treated cultures show no differences MOR immunoreactivity (A vs. A'). Morphine treatment, however, led to an enhancement of DOR immunoreactivity as compared to that in the control cultures (B' vs. B). Images were acquired on single confocal optical sections.


Figure 32. Effect of 48 hrs. morphine treatment on MOR and DOR immunoreactivity in rat cortical neurons in culture. Graph shows no significant changes in MOR immunoreactivity following chronic morphine treatment ("MOR"- *open vs. solid bar*) while DOR immunoreactivity is significantly enhanced as compared to that in the control cultures (***P<0.001)("DOR"- *open vs. solid bar*). "Integrated density/Area" (ID/A) refers to whole cell integrated densities measured from digitalized confocal images using Scion Image Software and standardized against their respective total cell surface area. Statistical analyses were done using Two-sample t-test.



DISCUSSION

The present study is the first to demonstrate a receptor-mediated internalization of opioid ligands in central neurons. The objectives were three-fold: 1) To describe the internalization and trafficking of fluorescent MOR and DOR agonists, namely fluo-DRM and fluo-DLT-I, in cortical neurons, 2) To determine the fate of their receptors following internalization, and 3) To determine the effects of chronic treatment with either naloxone or morphine on the above regulatory processes.

Before pursuing our first objective we proceeded to test: 1) the specificity of the ligands and 2) the specificity of the antibodies used. COS-7 cells transiently transfected with plasmid for either the μ or δ opioid receptors were used to this aim. First, our results showed that the ligands used in the present investigation were receptor-specific since only COS-7 cells that were transfected with pcDNA-MOR or with pcDNA-DOR were fluorescently labeled after exposure to fluo-DRM or fluo-DLT-I, respectively. Binding and internalization of both ligands were specific as this fluorescent labeling was abolished in the presence of the opioid receptor antagonist naloxone. Moreover, the lack of fluorescent signal in the non-transfected cells, as well as the absence of ligand cross-reactivity across the receptor subtypes, confirmed the specificity of both agonists. Secondly, primary and secondary antibodies used in this study were also found to be specific since no immunoreactivity was detected when the targeted epitope was absent. Thus, other than in COS-7 cells expressing the appropriate receptors, we did not observe immunolabeling in non-transfected control cells nor in transfected cells expressing the

opposite opioid receptor cDNA. The labeling method was also specific as the secondary antibodics, goat anti-rabbit or goat anti-mouse, did not label cells of any phenotype in the absence of the primary antibodies.

A third issue before proceeding with our study was to determine the phenotype of our cortical cultures and what proportion of these expressed MOR and/or DOR. Anti-MAP2 immunolabeling revealed an abundant number of morphologically welldifferentiated neurons in our primary culture. Phase contrast and astrocyte cell-specific rabbit anti-GFAP immunostaining also revealed abundant number of glial cells. Although glial cells were as abundant as the neurons in our cultures, we did not proceed with further investigations on this cell population.

Double immunocytochemical labeling with polyclonal rabbit anti-MOR and monoclonal mouse anti-MAP2 antibodies revealed that a relatively small sub-population (5.69%) of rat cortical neurons in culture expressed the MOR. Similar experiments using polyclonal rabbit anti-DOR antibody, showed that a larger sub-population (11.60%) of rat cortical neurons expressed DOR. Parallel experiments employing fluorescent ligand binding confirmed these immunocytochemical results, in that neuronal populations of equal size as those labeled immunocytochemically were found to bind fluo-DRM and fluo-DLT-I respectively. Unfortunately, pending the development of DOR and/or MOR antibodies raised in other species, the methods employed here did not allow us to determine whether cortical neurons co-expressed the two opioid receptors, as the selective antibodies for MOR and DOR were both raised in rabbit and the fluorescent binding experiments could not be combined with immunocytochemistry in this model system (our own unpublished observations). However, other unpublished evidences from our laboratory support the notion that there is indeed co-expression of MOR and DOR in a sub-population of cortical neurons.

The results conform radioligand, present vitro previous in to immunocytochemical and mRNA in situ hybridization studies, which reported MOR and DOR to be widely expressed in rat cerebral cortex (Cahill et al., 2000; Hiller et al., 1994; Mansour et al., 1994a; Mansour et al., 1994b; Mansour et al., 1994c; Mansour et al., 1993; Quirion et al., 1983; Sharif and Hughes, 1989). Furthermore, the size and shape of some of the neurons labeled for either MOR or DOR are consistent with reports on the expression of these 2 receptor subtypes by bipolar, bi-tufted, and pyramidal cells in rat ccrebral cortex (Cahill et al., 2000; Melone et al., 2000).

Consistent with previous findings in non-neuronal systems (Gaudriault et al., 1997; Keith et al., 1996), our study demonstrated that fluo-DRM and fluo-DLT-I were substantially internalized in cortical neurons 5 minutes after ligand exposure, as determined by the resistance of the fluorescent labeling to hypertonic acid wash. Internalization of these fluo-ligands doubled by 30 minutes, denoting that ligand internalization is time-dependent. Furthermore, the internalization process for both fluo-ligands was receptor-specific and receptor-mediated as it did not occur when the incubation was carried out in the presence of an excess of opioid receptor antagonist (i.e. naloxone). Previous experiments in our laboratory, also employing a primary cortical culture system, demonstrated internalization of fluorescent somatostatin in a similarly time-dependent, receptor subtype-mediated fashion (Stroh et al., 2000). However, no

report exists in the literature to date that addresses the fate of opioid ligands in central neurons. Thus, our study is the first one to report that MOR and DOR-specific ligands are internalized in central neurons via receptor-mediated mechanisms.

The disappearance of intracellular fluorescence in the presence of the endocytosis inhibitor, PAO, demonstrated that internalization of fluo-DRM and fluo-DLT-I via MOR and DOR, respectively, is clathrin-mediated. This is consistent with the knowledge that most GPCRs internalize via clathrin-coated pits (Grady et al., 1996; Silva et al., 1986, Boudin, 2000 #167; Zhang et al., 1996). Fluo-SRIF, a somatostatin peptide, has been shown to be internalized in cortical neurons via this same pathway (Stroh et al., 2000). Gaudriault et al. (Gaudriault et al., 1997) also demonstrated that fluo-DRM and fluo-DLT-I internalize in COS-7 cells transfected with either MOR or DOR, respectively, via a clathrin dependent mechanism, as PAO treatment inhibited this process, and acid wash in the presence of PAO abolished all fluorescent signals. Additionally, other studies in neurons in the case of MOR (Keith et al., 1998) and in non-neuronal systems in the case of DOR (Chu et al., 1997) have demonstrated that MOR and DOR internalize via clathrin-coated pits. Using confocal microscopy, Keith et al. reported colocalization of internalized MORs with transferrin receptors in mammalian brain, indicating that MOR internalization in vivo employs the same clathrin-mediated pathway as do transferrin receptors (Keith et al., 1998). Chu et al. observed that the rapid internalization of DORs could be specifically inhibited in cells expressing K44E mutant dynamin I, suggesting that type-specific internalization of opioid receptors was mediated by clathrin-coated pits (Chu et al., 1997).

The fluorescent "hot spots" observed inside perikarya and processes following internalization of fluo-DRM and fluo-DLT-I most likely represent endosomes. Indeed, earlier studies have clearly shown that internalized G protein-coupled receptors may accumulate within endosomes in neuronal cells (Bernard et al., 1998; reviewed in Bertrand et al., 1999; Dumartin et al., 1998). The latter studies investigators showed that there was a dramatic reduction in receptor abundance (55-70%) at the neuronal plasma membrane following administration of their cognate ligands, accompanied by a parallel increase of receptors in the endosomal compartment. It was further shown in HEK293 cells that etorphine (MOR agonist) caused a reduction in the fluorescence intensity of anti-MOR-labeled cells and a subsequent localization of MOR in transferrin receptorcontaining endosomes (Keith et al., 1996). Later studies by the same investigators confirmed these findings in in vivo rat brain (Keith et al., 1998). Sternini et al. also reported that following intraperitoneal injection of etorphine, there was an increase in MOR immunoreactivity within endosomes as detected by confocal microscopy (Sternini et al., 1996). The kinetics of ligand-induced receptor internalization, as detected by immunofluorescent cytometry, have been measured in HEK293 cells; the half life of etorphine-induced fluorescent antibody-labeled MOR internalization was found to be 6.0 \pm 1.7 min, whereas that of enkephalin-analogue induced fluorescent antibody-labeled DOR internalization was 5.9 ± 2.2 min (Keith et al., 1996).

The intra-neuronal distribution of internalized fluo-DRM and fluo-DLT-I varied markedly with time. Our results showed that whereas the accumulation of both fluo-DRM and fluo-DLT-I remained unchanged within neuronal processes across the 5 and 30 minutes time points, there was a very significant accumulation of both fluo-ligands in the

somatic compartment with time. Two possible explanations may account for such a phenomenon. It is possible that fluo-ligands internalized at the level of both soma and processes are being targeted to the somatic compartment with time. Alternatively, this increase in somatic labeling in the absence of a parallel increase in the labeling of processes may be the result of differences in the capacity of each compartment either to internalize or to accumulate fluo-ligands (i.e. because of different receptor recycling or targeting rates). Thus, it is possible that the observed lack of incremental increase in the amount of fluo-ligand along the processes between 5 and 30 minutes is due to saturation of internalization mechanism(s) in this compartment between the two time points. To test these hypotheses, a microtubular disruptor agent (nocodazole) was used in our ligandinternalization assay in order to determine whether retrograde transport of internalized ligand from processes to perikarya could account for the observed differences. It was observed that after nocodazole treatment, significantly less fluo-DLT-I accumulated in the somatic compartment at 30 minutes as compared to untreated controls. Furthermore, an increase in the amount of accumulated fluo-DLT-I was observed in neuronal processes at 30 minutes compared to untreated cells. The same observations were made for fluo-DRM when nocodazole was added in the ligand-binding assay, although quantitative analysis could not be performed due to the inadequate sample size. These results clearly indicate that: 1) internalization of both ligands occurs in both neuronal compartments, and 2) fluo-DLT-I, and presumably also fluo-DRM, internalize at the level of the processes and are subsequently targeted to the neuronal somatic compartment via a microtubule-dependent transport mechanism. By way of consequence, it may be surmised that the lack of increase in fluorescent labeling along the processes with time represents a

steady state between ligands that are internalized and ligands that are being transported to the soma.

