INVESTIGATING THE EFFECTS OF PERIPHERAL NERVE INJURY ON δ OPIOID RECEPTOR EXPRESSION AND FUNCTION: IMPLICATIONS FOR THE TREATMENT OF CHRONIC NEUROPATHIC PAIN

by

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Abstract

Neuropathic (NP) pain is a debilitating chronic pain disorder that is a challenge to diagnose and an even greater challenge to treat. Commonly described as burning, shooting or shock-like, NP pain is characteristically resistant to traditional analgesic therapy. This thesis project aimed to investigate the potential therapeutic benefit of delta opioid receptor (δOR)-selective agonists in the management of NP pain. In the current experiments, rats that underwent unilateral sciatic nerve injury displayed characteristic behavioural manifestations including cold and thermal hyperalgesia (exaggerated nociceptive response to a noxious stimulus) as well as tactile allodynia (nociceptive response to an innocuous stimulus) in the ipsilateral hind paw. The spinal administration of DLT, a δOR-selective agonist, dose-dependently reversed tactile allodynia in NP rats and attenuated cold and thermal hypersensitivities. Moreover, DLT produced greater antinociceptive effects in NP rats compared with controls in the cold water paw withdrawal, hot water tail flick, and thermal plantar box tests. Nerve injury-induced augmentation in δOR function was dependent on nociceptive afferents, since the effect was absent in NP rats that received neonatal treatment with capsaicin. Furthermore, it was not due to increased δOR biosynthesis as western blots and immunohistochromistry revealed no change in spinal δOR protein. We hypothesized that an alternative mechanism, such as redistribution of receptors within the neuron, may underlie δOR function changes. Using immunogold electron microscopy, we showed that nerve injury indeed increased the cell surface expression of δORs within dendritic profiles of the dorsal horn via redistribution of existing receptors. Interestingly, this event was observed bilaterally in the deep dorsal horn, with no effect in the superficial laminae. The mechanisms underlying nerve injury-induced δOR trafficking remain unclear however we may take cues from other δOR trafficking events. We showed that concomitant treatment of rats with morphine and a glial inhibitor prevented both the activation of spinal glia and the changes in δOR agonist effects observed with morphine alone, suggesting that glial activity contributes to morphine-induced δOR trafficking in vivo and may provide insight into the mechanisms underlying nerve injury-induced δOR trafficking. Collectively, these studies reveal an important role of δORs in modulating pain symptoms associated with nerve injury, supporting further exploration of δORs as novel therapeutic targets in the treatment of NP pain.
Co-Authorship

The research upon which this thesis is based was conducted by Sarah V. Holdridge under the supervision of Dr. Catherine M. Cahill. Ms. Stacey A. Armstrong performed the behavioural and immunohistochemical assays outlined in Chapter 3 and Ms. Anna M.W. Taylor provided preliminary data for this study. Ms. Lihua Xue provided technical support throughout the thesis project. Ms. Mariette Lavallee performed the ultrathin sectioning and counterstaining of spinal cord samples in Chapter 5.
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<th>Description</th>
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<tbody>
<tr>
<td>α</td>
<td>alpha</td>
</tr>
<tr>
<td>A</td>
<td>amygdala</td>
</tr>
<tr>
<td>AC</td>
<td>adenylyl cyclase</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AVM</td>
<td>arteriovenous malformation</td>
</tr>
<tr>
<td>BNTX</td>
<td>7-benzylidenenaltrexone</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BUBU</td>
<td>Tyr-D-Ser(O-t-butyl)-Gly-Phe-Leu-Thr</td>
</tr>
<tr>
<td>CCI</td>
<td>chronic constriction injury</td>
</tr>
<tr>
<td>CFA</td>
<td>complete Freund’s adjuvant</td>
</tr>
<tr>
<td>CGRP</td>
<td>calcitonin gene-related peptide</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CONTRA, C</td>
<td>contralateral</td>
</tr>
<tr>
<td>CPS</td>
<td>capsaicin</td>
</tr>
<tr>
<td>CTAP</td>
<td>D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂</td>
</tr>
<tr>
<td>CTOP</td>
<td>D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂</td>
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<tr>
<td>δ</td>
<td>delta</td>
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<tr>
<td>DADLE</td>
<td>Tyr-D-Ala-Gly-Phe-D-Leu</td>
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<td>DAMGO</td>
<td>Tyr-D-Ala-Gly-MePhe-Gly-ol</td>
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<td>Deltorphin I</td>
<td>Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH₂</td>
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<tr>
<td>Deltorphin II</td>
<td>Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH₂</td>
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<tr>
<td>DLP</td>
<td>dorsolateral pons</td>
</tr>
<tr>
<td>DLT</td>
<td>deltorphin II</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DPDPE</td>
<td>Tyr-D-Pen-Gly-Phe-D-Pen</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglion</td>
</tr>
<tr>
<td>DSLET</td>
<td>Tyr-D-Ser-Gly-Phe-Leu-Thr</td>
</tr>
<tr>
<td>DTLET</td>
<td>Tyr-D-Thr-Gly-Phe-Leu-Thr</td>
</tr>
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</table>
NMDA  N-methyl-D-aspartate  
NNH  number needed to harm  
NNT  number needed to treat  
Nor BNI  17,17’-bis(cyclo-propylmethyl)-6,6’,7,7’-tetrahydro-4,5,4’,5’-diepoxy-6,6’-(imino)[7,7’-bimorphinan]-3,3’, 14,14’-tetrol  
NP  neuropathic  
NPc  neuropathic contralateral  
NPi  neuropathic ipsilateral  
OR  opioid receptor  
ORL  opioid receptor-like  
PAG  periaqueductal gray  
PB  phosphate buffer  
PBS  phosphate buffered saline  
PF  propentofylline  
PFA  paraformaldehyde  
PKC  protein kinase C  
PMA  phorbol myristate acetate  
PN  parabrachial nucleus  
-R  receptor  
RF  reticular formation  
RNA  ribonucleic acid  
RT  room temperature  
RVM  rostral ventromedial medulla  
Sal  saline  
SB  spinobulbar  
s.c.  subcutaneous  
SCV  small clear vesicle  
s.d.  standard deviation  
SDS  sodium dodecyl sulfate  
s.e.m.  standard error of the mean  
ScN  sciatic nerve  
SN  solitary nucleus
SNC80  (+)-4-[(αR)-α-((2S,5R)-4-Allyl-2,5-dimethyl-1-piperainyl)-3-metholxybenzyl]-N,N-diethylbenzamide
SNRI  selective norepinephrine reuptake inhibitor
SSRI  selective serotonin reuptake inhibitor
SOM  somatostatin
SP  substance P
SSC  somatosensory cortex
ST  spinothalamic
(-)-TAN-67  2-methyl-4-αα-(3-hydroxyphenyl)-1,2,3,4,4a,5,2,12aa-octahydro-quinolino[2.3,3-g]isoquinoline
TBS  tris-buffered saline
Th  thalamus
TCA  tricyclic antidepressant
TIPPψ  H-Tyr-Ticψ[CH2-NH]Phe-Phe-OH
Trk  tyrosine kinase
TRPV1  transient receptor potential vanilloid 1
U-50,488  trans-2,3-dichloro-N-methyl-N[2-)1-pyrrolidinyl]-cyclohexyl]-benzeneacetamide
U-69,593  (5a,7α,8β)-(−)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspirol('5)dec-8-yl]phenyl-benzene acetamide
U-62,066  (5a,7α,8β)-(±)-3,4-dichloro-N-[7-(1-pyrrolidinyl)-1-oxaspirol('5)dec-8-y]-methan sulfonate
Veh  vehicle
WDR  wide dynamic range
VGCC  voltage-gated calcium channel
VLM  ventrolateral medulla
CHAPTER 1: INTRODUCTION

1.1 PAIN

1.1.1 A survival necessity

Pain is described as an unpleasant sensory or emotional experience associated with actual or potential tissue damage, or described in terms of such damage (IASP). It is an evolutionary adaptive mechanism that serves as warning of impending danger in the environment. The integration of danger signals by the brain and spinal cord results in motor commands which appropriately guide the body away from harm. In addition to the somatosensory pain experience, danger signals incur psychological, emotional, and autonomic responses which serve to deter dangerous behaviour and promote avoidance of harmful stimuli. Hence, pain is a multidimensional and highly subjective experience that is absolutely necessary for survival of the organism. The encoding and processing of noxious stimuli is termed, ‘nociception’.

1.1.2 Nociception

1.1.2.1 Primary sensory neurons

Noxious stimuli are detected and processed by a specialized group of primary afferent sensory neurons called nociceptors (Sherrington, 1906). Nociceptors transmit
information from skin, bones and joints, muscles, and viscera to the central nervous system (CNS). Some nociceptors respond selectively to particular noxious energy forms (ie. thermal, chemical, or mechanical), while a large proportion, termed polymodal nociceptors, is responsive to multiple sensory modalities (Belmonte and Cervero, 1996). Together, nociceptive neurons relay specific information about the intensity and location of the harmful stimulus to the brain.

Primary sensory neurons, also called dorsal root ganglion (DRG) cells, are so named because their somas are located in a collection of cell bodies along the dorsal spinal nerves. Sensory afferents are pseudo-unipolar neurons with a single axon emanating from the cell body which bifurcates into a peripheral and a central process. The peripheral axon provides the dendritic field which innervates skin, muscle, bone, and the internal organs and is receptive to a diversity of stimuli. The central axon travels along the dorsal root and synapses onto second order neurons in the spinal cord. Sensory neurons can be functionally classified based on several morphological characteristics. The cell body size, axon diameter, and the presence or absence of a myelin sheath are highly predictive factors of the types of stimuli to which a neuron will respond as well as the velocity with which inputs will be conducted. The large, heavily myelinated axons of large DRG cell bodies give rise to Aβ fibres which rapidly transmit innocuous proprioceptive information (> 12 m/s; Lawson et al, 1993). Noxious and thermal inputs are transduced by two groups of afferents: Aδ and C fibres. Aδ fibres are thinly myelinated with small- to medium-sized cell bodies that are thought to convey acute, sharp pain (1.3-2 m/s; Lawson et al, 1993), while small, unmyelinated C fibres produce a
dull and diffuse pain that is slightly delayed (< 1.3 m/s; Lee et al, 1986; Lawson et al, 1993; see Fig. 1.1). It is important to note that Aδ and C fibres also mediate innocuous thermal signals generated by cool or warm stimuli.

Nociceptive afferents can be further divided into two broad subpopulations: peptidergic and non-peptidergic. Non-peptidergic nociceptors, like all sensory neurons, convey signals to post-synaptic spinal cord neurons through the release of the excitatory amino acid neurotransmitters, L-glutamate and L-aspartate. In addition to L-glutamate, peptidergic nociceptors release neuropeptides such as substance P (SP), calcitonin gene-related peptide (CGRP), and somatostatin (SOM) which act on their endogenous targets – namely, the neurokinin-1 (NK-1), CGRP, and SOM receptors, respectively – expressed on post-synaptic profiles within the dorsal spinal cord. The two groups can be identified immunohistochemically using distinct cellular markers. Non-peptidergic nociceptors possess fluoride-resistant acid phosphatase (FRAP) activity, bind the *Griffonia simplicifolia* plant isolecitin B4 (IB4), and express the purinergic P2X3 ATP receptor while the peptidergic group can be identified by immunodetection of neuropeptides (Alvarez and Fyffe, 2000). The two cell populations also differ in neurotrophic support. Both peptidergic and non-peptidergic neurons respond to nerve growth factor (NGF) during development and hence express its receptor, tyrosine kinase (Trk) receptor A. Upon maturation, the non-peptidergic group undergoes a phenotypic switch whereby it becomes responsive to glial cell line-derived neurotrophic factor (GDNF) and expresses one or more of the GFRα1-4 receptors (Bennett et al, 1998). Approximately 40% of all DRG cells are peptidergic neurons – 50% of all C fibres and 20% of all Aδ fibres
Figure 1.1 Primary Sensory Neurons. The morphology-function relationship between $\alpha$β, $\alpha$δ and C fibres. (A) The cell diameter of, and amount of myelination surrounding, a dorsal root ganglion neuron correlates with both the velocity with which it conducts electrical impulses and the type of sensory information that it relays to the spinal cord. (B) $\alpha$δ and C fibre activity are involved in the processing of noxious stimulation. The types of information relayed by these two fibre groups are qualitatively and quantitatively distinct. For example, after stubbing one’s toe, $\alpha$δ fibres mediate the immediate and distinctly localized sharp pain upon impact, while C fibre activity mediates the delayed, dull, diffuse pain that ensues. Adapted from Julius and Basbaum, 2001.
(Lawson et al, 1996; McCarthy and Lawson, 1989). A considerable proportion of both peptidergic and non-peptidergic neurons expresses the transient receptor potential V1 (TRPV1) cation channel which plays a significant role in the transduction of heat and is the endogenous target for capsaicin, the pungent ingredient in chili peppers (Caterina et al, 1997; Guo et al, 1999). The central axon terminals of each fibre type can be distinguished ultrastructurally based on the morphology of neurotransmitter vesicles. Amino acid and small molecule neurotransmitters, such as L-glutamate and acetylcholine (ACh), are packaged into small, clear vesicles (SCV) while neuropeptides are contained within large dense core vesicles (LDCV; Edwards, 1998; Fig. 1.2). Although the classification is convenient, the distinction between peptidergic and non-peptidergic neurons is not absolute; a small but significant group of neurons expresses neuropeptides and binds IB4 (Alvarez and Fyffe, 2000; De Koninck and Ribeiro-da-Silva, 2008). Furthermore, alterations in the expression and cellular localization of these markers have been documented in response to nerve injury (Bennett et al, 1998; Hammond et al, 2004) and may contribute to the abnormal pain state that ensues.

1.1.2.2 Dorsal horn neurons

The spinal gray matter is divided into morphologically and functionally distinct regions called laminae of Rexed (1952, 1954; Fig. 1.3A). Laminae I-VI comprise the dorsal horn which serves as the major point of termination for all sensory afferent neurons. Most nociceptive afferents synapse onto neurons in laminae I (marginal layer), and II (substantia gelatinosa), so named for their localization within the gray matter and
Figure 1.2 Ultrastructural Detection of Peptidergic and Non-peptidergic axon terminals. Electron photomicrograph depicts a dendrite (centre; D) forming axodendritic synapses with a peptidergic axon terminal containing large dense core vesicles (left; A1) and a non-peptidergic axon terminal containing small clear vesicles (right; A2). Synapses appear as dark densities (arrow heads). Image obtained from Chapter 3 data collection. Scale bar: 0.5 μm.
Figure 1.3 Morphology of the Spinal Cord. (A) The spinal cord gray matter is divided into ten morphologically and functionally distinct regions called laminae of Rexed (I-X). Laminae I-VI comprise the dorsal horn. (B) The various groups of primary afferent fibre types exhibit distinct patterns of termination in the dorsal horn. Each lamina is comprised of distinct dorsal horn neurons which differentially process and relay sensory information to the brain. NF200: neurofilament 200; CGRP: calcitonin gene-related peptide; SP: substance P; IB4: isolecin B4. *Aδ fibres may express peptides such as CGRP and SP, or not (i.e. they can be either peptidergic or non-peptidergic).
gelatinous appearance, respectively. Together, laminae I-II make up the superficial dorsal horn. Superficial dorsal horn neurons receive noxious inputs from both peptidergic and non-peptidergic C fibres as well as Aδ fibres (Willis and Coggeshall, 1991; Fig. 1.3B). Laminae III-VI constitute the deeper dorsal horn which receives innocuous tactile information from large Aβ neurons. Lamina V neurons also receive noxious inputs from peptidergic C fibres and Aδ nociceptors (De Koninck and Ribeiro-da-Silva, 2008). Post-synaptic profiles in both the superficial and deep laminae are mainly intrinsic interneurons that form local circuits within the dorsal horn. The two areas communicate extensively through interneurons and via direct connections. Indeed, lamina I neurons are known to send dendritic processes ventrally to deeper laminae (Light et al, 1979; Woolf and Fitzgerald, 1983; Hylden et al, 1985), while lamina V neurons send extensive antenna-like dendritic projections to substantia gelatinosa neurons (Willis and Coggeshall, 1991). A relatively small portion of post-synaptic cells in these areas represent projection neurons (De Koninck and Ribeiro-da-Silva, 2008) which relay noxious inputs to supraspinal sites. In fact, while lamina I contains the highest density of projection neurons in the dorsal horn, less than 5% of post-synaptic profiles in this region actually leave the dorsal horn (Spike et al, 2003).

Similar to primary sensory afferents, dorsal horn neurons display functional selectivity. While some cells are selectively responsive to innocuous mechanical, noxious mechanical or thermal stimuli, other cells – aptly named wide dynamic range (WDR) neurons – respond to a variety of low- and high-threshold inputs (Price, 1988;
Willis, 1985) and may serve to integrate afferent inputs from the entire sensory spectrum (Wall, 1973). Wide dynamic range neurons are abundant in lamina V.

### 1.1.2.3 Synaptic neurotransmission

Upon stimulation, primary afferent neurons depolarize, sending a wave of electrical current along the axon, toward the central terminal in the dorsal horn (Fig. 1.4). This current stimulates the calcium-dependent release of L-glutamate via fusion of SCVs with the cell membrane and expulsion of its neurotransmitter contents by exocytosis. Fast excitatory neurotransmission is mediated by the binding of L-glutamate to post-synaptic ionotropic glutamate receptors such as α-amino-3-hydroxyl-5-methyl-4-isoxazole-proprionic acid (AMPA), kainite, and N-methyl-D-aspartate (NMDA) receptors. The opening of these channels allows the influx of cations like sodium and calcium, initiating depolarization of the post-synaptic cell and effectively propagating the signal. Peptidergic nociceptors also release neuropeptides, such as SP and CGRP, which bind to post-synaptic metabotropic receptors. The ensuing activation of second messenger signaling cascades leads to pro-nociceptive changes within the cell.

The conduction of afferent inputs is typically unidirectional, meaning that sensory information flows from peripheral dendrites toward the spinal cord. In addition to their central terminals, nociceptor axons give rise to peripheral terminals via collateral axon branches. Upon activation of the nociceptor, peripheral axon terminals can release neuropeptides locally and this may contribute to peripheral sensitization in pathological states via actions on surrounding neurons, immune cells, and vasculature (Snider and
Figure 1.4  Excitatory Synaptic Neurotransmission. Primary nociceptive neurons relay sensory information to dorsal horn neurons through excitatory synaptic neurotransmission. Upon stimulation of a nociceptive neuron, an electrical impulse travels down its axon toward the axon terminal (1). At the terminal, the electrical gradient stimulates the opening of voltage-gated calcium (Ca\(^{2+}\)) channels (VGCCs), through which Ca\(^{2+}\) flows into the cell (2). The increase in intracellular Ca\(^{2+}\) triggers the fusion of neurotransmitter vesicles with the presynaptic plasma membrane and subsequent release of vesicle contents via exocytosis (3). Neurotransmitter vesicles that contain non-peptidergic transmitters, such as L-glutamate, are called small clear vesicles (SCVs), while peptide transmitter-containing vesicles are called large dense core vesicles (LDCVs). Neurotransmitters diffuse across the synaptic cleft and interact with selective receptors on the postsynaptic membrane. L-glutamate binds to ionotropic receptors which permit the influx of cations such as sodium (Na\(^{+}\); 4), initiating membrane depolarization and propagating the noxious signal (5). CGRP and SP bind to metabotropic CGRP- and NK-1-receptors which activate adenylyl cyclase (AC) activity and lead to pro-nociceptive intracellular changes (6). Surrounding glial cells, such as astrocytes, modulate excitatory neurotransmission by removing excess L-glutamate from the synapse through the glutamate transporters, GLAST and GLT-1 (7).
Inflammation generated by neuronal activity is referred to as ‘neurogenic inflammation’ (Foreman, 1987; Lynn, 1996).

The transmission of neural signals is not solely dependent on neurons. Glial cells - which outnumber neurons in the CNS by a factor of 10 – subserve a variety of structural, metabolic and nutritive functions in addition to modulating neuronal communication (Stevens, 2003). Oligodendrocytes provide insulating myelin which surrounds axons and enhances electrical conductance. Astrocytes play significant roles in maintaining ion homeostasis in synaptic regions, ensheathing blood vessels to maintain the blood-brain barrier, and supplying extracellular matrix proteins, adhesion molecules, and neurotrophic factors to surrounding neurons. Microglia constitute approximately 20% of the total glial population and serve as resident immune cells in the CNS, belonging to the mononuclear phagocyte lineage (Vilhardt, 2004). Glial cells express a wide range of voltage-gated ion channels as well as membrane transporters for neurotransmitters such as L-glutamate. For example, glial GLAST and GLT-1 transporters remove excess glutamate from synapses to terminate excitatory neurotransmission at the site of release and to prevent glutamate spillover to extrasynaptic receptors (Marcaggi et al, 2003). Once regarded as mere support cells for CNS neurons, glial cells are increasingly recognized as an active third participant in the ‘tripartite’ synapse (Stevens, 2003). Known to actively communicate with neurons and with each other, glial cells play an important role in modulating synaptic activity.
1.1.2.4 Ascending nociceptive systems

Several ascending systems relay nociceptive information from the spinal cord to supraspinal sites (see Dostrovsky and Craig, 2006). Dorsal horn projections originate from the marginal zone as well as laminae IV-V, decussate at the dorsal and ventral commissures, and ascend in the contralateral white matter (Fig. 1.5). Two major pathways include the spinothalamic (ST) and spinobulbar (SB) tracts. ST tract neurons project to the somatosensory cortex via the thalamus and are largely responsible for the sensation of pain and temperature. SB tract neurons send projections to homeostatic and behavioural control centres such as brain stem catecholaminergic regions, periaqueductal gray (PAG), reticular formation, and the hippocampus and amygdala via the parabrachial nucleus. Together, these pathways mediate the emotional, behavioural, and autonomic responses to pain.

1.2 MODULATION OF PAIN

1.2.1 History of opioids

Opium is obtained from the seed pods of the Papaver somniferum poppy (Dhawan et al, 1996). Its analgesic and anti-diarrheal properties were well recognized in ancient Sumerian and Egyptian cultures. Opium was also widely used in religious rituals for its powerful euphoric effects (Kritikos and Papdaki, 1967). As such, opium has been used, and abused, for more than 5000 years. In 1806, the active ingredient of opium was isolated by the German chemist, Friedrich Sertürner, who named it morphine, after Morpheus, the god of dreams (Sertürner, 1806). Following the development of the
Figure 1.5 Ascending Nociceptive Systems. Nociceptive information is relayed from the periphery to the central nervous system via the dorsal horn of the spinal cord. Incoming nociceptive afferents synapse onto dorsal horn neurons which decussate and ascend via two major projection pathways: the spinothalamic (in green) and spinobulbar (in blue) tracts. A: amygdala; DLP: dorsolateral pons; H: hippocampus; LC: locus coeruleus; PAG: periaqueductal gray; PN: parabrachial nucleus; RF: reticular formation; SN: solitary nucleus; SSC: somatosensory cortex; Th: thalamus; VLM: ventrolateral medulla.
hypodermic syringe in the 1850s, medical practitioners began to use morphine for anesthesia during minor surgical procedures and for chronic pain (Brownstein, 1993). However, morphine was neither particularly safe nor free of abuse potential. The synthesis of diacetylmorphine, or heroin, by Bayer chemists in 1868 was originally heralded as the remedy to this problem. Ironically, heroin would prove equally addictive with a 2003 National Survey on Drug Use and Health estimating that 3.7 million Americans had misused heroin at some point in their lives (NIDA, 2005). Thus, the quest for superior analgesic alternatives persists today. Despite its shortcomings, morphine is still widely used for the clinical treatment of moderate to severe pain and remains the gold standard of comparison for new analgesic therapies.

