EXPRESSION OF CALCITONIN GENE-RELATED PEPTIDE AND SUBSTANCE P IN CULTURED DORSAL ROOT GANGLION NEURONS FOLLOWING CHRONIC EXPOSURE TO OPIATES

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Abstract

The mechanisms involved in morphine tolerance are poorly understood. It was reported that calcitonin gene-related peptide (CGRP) immunoreactivity was increased in the spinal dorsal horn during morphine tolerance and an *in vitro* repetitive morphine treatment was able to mimic the *in vivo* results by inducing increases in CGRP- and substance P (SP)-immunoreactive (IR) neurons in cultured dorsal root ganglia (DRG). The aim of this study was to validate the DRG cell culture model by establishing which subtypes of opioid receptors are involved in the induction of CGRP and SP in cultured rat DRG neurons and to examine the signaling pathway involved in the induction of the neuropeptides following repetitive opiate treatments.

Following treatment with any of the three opioid agonists [μ; DAMGO, δ; DPDPE, κ; U50488H], the number of CGRP- and SP-IR neurons increased significantly, in a concentration-dependent manner. Double-immunofluorescence staining showed that the three opioid receptors were colocalized with both of the pain-related neuropeptides. Protein kinase C (PKC)-IR was found to be significantly increased following a repetitive treatment with DAMGO. Double-immunofluorescence staining showed the colocalization of PKCα with CGRP or SP in cultured DRG neurons. Moreover, a combined treatment of the opioid with a PKC inhibitor was able to block the effects of the opioid on increased CGRP-like IR.

In conclusion, the data suggests that the three opioid receptors may be involved in the induction of CGRP and SP observed following chronic exposure to opiates and that PKC probably plays a role in the signaling pathway leading to the upregulation. These findings further validate the DRG cell culture as a suitable model to study intracellular pathways that govern changes seen following repetitive opioid treatments.

Résumé

Les mécanismes impliquées dans le développement de la tolérance à la morphine n'ont pas encore été clairement déterminés. Certains résultats ont démontrés que l'immunoréactivité du peptide relié au gène de la calcitonine (CGRP) augmentait dans la corne de l'épine dorsale chez des rat tolérants à la morphine. Des données comparables ont été obtenues *in vitro* alors qu'un traitement répété à la morphine a augmenté le nombre de neurones immunoreactifs au CGRP et à la substance P (SP) dans des neurones de la corne de l`épine dorsale en culture. Le but de ce mémoire était de valider ce modèle de culture cellulaire en déterminant quel sous-type de récepteurs opiacés sont impliqués dans l'augmentation de l`immunoréactivié au CGRP et à la SP. Un second objectif était d'examiner les mécanismes intracellulaires qui jouent un rôle dans l'induction des neuropeptides observé après un traitement répété avec un opiacé.

Suite à un traitement répété avec un des trois agonistes opiacés (μ; DAMGO, δ;DPDPE, κ;U50488H), le nombre de neurones immunoréactifs pour le CGRP et la SP est augmenté très significativement. Un double marquage a de plus démontré que les trois récepteurs opiacés et les deux peptides reliés à la douleur étaient co-localisées. Un traitement avec le DAMGO induit aussi une augmentation du nombre de neurones immunoréactifs pour la proteine kinase C (PKC). Une immunohistolocalisation double a aussi démontrée que la PKCα, le CGRP et la SP sont exprimés par les mêmes neurones. Finalement, un traitement combiné avec un opiacé et un inhibiteur de la PKC a bloqué l'effet des opiacés sur l`augmentation de l`immunoréactivité au CGRP.

En conclusion, nos résultats suggèrent que les trois sous-types de récepteurs opiacés sont capables d`induire l`expression du CGRP et de la SP suite à un traitement répété. Il semble aussi que la PKC soit impliquée dans les mécanismes intracellulaires qui sont responsables de ces changements.

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TABLE OF CONTENTS

TABLES AND FIGURES

A. TABLES

B. FIGURES

CHAPTER I - REVIEW OF THE LITERATURE

A. OPIOIDS

The opium poppy (*Papaver somniferum*) has been known and used in Asia and southeastern Europe for over 2000 years (for reviews see Brownstein *et al.*, 1993; Satoh and Minami, 1995; Dhawan *et al.*, 1996). Its juice was known to contain an agent that relieved pain and diarrhea, produced sleep or drowsiness, and in low doses produced a blissful or euphoric state. Only crude preparations were available for medical use until the isolation and purification of morphine by Serturer in 1805 who also gave it its name after the God of dreams, Morpheus. Following the invention of the hypodermic syringe and the hollow needle in the 1850s, the use of morphine was extended to surgical procedures, postoperative and chronic pain, and also as an adjunct to general anesthetics.

Unfortunately, it was realized that morphine had a potential for abuse as great as opium and was not safe for chronic use. Since that time a great deal of energy has been spent trying to develop safer and more efficacious, non-addictive opiates. In 1898, heroin was first synthesized and was claimed to be more potent than morphine and free from abuse potential. This was the first of such claims for novel opiates and still to date none of these have proven valid as all cause tolerance as well as physical and psychological dependence. In the 1930s and 1940s, meperidine and methadone were synthesized; these were the first opiates with structures different from that of morphine but with similar pharmacological profiles. Around the same time, Weijlard and Erikson (1942) produced nalorphine (*N*-allylnormorphine); the first opiate mixed agonist-antagonist. This compound had the ability to reverse the respiratory depression produced by morphine and to precipitate the withdrawal syndrome in addicts. Its discovery was important since it lead to the development of other compounds such as naloxone, which is a relatively pure opiate antagonist. By

the 1960s, it was evident that the actions of opiate agonists, antagonists and mixed agonist-antagonists could only be explained by actions on multiple opiate receptors (Portoghese, 1965). In 1971, Goldstein *et al.* attempted to demonstrate the existence of these receptors and to characterize them using radiolabeled drugs. However, their efforts failed because their radioligands were not of high specific activity. In 1973, three different groups showed the presence of stereospecific receptors for opioid drugs existed in the central nervous system (Pert and Snyder, 1973; Simon *et al.*, 1973; Terenius *et al.*, 1973). Since it seemed improbable that the animal brain would have evolved receptors for plant alkaloids to which it was never exposed, researchers postulated that these receptors must normally mediate the effects of endogenous material produced in the brain itself. Kosterlitz and colleagues (Hughes *et al.*, 1975) isolated the first such materials which were found to be short peptides with morphine-like properties, and termed enkephalins. Soon afterward, longer peptides with similar properties (endorphins, dynorphins) were discovered (Bradbury *et al.*, 1976; Pasternak *et al.*, 1976; Goldstein *et al.*, 1981). These are known collectively as endogenous opioids. These peptides are synthesized within the brain itself as large precursor proteins (proenkephalin, prodynorphin and proopriomelanocortin; Table 1; Nakanishi *et al.*, 1979; Noda *et al.*, 1982; Kakidani *et al.*, 1982), which contain the opioid peptides and other neuroendocrine peptides (ACTH, αMSH) as parts of their amino acid sequences. These precursors are expressed in several regions in the brain but are cleaved by different enzymes, thus giving rise to different products at different sites.

Table 1. Affinity (K_i – nM) of endogenous opiates.

LIGAND	μ [^{3}H] DAMGO	δ [^{3}H] naltrindole	κ [^{3}H]U-69,593
Leu-enkephalin	3.4	4.0	>1000
Met-enkephalin	0.65	1.7	>1000
Dynorphin	32	>1000	0.5
β-endorphin	1.0	1.0	52

From Raynor *et al.*, 1994.

The enkephalins are considered the endogeneous ligands for the δ class of opioid receptors and dynorphins are the endogenous ligands for the κ receptor. β-endorphin was found to bind with about equal affinity to both μ and δ receptors. Recently, two peptides isolated from brain, endormorphin-1 and endomorphin-2 showed the highest affinity and selectivity for the μ-opioid receptor (Zadina *et al.*, 1997). Further studies involving the pharmacological characterization of these endogenous peptides support the possibility that they may represent the endogenous μ receptor ligands (Zadina *et al.*, 1997; Goldberg *et al.*, 1998).

1. Opioid Receptors

The realization that many potential ligands were present in the central nervous system gave credibility to the hypothesis of the existence of more than one class of opioid receptors. This notion had been postulated when it was reported that nalorphine, an opiate antagonist, did not antagonize different narcotic analgesics to the same extent (Cox and Weinstock, 1964). Among the first piece of evidence for this came in 1976 when Martin *et al.* performed a detailed analysis of the neurophysiological and behavioral properties of several opiate compounds and looked for "cross tolerance" among these opiates in the dog. They proposed the existence of three opioid receptors named after the drugs used: μ (for morphine, which induced analgesia, miosis, bradycardia, hypothermia, indifference to environmental stimuli), κ (for ketocyclazocine, which induced miosis, sedation, depression of flexor reflexes) and σ (for *N*-allylnormetazocine, which induces mydriasis, tachycardia, delirium, increased respiration). Following the first discovery of native opioid peptides, Kosterlitz and his colleagues attempted to know on which opioid receptors the enkephalins were acting on (Lord *et al.*, 1977). They were able to demonstrate that opioid peptides appeared to be interacting with different populations of

opioid receptors in the mouse vas deferens and the guinea pig ileum. During these studies, they found that enkephalins showed a higher potency relative to morphine in the mouse vas deferens but lower potency relative to morphine in the guinea pig ileum. Following these observations they proposed that a fourth type of opioid receptor, the δ receptor, was present in the mouse vas deferens but absent in the guinea pig ileum. Other pharmacological evidence for multiple opioid receptors resulted from selective protection and inactivation experiments with alkylating and acylating agents (Robson *et al.*, 1979; Smith *et al.*, 1980).

a. Pharmacological classification

Opioids have a large number of actions, both centrally and peripherally. Although most opioids produce analgesia, they differ a greatly in their other effects. The discovery of different classes of opioid receptors has helped to explain this spectrum of effects. The synthesis of highly selective opioid agonists and antagonists has provided further evidence for the existence of opioid receptor sub-types. Since these bind with high affinity and specificity, they can be used for receptor binding as well as anatomical, receptor autoradiograpy, physiological and pharmacological studies. Three major classes of opioid receptors are recognized (for review see Adler *et al.*, 1990; Simon and Gioannini, 1993), with each class having its own pattern of affinity for the various endogenous and synthetic opioid agonists and antagonists. None of the opiate drugs appear to have absolute specificity for a single receptor type; rather, each drug has a major affinity for one type, with lesser degrees of affinity for the other types. The μ-opioid receptor binds with high affinity to morphine, naloxone and [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin (DAMGO) (Kosterlitz and Paterson, 1980). The δ-opioid receptor has high affinity for [D-Ala^2, D-Leu^5] enkephalin (DADLE) and the more selective ligand [D-Pen^2, D-Pen^5] enkephalin (DPDPE) (Mosberg *et al.*, 1983). The κ-

opioid receptor showed high affinity for the endogenous κ-agonist dynorphin A, the κ selective synthetic ligands (+-)-*trans*-3, 4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl) cyclohexyl] benzeneacetamide (U50488H) and (+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl bezeneacetamide (U69593) (von Voigtlander *et al.*, 1983; Lahti *et al.*, 1985). At present, seven subtypes of opioid receptor have been pharmacologically characterized: μ_1, μ_2 and μ_3 (Ling *et al.*, 1983; Martin, 1984; Pasternak *et al.*, 1986; Stefano *et al.*, 1993), κ_1, κ_2 and κ_3 (Clark *et al.*, 1989) and δ_1 and δ_2 (Mattia *et al.*, 1991, 1992; Zhu *et al.*, 1999). However, only three distinct clones (μ, δ and κ) have been characterized thus far. A fourth class was formerly believed to exist, the σ-receptor (Martin *et al.* 1976), but this is no longer considered to be an opioid receptor. A major difficulty in studying the pharmacological profile of opioid receptors is that most tissues studied co-express several subtypes of these receptors and many of the compounds used can cross-react with the other receptors to varying degrees. Hence, molecular studies have proved most useful to precisely identify and to pharmacologically characterize receptors.

b. Opioid Receptor Clones

Attempts to purify opioid receptors to homogeneity were thwarted by their paucity in most tissues and their lability after detergent solubilization. Many attempts were made to clone cDNAs encoding opioid receptors. In 1989, Schofield *et al.* isolated a cDNA encoding an opioid-binding protein with μ selectivity however the protein lacked transmembrane domains deemed necessary for signal transduction. In 1992, the isolation of another cDNA was obtained by expression cloning (Xie *et al.*, 1992) but the affinity of opioid ligands for this receptor expressed in COS cells was two orders of magnitude below the expected value and no subtype specificity could be shown. The structure of opioid receptors remained a mystery until 1993 when both Kieffer *et al.* and Evans *et al.* reported the isolation and pharmacological

characterization of the first high affinity opioid receptor, the mouse δ opioid receptor. Both groups isolated the cDNA encoding the δ opioid receptor by transfecting a cDNA library from NG-108-15 cells, which expresses a high level of δ receptors per cell, into COS cells. Kiefer *et al.* (1993) screened the transfected cells with a ^{3}H-labeled Tyr-D-Thr-Gly-Phe-Leu-Thr (DTLET) to find the presence of high-affinity binding sites with a K_D similar to the native δ receptor. Evans *et al.* (1993) used a similar method to isolate the same cDNA clone and they also showed that activation of their receptor with selective δ agonists could decrease cAMP levels in transfected COS cells. The cloned mouse receptor is 372 amino acid protein, whose sequence is related to that of the somatostatin receptor, among members of the G protein-coupled family of receptors.

Hybridization experiments with the δ-OR cDNA as a probe then allowed the isolation of cDNAs encoding another opioid-receptor subtype. The cloning of the mouse κ opioid receptor occurred during efforts to obtain mouse somatostatin receptor cDNA probes. Yasuda *et al.* (1993) while trying to isolate cDNAs encoding novel somatostatin receptors identified two closely related G protein-coupled receptor clones in an adult mouse brain cDNA library. One of these G protein-coupled receptor had the identical sequence of the δ opioid receptor that had been cloned by Evans *et al.* (1993). Preliminary binding studies with agonists selective for μ, δ, κ opioid receptors confirmed that one of the receptors was indeed the δ opioid receptor. The other G protein-coupled receptor was shown to be the κ opioid receptor since it was able to only bind to [^{3}H]naloxone and the κ-selective agonist [^{3}H]U-69-593. No specific binding of [^{3}H]naltrindole (δ), [^{3}H]DPDPE (δ), [^{3}H]DTG (μ), [^{3}H]dextromethorphan (μ) or [^{3}H]DAMGO (μ) was detected. The mouse κ receptor is a 380 amino acid protein having 70% amino acid similarity with the sequence of the mouse δ receptor. The sequences of these two receptors were most similar in the membrane-spanning segments and intracellular loops with

the amino- and carboxyl-termini showing differences in both sequence and length. This group also showed that both of these opioid receptors were able to mediate subtype-specific agonist-induced inhibition of adenylyl cyclase activity in COS-1 cells (Yasuda *et al.*, 1993). These findings suggested that the different opioid receptor classes originate from distinct gene products.

The first μ opioid receptor cDNA clone was described by Chen *et al.* (1993). The clone was identified from a rat brain cDNA library using a probe obtained from the same library through PCR amplification with two mouse δ opioid receptor primers corresponding to the region between the third transmembrane domain to the third cytoplasmic loop. Through the agonist and antagonist profile of the clone expressed in COS-7 cells, the authors were able to conclude that they had isolated a rat μ opioid receptor clone. The sequence of the 398 amino acid rat μ opioid receptor showed 58% and 57% amino acid identity with the sequences of the mouse δ and κ opioid receptors, respectively. Similar to the other groups, Chen *et al.* (1993) were able to show that the μ opioid receptor is functionally coupled to the inhibition of adenylyl cyclase. Around the same period, two groups (Minami *et al.*, 1994; Thompson *et al.*, 1993) were also successful in cloning the rat μ opioid receptor.

Once the opioid receptor clones were identified in rodents, the next step was to isolate the human counterparts since these are the ultimate targets of therapeutic opioid drugs. The clones for each human opioid receptor type were identified in 1994. Wang *et al.* (1994) screened a human cerebral cortex cDNA library using oligonucleotide fragments from the rat μ-opioid receptor and produced a human μ-opioid receptor clone. The protein sequence had 95 % amino acid identity to the rat μ receptor. The cloning of a cDNA for a human δ opioid receptor was described by Knapp *et al.* (1994) using hybridization screening methods. Two clones were identified, when they ligated them at an overlapping region, the assembled open reading frame encoded a 372 residue

protein having 93% amino acid identity to both the mouse and rat δ opioid receptors. The cDNA for a human κ opioid receptor was produced by Mansson *et al.* (1994) using a consensus 5' sense primer and 3'end antisense primer. The PCR product had an open reading frame encoding 380 amino acids with 91% amino acid identity to the cloned rat κ opioid receptor. The human μ-, δ-, and κ-opioid receptor genes are located on chromosomes 6q24-25 (Wang *et al.*, 1994), 1p34.3-36.1 (Befort *et al.*, 1994) and 8q11.2 (Yasuda *et al.*, 1994), respectively. One of the principal motivations for cloning opioid receptors was to provide information on their structures and to allow their detailed pharmacological characterization. With the availability of cloned opioid receptors, it permitted each receptor type to be examined independently of the others since cell lines can be generated that express only a single class of opioid receptor. Although only three opioid receptors have been cloned, there is evidence from binding and autoradiographic studies for further receptor heterogeneity. These multiple receptor subtypes must be a product of alternative splicing (Pan *et al.*, 1999), heterodimerization (Heyman *et al.*, 1989; Traynor and Elliott, 1993), differential post-translational modifications or alterations in the molecular environment of the receptor protein. A number of investigators have proposed that receptor-receptor interactions could form the basis for some of the opioid receptor subtypes (Porreca *et al.*, 1992; Xu *et al.*, 1993; Jordan and Devi, 1999). It has been shown that δ receptors heterodimerize with both μ (Gomes *et al.*, 2000) and κ receptors (Jordan and Devi, 1999) thus changing their ligand binding and signaling properties (Jordan and Devi, 1999; Jordan *et al.*, 2000; Gomes *et al.*, 2001). Another possibility could be the existence of other opioid receptor genes that still need to be cloned.

G-Protein Coupling

Hydrophobicity analyses of the deduced amino acid sequences of the three cloned opioid receptors indicated that these receptors have seven putative

transmembrane helices characteristic of the G-protein coupled receptor family. The comparison of the amino acid sequences of the three clones receptors shows that the sequences of membrane spanning segments 2, 3 and 7 are highly conserved whereas the sequences of segments 1, 4 and 5 are more divergent (Dohlman *et al.*, 1991). The sequences of the intracellular loops connecting them are similar in the three opioid receptors, but the extracellular connecting loops and -NH_2 and -COOH terminals are different in the three subtypes and presumably account for the distinct ligand affinities and effects. The high degree of sequence homology seen in the intracellular regions within the opioid receptor family suggests that they may interact with similar G proteins (for review see Law *et al.*, 2000). Hence, these receptors may share similar second messenger systems, since the intracellular loops are thought to be critical for the coupling of this receptor family to G proteins.

Second Messenger Systems

The physiological responses activated by opiates are mediated by multiple agonist-induced mechanisms, which in turn regulate various downstream signaling pathways (for review, see Jordan and Devi, 1998; Law *et al.*, 2000). G proteins link opioid receptors to second messengers that act to inhibit adenylyl cyclase activity (Sharma *et al.*, 1975; Yu *et al.*, 1986, 1990; for review, see Childers, 1993), decrease voltage-gated Ca^{2+} currents (Seward *et al.*, 1991; Surprenant *et al.*, 1990; for reviews, see North, 1986, 1993), regulate the MAP kinase pathway (Li and Chang, 1996) and increase receptor-gated K^+ current leading to hyperpolarization (Tatsumi *et al.*, 1990; for reviews, see North, 1986, 1993). These cellular effects have been shown to be modulated by protein kinases, including protein kinase A (PKA), protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase II (Yu *et al.*, 1996). Activity at opioid receptors stimulates the hydrolysis of phosphatidyinositol (PI) leading to an increased production of the intracellular messengers inositol trisphosphate

(IP_3) and diacylglycerol (DAG). Intracellular Ca^{2+} becomes increased in response to the production of IP_3 , which then promotes the release of intracellular Ca^{2+} from its stores in the endoplasmic reticulum (Womack *et al.*, 1988). The increased Ca^{2+} concentration and production of DAG leads to the stimulation of various forms of protein kinase C (Hug *et al.*, 1993). PKC has been implicated in signaling pathways that lead to the induction of various proto-oncogenes such as c-*fos* and c-*jun*, which encode for transcription factors controlling the expression of different genes that are involved in pain (Naranjo *et al.*, 1991; Li and Clark, 1999). Pertussin toxin abolishes the inhibition of adenylate cyclase by opioids in NG108-15 cells (Burns *et al.*, 1983), suggesting that G_i- (or G_o-) protein couples to the opioid receptors to exert these inhibitory effects. The reduction in Ca^{2+} currents and increases in K^+ conductance through opioid receptors was also blocked by pertussis toxin (Seward *et al.*, 1991; Surprenant *et al.*, 1990; Tatsumi *et al.*, 1990) further indicating the involvement of G_i- and/or G_o- proteins. The end result of opioid receptor activation is the hyperpolarization of the neuron bearing the receptor, with a concomitant reduction in its activity and its transmitter release. Opioids appear to act essentially as inhibitors of neuronal electrical activity, both spontaneous and evoked, and of neurotransmitter release.

2. Distribution of Opioid Receptors

The distribution of opioid receptors and opioid peptides is not uniform throughout the nervous system. There is some important overlap in the localization of each of these receptor types and their precise anatomical distributions vary markedly in a given species and across species.

μ Opioid Receptor

Autoradiographical studies with selective radioligands showed that the μ-opioid receptor is distributed throughout the neuraxis. The highest density of

receptors is present in the caudate putamen and the density diminished but remained important in the neocortex, nucleus accumbens, hippocampus and amygdala. Expression of μ-opioid receptor mRNA is intense in the superficial laminae of the dorsal horn (laminae I and II; Gouarderes *et al.*, 1991; Maekawa *et al.*, 1994) where nociceptive C and Aδ fibers of primary afferents terminate (Light *et al.*, 1977; Beese *et al.*, 1990) suggesting that the receptors play a role in the modulation of nociceptive information at postsynaptic sites of the primary afferents. Moderate amounts are observed in the periqueductal gray and raphe nuclei while low densities are found in the hypothalamus, preoptic area and globus pallidus (Waksman *et al.*, 1986; Hawkins *et al.*, 1988; Mansour *et al.*, 1988; for review see Mansour *et al.*, 1994). Immunohistochemical investigations using antibodies raised against the cloned μ-opioid receptor confirmed the autoradiographical data (Bourgoin *et al.*, 1994; Honda *et al.*, 1995; Mansour *et al.*, 1995; Arvidsson *et al.*, 1995b). Moreover, data at the ultrastuctural level (Arvidsson *et al.*, 1995b) demonstrated that the μ-opioid receptor is localized both pre- and postsynaptically in the dorsal horn. μ-Opioid receptor are also widely distributed in the peripheral nervous system, most notably in the myenteric neurons of the gut (Hutchison *et al.*, 1975) and the vas deferens (Lemaire *et al.*, 1978).

δ-Opioid Receptor

In the central nervous system, δ-opioid receptors have a more restricted distribution than the other opioid receptors and predominantly seen in forebrain structures. Autoradiographical studies with tritiated and radioiodinated ligands have shown that the highest receptor densities are found in the olfactory bulb, neocortex, caudate putamen and nucleus accumbens while the thalamus, hypothalamus and brainstem show much lower levels (Mansour *et al.*, 1988; Dupin *et al.*, 1991; Renda *et al.*, 1993; LeMoine *et al.*, 1994). These results were later confirmed in both rodents and primates using immunohistochemical studies with antibodies generated against portions of the δ-opioid receptor

(Dado *et al.*, 1993; Arvidsson *et al.*, 1995a; Bausch *et al.*, 1995; Honda *et al.*, 1995). In the rat spinal cord, the expression of δ-opioid receptor mRNA is in low to moderate amounts throughout laminae I-VI. Also, immunocytochemistry at the ultrastuctural level (Cheng *et al.*, 1995) demonstrated the existence of presynaptic δ-opioid receptor that could be responsible for the decrease in the release of neurotransmitters (substance P, CGRP, etc.) from the primary afferent nerves within the dorsal horn of the spinal cord (Bourgoin *et al*, 1994). It has been postulated that the μ- and δ-opioid receptors were closely related and possibly physically associated (Schoffelmer *et al.*, 1987) however an autoradiographic study in μ-opioid receptor knockout mouse showed no difference in selective ligand binding to δ-opioid receptor between the homozygous mutant and wild-type mouse (Chen *et al.*, 2000).