Centripetal trafficking of internalized opioid ligands demonstrated in this study is in keeping with several lines of evidences that demonstrated centripetal retrograde transport of various internalized neuropeptides to the perikarya. Castel et al. (Castel et al., 1994) observed that iodinated neurotensin internalized at the level of dopaminergic axon terminals in the neostriatum accumulated gradually in the ipsilateral substantia nigra two hours after injection of the ligand into the striatum. This phenomena was receptorspecific, as radioactivity in the soma was prevented by non-radioactive neurotensin, as well as microtubule-dependent since introduction of the microtubule disruptor colchicine prevented such transport. This latter study suggested that the appearance of radioactivity in the ipsilateral substantia nigra was dependent on the initial binding of this peptide to its receptor in the striatum, and that there was a microtubule-dependent mechanism responsible for its retrograde transport after internalization. Similar centripetal trafficking of neuropeptides were observed in our laboratory, whereby Faure et al. demonstrated that fluo-neurotensin was internalized via neurotensin-1 high-affinity neurotensin receptors within the dendrites of basal forebrain cholinergic neurons and ventral midbrain tegmental neurons and that the endocytosed ligand was targeted from these dendritic processes to the perinuclear region of the soma within endosome-like organelles (Faure et al., 1995).

Quantitative analysis of immunocytochemical results in the present study did not show an increase in MOR or DOR protein levels between 5 and 30 minutes of ligand

exposure. Moreover, despite the observed changes in the compartmental distribution of internalized fluo-DRM and fluo-DLT-I with time, no parallel changes in MOR and DOR distribution between somatic and processes compartments were detected in the present study. Unlike what was demonstrated for fluo-DRM and fluo-DLT-I, MOR and DOR did not appear to be mobilized intracellularly across neuronal compartments. It would seem that contrary to their cognate ligands neither receptor is retrogradely transported to the perikarya. One possible interpretation for these observations is that internalized receptors are recycled locally to the plasma membrane. This interpretation is congruent with the observed ongoing internalization of the ligands with time, although targeting of neosynthesized receptors from intracellular compartments to the membrane of processes may also account for this ongoing process. An alternative interpretation for the lack of apparent process to soma trafficking of internalized receptors is that the actual number of ligand-bound MOR or DOR that are mobilized, in comparison to the total number of immunoreactive MOR or DOR present in these neurons, is too small to be detectable by immunocytochemistry. Thus, any change in the distribution of MOR or DOR would go unperceived. The first interpretation for our observations, however, is more consistent with the present literature, which supports the concept of local receptor recycling. Several GPCRs have indeed been shown to be locally recycled. For example, it was observed that in the soma of enteric neurons, substance P-induced rapid, clathrin-mediated internalization of the substance P receptor (SPR) into early endosomes (Grady et al., 1996). This internalization was followed by a return of SPR immunoreactivity to the cell surface after 4-8 hours. It was further observed that this return of surface receptor was not prevented by cycloheximide- indicating that it was independent of new protein synthesisbut was prevented by acidotropic agents, suggesting that it required endosomal

acidification. Since endocytosis and recycling correlated with loss and recovery of functional binding sites for substance P, these observations were interpreted to suggest that the SPR recycled in the soma (Grady et al., 1996). Another example comes from an in vivo immunohistochemical study performed by Mantyh et al. on SPR in spinal cord neurons (Mantyh et al., 1995). Following somatosensory stimulation in the form of either a pinch or capsaicin injection, an increase in the number SPR-immunoreactive endosomes was seen in dendritic processes within lamina I and II. This increase was accompanied by a substantial decrease in SPR immunoreactivity on the plasma membrane of the same neuronal processes. By 60 minutes after capsaicin injection, the number of SPR-positive neuronal processes that showed high concentrations of internalized SPR-positive endosomes had returned to the normal unstimulated levels seen on the contralateral side, suggesting that agonist-induced SPR internalization were reversible and that SPRs were recycled back to the plasma membrane (Mantyh et al., 1995). At no point in time was accumulation of the internalized receptors observed in nerve cell bodies within either the same or deeper layers. No evidence has yet been reported in the literature to support local recycling of opioid receptors in neurons. Experimental assays using receptor immunocytochemistry and monensin (inhibitor of receptor recycling) at different time points are required for a more definite demonstration of opioid receptor recycling. Assays using cycloheximide would also be needed to rule out confusion with membrane targeting of newly synthesized opioid receptors. In the event that local opioid recycling is proven, it would definitely suggest that opioid ligands dissociate from their respective receptors early on (i.e. in endosomes along the processes), and are presumably targeted to soma while their receptors recycle locally.

CHRONIC NALOXONE TREATMENT

In the current study, 48 h treatment with the non-selective antagonist naloxone was shown to significantly increase the levels of MOR immunoreactivity in both somatic and processes compartments. By contrast, fluorescent ligand internalization assays showed no change in the amount of internalized fluo-DRM following chronic naloxone treatment. These results suggest that prolonged naloxone exposure induced an up-regulation of MOR proteins. However, the fact that the increase in MOR immunoreactivity did not result in a parallel increase in fluo-DRM internalization suggests that these additional receptors are not targeted to the plasma membrane and are therefore not available for ligand-induced internalization. The time frame of 48 h was chosen because it had been previously reported to be optimal for up-regulation of DOR mRNA levels in neuroblastoma cell lines following chronic naloxone treatment (Jenab et al., 1994). Moreover, increasing the time frame of naloxone treatment might be required for functional maturation and for membrane targeting of the neosynthesized receptor.

Previous studies using non-neuronal as well as *in vivo* neuronal cells have shown that treatment of cells with opioid antagonist naloxone causes an up-regulation of MOR binding sites. For example, it has been shown through ligand-binding assays in cell lines as well as *in vivo*, that both short-term and long-term naloxone exposure led to an increase in both MOR agonist binding and intracellular signaling (Zadina et al., 1994; Zaki et al., 2000). Furthermore, it was reported in a binding study using membrane preparations from mouse brains that long-term (i.e. 8 days) pre-treatment with opioid antagonists such as naloxone or naltrexone led to the enhancement of MOR agonist

binding that can not be reversed by concurrent agonist treatment, indicating that antagonist-induced up-regulation is a robust, receptor-mediated phenomenon (Yoburn et al., 1995). Autoradiographic studies also demonstrated that long-term treatment of mice with naloxone produced an up-regulation of μ , δ and κ -opioid receptor binding sites in many brain areas (Hyytia et al., 1999). Additional findings on changes at the cellular level comes from the group of Unterwald et al. (Unterwald et al., 1995) whose concurrent observations of an up-regulation of MOR in rat brain (as determined by radioligand binding assay) and a lack of increase in the levels of MOR mRNA after chronic naloxone treatment led them to suggest that up-regulation of MOR under such conditions was not the result of an increase in *de novo* MOR synthesis, but rather the result of an increased receptor targeting to the membrane. However, this hypothesis turned out to be true for certain regions of the rat brain only. Following 7 days of naltrexone administration (7-8 mg/kg/day) in rats, it was observed by the same investigators (Unterwald et al., 1998) that MOR immunoreactivity was significantly higher in the amygdala, thalamus, hippocampus, and interpeduncular nucleus as compared with the saline-treated control animals.

By contrast, in the present study, chronic naloxone treatment did not increase the density of immunoreactive DOR. At the time of our study, there existed no report in the literature that addressed specifically the effects of naloxone on DOR-expression in cortical neurons. Prompted by the present results, our laboratory pursued further investigations on this regard. Employing immunoblot experiments on neuronal membrane preparations from cortical cultures *in vitro* (using the same anti-DOR antibody), Cahill et al. observed an increase in the total amount of DOR protein following 48 hrs. of naloxone

exposure (Cahill et al., submitted). Therefore, the fact that we did not detect an enhancement of DOR immunoreactivity in the present study may be related to the limited sensitivity of the immunocytochemical as well as the digital image analysis method used. Although chronic naloxone treatment did not affect the amount of fluo-DLT-I internalized in whole neurons, there was a significant increased amount of fluorescent ligand internalized in processes as compared to non-treated neurons at both 5 and 30 minutes. These observations, taken together, suggest that chronic naloxone treatment does increase the number of DOR available for internalization, and that the upregulated DOR observed by Western blot are targeted to the plasma membrane.

Pharmacological studies have previously suggested that opioid antagonists can up-regulate DOR *in vivo* and *in vitro*. It was initially shown that membrane preparations from brains of animals treated chronically with an opioid antagonist increased the binding of ³H-naloxone (Hitzemann et al., 1974). Later, Barg et al. (Barg et al., 1984) confirmed such findings in chronic naloxone treated neuroblastoma-glioma (NG108-15) cell culture and demonstrated that the mechanism underlying such up-regulation was not based on an alteration of the interaction between the receptor and the adenylate cyclase–GTP-binding protein system. Further along this line, using a sensitive solution hybridization assay, Jenab et al. (Jenab and Inturrisi, 1994) demonstrated that DOR mRNA transcript levels in NG108-15 cells peaked at 24 to 48 hours of naloxone treatment producing a 3 fold increase in DOR mRNA levels. Other investigators similarly reported that chronic naloxone treatment can up-regulate DORs in both non-neuronal cells and in *in vivo* neuronal systems (Belcheva et al., 1991; Hyytia et al., 1999). An interesting hypothesis regarding the mechanism underlying this up-regulation came from a study performed by

the group of Morris et al. (Morris and Millan, 1991). In this latter study, it was observed that whereas naloxone, which possesses negative intrinsic activity at the DOR receptor, has the ability to induce both supersensitivity and receptor up-regulation, MR2266, an antagonist with neutral intrinsic activity, has not. This let them to conclude that negative intrinsic activity may be required for DOR up-regulation to occur.