1.2.2 Endogenous opioid system

1.2.2.1 Opioid receptors

The physiological activity observed following opiate drug administration led to the concept that opioids must interact with an endogenous receptor system (Pert et al, 1973; Simon et al, 1973; Terenius, 1973; Hughes, 1975). The existence of multiple opioid receptors was postulated by Goldstein et al (1971) and supported by radioligand binding studies which revealed differential binding site distribution in the CNS with various radiolabeled drugs (Hiller et al, 1973; Kuhr et al, 1973). Goldstein and colleagues later demonstrated agonist-specific effects in dogs (1976) and showed a lack of cross tolerance between morphine and ketocyclazocine, suggesting that these agonists interacted with distinct receptors. The nomenclature of three opioid receptor (OR) types
### Table 1.1 Opioid Receptors and Their Exogenous Selective Ligands

<table>
<thead>
<tr>
<th></th>
<th>µOR Non-peptides</th>
<th>δOR Non-peptides</th>
<th>κOR Non-peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Agonists</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morphone</td>
<td>SNC80</td>
<td>Ketocyclazocine</td>
<td></td>
</tr>
<tr>
<td>Fentanyl</td>
<td>(-)-TAN-67</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Etorphine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sufentanyle</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Methadone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Peptides</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAMGO</td>
<td>Deltorphin Ie</td>
<td>E-2078</td>
<td></td>
</tr>
<tr>
<td>Dermorphine</td>
<td>Deltorphin IIef</td>
<td></td>
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<tr>
<td></td>
<td>DPDPEG</td>
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<td></td>
<td>DSLETfg</td>
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<tr>
<td></td>
<td>DTLET</td>
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<tr>
<td></td>
<td>DADLEh</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>BUBU</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Antagonists</strong></td>
<td>CTOP</td>
<td>Naltrindole</td>
<td>NorBNI</td>
</tr>
<tr>
<td>CTAP</td>
<td>TIPψ</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BNTXj</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Naltibenk</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Available for clinical or veterinary use.*

*Affinity for µOR is 50x higher than for δOR (Emmerson et al, 1994).*

*Possesses antagonist activity at N-methyl-D-aspartate ionotropic glutamate receptors.*

*20x more potent at µOR than at κ or δ in [35S]-GTP-γ-S assay (Romero et al, 1999).*

*Exogenous to mammals; belongs to class of amphibian skin opioids (Erspamer et al, 1989; Richter et al, 1990; Lazarus et al, 1994).*

*Highly selective for δ₂ subtype in vivo (Portoghese et al, 1992a,b).*

*In vivo antinociceptive effects mediated via δ₁; modulatory effects on µOR via δ₂ (Vanderah et al, 1994).*

*Affinity for δOR is 2x higher than for µOR (James and Goldstein, 1984).*

*Naloxone and naltroxene are non-selective opioid receptor antagonists.*

*Selective for δ₁ in vivo (Dhawan et al, 1996)*

*Selective for δ₂ in vivo (Sofuoglu et al, 1991; Dhawan et al, 1996)*

**Abbreviations:** BNTX, 7-benzylidenenaltrexone; BUBU, Tyr-D-Ser(O-t-butyl)-Gly-Phe-Leu-Thr(O-t-butyl); CTAP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂; DADLE, Tyr-D-Ala-Gly-Phe-D-Leu; DAMGO, Tyr-D-Ala-Gly-MePhe-Gly-ol; Deltorphin I, Tyr-D-Ala-Phe-Asp-Val-Gly-Val-NH₂; Deltorphin II, Tyr-D-Ala-Phe-Glu-Val-Gly-Val-NH₂; DPDPE, Tyr-D-Pen-Gly-Phe-D-Pen; DSLET, Tyr-D-Ser-Gly-Phe-Leu-Thr; DTLET, Tyr-D-Thr-Gly-Phe-Leu-Thr; E-2078, [N-methyl-Tyr₁,N-methyl-Arg₁,D-Leu₈]dynorphin-(1-8) ethylamide; Nor BNI, 17,17'-bis(cyclopropylmethyl)-6,6',7,7'-tetrahydro-4,4',5',5'-diepoxy-6,6'-(iminotetramorphinan)-3,3', 14,14'-tetrol; SNC80, (±)-4-[(α-R)-α-[2S,5R]-4-allyl-2,5-dimethyl-1-piperazinyl]-2-methoxybenzyl]-N,N-diethylbenzamide; (-)-TAN-67, 2-methyl-4-αα-(3-hydroxyphenyl)-1,2,3,4,4a,5,12α-octahydro-quinolino[2,3,3-g]isoquinoline; TIPPψ, H-Tyr-Ticψ[CH₂-NH]Phe-Phe-OH; U-50,488, trans-2,3-dichloro-N-methyl-N-[2-[1-pyrrolidinyl]-cyclohexyl]-benzeneacetamide; U-69,593, (5α,7α,8β)⋅(-)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspirol(5′,5′)dec-8-yl]phenyl-benzene acetamide; U-62,066, (5α,7α,8β)⋅(±)-3,4-dichloro-N-[7-(1-pyrrolidinyl)-1-oxaspirol(5′)dec-8-yl]methan sulfonate.*
was based on the experimental paradigms in which they were identified. Selective binding by drugs defined the mu (µ; morphine) and kappa (κ; ketocyclazocine; Martin et al, 1976) receptors, while tissue-specific activity defined the delta (δ; contraction of the vas deferens; Lord et al, 1977) receptor (see Table 1.1 for receptor-selective agonists and antagonists). The δOR (Evans et al, 1992; Kieffer et al, 1992), κOR (Yasuda et al, 1993), and µOR (Chen et al, 1993) were cloned shortly thereafter. In addition, an opioid receptor-like receptor (ORL₁R) has been identified which shares 50-60% sequence homology with the µ, κ, and δORs. While the ORL₁R binds the endogenous κOR peptides, the dynorphins, it does so with low affinity (Zhang and Yu, 1995) and preferentially interacts with the non-opioid peptide, nociceptin (also known as orphanin FQ; Meunier et al, 1995; Reinscheid et al, 1995). Finally, several studies suggest the existence of additional opioid receptors such as epsilon (Wuster et al, 1979), lambda (Grevel et al, 1985), and zeta (Zagon et al, 1991); however these receptors are poorly characterized and will not be discussed further in this thesis.

Opioid receptors belong to the superfamily of G protein-coupled receptors (GPCR) with seven transmembrane helical domains connected by intra- and extracellular loops. The three opioid receptor types share 58-68% sequence homology with highly conserved transmembrane domains (Dhawan et al, 1996). In contrast, the amino terminus and extracellular loops show marked variation, accounting for selective agonist interactions. The intracellular loops are fairly conserved and provide sites for G protein interactions. The highly divergent intracellular carboxy terminal tail likely confers
selectivity in regulation by accessory and chaperone proteins, as well as kinases, such as protein kinases A and C, and the G protein-coupled receptor kinases (GRKs).

Pharmacological evidence supports the existence of multiple receptor subtypes; however there is little genetic basis for this since only three genes have been identified. Therefore, opioid receptor heterogeneity likely arises from alternative means. Indeed, each of the three opioid receptor genes contains introns following the first and fourth transmembrane domain-encoding exons (Yasuda et al, 1993; Bare et al, 1994; Min et al, 1994; Pasternak and Standifer, 1995), providing the potential for alternative RNA splicing and the translation of molecular variants. Additionally, opioid receptors undergo maturation and post-translational modifications in the trans-Golgi network. Variations in phosphorylation, palmitoylation, and glycosylation may confer differences in receptor regulation and function (Dhawan et al, 1996). Finally, recent studies have increasingly suggested that opioid receptors form functional complexes with one another (Jordan et al, 2000) and with other GPCRs such as α2 adrenoceptors (Stone et al, 1997; Jordan et al, 2003; Rios et al, 2004; Riedl et al, 2009). Moreover, the δ-κ (Jordan and Devi, 1999), μ-δ (Gomes et al, 2004; Rozenfeld and Devi, 2007) and α2-μ (Jordan et al, 2003) heterodimers exhibit unique pharmacology from that of either monomeric receptor alone, suggesting that oligomerization may underlie, in part, the complex diversity in opioid receptor function. In fact, the pharmacological properties of the δ-κ heterodimer are virtually identical to those of the putative κ2 OR subtype (Jordan and Devi, 1999).
1.2.2.2 **Opioid receptor localization**

Immunohistochemical (Elde et al, 1995; Mansour et al, 1995b), *in situ* mRNA hybridization (Mansour et al, 1994a;b; 1995a; Elde et al, 1995) and autoradiographic ligand binding (Atweh and Kuhar, 1977a;b;c; Herkanham and Pert, 1980; Mansour et al, 1988; Sharif and Hughes, 1989) studies indicate that the three OR types exhibit discrete but overlapping distribution throughout the central and peripheral nervous systems. Expressed in the cortex and numerous subcortical regions including the basal ganglia, limbic structures, thalamic nuclei, ventral tegmental area, raphe nuclei, as well as spinal cord and DRG neurons, ORs are well situated to modulate nociception, movement, emotion and reward, as well as endocrine functions (Mansour et al, 1995a). In the spinal cord, ORs are located on pre-synaptic axon terminals of primary afferents and on soma and dendrites of dorsal horn neurons (Besse et al, 1990; Dado et al, 1993; Arvidsson et al, 1995; Cheng et al, 1995; 1996; 1997; Zhang et al, 1998). In the periphery, there is some discordance regarding the DRG cell types that express ORs. Some studies report preferential expression in small diameter neurons (Ji et al, 1995; Wenk and Honda, 1999) while others report OR localization in both small and large DRG neurons (Pare et al, 2001; Wang and Wessendorf, 2001; Kabli and Cahill, 2007).

1.2.2.3 **Descending modulation of pain**

The *ascending* nociceptive pathways relay important information about potentially harmful stimuli from the periphery to the spinal cord and brain. Likewise, *descending* pain-modulating circuits are equally important adaptive mechanisms which
integrate somatic and psychological factors as well as contextual cues in order to suppress or enhance the pain experience in a manner that promotes survival. For example, the suppression of nocifensive reflexes in a dangerous or threatening environment would facilitate escape to safety, whereas the enhancement of such responses in the presence of tissue injury would promote rest and healing (Fields et al, 2006).

The PAG (Basbaum and Fields, 1978; Bandler and Keay, 1996) and rostral ventromedial medulla (RVM; Basbaum and Fields, 1978; Fields et al, 1991) serve as major relay centres which integrate both ascending and descending inputs (recall: ascending spinobulbar tract neurons relay nociceptive inputs from the dorsal horn to the PAG and to pontine and medullary catecholaminergic cell clusters adjacent to the PAG and RVM, respectively). The PAG integrates descending outflow from the cortex, basal forebrain, hypothalamus (Bandler and Keay, 1996) and amygdala (Aggleton, 1992) and sends excitatory projections to the RVM and dorsolateral pontine tegmentum (DLPT; Bandler and Shipley, 1994). In turn, the RVM coordinates inputs from the PAG and nucleus cuneiformis (Fields et al, 2006) and sends serotonergic and non-serotonergic inputs to the dorsal horn and DLPT. The role of serotonergic neurotransmission in the modulation of pain is complex as it can be both pro-nociceptive (Todd and Millar, 1983) and anti-nociceptive (Millan, 2002, Alhaider et al, 1991), likely dependent, in part, on the serotonin receptor subtype involved. The DLPT integrates inflow from the PAG and RVM and sends descending noradrenergic inputs to the dorsal spinal cord. The spinal release of norepinephrine produces significant antinociception via activation of $\alpha_2$-
adrenoceptors expressed on dorsal horn neurons. Spinal \( \alpha_2 \)-adrenoceptors also likely mediate the antinociceptive effects of clonidine observed in pre-clinical (Milne et al, 1985) and clinical (Eisenach et al, 1996) studies.

Opioid receptors are highly expressed in cortical and subcortical regions that contribute to descending networks. Accordingly, microinjection of OR agonists into the PAG, amygdala or insular cortex produces marked analgesia (Yaksh and Rudy, 1978). Opioid-mediated effects in the RVM are more complex as their activation leads to analgesia through both the direct inhibition of pain-facilitating neurons (‘on cells’) and the disinhibition of pain-inhibiting neurons (‘off cells’). Moreover, microinjection of OR antagonists at these supraspinal sites attenuates the analgesic effects of systemic opioids, suggesting that these brain regions are important sites of action for systemic opioids, in addition to the dorsal spinal cord.

1.2.2.4 Endogenous opioid peptides

The demonstration of selective opioid ligand binding in the early 1970s (Pert et al, 1973; Simon et al, 1973; Terenius, 1973; Hughes, 1975) prompted a concentrated search for the endogenous ligands which interact with these putative binding sites, later identified as the \( \delta \)OR, \( \kappa \)OR and \( \mu \)OR. The pentapeptides, Met- and Leu-enkephalin were the first opioid peptides isolated and sequenced (Hughes et al, 1975), followed by additional enkephalins (Pasternak et al, 1976), \( \beta \)-endorphins (Bradbury et al, 1976; Cox et al, 1976; Li and Chung, 1976), and dynorphin (Goldstein et al, 1981). These peptides are produced by the proteolytic cleavage of larger precursor proteins, namely proenkephalin A, proopiomelanocortin and prodynorphin, respectively. More recently,
the highly μOR-selective peptides, endomorphin-1 and -2, were described (Zadina et al, 1997) however their precursors are as yet unidentified.

In addition to pain inhibition, descending pain-modulating networks can facilitate pain, in part, through the release of an anti-opioid peptide called cholecystokinin (CCK). Cholecystokinin acts as a physiological opioid antagonist and its endogenous CNS target, the CCK₂ receptor, produces cellular effects opposite those of ORs (Crawley and Corwin, 1994). Spinal and supraspinal CCK₂ receptors reportedly contribute to nerve injury-induced hypersensitivity (Kovelowski et al, 2000) and their antagonism has been shown to enhance both morphine (Price et al, 1985) and placebo (Benedetti, 1996) analgesia in humans. Table 1.2 provides a list of endogenous opioid and anti-opioid peptides and precursors proteins.

1.2.2.5 Opioid receptor signaling

Opioid receptors transduce extracellular signals via interactions with the heterotrimeric G proteins (Oldham and Hamm, 2008 for recent review). Upon activation by agonist, opioid receptors associate with inhibitory G proteins and stimulate the exchange of GDP for GTP on the G protein nucleotide binding site. This exchange signals the dissociation of the Gα from the Gβγ subunits. Although most attention in the literature has been paid to the cellular effects mediated by the Gα subunit, it is well known that the Gβγ subunits interact with effector molecules as well (Avidor-Reiss et al, 1996; Clapham et al, 1997). The three OR types couple to the pertussis toxin (PTX)-sensitive G₁₁α and possibly PTX-resistant G₂ isoforms with varying selectivity (Connor and Christie, 1999 for review). Receptor activation attenuates the activity of adenylyl
Table 1.2  Endogenous Opioid and Anti-Opioid Peptides and Precursor Proteins

<table>
<thead>
<tr>
<th>Precursor Protein</th>
<th>Enkephalins</th>
<th>Endorphins</th>
<th>Dynorphins</th>
<th>Endomorphins</th>
<th>Cholecystokinin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proenkephalin A</td>
<td>Met- and Leu-</td>
<td>α- and β-</td>
<td>Dynorphin A and B</td>
<td>Unknown</td>
<td>Procholecystokinin</td>
</tr>
<tr>
<td>Proopiomelanocortin</td>
<td>enkephalin</td>
<td>endorphin</td>
<td>B</td>
<td>-2</td>
<td>Polypeptides (ie. CCK-58)</td>
</tr>
<tr>
<td>Prodynorphin (or Proenkephalin B)</td>
<td>Dynorphin A and B</td>
<td>Endomorphin-1 and -2</td>
<td>Polypeptides (ie. CCK-58)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Procholecystokinin</td>
<td>Procholecystokinin</td>
<td>Procholecystokinin</td>
<td>Procholecystokinin</td>
<td>Procholecystokinin</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Receptor Selectivity</th>
<th>Enkephalins</th>
<th>Endorphins</th>
<th>Dynorphins</th>
<th>Endomorphins</th>
<th>Cholecystokinin</th>
</tr>
</thead>
<tbody>
<tr>
<td>δOR, µOR</td>
<td>µOR, δOR</td>
<td>κOR</td>
<td>µOR</td>
<td>CCK2-R</td>
<td></td>
</tr>
</tbody>
</table>

22
Figure 1.6 Opioid Receptor Signalling. Opioid agonists produce antinociception at the level of the spinal cord through interaction with pre- and postsynaptic opioid receptors (ORs). Upon agonist activation, presynaptic ORs inhibit the activity of voltage-gated calcium (Ca\(^{2+}\)) channels (VGCC) and thus inhibit the Ca\(^{2+}\)-dependent release of excitatory and pro-nociceptive neurotransmitters into the synaptic cleft. Postsynaptic OR activation triggers the opening of inwardly-rectified potassium (K\(^{+}\)) channels (KIR) which, despite their name, typically allow the efflux of K\(^{+}\) ions with their concentration gradient, thus leading to hyperpolarization of the postsynaptic membrane. Additionally, postsynaptic ORs couple negatively to adenylyl cyclase (AC) and inhibit the changes in kinase activity and gene regulation which promote nociception. Cumulatively, these opioid-induced effects serve to inhibit the ascending pain signal.
cyclase (Burns et al, 1983; Johnson et al, 1994), inhibits the opening of voltage-gated calcium channels (Tsunoo et al, 1986), and increases potassium conductance through inwardly-rectified channels (North et al, 1987; Darlison et al, 1997; Schneider et al, 1998; Fig. 1.6). The latter actions inhibit pre-synaptic excitatory amino acid (Glaum et al, 1994; Grudt and Williams, 1994) and peptide (Suarez-Roca et al, 1992) neurotransmitter release and hyperpolarize the post-synaptic membrane, respectively. Additionally, opioid receptors exhibit complex interactions with kinase pathways such as protein kinase C (PKC; Lou and Pei, 1997) and mitogen-activated protein kinase (MAPK; Burt et al, 1996; Fukuda et al, 1996; Li and Chang, 1996, Trapaide et al, 2000), as well as inositol (1,4,5)-triphosphate-sensitive calcium stores (Chen et al, 1993; Johnson et al, 1994; Jin et al, 1994; Sánchez-Blázquez and Garzón, 1998) and phospholipase C (Misawa et al, 1990; Prather et al, 1995, Lee et al, 1998). When one also considers the extensive heterogeneity amongst G protein subunits – 18 α, 5 β, 7 γ isoforms (Rens-Domiano and Hamm, 1995) – it is no wonder that GPCRs like ORs mediate such a vast array of cellular actions. The specificity of cellular effects produced by OR activation is therefore largely dependent on the differentially localized expression of G proteins and other effector molecules within various cells and tissues, as well as within various regions of a single cell. Moreover, G protein-independent opioid activity has been suggested (Connor and Christie, 1999). Indeed, the heterodimerization of μ-δ ORs appears to promote a switch in signaling from G protein-mediated to arrestin-mediated (Rozenfeld and Devi, 2007).
1.2.2.6 Regulation of opioid receptor activity

Opioid receptor-mediated cellular effects can be regulated by modulating the number of receptors present at the neuronal plasma membrane (Appendix A). Opioid receptor cell surface expression, and therefore accessibility to extracellular ligand, can be modulated by several means. Non-exhaustively, such modulatory processes include i) receptor desensitization, internalization and resensitization, ii) receptor synthesis and maturation, and iii) receptor trafficking.

Receptor Desensitization, Internalization and Resensitization. Following agonist binding and the subsequent interactions with G proteins, OR signaling is impeded by the phosphorylation of the receptor by GRKs. Phosphorylated receptors attract arrestin proteins which serve to ‘arrest’ signaling by inhibiting further G protein coupling (Ferguson et al, 1996; But see Rozenfeld and Devi, 2007 for arrestin-mediated opioid signaling). Subsequently, chaperone proteins such as clatharin and dynamin are recruited to initiate endocytosis of the receptor-agonist complex (Ferguson et al, 1996; Zhang et al, 1999). The internalized receptor is then routed into one of two pathways: i) lysozymal degradation (Tsao et al, 2001); or ii) resensitization by recycling of the functional receptor back to the plasma membrane, where it can once again interact with ligands (Ferguson and Caron, 1998; von Zastrow, 2004 for review).

Receptor Synthesis and Maturation. The regulation of cell surface OR expression can also be achieved by modulating net synthesis and maturation of the receptor protein. Opioid receptor RNA synthesis is governed by promoters within DNA that are typically located upstream of the genes themselves. Flanking these promoter regions are numerous
putative binding sites for various transcription factors, which can either stimulate or inhibit gene transcription, demonstrating the inherent regulatory complexity of protein synthesis (Wei and Loh, 2002). Furthermore, while some factors bind ubiquitously to promoter regions in all OR genes, others exhibit receptor selectivity, conferring heterogeneity in the regulated expression of μOR, δOR, and κOR. Additionally, the net expression of ORs may be modulated through post-transcriptional events such as those that influence RNA stability and protein translation (Wei et al, 2000) as well as proper protein folding into tertiary and quaternary structures (Wannemacher et al, 2007).

**Receptor Trafficking: Focus on the δOR.** G protein coupled receptors are synthesized in the endoplasmic reticulum and exported to the trans Golgi network where they undergo post-translational modifications. Mature receptors are trafficked toward, and inserted into, the plasma membrane, where they are accessible for binding by extracellular ligands (Harter and Wieland, 1996; Claing et al, 2002; Appendix A). An interesting exception to this general mechanism is the δOR. In contrast to most GPCRs, including the μOR (Cheng et al, 1996a; 1996b; 1997; Van Bockstaele et al, 1996; Wang et al, 1997), the majority of mature δORs is retained intracellularly (Dado et al, 1993; Cheng et al, 1995; Zhang et al, 1998; Cahill et al, 2001a; 2001b; 2003b; Petäjä-Repo et al, 2002; Morinville et al, 2004). Consequently, the acute antinociceptive effects of δOR agonists are often minimal under basal conditions. However, the cell surface trafficking of δORs can be initiated by numerous stimuli including agonist exposure (Bao et al, 2003; Petäjä-Repo et al, 2002; 2006; Walwyn et al, 2005), chronic morphine treatment (Cahill et al, 2001b; Morinville et al, 2003; Hack et al, 2005; Lucido et al, 2005; Gendron
et al, 2006; Ma et al, 2006; Pradhan et al, 2006), and chronic peripheral inflammation (Cahill et al, 2003b; Morinville et al, 2004; Gendron et al, 2006). Accordingly, δOR agonist-mediated effects are enhanced under these conditions, suggesting that δORs are uniquely regulated and may represent a novel target for the pharmacological treatment of pathological pain (see Zhang et al, 2006; Appendix A). For a complete list of stimuli that induce δOR trafficking, see Table 1.3.

1.3 CHRONIC PAIN

Nociceptive pain is an adaptive mechanism which warns of actual or impending tissue damage. The sensory-discriminative, autonomic, and affective components of pain promote rest and healing after injury, as well as avoidance of potentially dangerous stimuli. Accordingly, nociceptive signaling diminishes when the tissue heals and/or when the danger is effectively removed. In contrast, pathological pain is often disproportionate to the extent of injury, may persist long after the tissue is healed or may arise in the absence of any obvious injury. Pathological pain is maladaptive and does not serve a protective function. Chronic non-cancer pain – lasting 6 months or longer (Russo, 1998) - affects an estimated 30% of Canadians and incurs health care costs upwards of $10 billion annually (Moulin et al, 2002). With an aging baby boomer population, seniors represent the fastest growing demographic in Canada and are projected to account for almost 25% of the Canadian population by 2041 (Health Canada, 2002). Moreover, the improved management of many age-related diseases, such as cardiovascular disease, has contributed to a longer life expectancy for Canadian men and
Table 1.3 *In vitro* and *in vivo* trafficking of δORs

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Cell Population</th>
<th>Mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vitro</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>δOR agonist</td>
<td>DRG culture neurons and PC12 cells</td>
<td>↑Cai through Ca2+ influx and release of IP3-sensitive intracellular stores (blocked by naltrindole)</td>
<td>Bao et al, 2003</td>
</tr>
<tr>
<td>δOR ligand</td>
<td>HEK293 cells transfected with δOR</td>
<td>Pharmacological chaperon possibly through receptor palmitoylation</td>
<td>Petäjä-Repo et al, 2002; 2006</td>
</tr>
<tr>
<td>Prolonged CTAP and brief δOR agonist</td>
<td>Isolated DRG neurons</td>
<td>µOR expression causes δOR intracellular retention</td>
<td>Walwyn et al, 2005</td>
</tr>
<tr>
<td>Prolonged or chronic morphine</td>
<td>GABA-containing neurons in PAG</td>
<td>µOR activation and β-arrestin-dependent</td>
<td>Hack et al, 2003</td>
</tr>
<tr>
<td></td>
<td>Nucleus accumbens, dorsal neostriatum, but not frontal cortex</td>
<td>?</td>
<td>Lucido et al, 2005</td>
</tr>
<tr>
<td></td>
<td>DRG neurons</td>
<td>?</td>
<td>Gendron et al, 2006; Ma et al, 2006</td>
</tr>
<tr>
<td></td>
<td>GABA-containing neurons in nucleus raphe magnus</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cortical culture neurons</td>
<td>µOR activation</td>
<td>Cahill et al, 2001b</td>
</tr>
<tr>
<td>K+</td>
<td>DRG culture</td>
<td>↑Cai through Ca2+ influx</td>
<td>Bao et al, 2003</td>
</tr>
<tr>
<td>K+ (NGF?)</td>
<td>PC12 cells</td>
<td>NGF causes δOR retention in <em>trans</em> Golgi complex</td>
<td>Kim and von Zastrow, 2003</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>Trigeminal ganglion culture neurons</td>
<td>PKC-independent (although δOR functional competence was PCK-dependent)</td>
<td>Patwardhan et al, 2005</td>
</tr>
<tr>
<td>Activation of P2X1R</td>
<td>DRG culture</td>
<td>↑Cai through Ca2+ influx and release of IP3-sensitive intracellular stores</td>
<td>Bao et al, 2003</td>
</tr>
<tr>
<td><em>In vitro</em> capsaicin</td>
<td>DRG culture</td>
<td>↑Cai through Ca2+ influx</td>
<td>Bao et al, 2003</td>
</tr>
<tr>
<td><strong>In Vivo</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prolonged or chronic morphine</td>
<td>Spinal cord neurons</td>
<td>µOR activation and primary afferent drive-dependent</td>
<td>Cahill et al, 2001b; Morinville et al, 2003; 2004b</td>
</tr>
<tr>
<td>In vivo capsaicin</td>
<td>Small DRG</td>
<td>?</td>
<td>Gendron et al, 2006</td>
</tr>
<tr>
<td>Chronic inflammation</td>
<td>Small and medium DRG neurons</td>
<td>?</td>
<td>Gendron et al, 2006</td>
</tr>
<tr>
<td></td>
<td>Spinal cord neurons</td>
<td>µOR activation</td>
<td>Cahill et al, 2001; Morinville et al, 2004a</td>
</tr>
<tr>
<td>Forced swim test</td>
<td>GABA-containing neurons in ventrolateral PAG</td>
<td>?</td>
<td>Commons et al, 2003</td>
</tr>
<tr>
<td>Unilateral dorsal rhizotomy</td>
<td>Spinal cord neurons</td>
<td>Primary afferent drive-dependent</td>
<td>Morinville et al, 2004b</td>
</tr>
</tbody>
</table>
This table summarizes stimuli shown to induce trafficking of δOR from intracellular compartments to neuronal plasma membranes in various cell types including neurons and transfected systems. The mechanisms identified in the trafficking event for individual studies are indicated.