κ-Opioid Receptor

The κ receptor demonstrates a third pattern, with its receptor binding sites found in the preoptic area, hypothalamus and caudate putamen (Mansour *et al.*, 1988). The distribution of κ-opioid receptor seems to vary widely across species (Quirion *et al.*, 1983; Zukin *et al.*, 1988; Boyle *et al.*, 1990; Rothman *et al.*, 1992). For example, in the rat, low levels of receptors are found throughout the cortex with the highest densities being observed in the nucleus accumbens and claustrum (Nock *et al.*, 1988). On the other hand, in the guinea pig, the highest density of κ-opioid receptors is found in the inner layers of the cerebral cortex and the substantia nigra. The mRNA is intensely expressed in the locus coeruleus of the mouse brain, whereas only a small number of cells express it in this region of the rat brain (Mansour *et al.*, 1994). Such species differences suggest differences in the roles of the κ-opioid receptor in several brain regions across the species. In the rat spinal cord (Maekawa *et al.*, 1994), high levels of κ-opioid receptor mRNA are expressed in laminae I and II

suggesting that these receptors play an important role in the modulation of nociceptive information at postsynaptic sites of primary afferents. The expression of the κ-opioid receptor mRNA is also expressed in important amounts throughout laminae III-VIII. The immunohistochemical localization of the κ-opioid receptor in both rats and guinea pig (Arvidsson *et al.*, 1995c) corresponds well with the distribution of its binding sites (Unterwald *et al.*, 1991) and the localization of its mRNA (Mansour *et al.*, 1994). Prominent staining for the κ-opioid receptor was seen in the ventral forebrain, hypothalamus, thalamus, posterior pituitary and midbrain (Arvidsson *et al.*, 1995c). At the ultrastrutural level, the κ-opioid receptor is mostly found postsynaptically in the somatodendritic compartment (Arvidsson *et al.*, 1995c). An autoradiographic study has demonstrated that a complete loss of μ-opioid receptors did not affect the distribution and expression levels of the κ-opioid receptor (Chen *et al.*, 2000).

Expression in the Dorsal Root Ganglia

In situ hybridization histochemistry have shown that in the dorsal root ganglia (DRG), where the cell bodies of primary afferent neurons are located, the expression of μ-, δ- and κ-opioid receptor mRNAs is intense (Maekawa *et al.*, 1994; Minami *et al.*, 1995). The μ-opioid receptor mRNA is highly expressed in 55% of DRG neurons while the δ- and κ-opioid receptor mRNAs are only present in 20% and 18% of DRG neurons, respectively. The dorsal horn, particularly laminae II and I, in all segments of the spinal cord was found to be enriched with the μ-opioid receptor (Kar and Quirion, 1995). The analgesic actions of opiates such as morphine and opioid peptides are attributed, in part, by their ability to inhibit the release of neurotransmitters from primary afferent terminals in the spinal dorsal horn. For example, double in situ hybridization histochemistry studies (Minami *et al.*, 1995) have shown that all three opioid receptors are present in substance P (SP)-containing neurons of the dorsal root ganglia, suggesting that opioid receptor agonists can act directly on the afferent

terminals of SP-containing neurons to modulate SP release. Also, other similar studies were able to show that some opioid receptors were colocalized with calcitonin gene-related peptide (CGRP), a peptide that enhances release of SP from primary afferents (Oku *et al.*, 1987; Dado *et al.*, 1993) and has a nociceptive effect (Xu *et al.*, 1990). Opiate receptors found in this region are associated with areas receiving small afferent primary fibers and are strategically placed to modulate noxious stimuli as well as explain some of the side effects of opiate administration.

3. Opioid Functions As Relevant To Pain

Although a plethora of studies on the physiological and behavioral effects of opioid peptides have been conducted, it is still difficult to fully characterize the functional roles of the different opioid receptor types. Recently, mice lacking opioid receptors were generated (Matthes *et al.*, 1996; Simonin *et al.*, 1998; Zhu *et al.*, 1999; for review see Kieffer, 1999) and have proven useful in providing insights into the involvement of opioid receptors in physiological and behavioral pain responses.

μ-Opioid Receptor

The wide distribution of μ-opioid receptors is consistent with its important roles in pain regulation and with many other physiological functions. μ-receptors found in spinal, supraspinal structures (Fang *et al.*, 1986; Porreca *et al.*, 1987) and the periphery (Stein, 1993) play an important role in nociception (Hansen *et al.*, 1984). μ agonists have the ability to block nociceptive responses to mechanical, thermal or chemical stimulations (Knapp *et al.*, 1989). Strong evidence indicates that the morphine-preferring μ-opiate receptor is the major site of action for the analgesic action of most clinically relevant analgesics. This observation was further proven in μ-opioid receptor knockout mice (Matthes *et al.*, 1996; Sora *et al.*, 1997a; Schuller *et al.*, 1999)

where morphine failed to reduce nociception in analgesia tests of homozygous mice while heterozygotes displayed right and downward shifts in morphine analgesia dose-effect relationships. Prototypical δ- and κ-agonists have been shown to function poorly without the presence of μ-opioid receptor (Sora *et al.*, 1997b) however, this notion remains controversial as other studies (Loh *et al.*, 1998) have shown that the effect of DPDPE (δ) and U50488 (κ) remained unchanged in the heterozygote and homozygote μ-opioid receptor knockout mice. Some of the other functions that seem to involve the μ-opioid receptor include respiration, cardiovascular functions, intestinal transit, feeding, learning and memory, locomotor activity, thermoregulation, hormone secretion, all of which are usually attenutated by agonistic activity (for review see Pasternak, 1988). μ receptor agonists have a pronounced respiratory depressant activity that likely involves a decreased sensitivity to CO_2. This is further demonstrated from the fact that the major acute toxicity associated with morphine is death from respiratory failure. The cardiovascular effects of the μ-opioid receptors are closely related to the respiratory effects and are mediated through central and peripheral receptors. Constipation, which has also been reported to be a major side effect of morphine use, is probably a result of μ receptor activity leading to a decrease in gastrointestinal secretions and motility.

When the μ-opiate, morphine, was injected into mice they exhibited increased locomotor activity (Micheal-Titus *et al.*, 1989), on the other hand, the homozygous μ-opioid receptor knockout mice was not affected by morphine injection (Tian *et al.*, 1997). Like most opioids, μ-opioids inhibit the thermoregulatory mechanisms which take place in the anterior hypothalamic region, leading to the impaired ability of the body temperature to remain constant. μ-agonists have also been known to affect hormonal release and neuroendocrine-related behaviors. These results were demonstrated by μ-agonist treatment leading to increased release of prolactin, growth hormone and corticosteroids while decreasing the release of luteinizing hormone (Pang *et al.*,

1977; Pfeiffer *et al.*, 1985; Kehoe *et al.*, 1993). Studies have supported the role of opioids in modulating the host defense system (Stefano *et al.*, 1996). This was further examined in μ-opioid receptor knockout mice which showed an enhanced production of the myeloid progenitors (Tian *et al.*, 1997) providing evidence that the μ-opioid receptor is involved in blood cell production, probably as a negative regulatory influence. The μ-opioid receptor also seems to be involved in the reproductive system since homozygous μ-opioid receptor knockout male mice displayed decreased sexual activity (Tian *et al.*, 1997). Although many studies have involved the μ-opioid receptor in many different physiological functions, the more recent knockout models have provided strong evidence for the receptor's role in analgesia, locomotor activity, constipation and respiration.

δ-Opioid Receptor

Similar to μ-opioid receptors, the δ-opioid receptor is involved in analgesia, gastrointestinal motility, olfaction, respiration and mood. The δ-opioid receptor is also associtated with tolerance (Kest *et al.*, 1996) and reproduction (Zhu and Pintar, 1998). A spinal administration of δ-opioid receptor agonists was effective in decreasing thermal and chemical stimuli (Schmauss *et al.*, 1984). The role of the δ-opioid receptor in analgesia was further elucidated in δ-opioid receptor knockout mouse where DPDPE was less sensitive in eliciting analgesia in homozygous mutant mice (Zhu *et al.*, 1999). The involvement of respiration was confirmed following an δ-agonist treatment that led to respiratory depression (Freye *et al.*, 1991). The δ-opioid receptors have been shown to be involved in analgesia, tolerance and the inhibition of gastrointestinal transit hence sharing many effects with the μ-opioid receptor.

κ-Opioid Receptor

κ-opioid receptors have been proposed to be involved in the regulation of different functions. Some of these include nociception, diuresis, feeding and neuroendocrine secretions (Hansen *et al.*, 1984; Desjardins *et al.*, 1990). In the limbic system κ-opioid receptors control mood and locomotion in opposition to μ- and δ-opioid receptors (Millan, 1990). However studies done in the κ-opioid receptor knockout mouse have shown that the absence of the κ-opioid receptor had little influence on this behaviour (Simonin *et al.*, 1998). The μ-, δ- and κ-opioid receptors have all been implicated in the mediation of analgesia while most studies have frequently attributed the mediation of nociception mostly to the μ receptors (Fang *et al.*, 1986; Dauge *et al.*, 1987), other studies have suggested that the κ-opioid receptors participate in the control of chemical, mechanical and thermal pain at the spinal level (Millan, 1990). These results are now controversial as the κ-opioid receptor deficient mouse displayed no change in their nociceptive threshold following mechanical, chemical and thermal stimulus (Simonin *et al.*, 1998). These same studies also demonstrated that these receptors play a role in the analgesic repsonses to opioids as the κ-agonist, U50488H, displayed a lack of antinociception in the hot plate and tail-flick tests in animals lacking κ-opioid receptors (Simonin *et al.*, 1998). Like the other two opioid receptors, κ-opioid receptors has also been implicated in the development of dependence following κ-agonist administration (Maldonado *et al.*, 1992).

B. Calcitonin gene-related peptide

Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide arising from the alternative splicing of the RNA transcript of the calcitonin gene (Rosenfeld *et al.*, 1983; MacIntyre *et al.*, 1992; Feurstein *et al.*, 1995;

Wimalawansa *et al.*, 1996; Wimalawansa *et al.*, 1997; for reviews see van Rossum *et al.*, 1997; Jacques *et al.*, 2000). CGRP is widely distributed in the central and peripheral nervous systems where it plays a role in many biological actions including cardioexcitatory effects (Brain *et al.*, 1985b, 1996), inhibition of gastric acid secretion (Hughes *et al.*, 1984) and food intake (Krahn *et al.*, 1984; Morley *et al.*, 1996). CGRP shares homology with a number of other peptides such as amylin and adrenomedullin (ADM) which are included in the CGRP family. Calcitonin (CT) was the first member within this family to be isolated and sequenced. Following the molecular cloning of the calcitonin gene, Rosenfeld *et al.* discovered CGRP by using a recombinant DNA (Amara *et al.*, 1982; Rosenfeld *et al.*, 1983). Subsequently, the related peptides amylin and adrenomedullin were isolated and found to share considerable homology with CGRP, 46% and 24% respectively. These single-chain peptides all have an amidated C-terminal and two N-terminal cysteines forming a disulfide bridge.

Two forms of the calcitonin gene-related peptide have been identified, CGRPα and CGRPβ (Bennett and Amara, 1992). Both of these genes encoding the two CGRP isoforms are located on chromosome 11 and are thought to have risen from gene duplication. CGRPα and CGRPβ are highly homologous differing by only 1 amino acid in rats and 3 amino acids in humans. Both isoforms are present in the rat nervous system and exhibit nearly identical pharmacological profiles (Amara *et al.*, 1985).

1. Calcitonin gene mRNA maturation

The calcitonin gene is comprised of six exons and encodes two different mRNAs that share an identical 5' sequence but have unique 3' sequences. Splicing of the first four exons generates calcitonin mRNA, which represents over 98% of the mature transcripts in thyroid C cells (Rosenfeld *et al.*, 1992).

In non-neuronal tissues the calcitonin mRNA encodes a 17,500 molecular weight calcitonin precursor protein, which is proteolytically processed to yield the Ca^{2+}-regulating hormone calcitonin. Alternative processing of the calcitonin gene results in the production of a mature transcript in neural tissue distinct from the predominant mRNA in thyroid cells. In the central and peripheral nervous systems, splicing of the fifth and sixth exons generates the mRNA encoding the 16,000 Dalton precursor of CGRPα (Rosenfeld *et al.*, 1992). The complex calcitonin/CGRP gene arose either by duplication and sequence divergence of the primordial calcitonin-like exon itself or as a consequence of gene duplication and rearrangement (Rosenfeld *et al.*, 1992). The expression of the calcitonin/CGRP gene is under control of hormones and second messengers such as glucocorticoids (Tverberg and Russo, 1992) and cAMP (Wind *et al.*, 1993). The complete identification and combination of the different factors contributing to the specificity of CGRP/calcitonin RNA splicing remains to be established.

2. CGRP receptors

A wide variety of biological functions have been described for CGRP. These effects are mediated through specific receptors. Through evaluation of the pharmacological properties of numerous CGRP fragments and analogues in several peripheral tissue preparations and in the brain, the existence of at least two CGRP receptor sub-types was proposed, namely $CGRP_1$ based on the strong antagonistic properties of the CGRP antagonist, $CGRP_{8-37}$, and $CGRP_2$, based on the strong agonistic properties of the linear analogue of CGRP, $[Cys(ACM)^{2,7}]$hCGRP and the weak binding affinity of $CGRP_{8-37}$. (Table 2; Dennis *et al.*, 1989; Dennis *et al.*, 1990; Quirion *et al.*, 1992, 1998; for review see Juaneda *et al.*, 2000).

Table 2. Classification of CGRP receptor subtypes.

	$CGRP_1$	$CGRP_2$
Potency of endogeneous homologues	CGRPα, CGRPβ > ADM > amylin	CGRPα, CGRPβ > ADM > amylin
Selective agonist	----	$[Cys(ACM)^{2,7}]$hCGRPα
Selective antagonist	$hCGRP_{8-37}$ $hCGRP_{9-37}$ $[Tyr^{27}, Asp^{31}, Pro^{34}, Phe^{35}]$-$CGRP_{27-37}$ $[Tyr^{27}, Pro^{34}, Phe^{35}]$-$CGRP_{27-37}$ BIBN4096BS	$hCGRP_{8-37}$

From Jacques *et al.*, 2000.

Various structure-activity studies have been conducted to develop smaller molecules that could act as antagonists and attempt to further demonstrate the existence of these receptor subtypes. To help this process, cell lines have been characterized as enriched with $CGRP_1$ receptors (eg. human SK-N-MC cells and rat L6 skeletal myocytes; Poyner *et al.*, 1995; Howitt *et al.*, 1997; Poyner *et al.*, 1998, Quirion *et al.*, 1998) or the $CGRP_2$ receptor (eg. COL-29 and HCA-7 colonic epithelium cells; Cox *et al.*, 1994; Poyner *et al.*, 1995; Quirion *et al.*, 1998). $[Asp^{31}, Pro^{34}, Phe^{35}]CGRP_{27-37}$ and $[Pro^{34}, Phe^{35}]CGRP_{27-37}$ are two smaller peptide antagonists that have been recently reported to bind to the $CGRP_1$ receptor expressed in SK-N-MC cells (Rist *et al.*, 1998). More recently, the first non-peptide CGRP receptor anatagonist, BIBN4096BS was reported (Doods *et al.*, 2000; Powell *et al.*, 2000).

Pharmacological classification

The C-terminal fragment of hCGRP, $hCGRP_{8-37}$, competes for $[^{125}I]$hCGRPα binding sites with very high affinity (IC_{50} = 0.5-1 nM) both in the CNS and peripheral membrane preparations (Dennis *et al.*, 1989, 1990). Its iodinated

counterpart, [^{125}I]hCGRP$_{8-37}$, binds with high affinity (K_D = 0.075-0.215 nM) to CGRP receptors in brain, atrium and vas deferens membrane preparations (van Rossum *et al.*, 1994). Shorter C-terminal fragments including CGRP$_{12-37}$ (Dennis *et al.*, 1989), CGRP$_{19-37}$ (Rovero *et al.*, 1992) and [Tyr^0]CGRP$_{28-37}$ (Chadker and Rattan, 1990) have also been defined as antagonists but with lower potencies than CGRP$_{8-37}$ (Dennis *et al.*, 1989, 1990; Quirion *et al.*, 1992). CGRP$_{8-37}$ usually does not induce any biological activity in neither guinea pig atrial or ileal preparations nor a variety of behavioural assays. However, CGRP$_{8-37}$ was able to inhibit some of the effects of hCGRPα in these preparations and is thus considered a relatively potent, competitive CGRP antagonist (Dennis *et al.*, 1989, 1990). The antagonistic effects of CGRP$_{8-37}$ were shown to be much weaker in the rat vas deferens (Dennis *et al.*, 1989, 1990). The differential antagonistic potencies of hCGRP$_{8-37}$ in various bioassays suggested the existence of multiple CGRP receptor subtypes. It was therefore proposed by Dennis *et al.* (1990) that the hCGRP$_{8-37}$-sensitive sites be classified as CGRP$_1$ receptors and the CGRP$_{8-37}$ rather resistant sites be classified as the CGRP$_2$ subtype (for review see Juaneda *et al.*, 2000). The linear analogue of hCGRP, [Cys(ACM)^{2,7}]hCGRP, binds to rat whole brain membranes with an affinity (3.0 ±1.4 nM) similar to the native peptide (2.4 ± 0.6 nM). This synthetic linear peptide does not induce the cardioexcitatory activity characteristic of hCGRP in atrial preparations. However, [Cys(ACM)^{2,7}]hCGRP does retain some of the agonistic properties of hCGRP in the rat vas deferens (Dennis *et al.*, 1989). These results suggested that [Cys(ACM)^{2,7}]hCGRP acts as a fairly selective agonist for the CGRP$_2$ receptor subtype located in tissues resistant to the antagonistic properties of hCGRP$_{8-37}$.

Most recently, BIBN4096BS, a non-peptide antagonist, demonstrated high affinity for the CGRP$_1$ receptor expressed in SK-N-MC cells (14.4 ± 6.3 pM) to block CGRPα-induced cAMP production in those cells (Doods *et al.*, 2000). In the rat atria, BIBN4096BS competitively antagonized the effects of

hCGRPα, hCGRPβ and rCGRPα with pA_2 values in the nM range showing a potency about ten-fold higher than $hCGRP_{8-37}$ (Wu *et al.*, 2000). In the rat vas deferens, BIBN4096BS was considerably less potent at inhibiting the effects of these various CGRP homologues (Doods *et al.*, 2000) therefore the non-peptidic antagonist has a pharmacological profile that discriminates more evidently between $CGRP_1$ and $CGRP_2$ receptor subtypes more than $CGRP_{8-37}$ (Wu *et al.*, 2000). The use of BIBN4096BS has suggested the existence of another CGRP receptor subtype in the rat vas deferens (Wu *et al.*, 2000). In this tissue preparation, the non-peptide antagonist weakly inhibited the effects of endogeneous CGRP ligands but had the ability to block the action of both ADM and $[Cys(Et)^{2,7}]$hCGRPα suggesting the presence of a receptor that is not activated by CGRPα or CGRPβ but by the linear peptide and ADM. This subtype would be different from the $CGRP_2$ receptor, which can bind the cyclic and linear forms of CGRP and ADM but is less sensitive to BIBN4096BS. Another CGRP receptor subtype could also be present in the rat colon where $[Cys(ACM)^{2,7}]$hCGRPα and $[Cys(Et)^{2,7}]$hCGRPα were unable to induce agonistic actvity (Esfandyari *et al.*, 2000), indicating that CGRP is mediating its activity through a receptor distinct from the $CGRP_1$ and $CGRP_2$ subtypes. The antagonistic properties of BIBN4096BS and other non-peptidic analogs are crucial to investigate and provide information on the different CGRP receptor subtypes.

G-Protein Coupling

Evidence from biochemical and pharmacological studies suggests that CGRP receptors belong to the family of G protein coupled receptors. Various reports have described the effect of GTP or its analogues on $[^{125}I]$CGRP binding affinity in a variety of tissue preparations (Roa *et al.*, 1991; Chatterjee *et al.*, 1991a, 1991b, 1993; van Rossum *et al.*, 1993). van Rossum *et al.* (1993) studied the effects of the non-hydrolizable GTP nucleotide analogue Gpp(NH)p in the brain, cerebellum, atria and vas deferens and showed that Gpp(NH)p

induced a shift to a lower affinity receptor state, as expected for G-protein coupled receptors.

Second Messenger Systems

Various reports have shown that CGRP's actions is most likely mediated through an increase in cAMP. Various tissue preparations such as cardiac muscle (Edwards *et al.*, 1991; Sun *et al.*, 1995, Wellman *et al.*, 1998), blood vessels (Wellman *et al.*, 1998), isolated rat thymocytes (Kurz *et al.*, 1995), rat cardiac myocytes (Chatterjee *et al.*, 1991b), primary cultures of neonatal rat spinal cord (Parsons *et al.*, 1997), heart and spleen (Sigrist *et al.*, 1986) show an increase in cAMP in the presence of CGRP. CGRP has also been shown to modulate K^+ channels *via* cAMP-dependent protein kinase, protein kinase A, in vascular smooth muscles (Miyoshi *et al.*, 1995) and guinea pig ureter (Santicioli *et al.*, 1995). CGRP-dependent increases in cAMP have been shown to be selectively antagonized by $CGRP_{8-37}$ (Parsons *et al.*, 1997; Yousufzai *et al.*, 1998). There is also evidence that CGRP can mediate its effects through nitric oxide synthesis and cGMP (Abdelrahman *et al.*, 1992). It appears that CGRP can activate different transduction signaling pathways.

CGRP Receptor Clones

Over the last few years, various putative CGRP receptor clones have been isolated (Kapas *et al.*, 1995; Aiyar *et al.*, 1996). The real identity of these receptor clones remained controversial since they shared very low homology to one another and do not seem to be expressed extensively in the CNS or on blood vessels where CGRP binding sites have been shown to be found (Wimalawansa *et al.*, 1993; van Rossum *et al.*, 1994). In 1995, Kapas *et al.* identified a canine orphan receptor, RDC-1, as a $CGRP_1$ receptor. Northern blot analysis showed that RDC-1 transcripts were highly expressed in the heart, kidney and thyroid, with much weaker signals in the brain and spleen (Libert *et al.*, 1989). Moreover, Tong *et al.* (1998) showed that the RDC-1 receptor

mRNA was observed in brain regions containing only low to very low levels of CGRP binding sites. Also, the complete loss of mRNA for RDC-1 using antisense oligonucleotides had no effect on CGRP-induced cAMP production (Hall *et al.*, 1998).

Another interesting GPCR clone was isolated from rat pulmonary blood vessels (Njuki *et al.*, 1993; Chang *et al.*, 1993; Fluhmann *et al.*, 1995; Aiyar *et al.*, 1996) and referred to as the calcitonin receptor like receptor (CRLR). Northern blot analysis revealed the expression of this putative $CGRP_1$ clone in cardiac myocytes and alveolar cells of the lung (Han *et al.*, 1997). The importance of CRLR as a CGRP receptor remained controversial until the discovery of RAMPs (McLatchie *et al.*, 1998). When CRLR was transfected in COS-7 cells, specific binding for CGRP was difficult to detect (Fluhmann *et al.*, 1995). HEK 293 cells expressing the clone showed higher but still low affinity for ^{125}I-CGRP-binding as well as an increase in cAMP in the response to CGRP (Aiyar *et al.*, 1996). This functional response to CGRP is competitively antagonized by $CGRP_{8-37}$ ($pA_2 = 7.57$; Aiyar *et al.*, 1996).