CHRONIC MORPHINE TREATMENT

Subjecting cortical neuronal cultures chronically treated with morphine to fluorescent internalization binding assays showed that there was a pronounced reduction of fluo-DRM internalization in morphine-treated neurons compared to untreated controls. This decrease in neuronal labeling was observed after both 5 and 30 minutes of fluorescent ligand exposure and was mainly attributable to a dramatic reduction in the fluo-DRM labeling of neuronal processes. The number of fluorescent hot spots was also significantly decreased in neuronal processes at both time points as compared to the controls. Parallel immunocytochemistry of MOR demonstrated no change in the overall MOR immunoreactivity between morphine treated and control untreated cultures. When somatic and processes compartments were analyzed separately, however, there was a significant increase in MOR immunoreactivity in neuronal somatas and a significant decrease in MOR immunoreactivity in the processes as compared to control cultures. This decrease in MOR immunoreactivity along the processes presumably reflects a reduction in surface receptor density within this compartment and is congruent with the observed decrease in fluo-DRM internalization along the processes following chronic morphine treatment. Our observation is in keeping with those of other *in vitro* neuronal

cell line studies, in which chronic morphine stimulation has been observed to lead to desensitization and down-regulation of MOR (Law et al., 1982; Zadina et al., 1993). To this effect, Law et al. observed a complete loss of morphine activity (as determined by a loss of adenylate cyclase activity) as well as a decrease in radioligand binding in NG108-15 hybrid cells treated with 100 µM morphine for 72 hours. Similar radioligand binding study performed on human neuroblastoma cell line SH-SY5Y chronically treated with morphine revealed a decrease in the binding but not in the affinity of MOR to [³H] DAMGO, a MOR-selective ligand (Zadina et al., 1993). The decrease in the number of receptors was related to time of exposure, with a half-maximum disappearance time (T1/2) of about 3 hr during the initial phase. The receptor decrease was near maximum at 24 hr with no further significant change up to 72 hours. In the present investigation, our choice of 48 hours morphine pre-treatment was based on the kinetics of MOR down-regulation reported in carlier studies (Law et al., 1982; Zadina et al., 1993). It would, however, be interesting to know whether longer treatment with morphine would result in further MOR regulatory changes.

On the other hand, the observed increase in MOR immunoreactivity observed within the somatic compartment could be a reflection of the somatic accumulation of peripheral down-regulated MORs that have been targeted centripetally for degradation in the lysosomes within the neuronal soma. Although this is yet to be proven, Keith et al. have suggested that the physiological role of receptor internalization may have the longterm aim at down-regulating receptor signaling by delivering these receptors to lysosomes for degradation. The increase of MOR immunoreactivity observed in the soma may also be attributable to an impairment in the delivery of MOR from the soma, where it is synthesized and stored, to the neuronal processes. Indeed, it has been reported for dopamine-1 receptors that chronic exposure to a functional hyperdopaminergic tone- as seen in homozygous dopamine-transporter gene knockout animals- resulted not only in a down-regulation of dopamine-1 receptors in dopaminergic neurons, but also in a longterm impairment of the delivery of these receptors to the cell plasma membrane (Bertrand et al., 1999). This was seen associated with an abnormal storage of dopamine-1 receptors in the soma, and particularly in the endoplasmic reticulum and the Golgi complex (Bertrand et al., 1999). In a similar way then, we postulate here that chronic high morphine tone may cause newly synthesized MOR to be abnormally retained in the somatic compartment of cortical neurons, impairing their and impairs its delivery to the neuronal periphery. The mechanism and functional implication(s) of this phenomenon have yet to be investigated.

At the concentration of morphine used in the chronic pre-treatment of our neuronal cultures, it is expected that morphine would activate DOR and would result in a down-regulation of the receptor. Indeed, although morphine displays the highest affinity towards MOR, a 10 μ M concentration also possesses some agonist action at DOR. As discussed previously, chronic morphine treatment resulted in a significant decrease in MOR immunoreactivity along neuronal processes with a concomitant reduction in fluo-DRM internalization within this compartment. By contrast, exposure of cortical neurons to high morphine concentrations not only did not decrease fluo-DLT-I internalization, but it resulted in a dramatic increase in the internalization of the δ agonist compared to that observed in the control untreated cultures. This effect was prevented by the addition of opioid receptor antagonist naloxone, demonstrating that it was a receptor-mediated

mechanism. Early observations at 5 minutes showed that there was a significant increase in the amount of fluo-DLT-I internalized in the somatic compartment while there was no change along the processes. By 30 minutes, a significant increase in the cell occupancy by fluo-DLT-I was detected along the processes of morphine-treated cultures and an even more dramatic increase in the amount of internalized fluo-DLT-I was observed in the somatic compartment of these neurons as compared to those seen in the untreated control cultures. These observations suggested to us that morphine treatment had caused either an increase in the levels of functional DOR- whether by de novo synthesis or by some cellular modifications in DOR processing- or an increase in internalization dynamics of its ligand or both. Our immunocytochemical results demonstrated that chronic morphine treatment actually increased DOR immunoreactive proteins in both somatic and processes compartments, suggesting that the observed increase in fluo-DLT-I internalization resulted at least in part from a morphine-induced up-regulation of DOR. However, recent studies from our laboratory further indicated that chronic activation of MOR by morphine selectively increased the plasma membrane targeting of DOR from intracellular stores, making more of the latter available for DOR agonist internalization (Cahill et al., unpublished). Indeed, electron microscopic studies using silver-intensified gold grains showed that, compared to control untreated cultures, the proportion of membrane associated over intracellular grains for each labeled neuron was significantly increased. This morphine-induced targeting enhancement is MOR-specific as the addition of the selective MOR antagonist CTOP to the morphine-treated cultures prevented the increase in fluo-DLT-I internalization. In light of these observations, we suggest here that the dramatic increase of fluo-DLT-I internalization in cortical neurons seen following chronic morphine treatment is attributable not only to an increase in the number of

functional DORs, but also to an increase in membrane targeting of this receptor via a morphine-induced MOR-specific effect.

In conclusion, this study is the first to demonstrate the internalization and targeting of opioid receptor ligands in neurons. We report that cortical neurons expressing MOR and DOR in vitro internalize fluo-DRM and fluo-DLT-I, respectively, in a time-dependent, receptor- and clathrin-mediated fashion, with kinetics comparable to those reported previously for MOR and DOR internalization in non-neuronal and neuronal systems. We also report that, following receptor-ligand internalization, both fluo-ligands dissociate from their receptors and are transported centripetally via a microtubule-dependent mechanism to the neuronal soma in endosomal-like vesicles while both MOR and DOR appear to be recycled locally. Furthermore, naloxone treatment of our primary cultures for 48 hours resulted in an up-regulation of MOR without a concomitant increase in fluo-DRM uptake nor a change in the dynamics of internalization and transport of the fluorescent ligand, suggesting that the up-regulated receptor was either not targeted to the plasma membrane or incompletely maturated. On the other hand, chronic naloxone treatment targets DORs to the plasma membrane without changing its level of expression, thereby increasing the amount of fluo-DLT-I internalization in both processes and somatic compartments. Additionally, we report that chronic morphine treatment of cortical neurons leads to a down-regulation of cell surface MORs which results in a decrease in ligand uptake and internalization of fluo-DRM. At the same time, we postulated here that chronic high morphine tone causes MOR to be abnormally retained in the somatic of the cortical neuron and impairs its delivery to the neuronal periphery. Lastly, chronic morphine treatment led to a functional up-regulation

of DOR that resulted in an enhanced internalization of fluo-DLT-I. In essence, chronic morphine treatment increases the availability of cell surface DOR available for internalization.

.

BIBLIOGRAPHY

- Abood, M. E., Noel, M. A., Farnsworth, J. S., and Tao, Q.: Molecular cloning and cxpression of a delta-opioid receptor from rat brain. J Neurosci Res 37 (6): 714-9, 1994.
- Aimone, L. D., and Yaksh, T. L.: Opioid modulation of capsaicin-evoked release of substance P from the rat spinal cord in vivo. Peptides 10: 1127-1131, 1989.
- Akil, H., Mayer, D. J., and Liebeskind, J. C.: Antagonism of stimulation-produced analgesia by naloxone, a narcotic antagonist. Science **191** (4230): 961-2, 1976.
- Anderson, K. M., and Peach, M. J.: Receptor binding and internalization of a unique biologically active angiotensin II-colloidal gold conjugate: morphological analysis of angiotensin II processing in isolated vascular strips. J Vasc Res 31 (1): 10-7, 1994.
- Arvidsson, U., Dado, R. J., Riedl, M., Lee, J. H., Law, P. Y., Loh, H. H., R., E., and Wessendorf, M. W.: δ-opioid receptor immunoreactivity: distribution in brainstem and spinal cord, and relationship to biogenic amines and enkephalin. Journal of Neuroscience 15: 1215-1235, 1995a.
- Arvidsson, U., Riedl, M., Chakrabarti, S., Lee, J. H., Nakano, A. H., Dado, R. J., Loh, H. H., Law, P. Y., Wessendorf, M. W., and Elde, R.: Distribution and targeting of a mu-opioid receptor (MOR1) in brain and spinal cord. J Neurosci 15 (5 Pt 1): 3328-41, 1995b.
- Ashworth, R., Yu, R., Nelson, E. J., Dermer, S., Gershengorn, M. C., and Hinkle, P. M.: Visualization of the thyrotropin-releasing hormone receptor and its ligand during endocytosis and recycling. Proc Natl Acad Sci U S A 92 (2): 512-6, 1995.