Adapted from Cahill et al, 2007.

**Abbreviations:** CTAP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH$_2$; DRG, dorsal root ganglion; IP$_3$, inositol (1,4,5)-triphosphate; NGF, nerve growth factor; PAG, periaqueductal gray; PKC, protein kinase C; PMA, phorbol myristate acetate.
women. The aging baby boomer population is projected to incur unprecedented demands on the health care system and will only exacerbate existing health care strains. Additionally, chronic pain is highly comorbid with mental health issues. Indeed, individuals living with persistent pain are four times more likely to suffer from anxiety or depression (Gureje et al, 1998). Thus, the implications of poorly managed chronic pain are enormous for not only direct health care expenditures and utilization of health system resources, but also for social and economic productivity.

Pain is widely considered one of the major determinants of quality of life (Rummans et al, 1998; Anderson et al, 1999), and nearly 50% of Americans visit a physician with a principal complaint of pain each year (Katz, 2002). However, pain is under-recognized and patient advocacy groups have identified numerous barriers to adequate pain management. A recent national survey aimed to assess pain curricula in 41 Canadian university health science programs including medicine, nursing, dentistry, pharmacy, physical therapy and occupational therapy, while veterinary programs were surveyed for comparison (Watt-Watson et al, 2007). Merely 32.5% of health science respondents could identify designated pain education content whereas all veterinary respondents reported mandatory pain training in their curricula. In fact, veterinary pain education - which included pain physiology, assessment, and management – exceeded medical pain education by 5 fold.

An additional barrier to adequate chronic pain control is the lack of pharmacotherapeutic agents which are both efficacious and safe for long-term use. Chronic pain has been increasingly recognized as an independent disease process unto
itself necessitating both symptom relief and mechanism-based therapy. As such, its undermanagement has prompted sustained clinical and preclinical efforts to uncover novel treatment strategies.

1.4 NEUROPATHIC PAIN

Neuropathic (NP) pain, resulting from a lesion of dysfunction of the peripheral or CNS, is a particularly debilitating chronic pain condition that is very difficult to treat. Neuropathic pain syndromes may be classed according to the etiology of nervous system damage (Table 1.4). These classes include mechanical nerve trauma, metabolic diseases such as diabetes, viral infections such as herpes zoster, neurotoxicity due to cancer chemotherapy, and inflammatory or immunologically-based nerve damage. These classifications are useful diagnostic tools; however they provide little information regarding the mechanism underlying the persistence of chronic NP pain. Moreover, the etiology of the disease has proven to be a poor index of whether a particular class of analgesic drugs will be effective in treating the pain (Woolf and Mannion, 1999).

Neuropathic pain adversely impacts functionality and quality of life, interfering with work, relationships, and hobbies (Nicholson, 2004). Furthermore, it is often comorbidly exhibited with poor sleep, depressed mood, and anxiety – each of which may both result from and exaggerate chronic pain symptoms. A recent population study emphasized the poor quality of life of NP patients; on a scale from 0 (death) to 1 (perfect health) the utility associated with NP pain was 0.42, a score lower than patients with osteoporosis, chronic obstructive pulmonary disorder, or irritable bowel syndrome
**Table 1.4 Classification of Neuropathic Pain Syndromes by Etiology**a,b

<table>
<thead>
<tr>
<th>Peripheral Etiologies</th>
<th>Central Etiologies</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mononeuropathies</strong></td>
<td><strong>Spinal Root/Dorsal Root Ganglion</strong></td>
</tr>
<tr>
<td>Trauma: compression, transection, post-thoracotomy, painful scars</td>
<td>Post-herpetic neuralgia</td>
</tr>
<tr>
<td>Diabetic mononeuropathy and amyotrophy</td>
<td>Trigeminal neuralgia</td>
</tr>
<tr>
<td>Malignant and radiation plexopathy</td>
<td>Prolapsed disc</td>
</tr>
<tr>
<td><strong>Polyneuropathies</strong></td>
<td>Surgical rhizotomy</td>
</tr>
<tr>
<td><strong>Metabolic/Nutritional:</strong></td>
<td>Tumour</td>
</tr>
<tr>
<td>Diabetic</td>
<td></td>
</tr>
<tr>
<td>Alcoholic</td>
<td></td>
</tr>
<tr>
<td>Beri-beri (vitamin B1 deficiency)</td>
<td></td>
</tr>
<tr>
<td><strong>Drugs/Toxic Metals:</strong></td>
<td></td>
</tr>
<tr>
<td>Isoniazidc</td>
<td>Post-herpetic neuralgia</td>
</tr>
<tr>
<td>Vincristine d</td>
<td>Trigeminal neuralgia</td>
</tr>
<tr>
<td>Cisplatin d</td>
<td>Prolapsed disc</td>
</tr>
<tr>
<td>Nitrofurantoin e</td>
<td>Surgical rhizotomy</td>
</tr>
<tr>
<td>Disulfiram f</td>
<td>Tumour</td>
</tr>
<tr>
<td>Clioquinolg</td>
<td></td>
</tr>
<tr>
<td>Thallium</td>
<td></td>
</tr>
<tr>
<td>Arsenic</td>
<td></td>
</tr>
<tr>
<td><strong>Infective:</strong></td>
<td></td>
</tr>
<tr>
<td>HIV</td>
<td></td>
</tr>
<tr>
<td>Acute inflammatory polyneuropathy</td>
<td></td>
</tr>
<tr>
<td>Guillain-Barre</td>
<td></td>
</tr>
<tr>
<td><strong>Hereditary:</strong></td>
<td></td>
</tr>
<tr>
<td>Fabry’s disease</td>
<td></td>
</tr>
<tr>
<td>Dominantly inherited sensory neuropathy</td>
<td></td>
</tr>
<tr>
<td><strong>Malignant:</strong></td>
<td></td>
</tr>
<tr>
<td>Myeloma</td>
<td></td>
</tr>
<tr>
<td>Carcinomatous</td>
<td></td>
</tr>
<tr>
<td><strong>Idiopathic small fibre neuropathy</strong></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

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aTable is intended to provide examples of etiologies and is not exhaustive.
bAdapted from Scadding, 2003.
cAnti-tuberculosis agent.
dCancer chemotherapeutic agent.
eAntibiotic agent.
fAnti-abuse agent for chronic alcoholism.
gAnti-fungal agent.

**Abbreviations:** AVM, arteriovenus malformation; HIV, human immunodeficiency virus.
(Gordon, 2005). Taken together, NP pain is considered one of the most encumbering diseases for patients, their families, and the healthcare system, yet no effective treatment or therapy is currently available. Neuropathic pain is often associated with abnormal pain sensations of a burning, throbbing, shooting, or lancinating character that can be continuous or paroxysmal (Dworkin, 2002). Characteristic symptoms include hyperalgesia (exaggerated nociceptive response to a noxious stimulus), allodynia (nociceptive response to an innocuous stimulus), and spontaneous pain (unevoked nociception). For a schematic representation of the terminology used to describe NP pain behaviours, and the ability of pharmacological agents to reverse them, see Fig. 1.7. It is important to note that these terms are not merely used semantically. Specifically, the dashed horizontal lines in Fig. 1.7 do not simply delineate the transition from low-frequency innocuous to high-frequency noxious stimulations. Largely, the sensory afferent fibres which respond to stimuli above or below the dashed lines comprise distinct groups of neurons with well-defined neurochemical and physiological characteristics as well as highly divergent post-synaptic contacts (Figs. 1.1 and 1.3B). Accordingly, the presentation of NP pain symptoms indicates the abnormal processing of sensory inputs and suggests altered neuronal physiology and/or synaptic rearrangement.

1.4.1 Current therapeutic strategies and challenges

Neuropathic pain is very poorly managed in the clinical setting. It is reportedly less responsive to traditional opioid agonist therapy, although this topic remains controversial. Some clinical reports suggest that opioids provide inadequate pain relief
Figure 1.7 Characteristic Neuropathic Pain Symptoms. Neuropathic pain symptoms commonly include hyperalgesia (exaggerated nociceptive response to a noxious stimulus), allodynia (nociceptive response to an innocuous stimulus), and spontaneous pain (unevoked nociception). The above graphs depict the relationship between stimulus intensity (x-axis) and the perception of the stimulus (y-axis) in non-injured (plotted black solid line) and neuropathic (plotted red solid line) rats for each hallmark symptom. Note that the horizontal dashed line represents the transition from innocuous to noxious as it is perceived by the subject and does not refer to the nature of the stimulus itself. The hollow block arrows depict changes in the stimulus-perception relationships following nerve injury which are interpreted as hyperalgesia, allodynia and spontaneous pain. The solid block arrows illustrate the reversal of these behaviours following administration of a drug while the plotted labels provide the terminologies used to describe such drug-induced effects. Adapted from Bridges et al, 2001.
(Kupers et al, 1991) and/or incur too many undesirable side effects (Arner and Myerson, 1988), while others suggest that opioids may be used effectively in the management of NP pain provided they are appropriately titrated and monitored (Portenoy et al, 1990; Benedetti et al, 1998; Attal et al, 2006) or when used in combination with non-opioid analgesics (Attal et al, 1006; Gilron et al, 2006). Nevertheless, this debate has prompted the extensive off-label use of non-traditional analgesic drugs. Table 1.5 outlines the current pharmacological treatment options for various types of NP pain as well as the number needed to treat (NNT) values. Based on clinical trial data, the NNT value is defined as the number of patients who must be treated with a particular drug in order for one patient to achieve a 50% reduction in pain scores. Hence, a low number indicates a highly efficacious drug; however, the NNT value does not account for toxicities incurred by drug administration that may hinder its use. Accordingly, the number needed to harm (NNH) value is a similar calculation which accounts for adverse drug reactions. A high NNH value indicates a low adverse reaction risk. While useful, NNH values are not often provided and are not as easily interpreted. Adverse reactions are frequently considered all-or-nothing events (ie. not graded responses) and are calculated independently for each type of adverse effect. Clinicians must therefore exercise discretion in assessing the risk:benefit ratio of a particular drug for each patient.

The hypersensitivity that ensues following tissue injury serves a protective role; nociceptive neurons continue to send noxious inputs to the brain until the tissue is adequately healed and no longer requires special attention. Acute nociceptive pain is well managed with traditional analgesic agents which typically act peripherally to directly or indirectly inhibit nociceptor activation (ie. steroidal and non-steroidal anti-
Table 1.5 Current Pharmacological Treatment Options for Various Types of Neuropathic Pain<sup>a</sup>

<table>
<thead>
<tr>
<th>Type of Neuropathic Pain</th>
<th>Drug Class</th>
<th>NNT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyneuropathy (ie. diabetic neuropathy)</td>
<td>Antidepressants</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>TCA</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>SNRI</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>SSRI</td>
<td>4.6 – 5.2</td>
</tr>
<tr>
<td></td>
<td>Anticonvulsants</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>Oxcarbazepine</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>Lamotrigine</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>Topiramate</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>Gabapentin</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>Opioids</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>Oxycodone</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>Tramadol</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>Anticonvulsants</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>Gapapentin</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td>Pregabalin</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>Valproate</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>Opioids</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>Strong opioids</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>Tramadol</td>
<td>4.8</td>
</tr>
<tr>
<td>Post-herpetic Neuralgia</td>
<td>Anticonvulsants</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>Carbamazepine</td>
<td>1.8</td>
</tr>
<tr>
<td>Trigeminal Neuralgia</td>
<td>Antidepressants</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>TCA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.7</td>
</tr>
</tbody>
</table>

<sup>a</sup>Compiled from Attal et al, 2006.

<sup>b</sup>For post-stroke pain, not for spinal cord injury pain.

**Abbreviations:** **NNT**, number needed to treat; **SNRI**, selective norepinephrine reuptake inhibitor; **SSRI**, selective serotonin reuptake inhibitor; **TCA**, tricyclic antidepressant.
inflammatory drugs, acetaminophen, cyclooxygenase-2 inhibitors, local anesthetics) as well as those that typically act centrally to intercept ascending pain signals (ie. opioids). The pharmacodynamic action of these agents involves the prevention or interruption of nociceptive signaling and their analgesic efficacy is therefore contingent on the intact sensory nervous system. However, in pain states arising from injury to the nervous system itself, neuronal physiology is altered such that traditional analgesic drugs are rendered less effective. It is therefore imperative to understand the physiological and pharmacological changes incurred by nerve injury in order to develop novel mechanism-based therapies.

1.4.2 Animal models for research purposes

The development of rodent models of experimental neuropathy in the late 1980s has greatly facilitated the preclinical investigation of the pathophysiological mechanisms underlying NP pain as well as the effectiveness with which novel analgesic drugs alleviate NP symptoms (Arniniello, 1999). Stimulus-evoked pain in rodents is inferred by measurements of response latencies and thresholds to thermal (hot/cold), tactile, and chemical (ie. capsaicin, formalin) stimuli. These stimuli are applied at intensities that are typically noxious or non-noxious to an uninjured rat as an index of hyperalgesia and allodynia in a nerve-injured rat, respectively. Additionally, spontaneous pain may be indicated by guarding behaviour, limping and impaired gait, licking or biting, and decreased locomotor activity. Moreover, the ability of novel compounds to alleviate NP pain behaviours can be assessed in these paradigms (Fig. 1.7). Numerous rodent models
of experimental nerve injury have been developed which simulate specific chronic NP pain conditions of varying etiologies (Table 1.6).

1.4.3 Mechanisms of neuropathic pain

1.4.3.1 Peripheral mechanisms of neuropathic pain

Hyperalgesia involves an enhancement in nociceptive signaling, and feasibly results from peripheral sensitization leading to hyperexcitability of primary sensory afferents (Bridges et al, 2001; Chung and Chung, 2002; Teng and Mekhail, 2003). Several mechanisms may lead to peripheral sensitization and each may contribute to the generation and persistence of NP pain. Alterations in sodium and calcium channel expression and distribution have been observed following peripheral nerve injury and these changes can alter the firing properties of injured and surrounding uninjured neurons (Matzner and Devor, 1994; Zimmerman, 2001; Chung and Chung, 2002). As a result, spontaneous ectopic activity and hyperactivity have been demonstrated in primary afferents, contributing to spontaneous and exaggerated pain (Lisney and Devor, 1987; Woolf and Mannion, 1999; Bridges et al, 2001; Zimmerman, 2001; Chung and Chung, 2002; Teng and Mekhail, 2003). The genesis of ectopic activity exacerbates pain transmission by causing excessive release of neuropeptides, sensitizing the peripheral sensory terminals of both injured and uninjured neurons. This sensitization is characterized by ongoing discharges and results in a lowered firing threshold, and an increased response to a given stimulus.
Table 1.6  Rodent Models of Experimental Nerve Injury

<table>
<thead>
<tr>
<th>Experimental Model</th>
<th>Details</th>
<th>Clinical Representation</th>
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</thead>
<tbody>
<tr>
<td><strong>Peripheral Neuropathy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic Constriction Injury</td>
<td>Loose ScN constriction by:</td>
<td>Chronic nerve compression (ie. carpal tunnel syndrome), disk herniation</td>
</tr>
<tr>
<td>Bennett and Xie, 1988</td>
<td>- 4 chromic gut sutures</td>
<td></td>
</tr>
<tr>
<td>Mosconi and Kruger, 1996</td>
<td>- polyethylene cuff</td>
<td></td>
</tr>
<tr>
<td>Spinal Nerve Ligation</td>
<td>Tight ligation of L₅ or L₆ spinal nerves</td>
<td>Nerve plexus injury, dorsal root injury</td>
</tr>
<tr>
<td>Kim and Chung, 1992</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spared Nerve Injury</td>
<td>Axotomy of tibial, common peroneal nerves (sural nerve intact)</td>
<td>Generalized nerve injury</td>
</tr>
<tr>
<td>Decosterd and Woolf, 2000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partial Sciatic Nerve Ligation</td>
<td>Tight ligation of approximately half of ScN fascicles</td>
<td>Accidental nerve bruise, gunshot-induced nerve injury</td>
</tr>
<tr>
<td>Seltzer et al, 1990</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dorsal Rhizotomya</td>
<td>Transection of dorsal root</td>
<td></td>
</tr>
<tr>
<td>Diabetic Neuropathy</td>
<td>Streptozotocin injection (i.p.)</td>
<td>Diabetic neuropathy</td>
</tr>
<tr>
<td>Courtiex et al, 1993</td>
<td></td>
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<tr>
<td>Post-herpetic Neuralgia</td>
<td>Varicella-zoster virus injection (s.c.)</td>
<td>Post-herpetic neuralgia</td>
</tr>
<tr>
<td>Fleetwood-Walker et al, 1999</td>
<td></td>
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<tr>
<td><strong>Central Neuropathy</strong></td>
<td></td>
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<tr>
<td>Allen, 1911</td>
<td>Trauma/contusion injury</td>
<td>Spinal cord injury</td>
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<tr>
<td>Hao et al, 1991</td>
<td>Ischemia</td>
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<tr>
<td>Yezierski et al, 1998</td>
<td>Excitotoxicity</td>
<td></td>
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<tr>
<td><strong>Abbreviations:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.p., intraperitoneal; s.c., subcutaneous;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ScN, sciatic nerve</td>
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</table>

*Not a commonly used model of nerve injury however dorsal rhizotomy can produce a temporary state of neuropathic pain due to the degeneration of primary nociceptive afferents and subsequent release of neuropeptides and other factors (Eschenfelder et al, 2000; Li et al, 2000). Surgical rhizotomy can similarly produce neuropathic pain in human patients (see Table 1.4).*
Accordingly, the administration of drugs which block ion channels have proven effective in alleviating NP pain symptoms in pre-clinical studies. For example, gabapentin, an anti-epileptic which binds the $\alpha_2\delta$ subunit of calcium channels, has been shown to have analgesic efficacy in both clinical (Curran and Wagstaff, 2003; Gilron et al, 2005) and basic pre-clinical (Matthews and Dickenson, 2002; Teng & Mekhail, 2003; Klugbauer et al, 2003; Hansen et al, 2004) studies.

Finally, coupling between the sympathetic and sensory nervous systems can result in what is termed ‘sympathetically maintained pain’ (Korenman and Devor, 1981; Woolf and Mannion, 1999; Bridges et al, 2001; Zimmerman, 2001; Teng and Mekhail, 2003). Following axotomy, increased expression of the $\alpha_{1B}$ and $\alpha_{2A}$ adrenoceptor subtypes in DRG neurons has been reported (Xie et al, 2001; Shi et al, 2000; Cho et al, 1997; Chen et al, 1996), rendering these neurons overly sensitive to circulating catecholamines (Woolf and Mannion, 1999). Furthermore, nerve injury triggers sprouting of sympathetic fibers into the DRG, providing catecholaminergic ligands to the increased population of adrenoceptors (McLachlan et al, 1993; Ramer et al, 1998; Ramer and Bisby, 1998; Woolf and Mannion, 1999; Bridges et al, 2001: Chung and Chung, 2002). Accordingly, sympathetic blocks have been successfully employed in the clinical treatment of NP pain for some patients (Bridges et al, 2001).

1.4.3.2 Central mechanisms of neuropathic pain

Allodynia involves a change in the quality of a sensation, and likely results from considerable reorganization in the spinal cord (Bridges et al, 2001). The persistent
activation of peripheral neurons can lead to anatomical and neurochemical changes in the CNS, altering the processing of pain signals (Teng and Mekhail, 2003). This central reorganization is termed neuroplasticity.

The increased afferent discharge associated with peripheral nerve injury leads to the development of a sustained state of hyperexcitability of dorsal horn neurons (Bridges et al, 2001). Activation of Aδ and C sensory neurons leads to release of glutamate and pro-nociceptive peptides like SP and CGRP in the dorsal horn of the spinal cord. This glutamate activates post-synaptic AMPA and kainite ligand-gated ion channels, slightly depolarizing the dorsal horn neurons (Woolf and Mannion, 1999; Woolf and Salter, 2000). Weak stimulation does not activate the NMDA receptor, as this channel is voltage-dependently blocked by magnesium (Mayer et al, 1984; Herron et al, 1985; Rothman et al, 1985; Teng and Mekhail, 2003). Upon greater or sustained stimulation, continuous activation of AMPA receptors leads to greater depolarization of the post-synaptic membrane, sufficient to remove the magnesium block. Subsequently, glutamate binding to NMDA receptors will open the channel to sodium and calcium ions. This phenomenon, called ‘wind-up’, increases the responsiveness of dorsal horn neurons to frequency-dependent stimulation (Dubner and Ruda, 1992; Woolf and Salter, 2000; Bridges et al, 2001; Teng and Mekhail, 2003). Repetitive episodes of wind-up result in long-term potentiation (LTP) of spinal cord synaptic transmission, contributing to clinical NP symptoms of hyperalgesia and allodynia (Dubner and Ruda, 1992; Woolf and Mannion, 1999; Woolf and Salter, 2000; Bridges et al, 2001). There is also evidence of activity-dependent receptor trafficking events leading to LTP. Light and electron
microscopy studies have revealed that intracellular and extra-synaptic AMPA receptors are targeted to the synapse (Barria and Malinow, 2002; Malenka et al, 2003), increasing synaptic strength, and likely contributing to central mechanisms of NP pain. Other reports suggest similar synaptic trafficking of NMDA receptors (Ehlers, 2005).

1.4.3.3 Role of spinal glia

Once considered mere support cells for CNS neurons, spinal glia are now recognized as performing vital and complex functions in response to physiological stressors. Peripheral sciatic nerve injury has repeatedly been shown to induce the hypertrophy, functional hyperactivity, and migration of astrocytes and microglia in the lumbar spinal cord (Jin et al, 2003; Raghavendra et al, 2002; 2003; Tsuda et al, 2003; 2005; Bursztajin et al, 2004; Zhuang et al, 2005; Narita et al, 2006; Tanga et al, 2006; Zhang and De Koninck, 2006; Zhuang et al, 2006; Moss et al, 2007). Furthermore, the suppression of glial activation by glial metabolic inhibitors prevents and/or reverses NP pain behaviours in nerve-injured rats (Raghavendra et al, 2003a; 2003b; Taraneh et al, 2006). Activated glia contribute to enhanced neuronal excitability and chronic pain through the release of pro-nociceptive cytokines and inflammatory mediators, as well as impaired glutamate reuptake mechanisms.

1.5 δ OPIOID RECEPTORS: A NOVEL DRUG TARGET

While the overwhelming majority of clinically available opioids are agonists at the μOR (see Table 1.1), a great body of evidence suggests an important role for δOR agonists in mediating antinociception. Delta OR selective agonists have been shown to

Several lines of preclinical evidence suggest that δOR may also be useful in the treatment of NP pain. Delta OR selective agonists were shown to inhibit neurogenic and nociceptive pain behaviours in rats following partial (Petrillo et al, 2003), crushed (Mika et al, 2001), tight ligation (Sohn et al, 2000) or chronic constriction (Desmeules et al, 1993; Nichols et al, 1995) nerve injuries, following ischemia-induced spinal cord injury (Hao et al, 1998a) and in streptozotocin-treated diabetic rats (Kamei et al, 1992; 1995; 1997). Another interesting experiment showed a reversal of NP allodynia following transplantation of immortalized enkephalin-expressing astrocyte cells into the lumbar spinal cord (Xu et al, 2008). In contrast, Lee and colleagues reported no antiallodynic effects of DPDPE, a δOR selective agonist, in spinal nerve ligated rats (Lee et al, 1995). Nevertheless, genetic deletion of the δOR (Nadal et al, 2006) as well as spinal administration of a δOR-selective antagonist (Hao et al, 1998b) produced enhanced NP pain behaviours, suggesting a tonic suppression of nerve injury pain by endogenous δOR activity.

As outlined in Table 1.5, tricyclic antidepressant drugs such as amitriptyline are widely used in the treatment of NP pain however their mechanism of analgesic action is
poorly understood and is presumed to be distinct from their antidepressant activity since the onset of therapeutic benefit is different for each condition. Interestingly, the anti-allodynic effects of amitriptyline and nortriptyline in NP mice were reversed by administration of a δOR antagonist and were absent in δOR null mutants (Benbouzid et al, 2008), suggesting a role for δOR in the therapeutic mechanism. While it is unclear whether δORs similarly play a role in the antidepressant actions of TCAs, several studies suggest both an anxiolytic (Baamonde et al, 1992; Filliol et al, 2000; Primeaux et al, 2006) and antidepressant (Jutkiewicz, 2006; Jutkiewicz et al, 2006; Narita et al, 2006) role for δORs. Given the incidence of comorbidity between chronic pain and anxiety and depression (Gureje et al, 1998), such dual-activity of δOR agonists is an intriguing therapeutic possibility.