Accessory Proteins

Recently, McLatchie *et al.* (1998) cloned a series of receptor-activity modifying proteins (RAMPs), a novel family of transmembrane proteins. The RAMP-1 was isolated from *Xenopus* oocytes and encoded a 148 amino acid single-domain protein. RAMP-1 is not, by itself, a CGRP receptor, as the expression of RAMP-1 in mammalian cells did not induce cAMP responses to CGRP or specific binding to ^{125}I-labelled CGRP. Neither RAMP-1 nor CRLR induced significant responses to CGRP when transfected alone, but the expression of both produced cells which conferred a $CGRP_1$-receptor-like profile to CRLR. Both CGRPα and $CGRP_{8-37}$ exhibited a high affinity for this receptor complex that has the ability to respond to CGRP by increasing intracellular cAMP levels. RDC-1 did not induce binding or responses to CGRP with or without expression of RAMP-1 further indicating that RDC-1 is

not a genuine CGRP receptor. The requirement of CRLR and RAMP-1 to reconstitute a CGRP receptor may explain why it has been difficult to use expression cloning for CGRP receptors. Furthermore, the prerequisite coexpression of CRLR and RAMP-1 for CGRP receptor function explains the failure of CRLR alone to function in oocytes and the observation that CRLR can only function as a CGRP receptor in certain cell lines which presumably express an endogeneous RAMP-1. It was shown by fluorescence-activated cell sorting (FACS) that RAMP-1 increased cell surface expression of CRLR and plays a prominent role in the intracellular translocation of the CRLR-maturing protein and its insertion into the plasma membrane. It is also believed that RAMP-1 is necessary for the terminal glycosylation of CRLR (McLatchie *et al.*, 1998). RAMP-1 is part of a family of receptor-activity modifying proteins. RAMP-2 and RAMP-3 have been cloned from SK-N-MC cell cDNA library and human spleen mRNA, respectively (McLatchie *et al.*, 1998). In contrast to RAMP-1, RAMP-2 and RAMP-3 are unable to potentiate the oocyte response to CGRP. In other cell lines, neither RAMP-2 nor RAMP-3 enabled CRLR to function as a CGRP receptor. However, the coexpression of RAMP-2 and CRLR resulted in a receptor complex that behaves as an ADM receptor with ADM-like peptides having much greater affinity than the CGRP derivatives (McLatchie *et al.*, 1998). This exciting discovery implies that the same G-protein coupled receptor can demonstrate different pharmacological profiles depending on the presence of chaperone proteins such as RAMPs, a novel interaction in the GPCR family.

Hence, RAMP proteins not only facilitate the intracellular translocation of the CRLR protein and its insertion into the membrane but they also significantly modify the pharmacological profile of CRLR (McLatchie *et al.*, 1998; Christopoulos *et al.*, 1999; Muff *et al.*, 1999). It has recently been shown that RAMPs can not only modulate the profile of the CRLR receptor but also a calcitonin receptor known as the human calcitonin receptor isotype 2 (hCTR2). hCTR2 usually behaves as a mammalian calcitonin receptor but in the presence

of RAMP1 or RAMP3, it forms a complex that behaves as an amylin receptor. (Muff *et al.*, 1999; Tilakaratne, *et al.*, 2000). Recent data has also involved RAMP1 in receptor desensitization through the modulation of protein kinase A in SK-N-MC cells (Drake *et al.*, 1999).

In 1996, Luebke *et al.* (1996) isolated a CGRP responsive protein from the guinea-pig organ of Corti and the cerebellum. This short hydrophilic protein (146 a.a.) does not belong to the typical seven transmembrane G-protein coupled receptors and it has no homology to any known class of proteins. RCP is referred to as the CGRP receptor component protein (CGRP-RCP). RCP seems to be a component that is required in oocytes and NIH3T3 cells to observe CGRP responsiveness,, this is further demonstrated with the use of specific RCP antisense which resulted in a decrease in receptor response (Rosenblatt *et al.*, 1998). A RCP homologue has recently been cloned from ciliary body and was shown to be involved in ensuring CGRP receptor signal transduction in that tissue by promoting coupling with G_S, leading to cAMP production (Rosenblatt *et al.*, 2000; Evans *et al.*, 2000). The CGRP receptor complex seems (likely $CGRP_1$) to composed of the CRLR protein, a RAMP that confers the pharmacological profile and properly translocates and inserts the CRLR protein into the plasma membrane, and a RCP that is involved in the signal transduction (for a recent review see Juaneda *et al.*, 2000).

3. Distribution of CGRP and its Binding Sites

Distribution of calcitonin gene-related peptides

Calcitonin gene-related peptide is widely distributed throughout the nervous system of vertebrates as well as invertebrates (Skofitsch *et al.*, 1985; Lee *et al.*, 1985; Kawai *et al.*, 1985; for review see Jacques *et al.*, 2000). In the periphery, CGRP is highly expressed in the bladder (Wimalawansa *et al.*, 1987a, 1992), pancreas (Wimalawansa, 1992), penis (Wimalawansa *et al.*, 1987a, 1992), skin (Brain *et al.*, 1986; Ishida-Yamamoto *et al.*, 1989), smooth

muscle layers of blood vessels (Rosenfeld *et al.*, 1983; Wimalawansa *et al.*, 1987a, 1992), and the thyroid gland (Wimalawansa *et al.*, 1987a, 1992). CGRP was also observed in low amounts in the lung (Rosenfeld *et al.*, 1983; Wimalawansa *et al.*, 1987a; Hokfelt *et al.*, 1992), gastrointestinal tract (GI) (Rosenfeld *et al.*, 1983; Hokfelt *et al.*, 1992), adrenal glands (Rosenfeld *et al.*, 1983; Hokfelt *et al.*, 1992) and in the heart (Rosenfeld *et al.*, 1983; Wimalawansa *et al.*, 1987a) where 4-fold higher levels were seen in the atria compared to the ventricles (Franco-Cereceda *et al.*, 1987b). Systemic capsaicin treatments in adult guinea pigs (Franco-Cereceda *et al.*, 1987b) and newborn rats (Wimalawansa, 1993) decrease CGRP-immunoreactivity in the circulation, cardiovascular tissues, lungs, GI tract, genitourinary tract and nervous tissues. This suggests that CGRP-immunoreactivity in the peripheral organs is associated with sensory neurons (Franco-Cereceda *et al.*, 1987b). α- and β-CGRP are detected in the circulation in both human plasma and cerebrospinal fluid (CSF; Wimalawansa *et al.*, 1987b, 1989). It is believed that the high circulating levels of CGRP originate from the thyroid and perivascular nerves suggesting that CGRP may play a role in the regulation of vascular tone (Grigis *et al.*, 1985; Zaidi *et al.*, 1986).

Central Nervous System and Spinal Cord

In the central nervous system, CGRP is expressed in discrete brain regions and in the spinal cord (Skofitsch *et al.*, 1985; Kawai *et al.*, 1985; Hokfelt *et al.*, 1992; Juaneda *et al.*, 2000; for review see Jacques *et al.*, 2000). The distribution of CGRP mRNA detected through *in situ* hybridization histochemistry (Amara *et al.*, 1985; Kresse *et al.*, 1995) revealed that the highest densities of neurons expressing CGRP mRNA were mainly found in the brainstem. More than 50% of the large sensory perikarya in the cranial trigeminal ganglion showed high levels of CGRP mRNA (Kresse *et al.*, 1995). The majority of α-motoneurons of the spinal cord at the cervical level were found to express high levels of CGRP mRNA (Kresse *et al.*, 1995). Most of

the neurons in the ventral horn of the cervical and lumbar regions of the spinal cord were positive for CGRP mRNA while up to 75% of the motoneurons of the ventral spinal cord were found to express CGRP mRNA (van Rossum *et al.*, 1997). Like the distribution of CGRP mRNA, CGRP-like immunoreactivity was widely but unevenly distributed in the rat brain. CGRP-immunoreactivity (CGRP-IR) can be observed in various areas of the hypothalamus, hippocampus, dendate gyrus and all cranial motor nuclei (Rosenfeld *et al.*, 1983; Skofitsch *et al.*, 1985; Hokfelt *et al.*, 1992; for review see Jacques *et al.*, 2001). Several hypothalamic nuclei demonstrated high amounts of CGRP-immunoreactvie fibers while at the level of the lower brainstem, high concentrations of CGRP-IR were located in the superficial layers of the sensory trigeminal areas (Jacques *et al.*, 2000). In humans, the pituitary shows high levels of CGRP with lower amounts in cerebral and cerebellar cortices (Tschopp *et al.*, 1985). In the spinal cord, CGRP-IR fibres form a dense network in lamina I/II of the spinal cord (Wiesenfeld-Hallin *et al.*, 1984; Skofitsch *et al.*, 1985; Wimalawansa *et al.*, 1987a). In humans, CGRP-IR fibers are concentrated in the spinal trigeminal nucleus and principal sensory trigeminal nucleus (Unger *et al.*, 1991).

Sensory System

The high levels of CGRP-immunoreactivity in the dorsal horn suggests a role for CGRP in sensory processes. CGRP is the most abundant peptide in neurons of the dorsal root ganglia (DRG) with approximately 50% of the cells showing immunoreactivity (Rosenfeld *et al.*, 1983; Skofitsch *et al.*, 1985; Wimalawansa *et al.*, 1987a; Lawson, 1995). The spinal cord contains a dense CGRP-IR innervation in the superficial layers of the dorsal horn (Gibson *et al.*, 1984). The CGRP-positive neurons are mostly unmyelinated or small-diameter myelinated neurons which constitute about 30% of the primary afferent axons of the Lissauer's tract, the major afferent input into the superficial laminae of the dorsal horn (Levine *et al.*, 1993). In spinal ganglia,

all substance P-immunoreactive (SP-IR) cell bodies also contain CGRP-immunoreactivity while not all CGRP-immunoreactive cells contain SP-immunoreactivity (Wiesenfeld-Hallin *et al.*, 1984; Lee *et al.*, 1985). CGRP and substance P are also colocalized in axonal buttons of the superficial dorsal horn (Ribeiro-Da-Silva, 1995). CGRP-immunoreactive neurons have their terminals in various areas of the spinal cord (laminae II V X and I; Gibson *et al.*, 1984) and brainstem (Rosenfeld *et al.*, 1983) and are believed to relay somatic cutaneous pain and temperature information. CGRP-rich fibers form part of the primary afferent nervous system, comprising capsaicin-sensitive A(δ) and C fiber afferent nerves (Franco-Cereceda *et al.*, 1987b). Sensory neurones are enriched in CGRPα, containing three to six times more CGRPα than CGRPβ (Gibson *et al.*, 1984; Mulderry *et al.*, 1988). Both CGRPα and CGRPβ mRNAs are located in the dorsal root ganglia (Mulderry *et al.*, 1988).

Distribution of calcitonin gene-related peptide binding sites

CGRP binding sites have been studied in various species including man and rat and are widely distributed throughout the nervous system (Skofitsch *et al.*, 1985; Tschopp *et al.*, 1985; Dennis *et al.*, 1991; Quirion *et al.*, 1992; for review see Jacques *et al.*, 2000). CGRP receptors are also present in abundance in the cardiovascular system (Sigrist *et al.*, 1986; Wimalawansa *et al.*, 1987a, 1992, 1993; Chang *et al.*, 2001), blood vessels (Sigrist *et al.*, 1986, Wimalawansa *et al.*, 1987a, 1992, 1993; Jansen-Olesen *et al.*, 2001), spleen (Sigrist *et al.*, 1986, Wimalawansa *et al.*, 1987a, 1992, 1993), penis (Wimalawansa *et al.*, 1987a, 1992, 1993), vas deferens (Dennis *et al.*, 1990), lungs (Wimalawansa, 1992) and adrenal gland (Wimalawansa *et al.*, 1987a, 1992, 1993). Moderate levels of binding are also observed in the pancreas (Wimalawansa, 1992) and bladder (Wimalawansa *et al.*, 1987, 1992; Burcher *et al.*, 2000). Negligible amounts are detected in kidneys (Wimalawansa, 1992), muscle and liver (Wimalawansa *et al.*, 1987a).

Nervous System

In the central system, high densities of CGRP binding sites are observed in the cerebellum, dorsal spinal cord, nucleus accumbens, amygdaloid complex, mammilary body, superior colliculus, inferior olive and temporal and frontal cortices (Dennis *et al.*, 1991; Wimalawansa, 1992; van Rossum *et al.*, 1997; for review see Jacques *et al.*, 2000). The substantia nigra, medulla, pons, striatum, hypothalamus, hippocampus, medial geniculate nucleus and inferior colliculus show intermediate levels of CGRP binding (Dennis *et al.*, 1991; Wimalawansa, 1992; van Rossum *et al.*, 1997; for review see Jacques *et al.*, 2000). In humans, binding sites for ^{125}I-CGRP are detected in high quantities in the cerebellar cortex, spinal cord and nucleus dentatus; intermediate levels in the inferior colliculus and substantia nigra while only low amounts in the hippocampus, amygdala, superior colliculus, thalamus, hypothalamus and globus pallidus (Tschopp *et al.*, 1985; Wimalawansa *et al.*, 1993; van Rossum *et al.*, 1997; for review see Jacques *et al.*, 2000).). The highest density of specific ^{125}I-hCGRPα binding in the rat spinal cord was observed around the central canal while the deeper dorsal horn and ventral horn showed moderate labeling (Yashpal *et al.*, 1992; Kar *et al.*, 1995). The supeficial dorsal horn and white matter showed relatively low densities of specific labeling. The widespread distribution of ^{125}I-hCGRP binding sites in the developing spinal cord suggests the possible involvement of this peptide and its receptor in the growth, development and normal maturation of the cord (Kar *et al.*, 1995). No binding was observed in the dorsal root ganglia of mature rats (Tschopp *et al.*, 1985; Wimalawansa *et al.*, 1993). Overall, the distribution of CGRP mRNA, CGRP-like immunoreactivity and CGRP binding sites throughout the brain correlated rather well.

4. CGRP Functions

Although the exact biological functions of CGRP have not been fully characterised, its anatomical distribution suggests roles in autonomic, somatosensory, integrative and motor functions (Rosenfeld *et al.*, 1983, 1992; Wimalawansa, 1996, 1997; van Rossum *et al.*, 1997; for review see Jacques *et al.*, 2000).

Nociception

CGRP-immunoreactive cells constitute 40-50% of dorsal root ganglia neurons (Levine *et al.*, 1993). Somatosensory systems containing CGRP originate from the trigeminal ganglion to terminate in the spinal trigeminal nucleus while those from the dorsal root ganglion project to the dorsal horn of the spinal cord (Alvarez *et al.*, 1993). CGRP is expressed by one-third of adult rat lumbar DRG neurons, many of which play a role in mediating pain or vasodilation (Ai *et al.*, 1998). The localization of CGRP in small dorsal root ganglion neurons and in most major sites of termination of nociceptors suggests that CGRP may participate in nociceptive transmission. Intrathecal injections of CGRP do not produce aversive reactions (Wiesenfeld-Hallin *et al.*, 1984) nor analgesic responses (Jolicoeur *et al.*, 1992). However, when injected along with substance P, it potentiates the scratching/biting behaviour observed following the administration of SP alone (Wiesenfeld-Hallin *et al.*, 1984), possibly by inhibiting the metabolic degradation of substance P (Le Greves *et al.*, 1989; Mao *et al.*, 1992). Intracerebroventricular (i.c.v.) administration of CGRP produces antinociceptive responses in acute pain assays such as tail-flick and hot-plate tests, an effect blocked by $CGRP_{8-37}$ (Bates *et al.*, 1984; Jolicoeur *et al.*, 1992). Injection of CGRP in peripheral tissues has the ability to elicit visceral pain (Friese *et al.*, 1997). Noxious thermal and mechanical stimulation have the ability to cause the release of CGRP in the superficial dorsal horn (Morton *et al.*, 1989). Furthermore, the

role of CGRP in nociception is further complicated by its relationship with opioids. Intrathecal injection of CGRP has the ability to decrease the analgesia produced by opioid agonists (Welch *et al.*, 1989). Studies in CGRP-deficient mice have shown that these rodents display an attenuated response to morphine analgesia, chemical pain and inflammation (Salmon *et al.*, 1999, 2001). Taken together, these results indicate that CGRP is involved in the complex process of pain signaling but its precise contribution remains to be established.

Neuropathic Pain

Modulation of CGRP and CGRP binding sites have also been shown following neuropathic injury. CGRP was shown to be decreased in the spinal cord following chronic constriction injury (Kajander *et al.*, 1995; Carlton *et al.*, 1996; Xu *et al.*, 1996). Garry *et al.* (1991) found no change in ^{125}I-hCGRP binding in the dorsal spinal cord of rats at 2, 5, 10 and 20 days following chronic constriction injury. However, the same group showed a large increase in ^{125}I-hCGRP binding ipsilateral to the lesion at 4 and 8 days following dorsal rhizotomy (Garry *et al.*, 1991). In arthritic rats, there was an increase in CGRP-IR fibers innervating the dorsal horn of the spinal cord (Kar *et al.*, 1994). The same group also showed that following unilateral section of the peripheral nerves lead to marked depletion of CGRP-IR fibers in the ipsilateral dorsal horn. Sciatic nerve section or facial nerve crushing in rat can induce an increase in the levels of immunoreactive CGRP and CGRP mRNA in axotomized motoneurons (Piehl *et al.*, 1993). As for inflammation, $CGRP_{8-37}$ increased the withdrawal latencies to both thermal and mechanical stimulation in rats with unilateral mononeuropathy (Yu *et al.*, 1996).

C. SUBSTANCE P

Substance P, an 11 amino acid neuropeptide, was discovered in 1931 by von Euler and Gaddum (1981) but it was not until 1971 that it was purified and its structure isolated by Leeman and colleagues (Chang *et al.*, 1970). It is a member of the tachykinin family along with neurokinin A (NKA), neurokinin B (NKB), neuropeptide K (NPK) and neuropeptide gamma (NP-γ; Tatemoto *et al.*, 1985; for review see Hokfelt *et al.*, 2001) who all share common amino acids in their C-terminal sequences. The gene organization for the preprotachykinin-A and preprotachykinin-B precursors are very similar (Kotani *et al.*, 1986). This suggests that the mammalian tachykinin system has acquired diversity through various cellular mechanisms including gene duplication, differential expression of duplicated genes, and alternative RNA splicing (Kotani *et al.*, 1986). The rat preprotachynin-I (PPT-I) gene mRNA is alternatively spliced in a tissue-specific manner to yield four different mRNA, alpha-, beta-, delta- and gamma-preprotachynin (Nawa *et al.*, 1984; MacDonald *et al.*, 1989; Lai *et al.*, 1998). Alpha-PPT is processed to the mature undecapeptide substance P. Beta-PPT is processed into various products including substance P, neurokinin A, neurokinin (3-10) and neuropeptide K while gamma-PPT (Kawaguchi *et al.*, 1986) gives rise to substance P, neurokinin A, neurokinin (3-10) and neuropeptide γ. A delta isoform of proprotachynin mRNA has also been identified in the rat intestine (Khan *et al.*, 1994), dorsal root ganglia (Harmar *et al.*, 1990) and human mononuclear phagocytes and lymphocytes (Lai *et al.*, 1998). The sequence analysis of the δ-PPT isoform predicts the existence of a novel tachykinin precursor polypeptide containing substance P. However, it is still to be determined if this new precursor is translatable (Lai *et al.*, 1998). The second mammalian tachykinin precursor, preprotachykinin-II (PPT-II) is processed to yield neurokinin B (Kotani *et al.*, 1986).

1. Neurokinin Receptors

Three neurokinin receptors have been isolated: neurokinin-1 (NK-1), neurokinin-2 (NK-2) and neurokinin-3 (NK-3; Nakanishi, 1991; Fong, 1996; for review see Hokfelt *et al.*, 2001). Neurokinin receptors belong to the G-protein-coupled receptor family (Routh *et al.*, 1995). Like all G-protein-coupled receptors, they have α-helical transmembrane domains, three extracellular loops, three cytoplasmic loops and a cytoplasmic C-terminal region. All three receptors are encoded in five exons that are very similar between the three genes. Thus, the neurokinin receptors share 54-66% homology in their transmembrane and cytoplasmic regions (Routh *et al.*, 1995). This considerable homology between the receptor subtypes is consistent with the fact that all three tachykinins have the ability to bind all three receptors (Quirion *et al.*, 1991). Substance P binds preferentially to the NK-1 receptor while neurokinin-A and neurokinin-B bind with higher affinity to the NK-2 and NK-3 receptors respectively (Routh *et al.*, 1995). More recently, a binding site for a NK-1 agonist septide, separate from substance P has lead to the suggestion of a distinct receptor subtype, possibly a different conformation of the NK-1 receptor (Maggi *et al.*, 1997; Ciucci *et al.*, 1998). Like many G protein-coupled receptors, their activation leads to intracellular signals via the IP3/diacylglycerol pathway resulting in modulation of intracellular [Ca^{2+}] and increased levels of cAMP (Nakajima *et al.*, 1991; Nakanishi, 1991; for review see Quartara *et al.*, 1997). More recently, it has been shown that the interaction of substance P with the NK-1 receptor can activate members of the mitogen-activated protein kinase (MAPK) such as ERK1/2 through the formation of β-arrestin-containing scaffolding complexes (DeFea *et al.*, 2000) leading to proliferative and antiapoptotic effects.

2. Distribution of Substance P and its binding sites

Substance P has a wide distribution and is likely involved in functions of the central nervous system, peripheral nervous system, visual system, gastrointestinal tract, endocrine system, skeletal system and the cardiovascular system (Pernow, 1983; Kiyama *et al.*, 1993; Goto *et al.*, 1998; for review see Hokfelt *et al.*, 2001). Substance P can be extracted from the brain of all vertebrate species from fish to mammals. Extensive radioimmunological and immunohistochemical studies have established the presence of SP in most parts of the central nervous system of all mammals (Hokfelt *et al.*, 1975; Cuello *et al.*, 1997; for review see Hokfelt *et al.*, 2001). The highest amounts are present in the mesencephalon, hypothalamus and preoptic area while much lower concentrations can be found in the cerebellum. Substance P immunoreactivity has been demonstrated in the nerve fibers of cerebral blood vessels (Shimizu *et al.*, 1999). Also, substance P immunoreactive nerve fibers have been localized in different tissues of the pancreas such as the islets of Langerhans, acinis, ducts and blood vessels (Schmidt *et al.*, 2000). In situ hybridization histochemistry was able to show that the NK-1 mRNA signal was present throughout the brain and spinal cord (Kiyama *et al.*, 1993). Strongly labeled neurons were observed in the olfactory bulb, caudate putamen and amygdala and locus coeruleus while poor localization of the NK-1 mRNA signal was found in the superficial part of the dorsal horn (Kiyama *et al.*, 1993).

In agreement with human and rat brains, a guinea-pig brain study showed that the most marked NK-1 receptor immunoreactivity was found in the caudate-putamen and widely distributed in diencephalic structures, thalamus, hypothalamus, amygdala as well as the mid- and hind brain (Yip and Chahl, 2000). Substance P receptors have also demonstrated to exist in the cerebral blood vessels and the cranial ganglia that innervate these vessels (Shimizu *et al.*, 1999). Neurokinin receptors have also been located outside the central nervous system through in situ hybridization and immunohistochemisty.

Numerous studies has been able to show that the NK-1, NK-2 and NK-3 receptors are expressed in multiple cell types found in the ileum and colon (Smith *et al.*, 1998; Renzi *et al.*, 2000; Vannucchi and Faussone-Pellegrini, 2000). Autoradiographic studies have also shown that both the NK-1 and NK-2 receptor are located in different cell types of the urinary bladder (Burcher *et al.*, 2000). The NK-1, NK-2 and NK-3 receptors have also been localized in the rat and rabbit retina (Denis *et al.*, 1991; Oyamada *et al.*, 1999) and in rat kidney tissues (Chen and Hoover, 1995). NK-1 receptor immunoreactive structures have been observed in rat bone tissues such as osteocytes and osteoblasts (Goto *et al.*, 1998). The NK-3 receptor is predominantly expressed in the central nervous system however there are many species differences in NK-3 receptor pharmacology. The autoradiographic distribution of NK-3 in the rat brain revealed that it was highly concentrated in mid-cortical layers, supraoptic nucleus, zona incerta and amygdala with lower levels observed in the caudate-putamen and cerebellum (Dam *et al.*, 1990). A study that compared NK-3 receptor distribution in guinea-pig, gerbil and rat brain was able to show that in all three species the NK-3 receptor was similarly distributed in the cerebral cortex, the amygdaloid complex and the zona incerta (Lanlois *et al.*, 2001). Outside these structures, each species revealed a specific distribution pattern of NK-3 receptors (Langlois *et al.*, 2001). Other studies in the guinea-pig brain have provided similar results, showing the NK-3 receptor immunoreactivity located in both the superficial and deep layers of the cortex (Yip and Chahl, 2000). The distribution of NK-3 receptor-like immunoreactivity has been shown to colocalize well with the presence of NK-3 receptor mRNA on neuronal cell bodies and dendrites of many structures including the cortices, hypothalamic areas and the amygdala (Ding *et al.*, 1996).