- Barg, J., Levy, R., and Simantov, R.: Up-regulation of opiate receptors by opiate antagonists in neuroblastoma-glioma cell culture: the possibility of interaction with guanosine triphosphate-binding proteins. Neurosci Lett **50** (1-3): 133-7, 1984.
- Bausch, S. B., Patterson, T. A., Appleyard, S. M., and Chavkin, C.: Immunocytochemical localization of delta opioid receptor in mouse brain. Journal of Chemical Neuroanatomy 8: 175-189, 1995.
- Befort, K., Tabbara, L., Kling, D., Maigret, B., and Kieffer, B. L.: Role of aromatic transmembrane residues of the delta-opioid receptor in ligand recognition. J Biol Chem 271 (17): 10161-8, 1996.
- Belcheva, M. M., Barg, J., McHale, R. J., Gao, X. M., Chuang, D. M., and Coscia, C. J.: Up-regulation of delta opioid receptors in neuroblastoma hybrid cells: evidence for differences in the mechanisms of action of sodium butyrate and naltrexone. J Pharmacol Exp Ther 259 (1): 302-9, 1991.
- Benovic, J. L., Kuhn, H., Weyand, I., Codina, J., Caron, M. G., and Lefkowitz, R. J.:
 Functional desensitization of the isolated beta-adrenergic receptor by the betaadrenergic receptor kinase: potential role of an analog of the retinal protein arrestin (48-kDa protein). Proc Natl Acad Sci U S A 84 (24): 8879-82, 1987.
- Bernard, V., Laribi, O., Levey, A. I., and Bloch, B.: Subcellular redistribution of m2 muscarinic acetylcholine receptors in striatal interneurons in vivo after acute cholinergic stimulation. J Neurosci 18 (23): 10207-18, 1998.
- Bertagna, X.: Proopiomelanocortin-derived peptides. Endocrinol Metab Clin North Am 23 (3): 467-85, 1994.
- Bertrand, B., Dumartin, B., and Bernard, V.: In vivo regulation of intraneuronal trafficking of G-protein-coupled receptors for neurotransmitters. TIPS 20 (8): 315-

- Bewley, T. H.: An introduction to drug dependence. Br J Psychiatry Spec (9): 225-38, 1975.
- Bishop, N. E.: An Update on Non-clathrin-coated Endocytosis. Rev Med Virol 7 (4): 199-209, 1997.
- Bodnar, R. J., Williams, C. L., Lee, S. J., and Pasternak, G. W.: Role of μ₁-opioids receptors in supraspinal opiate analgesia: a microinjection study. Brain Research 447: 25-34, 1988.
- Burford, N. T., Tolbert, L. M., and Sadee, W.: Specific G protein activation and muopioid receptor internalization caused by morphine, DAMGO and endomorphin I. Eur J Pharmacol 342 (1): 123-6, 1998.
- Cahill, C. M., McClellan, K. A., Morinville, A., Hoffert, C., Hubatsch, D., O'Donnell, D., and Beaudet, A.: Immunocytochemical distribution of delta opioid receptors in rat CNS: antigen-specific cellular compartmentalization. Journal of Comparative Neurology Submitted, 2000.
- Cahill, C. M., Morinville, A., Lee, M.-C., Vincent, J.-P., Collier, B., and Beaudet, A.: Prolonged morphine treatment targets delta opioid receptors to neuronal plasma membranes and enhances delta-mediated antinociception. paper submitted.
- Carman, C. V., and Benovic, J. L.: G-protein-coupled receptors: turn-ons and turn-offs. Curr Opin Neurobiol 8 (3): 335-44, 1998.
- Castel, M. N., Beaudet, A., and Laduron, P. M.: Retrograde axonal transport of neurotensin in rat nigrostriatal dopaminergic neurons. Modulation during ageing and possible physiological role. Biochem Pharmacol 47 (1): 53-62, 1994.

- Chen, Y., Mestek, A., Liu, J., Hurley, J. A., and Yu, L.: Molecular cloning and functional expression of a mu-opioid receptor from rat brain. Mol Pharmacol 44 (1): 8-12, 1993.
- Cheng, P. Y., Wu, D., Decena, J., Soong, Y., McCabe, S., and Szeto, H.H.: Opioid induced stimulation of fetal respiratory activity by (D-Ala²)deltorphin I. European Journal of Pharmacology 230: 85-88, 1993.
- Childers, S. R.: Opioid receptors: pinning down the opiate targets. Current Biology 7: R695-R697, 1997.
- Chu, P., Murray, S., Lissin, D., and von Zastrow, M.: Delta and kappa opioid receptors are differentially regulated by dynamin-dependent endocytosis when activated by the same alkaloid agonist. J Biol Chem 272 (43): 27124-30, 1997.
- Cowan, A., Zhu, X. Z., Mosberg, H. I., Omnaas, J. R., and Porreca, F.: Direct dependence studies in rats with agents selective for different types of opioid receptor. J Pharmacol Exp Ther 246 (3): 950-5, 1988.
- Cvejic, S., and Devi, L. A.: Dimerization of the delta opioid receptor: implication for a role in receptor internalization. J Biol Chem 272 (43): 26959-64, 1997.
- Dado, R. J., Law, P. Y., Loh, H. H., and Elde, R.: Immunofluorescent identification of a delta (δ) opioid receptor on primary afferent nerve terminals. Neuroreport 5: 341-344, 1993.
- Daniels, G. M., and Amara, S. G.: Regulated trafficking of the human dopamine transporter. Clathrin-mediated internalization and lysosomal degradation in response to phorbol esters. J Biol Chem 274 (50): 35794-801, 1999.
- Defagot, M. C., and Antonelli, M. C.: Autoradiographic localization of the putative D4

dopamine receptor in rat brain. Neurochem Res 22 (4): 401-7, 1997.

- Delay-Goyet, P., Zayac, J. M., and Roques, B. P.: Improved quantitative radioautography of rat brain delta-opioid binding sites using [³H]DSTBU-LET, a new highly potent and selective linear enkephalin analogue. Neurochemistry International 16: 341-368, 1990.
- Delfs, J. M., Kong, H., Mestek, A., Chen, Y., Yu, L., Reisine, T., and Chesselet, M. F.: Expression of mu opioid receptor mRNA in rat brain: an in situ hybridization study at the single cell level. J Comp Neurol **345** (1): 46-68, 1994.
- Dhawan, B. N., Cesselin, F., Raghubir, R., Reisine, T., Bradley, P. B., Portoghese, P. S., and Hamon, M.: International Union of Pharmacology. XII. Classification of opioid receptors. Pharmacol Rev 48 (4): 567-92, 1996.
- Dickenson, A. H.: Plasticity: Interventions for opioids and other pharmacological interventions in specific pain states. Behavioral Brain Science 20: 392-403, 1997.
- Dickerson, I. M., and Noel, G.: Tissue specific peptide processing. In Peptide Biosynthesis and Processing, ed. by L. D. Fricker, pp. 71-109, CRC, 1991.
- Ding, Y. Q., Kancko, T., Nomura, S., and Mizuno, N.: Immunohistochemical localization of mu-opioid receptors in the central nervous system of the rat. Journal of Comparative Neurology **367** (3): 375-402, 1996.
- Douglas, A. J., Dyc, S., Leng, G., Russell, J. A., and Bicknell, R. J.: Endogenous opioid regulation of oxytocin secretion through pregnancy in the rat. Journal of Neuroendocrinology 5: 307-314, 1993.
- Dournaud, P., Boudin, H., Schronbrunn, A., Tannenbaum, G., and Beaudet, A.: Interrelationships between somatostatin sst2A receptors and somatostatincontaining axons in rat brain: evidence for regulation of cell surface receptors by

- Dumartin, B., Caille, I., Gonon, F., and Bloch, B.: Internalization of D1 dopamine receptor in striatal neurons in vivo as evidence of activation by dopamine agonists. J Neurosci 18 (5): 1650-61, 1998.
- Evans, C. J., Keith, D. E., Jr., Morrison, H., Magendzo, K., and Edwards, R. H.: Cloning of a delta opioid receptor by functional expression [see comments]. Science 258 (5090): 1952-5, 1992.
- Faure, M. P., Alonso, A., Nouel, D., Gaudriault, G., Dennis, M., Vincent, J. P., and Beaudet, A.: Somatodendritic internalization and perinuclear targeting of neurotensin in the mammalian brain. J Neurosci 15 (6): 4140-7, 1995.
- Ferguson, S. S., Barak, L. S., Zhang, J., and Caron, M. G.: G-protein-coupled receptor regulation: role of G-protein-coupled receptor kinases and arrestins. Can J Physiol Pharmacol 74 (10): 1095-110, 1996a.
- Ferguson, S. S., Downey, W. E., 3rd, Colapietro, A. M., Barak, L. S., Menard, L., and Caron, M. G.: Role of beta-arrestin in mediating agonist-promoted G proteincoupled receptor internalization. Science 271 (5247): 363-6, 1996b.
- Ferguson, S. S., Menard, L., Barak, L. S., Koch, W. J., Colapietro, A. M., and Caron, M.
 G.: Role of phosphorylation in agonist-promoted beta 2-adrenergic receptor sequestration. Rescue of a sequestration-defective mutant receptor by beta ARK1.
 J Biol Chem 270 (42): 24782-9, 1995.
- Ferrante, F. M.: Principles of opioid pharmacotherapy: practical implications of basic mechanisms. J Pain Symptom Manage 11 (5): 265-73, 1996.
- Ferrari, S. L., Behar, V., Chorev, M., Rosenblatt, M., and Bisello, A.: Endocytosis of

ligand-human parathyroid hormone receptor 1 complexes is protein kinase Cdependent and involves beta-arrestin2. Real-time monitoring by fluorescence microscopy. J Biol Chem **274** (42): 29968-75, 1999.