Finally, preclinical studies suggest that δOR agonists produce antinociception with a lower incidence of adverse effects as compared with µOR agonists (Porreca et al, 1984). Indeed, administration of δOR agonists produces minimal induction of physical dependence (Cowan et al, 1988) with a lower abuse potential (Mika et al, 2001). Together with a lower propensity for respiratory (Kiritsy-Roy et al, 1989; May et al, 1989; Szeto et al, 1999), cognitive (Sharif and Hughes, 1989), and gastro-intestinal (Shook et al, 1987) impairments than their µ counterparts, agents which selectively activate the δOR represent a promising class of drug for the treatment of chronic pain.
1.6 RESEARCH STATEMENT AND OBJECTIVES

Neuropathic pain is seriously undermanaged in the clinical setting. Clinically available opioid drugs, such as morphine, show reduced efficacy in NP pain patients. Preclinical evidence suggests that δOR agonists are attractive analgesic alternatives with more favourable side effect profiles. The following thesis will address the **GENERAL HYPOTHESIS** that spinal δORs play an important role in modulating nociceptive neurotransmission following nerve injury and agonists selective for the δOR represent a promising new class of drugs for the treatment of chronic NP pain.

Neuropathic pain is a debilitating chronic pain disorder considered to be inherently resistant to therapy with traditional analgesics. However, pharmacological and physiological evidence increasingly suggests a role for δOR agonists in modulating NP pain symptoms. Thus, **OBJECTIVE 1** aimed to examine the antihyperalgesic and antiallodynic effects of a spinal δOR agonist in a rodent model of NP pain. The effect of chronic constriction of the sciatic nerve on δOR protein expression in the lumbar dorsal spinal cord was assessed by western blotting techniques.

The mechanisms underlying nerve injury-induced changes in δOR function following nerve injury are unclear; however previous reports of augmented δOR function have linked these changes to primary sensory afferent inputs (Morinville et al, 2004). Therefore, **OBJECTIVE 2** aimed to assess the contribution of capsaicin-sensitive primary afferent fibres to δOR functional competence following peripheral nerve injury using immunohistochemical and behavioural techniques.
Changes in δOR antinociception have previously been linked to changes in subcellular distribution of the receptor. Indeed, the δOR is primarily localized to intracellular sites (Cheng et al, 1995; 1997; Arvidsson et al, 1995; Cahill et al, 2001a). Trafficking of δORs from internal stores toward the plasma membrane can be initiated by various pro-inflammatory (Cahill et al, 2003b; Morinville et al, 2004; Patwardhan et al, 2005) and pro-nociceptive (Guan et al, 2005, Gendron et al, 2006) stimuli and this cell surface recruitment has correlated with increased δOR functional competence. Therefore, the aim of OBJECTIVE 3 was to assess the effect of peripheral nerve injury on the subcellular localization of δORs in post-synaptic profiles within the dorsal horn using immunogold electron microscopy. The thermal antihyperalgesic effects of a δOR agonist were assessed following spinal administration.

Previous studies have demonstrated that prolonged morphine treatment in vivo induces the translocation of δORs from intracellular compartments to neuronal plasma membranes and this trafficking event correlates with an increased functional competence of the receptor. The mechanism underlying this phenomenon is unknown; however chronic morphine treatment has been shown to involve the activation and hypertrophy of spinal glial cells. Therefore, OBJECTIVE 4 aimed to assess whether activated glia are associated with the enhanced δOR agonist-mediated antinociception observed following prolonged morphine treatment. To meet this objective, morphine-treated rats received, or not, concomitant administration of propentofylline, a glial metabolic inhibitor. The inhibitory effects of propentofylline on glial activation and morphine-induced δOR
functional enhancement were assessed using immunohistochemical and behavioural assays, respectively.

Collectively, these studies will elucidate the role of spinal δORs following peripheral nerve injury and expand our understanding of the mechanisms which contribute to chronic neuropathic pain. Such an understanding will undoubtedly uncover novel and effective mechanism-based therapeutic strategies for patients suffering from this debilitating chronic pain disorder.
CHAPTER 2:
SPINAL ADMINISTRATION OF A δ OPIOID RECEPTOR AGONIST
ATTENUATES HYPERALGESIA AND ALLODYNIA IN A RAT
MODEL OF NEUROPATHIC PAIN

This work has been published in the European Journal of Pain:


2.1 INTRODUCTION

Neuropathic pain (NP) is a debilitating chronic pain disorder involving a peripheral and/or central nervous system lesion. Characterized by the occurrence of allodynia (pain evoked by a normally innocuous stimulus), and hyperalgesia (increased sensitivity to noxious stimuli), NP pain is estimated to affect more than 2-3 % of North Americans (Gilron et al, 2006). Yet despite its prevalence and adverse impact on functionality and quality of life, it remains a challenge for physicians to treat. The clinically available opioids, such as morphine, are agonists at the µ opioid receptor (OR), and although partially effective in alleviating symptoms of NP pain (Gilron et al, 2006), they elicit several adverse effects such as gastro-intestinal disturbances and sedation (Shook et al, 1987). In contrast, pre-clinical studies suggest that δOR agonists are capable of producing analgesia with a lower incidence of adverse effects (Porreca et al, 1984; Mika et al, 2001). Indeed, administration of δOR agonists produces minimal
induction of dependence (Cowen et al., 1988) with lower abuse potential (Mika et al., 2001). Together with a lower propensity for respiratory (Kiritsy–Roy et al, 1989; May et al, 1989; Szeto et al, 1999), cognitive (Sharif and Hughes, 1989), and gastro-intestinal (Shook et al, 1987) impairments than their µ counterparts, agents which selectively activate the δOR represent a promising class of drugs for the treatment of chronic pain.

The analgesic effectiveness of selective δOR agonists has been demonstrated in numerous pharmacological studies of acute and persistent pain (Stewart and Hammond, 1993; Glaum et al, 1994; Fraser et al, 2000); however studies examining the role of δORs in NP pain states encouragingly support further determination of the effectiveness of selective δOR agonists in animal models of NP pain. Hence, administration of selective δOR agonists was shown to alleviate allodynia and/or hyperalgesia in various rat models of neuropathic pain induced by nerve injury following spinal administration (Nichols et al, 1995; Mika et al, 2001). Furthermore, δOR agonists elicited antihyperalgesic effects in rats with experimental diabetic neuropathy (Kamei et al, 1997; Chen and Pan, 2003). Nevertheless, there exist other studies that report the lack of effect of δOR agonists in attenuating neuropathic pain symptoms, wherein mechanical allodynia induced by spinal nerve ligation was unaltered by selective δOR agonists (Lee et al, 1995). Similarly, there are inconsistent reports on changes in δOR expression following nerve injury. While autoradiographic binding (Stevens et al, 1991; Besse et al, 1992) and immunohistochemical (Zhang et al, 1998; Stone et al, 2004) studies report no change or decreased δOR expression following nerve injury, Zaratin and colleagues (1998) observed an increase in δOR mRNA. However, a recent study utilizing genetically
modified mice reported enhanced neuropathy-induced hypersensitivity in δOR knock-out mice compared to wild-type littermates (Nadal et al, 2006), suggesting a role of δORs in modulating neuropathic pain.

The antinociceptive potential of δOR agonists, together with a lower incidence of adverse effects than μOR agonists, makes the δOR an attractive target in the pharmacological treatment of chronic pain syndromes. However, little is known about the efficacy of δOR agonists in alleviating NP pain symptoms, nor about the functional changes in receptor expression, targeting, and pharmacology that may occur in NP pain states. It is therefore important to explore the functional role of the δOR following nerve injury. In the present study, we aimed to investigate the antihyperalgesic and antiallodynic effectiveness of a spinally administered δOR agonist following sciatic nerve injury. Quantitative experiments aimed to determine protein expression were performed to assess nerve injury-induced changes in δOR in the dorsal spinal cord. Some of the data have already been published in abstract form (Holdridge et al, 2005).

2.2 METHODS

2.2.1 Animals

Experiments were performed on adult male Sprague Dawley rats (225-250 g; Charles River, Quebec, Canada) housed in groups of two. Rats were maintained on a 12/12 h light/dark cycle and were given ad libitum access to food and water. Experiments were carried out during the light cycle according to protocols approved by
the Queen’s University Animal Care Committee and in accordance with guidelines set forth by the Canadian Council on Animal Care and the International Association for the Study of Pain Committee for Research and Ethical Issues.

2.2.2 Induction of chronic neuropathic pain

Chronic constriction injury (CCI) was accomplished by a slight modification of methods previously described by Mosconi and Kruger (1996). Rats were anaesthetized by isofluorane inhalation (induced at 5 L/min, maintained at 2 L/min) and their left hind legs and hips shaved clean of hair. A small incision was made in the left hind leg, distal to the hip, along its longitudinal axis. The underlying muscle was bluntly dissected to expose the sciatic nerve. The nerve was freed of connective tissue and a fixed diameter (2 mm) cuff assembled from polyethylene 90 tubing was placed around the nerve proximal to its bifurcation. The muscle and dermal wounds were closed with single non-continuous stitches using Monocryl 3-0 suture thread. The rats were given Tribrissen injectable (0.02 ml/kg) antibiotic and 5 ml lactated ringer subcutaneously. Furacin antibiotic was applied topically to the incision site. Rats were monitored until awakening from the anesthetic and then returned to their home cages. This model is a modified version of that described by Bennett and Xie (1988) in which 4 chromic gut ligatures are tied loosely around the sciatic nerve. The modified version has been employed in the current study as it produces nociceptive behaviours reminiscent of those reported clinically, such as thermal hyperalgesia and mechanical allodynia, with reportedly less variability as compared to its predecessor. Indeed, the use of a cuff of fixed diameter ensures a consistent constriction of the nerve and negates the possibility of variation in
tightness of chromic gut ligatures between individual ligatures and between operators. Furthermore, the Mosconi and Kruger model has been used extensively in the mechanistic and pharmacological characterization of neuropathic pain (Fisher et al, 1998; Cahill and Coderre, 2002; Cahill et al, 2003; Coull et al, 2003; 2005). Sham-operated rats received identical surgeries without manipulation of the nerve and were used, along with naïve rats, as behavioural controls. Post-surgical behavioural testing revealed no differences between sham-operated and naïve rats (p = 0.2262 for cold hyperalgesia; p = 0.1669 for mechanical allodynia) and as such, data from the two groups were pooled to form the control group.

2.2.3 Behavioural testing

Separate groups of rats were used in each behavioural testing paradigm. All rats underwent pre-surgical behavioural testing in their respective paradigms, to establish baseline values to which post-surgical and post-drug values could be compared. Rats were then divided into three groups, those that underwent sciatic nerve constriction, those undergoing sham surgery, and naïve rats. Intrathecal (i.t.) administration of all drugs (30 µl volume) was accomplished by way of lumbar puncture between the L4 and L5 vertebrae under brief halothane anesthesia. Successful drug placement was confirmed by a vigorous tail flick upon injection. Antiallodynic and antihyperalgesic effects of the selective δOR agonist D-[Ala², Glu⁴]deltorphin II (deltorphin II; Sigma, St. Louis, MO, USA) were assessed at 20 minutes following injection, as preliminary experiments in our laboratory have revealed this time point to correspond with peak analgesic effect. To assess the antinociceptive stress response following the lumbar puncture procedure,
saline vehicle was administered and the effects observed at 20 min post-injection. Moreover, the receptor selectivity of deltorphin II-mediated antinociceptive effects was assessed by co-administration of deltorphin II with the δOR-selective antagonist, naltrindole (NALT; Sigma) in a 1:2 molar ratio (10 µg DLT: 11.52 µg NALT).

2.2.3.1 Noxious cold testing

Withdrawal latencies from noxious cold were assessed in CCI and age-matched control rats as previously described (Cahill and Coderre, 1998). An open-ended clear Plexiglas® cylinder was placed end-up into a cold water bath, maintained at 1 °C, with a depth of 1 cm. Rats were placed into the bath and the latency to respond was measured. Neuropathic rats responded by elevating their injured paw out of contact with the water. A cut-off latency of 180 sec was imposed to prevent tissue damage in the event that the rats did not respond. Rats were removed from the cold stimulus upon responding or reaching the cut-off latency.

2.2.3.2 Innocuous mechanical testing

Mechanical withdrawal responses to von Frey filament stimulation were assessed in CCI and control rats as previously described by Chaplan et al. (1994). Rats were placed under opaque Plexiglas® boxes positioned on a wire grid platform (5 mm x 5 mm mesh), through which von Frey filaments were applied to the plantar surface of the hind paw in an up-down fashion. In brief, filaments were applied in either ascending or descending force as necessary in order to most accurately determine the threshold of response. The intensity of stimuli ranged from 0.25 g to 15 g. From the resulting
response patterns, the 50% response thresholds (g) were calculated. Paw withdrawal thresholds are expressed as 50% withdrawal thresholds or converted to percentage of maximum possible effect (% M.P.E.) according to the following formula:

\[
\% \text{ M.P.E.} = \frac{(\text{post-drug latency} - \text{baseline})}{(\text{cut-off latency} - \text{baseline})} \times 100
\]

2.2.4 Western blotting

Neuropathic and naïve rats were sacrificed by decapitation under light halothane anesthesia and their spinal cords were quickly removed by spinal ejection. The lumbar spinal cord was isolated and cut longitudinally into dorsal and ventral segments. The dorsal segment was then hemisected into ipsi- and contralateral segments and homogenized with a Polytron in buffer A containing 50 mM Trisma base, pH 7.4 and 4 mM ethylenediammine-tetraacetic acid (EDTA) with protease inhibitors (Complete™ Protease Inhibitor Tablets, Roche Molecular Biochemicals, Laval, Quebec, Canada; Phenylmethylsulfonyl Fluoride, Sigma-Aldrich, St. Louis, MO, USA). The samples were centrifuged at 4 °C for 10 min at 1000 rpm (Beckmann). The supernatant was collected and centrifuged at 4 °C for 30 min at 50,000 rpm. The pellets were resuspended in buffer B containing 50 mM Trisma base, pH 7.4 and 0.2 mM EDTA with protease inhibitors by vortexing and sonication for 5 sec.

Protein content was determined (Bradford, 1976) and samples were denatured using 6x Laemmli sample buffer (0.375 mM Trisma base, pH 6.8, 30% v/v glycerol, 12%
v/v 2-β-mercaptoethanol, 12% w/v sodium dodecyl sulfate (SDS), 0.2% w/v bromophenol blue) and then vortexed for 30 min at room temperature (RT). Denatured samples were stored at -20 °C. Unused tissue samples were stored at -80 °C for denaturing at a later date.

Samples were loaded (45 µg protein) and resolved using 8% Tris-Glycine pre-cast gels (Novex, San Diego, CA, USA) and the proteins were electroblotted onto nitrocellulose membranes (BioRad Laboratories, Richmond, CA, USA). A Biotinylated Protein Ladder (Cell Signalling Technology) and Kaleidoscope Prestained Standards (BioRad Laboratories, Richmond, CA, USA) were used to calibrate gel migration.

Nitrocellulose membranes were incubated for 1 hour at RT with blocking solution (1% bovine albumin serum, 1% chicken ovalbumin in 26 mM Trisma buffered saline (TBS) containing 0.075% Tween 20) and then overnight with δOR antibody (Chemicon, Temecular, CA, USA; lots 23040417, 24040710) at 0.13 µg/ml in blocking solution at 4 °C. An HRP-conjugated goat anti-rabbit secondary antibody (Amersham Pharmacia Biotech) diluted 1:4000 and an HRP-conjugated anti-biotin antibody (New England Biolabs) diluted 1:10,000 in 26 mM TBS containing 0.075% Tween 20 and 5% powdered milk, were used to visualize the bound primary antibodies and the biotinylated protein ladder, respectively. Secondary antibody incubation was carried out for 1 hour at RT. The membranes were subsequently exposed to chemiluminescent reagents (Amersham Pharmacia Biotech) and developed onto hyperfilm. All membranes were routinely stripped and re-probed for β-actin to normalize the immunoreactive band density for minor differences in protein loading. Blots were digitized with a Hewlett-Packard 4570c
Scion Image software (NIH) was used to measure integrated densities of immunoreactive bands. A calibration curve was calculated using the distance traveled by the biotinylated protein ladder and the molecular weights of immunoreactive bands were then estimated by extrapolation. The specificity of the Chemicon δOR antisera was confirmed by pre-adsorption of the antisera with an appropriate antigenic peptide (20 µg/ml of peptide for 0.1 µg/ml antibody).

2.2.5 Statistical analyses

All behavioural data are expressed as means ± standard error of the mean (s.e.m.) and molecular data as means ± standard deviation (s.d.). Statistical analyses were performed using one way analysis of variance (ANOVA) followed by the Tukey’s multiple comparison test for post-hoc, or by unpaired t-test, as applicable. All graphs were generated and statistical analyses performed using GraphPad Prism software 3.01 (San Diego, CA, USA).

For gel electrophoresis experiments, immunoblots are representative of experiments performed on $n = 3$ per condition obtained from separate groups of animals. Additionally, each set of samples was run in duplicate and averaged to represent an $n$ value of one. All behavioural data were performed on $n = 6-7$ per group.
2.3 RESULTS

2.3.1 Behavioural observations

Neuropathic rats developed characteristic postural manifestations which were evident in the ipsilateral hind leg only. These rats displayed cupped hind paws, which they held in what appeared to be a protective manner, bearing more of their body weight on the contralateral side. Following surgery, CCI rats were not hindered in their ability to retrieve food and water or in their social interaction with cagemates. Sham-operated rats recovered quickly from surgery and displayed no postural manifestations. Furthermore, surgery rats displayed no obvious changes in weight gain or grooming behaviour compared to naive rats.

2.3.1.1 Antihyperalgesic effects of δOR agonist in cold testing

Figure 2.1A illustrates the time course of cold-induced nociceptive responses in control and CCI rats. On days 7 and 14 following surgery, CCI rats displayed a significant decrease in the latency to withdraw the ipsilateral hind paw from a noxious cold stimulus, as compared with pre-surgical baseline values ($F_{3,15} = 9.128, p = 0.0011$). This decrease in cold thermal latency was interpreted as hyperalgesia and was exhibited in the ipsilateral hind paw, but not the contralateral side. Control animals showed no change in withdrawal thresholds throughout the 2-week testing period. Acute i.t. administration of deltorphin II (10 µg) on day 14 following surgery produced significant increases in withdrawal latencies in both control ($F_{3,18} = 11.28, p < 0.05$) and CCI ($F_{3,15} =$
Figure 2.1  (A) Cold withdrawal latencies using the noxious cold water test were assessed in control and CCI rats.  (B) Anti-hyperalgesic effects of i.t. administration of deltorphin II (10 µg) were assessed at 20 min following injection on day 14 post-surgery in control and CCI rats.  Mean cold withdrawal latencies following drug administration were converted to % values of respective pre-drug day 14 latencies.  All data represent means ± s.e.m. for n = 6-7 per group.  Data in panel A were analyzed by a one-way ANOVA, followed by Tukey’s post hoc multiple comparison test.  Data in panel B were analyzed by a two-tailed, unpaired t-test.  The asterisk denotes significant difference from respective baseline value (in A) or from control value (in B); *P < 0.05, **P < 0.01.  The number sign denotes significant difference from respective pre-drug value on day 14; # P < 0.05. DELT: deltorphin II; B: baseline.
9.128, p < 0.01) rats as compared to pre-drug values on the same day. Deltorphin II reversed cold hyperalgesia in CCI rats, producing latencies that were not significantly different from baseline values. Furthermore, CCI post-drug latencies were not significantly different from pre-drug values of control rats on day 14, indicating a return to normal nociceptive levels in CCI rats. When post-drug latency was converted to a % value of the pre-drug latency, Deltorphin II was shown to have a significantly greater effect in CCI rats compared to controls (unpaired t-test, p = 0.0089; Fig. 2.1B).

2.3.1.2 **Antiallodynic effects of δOR agonist in mechanical testing**

Prior to surgery, all rats were unresponsive up to the maximum tactile force of 15.0 g, indicating the innocuous nature of the stimulus. Following surgery, CCI rats displayed a significant decrease in mechanical withdrawal thresholds in the ipsilateral hind paw interpreted as the development of mechanical allodynia, with no change in withdrawal thresholds on the contralateral hind paw over time ($F_{13,52} = 6.117$; Fig. 2.2A). Control animals remained unresponsive to the von Frey filament-stimulation throughout the 2-week testing period (data not shown). Acute i.t. administration of vehicle had no effect on withdrawal thresholds in CCI rats (Fig. 2.2C). In contrast, acute i.t. administration of deltorphin II (3 – 30 µg) on day 14 post-CCI surgery produced a dose-dependent increase in mechanical withdrawal thresholds in CCI rats (Fig. 2.2B). Post-drug 50% withdrawal thresholds were significantly higher than pre-drug values at doses of 10 µg or more ($F_{5,31} = 15.21$, p < 0.05 for 10 µg, p < 0.01 for 15, p < 0.001 for 30 µg). Co-administration of deltorphin II with the δOR-selective antagonist, naltrindole,
Figure 2.2  (A) Mechanical withdrawal responses were assessed using calibrated von Frey filaments in CCI rats ($n=7$). (B) Effects of i.t. deltorphin II were assessed on day 14 post-surgery in CCI rats (3, 10, 15, 30 $\mu$g; $n=4-9$ per dose). Data were transformed to percentage of maximum possible effect (M.P.E.). (C) The effects of vehicle (saline) and co-administration of deltorphin II and the selective $\delta$OR antagonist naltrindole (in a 1:2 DELT:NALT molar ratio; 10 $\mu$g DELT, 11.52 $\mu$g NALT) on mechanical withdrawal thresholds are presented. Mean 50% withdrawal thresholds were converted to % M.P.E.s and compared to that of deltorphin II (30 $\mu$g, i.t.) alone. Data represent means ± s.e.m. Statistical analyses were performed by a one-way ANOVA, followed by Tukey’s post-hoc multiple comparison test. The asterisk denotes significant difference from baseline value at day 0 (in A) or from deltorphin II (10 $\mu$g; in C); *$P<0.05$, **$P<0.01$. Ipsi: ipsilateral; contra: contralateral; B: baseline; Pre: pre-drug; DELT: deltorphin II; NALT: naltrindole.
produced no significant effect on withdrawal thresholds, demonstrating that deltorphin II was mediating its antiallodynic effects via activation of the δOR (Fig. 2.2C).

2.3.2  Effect of chronic constriction injury on δOR protein levels

To assess changes in δOR expression in the dorsal spinal cord, western blotting techniques were used to quantify total protein in membranes prepared from lumbar spinal cord segments of naïve and day 14 CCI rats. Immunoreactive bands were observed at estimated molecular weights of 52 and 75 kDa, consistent with earlier reports using the same antisera (Cahill et al, 2001; Fig. 2.3A). Immunospecificity was confirmed by the absence of immunoreactive bands when the membrane was pre-incubated with antigenic peptide prior to antibody (data not shown). Following quantification of δOR-immunoreactive bands, membranes were stripped and re-probed for β-actin, a housekeeping protein. To normalize differences in protein-loading, all data were expressed as δOR/β-actin ratios. No significant change in the integrated density ratios of either molecular weight band was observed at day 14 following nerve injury in the ipsilateral or contralateral spinal cord when compared to control ($F_{2,6} = 2.497$ for 52 kDa; $F_{2,6} = 2.340$ for 75 kDa; Fig. 2.3B).

2.4  DISCUSSION

The present study reveals promising evidence for the use of δOR-selective agonists in alleviating pain symptoms associated with peripheral nerve injury. The
Figure 2.3  (A) Identification of the δOR protein by western blotting. Membranes from dorsal spinal cord tissues of control and CCI rats were isolated and proteins resolved using 8% Tris-Glycine gels followed by electroblotting onto nitrocellulose membranes. Immunoblot analysis revealed two specific immunoreactive bands with estimated molecular weights of approximately 52 and 75 kDa. Membranes were subsequently stripped and reprobed for β-actin housekeeping protein.  (B) Integrated density values of immunoreactive bands were converted to ratios of δOR to β-actin and are expressed as means ± s.d. for n = 3 per condition. One-way ANOVA analyses revealed no significant difference in δOR immunoreactive band densities between CCI ipsilateral (CCIi), CCI contralateral (CCIc), and control (C).
current behavioural experiments involving a noxious cold stimulus revealed that acute spinal administration of deltorphin II produced marked antihyperalgesic actions in CCI rats. Indeed, following surgery, CCI rats displayed significant reductions in withdrawal latencies in response to noxious cold water on days 7 and 14 while control rats showed no changes in latencies throughout the testing period. Intrathecal administration of the selective δOR agonist deltorphin II returned withdrawal latencies in CCI rats to presurgical levels. These results complement previous observations that i.t. deltorphin II dose-dependently prolonged response latencies in the cold-water allodynia and the cold-water tail flick tests in rats following sciatic nerve crush (Mika et al, 2001). Delta OR agonist-mediated antihyperalgesic effects have also been observed supraspinally where microinjection of [D-Pen², D-Pen⁵]-enkephalin (DPDPE) into the periaqueductal gray reversed cold allodynia following tight ligation of the tibial and sural nerves (Sohn et al, 2000). These findings contrast studies reporting that nerve injury resulted in a loss in potency of morphine (Kamei et al, 1992; Ossipov et al, 1995) and fentanyl (Zurek et al, 2001) following either systemic or spinal administration. Moreover, our data reveal a significantly enhanced effect of deltorphin II in CCI rats compared to controls. Granted, our experiments assessed the antinociceptive effects at only one dose, however the observation is not unique to our study. Kamei and colleagues (1995; 1997) reported enhanced δOR-mediated analgesia in rats with experimental diabetic neuropathy as compared to control rats. While no changes in δOR protein were observed in the spinal cord following CCI, a recent study from our laboratory reported a bilateral increase in δOR protein in the DRGs of CCI rats compared to controls (Kabli and Cahill, 2007).
Deltorphin delivered intrathecally may diffuse toward and act at the DRG, which may in part explain the enhanced effects of spinally administered deltorphin following CCI. Furthermore, enhanced δOR agonist effects have also been observed in other chronic pain models such as inflammation induced by complete Freund’s adjuvant (Fraser et al, 2000; Hurley and Hammond, 2000; Cahill et al, 2003; Morinville et al, 2004) or by carrageenan (Hylden et al, 1991). Despite different induction methods, similar mechanisms may underlie the chronicity of both neuropathic and inflammatory pains including nociceptor activation-dependent changes (Dubner and Ruda, 1992; Woolf and Mannion, 1999; Woolf and Salter, 2000; Bridges et al, 2001), a mechanism also proposed to induce δOR membrane-targeting and subsequently enhance δOR agonist function (Gendron et al. 2006).