Distribution of Substance P and its binding sites in the spinal cord

Substance P is highly concentrated in superficial layers (I-III) of the dorsal horn where most primary afferent fibres terminate. The neuropeptide occurs in higher concentrations in the primary sensory fibres that terminate in regions of the dorsal horn where nociception has been shown to be integrated (Hokfelt *et al.*, 1975). Substance P is synthesized in nociceptive primary sensory neurons sensory neurons, which send C- and Aδ fibers to the dorsal horn projections in laminae I nad IV-V. Unilateral rhyzotomy results in a dramatic decrease in substance P content further suggesting its presence in primary afferent terminals (Takahashi *et al.*, 1975; Hokfelt *et al.*, 1975; Kajander *et al.*, 1995). Substance P-like immunoreactivity has been found in 18-20% of rat lumbar DRG neurons (Lawson, 1992). The highest density of substance P binding sites was observed in the superficial dorsal horn and in the region surrounding the central canal (Rossler *et al.*, 1993; Kar *et al.*, 1995). Moderate to low densities were detected in deeper laminae and in the ventral horn. Using autoradiography, it was found that NK-1 binding sites were localized in the superficial layers of the dorsal horn particularly laminae I and II whereas lower densities were observed in the deeper laminae (Yashpal *et al.*, 1991; Kar and Quirion, 1995). These data were then confirmed using NK-1 receptor immunohistochemisty (Vigna *et al.*, 1994) and in situ hybridization (Schafer *et al.*, 1993). Immunohistochemisty done with antibodies raised against the rat NK-2 receptor were able to show that the receptor can be found in both the dorsal and ventral horn of the spinal cord with the densest labelling was in the outer part of laminae I (Zerari *et al.*, 1998). The NK-3 receptors have also been shown to be localized in the spinal cord through receptor autoradiography (Beresford *et al.*, 1992; Kar and Quirion, 1995). In situ hybridization and immunohistochemistry techniques have shown that the NK-3 receptor is expressed in the spinal trigeminal nucleus and in the spinal dorsal horn (Ding *et al.*, 1996).

3. Substance P Functions

In addition to their role in sensory transmission, tachykinins have been implicated in a variety of CNS functions such as the control of motor activities, autonomic and endocrine functions and memory processing. This section will concentrate on the role of substance P in sensory processing (for review see Cuello, 1993). The involvement of substance P in the development of chronic pain has been studied in some details. It has been shown that substance P and its binding sites are altered in various models of chronic pain. In models of inflammation, it has been demonstrated that substance P immunoreactivity (Smith *et al.*, 1992) and preprotachykinin mRNA (Donaldson *et al.*, 1992) were increased in dorsal root ganglia neurons. An increase in the release of substance P (Oku *et al.*, 1987; Garry *et al.*, 1992) and the expression of its binding sites has also been demonstrated (Abbadie *et al.*, 1996). Differential effects have been observed in models of neuropathic pain. Substance P immunoreactivity was significantly increased in the dorsal horn (Takahashi *et al.*, 1975; Hokfelt *et al.*, 1975) following neuropathy while substance P receptor (NK-1) immunoreactivity was increased in nerve injury models of chronic pain (Abbadie *et al.*, 1996). Hence, substance P has been proposed as a pain transmitter (Henry, 1976; De Koninck *et al.*, 1992). Substance P has been shown to excite nociceptive dorsal horn neurons (Henry, 1976) and to be released in the spinal cord following activation of the primary sensory fibres (Theriault *et al.*, 1979). Nociceptive stimulation can trigger the release of substance P from C-afferent terminals in the marginal layers of the spinal cord (Duggan *et al.*, 1987) and evoke slow excitatory postsynaptic potentials in sensory neurons of the dorsal horn (De Koninck and Henry, 1991). Also, treatment with a substance P receptor antagonist has been able to block nociceptive responses (De Koninck and Henry, 1991). Recently, the development of NK-1 receptor knockout mice (Bozic *et al.*, 1996) has allowed the involvement of the NK-1 receptor in pathophysiological states to be more

closely examined. This group was able to demonstrate that the neurogenic response to capsaicin applied topically to the ear was reduced in NK-1 receptor knockout mice. Other reports have shown that the neurokinin receptors are involved in pain response (De Felipe *et al.*, 1998; Mansikka *et al.*, 2000). Using a mice with a targeted deletion of the tachykinin-1 gene, a group was able to show that mice that were incapable of producing substance P displayed no significant responses to a formalin injection and had an increased pain threshold in the hotplate test (Zimmer *et al.*, 1998). Using similar techniques, it was shown that the behavioral response to moderate to intense pain was significantly reduced when the neurogenic inflammation that results from the peripheral release of substance P was absent in mutant mice (Ahluwalia *et al.*, 1998; Cao *et al.*, 1998; Laird *et al.*, 1998).

D. GALANIN

Galanin is a 29-30 amino acid peptide isolated from the porcine upper small intestine (Tatemoto *et al.*, 1983). Its name is derived from its N-terminal glycine and C-terminal alanine. The C-terminal region showed some sequence similarity to other neuropeptides such as substance P and gonadotrophin-releasing hormone. Galanin is a phylogenetically old peptide and was well conserved throughout evolution; human, porcine and rat galanin showing 90% homology. All galanin sequences determined so far consist of 29 amino acids except the human galanin, which is comprised of 30 amino acids. The first 15 N-terminal residues are fully conserved while the C-terminal portion shows a higher degree of variability. This neuropeptide has a wide distribution in tissues such as brain, spinal cord and gut, and can regulate numerous functions including endocrine secretions (Bauer *et al.*, 1986), ingestive behaviour (Crawley *et al.*, 1990), nociception (Kask *et al.*, 1997) and memory (Kask *et al.*, 1997; for review see Branchek *et al.*, 2000).

Galanin Receptor Sub-Types

Three galanin receptors have so far been cloned, GalR-1, GalR-2 and GalR-3.

GalR-1 A cDNA coding for a human galanin receptor was isolated from a Bowes melanoma cell line (Habert-Ortoli *et al.*, 1994). It is a 349 amino acid protein with 7 putative hydrophobic transmembrane domains and it shows significant homology to the members of the guanine nucleotide binding protein-coupled receptor family. The cloned receptor expressed in COS-cells specifically binds human, porcine and rat galanin with high affinity (K_D = 0.8 ±0.2 nM). The primary sequence shows homology (30%) to the human somatostatin and human delta opioid receptors. A rat GALR-1 homologue comprising of 346 amino acids and showing 92% similarity with its human counter part has been cloned from brain (Burgevin *et al.*, 1995). The cloned GALR-1 has the ability upon activation to reduce cAMP concentrations (Habert-Ortoli *et al.*, 1994; Burgevin *et al.*, 1995), opens K^+ channels (Smith *et al.*, 1998) and stimulates MAP kinase activity (Wang *et al.*, 1998; for review see Branchek *et al.*, 2000).

GalR-2 The GalR-2 receptor cDNA was isolated originally from the rat hypothalamus (Smith *et al.*, 1997; Wang *et al.*, 1997). The receptor is 372 amino acids in length G-protein-coupled receptor that shares 40% homology with the rat GalR-1 receptor and 55% with the rat GalR-3. The human GalR-2 was then cloned and found to contain 387 amino acids with only 85% similarity to the rat GalR-2 (Borowsky *et al.*, 1998). ^{125}I-human galanin binds with high affinity to the GalR-2 receptor expressed in COS-1 cells (K_D = 0.59 nM). Rat GalR-1 and GalR-2 share similar pharmacological profiles in that they both possess high affinity for full-length and N-terminal fragments of galanin (Smith *et al.*, 1997, 1998). Their pharmacological differ in their affinities for other galanin fragment such as galanin$_{2-30}$ for which GalR-2 has a low affinity (Wang *et al.*, 1997). Activation of the cloned GalR-2 receptor by

galanin leads to the stimulation of multiple intracellular events. A major pathway seems to involve phospholipase C since GalR-2 mediates inositol phosphate hydrolysis (Smith *et al.*, 1997; Borowsky *et al.*, 1998) and intracellular Ca^{2+} mobilization (Smith *et al.*, 1997; Borowsky *et al.*, 1998) as well as the inhibition of cAMP accumulation (Wang *et al.*, 1998; for review see Branchek *et al.*, 2000). GalR-2 has also been shown to mediate MAPK activity (Wang *et al.*, 1998).

GalR-3 The third cloned galanin receptor was initially cloned from rat through both expression and homology cloning approaches (Smith *et al.*, 1997; Wang *et al.*, 1997). The rat GalR-3 cDNA encodes a G protein-coupled receptor of 370 residues. The human GalR-3 was subsequently cloned from a human genomic library based on its similarity to human GalR-1 and GalR-2 (Smith *et al.*, 1998). The human counterpart is comprised of 368 amino acids and shares 90% homology to the rat GalR-3. The pharmacology profile of GalR-3 shares similarities with both GalR-1 and GalR-2. Both GalR-3 and GalR-2 have weak selectivity for porcine galanin compare to $galanin_{2-30}$ (Wang *et al.*, 1997; Smith *et al.*, 1998; for review see Branchek *et al.*, 2000). Activation of GalR-3 leads to the activation of an inward K^+ current when transfected into Xenopus oocytes (Smith *et al.*, 1998) and inhibition of adenylate cyclase.

1. Distribution of Galanin and its Binding Sites

Galanin is abundant in both the central and peripheral nervous systems as well as in the intestine. In the central nervous system, galanin-immunoreactive (Gal-IR) structures were observed in high levels in the superficial layers and interneurons of the spinal cord (Melander *et al.*, 1986). In the rat brain, the highest concentrations were observed in posterior pituitary and the hypothalamus (Ch'ng *et al.*, 1985). In the peripheral nervous system, sensory dorsal root ganglion cells (DRG) show moderate levels of galanin-

immunoreactivity. Immunocytochemistry has also revealed a dense network of galanin-immunoreactivity throughout the rat gastrointestinal tract (Ekblad *et al.*, 1985). GalR-1 mRNA has been detected in the central and peripheral nervous system with the highest expression seen in the hypothalamus, amygdala as well as the spinal cord and dorsal root ganglia (Waters and Krause, 2000). Other in situ hybridization studies have confirmed these results and showed that intense hybridizations signals were also observed in the nucleus of olfactory tract, hippocampus and the parabrachial nucleus (Gustafson *et al.*, 1996). GalR-2 mRNA was highly expressed in hypothalamus, dorsal root ganglia as well as tissues such as the kidneys, heart and intestine (Borowsky *et al.*, 1998; Waters and Krause, 2000). The GalR-2 transcript was also located in abundance in regions of the hippocampus, cortex, Purkinje cells and brainstem nuclei as well as in the anterior lobes of the pituitary (Depczynski *et al.*, 1998; O'Donnell *et al.*, 1999). GalR-3 mRNA was also widely distributed at low to moderate levels in many central and peripheral tissues with abundant expression in the hypothalamus, pituitary olfactory cortex, the hippocampal CA regions and the dendate gyrus (Kolakowski *et al.*, 1998; Smith *et al.*, 1998; Waters and Krause, 2000). GalR-3 mRNA signal was also observed in testis, adrenal gland and pancreas (Kolakowski *et al.*, 1998).

Galanin Binding Sites in the Spinal Cord

Galanin binding sites show a widespread distribution in the nervous system (Melander *et al.*, 1986) and in neurons innervating the GI tract (King *et al.*, 1989). Their distribution seems to be well conserved among different species (Ma *et al.*, 1997). Autoradiographic mapping show that the expression of galanin binding sites is in good correlation with the distribution of galainin-like immunoreactivity. In the dorsal spinal cord, the highest density of labelling was observed in superficial layers (laminae I and II) of the dorsal horn wile moderate labeling was detected around the laminae III-IV (Melander *et al.*,

1986; Kar *et al.*, 1995). GALR-1 can be observed in about 20% of all neurons of L4 and L4 DRGs in the rat (Xu *et al.*, 1997). Under normal circumstances the presence of galanin can be observed in low numbers of dorsal root ganglion (DRG) neurons and in neurons of the superficial dorsal horn (Zhang *et al.*, 1995). Northern blot analysis revealed a more widespread distribution for GalR-2 suggesting a broader functional range than for GalR-1. GalR-2 mRNA was found to be in high abundance in the dorsal root ganglia relative to other brain regions (Ahmad *et al.*, 1998; O'Donnell *et al.*, 1999). The hybridizaiton signal is mostly found on small and intermediate primary sensory neurons (O'Donnell *et al.*, 1999). Nearly 25% of all DRG neurons were found to be GALR-2 receptor mRNA-positive (Shi *et al.*, 1997). They are mainly small neurons that also contain substance P and CGRP and which store galanin into large dense-core vesicles (LDCVs) in the Golgi complex (Zhang *et al.*, 1995). It has also been shown that approximately 50% of all dorsal root axons contain galanin-immunoreactivity (Klein *et al.*, 1990). These results suggest that galanin binding sites in the superficial dorsal horn found on primary afferents and local neurons have the ability to play a role at the spinal level.

2. Galanin Functions

Although galanin is involved in ingestive behaviour (Crawley *et al.*, 1990), cognition (Wrenn and Crawley, 2001) and the neuroendocrine system (Bauer *et al.*, 1986; Kask *et al.*, 1997), this section will focus on the involvement of galanin within the sensory system (Wiesenfeld-Hallin *et al.*, 1992; for review see Wiesenfeld-Hallin and Xu, 1998). Galanin has been shown to be inhibitory to excitatory peptides in the spinal cord and to produce a tonic inhibition of spinal cord neuronal excitability (Xu *et al.*, 1989, 2000; for review see Wiesenfeld-Hallin and Xu, 1998). Since galanin is co-stored with SP and/or CGRP in the same LDCVs in DRG neurons, it can be suggested that galanin can be released concomitantly with SP and/or CGRP. For example, galanin antagonized the effect of substance P on the nociceptive flexor reflex in the rat

(Xu *et al.*, 1989). Galanin has been shown to have weak analgesic effects in acute pain assays such as mechanical and thermal tests when administered intrathecally in mice (Post *et al.*, 1988) and rats (Wiesenfeld-Hallin *et al.*, 1993). A low dose of galanin, not antinociceptive by itself, was also shown to potentiate the antinociceptive effects of morphine in the hot plate test (Wiesenfeld-Hallin *et al.*, 1990). Galanin has also been shown to be modulated in models of chronic pain, however, this issue remains controversial. Some studies have reported a decrease in galanin binding sites following sciatic nerve cut (Kar *et al.*, 1994) while others have reported no changes in the distribution or intensity of galanin binding sites (Zhang *et al.*, 1995). Also, galanin-immunoreactivity has been shown to be significantly increased in the superficial laminae of the dorsal horn following chronic constriction injury, partial injury and complete transection of the sciatic nerve (Wiesenfeld-Hallin *et al.*, 1992; Carlton *et al.*, 1996; Ma *et al.*, 1997, Hu and McLachlan, 2000; for review see Xu *et al.*, 2000) with a similar increase in galanin mRNA levels (Villar *et al.*, 1989). Recent work studying GalR-2 mRNA in rat dorsal root ganglia following peripheral tissue inflammation and axotomy showed that there was a strong increase in the number and density of GalR-2 mRNA-positive neurons after injury (Shi *et al.*, 1997). After axotomy about 70% of the neurons were shown to express galanin (Zhang *et al.*, 1993). A study showed that axonal blockade could be involved in this modulation (Kashiba *et al.*, 1992). A local application of colchicine, a blocker of axonal transport, on the sciatic nerve caused an upregulation of galanin in dorsal root ganglia. Galanin seems to occur normally in small primary sensory neurons and is present in 2-3% of DRG cells however following peripheral nerve injury or inflammation, expression of galanin in primary afferents and spinal cord is upregulated with 40-50% of all DRG neurons positive for galanin (Hokfelt *et al.*, 1994; Hu and McLachlan, 2000; for review see Xu *et al.*, 2000). These different results suggest that galanin-related mechanisms may participate in complex adaptive responses in dorsal root ganglia after inflammation and nerve

injury. Furthermore, the generation of mice carrying a loss-of-function mutation in the galanin gene (Wynick *et al.*, 1998) has further elucidated the role of galanin in pain by showing that the absence of galanin in development causes an attenuation in chronic neuropathic pain behavior (Kerr *et al.*, 2000). This decrease in pain function was likely due to developmental deficits in the small peptidergic DRG neurons of the galanin mutant animals (Holmes *et al.*, 2000).

E. NEUROPEPTIDE Y

Neuropeptide Y (NPY) is a 36-amino acid peptide that belongs to a large family of central and peripheral peptides including peptide YY (PYY) and pancreatic polypeptides (PP; Tatemoto *et al.*, 1982). All of the NPY-family peptides contain the key residues to adopt a so-called PP-fold, which is characterized by an extended proline helix with three prolines, a turn, and an alpha helix with two tyrosines interdigitating with the three prolines. Despite its relatively large size, NPY has been well conserved throughout evolution and shows a high degree of sequence conservation among a wide variety of vertebrates (Larhammar *et al.*, 1993). NPY has been shown to exert many different effects on peripheral (blood vessels, heart, airways, gastrointestinal tract, kidney, pancreas, thyroid gland, platelets, mast cells, and sympathetic, parasymapathetic and sensory nerves) and central (pituitary hormone release, behavior, central autonomic control) targets (McDonald *et al.*, 1988; Wahlestedt *et al.*, 1989; Dumont *et al.*, 1992; for review see Dumont *et al.*, 2000).

Neuropeptide Y Receptors

Five distinct NPY receptors have been cloned (Y_1, Y_2, Y_4, Y_5 and y_6; for review see Dumont *et al.*, 2000) which have been shown to belong the G protein-

coupled receptor family. The pharmacological profile of a sixth NPY receptor has been demonstrated but as yet to be cloned.

Y_1 Receptor

In 1990, a cDNA was isolated from rats that was thought to encode an orphan receptor (Eva *et al.*, 1990; Krause *et al.*, 1992). The mRNA distribution of this receptor was very similar to the reported distribution of the Y_1-like receptors in the rat brain (Dumont *et al.*, 1990) and hence became known as the Y_1 receptor subtype. Shortly after species homologs from mice (Eva *et al.*, 1992) and humans (Larhammar *et al.*, 1992) were identified. NPY and PYY have been shown to be the most potent peptides at the Y_1 subtype receptor with NPY having the highest potency. (Krause *et al.*, 1992). SK-N-MC (Wieland *et al.*, 1995) and human erythroleukemia (Feth *et al.*, 1992) are two human cell lines that contain homogeneous populations of Y_1 receptor.

Y_2 Receptor

The cDNA for the Y_2 receptor was first isolated from human SMS-KAN cells (Rose *et al.*, 1995) and then from human brain cDNA libraries (Gerald *et al.*, 1995) and a human neuroblastoma cell line (Rimland *et al.*, 1996). The Y_2 receptor has now been cloned from other species such as rat (St-Pierre *et al.*, 1998) and mouse (Nakamura *et al.*, 1996) to reveal high homolgy between the different species. For this receptor NPY and PYY are both equally potent at binding the receptor (Rose *et al.*, 1995). SMS-KAN cells as well as rabbit kidney cells are two cells lines that contain a high amount of the Y_2 receptor subtype (Wieland *et al.*, 1995).

Y_4 Receptor

Using sequence homology and screening with a NPY Y_1 receptor probe, the Y_4 receptor was first isolated from a human genomic library and named "PP1" (Lundell *et al.*, 1995). Homologs from rat (Gerald *et al.*, 1996) and mice (Gregor *et al.*, 1996) were cloned shortly afterwards. The main attribute that characterizes the Y_4 receptor is its very high affinity for PP (Gregor *et al.*, 1996). This is in contrast to the low affinity of the PPs for the Y_1 and Y_2 receptor subtypes (Michel *et al.*, 1998). The Y_4 receptor mRNA was found to be mainly expressed in the colon, small intestine and prostate while different CNS regions displayed low expression levels (Lundell *et al.*, 1995).

Y_5 Receptor

More recently, a Y_1-like receptor cDNA was cloned from rats and humans encoding a protein of 456 amino acids (Gerald *et al.*, 1996). The gene seems to be found on the same chromosome as Y_1 but in the opposite orientation (Gerald *et al.*, 1996). This receptor has also been cloned from mouse (Nakamura *et al.*, 1997) and dog (Borowsky *et al.*, 1998). This receptor showed a high affinity to NPY and PYY when expressed in 293 cells and coupled to the inhibition of cAMP accumulation (Gerald *et al.*, 1996). Another characteristic of this subtype is its high affinity for human PP but not for rat PP (Michel *et al.*, 1998). The mRNA for this receptor were found, through Northern blotting and in situ hybridization, to be detected in the testis and in different brain areas known to regulate food intake (Gerald *et al.*, 1996).

y_6 Receptor

The y_6 receptor subtype clone came from mouse DNA and encodes a 371 amino acid protein (Weinberg *et al.*, 1996). This receptor has been referred to as the Y_5 (Weinberg *et al.*, 1996), PP_2 (Gregor *et al.*, 1996) and Y_{2b} (Matsumoto *et al.*, 1996) depending on the author. Homologues of this receptor have been isolated from rabbit, monkey and humans (Gregor *et al.*, 1996). The order of potency of the different NPY peptides and fragments for

this receptor has remained controversial, although it seems to have a similar pharmacological profile to the Y_5 receptor (Weinberg *et al.*, 1996). The primate gene sequences of the protein differs from the ones in rabbits and rodents by a frame shift mutation which results in a stop codon and a truncated protein (Gregor *et al.*, 1996). The expression of a functional protein from the monkey or the human clone has not resulted in a functional receptor despite the mRNA being present in many different human tissues (Gregor *et al.*, 1996). The physiological function of this truncated protein is still unknown.

Y_3 Receptor

Many pharmacological studies have proposed the existence of a sixth NPY receptor that depicts a high affinity for NPY but not PYY (Dumont *et al.*, 1993, 1994; for review see Dumont *et al.*, 2000). The cloning of a bovine Y_3 receptor has been reported (Rimland *et al.*, 1991) however it was found not to be a NPY receptor (Herzog *et al.*, 1993; for review see Dumont *et al.*, 2000).

1. Distribution of Neuropeptide Y and its Binding Sites

NPY and its receptors are abundantly present in many regions of the mammalian brain, in fact, it is believed to be the most abundant and widely distributed neuropeptide present in the mammalian central and peripheral nervous system. NPY-like immunoreactivity (NPY-IR) has been found to be most present in the amygdala, nucleus accumbens and basal ganglia while a more moderate expression was found in the hypothalamus, hippocampus, septal nuclei, cortex and periaqueductal grey (Adrian *et al.*, 1983; Schwartzberg *et al.*, 1990; for review see Dumont *et al.*, 2000). Autoradiographic studies in the rat brain have demonstrated the presence of NPY binding sites mostly concentrated in the hippocampus, cortex, spinal cord and thalamic nuclei with lower levels seen in the striatum, hypothalamus and in the cerebellum (Ohkubo *et al.*, 1990; Dumont *et al.*, 1993b; Jacques *et al.*, 1996, 1997, 1998; for review see Dumont *et al.*, 2000) with significant species

differences existing (Dumont *et al.*, 1998). Other than the brain, radioligand receptor studies with iodinated or tritiated NPY have shown the evidence of binding sites in the vasculature, heart, kidneys, spleen and uvea (Wahlestedt *et al.*, 1990).

Distribution of NPY Receptor Subtypes in Rat Spinal Cord

NPY is widely distributed in the brain and it has also been identified in human, lemur and rat spinal cord, where it seemed to be concentrated in the dorsal horn region (Allen *et al.*, 1983; Gibson *et al.*, 1984; Rowan *et al.*, 1993). NPY binding sites have been found in the trigeminal and dorsal root ganglia of rat, rabbit and monkey (Mantyh *et al.*, 1994). Also, significant amounts of NPY binding sites are expressed in laminae I and II of the dorsal horn (Kar and Quirion, 1992). Moderate to high amounts of Y_1 receptor subtype labeling was observed in laminae I and II of the dorsal horn of the cervical, thoracic, lumbar and sacral segments of the spinal cord (Zhang *et al.*, 1995; Dumont *et al.*, 2000; Migita *et al.*, 2001). In contrast to the Y_1 receptor distribution confined to laminae I and II, the distribution of the Y_2 receptor was observed in moderate amounts in most laminae of the dorsal horn and ventral horns of the cervical, thoracic, lumbar and sacral spinal cord (Dumont *et al.*, 2000). The distribution of the Y_5 receptor has been studied (Dumont *et al.*, 1998; Jacques *et al.*, 1998) but no information is available on its distribution in the spinal cord. No detailed information regarding the presence of the Y_4 in the spinal cord is available. Similar to the distribution of the Y_1 receptors, Y_1 receptor mRNA-positive neurons were also observed in laminae I and II of the dorsal horn of the spinal cord (Dumont *et al.*, 2000). No positive hybridization signals for the Y_2 receptor mRNA were detected in the dorsal horn of the spinal cord suggesting that the Y_2 receptor protein that were detected in the dorsal horn are likely projection neurons originating from the dorsal root ganglia (Dumont *et al.*, 2000).