- Fox, D. A., and Burks, T. F.: Roles of central and peripheral mu, delta and kappa opioid receptors in the mediation of gastric acid secretory effects in the rat. Journal of Pharmacology and Experimental Therapeutics **244**: 456-462, 1988.
- Fukuda, K., Kato, S., Morikawa, H., Shoda, T., and Mori, K.: Functional coupling of the delta-, mu-, and kappa-opioid receptors to mitogen-activated protein kinase and arachidonate release in Chinese hamster ovary cells. J Neurochem 67 (3): 1309-16, 1996.
- Gagnon, A. W., Kallal, L., and Benovic, J. L.: Role of clathrin-mediated endocytosis in agonist-induced down-regulation of the beta2-adrenergic receptor. J Biol Chem 273 (12): 6976-81, 1998.
- Gaudriault, G., Nouel, D., Dal Farra, C., Beaudet, A., and Vincent, J. P.: Receptorinduced internalization of selective peptidic mu and delta opioid ligands. J Biol Chem 272 (5): 2880-8, 1997.
- George, S. R., Fan, T., Xie, Z., Tse, R., Tam, V., Varghese, G., and O'Dowd, B. F.: Oligomerization of mu- and delta-opioid receptors. Generation of novel functional properties. J Biol Chem 275 (34): 26128-35, 2000.
- Gibson, A. E., Noel, R. J., Herlihy, J. T., and Ward, W. F.: Phenylarsine oxide inhibition of endocytosis: effects on asialofetuin internalization. Am J Physiol 257 (2 Pt 1): C182-4., 1989.
- Gilbert, P. E., and Martin, W. R.: The effects of morphine and nalorphine-like drugs in the nondependent, morphine-dependent and cyclazocine-dependent chronic spinal

dog. J Pharmacol Exp Ther 198 (1): 66-82, 1976.

- Goldstein, A. and James, I. F.: Multiple opioid receptors. Criteria for identification and classification. Trends Pharmacol Sci 5: 503-505, 1984.
- Goodman, O. B., Jr., Krupnick, J. G., Gurevich, V. V., Benovic, J. L., and Keen, J. H.: Arrestin/clathrin interaction. Localization of the arrestin binding locus to the clathrin terminal domain. J Biol Chem 272 (23): 15017-22, 1997.
- Gouldson, P. R., Snell, C. R., Bywater, R. P., Higgs, C., and Reynolds, C. A.: Domain swapping in G-protein coupled receptor dimers. Protein Eng 11 (12): 1181-93, 1998.
- Grady, E. F., Slice, L. W., Brant, W. O., Walsh, J. H., Payan, D. G., and Bunnett, N. W.: Direct observation of endocytosis of gastrin releasing peptide and its receptor. J Biol Chem 270 (9): 4603-11, 1995.
- Grady, E. F., Gamp, P. D., Jones, E., Baluk, P., McDonald, D. M., Payan, D. G., and Bunnett, N. W.: Endocytosis and recycling of neurokinin-1 receptors in enteric neurons. Neuroscience 75 (4): 1239-54, 1996.
- Grisel, J. E., Mogil, J. S., Belknap, J. K., and Grandy, D. K.: Orphanin FQ acts as a supraspinal, but not a spinal, anti-opioid peptide. Neuroreport 7 (13): 2125-9, 1996.
- Haasemann, M., Cartaud, J., Muller-Esterl, W., and Dunia, I.: Agonist-induced redistribution of bradykinin B2 receptor in caveolae. J Cell Sci 111 (Pt 7): 917-28, 1998.
- Hackler, L., Zadina, J. E., Ge, L. J., and Kastin, A. J.: Isolation of relative large amounts of endomorphin-1 and endomorphin-2 from human brain cortex. Peptides 18 (10): 1635-1639, 1997.

- Hamon, G., and Jouquey, S.: Kappa agonists and vasopressin secretion. Hormone Research 34: 129-132, 1990.
- Hassan, A. H., Ableitner, A., Stein, C., and Herz, A.: Inflammation of the rat paw enhances axonal transport of opioid receptors in the sciatic nerve and increases their density in the inflamed tissue. Neuroscience **55** (1): 185-95, 1993.
- Hc, L., and Lee, N. M.: Delta opioid receptor enhancement of mu opioid receptorinduced antinociception in spinal cord. J Pharmacol Exp Ther 285 (3): 1181-6, 1998.
- Hebert, T. E., and Bouvier, M.: Structural and functional aspects of G protein-coupled receptor oligomerization. Biochem Cell Biol 76 (1): 1-11, 1998.
- Henderson, G., and McKnight, A. T.: The orphan opioid receptor and its endogenous ligand--nociceptin/orphanin FQ. Trends Pharmacol Sci 18 (8): 293-300, 1997.
- Hiller, J. M., Zhang, Y., Bing, G., Gioannini, T. L., Stone, E. A., and Simon, E. J.: Immunohistochemical localization of the mu-opioid receptors in the rat brain using antibodies generated against a peptide sequence in a purified mu-opioid binding protein. Neuroscience 62 (3): 829-841, 1994.
- Hinshaw, J. E.: Dynamin and Its Role in Membrane Fission. Annu Rev Cell Dev Biol 16: 483-519, 2000.
- Hirota, N., Kuraishi, Y., Hino, Y., Sato, Y., Satoh, M., and Takagi, H.: Met-enkephalin and morphine but not dynorphin inhibit noxious stimuli-induced release of substance P from rabbit dorsal horn in situ. Neuropharmacology 24: 567-570, 1985.

- Hirst, J., and Robinson, M. S.: Clathrin and adaptors. Biochim Biophys Acta 1404 (1-2): 173-93, 1998.
- Hitzemann, R. J., Hitzemann, B. A., and Loh, H. H.: Binding of 3H-naloxone in the mouse brain: effect of ions and tolerance development. Life Sci 14 (12): 2393-404, 1974.
- Hollt, V.: Opioid peptide genes: structure and regulation. *In* Neurobiology of Opioids, ed. by O. F. X. Almeida and T. S. Shippenberg, pp. 11-51, Springer-Verlag, 1991.
- Hughes, J., Smith, T. W., Kosterlitz, H. W., Fothergill, L. A., Morgan, B. A., and Morris,
 H. R.: Identification of two related pentapeptides from the brain with potent opiate agonist activity. Nature 258 (5536): 577-80, 1975.
- Hyytia, P., Ingman, K., Soini, S. L., Laitinen, J. T., and Korpi, E. R.: Effects of continuous opioid receptor blockade on alcohol intake and up-regulation of opioid receptor subtype signalling in a genetic model of high alcohol drinking. Naunyn Schmiedebergs Arch Pharmacol 360 (4): 391-401, 1999.
- Jenab, S., and Inturrisi, C. E.: Ethanol and naloxone differentially upregulate delta opioid receptor gene expression in neuroblastoma hybrid (NG108-15) cells. Brain Res Mol Brain Res 27 (1): 95-102, 1994.
- Jennes, L., Bronson, D., Stumpf, W. E., and Conn, P. M.: Evidence for an association between calmodulin and membrane patches containing gonadotropin-releasing hormone--receptor complexes in cultured gonadotropes. Cell Tissue Res 239 (2): 311-5, 1985.
- Ji, R. R., Zhang, Q., Law, P. Y., Low, H. H., Elde, R., and Hökfelt, T.: Expression of mu-, delta-, and kappa-opioid receptor-like immunoreactivities in rat dorsal root

ganglia after carrageenan-induced inflammation. Journal of Neuroscience 15: 8156-8166, 1995.

- Jiang, Q., Mosberg, H. I., and Porreca, F.: Selective modulation of morphine antinociception, but not development of tolerance, by delta receptor agonists. Eur J Pharmacol 186 (1): 137-41, 1990.
- Jordan, B. A., and Devi, L. A.: G-protein-coupled receptor heterodimerization modulates receptor function. Nature **399** (6737): 697-700, 1999.
- Kakidani, H., Furutani, Y., Takahashi, H., Noda, M., Morimoto, Y., Hirose, T., Asai, M., Inayama, S., Nakanishi, S., and Numa, S.: Cloning and sequence analysis of cDNA for porcine beta-neo-endorphin/dynorphin precursor. Nature 298 (5871): 245-9., 1982.
- Kcith, D. E., Anton, B., Murray, S. R., Zaki, P. A., Chu, P. C., Lissin, D. V., Monteillet-Agius, G., Stewart, P. L., Evans, C. J., and von Zastrow, M.: mu-Opioid receptor internalization: opiate drugs have differential effects on a conserved endocytic mechanism in vitro and in the mammalian brain. Mol Pharmacol 53 (3): 377-84, 1998.
- Keith, D. E., Murray, S. R., Zaki, P. A., Chu, P. C., Lissin, D. V., Kang, L., Evans, C. J., and von Zastrow, M.: Morphine activates opioid receptors without causing their rapid internalization. J Biol Chem 271 (32): 19021-4, 1996.
- Kieffer, B. L., Befort, K., Gaveriaux-Ruff, C., and Hirth, C. G.: The delta-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization [published erratum appears in Proc Natl Acad Sci U S A 1994 Feb 1;91(3):1193]. Proc Natl Acad Sci U S A 89 (24): 12048-52, 1992.
- Knapp, R. J., Malatynska, E., Fang, L., Li, X., Babin, E., Nguyen, M., Santoro, G.,

Varga, E. V., Hruby, V. J., Roeske, W. R., and et al.: Identification of a human delta opioid receptor: cloning and expression. Life Sci 54 (25): L463-9, 1994.