The antiallodynic effects of deltorphin II were similarly assessed following nerve injury. Following surgery, CCI rats displayed significant decreases in mechanical withdrawal thresholds, indicating a hypersensitivity to innocuous light touch. The allodynic behaviour was observed in the ipsilateral hind paw only, and was absent in control animals. Spinal administration of deltorphin II dose-dependently reversed allodynia in a naltrindole-sensitive manner. Others similarly report a reversal of nerve injury-induced tactile allodynia following spinal (Nichols et al, 1995) and supraspinal (Sohn et al, 2000) administration of deltorphin II and DPDPE, respectively. Furthermore, Desmeules and colleagues (1993) observed a significant antinociceptive effect of the δOR-selective agonist Tyr-D-Ser(O-t-butyl)-Gly-Phe-Leu-Thr (BUBU) in the paw pressure test in CCI rats. Intravenously administered BUBU produced anti-allodynic and
analgesic effects, as exhibited by a return to and increase from presurgical baseline vocalization thresholds. An additional interesting study employed the i.t. transplantation of immortalized rat astrocytic cells genetically modified by human preproenkephalin gene (An et al, 2005). Spinal injection of these cells on day 7 following spared nerve injury alleviated tactile alldynia as compared with control rats that received unmodified cells, and these effects persisted for 5 weeks. Moreover, several lines of evidence suggest a role for endogenous δ opioid peptides in modulating NP pain symptoms. Following ischemic spinal cord injury, most rats displayed tactile alldynia that was reversed with i.t. DPDPE (Hao et al, 1998a). Interesting, those rats that did not display alldynia spontaneously, exhibited such behaviour upon administration of naltrindole, while this antagonist had no effect in naïve animals (Hao et al, 1998b). This suggests that following nerve injury, nociceptive levels may be under tonic inhibition via δOR activation by endogenous peptides. Furthermore, δOR knock-out mice displayed enhanced allodynic behaviour following partial sciatic nerve ligation, compared to wild type littermates (Nadal et al, 2006). Contrarily, Lee and colleagues (1995) reported no alleviation of neuropathic alldynia following i.t. or intracerebroventricular administration of DPDPE. The basis for these conflicting data is unclear at present. Nevertheless, δOR agonists have also been shown to reverse alldynia induced by spinal administration of dynorphinA where (+)-4-[(αR)-α-(2S,5R)-4-Allyl-2,5-dimethyl-1-piperainyl)-3-metholxybenzyl]-N,N-diethylbenzamide (SNC80) dose-dependently alleviated tactile alldynia (Kawaraguchi et al, 1998).
Numerous studies suggest that µOR agonists, like morphine, show decreased analgesic potency in NP states (Bian et al, 1995; Ossipov et al, 1995; Idänpään-Heikkila and Guilgaund, 1999; Kim et al, 2003; Rashid et al, 2004). One proposed mechanism for the loss of opioid analgesia is the nerve injury-induced reduction in µOR expression in the dorsal spinal cord (Stevens et al, 1991; Porreca et al, 1998; Kohno et al, 2005). Since the current behavioural experiments revealed no such loss in δOR-mediated effects, we aimed to investigate changes in δOR expression that may occur in response to nerve injury. Previous studies on the topic are inconclusive. While some groups report a decrease in δOR-immunoreactivity (Stone et al, 2004) or binding (Stevens et al, 1991) following nerve injury, others report no change (Besse et al, 1992) or increased δOR protein (Kabli and Cahill, 2007) and mRNA (Zaratin et al, 1998). In the current study, spinal δOR protein levels in naïve and CCI rats were assessed by western blotting techniques. The antisera utilized for δOR immunodetection recognized an N-terminal sequence of the protein and revealed two specific immunoreactive bands at estimated molecular weights of 52 and 75 kDa. This was consistent with earlier reports using the same antibody (Cahill et al, 2001), the specificity of which was confirmed as preadsorption of the antisera with an antigenic peptide abolished both immunoreactive bands. Different post-translational modifications of the δOR such as homo- and heterodimerization (Cvejic and Devi, 1997; Jordan and Devi, 1999; George et al, 2000) and glycosylations (Belcheva, 1996; Miller, 1998) may account for δOR variants detected at the protein level. The current protein analyses revealed no changes in δOR protein expression in the dorsal spinal cord following CCI. Notwithstanding the minimal effects
on δOR expression, nerve injury may induce changes in receptor function and future studies will investigate this possibility.

### 2.5 CONCLUSION

In the current study, we have demonstrated that spinal administration of deltorphin II effectively attenuates nerve injury-induced cold hyperalgesia and mechanical allodynia in the chronic constriction injury model while western blotting experiments revealed no alterations in δOR protein biosynthesis. These data reveal a clinically relevant capacity of δOR agonists to suppress hyperexcitable nociceptive transmission to normal physiological levels and suggest an important role of δORs in modulating pain symptoms associated with nerve injury, supporting further exploration of δORs as novel therapeutic targets in the treatment of NP pain.
CHAPTER 3:
NERVE INJURY ENHANCES δ OPIOID RECEPTOR AGONIST EFFECTS: INVOLVEMENT OF CAPSAICIN-SENSITIVE PRIMARY AFFERENT FIBRES

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3.1 INTRODUCTION

Opiate drugs are highly efficacious analgesic agents which modulate the signaling of noxious inputs via interactions with the endogenous opioid receptors: mu (µOR), delta (δOR), and kappa (κOR). The overwhelming majority of clinically available opioid analgesics acts selectively at the µOR and represents the mainstay in treating most types of moderate to severe pain. Neuropathic (NP) pain, an abnormal pain state arising from injury to a peripheral or central nerve, produces characteristic hyperalgesia and allodynia which is reportedly less responsive to µOR agonist therapy, though this topic remains controversial. Some clinical reports suggest that opioids provide inadequate pain relief (Kupers et al, 1991) and/or incur too many undesirable side effects (Arner and Meyerson, 1988), while others suggest that opioids may be used effectively in the management of
NP pain provided they are appropriately titrated and monitored (Portenoy et al, 1990; Benedetti et al, 1998; Attal et al, 2006) or when used in combination with non-opioid analgesics (Attal et al, 2006; Gilron et al, 2006). Rodent models of NP pain employed to investigate the usefulness of opioids have also yielded conflicting results (Mao et al, 1995; Kouya et al, 2002; Erichsen et al, 2005). Several studies concluded that μOR agonists are ineffective following nerve injury (Ossipov et al, 1995; Mao et al, 1995; Zurek et al, 2001) however these observations may depend on the site of administration (Kamei et al, 1992; Bian et al, 1995; Lee et al, 1995; Pertovaara and Wei, 2001; Zurek et al, 2001) and on the particular μOR agonist employed (Nichols et al, 1995; Przewlocka et al, 1999). Nevertheless, NP pain remains poorly managed in the clinic, prompting a sustained research effort aimed at better understanding the opioid system following nerve injury.

Increasing evidence suggests a role for δOR agonists in the management of chronic pain states. Delta selective agonists were shown to be antihyperalgesic in models of persistent inflammation following spinal (Hylden et al, 1991; Stewart and Hammond, 1994; Cahill et al, 2003) and supraspinal (Fraser et al, 2000; Hurley and Hammond, 2000) administration. Moreover, the antinociceptive effectiveness of δOR ligands is enhanced during chronic inflammatory pain (Fraser et al, 2000; Hurley and Hammond, 2000; Cahill et al, 2003) and this functional potentiation may be due to mobilization of internal δOR stores towards neuronal plasma membranes, enhancing agonist accessability (Cahill et al, 2003; Morinville et al, 2004). We recently reported that hyperalgesia and allodynia in rats with chronic constriction nerve injuries were attenuated following local
(Kabli and Cahill, 2007) and spinal (Holdridge and Cahill, 2007a) administration of deltorphin II, a δOR selective agonist. Moreover, the effectiveness with which intrathecal deltorphin II enhanced latencies to noxious cold water was enhanced in nerve-injured rats compared with controls. Delta OR selective agonists similarly inhibited neurogenic and nociceptive pain behaviours in rats following partial (Petrillo et al, 2003), crushed (Mika et al, 2001), tight ligation (Sohn et al, 2000) or chronic constriction (Desmeules et al, 1993; Nichols et al, 1995) nerve injuries, as well as in ischemia-induced spinal cord injury (Hao et al, 1998a) and in streptozotocin-induced diabetic neuropathy (Kamei et al, 1992; 1995; 1997). Another interesting experiment showed a reversal of NP allodynia following transplantation of immortalized enkephalin-expressing astrocyte cells into the lumbar spinal cord (Xu et al, 2008). In contrast, Lee and colleagues reported no antiallodynic effects of DPDPE, a δOR-1 selective agonist, in spinal nerve ligated rats (Lee et al, 1995). Nevertheless, genetic deletion of the δOR (Nadal et al, 2006) as well as spinal administration of a δOR-selective antagonist (Hao et al, 1998b), produced enhanced NP pain behaviours, suggesting a tonic suppression of nerve injury pain by endogenous δOR activity.

In the current study, we aimed to compare the acute analgesic effects of intrathecally administered morphine and deltorphin in rats following chronic constriction injury. Previous reports of augmented δOR function have linked these changes to primary sensory afferent inputs (Morinville et al, 2004) and, as such, we investigated the role of capsaicin-sensitive afferents in the δOR functional competence following nerve injury by neonatal treatment with capsaicin. Immunohistochemical techniques were used
to visualize changes in the expression of δOR as well as nociceptive molecular markers induced by nerve injury and by neonatal capsaicin treatment. The antinociceptive effects of spinal deltorphin were examined in these animals using the hot water tail flick test. Portions of this manuscript have been published previously in abstract form (Holdridge and Cahill, 2008).

3.2 METHODS

3.2.1 Animals

Behavioural and immunohistochemical experiments were carried out using adult male Sprague Dawley rats (200-225 g at surgery; Charles River, Que., Canada). Animals were maintained on a 12/12 h light/dark cycle and were provided ad libitum access to food and water. All experimental protocols were approved by the Queen’s University Animal Care Committee and were performed in accordance with guidelines set by the Canadian Council on Animal Care and the International Association for the Study of Pain Committee for Research and Ethical Issues.

3.2.2 Induction of chronic neuropathic pain

Neuropathic injury was accomplished by chronic constriction of the left sciatic nerve as previously described (Mosconi and Kruger, 1996). Briefly, animals were anesthetized by isoflurane inhalation (5 L/min induction; 2 L/min maintenance). A small skin incision was made on the upper thigh and the underlying muscle was bluntly
dissected. A fixed-diameter polyethylene cuff of 2 mm length was placed loosely around the common sciatic nerve proximal to its bifurcation into the tibial and sural nerves. The muscle and dermal wounds were sutured closed. Control animals received no surgery. Thermal pain testing or tissue harvesting were conducted on post-surgical day 7. Assessment of mechanical withdrawal thresholds was conducted pre-surgically and on post-surgical day 14.

### 3.2.3 Acute thermal pain test

The latency to a vigorous tail flick following emersion of the distal tail into noxious 52 °C water was measured. Three baseline values were recorded and their summation imposed as a cut-off during post-drug trials. The analgesic effects of the classic µOR agonist, morphine sulfate (MS; 0.1, 1, 5, 10 µg in 30 µl saline), or the δOR-selective agonist, D-[Ala², Glu⁴]deltorphin II (DLT; 3.75, 7.5, 10, 30 µg in 30 µl saline; Sigma, St. Louis, MO, USA) were assessed following intrathecal administration by acute lumbar puncture under brief halothane anesthesia. Thermal withdrawal latencies were measured at 10 minute intervals following drug injection. Analgesic drug effect was assessed by transforming raw latency data into percentage of the maximum possible effect (% M.P.E.) values as follows:

\[
\% \text{ M.P.E.} = \frac{\text{Test Latency} - \text{Baseline Latency}}{\text{Cut-off} - \text{Baseline Latency}} \times 100 \%
\]

Dose-response curves were generated using the % M.P.E. values at 30 and 20 minutes for morphine and deltorphin, respectively, as these time points represented peak analgesic
responses. Dose-response curves and analgesic time-courses were analyzed by comparison of mean % M.P.E. values at each dose by two-tailed unpaired \( t \)-test. All data represent means ± s.e.m. for \( n = 4 - 9 \) per group.

### 3.2.4 Neonatal capsaicin treatment

In order to assess the role of capsaicin-sensitive nociceptive afferents, rat pups were anesthetized on ice and administered capsaicin or vehicle on the first and second post-natal days (25 mg/kg and 50 mg/kg in 50 µl 60% DMSO, s.c.; Sigma). Pups were housed with the mother until weaning at 21 days, after which they were housed in pairs. At approximately 215 g body weight (6 weeks of age), mature male rats underwent NP surgery, or not, for behavioural and immunohistochemical analyses.

### 3.2.5 Immunohistochemistry for fluorescent microscopy

To assess the loss of nociceptive afferent cell populations in adult rats following neonatal capsaicin treatment, fluorescent detection of specific cell markers was carried out using immunohistochemical techniques. Rats were anesthetized with sodium pentobarbital (75 mg/kg, i.p.) and perfusion-fixed through the aortic arch with 500 ml 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.4). Lumbar spinal cords were isolated and post-fixed in 4% PFA in 0.1 M PB for 30 min at 4 °C, followed by cryoprotection in 30% sucrose in 0.1 M PB for 48 h at 4 °C. Spinal cords were cut into 40 µm transverse sections on a freezing sledge microtome and free-floating sections were processed for immunodetection of calcitonin gene-related peptide (CGRP; 1:1000, Bachem, Torrance, CA), substance P (SP; 1:1000, Chemicon, Temecula, CA), the
*Griffonia simplicifolia* isolectin B4 (IB4; 1:200, Sigma), and δOR (1:50; Santa Cruz; Cat No. SC-9111, Lot No. L0904). Briefly, sections were rinsed in 0.1 M tris-buffered saline (TBS) and then incubated in 3% normal goat serum (NGS) in 0.1 M TBS for 1 h at room temperature. Sections were then incubated with the primary antibodies diluted in 1% NGS in 0.1 M TBS as follows: anti-δOR and anti-SP for 48 h at 4 °C, anti-CGRP for 24 h at 4 °C. Following rinses with 0.1 M TBS, sections were incubated with the appropriate secondary antisera conjugated to either Alexa-594 or -488 fluorophores (all 1:200, Molecular Probes, Invitrogen, ON, CA) for 2 h at room temperature in the dark. The lectin antibody used to label IB4 was directly conjugated to FITC and was administered in 1% NGS in 0.1 M TBS at the same time as the secondary antisera. Finally, sections were rinsed with 0.1 M TBS, mounted onto glass Superfrost microscope slides and coverslipped with Aquamount (Polysciences, Canada). Spinal cord sections were visualized on a Leica DM 4000 microscope (5 x magnification; Leica Microsystems Inc, Ontario, Canada) and imaged using a Leica DFC 350 FX digital camera (Leica Microsystems) and OpenLab 4.01 software (Improvision / Quorum Technologies, Guelph, CA). Quantification of immunolabeling was carried out by densometric measurement of mean density values using Image J software (NIH). Four spinal cord sections were randomly selected from *n* = 2–4 rats in each treatment group. Mean intensity values in the dorsal horn region of all treatment groups are expressed as means ± s.e.m. and were compared by one-way ANOVA followed by Bonferroni’s post-hoc multiple comparison test (MCT).
3.2.6 Innocuous mechanical test

Paw withdrawal thresholds to innocuous mechanical stimulation were assessed using von Frey filaments as previously described (Chaplan et al, 1994). Rats were placed under opaque boxes on a wire mesh grid through which von Frey filaments were applied to the plantar surface of the hind paws in an up-down fashion (Holdridge and Cahill, 2007a). The stimulus intensity ranged from 0.25 to 15 g. A non-response was recorded as the 15 g maximum value. The withdrawal response pattern was used to calculate the 50% paw withdrawal threshold. The antiallodynic effects of DLT (10 µg, i.t.) were assessed at 20 min post-injection and converted to % M.P.E. values as outlined above. To assess the effect of neonatal capsaicin treatment on the development of allodynia and on the antiallodynic effects of DLT, data obtained from capsaicin-treated NP rats in the current study were compared with data from untreated NP rats published previously by us (Holdridge and Cahill, 2007a). Changes in withdrawal thresholds within capsaicin-treated rats over time were analyzed by one-way ANOVA followed by Bonferroni’s post-hoc MCT. Withdrawal thresholds and % M.P.E. values were compared between capsaicin-treated and untreated NP rats by two-tailed unpaired t-tests. All data represent means ± s.e.m. for n = 5-8 per group.

3.2.7 Data analyses

All data were calculated, analyzed and graphed using Excel XP (Microsoft Corporation, Redmond, WA, USA) and Prism 5.0 (GraphPad Software, San Diego, CA, USA).
3.3 RESULTS

3.3.1 Effects of peripheral nerve injury on morphine- and deltorphin-mediated antinociception

The antinociceptive effects of intrathecal morphine or deltorphin were assessed using the hot water tail flick test on post-surgical day 7 (Fig. 3.1). Both drugs produced dose-dependent analgesia in both naïve and NP rats. Morphine- and deltorphin-induced antinociception peaked at 30 and 20 minutes post-injection, respectively, and thus, the % M.P.E. values were calculated at those time points to generate dose-response curves. Peripheral nerve injury produced a rightward shift in the morphine dose-response curve which was statistically significant at 5 µg ($p = 0.0101$; Fig. 3.1A). In contrast, the dose-response curve for deltorphin was shifted to the left in NP rats compared to controls, achieving significance at 10 µg ($p = 0.0234$; Fig. 3.1C). Accordingly, the analgesic time-courses were plotted for 5 µg morphine (Fig. 3.1B) and 10 µg deltorphin (Fig. 3.1D). Morphine produced significantly less antinociception in NP rats throughout the 60 minute time course (10 min: $p = 0.0184$; 20 min: $p = 0.0004$; 30 min: $p = 0.0101$; 40 min: $p = 0.0003$). However, the deltorphin-mediated antinociceptive effects were enhanced in NP rats compared with controls (20 min: $p = 0.0234$; 40 min: $p = 0.0095$).
Figure 3.1 The acute antinociceptive effects of intrathecal morphine sulfate (MS) and deltorphin II (DLT) were evaluated using the hot water tail flick test. Tail flick latencies were measured prior to and every 10 min following injection in naïve and day 7 neuropathic (NP) rats. The percentage of maximum possible effect (% M.P.E.) was calculated at the time of peak antinociception for MS (30 min) and DLT (20 min) and these data were used to plot drug-response relationship curves (A, C). Time course of antinociception is shown for MS (5 µg; B) and DLT (10 µg; D). Data represent means ± s.e.m. for n = 4-9 per group. Statistical analyses by two-tailed, unpaired t-test at each dose (*p < 0.05).
3.3.2 General observations of rats treated with capsaicin

3.3.2.1 Neonatal injections

Upon injection with capsaicin, rat pups generally vocalized and writhed for several minutes. Following injection, pups often exhibited mild respiratory depression and were stimulated to breathe by administration of oxygen. This reaction proceeded for 10 to 15 minutes post-injection, after which pups appeared normal. No reaction was observed in pups treated with vehicle. In both groups, the dam received the pups normally following injections.

3.3.2.2 Effects of neonatal capsaicin on general health of adult rats

Vehicle-treated rats were indistinguishable from untreated controls. Capsaicin-treated rats developed cutaneous lesions on the head and neck area, likely a result of trophic disturbances (Carrillo et al, 1998). Otherwise, capsaicin produced no observable changes in markers of overall health such as growth rate, as assessed by weekly measurements of body mass (data not shown).

3.3.3 Effects of nerve injury and neonatal capsaicin treatment on expression of nociceptive markers

3.3.3.1 Isolectin B4

Spinal IB4 immunoreactivity (-IR) was observed most intensely in inner lamina II within the dorsal gray matter of vehicle-treated naïve rats (Fig. 3.2A). Some labeling was
Figure 3.2 The effects of neonatal capsaicin treatment on nociceptive afferent markers were assessed in lumbar spinal cords of adult naïve and day 7 neuropathic (NP) rats by fluorescent immunohistochemistry. (A) Representative low magnification photomicrographs of IB4-, CGRP-, and SP-immunoreactive labeling. Left side is ipsilateral to site of nerve injury. Scale bar = 1 mm applies to all. (B) Mean intensity of immunoreactive labeling was quantified for ipsilateral (upper panel) and contralateral (lower panel) dorsal horn regions. Data represent means ± s.e.m. for 4 sections per rat and n = 2–4 rats per condition. Statistical analyses by one-way ANOVA followed by Bonferroni’s post-hoc MCT. Asterisk denotes significant difference from vehicle-treated naïve (*p < 0.05; **p < 0.01; ***p < 0.001). CPS: capsaicin-treated; Veh: DMSO vehicle-treated.
also evident in white matter regions likely corresponding to the path of incoming afferent fibers. Mean density values were compared between groups for ipsilateral ($F_{3,44} = 60.92$, $p < 0.0001$) and contralateral ($F_{3,44} = 63.41$, $p < 0.0001$) dorsal horn regions (Fig. 3.2B). Following nerve injury, a substantial bilateral reduction in IB4-IR (~26% and ~22% in ipsilateral and contralateral spinal cord, respectively) was observed which was most apparent in the medial region of inner lamina II (ipsi: $p < 0.001$; contra: $p < 0.001$). Neonatal capsaicin treatment decreased spinal IB4-IR by ~53% compared with vehicle-treated naïve rats ($p < 0.001$). This decrease was observed throughout inner lamina II and was particularly evident in the lateral region. Nerve injury did not produce any further decrease in IB4-IR in capsaicin-treated rats.

### 3.3.3.2 Substance P

Spinal SP-IR was evident in laminae I and II with some white matter labeling presumably corresponding to incoming afferent fibers which terminate in the deeper dorsal horn, where sparse axonal SP-IR was present (Fig. 3.2A). Nerve injury did not produce an observable change in SP-IR labeling. Neonatal treatment with capsaicin produced a significant reduction in SP-IR (~26%) in the dorsal horn (ipsi: $F_{3,44} = 6.511$, $p < 0.05$; contra: $F_{3,44} = 7.888$, $p < 0.05$). Nerve injury did not alter SP-IR labeling in capsaicin-treated rats.

### 3.3.3.3 Calcitonin gene-related peptide

Spinal CGRP-IR was observed primarily in lamina I and outer lamina II with some labeled axons traversing into deeper dorsal horn laminae (Fig. 3.2A). Nerve injury
produced no significant effect on CGRP-IR in either ipsilateral or contralateral dorsal horn regions (Fig. 3.2B). Neonatal capsaicin treatment significantly reduced CGRP-IR (~40%) in the superficial dorsal horn (ipsi: $F_{3,28} = 6.407, p < 0.005$; contra: $F_{3,28} = 16.60, p < 0.001$) and abolished CGRP-IR axonal labeling in deeper laminae. Nerve injury produced no change in CGRP-IR in capsaicin-treated animals.