2. Neuropeptide Y Functions

Although NPY and its binding sites are widely distributed and have been shown to be involved in many vital functions such as circadian rhythms (Calza *et al.*, 1990), body temperatures (Jolicoeur *et al.*, 1995), sexual behavior (Kalra *et al.*, 1988), appetite (Kalra *et al.*, 1988; Quirion *et al.*, 1990) and neuroendocrine secretions (Dumont *et al.*, 1992, Wettstein *et al.*, 1995; for review see Dumont *et al.*, 2000), the role of NPY on the sensory system will be closely examined.

As mentioned previously, NPY and its receptors have been found in mammalian spinal cord concentrated in the spinal dorsal horn region (Allen *et al.*, 1983) as well as in the trigeminal and dorsal root ganglia (Mantyh *et al.*, 1994). A study has shown that NPY had the ability to produce an antinociceptive effect when applied onto the spinal cord of rats (Hua *et al.*, 1991). This effect occurs, at least in part, due to an inhibition of substance P release in primary afferent fibres (Hua *et al.*, 1991). NPY, PYY and a number of their C-terminal fragments were administered and produced a dose-related antinociception effect as measured by the 52.5°C hot plate test and electric tail stimulation (Mellado *et al.*, 1996; Broqua *et al.*, 1996). The antinociceptive activities of NPY and its analogs have shown that this action lies with the Y_1 rather than the Y_2 or Y_3 receptor subtypes (Broqua *et al.*, 1996). NPY and Y_1 agonists produced a dose-dependent and complete suppression of acetic acid-induced writhing (Broqua *et al.*, 1996). This seems to provide evidence to the notion that NPY and its related peptides have the ability to induce receptor-mediated effects in the spinal cord. A similar experiment was performed where NPY agonists were administered to rats through a lateral ventricular injection. However, in this study there was no modification of the nociceptive threshold assessed by the warm-water tail flick test (Heilig *et al.*, 1992). An intra-periaqueductal grey injection of NPY induced significant increases in hindpaw withdrawal latency to thermal and mechanical stimulation n rats with inflammation (Wang *et al.*, 2001).

Complementary to this issue, NPY has been shown to be modulated in models of chronic pain. In a peripheral tissue inflammation model where the animals were injected with complete Freud's adjuvant, there was a marked increase in NPY mRNA expression observed in the dorsal horn as well as an increase in NPY Y_1 receptor mRNA expression (Ji *et al.*, 1994). The concentrations of NPY and Y_2 receptor mRNA were markedly up-regulated in spinal gray matter and dorsal root ganglion following sciatic nerve transection in Sprague-Dawley rats (Wakisaka *et al.*, 1992; Ma and Bisby, 1998; for review see Dumont *et al.*, 2000).

More recently, the development of an NPY-transgenic mice (Bannon *et al.*, 2000) and rat (Thorsell *et al.*, 2000) have attempted to elucidate the role of NPY in nociception. Y1 (-/-) mice were normal in being able to develop hyperalgesia to thermal, cutaneous and visceral pain and mechanical hypersensitivity (Naveilhan *et al.*, 2001). Neuropathic pain in these mice was increased and the mice showed a complete absence of the pharmacological analgesic effects of NPY (Naveilhan *et al.*, 2001). The Y_1 receptor may be required in the periphery for substance P release and subsequent neurogenic inflammation and plasma leakage (Naveilhan *et al.*, 2001).

Although, NPY and its receptors are found in the sensory system, their involvement in nociception is still uncertain.

F. MORPHINE TOLERANCE

Morphine is used widely in the management of pain and although it is useful as an analgesic, its application to treat chronic pain is limited by the development to its antinociceptive properties (Johnstone *et al.*, 1992). The development of tolerance necessitates an increase in drug doses, which results in serious side effects. Considerable efforts have been made in order to

understand the modifications occurring in the central nervous system (CNS) during the long-term administration of opioids, particularly during development of tolerance to their antinociceptive effects. Tolerance is described as a diminution of effects after exposure to a drug or the need for a higher dose of a drug to maintain a given response (Koob *et al.*, 1992). Exposure to a drug is thought to be the "driving force" for the development of tolerance and the need for a higher dose due to progressing pathology should not be considered as tolerance. It is well known that chronic morphine administration produces tolerance to the analgesic, thermal, respiratory depressant, euphoric, locomotor depressant and stimulant effects of the drug (Bhargava, 1994). Opioids produce their pharmacological effects through three types of receptors, μ, δ and κ opioid receptors located in several regions of the brain and spinal cord (Fowler *et al.*, 1994, Bhargava, 1994). Investigators first hypothesized that tolerance occurred primarily through critical alterations in opiate receptors as well as endogenous opiate systems. However, the nature of these critical alterations is not yet resolved as some authors show no change (Hollt *et al.*, 1975; Gouarderes *et al.*, 1993), an upregulation (Pert *et al.*, 1976; Brady *et al.*, 1989; Rothman *et al.*, 1991), a downregulation (Werling *et al.*, 1989; Bhargava *et al.*, 1990) or a desensitization (Nestler, 1993; Childers, S., 1991) of the opioid receptors. Consequently, other mechanisms that could possibly be involved in the modulation of tolerance have been studied. These include changes in neurotransmitter systems that appear to be the target of opiates and are closely involved in the transmission of nociceptive signals (Yaksh *et al.*, 1985; Gouarderes *et al.*, 1993). One of these neuropeptides is substance P since it is thought that opiates act partly by inhibiting the release of SP *in vivo* (Yaksh *et al.*, 1980). This suggests that SP and neurokinin-I/SP receptor sites could be modified during the development of tolerance. However, it was shown that SP receptors were not significantly altered during morphine tolerance (Gouarderes *et al.*, 1993; Menard *et al.*, 1995a).

As described above, CGRP has been shown to be involved in nociception and to be colocalized with substance P in dorsal root ganglia (Lee *et al.*, 1985) and superficial dorsal horn (Plenderleith *et al.*, 1990; Ribeiro-da-Silva, 1995). Important amounts of CGRP binding sites are concentrated in the dorsal horn of the spinal cord (Yashpal *et al.*, 1992). More importantly, CGRP inhibits the antinociception produced by morphine (Welch *et al.*, 1989) while morphine is able to inhibit the release of CGRP in the spinal cord (Pohl *et al.*, 1989). Thus, it seemed plausible that CGRP may have a role in the development of tolerance in animals administered a chronic intrathecal infusion of morphine. Menard *et al.* (1995b) investigated the possible involvement of various neuropeptides including CGRP, substance P, galanin and neuropeptide Y and their receptors in the dorsal horn of the spinal cord during the development of tolerance to the antinociceptive action of intrathecal morphine. Tolerance to the antinociceptive effect of morphine was verified with the tail-immersion test. By the 5^{th} day of treatment, the withdrawal latencies were not significantly different from controls indicating the beginning of tolerance to the effects of morphine. Tolerance to morphine analgesia persisted up to the last day of infusion. A marked increase in CGRP immunostaining was observed in the superficial laminae (I & II) of the spinal cord following 5 days of treatment. Substance P immunostaining was slightly increased after 5 days only while no change was observed in galanin immunostaining. Similar changes were observed following the chronic infusion of the delta agonist DPDPE, [D-Pen2, D-Pen 5], but not of the kappa agonist, U-50488H (Menard *et al.*, 1995b). [^{125}I]BH-SP, [^{125}I]galanin, [^{125}I]neurotensin and [^{125}I]NPY receptor binding sites in the dorsal horn of morphine-treated animals were not significantly different from saline-treated animals on the 5^{th} day of infusion. On the basis of these results, Menard *et al.* (1995a, b) postulated that chronic morphine administration is associated with desensitization to the inhibitory effect of morphine on the release of CGRP. Consequently, there is an increase in CGRP along with a concomitant downregulation of its receptors. Moreover, Menard

et al. (1996) have shown that the potent CGRP antagonist, $hCGRP_{8-37}$, could prevent the development of morphine tolerance in acute pain models such as the paw pressure and tail-immersion tests as well as the changes in hCGRPα immunostaining and binding sites (Menard *et al.*, 1996). More recently BIBN4096BS, a potent non-peptide antagonist of the CGRP receptor was shown to similarly block the development of tolerance to the antinociceptive effect of morphine (Powell *et al.*, 2000).

G. DORSAL ROOT GANGLION CULTURES

The mechanisms underlying the development of tolerance to the analgesic effects of opiates are not clearly understood. Neuronal adaptation following chronic exposure to morphine is thought to be one of the possibilities. It has been suggested that pain-related neuropeptides such as CGRP and substance P could be involved in the development of tolerance to opioids (Menard *et al.*, 1995a, 1995b, 1996). Chronic morphine exposure seems to induce the over-expression of CGRP in dorsal root ganglion (DRG) neurons since the CGRP immunoreactivity in the dorsal horn originates exclusively from primary sensory afferents (Chung *et al.*, 1988; Traub *et al.*, 1989). The terminals of nociceptive sensory neurons that project to the superficial layers of the dorsal horn represent one site that has been proposed to mediate opioid analgesia at the level of the spinal cord. The increase in CGRP in primary sensory afferents likely promotes the release of CGRP in the dorsal horn, thus facilitating nociception transmission. Using a DRG cell culture model, it was recently shown that an *in vitro* treatment with morphine can mimic the *in vivo* findings that showed an increase in CGRP-like immunostaining in morphine tolerant animals (Ma *et al.*, 2000). Following a repetitive exposure to morphine sulfate for 6 days, the number of CGRP- and SP- immunoreactive neurons in cultured DRG was significantly increased. The apparent increase in the number of CGRP- and SP-immunoreactive neurons observed following morphine treatment was blocked by naloxone, demonstrating the involvement of genuine

opioid receptors. The aim of that study was not only to mimic some previous *in vivo* results obtained in morphine tolerant rats but also to develop a simple *in vitro* model that could be used to investigate the intracellular events triggered by chronic morphine exposure *in vivo*. Earlier data has suggested that protein kinase C (PKC) plays a role in both neuropathic pain (Malmberg *et al.*, 1997) and in morphine tolerance (Narita *et al.*, 1994; Mayer *et al.*, 1995). Accordingly, cultured DRG neurons, which express CGRP, SP and opioid receptors may represent a suitable model to study intracellular pathways leading to morphine induction of CGRP and SP expression. This is particularly important since these events seem to be associated with the development of tolerance to morphine's antinociceptive effects.

H. AIM OF THESIS

The main objective of this thesis was thus to determine which subtype of opioid receptors is involved in the induction of CGRP- and SP-like IR that is observed following a repetitive treatment in DRG cultures. A second aim was to further validate the DRG cell culture as a suitable model to study the intracellular signal transduction events which could lead to morphine tolerance We particularly focused on the proposed role of protein kinase C (PKC) in the development of morphine tolerance, and its implication in the upregulation of CGRP- and SP-like IR in the DRG cell culture model.

CHAPTER 2 - MATERIALS AND METHODS

A. Primary Cell Culture Dorsal Root Ganglion (DRG) Neurons

Materials

Sprague-Dawley rats (male, 3 months old, body weight 225-275g) were obtained from Charles River (St-Constant, Quebec, Canada). Animals were maintained according to protocols and guidelines of the Canadian Council on Animal Care and the McGill University Animal Care Committee. Hank's balance salt solution (HBSS), Dubelco modified Eagle medium (DMEM), Ham's F12 solution, HEPES buffer solution (1M), the heat inactivated fetal bovine serum (FBS), the L-D-polylysine, the 2.5% trypsin and the 96-well plates were all purchased from GIBCO/BRL (Burlington, Ontario, Canada). The cell strainers (75μm) were obtained from Becton Dickinson Labware (Franklin Lakes, NJ, USA). The type II 2.5% collagenase was obtained from Cedarlane Lab Ltd. (Hornby, Ontario, Canada). All of the dissection instruments were purchased from Fine Point Instruments Inc. (Vancouver, BC, Canada).

Methods

Preparation of the Solutions

Hanks Balance Salt Solution (HBSS)

HBSS	100 ml
HEPES buffer soln. (1M)	1 ml
Penicillin/streptomycin	1:1000

The solution was prepared prior to the dissection and kept at 4°C for a few days.

Dubelco Modified Eagle Medium (DMEM)

DMEM	10 ml
HEPES buffer soln. (1M)	100 ml
Penicillin/streptomycin	50 µl
Fetal bovine serum	1 ml

The solution was prepared prior to the dissection and kept at 4°C for a few days.

L-D-polylysine solution

L-D-polylysine	1 ml
Distilled H_2O	9 ml

The solution is prepared prior to the dissection and can be kept at 4°C for a few weeks.

Coating of the Cell Culture Plate

About 1 hr. prior to seeding of the cells in the culture plate, 1 ml of the L-D-polylysine solution is added to each well for coating purposes. At the end of the 1-hr. period, the wells are rinsed twice with distilled H_2O before being ready for seeding.

Dissection

Prior to dissection all the instruments were immersed into 70% ethanol for at least 1 hour. Animals were decapitated. The back area of the animal was cleaned with 70% ethanol and then the backbone was cut out with a large pair of scissors. Using the bone breaker instrument along with forceps and small scissors, the DRGs (40-50) from the cervical, thoracic, lumbar and sacral levels were removed aseptically and collected in Hank's balanced salt solution (HBSS). After being minced into small pieces, DRG tissues was digested in 0.25% collagenase in Ham's F12 medium at 37°C for 45 min followed by a 15 min incubation in HBSS containing 0.25% trypsin. Tissue was then triturated

using a thin flame-polished pipet in Dubelco Modified Eagle Medium (DMEM) containing 1% HEPES buffer solution, penicillin/streptomycin (1:200) and 10% heat inactivated fetal bovine serum (FBS). Cells were then subjected to density gradient centrifugation at 400g for 10 min. The resulting pellet was resuspended in DMEM and the cell suspension was filtered through a cell strainer (75μm). DRG cells were seeded in a 96-well culture plate, yielding a cell density of $5x10^4$ cells/well, and cultured in a humid incubator at 37°C with 5% CO_2 and 95% O_2 for up to 8 days.

B. Opioid Treatments

Materials

DAMGO (μ agonist - Tyr-D-Ala-Gly-N-Methyl-Phe-Gly-ol), CTOP (μ antagonist -[D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr] amide, DPDPE (δ agonist - Tyr-D-Pen-Gly-Phe-D-Pen), naltrindole (δ antagonist - naltrindole hydrochloride), U50488H (κ agonist -(trans-(+-)3,4-dichloro-N-methyl-N-2-[1-pyrrolidinyl]cyclohexyl)-benzeneacetamide) and n-BNI (κ antagonist - nor-binaltorphimine dihydrochloride) were all purchased from Sigma Aldrich (St-Louis, MO, USA)

Methods

Treatment

Two days after seeding, the culture medium (DMEM) was changed. DAMGO (μ agonist), CTOP (μ antagonist), DPDPE (δ agonist), naltrindole (δ antagonist), U50488H (κ agonist) and n-BNI (κ antagonist) dissolved in culture medium at various concentrations (10, 20 and 30 μM) were added. In control wells only culture medium was added (vehicle treatment). Culture medium, opioid agonists and antagonists were changed every other day. For the combined treatment of the specific agonist and antagonist, two days after seeding, the culture medium was changed and 10, 20 or 30 μM antagonist was

given. Thirty minutes later, 10, 20 or 30 μM of the corresponding agonist was then added. Subsequently, culture medium was changed every other day (48 hrs.), the agonist and antagonist being added simultaneously.

Four separate experiments were carried out for each receptor subtype. For each experiment, new primary cultures were prepared under standard conditions. Each treatment was repeated in 4 different wells.

C. Opioid Treatment/Protein kinase C Inhibition

Materials

The PKC inhibitor chlerythrine chloride was purchased from Biomolecular Research Laboratory (Plymouth Meeting, PA, USA) and GO6976 from Calbiochem (La Jolla, CA, USA).

Methods

Treatment

Two days after seeding the culture medium (DMEM) was changed. DAMGO (30 μM) dissolved in culture medium was added. In control cells only culture medium was added (vehicle treatment). The protein kinase C (PKC) inhibitors, chelerythrine chloride (a non-selective PKC inhibitor; Herbert *et al.*, 1990) and GO6976 (an inhibitor of the α, β and γ PKC isoenzymes; Qatsha *et al.*, 1993) were diluted in DMEM to concentrations of 5 μM. For the combined treatment with DAMGO and the PKC inhibitors (chelerythrine chloride or GO6976), two days after seeding, the culture medium was changed and DAMGO (30 μM) and one of the PKC inhibitors (5 μM) were added simultaneously.

Four separate experiments were carried out for each receptor subtype. For each experiment, new primary cultures were prepared under standard conditions. Each treatment was repeated in 8 different wells.

D. Immunocytochemistry

Materials

Rabbit anti-CGRP, NPY and galanin antibodies were purchased from Peninsula Laboratories (Belmont, CA, USA). The primary antibody raised against substance P was purchased from Chemicon (Temecula, CA, USA) while the anti-PKCα antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The normal goat serum (NGS), goat anti-rabbit IgG and the Elite Vectastain ABC kit were obtained from Vector Laboratories (Burlingame, CA, USA). The glucose oxidase, 3,3'-diaminobenzidine (DAB) and glycerol was purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). All other chemicals were of analytical grade and obtained from VWR Canlab (Mississaugua, Ontario, Canada).

Immunostaining

Immunostaining of the DRG neurons in culture were performed after 6 days of opioid treatments according to the protocol described below. The same protocol was used for CGRP, SP, galanin, NPY and PKCα immunostaining.

Methods

Preparation of solutions

Sodium Sorenson's phosphate buffer (PB) (0.2 M)

Monobasic sodium phosphate (NaH_2PO_4)	12.69 g
Dibasic sodium phosphate (Na_2HPO_4)	43.74 g
Distilled H_2O	2 L

The 0.2 M phosphate buffer solution was kept at room temperature for a few weeks.

Phosphate Buffered Saline / Triton Solution (PBS+T, 0.1M)

Phosphate buffer (PB)	50 ml/l
NaCl	8.8 g/l
KCl	0.2 g/l
Triton-X 100	20 ml

Water was added to a final volume of one litre and the pH was adjusted to 7.4. This solution was kept at room temperature. PBS+T 0.1M was prepared prior to the experiment by mixing 100ml of PBS+T 1M with 900ml of H_2O.

4% Paraformaldehyde in 0.1M Phosphate Buffer (4%PFA)

Under fumehood, a solution of 80g/l of PFA in H_2O was heated to 60°C while stirring constantly. Drops of 1M NaOH was added until the solution became clear. It was then cooled on ice and filtered under vacuum. This 8% solution was kept at 4°C for a few weeks. Prior to use, equal parts of 8% paraformaldehyde and 0.2M PB were mixed.

Antisera Solutions

The rabbit anti-CGRP, SP, galanin and NPY antiserum were diluted 1:4000 in 0.01 PBS+T. The anti-rabbit IgG was diluted in PBS+T at 1:25 and the PKCα antibody was diluted 1:2000.

Acetate Buffer

A stock solution of 0.2M acetate buffer was prepared by mixing 13.61g of sodium acetate in 500 ml of H_2O. The pH was adjusted to 6.0 using a 10% solution of acetic acid. The 0.1M acetate buffer was diluted just before the experiment in an equal part of H_2O.

3,3'Diaminobenzidine Solution (DAB)

The DAB solution was prepared in the following manner:

3,3'diaminobenzidine	25 mg
PBS	50 ml
H_2O_2 (30%)	50 µl

This solution was prepared by adding the hydrogen peroxide and the 3,3'DAB and then diluting in PBS and filtering. The solution was then kept at -20°C for a few weeks.

Glucose Oxidase-3,3'Diaminobenzidine Nickel Solution

0.1M Acetic buffer pH 6.0	25 ml
Nickel ammonium sulfate	0.625 g
10% diaminobenzidine (DAB)	1.5 ml
D-glucose	50 mg

The solution was prepared and used immediately.

Immunostaining

Fixation: After 6 days of treatment, DRG cells were fixed in 4% paraformaldehyde in 0.1M phosphate buffer for 20 min. The cells were then washed and rinsed with PBS+T twice.

Preincubation: The DRG cells were pre-treated for 15 min. with 0.3% H_2O_2 and 10% normal goat serum (NGS) in 0.01M phosphate buffered saline containing 0.3% Triton-X 100 (PBS+T) to block endogenous peroxidase. The cells were then incubated with 50% ethanol for 30 min. to further remove endogenous peroxidase and render the plasma membrane permeable to allow penetration. An incubation with 10% NGS for 1 hr. was then performed to block against non-specific staining. Between each incubation, cells were washed in PBS+T twice.

Incubation with primary antisera: The DRG cells were then incubated for 72 hrs. with antisera to rat CGRP (1:4000), SP (1:4000), Galanin (1:4000), NPY (1:4000) or PKCα (1:2000) diluted in PBS+T containing 1% NGS. The cells were then washed in PBS+T twice.

Incubation with secondary antibody: The cells were then incubated for 2 hr. with a biotynilated goat anti-rabbit IgG (1:2000) diluted in PBS+T containing 1% NGS.

Visualization: The cultured cells were subsequently processed using the Elite Vectastain ABC kit (Vector) according to the manufacturer's instructions. The cells were incubated for 1 hr. in a solution containing 1% Solution A and 1% Solution B diluted in PBS+T with 1% NGS. Finally, immunoprecipitates were developed and revealed using a 3,3'-diaminobenzedine method with the glucose oxidase-3,3'-diaminobenzidine-nickel solution. All cultured cells were washed thoroughly with the sodium acetate buffer before being placed and kept in 70% glycerol.

Quantification: All quantitative analysis were performed using a Nikon F-601M inverted phase microscope. All cells were counted with the help of a delineated area in the camera-viewing field under 100X magnification. Fields of approximately 1 mm^2 in which the immunopositive cells were counted, were randomly chosen. The average number of counted cells was determined from 10 different fields in each well. The mean number of counted cells was obtained from the different wells (4-8) for each treatment.

Statistical Analysis: Values were compared statistically using one-way ANOVA and the post-hoc Student-Newman-Keuls multiple comparison method (Graphpad, Prism III) to compare every treatment group to the control (vehicle treatment) with $p<0.05$ being considered significant.

E. DOUBLE IMMUNOFLUORESCENCE STAINING

Materials

Rabbit anti-CGRP was purchased from Peninsula Laboratories (Belmont, CA, USA). The primary antibody raised against substance P, and the μ- and δ-opioid receptors were obtained from Chemicon (Temecula, CA, USA) while the anti-PKCα antibody was purchased from Transduction Laboratory (Mississauga, Ontario, Canada). The rabbit anti-κ-opioid receptor was kindly provided by Dr. S. Watson (Mental Health Research Institute, University of Michigan, MI, USA). Normal donkey serum (NDS), the donkey anti-rabbit IgG conjugated with fluorescein (FITC) and the goat anti-mouse IgG conjugated with rhodamine (TRITC) were obtained from Jackson Immunoresearch Inc. (West Grove, PA, USA). The normal goat serum (NGS) and the anti-fading mounting material Vectastain was obtained from Vector Laboratories (Burlingame, CA, USA). The laser confocal microscope is a TE300 from Nikon (New York, NY, USA).

Methods

Fixation: Following 6 days of treatment with the various opioid agonist, cultured DRG cells were fixed with 4% paraformaldehyde in 0.1M PB for 20 min. The cells were then washed and rinsed with PBS+T twice. The cells were then incubated with 50% ethanol for 30 min. to render the plasma membrane permeable to allow immunostaining

Fluorescein Staining (green):

Preincubation: An incubation with 10% NDS for 1 hr. was then performed to block against non-specific staining. Between each incubation, cells were washed in PBS+T twice.

Incubation with primary antisera: The DRG cells were then incubated for 72 hrs. with antisera to rat CGRP (1:500) or SP (1:500) diluted in PBS+T containing 1% NDS. The cells were then washed in PBS+T twice.

Incubation with the secondary antibody: The cells were then incubated for 2 hrs. with a donkey anti-rabbit IgG conjugated with fluorescein (FITC; 1:50) diluted in PBS+T containing 1% NDS. Once FITC-labeled CGRP- or SP-positive cells were observed under epifluorescence microscope with blue light, the cells were further processed.