- Koenig, J. A., Edwardson, J. M., and Humphrey, P. P.: Somatostatin receptors in Neuro2A neuroblastoma cells: ligand internalization. Br J Pharmacol 120 (1): 52-9, 1997.
- Kosterlitz, H. W., and Waterfield, A. A.: Differential effects of drugs on the acetylcholine output from the myenteric plexus and the responses of the longitudinal muscle of the guinea-pig ileum. Br J Pharmacol 46 (3): 569P-570P, 1972.
- Krueger, K. M., Daaka, Y., Pitcher, J. A., and Lefkowitz, R. J.: The role of sequestration in G protein-coupled receptor resensitization. Regulation of beta2-adrenergic receptor dephosphorylation by vesicular acidification. J Biol Chem 272 (1): 5-8, 1997.
- Laporte, S. A., Oakley, R. H., Holt, J. A., Barak, L. S., and Caron, M. G.: The interaction of beta-arrestin with the AP-2 adaptor is required for the clustering of beta 2adrenergic receptor into clathrin-coated pits. J Biol Chem **275** (30): 23120-6, 2000.
- Larkin, J. M., Donzell, W. C., and Anderson, R. G.: Potassium-dependent assembly of coated pits: new coated pits form as planar clathrin lattices. J Cell Biol 103 (6 Pt 2): 2619-27, 1986.
- Law, P. Y., Hom, D. S., and Loh, H. H.: Loss of opiate receptor activity in neuroblastoma X glioma NG108-15 hybrid cells after chronic opiate treatment. A multiple-step process. Mol Pharmacol 22 (1): 1-4, 1982.
- Le Gouill, C., Parent, J. L., Rola-Pleszczynski, M., and Stankova, J.: Structural and functional requirements for agonist-induced internalization of the human plateletactivating factor receptor. J Biol Chem 272 (34): 21289-95, 1997.

- Lefkowitz, R. J., and Caron, M. G.: Ciba-Geigy award for outstanding research. Regulation of adrenergic receptor function by phosphorylation. J Mol Cell Cardiol 18 (9): 885-95, 1986.
- Lin, X., Loh, H. H., Law, P. Y., Wessendorf, M. W., and al., e.: The kappa-opioid receptor is primarily postsynaptic: combined immunohistochemical localization of the receptor and endogenous opioids. Proceedings of the National Academy of Sciences of the United States of America. 92 (11): 5062-5066, 1995.
- Liu, P., Ying, Y., Ko, Y. G., and Anderson, R. G.: Localization of platelet-derived growth factor-stimulated phosphorylation cascade to caveolae. J Biol Chem 271 (17): 10299-303, 1996.
- Lohse, M. J., Benovic, J. L., Codina, J., Caron, M. G., and Lefkowitz, R. J.: beta-Arrestin: a protein that regulates beta-adrenergic receptor function. Science 248 (4962): 1547-50, 1990.
- Lord, J. A., Waterfield, A. A., Hughes, J., and Kosterlitz, H. W.: Endogenous opioid peptides: multiple agonists and receptors. Nature **267** (5611): 495-9, 1977.
- Lukoff, I. F.: Consequences of use. Heroin and other narcotics. NIDA Res Monogr 16: 195-227, 1977.
- Luttrell, L. M., Daaka, Y., Della Rocca, G. J., and Lefkowitz, R. J.: G protein-coupled receptors mediate two functionally distinct pathways of tyrosine phosphorylation in rat 1a fibroblasts. She phosphorylation and receptor endocytosis correlate with activation of Erk kinases. J Biol Chem **272** (50): 31648-56, 1997.
- Mackawa, K., Minami, M., Yabuuchi, K., Toya, T., Katao, Y., Hosoi, Y., Onogi, T., and Satoh, M.: In situ hybridization study of mu- and kappa-opioid receptor mRNAs in the rat spinal cord and dorsal root ganglia. Neurosci Lett **168** (1-2): 97-100, 1994.

- Malmberg, A. B., and Yaksh, T. L.: Isobolographic and dose-response analyses of the interaction between intrathecal mu and delta agonists: effects of naltrindole and its benzofuran analog (NTB). J Pharmacol Exp Ther **263** (1): 264-75, 1992.
- Mansour, A., Burke, S., Pavlic, R. J., Akil, H., and Watson, S. J.: Immunohistochemical localization of the cloned kappa 1 receptor in the rat CNS and pituitary. Neuroscience 71 (3): 671-690, 1996.
- Mansour, A., Fox, C. A., Akil, H., and Watson, S. J.: Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. Trends Neurosci 18 (1): 22-9, 1995a.
- Mansour, A., Fox, C. A., Burke, S., Akil, H., and Watson, S. J.: Immunohistochemical localization of the cloned mu opioid receptor in the rat CNS. Journal of Chemical Neuroanatomy 8 (4): 283-305, 1995b.
- Mansour, A., Fox, C. A., Burke, S., Meng, F., Thompson, R. C., Akil, H., and Watson, S. J.: Mu, delta, and kappa opioid receptor mRNA expression in the rat CNS: an in situ hybridization study. J Comp Neurol 350 (3): 412-38, 1994a.
- Mansour, A., Fox, C. A., Meng, F., Akil, H., and Watson, S. J.: Kappa 1 receptor mRNA distribution in the rat CNS: comparison to kappa receptor binding and prodynorphin mRNA. Mol Cell Neurosci 5 (2): 124-44, 1994b.
- Mansour, A., Fox, C. A., Thompson, R. C., Akil, H., and Watson, S. J.: mu-Opioid receptor mRNA expression in the rat CNS: comparison to mu-receptor binding. Brain Res 643 (1-2): 245-65, 1994c.
- Mansour, A., Khachaturian, H., Lewis, M. E., Akil, H., and Watson, S. J.: Autoradiographic differentiation of mu, delta and kappa opioid receptors int the rat forebrain and midbrain. Journal of Neuroscience 7: 2445-2464, 1987.

- Mansour, A., Khachaturian, H., Lewis, M. E., Akil, H., and Watson, S. J.: Anatomy of CNS opioid receptors. Trends Neurosci 11 (7): 308-14, 1988.
- Mansour, A., Thompson, R. C., Akil, H., and Watson, S. J.: Delta opioid receptor mRNA distribution in the brain: comparison to delta receptor binding and proenkephalin mRNA. J Chem Neuroanat 6 (6): 351-62, 1993.
- Mantyh, P. W., DeMaster, E., Malhotra, A., Ghilardi, J. R., Rogers, S. D., Mantyh, C. R., Liu, H., Basbaum, A. I., Vigna, S. R., Maggio, J. E., and et al.: Receptor endocytosis and dendrite reshaping in spinal neurons after somatosensory stimulation. Science 268 (5217): 1629-32, 1995.
- Martin, W. R., Eades, C. G., Thompson, J. A., Huppler, R. E., and Gilbert, P. E.: The effects of morphine- and nalorphine- like drugs in the nondependent and morphine-dependent chronic spinal dog. J Pharmacol Exp Ther **197** (3): 517-32, 1976.
- Martin-Schild, S., Zadina, J. E., Gerall, A. A., Vigh, S., and Kastin, A. J.: Localization of endomorphin-2-like immunoreactivity in the rat medulla and spinal cord. Peptides 18 (10): 1641-9, 1997.
- Melone, M., Brecha, N. C., Sternini, C., Evans, C., and Conti, F.: Etorphine increases the number of μ-opioid receptor positive cells in the cerebral cortex. Neuroscience 100 (3): 439-443, 2000.
- Meunier, J. C., Mollereau, C., Toll, L., Suaudeau, C., Moisand, C., Alvinerie, P., Butour, J. L., Guillemot, J. C., Ferrara, P., Monsarrat, B., and et al.: Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor [see comments]. Nature 377 (6549): 532-5, 1995.


- McConalogue, K., Grady, E. F., Minnis, J., Balestra, B., Tonini, M., Brecha, N. C., Bunnett, N. W., and Sternini, C.: Activation and internalization of the mu-opioid receptor by the newly discovered endogenous agonists, endomorphin-1 and endomorphin-2. Neuroscience **90** (3): 1051-9, 1999.
- Menard, L., Ferguson, S. S., Barak, L. S., Bertrand, L., Premont, R. T., Colapietro, A. M., Lefkowitz, R. J., and Caron, M. G.: Members of the G protein-coupled receptor kinase family that phosphorylate the beta2-adrenergic receptor facilitate sequestration. Biochemistry 35 (13): 4155-60, 1996.
- Menard, L., Ferguson, S. S., Zhang, J., Lin, F. T., Lefkowitz, R. J., Caron, M. G., and Barak, L. S.: Synergistic regulation of beta2-adrenergic receptor sequestration: intracellular complement of beta-adrenergic receptor kinase and beta-arrestin determine kinetics of internalization. Mol Pharmacol 51 (5): 800-8, 1997.
- Meng, F., Taylor, L. P., Hoversten, M. T., Ueda, Y., Ardati, A., Reinscheid, R. K., Monsma, F. J., Watson, S. J., Civelli, O., and Akil, H.: Moving from the orphanin FQ receptor to an opioid receptor using four point mutations. J Biol Chem 271 (50): 32016-20, 1996.
- Mentlein, R.: Dipeptidyl-peptidase IV (CD26)--role in the inactivation of regulatory peptides. Regul Pept 85 (1): 9-24, 1999.
- Meunier, J. C., Mollereau, C., Toll, L., Suaudeau, C., Moisand, C., Alvineric, P., Butour, J. L., Guillemot, J. C., Ferrara, P., Monsarrat, B., and et al.: Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor [see comments]. Nature 377 (6549): 532-5, 1995.
- Millan, M. J.: κ-opioid receptors and analgesia. Trends in Pharmacological Science 11: 70-76, 1990.
- Minami, M., Maekawa, K., Yabuuchi, K., and Satoh, M.: Double in situ hybridization

study on co-existance of mu, delta-, and kappa-opioid receptor mRNAs with preprotachykinin A mRNA in the rat dorsal root ganglia. Molecular Brain Research **30**: 203-210, 1995.