### 3.3.4 Effects of neonatal capsaicin treatment on nerve injury-induced changes in δOR agonist effects

#### 3.3.4.1 Acute thermal pain test

Peripheral nerve injury did not produce thermal hypersensitivity in vehicle-treated rats since hot water tail flick latencies were similar to vehicle-treated controls (Fig. 3.3A). Furthermore, neonatal capsaicin treatment did not alter baseline thermal nociception in naïve or NP rats. The antinociceptive effects of spinal deltorphin were similar in vehicle- and capsaicin-treated naïve rats (Fig. 3.3B), indicating that capsaicin treatment did not alter δOR agonist activity in control rats. In vehicle-treated rats, nerve injury produced a significant increase in DLT-mediated antinociception (unpaired $t$-test, $p = 0.0188$), consistent with data presented in Fig. 3.1, but this increase was absent in NP rats that received neonatal capsaicin, suggesting a role for capsaicin-sensitive primary afferents in the nerve injury-induced effects on δOR function.
Figure 3.3  The effects of neonatal capsaicin treatment on δOR-mediated antinociception in the hot water tail flick test in adult naïve and day 7 neuropathic (NP) rats.  (A) Comparison of pre-drug withdrawal latencies.  (B) The percentage of maximum possible effect (% M.P.E.) was calculated at 20 min following intrathecal injection of 10 µg deltorphin II (DLT). Statistical analyses by two-tailed, unpaired t-test vs. vehicle-treated naïve (*p < 0.05). CPS: capsaicin-treated; Veh: DMSO vehicle-treated.
3.3.4.2 Innocuous mechanical test

Prior to surgery, all rats were unresponsive up to the maximum tactile force of 15 g. At 14 days post-surgery, untreated rats displayed tactile allodynia in the ipsilateral hindpaw (data from Holdridge and Cahill, 2007a; $F_{2,18} = 22.34, p < 0.001$; Fig. 3.4) which was reversed by 10 µg DLT. Similarly, capsaicin-treated NP rats in the current study displayed a significant decrease in mechanical withdrawal thresholds in the ipsilateral hind paw on day 14, indicative of tactile alldynia ($F_{2,14} = 10.77, p < 0.01$). No such sensitivity was evident in the contralateral paw. Furthermore, 10 µg DLT attenuated this hypersensitivity, restoring withdrawal thresholds to values not different from baseline thresholds. Withdrawal thresholds and % M.P.E. values were similar between untreated and capsaicin-treated rats, indicating that neonatal capsaicin treatment altered neither the nerve injury-induced development of tactile alldynia nor the antiallodynic effects of DLT.

3.3.5 Effects of nerve injury and neonatal capsaicin treatment on δOR protein expression

Spinal δOR-IR was observed throughout the gray matter, including many small cells in the superficial laminae, slightly larger cells in the deeper dorsal laminae, and some large motor neurons in the ventral horn (Fig. 3.5A). Nerve injury produced no change in δOR-IR in the superficial or deep dorsal horn in vehicle-treated rats (Fig. 3.5B). Neonatal capsaicin treatment similarly did not alter spinal δOR-IR in naïve rats.
Figure 3.4  Mechanical withdrawal responses were assessed using calibrated von Frey filaments in day 14 neuropathic rats treated neonatally with capsaicin (full bars) or not (hallow bars; n = 5-8 per group). The response pattern was used to calculate the 50 percent paw withdrawal threshold (50% P.W.T.) The antiallodynic effects of 10 µg deltorphin II (DLT) were assessed 20 min following injection. The percentage of maximum possible effect (% M.P.E.) was then calculated and is plotted on the right y-axis. Statistical analyses by two-tailed, unpaired *t*-test vs. vehicle-treated naïve (*p < 0.05). CPS: capsaicin-treated; Veh: DMSO vehicle treated. B: presurgical baseline.
Figure 3.5 The effects of neonatal capsaicin treatment on δOR-immunoreactivity were assessed in the lumbar spinal cords of naïve and day 7 neuropathic (NP) rats. (A) Representative low magnification photomicrographs of δOR-immunoreactive labeling. Left side is ipsilateral to site of nerve injury. Scale bar = 1 mm applies to all. (B) Mean intensity of immunoreactive labeling was quantified for the superficial (left panel) and deep (right panel) dorsal horn regions of the ipsilateral (upper panel) and contralateral (lower panel) spinal cord. Data represent means ± s.e.m. for 4 sections per rat and n = 2–4 rats per condition. Statistical analyses by one-way ANOVA followed by Bonferroni’s post-hoc MCT. Asterisk denotes significant difference from vehicle-treated naïve (*p < 0.05; **p < 0.01). CPS: capsaicin-treated; Veh: DMSO vehicle-treated.
Moreover, there were no changes in δOR-IR in capsaicin-treated NP rats ipsilateral to nerve injury when compared to either vehicle-treated NP rats or capsaicin-treated naive animals. Interestingly, there was a decrease in δOR expression contralateral to nerve injury in capsaicin-treated NP rats compared with vehicle-treated naïve rats (superficial dorsal horn: $F_{3,39} = 4.198, p < 0.05$; deep dorsal horn: $F_{3,44} = 5.765, p < 0.01$).

### 3.4 DISCUSSION

The current study demonstrates that spinal administration of a δOR-selective agonist produces enhanced thermal antinociception in rats following peripheral nerve injury. This finding contrasts the observation that µOR-mediated antinociceptive effects are diminished in nerve-injured rats and highlights the differential regulatory mechanisms of each OR type under neuropathic conditions. The increase in δOR function is not due to increased δOR biosynthesis since δOR protein expression was not altered (Holdridge and Cahill, 2007a; current study). However, it was effectively abolished in rats that had been treated neonatally with the neurotoxin, capsaicin, suggesting a role for capsaicin-sensitive primary afferent fibers in precipitating the nerve injury-induced changes in δOR thermal antinociceptive effects. Furthermore, the development of allodynia and the ability of DLT to attenuate allodynic behaviour were not altered following capsaicin treatment, suggesting that thermal nociception and tactile allodynia involve different mechanisms and are differentially modulated by δOR in nerve-injured states.
Our study first aimed to compare the acute antinociceptive effects of intrathecal morphine and deltorphin in nerve-injured compared to control rats. Consistent with previous reports (Mao et al., 1995; Ossipov et al., 1995; Zurek et al., 2001), the morphine analgesic dose-response relationship was shifted to the right in NP compared with naïve rats, indicating a loss of µOR-mediated activity. In contrast, the effects of DLT were augmented following nerve injury, suggesting an increase in δOR expression and/or function at the spinal level. These results were recently supported by electrophysiological recordings from nerve ligated rats in which DLT was more effective in silencing C fibre-evoked responses in ligated rats compared with controls (Buesa et al., 2008). We and others have shown that administration of δOR agonists *in vivo* alleviates thermal (Shinoda et al., 2007), cold (Sohn et al., 2000; Mika et al., 2001; Holdridge and Cahill, 2007a) and tactile (Desmeules et al., 1993; Nichols et al., 1995; Sohn et al., 2000; Holdridge and Cahill, 2007; Kabli and Cahill, 2008) hypersensitivities associated with the injured hind paws of NP rats. In the current study, as previously reported by Ossipov and colleagues (1995), baseline thermal nociceptive latencies were similar in nerve-injured and control animals, indicating no neurogenic hypersensitivity in the tail. Thus, the µOR and δOR agonist effects reflect antinociception, rather than antiallodynia or antihyperalgesia. The leftward-shift in DLT-mediated antinociception in the tail suggests that injury to the sciatic nerve may in fact alter δOR pharmacology not only in the lumbar spinal cord, but also in adjacent sacral spinal segments which do not receive direct inputs from injured neurons. Consistent with this notion, Malan and colleagues reported increased dynorphin A levels in the lumbar spinal cord receiving inputs from injured
spinal nerves following L5/L6 ligation as well as in the rostral lumbar and sacral segments (2000), suggesting that primary afferent nerve injury can precipitate extrasegmental changes. Kamei et al. showed that δOR agonists attenuated acute nociceptive responses in the tails of streptozotocin-treated diabetic NP rats with comparable (1992) or enhanced (1995; 1997) effectiveness as compared with non-injured animals. It is unclear whether the diabetic rats in the aforementioned studies exhibited hyperalgesia in the tail however another study which employed the streptozotocin model reported no such hypersensitivity in the same testing paradigm (Zurek et al, 2001). Furthermore, δOR-mediated inhibition of acetic acid-induced abdominal constrictions was enhanced in diabetic rats despite similar pre-drug baseline constrictions within the diabetic and non-diabetic groups (Kamei et al, 1995). Therefore, δOR function may increase in the absence of localized injury. Such a globalized change in δOR function under pathological conditions indeed reflects favourably on the clinical development of δOR agonists as novel analgesic agents.

The mechanisms underlying nerve injury-induced changes in δOR function are unclear. Previous studies have reported increased δOR agonist effects in response to a variety of stimuli including agonist exposure (Cahill et al, 2001a; Bao et al, 2003; Morinville et al, 2003; Morinville et al, 2004a, Guan et al, 2005; Hack et al, 2005; Lucido et al, 2005; Gendron et al, 2006; Ma et al, 2006), as well as pro-inflammatory (Cahill et al, 2003; Patwardhan et al, 2005) and pro-nociceptive (Guan et al, 2005, Gendron et al, 2006) stimulation. The mechanisms underlying these events remain unclear, however, δOR cell surface recruitment in response to morphine treatment in vivo was shown to be
dependent upon the integrity of primary afferent neurons, as it was not evident following dorsal rhizotomy (Morinville et al, 2004a). In this study, deafferentation itself produced an increase in δOR cell surface expression in deep dorsal horn neurons on the side of sensory denervation, suggesting a role for injured sensory neurons. In the current study, we aimed to investigate the role of capsaicin-sensitive nociceptive afferents in the nerve injury-induced change in δOR agonist effects. Accordingly, we treated neonatal rat pups with the neurotoxin, capsaicin, which leads to selective destruction of unmyelinated C fibres (Scadding, 1980). We used fluorescent immunohistochemical detection to assess changes in cellular markers following both nerve injury and neonatal capsaicin treatment. Consistent with previous reports (Molander et al, 1996; Bennett et al, 1998; Wang et al, 2003; Hammond et al, 2004, Pezet et al, 2006), we observed a substantial decrease in spinal IB4-immunolabeling following peripheral nerve injury. Interestingly, this effect was bilateral and, to our knowledge, the current study is the first to assess contralateral changes in IB4-IR. In contrast to previous studies (Noguchi et al, 2003; Hammond et al, 2004), we did not detect a significant change in CGRP-IR following nerve injury. CGRP-IR appeared to decrease modestly however this change was not statistically significant. The reason for this discrepancy is unclear however it may involve the NP model employed. The aforementioned studies measured CGRP mRNA (Noguchi et al, 2003) and protein (Hammond et al, 2004) following complete sciatic nerve transection and spinal nerve ligation, respectively, and assessed changes in the DRG cell bodies that may not correlate precisely with changes at the central terminals. Following neonatal treatment with capsaicin, we observed a significant decrease in immunoreactivity for IB4,
SP and CGRP which is consistent with previous literature (Nagy et al, 1980; 1981; 1983b; Cuello et al, 1981; Helke et al, 1981; Jancsó et al, 1981; Kashiba et al, 1990; Meller et al, 1992; Noguchi et al, 1993; Pohl et al, 1990; Torsney et al, 2000), confirming the loss of these afferent fibre subpopulations. The age of rats upon sacrifice (7 weeks) was determined by the body mass range optimal for NP surgery; however there is evidence that capsaicin-induced loss of CGRP-IR is most pronounced at earlier time points and has already begun to normalize after 6 weeks of age (Hammond and Ruda, 1991). No additional loss of IB4-IR was observed following nerve injury in capsaicin-treated rats. It is possible that lectin-binding fibres subject to loss by nerve injury had already been destroyed by capsaicin treatment, given that the loss of IB4-IR incurred by capsaicin was approximately twice that incurred by nerve injury.

To determine the role of capsaicin-sensitive afferents in the current behavioural observations, the antinociceptive effects of DLT were assessed in NP and naïve rats that received treatment neonatally with capsaicin or vehicle. Despite the loss of nociceptive afferentation observed by immunohistochemistry, capsaicin-treated rats displayed no behavioural thermal hyposensitivity compared with vehicle-treated rats in the hot water tail flick test. The literature includes various reports of capsaicin-induced depletion of nociceptive neuropeptides accompanied by prolonged thermal latencies and/or increased mechanical thresholds (Holzer et al, 1979; Nagy et al, 1980; Cervero et al, 1981; Saumet et al, 1982; Nagy et al, 1983a; Doucette et al, 1987) as well as reports of no changes in nociceptive behaviours (Cervero et al, 1981; Buck et al, 1982; Welk et al, 1984; Nagy et al, 1986; Doucette et al, 1987). These apparent contradictions likely reflect both the
dose-dependency of capsaicin-induced changes (Nagy et al, 1983a; 1983b) as well as the relative sensitivities with which different behavioural tests assess nociceptive thresholds and distinguish between sensory modalities. Furthermore, Doucette and colleagues reported regionally-specific nociceptive changes despite systemic administration of capsaicin (1987), highlighting the complex nature of this neurotoxicity. In the current study, nerve injury produced enhanced δOR agonist-induced antinociception in the hot water tail flick test. Neonatal treatment with capsaicin abolished this δOR functional enhancement, indicating a role for capsaicin-sensitive fibres in precipitating δOR changes. The mechanistic details of this involvement, however, are unclear. It is unlikely that the nerve injury-induced loss of lectin-binding neurons directly contributes to enhanced δOR function since capsaicin treatment returned δOR function to normal levels with a more substantial loss in this fibre type. Rather, a separate and, as yet unidentified, subpopulation of capsaicin-sensitive primary afferents may modulate δOR function via direct or indirect effects on OR expression, agonist affinity or G protein coupling, or cell surface targeting. Indeed, spinal expression of the transient receptor potential vanilloid 1 (TRPV1) cation channel, the endogenous receptor target of capsaicin, was shown to be significantly enhanced in rats with CCI (Kanai et al, 2005). It is tempting to speculate that this increase would be sensitive to neonatal treatment with capsaicin and further experiments will investigate this possibility.

We (Holdridge and Cahill, 2007a; Kabli and Cahill, 2007) and others (Desmeules et al, 1993; Nichols et al, 1995; Sohn et al, 2000) have demonstrated the antiallodynic effects of δOR agonists in rodent models of NP pain. We were therefore interested to see
if neonatal capsaicin treatment would alter the development of allodynia in NP rats or the
ability of DLT to attenuate allodynic behaviour. There were no differences in paw
withdrawal thresholds between capsaicin-treated NP rats in the current study and
untreated NP rats tested previously (Holdridge and Cahill, 2007a). This suggests that the
development of tactile allodynia does not involve capsaicin-sensitive primary afferents,
or, alternatively, the capsaicin dosing regimen used currently was insufficient to produce
such changes. Indeed, thermal nociception was similarly unaltered in the current study
and this observation is likely attributable to the dose-dependent nature of capsaicin-
induced effects. Furthermore, in contrast with DLT-mediated thermal antinociception,
the antiallodynic effects of DLT were not altered by neonatal treatment with capsaicin,
suggesting that these pain symptoms are indeed mechanistically distinct and that δOR
function is differentially regulated in each condition. Interestingly, an additional study
reported a paradoxical increase in δOR-mediated attenuation of mechano-nociception
following treatment with resiniferatoxin, an ultrapotent agonist at TRPV1 receptors
which leads to selective destruction of fibers that express these receptors (Chen and Pan,
2008). The means by which this occurs are unclear and serve to highlight the complex
regulatory mechanisms governing δOR function.

The fate of the opioid system following peripheral nerve injury is an ardently
contested debate. A general impairment in opioidergic activity has been proposed as one
mechanism underlying the symptoms of, and the treatment challenges posed by,
neuropathic injury (see Zimmermann, 2001 for review). While a large proportion of the
studies investigating this topic have yielded conflicting results and have focused
predominantly on the μOR, relatively fewer studies have explored the δOR under such conditions. In the current study, we observed no change in δOR expression in the spinal cord following nerve injury, suggesting that increased δOR function in the nerve-injured state is not due to increased biosynthesis of the δOR protein. While capsaicin produced no changes in δOR-IR in naïve rats or in the ipsilateral spinal cords of NP rats, it produced a contralateral decrease in δOR-IR in nerve-injured animals. This observation is intriguing and the mechanisms underlying it are unclear however it is unlikely to account for the abolishment of nerve injury-induced changes in δOR function in the hot water tail flick test since DLT-mediated antinociception was similar between vehicle-treated naïve rats, capsaicin treated naïve rats, and capsaicin-treated NP rats. Thus, it is improbable that the attenuation of enhanced δOR agonist effects by capsaicin is simply due to a loss of presynaptic δOR expression resulting from deafferentation. Furthermore, we cannot conclude whether the nerve injury-induced enhancement in δOR function is due to changes at presynaptic or postsynaptic sites. Indeed, δOR functionality may be altered in the central terminals of capsaicin-sensitive primary afferents or, alternatively, in dorsal horn neurons which receive inputs from these neurons. Earlier studies from our laboratory showed a bilateral increase in peripheral (Kabli and Cahill, 2007) but not spinal (Holdridge and Cahill, 2007a) δOR protein expression following nerve injury while others report an ipsilateral decrease in the superficial dorsal horn (Robertson et al, 1999; Stone et al, 2004). Autoradiographic binding experiments were also conducted in rat spinal cords following CCI using selective δOR ligands. Stevens and colleagues (1991) showed a bilateral decrease in ³H-DPDPE binding in laminae I-II and V while
Besse et al (1992) reported a decreased ipsilateral to contralateral $^3$H-DTLET binding ratio in laminae I-II. In the latter study, however, this ratio may be more indicative of increased contralateral binding than of decreased ipsilateral binding. Given these perplexing and contradictory reports, one must consider several points. Firstly, previous immunohistochemical, in situ hybridization and ligand binding studies have described vastly different δOR expression patterns in the spinal cord tissue (Besse et al, 1992; Cahill et al, 2001a; Chabot-Doré et al, 2008; Mennicken et al, 2003; Morinville et al, 2004; Stone et al, 2004). These differences suggest multiple molecular species of the δOR – which may be differentially modulated by nerve injury - as well as disparate ligand and antisera binding affinities to the putative molecular variants. Secondly, regulation of δOR function in response to physiological stressors has been shown to involve not only changes in total mRNA or protein expression (Cahill et al, 2003) but also in cell surface targeting of the receptor from internal stores (Cahill et al, 2001a; 2003; Commons, 2003; Morinville et al, 2003; Morinville et al, 2004a; Lucido et al, 2005; Gendron et al, 2006). Given the predominantly intracellular localization of δORs (Arvidsson et al, 1995; Cheng et al, 1995; 1997; Svingos et al 1995; Zhang et al, 1998; Cahill et al, 2001a; Bao et al, 2003) and the numerous examples of stimulus-induced mobilization towards the membrane, it is entirely possible that modest changes in protein expression are not the principal determinant of δOR function following nerve injury, but rather the modulation of cell surface expression and, consequently, accessibility to extracellular ligands. Additional studies are required to fully understand the role of δORs
following nerve injury and should employ methodologies that account for subcellular distribution.

3.5 CONCLUSION

In summary, we have demonstrated that the thermal antinociceptive effects of a δOR-selective agonist are enhanced in rats following peripheral nerve injury. The increase in δOR function does not appear to be due to increased δOR biosynthesis since protein expression was not altered. The effect is sensitive to neonatal treatment with capsaicin, suggesting a role for capsaicin-sensitive primary afferents in driving these changes. In contrast, δOR-mediated antiallodynic effects were not altered by neonatal capsaicin, suggesting different underlying mechanisms. While additional studies are necessary to more fully understand the mechanisms underlying δOR functional changes in nerve injured states, the potential for δOR agonists as a clinical therapeutic strategy is promising.
CHAPTER 4:
PERIPHERAL NERVE INJURY INDUCES δ OPIOID RECEPTOR CELL SURFACE TRAFFICKING IN DORSAL HORN NEURONS

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Holdridge S.V., Cahill C.M. (2009)

4.1 INTRODUCTION

Nearly 50% of Americans visit a physician with a principal complaint of pain each year (Katz, 2002), yet only relatively recently has chronic pain been recognized as an independent disease process necessitating both symptom relief and mechanism-based therapy. Poorly controlled pain can be extremely debilitating and can have a profoundly negative impact on overall quality of life. Neuropathic (NP) pain, resulting from injury to a peripheral or central nerve, is particularly undermanaged in the clinical setting. It is characterized by hyperalgesia (increased response to a noxious stimulus), allodynia (nociceptive response to an innocuous stimulus), and spontaneous pain (unevoked nociception). First line therapies include traditional mu opioid receptor (µOR) agonists as well as non-traditional analgesic drugs such as tricyclic antidepressants and anticonvulsants (Attal et al, 2006). However, none of these agents is particularly useful,
prompting a sustained and collaborative research effort with the goal of developing novel treatment strategies.

Recent evidence from ours and other laboratories has identified the delta opioid receptor (\(\delta\)OR) as a promising new target for pharmacological intervention. Delta OR selective agonists have been shown to produce antinociception in acute pain tests (Porreca et al, 1984; Mattia et al, 1991; 1992; Stewart and Hammond, 1993) as well as in models of persistent inflammatory (Hylden et al, 1991; Stewart and Hammond, 1994; Fraser et al, 2000; Hurley and Hammond, 2000; Cahill et al, 2003b) and neuropathic (Desmeules et al, 1993; Nichols et al, 1995; Mika et al, 2001; Sohn et al, 2000; Petrillo et al, 2003) pain. Indeed, we recently reported that deltorphin II, a selective \(\delta\)OR agonist, alleviated nociceptive (Holdridge and Cahill, 2008) and neurogenic pain following local (Kabli and Cahill, 2007) and spinal (Holdridge and Cahill, 2007a) administration. Furthermore, behavioral (Holdridge and Cahill, 2007a; Holdridge and Cahill, 2008) and electrophysiological (Buesa et al, 2008) studies report an increase in \(\delta\)OR functional competence in nerve-injured animals compared with controls. Changes in \(\delta\)OR antinociception have previously been linked to changes in subcellular distribution of the receptor. Indeed, the \(\delta\)OR is primarily localized to intracellular sites (Arvidsson et al, 1995; Cheng et al, 1995; 1997; Cahill et al, 2001a). Trafficking of \(\delta\)ORs from internal stores toward the plasma membrane can be initiated by various pro-inflammatory (Cahill et al, 2003b; Morinville et al, 2004; Patwardhan et al, 2005) and pro-nociceptive (Guan et al, 2005, Gendron et al, 2006) stimuli and this cell surface recruitment has correlated with increased \(\delta\)OR functional competence.
In the current study, we aimed to investigate whether peripheral nerve injury produces changes in cell surface expression of δORs in the rat dorsal spinal cord. Acute thermal nociception was assessed over a 2-week period in rats that underwent either chronic constriction injury or sham surgery. On day 14, the antinociceptive effects of a δOR agonist were assessed. To investigate cell surface expression, the subcellular distribution of δORs was assessed in laminae I-II and V dendrites by immunogold electron microscopy. Portions of this manuscript have been published previously in abstract form (Holdridge and Cahill, 2007b; Holdridge et al, 2007).

4.2 METHODS

4.2.1 Animals

All experiments were performed on adult male Sprague Dawley rats (200-225 g at time of surgery; Charles River, Quebec, Canada) housed in groups of two. Rats were maintained on a reversed 12/12 h light/dark cycle and were given ad libitum access to food and water. Experiments were carried out during the dark cycle according to protocols approved by the Queen’s University Animal Care Committee and in accordance with guidelines set forth by the Canadian Council on Animal Care and the International Association for the Study of Pain Committee for Research and Ethical Issues.
4.2.2 Induction of chronic neuropathic pain

Neuropathic injury was accomplished by chronic constriction injury (CCI) to the left sciatic nerve as previously described (Mosconi and Kruger, 1996). Briefly, rats were anesthetized by isoflurane inhalation (5 L/min induction; 2 L/min maintenance). A small incision was made on the upper thigh and the underlying muscle was bluntly dissected. A fixed-diameter polyethylene cuff of 2 mm length was placed loosely around the common sciatic nerve proximal to its bifurcation into the tibial and sural nerves. The muscle and dermal wounds were sutured closed. Control animals received sham operation in which identical surgical procedures were used without manipulation of the nerve. Behavioral testing was conducted prior to and at 7 and 14 days following surgery. A separate group of animals was used for tissue harvest on day 14 post-surgery.

4.2.3 Acute thermal nociception

Acute thermal withdrawal latencies were assessed in the hind paws of rats as previously described (Hargreaves et al, 1988). Rats were placed in clear plastic boxes on a glass surface and were allowed to habituate for 15 minutes prior to testing. A noxious radiant heat source was applied to the plantar surface of the contralateral, and then ipsilateral, hind paws (IITC Life Science, Woodland Hills, CA, USA). Paw withdrawal latencies were measured to the nearest 0.1 sec using a digital timer connected to the heat source. The radiant beam intensity was set at 70 to yield baseline withdrawal latencies of 3-5 sec. Rats were tested prior to surgery and on days 7 and 14 following CCI or sham
surgery. On each of these days, the average of 3 values measured at 10 min intervals was used.