Rhodamine Staining (red):

Preincubation: The cells were incubated with 10% NGS for 1 hour to block against non-specific staining. _

Incubation with primary antisera: The cells were washed with PBS+T twice. The cultured DRG cells were incubated in mouse anti-PKCα (1:200), anti-μ-opioid receptor (1:500), anti-δ-opioid receptor (1:500) or anti-κ-opioid receptor (1:500) diluted in PBS+T containing 1% NGS for 72 hrs. The cells were then washed in PBS+T twice.

Incubation with secondary antibody: The cells were then incubated for 2hrs. with goat anti-mouse IgG conjugated with rhodamine (TRITC; 1:50) diluted in PBS+T containing 1% NGS. Once rhodamine-labeled positive cells were observed under epifluorescence microscope with green light, the cells were covered with anti-fading mounting material (Vectastain) and observed under a laser confocal microscope for colocalization.

CHAPTER 3 - RESULTS

A) Neuropeptide expression in DRG cultures treated with opioid agonists.

1. Immunocytochemistry Data

a) CGRP-IR Neurons

In vehicle- and opioid-treated DRG cultures, numerous CGRP-IR neurons were observed. More abundant CGRP-IR cells were observed in opioid agonists (10, 20 and 30 μM) treated DRG cultures than in the vehicle-treated DRG cultures. Figure 1 shows CGRP-like immunostained neurons in DRG cultures following specific opioid agonist treatment (μ, δ or κ) for 6 days at a concentration of 20 μM. In both vehicle- and opioid-treated DRG cultures, CGRP-IR cells are of various sizes and many express multiple long branches.

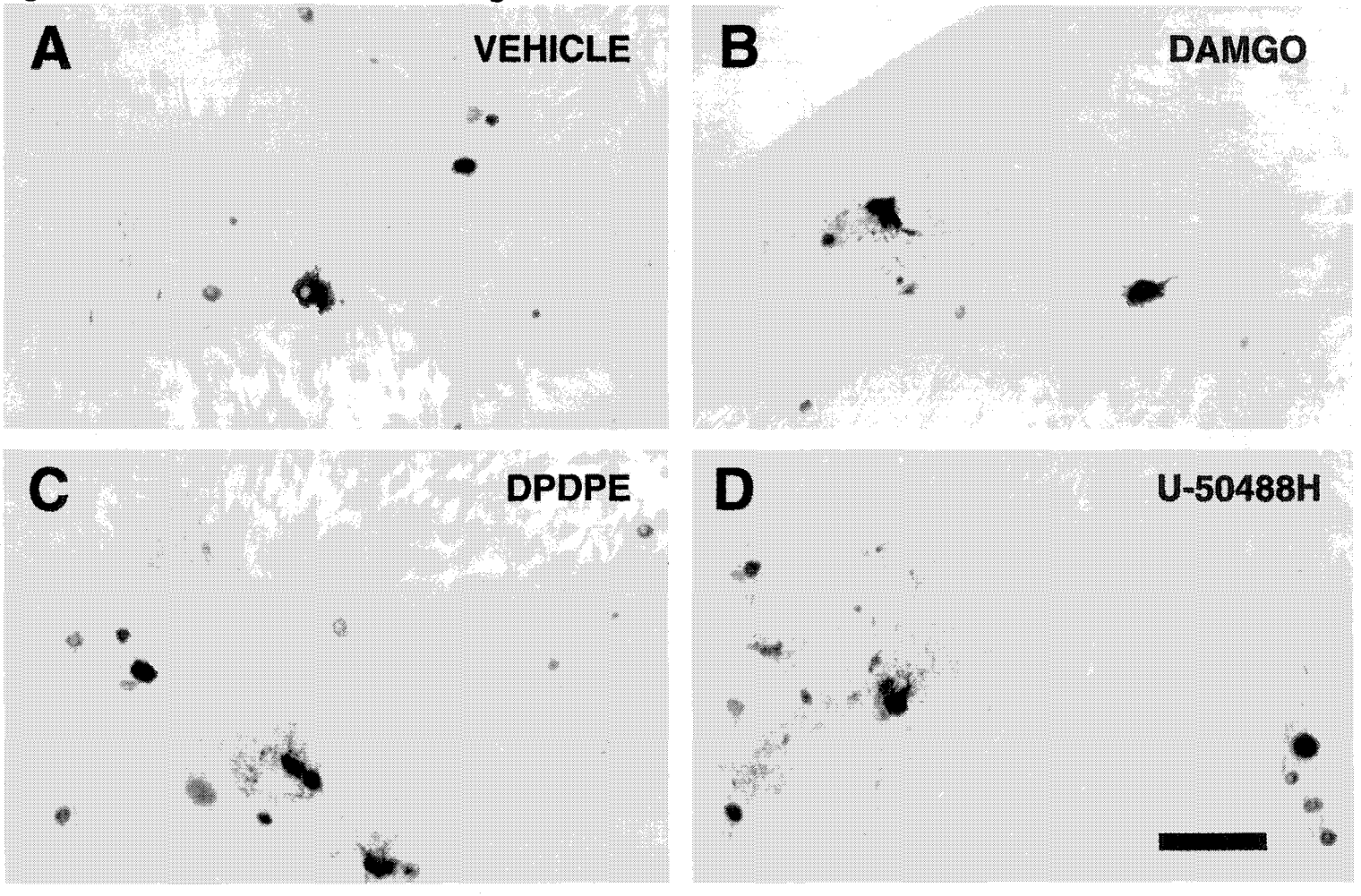
Following page:

Figure 1. CGRP-like Immunostaining

Photomicrographs of CGRP-IR neurons in cultured DRG. Following agonist treatment (30 μM: B; DAMGO, C; DPDPE or D; U50488H) for 6 days, a greater number of CGRP-IR neurons were observed in DRG cultures when compared to vehicle-treated controls (A). CGRP-IR neurons were of various sizes. Many CGRP-IR neurons exhibited multiple long branches. Scale bar, 100 μm.

Quantitatively, following treatment with 10, 20 and 30 μM DAMGO (μ-opioid agonist), the amount of CGRP-IR neurons in cultured DRG was significantly increased compared to vehicle treatment in a concentration-dependent manner (Figure 2). When the μ-opioid antagonist, CTOP, was administered

Figure 1 - CGRP-like Immunostaining



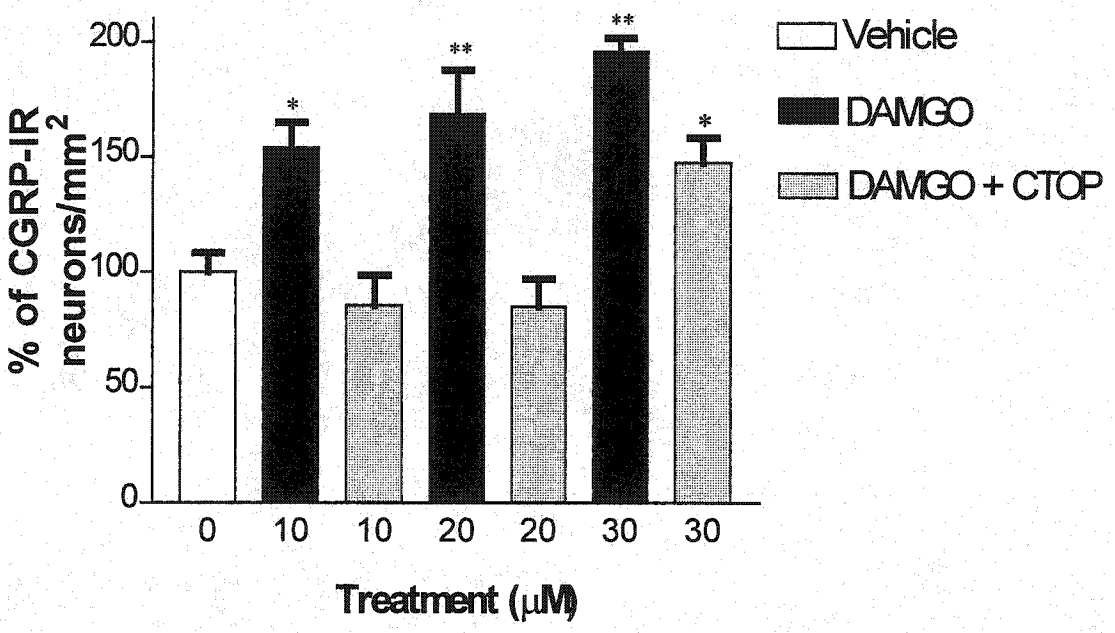


Figure 2. Cells in DRG cultures expressing CGRP-IR following exposure to DAMGO and the effect of a co-treatment with CTOP.

Mean number (±SEM, 4 different wells, repeated 3 times) of CGRP-IR neurons per 1 mm^2 in cultured DRG. Following a DAMGO treatment (10, 20 and 30 μM) for 6 days, the mean number of CGRP-IR neurons was significantly increased when compared to control. Similarly, following a DAMGO treatment (10, 20 and 30 μM) plus CTOP (10, 20 and 30 μM) treatment for 6 days, the mean number of CGRP-IR neurons was significantly different from the DAMGO-treated cultures. *$P<0.05$; **$P<0.01$, comparison between treatment and vehicle-treated controls.

concomitantly with DAMGO (both at the same concentrations), the increases in CGRP-IR neurons were significantly blocked. A co-treatment with CTOP and DAMGO blocked the stimulatory effect of DAMGO on the increases of CGRP-IR neurons in the DRG culture

A treatment with 10, 20 and 30 μM DPDPE (δ-opioid agonist) also lead to increases in CGRP-IR neurons in DRG cultures (Figure 3). When the δ-opioid antagonist, naltrindole, was administered concomitantly with DPDPE (both at the same concentrations), the increases in CGRP-IR neurons were attenuated to a level similar to control. A co-treatment with naltrindole and DPDPE was able to block the stimulatory effect of the δ-opioid agonist on the number of CGRP-IR neurons in DRG cultures.

Similar concentration-dependent increases in CGRP-IR were also observed following a repetitive U50, 488H (κ-opioid agonist) treatment (Figure 4). Large increases in CGRP-IR neurons were seen following 10, 20 and 30 μM treatments with U50, 488H compared to the vehicle. These increases in CGRP-IR were blocked when DRG cultures were treated with both U50, 488H and n-BNI (the κ-opioid antagonist).

b) SP-IR Neurons

SP-IR neurons were also present in vehicle- and opioid agonists-treated DRG cultures. A greater number of SP-IR neurons were observed following repetitive treatments with the different opioid agonists (DAMGO, DPDPE and U50, 488H) (Figure 5). Quantitatively, when treated with concentrations of 10, 20 and 30 μM of the opioid agonists, there was a concentration-dependent increase in the relative number of SP-IR neurons in DRG cultures which was significantly higher than that seen in vehicle treated DRG neurons.

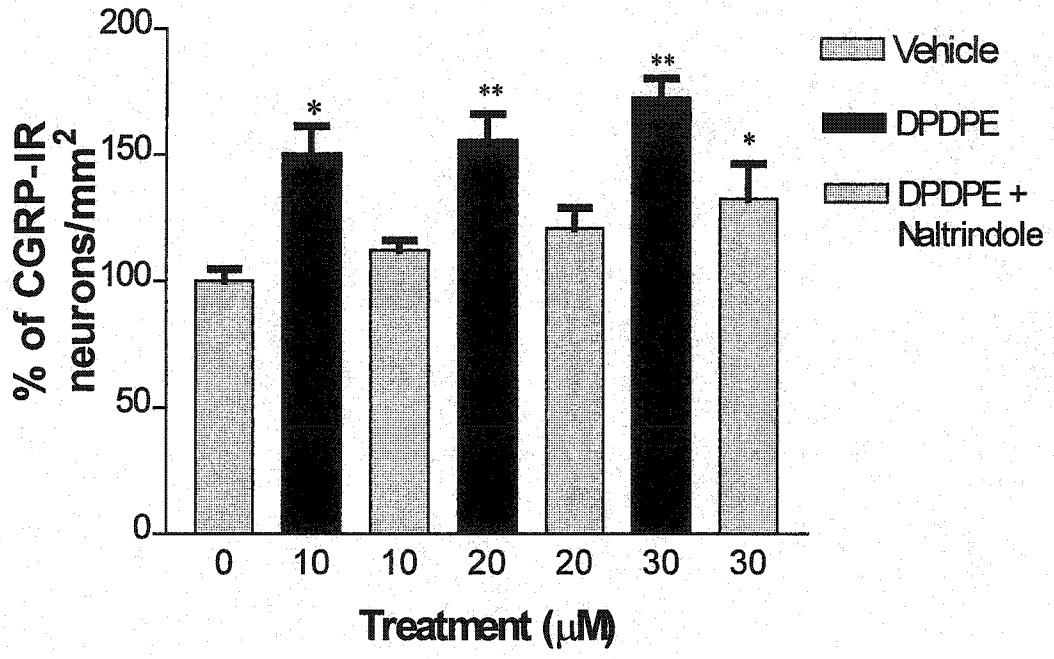


Figure 3. Cells in DRG cultures expressing CGRP-IR following exposure to DPDPE and the effect of a co-treatment with naltrindole.

Mean number (±SEM, 4 different wells, repeated 3 times) of CGRP-IR neurons per 1 mm^2 in cultured DRG. Following a DPDPE treatment (10, 20 and 30 μM) for 6 days, the mean number of CGRP-IR neurons was significantly increased when compared to control. Similarly, following a DPDPE treatment (10, 20 and 30 μM) plus naltrindole (10, 20 and 30 μM) treatment for 6 days, the mean number of CGRP-IR neurons was significantly different from the DPDPE-treated cultures. *$P<0.05$; **$P<0.01$, comparison between treatment and vehicle-treated controls.

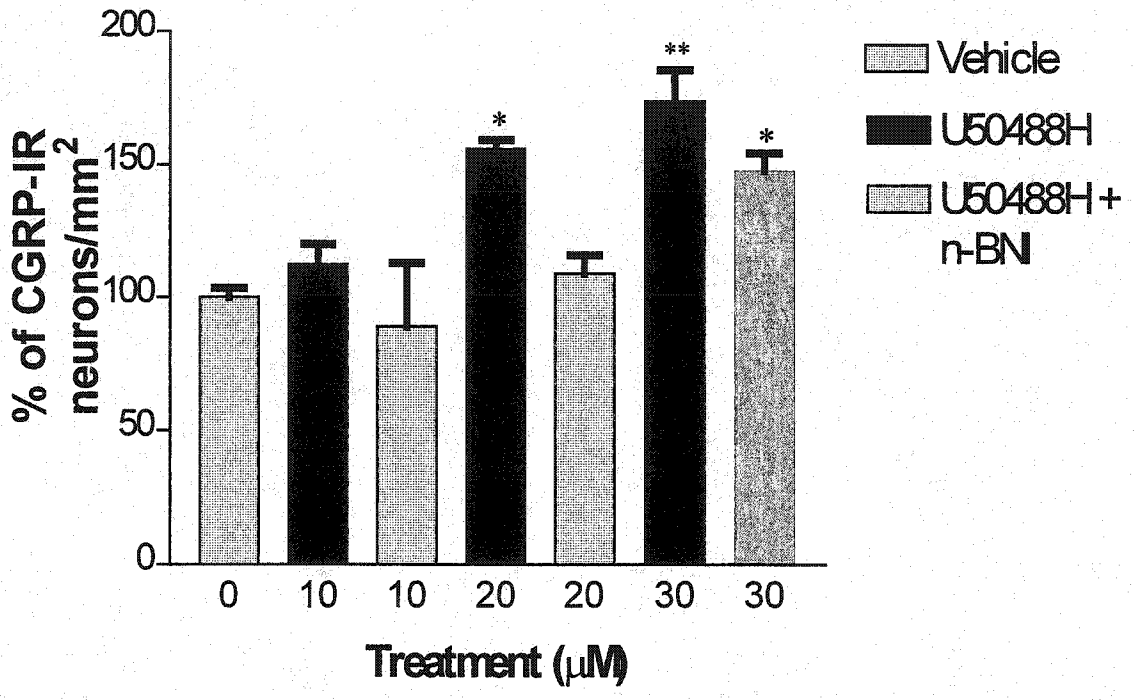


Figure 4. Cells in DRG cultures expressing CGRP-IR following exposure to U50488H and the effect of a co-treatment with n-BNI.

Mean number (±SEM, 4 different wells, repeated 3 times) of CGRP-IR neurons per 1 mm^2 in cultured DRG. Following a U50488H treatment (10, 20 and 30 μM) for 6 days, the mean number of CGRP-IR neurons was significantly increased when compared to control. Similarly, following a U50488H treatment (10, 20 and 30 μM) plus n-BNI (10, 20 and 30 μM) treatment for 6 days, the mean number of CGRP-IR neurons was significantly different from the U50488H-treated cultures. *$P<0.05$; **$P<0.01$, comparison between treatment and vehicle-treated controls.

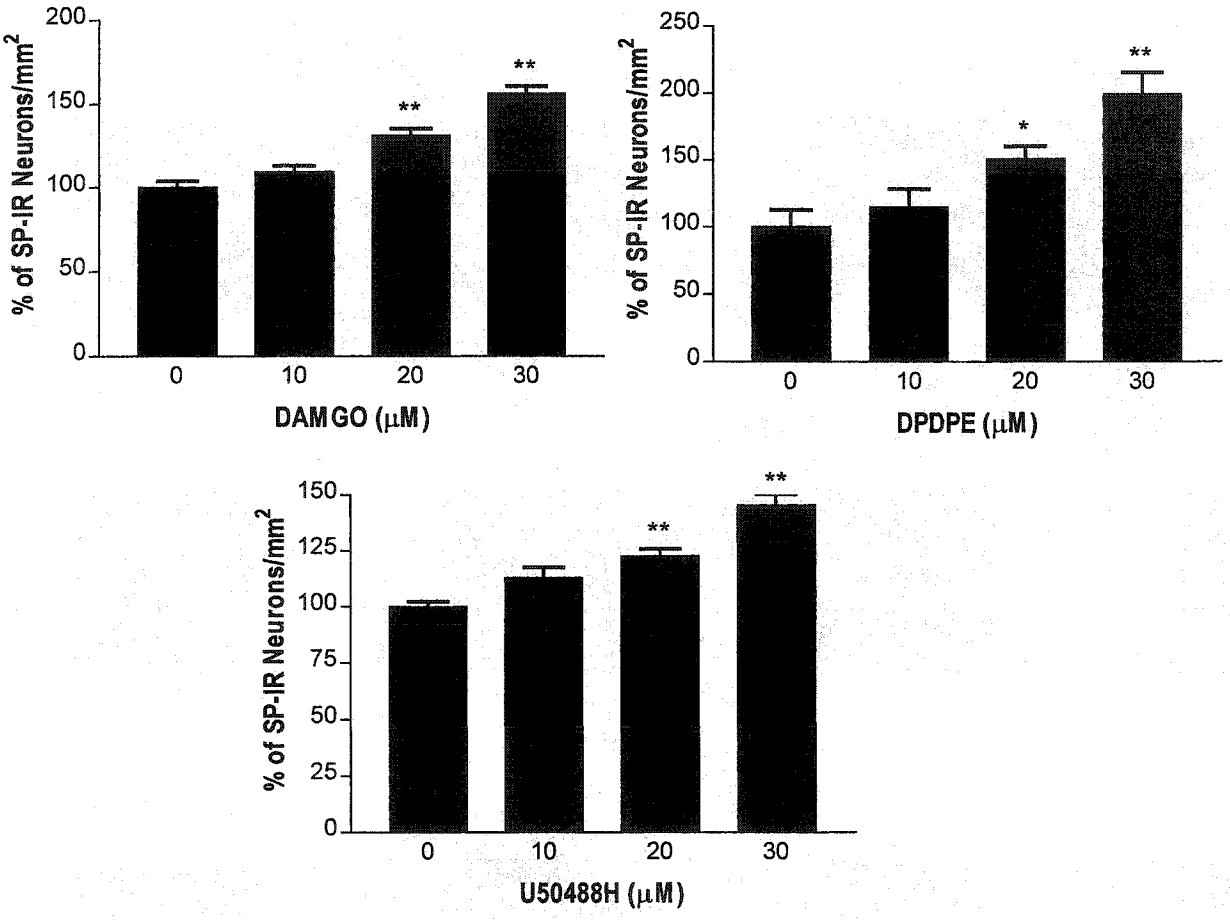


Figure 5. Cells in DRG cultures expressing SP-IR following exposure to opioid agonists.

Mean number (±SEM, 4 different wells, repeated 3 times) of SP-IR neurons per 1 mm^2 in cultured DRG. Following treatment with one of the three opioid agonists (μ: DAMGO, δ: DPDPE and κ: U50488H; 10, 20 and 30 μM) for 6 days, the mean number of SP-IR cultured DRG neurons was significantly increased when compared with controls (*$P<0.05$, **$P<0.01$). In some cases, no significant differences were seen at the lower concentration (10 μM).

c) NPY-IR Neurons

NPY-IR neurons were also present in vehicle- and opiate-treated DRG cultures. However, the number of NPY-IR neurons seen in opiate-treated (DAMGO, DPDPE and U50, 488H) cultures were not significantly different from that of NPY-IR neurons observed in vehicle-treated DRG cultures (Figure 6).

d) Galanin-IR Neurons

Following repetitive opioid-agonist (DAMGO, DPDPE and U50, 488H) exposure, the number of galanin-IR neurons was examined in vehicle- and opiate-treated DRG neurons (Figure 7). There were no significant differences between vehicle and treated groups.

B) Colocalization of CGRP and SP-IR and opioid receptors

1. Double Immunofluorescence Staining

DRG cultures treated repetitively with an opioid agonist (30 μM; DAMGO or DPDPE or U50, 488H) were double-immunostained using specific antibodies for CGRP or SP and the various opioid receptors (μ, δ and κ). In accordance with the results stated above, the number of both CGRP- and SP-IR neurons were remarkably increased post-treatment.

a) μ-Opioid Receptor

The majority of CGRP-IR neurons co-expressed the μ-opioid receptor (μ-OR), whereas only a few of the μ-OR-IR neurons were CGRP positive (Figure 8). Both CGRP-IR and μ-OR-IR neurons were found throughout the cultured neurons with low staining in the nucleus and as expected higher staining in the cytoplasm and at the plasma membrane. Similar results were observed for SP. Most SP-IR neurons co-expressed μ-OR while only a fraction of μ-OR-IR neurons expressed SP.

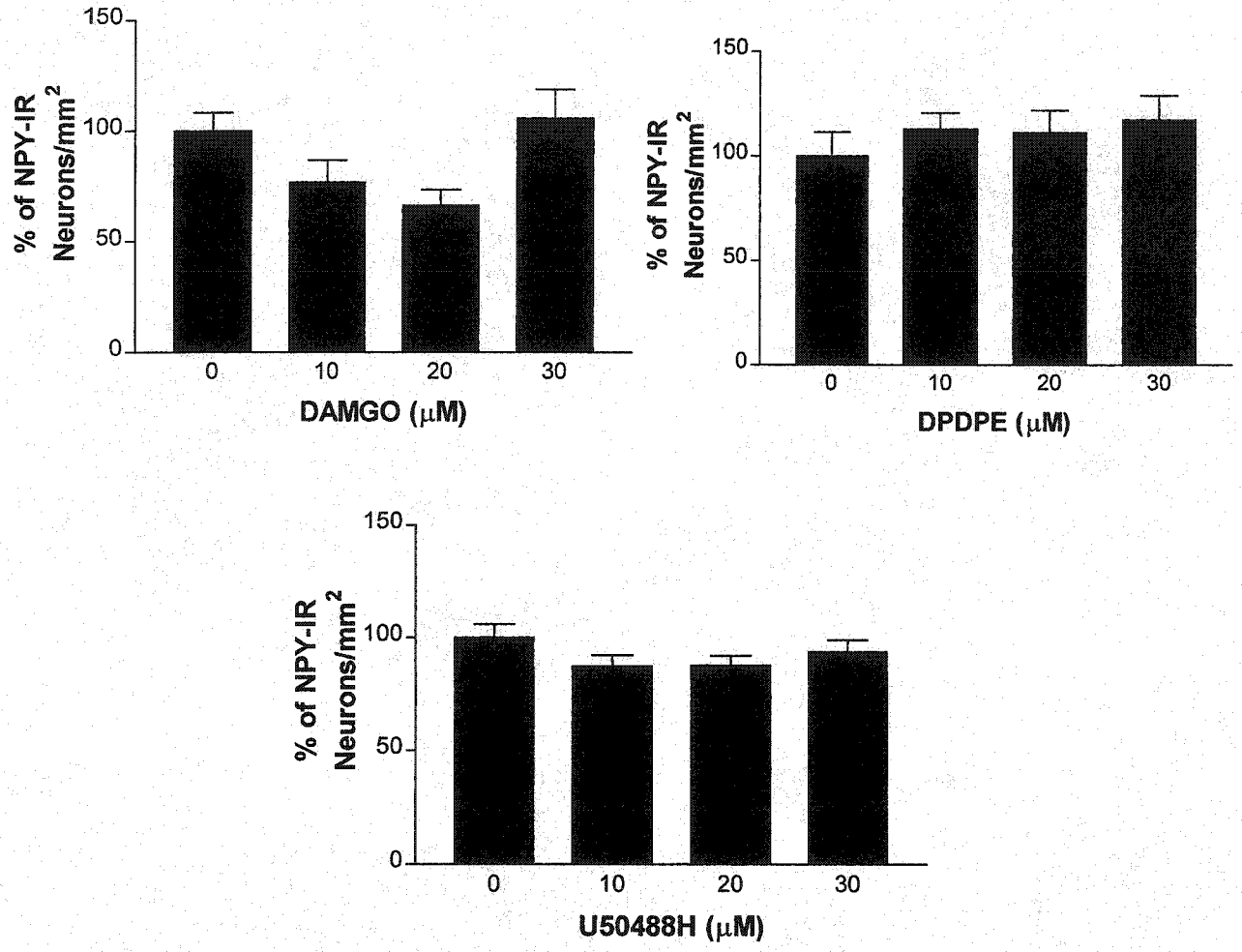


Figure 6. Cells in DRG cultures expressing NPY-IR following exposure to opioid agonists.