- Moriwaki, A., Wang, J. B., Svingos, A., van Bockstaele, E., Cheng, P., Pickel, V., and Uhl, G. R.: mu Opiate receptor immunoreactivity in rat central nervous system. Neurochemical Research 21 (11): 1315-1331, 1996.
- Morris, B. J., and Millan, M. J.: Inability of an opioid antagonist lacking negative intrinsic activity to induce opioid receptor up-regulation in vivo. Br J Pharmacol **102** (4): 883-6, 1991.
- Mulder, A. H., Hogenboom, F., Wardeh, G., and Schoffelmeer, A. N.: Morphine and enkephalins potently inhibit [3H]noradrenaline release from rat brain cortex synaptosomes: further evidence for a presynaptic localization of mu-opioid receptors. J Neurochem 48 (4): 1043-7, 1987.
- Mulder, A. H., Wardeh, G., Hogenboom, F., and Frankhuyzen, A. L.: Kappa- and deltaopioid receptor agonists differentially inhibit striatal dopamine and acetylcholine release. Nature **308** (5956): 278-80, 1984.
- Mulder, A. H., Wardeh, G., Hogenboom, F., Kazmierski, W., Hruby, V. J., and Schoffelmeer, A. N.: Cyclic somatostatin analogues as potent antagonists at mu-, but not delta- and kappa-opioid receptors mediating presynaptic inhibition of neurotransmitter release in the brain. Eur J Pharmacol **205** (1): 1-6, 1991.
- Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A. C., Cohen, S. N., and Numa, S.: Nucleotide sequence of cloned cDNA for bovine corticotropin-beta-lipotropin precursor. Nature 278 (5703): 423-7., 1979.
- Nestler, E. J.: Molecular mechanisms of drug addiction. Journal of Neuroscience 12: 2439-2450, 1992.

- Noda, M., Furutani, Y., Takahashi, H., Toyosato, M., Hirose, T., Inayama, S., Nakanishi, S., and Numa, S.: Cloning and sequence analysis of cDNA for bovine adrenal preproenkephalin. Nature 295 (5846): 202-6., 1982
- North, R. A., Williams, J. T., Surprenant, A., and Christie, M. J.: Mu and delta receptors belong to a family of receptors that are coupled to potassium channels. Proc Natl Acad Sci U S A 84 (15): 5487-91, 1987.
- Olson, G. A., Olson, R. D., and Kastin, A. J.: Endogenous opiates: 1994. Peptides 16 (8): 1517-55, 1995.
- Ossipov, M. H., Lopcz, Y., Nichols, M. L., Bian, D., and Porreca, F.: Inhibition by spinal morphine of the tail-flick response is attenuated in rats with nerve ligation injury. Neurosci Lett **199** (2): 83-6, 1995.
- Pan, Y. X., Xu, J., Wan, B. L., Zuckerman, A., and Pasternak, G. W.: Identification and differential regional expression of KOR-3/ORL-1 gene splice variants in mouse brain. FEBS Lett 435 (1): 65-8, 1998.
- Parton, R. G., Joggerst, B., and Simons, K.: Regulated internalization of cavcolae. J Cell Biol 127 (5): 1199-215, 1994.
- Pasternak, G. W.: Pharmacological mechanisms of opioid analgesics. Clinical Neuropharmacology 16: 1-18, 1993.
- Pasternak, G. W., Pan, Y. X., and Cheng, J.: Correlating the pharmacology and molecular biology of opioid receptors. Cloning and antisense mapping a kappa 3-related opiate receptor. Ann N Y Acad Sci 757: 332-8, 1995.
- Pcarse, B. M., Smith, C. J., and Owen, D. J.: Clathrin coat construction in endocytosis. Curr Opin Struct Biol 10 (2): 220-8, 2000.

- Perlman, J. H., Nussenzveig, D. R., Osman, R., and Gershengorn, M. C.: Thyrotropinreleasing hormone binding to the mouse pituitary receptor does not involve ionic interactions. A model for neutral peptide binding to G protein-coupled receptors. J. Biol. Chem. 267: 24413-24417
- Pert, C. B., and S.H., S.: Opiate receptor: Its demonstration in nervous tissue. Science 179: 1011-1014, 1973.
- Pippig, S., Andexinger, S., and Lohse, M. J.: Sequestration and recycling of beta 2adrenergic receptors permit receptor resensitization. Mol Pharmacol 47 (4): 666-76, 1995.
- Premont, R. T., Inglese, J., and Lefkowitz, R. J.: Protein kinases that phosphorylate activated G protein-coupled receptors. Faseb J 9 (2): 175-82, 1995.
- Probst, W. C., Snyder, L. A., Schuster, D. I., Brosius, J., and Sealfon, S. C.: Sequence alignment of the G-protein coupled receptor superfamily. DNA Cell Biol 11 (1): 1-20, 1992.
- Przewlocki, R., Labuz, D., Mika, J., Przewlocka, B., Tomboly, C., and Toth, G.: Pain inhibition by endomorphins. Ann N Y Acad Sci 897: 154-64, 1999.
- Quirion, R., Zajac, J. M., Morgat, J. L., and Roques, B. P.: Autoradiographic distribution of mu and delta opiate receptors in rat brain using highly selective ligands. Life Sci 33 (Suppl 1): 227-30, 1983.
- Rankin, M. L., Alvania, R. S., Gleason, E. L., and Bruch, R. C.: Internalization of G protein-coupled receptors in single olfactory receptor neurons. J Neurochem 72 (2): 541-8, 1999.



- Raposo, G., Dunia, I., Delavier-Klutchko, C., Kaveri, S., Strosberg, A. D., and Benedetti,
 E. L.: Internalization of beta-adrenergic receptor in A431 cells involves non-coated vesicles. Eur J Cell Biol 50 (2): 340-52, 1989.
- Reinscheid, R. K., Nothacker, H. P., Bourson, A., Ardati, A., Henningsen, R. A., Bunzow, J. R., Grandy, D. K., Langen, H., Monsma, F. J., Jr., and Civelli, O.: Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. Science 270 (5237): 792-4, 1995.
- Rocttger, B. F., Rentsch, R. U., Hadac, E. M., Hellen, E. H., Burghardt, T. P., and Miller,
 L. J.: Insulation of a G protein-coupled receptor on the plasmalemmal surface of the pancreatic acinar cell. J Cell Biol 130 (3): 579-90, 1995a.
- Rocttger, B. F., Rentsch, R. U., Pinon, D., Holicky, E., Hadac, E., Larkin, J. M., and Miller, L. J.: Dual pathways of internalization of the cholecystokinin receptor. J Cell Biol 128 (6): 1029-41, 1995b.
- Rossi, G. C., Leventhal, L., Bolan, E., and Pasternak, G. W.: Pharmacological characterization of orphanin FQ/nociceptin and its fragments. J Pharmacol Exp Ther 282 (2): 858-65, 1997.
- Roth, B. L., Palvimaki, E. P., Berry, S., Khan, N., Sachs, N., Uluer, A., and Choudhary,
 M. S.: 5-Hydroxytryptamine2A (5-HT2A) receptor desensitization can occur without down-regulation. J Pharmacol Exp Ther 275 (3): 1638-46, 1995.
- Sarret, P., Nouel, D., Dal Farra, C., Vincent, J. P., Beaudet, A., and Mazella, J.: Receptormediated internalization is critical for the inhibition of the expression of growth hormone by somatostatin in the pituitary cell line AtT-20. J Biol Chem **274** (27): 19294-300, 1999.

Schafer, M. K., Bette, M., Romeo, H., Schwaeble, W., and Weihe, E.: Localization of

kappa-opioid receptor mRNA in neuronal subpopulations of rat sensory ganglia and spinal cord. Neurosci Lett 167 (1-2): 137-40, 1994.

- Schafer, M. K.-H., et al.: Distribution of opioids in brain and peripheral tissue. In Neurobiology of opioids, ed. by O. F. X. Almeida and T. S. Shippenberg, pp. 53-71, Springer-Verlag.
- Schlador, M. L., and Nathanson, N. M.: Synergistic regulation of m2 muscarinic acetylcholine receptor desensitization and sequestration by G protein-coupled receptor kinase-2 and beta-arrestin-1. J Biol Chem 272 (30): 18882-90, 1997.
- Schreff, M., Schulz, S., Wiborny, D., and Hollt, V.: Immunofluorescent identification of endomorphin-2-containing nerve fibers and terminals in the rat brain and spinal cord. Neuroreport 9 (6): 1031-4, 1998.
- Sever, S., Damke, H., and Schmid, S. L.: Dynamin:GTP controls the formation of constricted coated pits, the rate limiting step in clathrin-mediated endocytosis. J Cell Biol 150 (5): 1137-48, 2000.
- Sharif, N. A., and Hughes, J.: Discrete mapping of brain Mu and delta opioid receptors using selective peptides: quantitative autoradiography, species differences and comparison with kappa receptors. Peptides 10 (3): 499-522, 1989.
- Sheldon, R. J., Riviere, P. J., Malarchik, M. E., Moseberg, H. I., Burks, T. F., and Porreca, F.: Opioid regulation of mucosal ion transport in the mouse isolated jejunum. J Pharmacol Exp Ther **253** (1): 144-51, 1990.
- Shields, D.: Signals for intracellular protein sorting: implication for peptide hormone precursor processing and secretion. *In* Peptide Biosynthesis and Processing, ed. by L. D. Fricker, pp. 37-70, CRC, 1991.