4.2.4 Antihyperalgesic effects of a δOR agonist

The antihyperalgesic effects of D-[Ala²,Glu⁴]deltorphin II (DLT; Tocris Bioscience, Ellisville, MO, USA) were assessed in NP and sham rats on day 14. Under brief isoflurane anesthesia, 10 µg DLT dissolved in 30 µl 0.9% saline was administered intrathecally by lumbar puncture between the 5th and 6th lumbar vertebrae. Successful injection was confirmed by a tail flick. Saline (30 µl) was delivered in a similar manner as a control for drug administration. Thermal withdrawal latencies were measured at 20, 30, 40 and 50 minutes following injection for n = 5-7 rats per treatment. A cut-off latency of 3 times the pre-drug value was imposed to prevent tissue damage. Antihyperalgesic drug effect was assessed by transforming raw latency data into percentage of the maximum possible effect (% M.P.E.) values as follows:

\[
\% \text{ M.P.E.} = \frac{\text{Test Latency} - \text{Baseline Latency}}{\text{Cut-off} - \text{Baseline Latency}} \times 100\%
\]

Data represent means ± standard error of the mean (s.e.m.). All behavioural data were analyzed by one- or two-way analysis of variance (ANOVA) followed by Dunnett’s post hoc test or Bonferroni multiple comparison test (MCT) where appropriate. Details of each analysis are provided in the results section. All data were calculated, analyzed and graphed using Excel XP (Microsoft Corporation, Redmond, WA, USA) and Prism 5.0 (GraphPad Software, San Diego, CA, USA).
4.2.5 Subcellular distribution of δOR

Day 14 NP and sham-operated rats were deeply anesthetized with sodium pentobarbital (75 mg/kg, i.p.; MTC Pharmaceuticals, Cambridge, ON, CAN) and transaortically perfused at 45 ml/min with 100 ml heparinized saline (6 U/ml heparin), followed by a solution containing 3.75% acrolein and 2% paraformaldehyde (PFA) in 60 ml 0.1 M phosphate buffer (PB), pH 7.4, and finally with 400 ml 2% PFA in 0.1 M PB. Lumbar spinal cords were removed and post-fixed in the same 2% PFA solution for 30 min at 4 °C. Transverse sections (50 µm) were cut using a vibratome and collected into ice-cold 0.1 M PB. Free-floating sections were then processed for δOR immunogold labeling as described previously (Cahill et al, 2001a; Morinville et al, 2003). Sections were incubated with 1% sodium borohydride in PB for 30 min to neutralize free aldehyde groups to improve antibody recognition. Following copious rinses with PB, sections were cryoprotected in 30% sucrose with 3% glycerol in PB for 30 min, and then snap frozen with isopentane (-70 °C) and liquid nitrogen in rapid succession, followed by thawing in PB at room temperature. Sections were rinsed with 0.1 M TBS and then blocked with 3% normal goat serum (NGS) in TBS for 30 min. They were then incubated with δOR antiserum (AB1560, Chemicon Temecula, CA, USA; lot numbers 0506000583, 0509011803) diluted to 1:5000 in TBS containing 0.5% NGS for 36 h at 4 °C. Control sections were processed in the absence of primary antibody. After rinsing, sections were incubated with colloidal gold (1 nm)-conjugated goat anti-rabbit IgG (AuroProbe One GAR, Amersham Pharmacia Biotech, Baie D’Urfé, QC, CAN) diluted to 1:200 in 0.01 M phosphate buffered-saline (PBS), pH 7.4, containing 2% gelatin and
8% bovine serum albumin. After thorough rinsing, sections were fixed with 2% glutaraldehyde in PBS for 10 min and then rinsed with PB. Immunogold particles were amplified by silver intensification for 10 min (IntenSE M Silver Enhancement Kit, Amersham Pharmacia Biotech) following which the reaction was stopped by application of 0.2 M citrate buffer, pH 7.4. Sections were post-fixed and stained for 40 min with 2% osmium tetroxide in PB, rinsed copiously with PB, dehydrated in graded alcohols, and finally embedded in Epon 812 blocks. Ultrathin sections (80 nm) were collected onto copper grids and counter-stained with uranyl acetate and lead citrate for visualization with a Hitachi transmission electron microscope at 15,000x magnification. Negatives were digitized using an Epson Perfection 4990 Photo scanner and images were processed using Photoshop 7.0 (Adobe Systems Inc., San Jose, CA, USA).

Quantification of δOR subcellular distribution was accomplished using ImageJ 1.36b (National Institutes of Health, USA). A minimum of 40 immunopositive dendritic profiles with 3 or more gold particles were imaged within laminae I-II and V from the ipsilateral and contralateral dorsal horn regions for \( n = 2-3 \) experiments per condition. The copper grids were coded and the experimenter was blinded to the sample conditions. The perimeter and cross-sectional area of each dendrite was measured. Within each dendrite, gold particles were counted and classified as being either plasma membrane-associated or cytosolic. Particles were considered membrane-associated if they were overlying or in direct contact with the plasma membrane, while all other particles were considered intracellular. For those gold particles deemed intracellular, the linear distance linking the particle to the closest segment of plasma membrane was measured. From
these data, the following calculations were made: i) average number of gold particles counted per dendrite; ii) percentage of total gold particles associated with the plasma membrane; iii) percentage of total dendrites with plasma membrane-associated gold particles; iv) average distance of intracellular particles from the plasma membrane. All data represent means ± s.e.m. Data were analyzed by one-way ANOVA followed by Tukey’s MCT or unpaired two-tailed t-test where appropriate. Details of each analysis are provided in the results section. All data calculations, analyses and graphs were prepared using Excel XP and Prism 5.0.

4.3 RESULTS

4.3.1 Development of thermal hyperalgesia

We first assessed thermal nociception in rats prior to, and following, nerve injury or sham surgery using the Hargreaves thermal plantar box. A time course of altered sensitivities to radiant heat is shown in Fig. 4.1. All rats responded by briskly lifting the paw away from the radiant beam, at which point the beam was turned off and the response latency measured. Nerve injury produced thermal hypersensitivity in the ipsilateral hind paw compared with the contralateral paw as evidenced by a significantly shorter latency on days 7 and 14 following surgery (Fig. 4.1A; 2-way ANOVA, Bonferroni MCT; $F_{1,36} = 16.92, p < 0.001$). Sham animals displayed no hypersensitivity in the operated paw throughout the testing period (Fig. 4.1B; 2-way ANOVA, $F_{1,30} = 0.004452, p > 0.05$). There were no differences in latencies between the contralateral
Figure 4.1 Thermal withdrawal latencies were assessed in the ipsilateral (closed bars) and contralateral (open bars) hind paws of sham (A) and neuropathic (NP; B) rats over a 2-week period \((n = 5-7\) per group). Baseline latencies were measured prior to surgery. Data represent means \(\pm\) s.e.m. Statistical analyses by 2-way ANOVA, Bonferroni MCT. Asterisks denote significant difference from respective contralateral hind paw, \(\ast p < 0.05, \ast\ast p < 0.01\).
paws of NP rats and the paws of sham-operated rats at any time point (2-way ANOVA, \( F_{2,48} = 0.08703, p > 0.05 \)). No visible redness or evidence of tissue injury was present on tested paws during the testing paradigm.

4.3.2 Antihyperalgesic effects of a \( \delta \)OR agonist

To determine the ability of a \( \delta \)OR agonist to attenuate thermal nociceptive responses, 10 µg was administered intrathecally on day 14 following surgery. Deltorphin significantly increased thermal withdrawal latencies in the ipsilateral (1-way ANOVA, Dunnett’s post hoc test, \( F_{4,30} = 5.615, p < 0.01 \); Fig. 4.2A) and contralateral (1-way ANOVA, Dunnett’s post hoc test, \( F_{4,30} = 2.717, p < 0.05 \); Fig. 4.2C) hind paws of NP rats. The same dose in sham rats produced no substantial increase in withdrawal latencies on either the ipsilateral (1-way ANOVA, \( F_{4,24} = 0.7326, p > 0.05 \); Fig. 4.2A) or contralateral (1-way ANOVA, \( F_{4,24} = 1.193, p > 0.05 \); Fig. 4.2C) side. Post-drug values were not different between groups throughout the 50 minute time course (2-way ANOVA, \( F_{3,86} = 0.9961, p > 0.05 \)), indicating that DLT restored normal thermal nociception in NP rats. Thermal latencies were converted to % M.P.E. values and are plotted in figures 4.2B and D. Deltorphin produced significant antihyperalgesia in NP ipsilateral paws compared with saline at 20, 30, and 50 minutes post-injection (2-way ANOVA, Bonferroni MCT, \( F_{12,3} = 16.52, p < 0.001 \)) and analgesia in NP contralateral paws at 20 and 50 minutes (2-way ANOVA, Bonferroni MCT, \( F_{12,3} = 5.854, p < 0.01 \)). The antinociceptive effects of DLT in sham rats were not different from saline. The
Figure 4.2 Antinociceptive effects of D-[Ala²,Glu⁴]deltorphin II (DLT; 10 µg/30 µl, i.t.) were assessed in the ipsilateral (top panel) and contralateral (bottom panel) hind paws of sham (open bars/squares) and neuropathic (closed bars/squares) rats on day 14 post-surgery. Saline (30 µl, i.t.; dashed lines) was administered as a control. Thermal withdrawal latencies were measured prior to and at 10 minute intervals beginning at 20 minutes following DLT injection (A, C). The percentages of maximum possible effect (% M.P.E.) were calculated from the raw latency data (B, D). Data represent means ± s.e.m. Statistical analyses of latency data by 1-way ANOVA, Dunnett’s post hoc test within neuropathic or sham groups and of % M.P.E. data by 2-way ANOVA, Bonferroni MCT between all groups. Asterisks denote significant difference from respective pre-drug or saline value, *p < 0.05, **p < 0.01, ***p < 0.001. Number signs denote significant difference from sham-DLT, #p < 0.05, ##p < 0.01, ###p < 0.001.
ipsilateral effects of DLT were significantly greater in NP hind paws than in sham paws, whereas the contralateral effects were not significantly different. The effects of saline were negligible in both groups, indicating no effect of lumbar puncture and injection itself.

4.3.3 Effects of nerve injury on δOR subcellular localization

To determine the effects of nerve injury on δOR subcellular localization, the distribution of silver-intensified gold particles was assessed in dendritic profiles within the dorsal spinal cords of NP and sham rats. Consistent with previous reports (Cahill et al, 2001a; b), some gold particles were detected on plasma membranes; however, the vast majority was intracellularly localized within perikaryal and dendritic profiles, axons and, to a lesser degree, axon terminals. Some gold particles were observed in association with endoplasmic reticulum, golgi networks, and mitochondria, while many did not appear to be associated with any defined cellular structure. The majority of plasma membrane-associated δORs was found at extra-synaptic sites. The membrane perimeters and cross-sectional areas of dendritic profiles sampled from laminae I-II and V are outlined in Table 4.1. Only dendrites of sizes which completely conformed to the constant region-of-interest boundary on the electron microscope at 15,000x magnification were imaged. Most profiles were of similar size and shape; however the dendrites sampled from NP ipsilateral (NPi) laminae I-II regions were significantly smaller than those sampled from sham and NP contralateral (NPc) rats in the same laminar regions. The smaller size of NPi dendrites yielded artificially high δOR quantitative values (i.e. gold particles per unit
Table 4.1  Details of immunolabeled dendritic profiles sampled in each experimental group.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total number of dendritic profiles sampled$^a$</th>
<th>Average dendritic perimeter (µm)</th>
<th>Average dendritic cross-sectional area (µm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAMi</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I,II</td>
<td>85</td>
<td>9.39 ± 0.305</td>
<td>3.60 ± 0.208*</td>
</tr>
<tr>
<td>V</td>
<td>99</td>
<td>10.57 ± 0.263</td>
<td>4.14 ± 0.196</td>
</tr>
<tr>
<td>NPi</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I,II</td>
<td>132</td>
<td>8.76 ± 0.251</td>
<td>3.00 ± 0.159</td>
</tr>
<tr>
<td>V</td>
<td>133</td>
<td>10.05 ± 0.219</td>
<td>3.97 ± 0.174</td>
</tr>
<tr>
<td>NPc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I,II</td>
<td>88</td>
<td>9.99 ± 0.255**</td>
<td>3.87 ± 0.179**</td>
</tr>
<tr>
<td>V</td>
<td>102</td>
<td>10.50 ± 0.259</td>
<td>4.35 ± 0.197</td>
</tr>
</tbody>
</table>

$^a$From 5-7 serial ultrathin sections per animal for $n = 2-3$ animals

Roman numerals indicate laminar region from which dendrites were sampled within the dorsal horn. Statistical analyses between values within a laminar region were performed by one-way ANOVA, Bonferroni MCT. Asterisks denote significant difference from NPi laminae I,II; *$p < 0.05$, **$p < 0.01$. SHAMi: sham ipsilateral; NPi: neuropathic ipsilateral; NPc: neuropathic contralateral.
length membrane, particles per unit² area) which we felt were more reflective of dendritic size than of real differences in δOR expression. For this reason, δOR expression is normalized and expressed as a ‘per dendrite’ value in the current study. There were no differences in dendritic size amongst samples from the deeper dorsal horn.

Quantitative analyses of δOR ultrastructural distribution revealed a bilateral increase in cell surface expression of gold labeled δORs in lamina V, but not I-II, dendrites following unilateral nerve injury. Representative electron photomicrographs from laminae I-II and V are displayed in figures 4.3 and 4.4, respectively. The percentage of total gold particles associated with the plasma membranes of lamina V profiles from NP rats (NPi: 16.6%, NPC: 17.4%) was significantly higher than that of sham rats (11.1%; 1-way ANOVA, $F_{2,331} = 3.350, p < 0.05$; t-test vs sham: NPi $p < 0.05$, NPC $p < 0.05$; Fig. 4.5A). This change translates into a 50% and 57% increase in membrane-bound δOR in the ipsilateral and contralateral dorsal horns, respectively. There was no effect of injury on the total number of gold particles counted per dendrite (1-way ANOVA, $p > 0.05$; Fig. 4.5B), indicating that the increase in cell surface δOR expression was likely a result of redistribution rather than of increased biosynthesis. The density of δORs on the plasma membrane was similarly higher in NP rats than in sham rats (1-way ANOVA, Tukey’s MCT, sham vs NPC $p < 0.05$; t-test vs sham: NPi $p < 0.05$, NPC $p < 0.05$; Fig. 4.5C). To assess the overall distribution of δORs within dendritic profiles, the linear distance of each gold particle from the plasma membrane was measured. The distance was significantly shorter in the NP rats compared with shams (1-way ANOVA, $F_{2,1280} = 3.078, p < 0.05$; t-test vs sham: NPi $p < 0.05$, NPC $p < 0.05$; Fig. 4.5D).
Figure 4.3  Electron photomicrographs showing the subcellular localization of δOR immunoreactivity within laminae I-II dendrites from sham and day 14 neuropathic (NPi: ipsilateral; NPC: contralateral) rats. Silver-intensified gold particles occur at predominantly intracellular sites in all groups. Arrows indicate gold particles associated with the plasma membrane. D, dendrite; At, axon terminal. Scale bar = 2 µm applies to all.
Figure 4.4  Electron photomicrographs showing the subcellular localization of δOR immunoreactivity within lamina V dendrites from sham and day 14 neuropathic (NPi: ipsilateral; NPC: contralateral) rats. Silver-intensified gold particles occur at predominantly intracellular sites in all groups; however nerve injury increases cell surface density of δOR on both the ipsilateral and contralateral sides. Arrows indicate gold particles associated with the plasma membrane. D, dendrite; At, axon terminal. Scale bar = 2 µm applies to all.
Figure 4.5 Quantitative analyses of δOR immunogold labeling in the dorsal spinal cords of sham (open bars) and day 14 NP (closed bars: ipsilateral; shaded bars: contralateral) rats. (A) Percentage of total gold particles that was associated with the plasma membrane. (B) Total number of gold particles counted in each dendritic profile. (C) Average number of plasma membrane-associated gold particles in each dendritic profile. (D) Average linear distance linking gold particles with the nearest segment of plasma membrane. Roman numerals indicate laminar region from which dendrites were sampled within the dorsal horn. Data represent means ± s.e.m. Statistical analyses within each laminar region by 1-way ANOVA, Tukey’s MCT test and by two-tailed, unpaired t-test. Asterisks denote significant difference from sham value by Tukey’s MCT. * \( p < 0.05 \). Number signs denote significant difference from sham value by t-test. # \( p < 0.05 \).
4.5D), suggesting an overall mobilization of δORs from intracellular compartments toward the plasma membrane following nerve injury. Finally, the percentage of dendrites sampled which expressed one or more gold particles on the cell surface was calculated. In sham rats, 36.0% of dendrites expressed δOR on the plasma membrane. Following nerve injury, this population increased significantly to 51.2% in the ipsilateral dorsal horn (1-way ANOVA, Tukey’s MCT, $F_{2,4} = 9.488$, sham vs NPi $p < 0.05$; data not shown). The percentage of such dendrites in the contralateral spinal cord of NP rats also increased (49.8%); however this value failed to reach significance. Nerve injury did not produce any changes in δOR subcellular distribution within laminae I-II dendrites on either side of the spinal cord, as assessed by analyses identical to those outlined above (Fig. 4.5).

4.4 DISCUSSION

The present study demonstrates that spinal administration of a selective δOR agonist reversed hyperalgesia in the ipsilateral hind paws of NP rats and produced antinociception in the contralateral paws. No antinociceptive effects were observed in sham-operated rats. This enhancement in δOR function correlated with a bilateral increase in cell surface expression of δOR in lamina V dendritic profiles, with no such increase in lamina I-II dendrites. The increased cell surface expression reflected a redistribution of δORs from intracellular compartments to the neuronal plasma membrane, with no overall increase in the total number of δORs within these post-synaptic profiles.
4.4.1 Antinociceptive effects of a δOR agonist

As previously reported (Cahill et al, 2003a; Shinoda et al, 2007), unilateral nerve injury produced thermal hypersensitivity in the ipsilateral hind paw, as indicated by a significant reduction in withdrawal latencies in response to noxious heat. Sham-operated rats exhibited no changes in thermal responsiveness. The antihyperalgesic effects of DLT observed in NP rats in the current study are in accordance with previous reports that δOR agonists alleviated nerve injury-induced hypersensitivities to noxious thermal (Petrillo et al, 2003; Shinoda et al, 2007) and cold (Sohn et al, 2000; Mika et al, 2001; Holdridge and Cahill, 2007a) stimuli following peripheral, spinal, or supraspinal administration. Antinociceptive effects were also observed in the contralateral paws of NP rats, but were completely absent in sham rats. We similarly showed that local subcutaneous administration of DLT into the dorsum of the hind paw failed to increase paw withdrawal latencies in naïve animals in the same testing paradigm (Kabli and Cahill, 2007). These findings are interesting since we previously showed that DLT, at the same dose as used currently (10 µg, i.t.), produced marked analgesia in the hot water tail flick test in both naïve and NP rats, albeit the effect was greater in rats with nerve injury (Holdridge and Cahill, 2008). This suggests that thermal nociceptive inputs from these sites are differentially modulated by δOR. Indeed, a study by Stewart and Hammond (1993) supports this idea. Intrathecal DLT dose-dependently increased tail flick latencies but not paw withdrawal latencies in response to noxious heat in uninjured animals. They postulated that while the putative δOR-2 subtype – proposed by pharmacological studies and selectively responsive to DLT – may suppress the tail flick reflex, the involvement of
both δOR-1 and δOR-2 are required for attenuation of thermal paw responses. Moreover, the same group showed that spinal and supraspinal administration of DLT did attenuate thermal responsiveness when rats were inflicted with peripheral inflammation, increasing paw withdrawal latencies not only in the injured paw (Stewart and Hammond, 1994) but also in the uninflamed paw (Hurley and Hammond, 2000). Taken together, these observations suggest that δORs may undergo changes which augment endogenous function and act to suppress persistent pain in chronically injured rats. Indeed, thermal and mechanical hypersensitivities were significantly enhanced in δOR null mutant mice following peripheral nerve injury (Nadal et al, 2006) and peripheral inflammation (Gavériaux-Ruff et al, 2008). Consistent with the current study, acute thermal nociception in the hind paw was not altered by δOR gene deletion in sham rats. Hao and colleagues reported that δOR antagonism by naltrindole enhanced neuropathic pain behaviours in rats with ischemic spinal cord injuries but did not affect nociceptive thresholds in uninjured rats (1998b). These studies highlight an important role of the δOR in modulating neuropathic pain and underscore its potential as a novel pharmacological target. Indeed, Kamei et al. showed that δOR agonists produced greater attenuation of thermal nociception (1997) and acetic acid-induced abdominal constrictions (1995) in streptozotocin-treated diabetic rats compared with controls. Moreover, recent electrophysiological data revealed that DLT was more effective in attenuating c fibre-evoked responses in spinal nerve ligated rats than in non-ligated controls (Buesa et al, 2008).
4.4.2 Nerve injury-induced changes in δOR subcellular distribution

The mechanisms underlying δOR functional enhancement following nerve injury are unclear and we thus decided to assess the effects of nerve injury on subcellular δOR distribution within lumbar spinal cord neurons using immunogold electron microscopy. We examined δOR expression in two dorsal horn regions which act as important relay sites in the processing of nociceptive information: laminae I-II and V. The superficial dorsal horn is the major point of termination of most unmyelinated peptidergic and non-peptidergic c fibres as well as thinly myelinated Aδ fibres while the deeper horn receives afferent inputs from large myelinated A fibres, Aδ and peptidergic c fibres (Ribeiro-da-Silva and De Koninck, 2008). In the current study, some δOR labeling was evident in axons and axon terminals while a larger proportion was observed in association with neuronal perikarya and dendrites. In all neuronal profiles, δORs were localized to predominantly intracellular sites, with very few receptors located on the plasma membrane under basal conditions. This is consistent with previous ultrastructural data and with the current behavioral observation that DLT produced no antinociceptive effects in sham-operated rats. However, reports on the cellular distribution of spinal δORs are less congruent. Several electron immunohistochemical studies observed predominantly presynaptic δORs which were localized to large dense core vesicles in the terminals of nociceptive primary afferents (Dado et al, 1993; Cheng et al, 1995; Zhang et al, 1998) while others report more varied distribution of the δOR within pre- and post-synaptic profiles (Cheng et al, 1995; Cahill et al, 2001a; 2001b; 2003; Morinville et al, 2004). This discrepancy likely reflects differences in antisera recognition and may indicate the
existence of multiple δOR molecular variants which are differentially packaged and intracelluarly trafficked and which may, in fact, be disparately modulated by nerve injury. In the current experiments, we assessed δOR distribution within dendritic profiles. Nerve injury produced no change in total expression or in subcellular distribution of δORs within laminae I-II dendrites. In contrast, δOR cell surface expression increased by 50% within lamina V dendrites and this effect was observed on both sides of the spinal cord. This increase did not correlate with any increase in the average number of δORs per dendrite and therefore reflects a redistribution of existing δORs from intracellular sites toward the plasma membrane. Post-synaptic profiles in this region represent mainly intrinsic interneurons (Ribeiro-da-Silva and De Koninck, 2008) as well as projection neurons of the spinothalamic (ST) and dorsal column ascending tracts (Willis and Coggeshall, 1991). Accordingly, δORs observed over these profiles are well situated to modulate the transmission of nociceptive inputs and their recruitment to the cell membrane following nerve injury could underlie the enhanced antinociceptive effects of DLT in NP rats. Indeed, a mobilization of reserve receptors to the plasma membrane would increase receptor accessibility and facilitate endogenous opioid peptide binding during chronic pain states. It is interesting that the δOR trafficking event was bilateral. Unilateral arthritic (Donaldson, 1999) and nerve (Koltzenburg et al, 1999; Lowrie, 1999) injuries have previously been shown to precipitate neurochemical and anatomical changes on the contralateral side. Notably, early neuroanatomical reports described axonal communications of lamina V neurons with the contralateral cord by way of dorsal and ventral commissural interneurons in the lumbar region (Willis and
Coggeshall, 1991) and this has been proposed as a potential mechanism of contralateral changes following unilateral injuries (Koltzenburg et al, 1999). Indeed, unilateral injection of complete Freund’s adjuvant (CFA) produced bilateral increases in spinal δOR mRNA, although only the ipsilateral increase translated into increased δOR protein at the time point assessed (Cahill et al, 2003b). Equally intriguing in the current study was that δOR trafficking was evident only within lamina V dendrites. While laminae I-II neurons respond chiefly to nociceptors, lamina V neurons also receive both direct and indirect nociceptive and non-nociceptive inputs from the periphery and synapse extensively with neurons of the substantia gelatinosa via antenna-like dorsal dendritic projections (Willis and Coggeshall, 1991). Whereas some lamina V ST tract cells are selectively responsive to innocuous mechanical, noxious mechanical or thermal stimuli, other cells – aptly named wide dynamic range (WDR) neurons – respond to a variety of low- and high-threshold inputs (Price, 1988; Willis, 1985) and may serve to integrate afferent inputs from the entire sensory spectrum (Wall, 1973). Additionally, lamina V ST tract neurons display ‘wind up’ activity in response to repetitive C fibre inputs (Price et al, 1978). Thus, WDR neurons may contribute to the hypersensitive and multimodal symptoms of neuropathic pain (Pertovaara et al, 1997; Chu et al, 2004; Sotgiu et al, 2006). Interestingly, while prolonged morphine treatment resulted in δOR trafficking throughout the dorsal horn (Cahill et al, 2001b; Morinville et al, 2004), the δOR expressional and functional changes induced by CFA injection (Cahill et al, 2003b) and by dorsal rhizotomy (Morinville et al, 2004) were most pronounced in lamina V,
suggesting an important role of δOR within this laminar region in modulating nociception following a peripheral injury.