Mean number (±SEM, 4 different wells, repeated 3 times) of NPY-IR neurons per 1 mm^2 in cultured DRG. Following treatment with one of the three opioid agonists (μ: DAMGO, δ: DPDPE and κ: U50488H; 10, 20 and 30 μM) for 6 days, the mean number of NPY-IR cultured DRG neurons was not significantly increased when compared with controls.

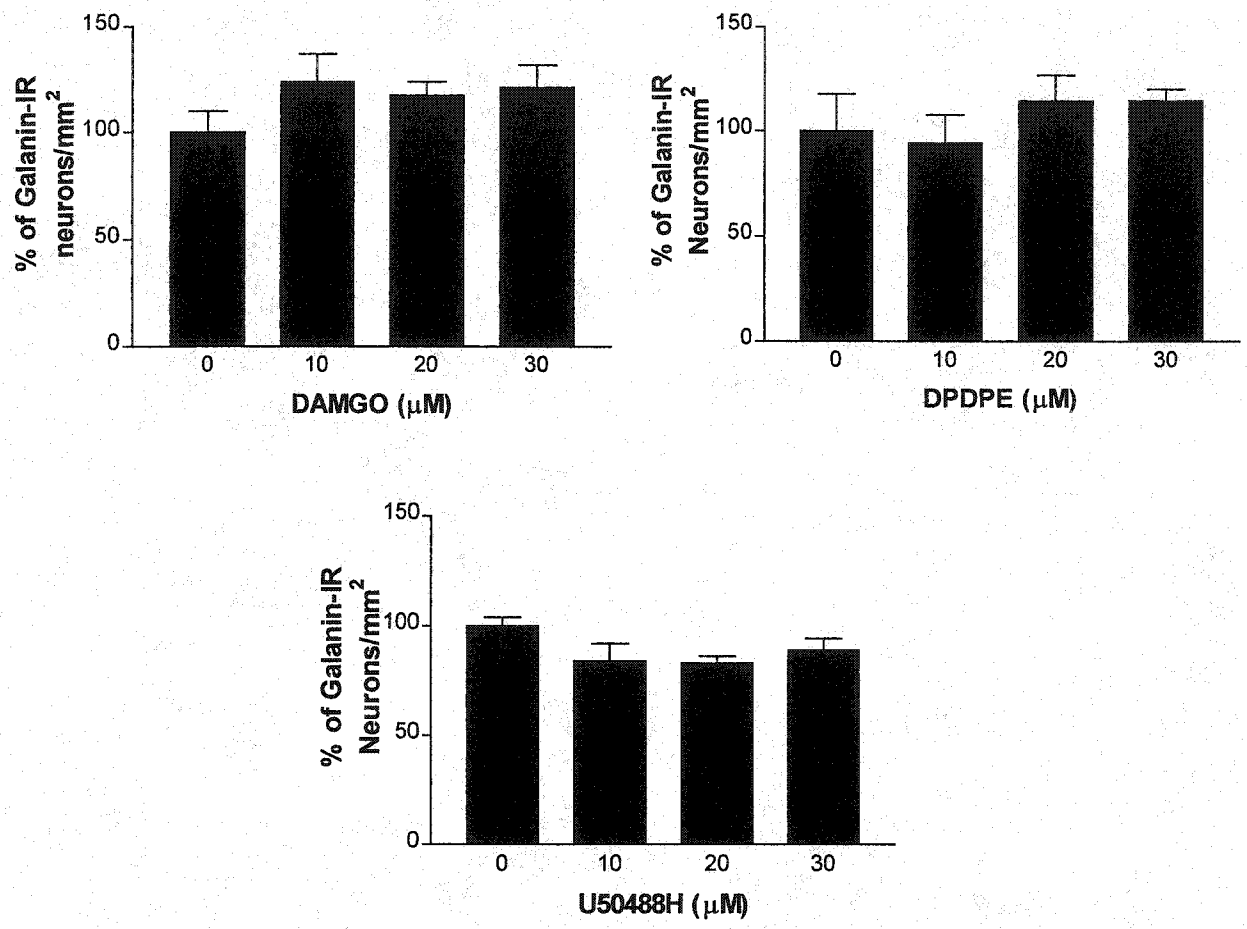


Figure 7. Cells in DRG cultures expressing galanin-IR following exposure to opioid agonists.

Mean number (±SEM, 4 different wells, repeated 3 times) of galanin-IR neurons per 1 mm^2 in cultured DRG. Following treatment with one of the three opioid agonists (μ: DAMGO, δ: DPDPE and κ: U50488H; 10, 20 and 30 μM) for 6 days, the mean number of galanin-IR cultured DRG neurons was not significantly increased when compared with controls.

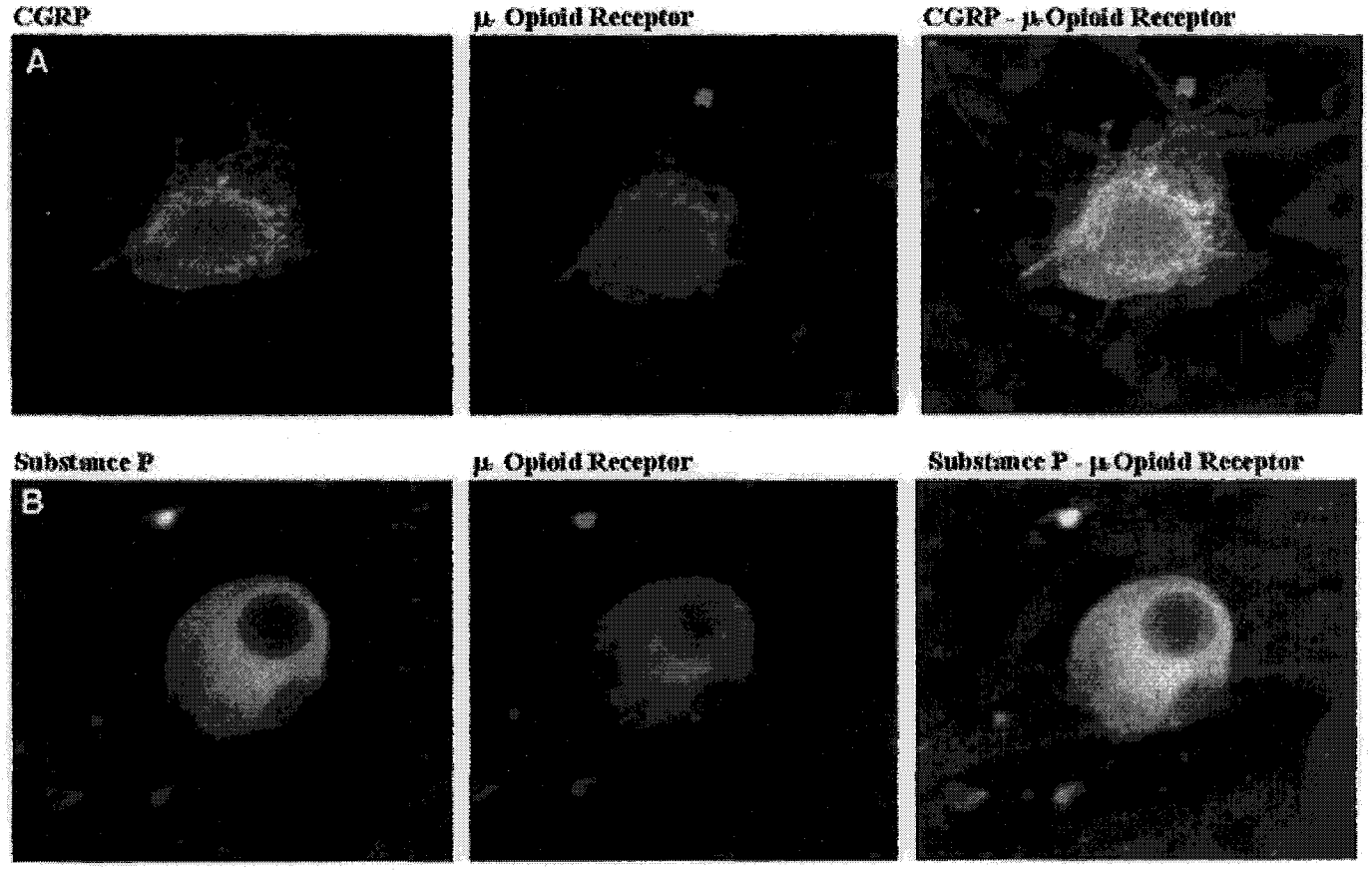


Figure 8. Cultured DRG neurons expressing CGRP-, SP- and the mu-opioid receptor- like IR.

Previous page:

Figure 8. Cultured DRG neurons expressing CGRP-, SP- and the μ-opioid receptor- like IR.

Color confocal photomicrographs of double immunofluorescence staining of cultured DRG neurons expressing CGRP (green)-, SP (green)- and μ-OR-IR in DAMGO-treated (30 μM) cells.

b) δ-Opioid Receptor

DRG cultures treated with DPDPE (30 μM) for 6 days demonstrated CGRP-IR, SP-IR and δ-opioid receptor-IR when immunostained with specific antibodies (Figure 9). The majority of CGRP-IR neurons δ-OR-immunopositive and vice-versa. A similar distribution of peptides and receptors was seen, the neuropeptides being absent from the nucleus but evenly distributed throughout the cytoplasm, while the δ-OR-IR seemed to be present throughout the cell but with lower intensity in the nucleus. SP-IR neurons also coexpressed δ-OR, while the majority of δ-OR-IR neurons were also SP-IR immunopositive.

Following page:

Figure 9. Cultured DRG neurons expressing CGRP-, SP- and the δ-opioid receptor-like IR.

Color confocal photomicrographs of double immunofluorescence staining of cultured DRG neurons expressing CGRP (green)-, SP (green)- and δ-OR-IR in DPDPE-treated (30 μM) cells. Note that almost all CGRP-IR and SP-IR neurons colocalized with μ-OR-IR and vice-versa.

c) κ-Opioid Receptor

Following treatment with U50, 488H (30 μM), DRG cultures were investigated for the colocalization of the κ-opioid receptor (κ-OR) with CGRP and SP (Figure 10). In accordance with previous results, most CGRP- and SP-IR neurons expressed the κ-OR and vice-versa. The distribution of the κ-OR was

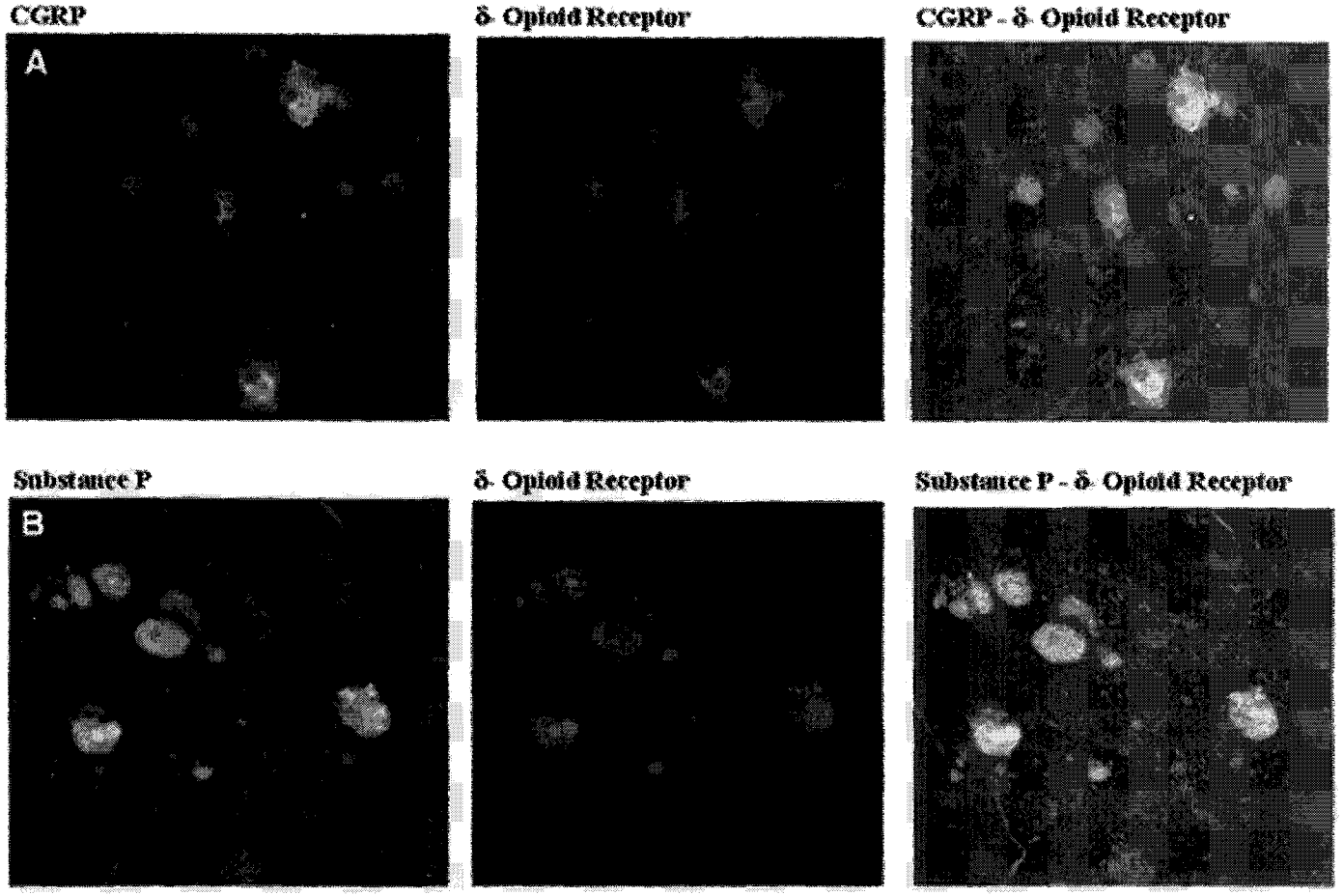


Figure 9. Cultured DRG neurons expressing CGRP-, SP- and the delta-opioid receptor-like IR.

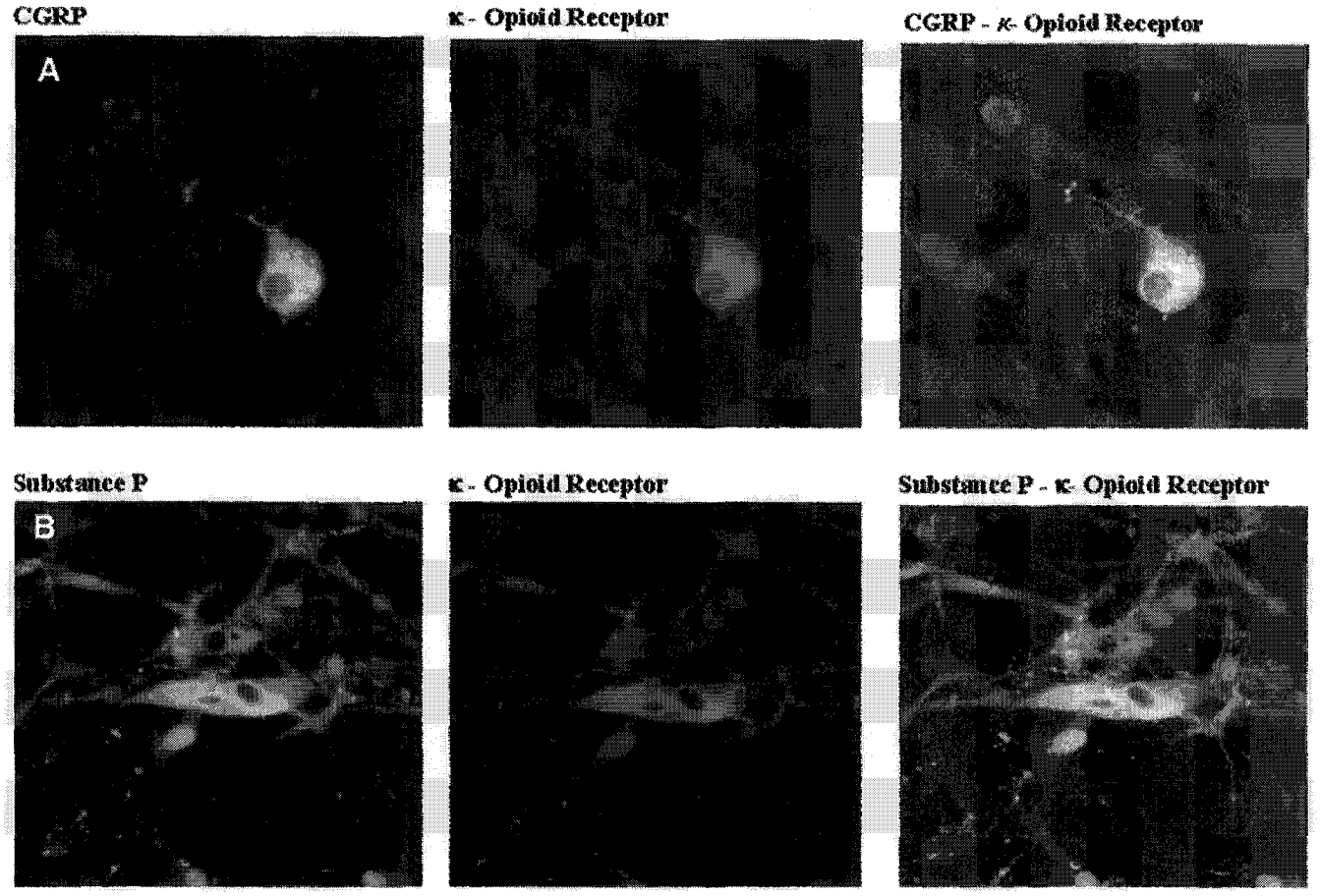


Figure 10. Cultured DRG neurons expressing CGRP-, SP- and the kappa-opioid receptor-like IR.

similar to that of other opioid receptors with staining throughout the neuron but at a lower concentration within the nucleus.

Previous page:

Figure 10. Cultured DRG neurons expressing CGRP-, SP- and the κ-opioid receptor-like IR.

Color confocal photomicrographs of double immunofluorescence staining of cultured DRG neurons expressing CGRP (green)-, SP (green)- and κ-OR-IR in U50488H-treated (30 μM) cells. Note that almost all CGRP-IR and SP-IR neurons colocalized with κ-OR-IR and vice-versa.

C) PROTEIN KINASE C (PKC)-IR, OPIOID EFFECTS AND CGRP AND SP STAINING

1. Double Immunofluorescence Staining

DRG cultures treated with DAMGO (20 μM) for 6 days were double-immunostained using specific CGRP, SP and PKCα antibodies (Figure 11). Both the numbers of CGRP- and PKCα-IR neurons were increased post-treatment. The majority of CGRP-IR neurons co-expressed PKCα-IR and vice-versa. Similar results were obtained with substance P. The lower panel of the figure shows the colocalization of two different antibodies to PKCα, one directed against rat PKCα (PKC_R) while the other is directed against the mouse enzyme (PKC_M). Both antibodies show same level of staining.

Following page:

Figure 11. Cultured DRG neurons expressing CGRP-, SP and PKCα-like IR.

Color confocal photomicrographs of double immunofluorescence staining of cultured DRG neurons expressing CGRP (green)-, SP (green)- and PKCα-IR in DAMGO-treated (30 μM) cells. Note that almost all CGRP-IR and SP-IR neurons colocalized with PKCα-IR and vice-versa. The lower panel of the

Figure 11. Colocalization of PKC with pain-related neuropeptides

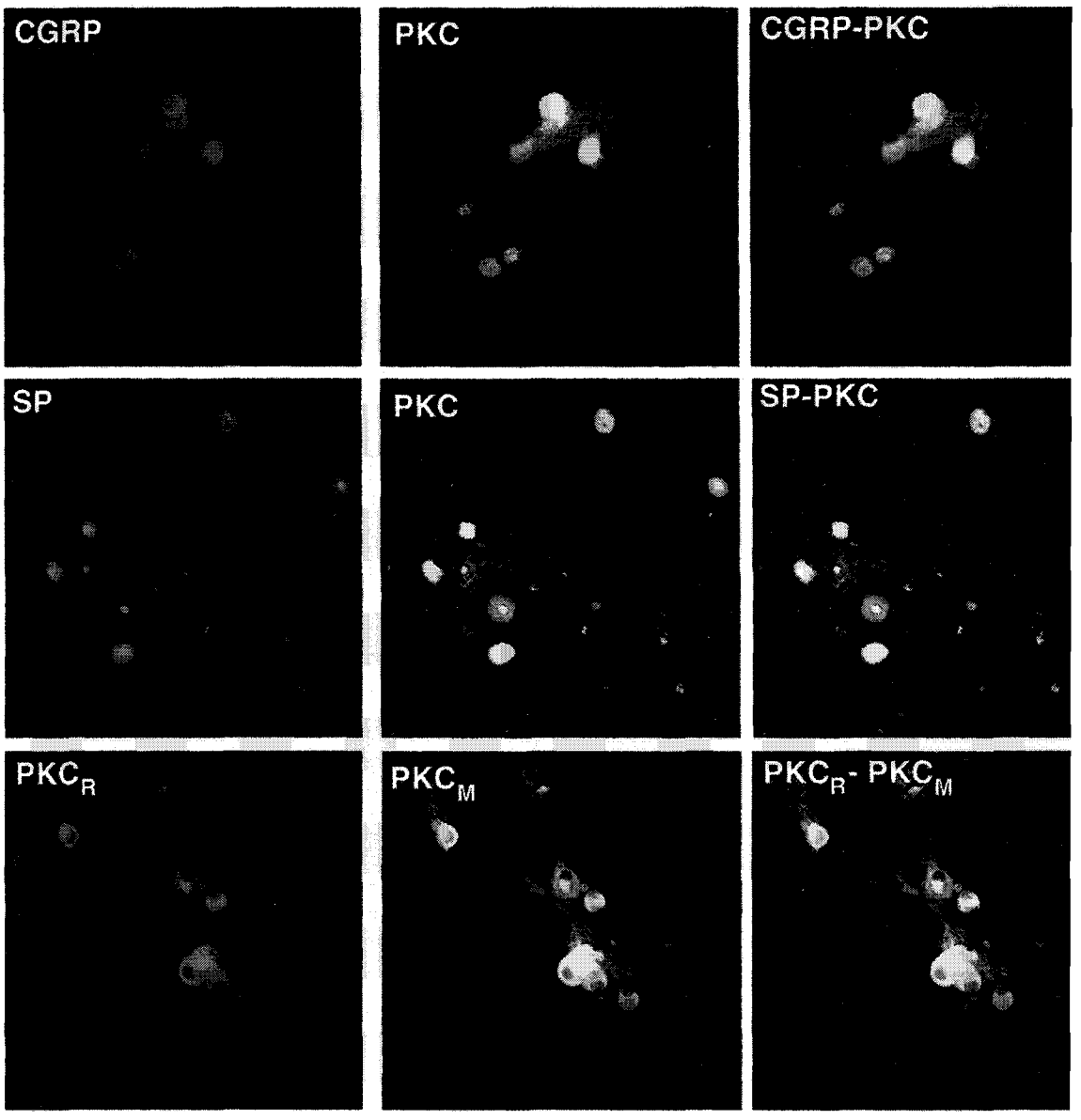


figure shows the colocalization of two different PKCα antibodies from different species (rat; PKC_R, mouse;PKC_M).