Silva, W. I., Andres, A., Schook, W., and Puszkin, S.: Evidence for the presence of

muscarinic acetylcholine receptors in bovine brain coated vesicles. J Biol Chem **261** (31): 14788-96, 1986.

- Simon, E. J., and Hiller, J. M.: Opioid peptides and opioid receptors. In Basic Neurochemistry: Molecular, Cellular, and Medical Aspects., ed. by G. J. Siegel., et al., pp. 321-339, Raven Press, 1994.
- Sternini, C., Spann, M., Anton, B., Keith, D. E., Jr., Bunnett, N. W., von Zastrow, M., Evans, C., and Brecha, N. C.: Agonist-selective endocytosis of mu opioid receptor by neurons in vivo. Proc Natl Acad Sci U S A 93 (17): 9241-6, 1996.
- Stroh, T., Jackson, A. C., Dal Farra, C., Schonbrunn, A., Vincent, J. P., and Beaudet, A.: Receptor-mediated internalization of somatostatin in rat cortical and hippocampal neurons. Synapse 38: 177-186, 2000.
- Takatsuki, A., Muroi, M., and Yamasaki, M.: [Inhibitors of intracellular translocation of glycoproteins]. Tanpakushitsu Kakusan Koso **38** (11): 1704-16., 1993.
- Temple, A., and Zukin, R. S.: Neuroanatomical patterns of the mu, delta and kappa opioid receptors of rat brain as determined by quantitative in vitro autoradiography. Proceedings of the National Academy of Science. USA. 84: 4308-4312, 1987.
- Terenius, L.: Stereospecific interaction between narcotic analgesics and a synaptic plasma membrane fraction of rat cerebral cortex. Acta of Pharmacology and Toxicology **32**: 317-320, 1973.
- Thompson, R. C., Mansour, A., Akil, H., and Watson, S. J.: Cloning and pharmacological characterization of a rat mu opioid receptor. Neuron **11**, 903-913, 1993.

Trapaidze, N., and Devi, A. L.: Agonist-independent internalization of Delta Opioid

Receptors: A role for C-tail in constitutive receptor endocytosis. J. Neurochem. submitted., 1999.

- Trapaidze, N., Gomes, I., Bansinath, M., and Devi, L. A.: Recycling and resensitization of delta opioid receptors. DNA Cell Biol 19 (4): 195-204, 2000.
- Trapaidze, N., Keith, D. E., Cvejic, S., Evans, C. J., and Devi, L. A.: Sequestration of the delta opioid receptor. Role of the C terminus in agonist-mediated internalization. J Biol Chem 271 (46): 29279-85, 1996.
- Tsao, P. I., and Zastrow, M.: Downregulation of G protein-coupled receptors. Curr Opin Neurobiol 10 (3): 365-9, 2000a.
- Tsao, P. I., and von Zastrow, M.: Type-specific sorting of G protein-coupled receptors after endocytosis. J Biol Chem 275 (15): 11130-40, 2000b.
- Tsuga, H., Kameyama, K., Haga, T., Kurose, H., and Nagao, T.: Sequestration of muscarinic acetylcholine receptor m2 subtypes. Facilitation by G protein-coupled receptor kinase (GRK2) and attenuation by a dominant-negative mutant of GRK2. J Biol Chem 269 (51): 32522-7, 1994.
- Unterwald, E. M., Anton, B., To, T., Lam, H., and Evans, C. J.: Quantitative immunolocalization of mu opioid receptors: regulation by naltrexone. Neuroscience 85 (3): 897-905, 1998.
- Unterwald, E. M., Rubenfeld, J. M., Imai, Y., Wang, J. B., Uhl, G. R., and Kreek, M. J.: Chronic opioid antagonist administration upregulates mu opioid receptor binding without altering mu opioid receptor mRNA levels. Brain Res Mol Brain Res 33 (2): 351-5, 1995.
- Vandenbulcke, F., Nouel, D., Vincent, J., Mazella, J., and Beaudet, A.: Ligand-induced internalization of neurotensin in transfected COS-7 cells: differential intracellular

trafficking of ligand and receptor [In Process Citation]. J Cell Sci 113 (Pt 17): 2963-75, 2000.

- Vogler, O., Nolte, B., Voss, M., Schmidt, M., Jakobs, K. H., and van Koppen, C. J.: Regulation of muscarinic acetylcholine receptor sequestration and function by beta-arrestin. J Biol Chem 274 (18): 12333-8, 1999.
- von Zastrow, M., Keith, D. E., Zaki, P., and Evans, C. J.: Morphine and opioid peptide cause opposing effects on the endocytic trafficking of opioid receptors. Regulatory Peptides 54: 315-316, 1994.
- von Zastrow, M., Link, R., Daunt, D., Barsh, G., and Kobilka, B.: Subtype-specific differences in the intracellular sorting of G protein-coupled receptors. J Biol Chem **268** (2): 763-6, 1993.
- Wang, J. B., Johnson, P. S., Imai, Y., Persico, A. M., Ozenberger, B. A., Eppler, C. M., and Uhl, G. R.: cDNA cloning of an orphan opiate receptor gene family member and its splice variant. FEBS Lett 348 (1): 75-9, 1994.
- Waterfield, A. A., and Kosterlitz, H. W.: Stereospecific increase by narcotic antagonists of evoked acetylcholine output in guinea-pig ileum. Life Sci 16 (12): 1787-92, 1975.
- Way, E. L.: Review and overview of four decades of opiate research. Adv Biochem Psychopharmacol 20: 3-27, 1979.
- Weiss, A., and Schlessinger, J.: Switching signals on or off by receptor dimerization. Cell **94** (3): 277-80, 1998.
- Whistler, J. L., Chuang, H. H., Chu, P., Jan, L. Y., and von Zastrow, M.: Functional dissociation of mu opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction [see comments]. Neuron 23 (4): 737-46, 1999.

- White, J. H., Wise, A., Main, M. J., Green, A., Fraser, N. J., Disney, G. H., Barnes, A. A., Emson, P., Foord, S. M., and Marshall, F. H.: Heterodimerization is required for the formation of a functional GABA(B) receptor [see comments]. Nature 396 (6712): 679-82, 1998.
- Willingham, M. C., Haigler, H. T., Fitzgerald, D. J., Gallo, M. G., Rutherford, A. V., and Pastan, I. H.: The morphologic pathway of binding and internalization of epidermal growth factor in cultured cells. Studies on A431, KB, and 3T3 cells, using multiple methods of labelling. Exp Cell Res 146 (1): 163-75, 1983.
- Yamashiro, D. J., and Maxfield, F. R.: Acidification of endocytic compartments and the intracellular pathways of ligands and receptors. J Cell Biochem 26 (4): 231-46, 1984.
- Yoburn, B. C., Shah, S., Chan, K., Duttaroy, A., and Davis, T.: Supersensitivity to opioid analgesics following chronic opioid antagonist treatment: relationship to receptor selectivity. Pharmacol Biochem Behav **51** (2-3): 535-9, 1995.
- Yu, W., Centonze, V. E., Ahmad, F. J., and Baas, P. W.: Microtubule nucleation and release from the neuronal centrosome. J Cell Biol 122 (2): 349-59., 1993.
- Zadina, J. E., Chang, S. L., Ge, L. J., and Kastin, A. J.: Mu opiate receptor downregulation by morphine and up-regulation by naloxone in SH-SY5Y human neuroblastoma cells. J Pharmacol Exp Ther 265 (1): 254-62, 1993.
- Zadina, J. E., Harrison, L. M., Ge, L. J., Kastin, A. J., and Chang, S. L.: Differential regulation of mu and delta opiate receptors by morphine, selective agonists and antagonists and differentiating agents in SH-SY5Y human neuroblastoma cells. J Pharmacol Exp Ther 270 (3): 1086-96, 1994.

Zadina, J. E., Martin-Schild, S., Gerall, A. A., Kastin, A. J., Hackler, L., Ge, L. J., and

Zhang, X.: Endomorphins: novel endogenous mu-opiate receptor agonists in regions of high mu-opiate receptor density. Ann N Y Acad Sci **897:** 136-44, 1999.

- Zaki, P. A., Bilsky, E. J., Vanderah, T. W., Lai, J., Evans, C. J., and Porreca, F.: Opioid receptor types and subtypes: the delta receptor as a model. Annu Rev Pharmacol Toxicol 36: 379-401, 1996.
- Zaki, P. A., Keith, D. E., Jr., Brine, G. A., Carroll, F. I., and Evans, C. J.: Ligand-induced changes in surface mu-opioid receptor number: relationship to G protein activation? J Pharmacol Exp Ther **292** (3): 1127-34, 2000.
- Zhang, J., Ferguson, S. S. G., Barak, L. S., Menard, L., and Caron, M. G.: Dynamin and beta-arrestin reveal distinct mechanisms for G protein-coupled receptor internalization. J Biol Chem 271 (31): 18302-5, 1996
- Zhang, J., Ferguson, S. S., Law, P. Y., Barak, L. S., and Caron, M. G.: Agonist-specific regulation of delta-opioid receptor trafficking by G protein-coupled receptor kinase and beta-arrestin. J Recept Signal Transduct Res 19 (1-4): 301-13, 1999.