2. Immunocytochemistry

a) PKCα-IR Neurons

Many PKCα-IR neurons were observed in vehicle and DAMGO-treated DRG cultures. A greater amount of PKCα-IR neurons were quantitated following repetitive treatment with μ agonist (10, 20 and 30 μM) for 6 days. There was a significant concentration-dependent increase in PKCα-IR neurons following treatment compared to vehicle-treated DRG cultured neurons (Figure 12).

b) CGRP-IR following PKC inhibition

Following a co-treatment with the protein kinase C inhibitors chelerythrine chloride or GO6976 (5 μM) and DAMGO (30 μM) for 6 days, CGRP-IR neurons were quantitated (Figure 13). As previously, there was a significant increase in CGRP-IR neurons when DRG neurons were treated with 30 μM DAMGO. PKC inhibitors blocked the effects of DAMGO on CGRP-IR. Chelerythrine chloride (5 μM) blocked the stimulatory effect of DAMGO on the number of CGRP-IR neurons. GO6976 (5 μM) had a weaker blocking effect than chelerythrine chloride in inhibiting the increases in CGRP-IR neurons (Figure 13). The increase in CGRP-IR following inhibition with GO6976 became non-significant compared to control showing that the PKC inhibitor had an inhibitory effect on the amount of CGRP-IR neurons in DRG culture treated with DAMGO.

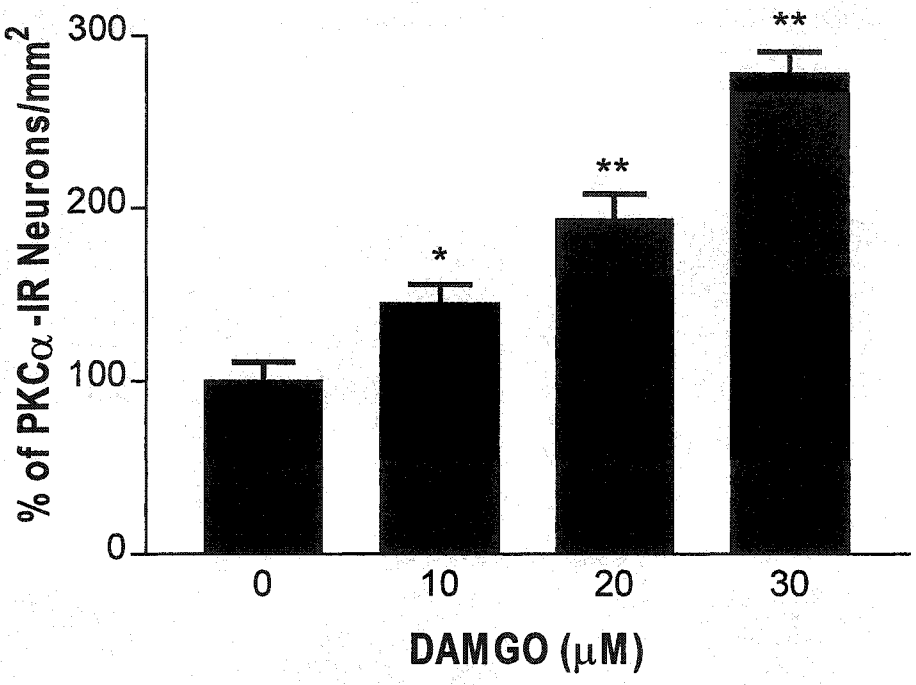
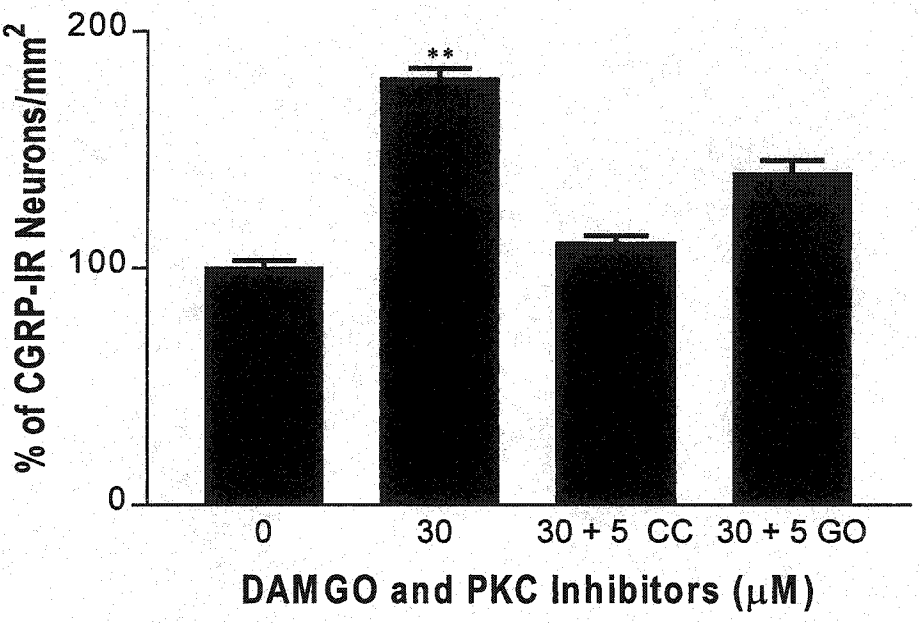


Figure 12. Cells in DRG cultures expressing PKCα-IR following exposure to DAMGO.

Mean number (±SEM, 4 different wells, repeated 3 times) of PKCα-IR neurons per 1 mm^2 in cultured DRG. Following a DAMGO treatment (10, 20 and 30 μM) for 6 days, the mean number of PKCα-IR neurons was significantly increased when compared to control (*P<0.05, **P<0.01).



CC: chelerythrine chloride – inhibits all PKC subtypes

GO: GO6976 – inhibits PKC β, γ and ε subtypes

Figure 13. Cells in DRG cultures expressing CGRP-IR following exposure to DAMGO and PKC inhibitors. Mean number (±SEM, 4 different wells, repeated 3 times) of CGRP-IR neurons per 1 mm^2 in cultured DRG. Following a DAMGO treatment (30 μM) for 6 days, the mean number of CGRP-IR neurons was significantly increased when compared to control. The concomitant use of PKC inhibitors (GO6976 and chelerythrine chloride) with DAMGO inhibited the increase in CGRP-IR neurons that is observed following exposure to morphine.

CHAPTER 4 - DISCUSSION

There is accumulating evidence that the neuropeptide CGRP plays a prominent role in nociceptive mechanisms. For example, CGRP is found in areas known to be important to sensory processes (Skofitsch *et al.*, 1985; Franco-Cereceda *et al.*, 1987; Wimalawansa *et al.*, 1987; Lawson *et al.*, 1995) such as 40% of the sensory neurons of the dorsal root ganglia and in both peripheral Aδ and C fibers of the primary afferent nerves of the spinal cord. CGRP has also been found to be colocalized with substance P, another important nociceptive neuropeptide, in dorsal root ganglia (Wiesenfeld-Hallin *et al.*, 1984; Lee *et al.*, 1985) and superficial dorsal horn (Plenderleith *et al.*, 1990; Ribeiro-da-Silva, 1995). In acute pain models, Menard *et al.* (1995a) have reported a modulation of CGRP binding sites and CGRP-like immunoreactivity following the development of tolerance to morphine. In addition, the CGRP antagonist, $CGRP_{8-37}$ was shown to prevent the development of tolerance to morphine as well as the modulation of CGRP binding sites and immunoreactivity that are usually seen in tolerant animals (Menard *et al.*, 1996). More recently BIBN4096BS, a potent non-peptide antagonist of the CGRP receptor was similarly shown to block the development of tolerance to the antinociceptive effect of morphine (Powell *et al.*, 2000). Using a CGRP-deficient mice, Salmon *et al.*, (2001) were able to show that the mice lacking CGRP displayed an attenuated response to both chemical pain and inflammation. Using a DRG cell culture and an *in vitro* treatment with morphine, Ma *et al.* (2000) were able to mimic these *in vivo* findings. Overall these data indicate that CGRP likely plays a prominent role in the production and transmission of pain signals. These results help to validate the DRG cell culture model as a simple *in vitro* approach to investigate intracellular pathways that lead to opioid modulation of CGRP and SP expression and their possible role on the development of tolerance to morphine's antinociceptive effects.

The main aim of this thesis was to establish which subtype of opioid receptors are involved in the induction of CGRP and SP in cultured DRG neurons. It has been reported that the μ-, δ- and κ-opioid receptor mRNAs are expressed in DRG neurons (Maekawa *et al.*, 1994; Minami *et al.*, 1995) and colocalized with preprotachykinin α/SP mRNA in the rat DRG (Minami *et al.*, 1995). Moreover, μ-opioid receptor immunoreactivity has been shown to be colocalized with CGRP and SP immunoreactivities in primary sensory neurons (Li *et al.*, 1998). Additional neuropeptides believed to be involved in nociception but not in morphine tolerance, such as galanin and neuropeptide Y, were also studied to assess the specificity of the changes observed with CGRP and SP. A second aim of this thesis was to further validate the DRG cell culture model as a representative *in vitro* model that expresses CGRP, SP and the three opioid receptors so that it is suitable to investigate further the various intracellular events that are triggered by chronic morphine exposure. Another goal of this thesis was to utilize the DRG cell culture model to begin studying the possible role of protein kinase C (PKC) in the development of morphine tolerance and its implication in the upregulation of CGRP and SP. Activation of PKC by Ca^{2+} and diacylglycerol (DAG) involves the translocation of the enzyme from the cytosol to the plasma membrane (Hug *et al.*, 1993). PKC phosphorylates substrate proteins that can contribute to cellular processes such as neurotransmitter release and transduction (Nishizuka, 1986). It was previously shown that there was an increase in membrane-associated PKC in the spinal cord in response to nerve constriction injury (Mao *et al.*, 1992b, 1993). Earlier *in vivo* data also suggested that PKC plays a role in both neuropathic pain (Malmberg *et al.*, 1997) as well as in morphine tolerance (Narita *et al.*, 1994a, 1994b; Mayer *et al.*, 1995). In these studies, the authors were able to show that PKC activity was increased following chronic morphine administration (Narita *et al.*, 1994b) and that the use of H-7, a PKC inhibitor, was able to inhibit the development of morphine tolerance (Narita *et al.*, 1994a).

A. NEUROPEPTIDE EXPRESSION IN DRG CULTURES TREATED WITH VARIOUS OPIOID AGONISTS

It was previously demonstrated that repetitive morphine exposure induced increases of CGRP-IR and SP-IR neurons in DRG cultures (Ma *et al.*, 2000), extending earlier *in vivo* findings that CGRP-like immunoreactivity was increased in the dorsal horn of morphine-tolerant rats (Menard *et al.*, 1995a, 1995b, 1996). In these studies, the number of NPY- and GAL-IR neurons was not modified in morphine treated DRG cultures or animals, suggesting the specificity of morphine induction for CGRP and SP. More recently, it has been shown that the exposure of DRG cultures to the CGRP antagonist, $CGRP_{8-37}$, was able to block the effect of morphine on CGRP-IR increases (Powell *et al.*, 2001). These results are in accordance with our previous *in vivo* results and also demonstrate that CGRP receptors are present in DRG cultures. In the present study, it was shown that all three opioid receptors could be involved in the induction of CGRP and SP occurring following repetitive opiate exposure. There was significant increases (up to 100%) of CGRP-IR neurons in DRG cultures upon repetitive treatment for 6 days with different concentrations of various opioid receptor agonists. DAMGO (μ), DPDPE (δ) and U50, 488H (κ) all induced concentration-dependent increases in the level of CGRP-IR neurons. For reasons yet still undetermined, neuronal primary cell cultures require massive doses when treated with peptides (Mount *et al.*, 1991; Ma *et al.*, 2000). Since large doses of agonists were required to elicit the increases in CGRP, it was suspected that the increase in CGRP-IR might not be due to the unique activation of a single opioid receptor sub-type. Hence, a concomitant treatment with their respective antagonist was used to show that the modulation of the neuropeptide was not due to cross-activation of multiple receptor sub-types. Indeed, the significant increases in CGRP-IR neurons induced by the specific opioid agonists was blocked by each specific sub-type antagonists

(CTOP (μ), naltrindole (δ) and n-BNI (κ)), indicating the respective involvement of each of the three opioid receptors (μ, δ and κ) in the observed increase in CGRP-IR.

The repetitive treatment with each specific opioid agonist not only induced concentration-dependent increases in CGRP-like IR but also in SP-like IR in cultured DRG neurons. Similar results were reported earlier using morphine (Ma *et al.*, 2000). SP has been the focus of much interest in studying the mechanisms possibly involved in the development of tolerance to morphine because of its well established role in nociceptive transmission in the spinal dorsal horn (Henry, 1976, Krause *et al.*, 1995) and in functional synergistic effects with CGRP in primary afferents in the dorsal horn (Woolf *et al.*, 1986). In the *in vivo* studies where morphine tolerance was induced through a continuous intrathecal infusion of morphine for 7 days, non-significant changes in SP related markers (immunoreactivity and binding sites) were observed (Menard *et al.*, 1995a, 1995b). Moreover, it was recently reported that a selective non-peptide neurokinin-1 receptor antagonist, SR 14033, significantly blocked the development of morphine tolerance (Powell *et al.*, 2000b). These *in vivo* and *in vitro* results suggest that as for CGRP, SP may play a role in the development of tolerance to opioids. The present findings that all three opioid receptors may induce the production of SP in DRG neurons further confirm that all three opioid receptors induce similar neuropeptide changes in DRG neurons following repetitive opiate exposure.

In accordance with previous *in vivo* and *in vitro* studies, GAL and NPY-IR neurons were not affected when the DRG cultures were subjected to repetitive opioid agonist treatment establishing further the specificity of opioids induction of CGRP and SP in our model.

B. VALIDATION OF THE DORSAL ROOT GANLION CELL CULTURE MODEL

An important objective of our studies was to further validate the DRG cell culture model that would permit, in future projects, the precise dissection of the cellular and molecular mechanisms involved in morphine induction of CGRP and SP expression following repetitive exposure. DRG cultures have previously been performed using ganglia from rat embryos where it was possible to identify and characterize all three opioid receptors (Vaysee *et al.*, 1990; Chen *et al.*, 1997). Ma *et al.* (2000) previously showed that post-natal cultured DRG neurons expressed CGRP, SP and opioid receptors by demonstrating that repeated morphine exposure modulated levels of CGRP and SP in DRG cultured neurons. The use of primary cultures of sensory neurons as a model system has an advantage in that a relatively homogeneous population of nondividing neuronal cells is used and are functionally important in mediating opioid analgesia at the level of the spinal cord. In order to show that all three opioid receptors are present in DRG cultured neurons and that each of them is able to modulate CGRP- and SP-immunoreactivity, it was critical to demonstrate that each opioid receptor subtype was indeed expressed in the DRG cultured neurons under our assay conditions and that they were at least partly colocalized with CGRP-/SP-IR neurons.

Double immunostaining studies showed that all three opioid receptors are present in DRG neurons and that they are colocalized with both CGRP- and SP-like IR cells. These data are among the first evidence for the presence and distribution of all three opioid receptor-like immunoreactivity in cultured DRG neurons. These opioid receptors have an almost identical distribution in DRG neurons however the μ-opioid receptor seems to be more abundant than the other two receptors. Most CGRP- and SP-IR neurons co-expressed the μ-OR-IR while only a fraction of the μ-OR-IR neurons were positive for the immunoreactivity of the neuropeptides.

Accordingly, DRG neurons in culture express CGRP, SP and all three subtypes of opioid receptors. Thus, they represent a suitable model to study cellular and molecular mechanisms that are involved in the opioid modulation of CGRP and SP expression following repetitive exposure.

C. POSSIBLE IMPLICATION OF PROTEIN KINASE C IN THE MODULATION BY OPIOIDS OF CGRP AND SP

There is evidence to suggest that PKC is involved in pain transmission at the spinal level. The PKC family of serine/threonine kinases is composed of at least 10 isoforms (α, β1, βII, γ, δ, ε, ξ, η, θ and λ; Nishizuka, 1992). All PKC isoforms exist in an inactive state within the cytosol and become translocated to the plasma membrane by various stimuli, including activation of G protein-coupled receptors (Nishizuka, 1992). There are controversies as to which isozymes are present in specific brain regions. Nevertheless immunocytochemical results seem to indicate that most PKC isozymes are present in the superficial layers of the dorsal horn (Mori *et al.*, 1990; Malmberg *et al.*, 1997). Thus far, PKC isozymes α, βI, βII and γ have been found in high concentrations in the superficial laminae of the dorsal horn (Martin *et al.*, 1999). All PKC isozymes except for γ have been identified in dorsal root ganglions. Their presence in the spinal cord is an indication that they could play a role in the processing of pain. Both pharmacological and anatomical studies have shown that the translocation and activation of most PKC enzymes in neurons of the spinal cord dorsal horn are involved in persistent pain produced by chemical stimulation such as formalin and mechanical hyperalgesia (Yashpal *et al.*, 1995). Furthermore, intrathecal injection of phorbol ester, a non-specific PKC activator, in awake rats evoked mechanical allodynia and thermal hyperalgesia (Palecek *et al.*, 1999). Moreover, following nerve injury (Coderre, 1992; Mao *et al.*, 1992) and inflammation (Martin *et al.*,

1999), PKC is activated in dorsal horn neurons. Also, the inhibition of spinal PKC reduced nerve injury-induced tactile allodynia in neuropathic rats (Hua *et al.*, 1999). Similarly, an intrathecal pretreatment of mice with the specific inhibitor, calphostin C, prevented the development and expression of acute antinociceptive tolerance following repeated spinal applications of deltorphin-II (Narita *et al.*, 1996). The same group also showed that a repeated administration of rats with morphine enhanced PKC activity in the brain with a time course similar to the development of tolerance to morphine (Narita *et al.*, 1994b, 1994c). Only a few specific PKC enzymes have been examined. PKCγ was shown to be potentially involved in neuropathic pain and its immunoreactivity was increased in the dorsal horn following nerve injury (Mao *et al.*, 1995). In PKCγ null mice, pain responses, tissue swelling and plasma extravasation were attenutated in the formalin test (Malmberg *et al.*, 1997). PKCε has recently been found to be present in most DRG neurons of adult mice and rats (Khasar *et al.*, 1999). The same group used a PKCε null mice to show this isozyme was required for the full expression of carrageenan-induced hyperalgesia.

In the present study, the role of PKC in opioid-induced up-regulation of CGRP and SP was examined. Since PKCγ is absent in DRG (Malmberg *et al.*, 1997) only PKCα expression was examined in DRG cultures. First by using double-immunofluorescence staining, it was possible to replicate earlier results showing the colocalization of CGRP-IR and PKCα-IR in the majority of CGRP-positive neurons (Ma *et al.*, 2001) and to also demonstrate that SP-IR neurons also colocalized with PKCα-IR neurons in most SP-positive DRG neurons.

By establishing that the pain-related neuropeptides were colocalized with PKCα, we examined next if changes occurred in PKCα following repetitive opioid exposure. When the cultured DRG neurons were treated repeatedly with DAMGO, there was a concentration-dependent increase in PKCα-IR neurons.

Taken together, these results may be taken as an indication that upon chronic opioid exposure there is a concentration-dependent increase in PKC-IR and possibly PKC activity, although functional enzymatic studies will be required to establish this hypothesis.

To further examine the role that PKC could play in the induction of CGRP, it was found that the PKC inhibitors, chelerythrine chloride and GO6976 were able to reverse the effects of DAMGO on the induction of CGRP-like IR in cultured DRG neurons. The use of chelerythrine has important limitations since it inhibits all PKC isoforms and thus could be toxic to DRG neurons explaining the apparent decrease in CGRP-IR. However, GO6976, which only inhibits a subset of the PKC isoenzymes (α, β, μ and γ), blocked the increase in CGRP-IR providing additional evidence that various PKC isoenzymes are present in DRG neurons and are likely involved in the opioid induction of pain-related neuropeptides following chronic exposure.

Taken together, our results suggest that PKC signaling (likely of the α, β, μ or γ isoenzyme-type, according to the results obtained with GO6976) is involved in opioid- induction of CGRP-IR in cultured DRG neurons.

D. FUTURE STUDIES

Previous *in vivo* studies have shown that in parallel to the increase in CGRP-like immunoreactivity in the dorsal horn of morphine-tolerant rats, significant decreases in CGRP receptor binding sites in the spinal dorsal horn also occured. It is possible that the observed increases in CGRP levels in primary sensory afferent are due to inhibited release by chronic opiate exposure, a compensatory up-regulation in CGRP receptor levels is then observed. It is likely that a repetitive opiate treatment may produce increases in CGRP and SP synthesis. An *in situ* hybridization study would be useful to elucidate if CGRP and

preprotachykinin mRNAs are modulated by chronic opiate exposure in DRG cultured neurons and *in vivo*. The role of desensitization of opioid receptors upon agonist activation in relation to the regulation of neuropeptides should also be examined to shed further light on the mechanism and consequences of desensitization. It would be interesting to quantify the number of opioid receptors on the membrane either by staining or by binding prior and before the upregulation of neuropeptides.

Further studies examining this topic should aim at determining the intracellular events triggered by repetitive opiate exposure that lead to the upregulated expression of CGRP and SP. I have shown here that there is an increase in PKCα-IR and possibly activity upon opiate treatment. It would be interesting to look at the relation between PKCα-IR and its translocation and activity in the immunoreactive neurons. Similar studies could be done in the DRG to look at the influence of PKC on the levels of SP, galanin and NPY following chronic morphine exposure. A similar approach could be taken to study the role of desensitization of opioid receptors in the up-regulation of PKCα. It would also be worthwhile to establish which pathways are activated by PKC to eventually lead to the enhanced expression of pain-related neuropeptides. There are over 20 PKC isoenzymes reported in the literature, it would thus be important to identify and define the role of the key isoenzymes involved. This would also help delineate the relationships with the other signaling pathways affected by opiate receptors (i.e. MAPK, cAMP, CaM kinase) as well as examine which pathways have an effect on gene expression.

Many transcription factors, including cFos, Fos B and CREB have been located in the central nervous system and may be involved in pain processing. These transcription factors would be interesting to observe in the spinal cord and DRG under chronic opiate exposure. Immunohistochemical studies would be able to identify in which regions they are expressed and may play a role. It would then be worthwhile to search for target genes for these different

transcription factors. It would then be possible to use transcription factor expression as an index of gene regulation.

Once some pathways are defined, their importance could be clarified in *in vivo* experiments. We could determine if specific opiates or dosing schedules are more likely than others to cause changes in neuropeptide expression and signaling pathways. For example one could monitor animals for changes in opiate tolerance and in neuropeptide-immunoreactivity following acute and chronic exposure. Also, whether chronic morphine treatment could change the PKC activity in smaller and more anatomically defined brain regions is worth investigating. These studies could help determine which specific neuropeptides and signaling pathways play a role in hyperalgesia and allodynia in neuropathic states. This could help us in determining if there is a change in the population of C, Aδ or Aβ neurons and to investigate possible differences in morphological distribution of immunoreactive neurons following opiate treatment. Likewise, we could be able to determine if agents that limit or reverse tolerance to the analgesic effects of opioids do so by influencing transcription factor or neuropeptide expression. A parallel behavioral study could then help define the role of these neuropeptides and signal pathways in the occurrence of dependence and withdrawal.

CHAPTER 5- CONCLUSION

The DRG neuronal cell culture provides a simple, reliable *in vitro* model to study cellular and molecular mechanisms that could be involved in the development of morphine tolerance occurring *in vivo* following repeated exposure. My studies have shown that DRG neurons express different neuropeptides (CGRP, SP, NPY and galanin), the three main subtypes of opioid receptors (μ, δ and κ) and also different PKC isoenzymes.

Moreover, my data suggest that μ-, δ- and κ-opioid receptors could be involved in the opiate induction of CGRP and SP, likely via a PKC pathway. These findings further confirm previous *in vivo* and *in vitro* studies that CGRP immunoreactivity is markedly increased in the dorsal horn and dorsal root ganglia of rats chronically exposed to morphine. These results also lend support to the existence of an interaction between CGRP and the development of tolerance to the antinociceptive effects of opiates in the rat spinal cord.

Whereas the precise mechanisms responsible for the modulation of CGRP levels and its receptors remains to be established, these findings provide new research avenues and some understanding of changes that occur following repetitive exposure to opiates.

CHAPTER 6 - REFERENCES

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