We indeed observed a shift in compartmentalization which produced a 50% increase in δOR cell surface expression; however, one pertinent question remains: what is the large remaining pool of intracellular δORs doing inside the cell? What function are these δORs carrying out? Surely the fate of these receptors in the absence of a definitive physiological stressor or injury is not merely to sit idle and await degradation. In actuality, the current ultrastructural data likely represent static ‘snap shots’ of what is actually a highly dynamic regulatory mechanism at work. Cell surface trafficking of δORs can be initiated by numerous in vitro and in vivo stimuli (see Appendix A). The molecular mechanisms underlying these trafficking events remain unclear; however they may entail regulation of molecular chaperones or accessory proteins which facilitate G protein-coupled receptor transport including the arrestins, homer proteins, and receptor activity modifying proteins, among many others (see Drake et al, 2006 for review). Further studies will be necessary to investigate the mechanisms involved in nerve injury-induced δOR translocation.

4.5 CONCLUSION

In the current study, we demonstrated that intrathecal administration of a δOR agonist attenuates nerve injury-induced thermal hyperalgesia in the ipsilateral hind paw and produces antinociception in the contralateral hind paw of NP rats. In contrast, no
antinociceptive effects were observed in sham-operated rats. Electron immunohistochemical analyses revealed an outward mobilization of δORs following nerve injury, thus increasing cell surface expression and ligand accessibility, and this trafficking event may underlie the enhancement in δOR agonist effects. Taken together, these data reveal the unique regulation of δOR following peripheral nerve injury and suggest that agonists at this receptor may be useful tools in treating neuropathic pain.
CHAPTER 5:
BEHAVIOURAL AND MORPHOLOGICAL EVIDENCE FOR THE INVOLVEMENT OF GLIAL CELL ACTIVATION IN δ OPIOID RECEPTOR FUNCTION: IMPLICATIONS FOR THE DEVELOPMENT OF OPIOID TOLERANCE

This work has been published in Molecular Pain:


5.1 INTRODUCTION

The opioid system, comprised of multiple highly homologous receptor families and their endogenous opioid peptide ligands, is fundamental to the modulation of the sensory and affective aspects of pain (Fields and Basbaum, 1994). Three classes of opioid receptors (ORs) have been identified through molecular and pharmacological techniques, namely the mu (µ), delta (δ), and kappa (κ) ORs (reviewed by Dhawan et al, 1996; Kieffer, 1999). Morphine, a classical µOR agonist with remarkable analgesic efficacy, is the current gold standard in the clinical treatment of moderate to severe pain; however, its use in the management of chronic pain may be restricted by the development of analgesic tolerance and the unwanted side effects associated with dose escalation. As such, understanding the mechanisms underlying opioid tolerance has become the primary
focus of an extensive research effort with the aim of uncovering novel therapeutic strategies to treat persistent, unremitting pain.

A growing body of evidence identifies the δOR as an instrumental player in the development of morphine-induced analgesic tolerance (reviewed by Zhang et al, 2006). Thus, concomitant administration of δOR antagonists with morphine (Abdelhamid et al, 1991; Fundytus et al, 1995; Wells et al, 2001; Riba et al, 2002; Roy et al, 2005) or antisense oligodeoxynucleotide treatment directed against the δOR (Kest et al, 1996) partially blocked the development of tolerance to morphine antinociceptive effects. In agreement with this data, δOR null mutant mice had a lower propensity to develop antinociceptive tolerance to morphine compared to their wild type littermates (Zhu et al, 1999; Nitsche et al, 2002). The mechanism by which δOR modulates μOR analgesic tolerance is not presently known, however, complex interactions between μ and δORs are likely to be relevant in eliciting various opioid-induced physiological responses. For example, direct coupling of μ-δORs in the form of hetero-oligomers has been demonstrated in both expression systems and spinal cord tissue (Gomes et al, 2004), which was proposed to underlie the antinociceptive synergy between μ and δOR agonists. We, and others, have also demonstrated that chronic activation of the μOR induces a translocation of δORs from intracellular compartments to neuronal plasma membranes and this phenomenon is correlated with an increase in δOR functional competence (Cahill et al, 2001; Ma and Zhao, 2002; Morinville et al, 2003; 2004; Hack et al, 2005). Taken together, the activation and translocation of δORs may represent an important
intermediary step in the development of morphine tolerance; however the mechanism underlying this trafficking remains unknown.

Several studies suggest an intimate and interactive relationship between opioids and glial cells. Once regarded as mere supports cells for CNS neurons, glial cells are now recognized as performing vital and complex functions in response to physiological stressors. Indeed, spinal glial activation has been observed in a number of pathological states including Alzheimer’s (Itagaki et al, 1989; Streit, 2002) and Parkinson’s (Du et al, 2001) diseases, HIV-associated dementia (Stoler et al, 1986; Kibayashi et al, 1996; Garden, 2002), as well as several persistent pain syndromes (Raghavendra et al, 2002: 2003a; 2003b; 2004b; Tsuda et al, 2003; 2005). Moreover, spinal glial cell activation has been linked to the development of opioid tolerance. Chronic morphine treatment was reported to activate microglial (Raghavendra et al, 2004a) and astrocytic (Song and Zhao, 2001; Raghavendra et al, 2004a) cells and to increase pro-inflammatory cytokine levels (Raghavendra et al, 2004a) in the lumbar spinal cords of tolerant rats. Accordingly, co-administration of a glial modulatory agent with morphine attenuated the spinal immune response and inhibited the loss of morphine analgesic potency (Song and Zhao, 2001; Raghavendra et al, 2004a), suggesting that spinal glia may contribute to mechanisms responsible for opioid tolerance.

In the current study, we aimed to investigate the functional relationship between δORs and glial cells following prolonged chronic morphine administration. We employed immunohistochemical techniques as well as a behavioural nociceptive paradigm to assess whether prolonged morphine treatment is associated with the
activation of spinal glial, and if indeed so, whether this spinal immune response is requisite for the observed enhancement in δOR-mediated antinociception.

5.2 METHODS

5.2.1 Animals

Adult male Sprague-Dawley rats (220-300 g; Charles River, Québec, Canada), were housed in groups of two with ad libitum access to food and water, and maintained on a reverse 12/12 h light/dark cycle. All behavioural experiments were performed during the dark phase of the cycle, and animals were handled prior to experimentation in order to reduce stress-related analgesia. All experimental protocols were approved by the Queen’s University Animal Care Committee, and complied with the policies and directives of the Canadian Council on Animal Care and the International Association for the Study of Pain.

5.2.2 Drug treatments

Rats were separated into one of four groups receiving i) morphine and intrathecal saline, ii) morphine and intrathecal propentofylline (inhibitor of glial activation), iii) intrathecal propentofylline alone, or iv) intrathecal saline alone (control group). Morphine sulfate (MS) was administered every 12 h by subcutaneous injections of increasing doses (5, 8, 10, 15 mg/kg in saline; Sabex, Kingston General Hospital, Kingston, Ontario, Canada). This treatment protocol was previously shown to induce the trafficking of
δORs from intracellular compartments to neuronal plasma membranes in cultured cortical neurons as well as in the spinal cord (Cahill et al, 2001). Propentofylline and saline (10 µg/30 µl diluted in saline and 30 µl, respectively; Sigma, St. Louis, MO, USA) were administered by intrathecal injection via lumbar puncture between the L4 and L5 vertebrae under brief isoflurane anesthesia every 24 hours for 5 days based on drug administration protocols required to block morphine tolerance (Raghavendra et al, 2004a). Successful drug placement was confirmed by a vigorous tail flick upon injection. All experiments were performed 12 hours following the final morphine injection.

5.2.3 Double-labeling fluorescent immunohistochemistry for confocal microscopy

Rats (n = 3 per group) were deeply anesthetized with sodium pentobarbital (75 mg/kg, i.p.; MTC Pharmaceuticals, Cambridge, Ontario, Canada) and transaortically perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; 500 ml, pH 7.4). The spinal cords were removed by spinal ejection and post-fixed in the above fixative for 30 minutes at room temperature and cryoprotected in 30% sucrose in 0.2 M PB for 48 hours at 4 °C. Lumbar segments were isolated and cut into 40 µm transverse sections on a freezing sledge microtome and collected in 0.1 M Tris-buffered saline (TBS; pH 7.4). Free-floating sections were incubated in a blocking solution containing 10% NGS, 10% BSA followed by incubation with a rabbit polyclonal antisera recognizing glial fibrillary acidic protein (GFAP; 1:2500 working dilution; DakoCytomation, Ontario, Canada) to label activated astrocytes and a mouse monoclonal antisera recognizing OX42 (1:1000 working dilution; Serotec, NC, USA) to label CD3/CD11B receptors on activated microglia. Spinal cord sections were incubated
overnight at 4 °C with both primary antibodies, followed by incubation with goat anti-
mouse and goat anti-rabbit secondary antibodies (both 1:200 working dilution; Molecular
Probes, Invitrogen, Ontario, Canada) conjugated to Alexa 594 and Alexa 488
fluorophores, respectively. To assess non-specific labeling, control sections were
processed in the absence of primary antibodies. Sections were mounted on glass slides,
air-dried, and cover-slipped using Aquamount (Fisher Scientific, Ontario, Canada).

Immunoreactive cells were visualized in the deep dorsal horn using the Leica TCS
SP2 multi photon confocal microscope (100x magnification; Leica Microsystems Inc,
Ontario, Canada) and images acquired and digitalized for quantitative analysis with Leica
Confocal Software. Twenty-five to thirty-five serial images were captured in 0.75 µm
increments throughout the z plane using identical acquisition parameters and x-y
coordinates for each of 12-20 immunoreactive cells per rat for n = 3 rats per experimental
group. The serial images were then stacked and reconstructed in three dimensions using
Image-Pro Plus v5.0 software (MediaCybernetics, MD, USA). Total cell volume (in
pixels$^3$) and cell surface area (in pixels$^2$) were calculated for each cell based on the three
dimensional cell reconstruction. Statistical analyses were performed using Excel XP
(Microsoft, Ontario, Canada) and Prism 4.01 (Graph Pad, San Diego, CA). The average
volume and surface area for cells within each treatment group were calculated and
expressed as means ± s.e.m. These values were compared by one-way ANOVA followed
by Tukey's post-hoc multiple comparison test. P values less that 0.05 were considered
significant.
5.2.4 Behavioural tail flick assay

The effects of a selective δOR agonist, [D-Ala]\(^2\)-deltorphin II (DLT; 10 µg/30 µl) DLT (10 µg/30 µl [i.t.]; Sigma), on thermal nociceptive responses were assessed using the hot water tail flick assay (Morinville et al, 2003). The distal 5 cm segment of the rat's tail was immersed in noxious 52 °C water, and the latency to a vigorous tail flick was measured. For \( n = 6 \) per group, three baseline latencies were measured prior to DLT injection in order to determine the normal nociceptive responses of the animals. A cut-off latency of four times the average baseline response threshold was imposed to avoid tissue damage in the event that the animal became unresponsive following DLT injection. Rats were then injected intrathecally with DLT. Thermal latencies were measured every 10 minutes following drug administration for 50 minutes. The percentage of maximum possible effect (% M.P.E.) of DLT was calculated at the 30 minute time point, as this time point corresponded with the maximum analgesic effect of DLT.

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% \text{M.P.E.} = \frac{\text{post-drug latency} - \text{baseline}}{\text{cut-off latency} - \text{baseline}} \times 100
\]

The thermal latencies to respond were analyzed by two-way ANOVA followed by Bonferroni post-hoc and the transformed % M.P.E. data were analyzed by one-way ANOVA followed by Tukey's post-hoc multiple comparison test. All values are expressed as means ± s.e.m. P values less than 0.05 were considered significant.
5.3 RESULTS

5.3.1 Prolonged morphine treatment induces spinal astrocytic and microglial hypertrophy

The effects of morphine treatment (5-15 mg/kg every 12 h; s.c.) on spinal astrocytes and microglia were assessed by fluorescent immunohistochemistry for confocal microscopy. Lumbar spinal cord segments from rats treated, or not, with morphine and/or the glial modulating drug, propentofylline (10 µg/30 µl; intrathecally [i.t.]), were processed for fluorescent detection of both GFAP and OX42, markers of astrocytes and microglia, respectively. Morphine treatment produced a significant increase in astrocytic ($F_{3,189} = 23.79$, $p = 0.0015$; Fig. 5.1) and microglial ($F_{3,101} = 8.403$, $p < 0.0001$; Fig. 5.1) cell volume as compared with saline-treated rats, indicating glial cell hypertrophy. Similarly, the surface areas of both astrocytic and microglial cells were significantly greater following morphine treatment as compared with saline-treated rats ($F_{3,189} = 18.70$, $p = < 0.0001$ for GFAP; $F_{3,101} = 10.32$, $p = < 0.0001$ for OX42; Table 5.1). Chronic intrathecal propentofylline administration effectively attenuated this morphine-induced hypertrophy, inhibiting both the increases in cell volume and in cell surface area. Propentofylline administration alone had no effect on astrocytic cell size as it produced no change in GFAP-immunoreactivity compared with controls. Interestingly, propentofylline alone significantly increased the cell volumes and surface areas of OX42-immunoreactive cells, indicating an effect on microglia which was independent of morphine treatment.
Figure 5.1 Detection of GFAP (panel A) and OX42 (panel B) in the dorsal lumbar spinal cords of rats treated, or not, with morphine was performed by fluorescent immunohistochemistry and photomicrographs were acquired by confocal microscopy. Displayed are representative three dimensional images of immunoreactive cells from rats receiving intrathecal saline (i, vi), morphine and intrathecal saline (ii, vii), morphine and intrathecal propentofylline (iii, viii), or intrathecal propentofylline alone (iv, ix). Morphine treatment produced a significant increase in both astrocytic and microglial cell volumes as compared with control. This hypertrophy was attenuated by coadministration of morphine with propentofylline. While propentofylline alone had no effect on GFAP-immunostaining, it significantly enhanced OX42-immunoreactive cell size. Data represent means ± s.e.m. for n = 12-20 cells per rat from n = 3 rats per group. Statistical analyses were performed by a one-way ANOVA followed by Tukey’s post-hoc multiple comparison test. The asterisks denote significant difference from saline-treated rats. * = p < 0.05, ** = p < 0.01, *** = p < 0.001. MS: morphine sulfate; PF: propentofylline; Sal: saline. Scale bar, 30 µm.
Table 5.1

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Saline + MS</th>
<th>PF + MS</th>
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<tr>
<td>GFAP</td>
<td></td>
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<td></td>
<td>41843.41 ± 1603.904 ***</td>
<td>57440.11 ± 1960.131</td>
<td>48338.16 ± 1667.994 **</td>
<td>43235.54 ± 1369.27 ***</td>
</tr>
<tr>
<td>OX42</td>
<td>50418.92 ± 2441.907 ***</td>
<td>79908.27 ± 6287.605</td>
<td>55693.48 ± 4137.721 **</td>
<td>67083.38 ± 3756.654</td>
</tr>
</tbody>
</table>

Total cell surface area in pixels² was calculated from the three dimensional reconstructed images of GFAP- and OX42-immunopositive cells within the dorsal spinal cords of rats treated with saline, saline and morphine, propentofylline and morphine, or propentofylline alone. Data represent means ± s.e.m. for n = 12-20 cells per rat from n = 3 rats per experimental group. Statistical analyses were performed by one-way ANOVA followed by Tukey’s post-hoc multiple comparison test. The asterisks denote significant difference from rats treated with saline and morphine. ** = p < 0.01, *** = p < 0.001. MS: morphine sulfate; PF: propentofylline.
5.3.2 Morphine-induced enhancement in δOR-mediated antinociception is attenuated by propentofylline

The acute effects of the DLT on thermal nociceptive thresholds in rats receiving chronic morphine, with or without the concomitant administration of propentofylline or vehicle are depicted in Figure 5.2. Baseline latencies were similar in all treatment groups, indicating no effect of pretreatment on normal thermal nociception (Figure 5.2A). Rats that received prolonged morphine treatment had significantly higher latencies at 30 minutes post-DLT injection when compared to rats pretreated with saline (Treatment $F_{3,15} = 8.739$, $p < 0.001$; Time $F_{5,15} = 19.41$, $p < 0.001$), indicating a morphine-induced enhancement in δOR-mediated analgesia. Concomitant treatment of morphine with propentofylline significantly attenuated the morphine-induced enhancement in δOR ligand effects. Animals pretreated with morphine displayed significantly greater %M.P.E. values than did saline-treated animals ($F_{3,20} = 6.700$, $p = 0.0026$; Figure 5.2B). The enhancement in DLT-mediated analgesia following morphine treatment was blocked when animals were co-treated with propentofylline. These animals displayed % M.P.E.s similar to control animals, indicating no change in δOR activity. Chronic administration of propentofylline alone had no effect on the analgesic effects of DLT, yielding % M.P.E.s similar to control animals.
Figure 5.2 The antinociceptive effects of DLT (10 µg, i.t.) were assessed in the tail flick acute thermal pain test. Rats chronically treated with morphine exhibited enhanced δOR-mediated analgesia as compared with controls and this enhancement was blocked by chronic co-administration of morphine with the glial modulatory agent, propentofylline. All testing was performed 12 h following the final morphine injection. (A) The latencies to respond with a brisk tail flick were measured prior to and at 10 minute intervals following DLT administration for 50 minutes. Three pre-drug latencies were averaged to obtain a baseline latency value for each rat. (B) Mean tail flick latencies at 30 minutes post-DLT injection were converted to % M.P.E. values. Statistical analyses of thermal latencies were performed by two-way ANOVA followed by Bonferroni post-hoc while statistics for transformed % M.P.E. data were accomplished by one-way ANOVA followed by Tukey’s post-hoc multiple comparison test. Data represent means ± s.e.m. for n = 5-6 rats per group. The asterisk denotes a significant difference from saline-treated rats. * = p < 0.05. 0: Baseline prior to drug administration; MS: morphine sulfate; PF: propentofylline.
5.4 DISCUSSION

Opioid agonists are highly efficacious analgesics; however their clinical use is limited by the incidence of adverse effects, particularly the development of analgesic tolerance following repeated use. A growing body of evidence identifies an important role for the δOR in modulatingterone tolerance (Abdelhamid et al, 1991; Fundytus et al, 1995; Kest et al, 1996; Wells et al, 2001; Riba et al, 2002; Roy et al, 2005) and this phenomenon may involve the trafficking of δORs from internal stores toward the neuronal plasma membrane, thereby enhancing the effects of δOR-selective ligands (Cahill et al, 2001; Ma and Zhao, 2002; Morinville et al, 2003; 2004; Hack et al, 2005; Zhang et al, 2006; Cahill et al, 2007). The mechanism by which this contributes to morphine tolerance is unknown; however recent studies support an active role for spinal glia following chronic morphine treatment (Song and Zhao, 2001; Raghavendra et al, 2004a). In the current study, we investigated the relationship between δORs and glial activation and indeed demonstrate a functional role for spinal glia in morphine-induced changes in δOR agonist effects. Moreover, administration of a glial inhibitor effectively blocked these changes in δOR function.

The involvement of spinal glia in the modulation of morphine analgesia has been demonstrated in both preclinical (Lazriev et al, 2001; Song and Zhao, 2001; Raghavendra et al, 2002; 2003b; 2004a; Narita et al, 2004; Johnston and Westbrook, 2005) and clinical (Lu et al, 2004) studies. We hypothesized that the recruitment of glial cells is a gradual response to long-term morphine administration and may be detectable at time points earlier than those at which analgesic tolerance is established. We therefore assessed the
spinal immune response using a 48 h morphine dosing schedule; one which has been shown to have substantive effects on δOR trafficking and function (Cahill et al, 2001; Morinville et al, 2003; 2004; Lucido et al, 2005). This dosing regimen does not produce a state of tolerance (Morinville et al, 2003); however it may initiate mechanisms involved early in the cascade of events leading to opioid tolerance. In developing a means of assessing the three dimensional structures of GFAP- and OX42-immunoreactive cells, we observed significant increases in cell volume and surface area of fluorescent GFAP- and OX42-immunoreactive cells in the dorsal spinal cord following prolonged morphine treatment. These results are in accordance with previous studies (Song and Zhao, 2001; Raghavendra et al, 2004a) illustrating the recruitment of glia in the events precipitating opioid tolerance. Morphine-induced glial hypertrophy was attenuated by co-administration with propentofylline. Interestingly, while propentofylline administration alone had no effect on astrocytes, it produced significant microglial hypertrophy in comparison with saline-treated rats. It is not clear why this occurs, since the combination of morphine and propentofylline did not show such an effect. The neuroprotective role of microglia in the CNS is well known and this cell population is very much attuned to its microenvironment, responding swiftly to even subtle physiological changes (Kreutzberg, 1996). It is possible that the localized administration of an exogenous compound into the spinal canal, in the absence of any ‘pathological’ events, was sufficient to produce a microglial response, although such an observation has not been reported previously (Raghavendra et al, 2004a). Nevertheless, additional functional studies are necessary to determine whether this propentofylline-induced increase in cell size was indeed
accompanied by an inflammatory response. Despite microglial hypertrophy, however, neither baseline tail flick latencies nor deltorphin-mediated analgesia were altered following propentofylline administration alone, suggesting that this increase in microglial cell size was not functionally relevant in our study.

Activation of both glial cells and δORs appears to be important in the mechanisms of morphine tolerance, however it is unknown whether these two events are mutually exclusive or if, in fact, they represent important and related intermediary steps in the development of tolerance. Previous studies demonstrate that δORs are trafficked from internal stores toward the neuronal plasma membrane following morphine treatment, correlating with an increased functional competence of the receptor (Cahill et al, 2001; Morinville et al, 2003; 2004); however it is not known if the spinal immune response observed following morphine is requisite for this δOR trafficking event. Therefore, our second series of experiments aimed to examine the functional role of spinal glia in morphine-induced changes in δOR function. Consistent with earlier reports (Melchiorri et al, 1992; Cahill et al, 2001; Morinville et al, 2003; Gendron et al, 2006), we observed a significant augmentation in δOR-mediated effects in rats treated with morphine. This enhancement was effectively blocked by co-administration of morphine with propentofylline, demonstrating an integral role of spinal glial activation in the functional changes in δOR.

Taken together with previous reports that glial inhibition prevents the development of morphine tolerance (Song and Zhao, 2001; Raghavendra et al, 2003b; 2004a), this study provides additional evidence for the role of δORs in opioid tolerance
and suggests that glial activity may precipitate changes in the δOR, including receptor trafficking. Glial cell activity has been documented to modulate the trafficking of ionotropic channels such as AMPA receptors (Leonoudakis et al., 2004; Stellwagen and Malenka, 2006); however the current study is the first to our knowledge to suggest such a modulation of a G protein coupled receptor. The precise mechanism by which glial-modulated functional changes in δOR may occur is unclear; however two possible mechanisms include i) increased efficiency with which the receptor couples to intracellular signaling cascades, and/or ii) enhanced cell surface expression of the receptor. Future experiments will be required to investigate these possibilities.

5.5 CONCLUSION

In the present study, we demonstrate a relationship between δOR function and spinal glial activation. Indeed, prolonged administration of morphine induced the activation of astrocytic and microglial cells in the lumbar spinal cord, which correlated with enhanced antinociceptive effectiveness of a δOR agonist. Moreover, attenuation of glial activation with propentofylline, a glial inhibitor, attenuated the enhancement of δOR agonist-mediated effects. These data support an intimate relationship between glial and opioidergic function and provide insight into the mechanisms by which opioid analgesic tolerance develops.
CHAPTER 6:  
DISCUSSION

6.1 SUMMARY

Neuropathic pain is an exceptionally debilitating chronic pain disorder that is a challenge to diagnose and an even greater challenge to treat. A variety of etiologies have been identified (Table 1.4) and diagnoses are primarily based on medical histories and physical examinations (Gilron et al, 2006), however the diagnosis itself provides little insight into the underlying mechanisms which contribute to NP pain. Moreover, the etiology of the disease has proven to be a poor index of whether a particular class of analgesic drugs will be effective in treating the pain (Woolf and Mannion, 1999). Commonly described as burning, shooting or shock-like, NP pain is characteristically resistant to traditional analgesic therapy and clinical management often includes the use of nontraditional analgesics such as antidepressants and anticonvulsants (Table 1.5). However, these treatment strategies often do not offer adequate pain relief or tolerability. This thesis project aimed to investigate the potential therapeutic benefit of δOR-selective agonists in the management of NP pain.

In the current series of experiments, rats that underwent sciatic nerve injury displayed characteristic postures and behavioural manifestations including cold and thermal hyperalgesia as well as tactile allodynia in the ipsilateral hind paw. The spinal administration of DLT, a δOR-selective agonist, dose-dependently reversed tactile
allodynia in NP rats and attenuated cold and thermal hypersensitivities. Moreover, DLT produced greater antinociceptive effects in NP rats compared with controls in the cold water paw withdrawal, hot water tail flick, and Hargreaves thermal plantar box tests. This increase in δOR functional competence did not appear to be due to increased δOR biosynthesis as western blots revealed no change in spinal δOR protein. We hypothesized that an alternative mechanism, such as redistribution of receptors within the neuron, may underlie δOR function changes.

Previous reports of augmented δOR function have linked these changes to primary sensory afferent inputs (Morinville et al, 2004) and, as such, we investigated the role of capsaicin-sensitive afferents in altered δOR functional competence following nerve injury. In contrast with vehicle-treated NP rats, NP rats that were treated neonatally with capsaicin showed no increase in δOR agonist effects in the hot water tail flick test, suggesting that primary nociceptive afferent drive is required for nerve injury-induced δOR changes. While nerve injury decreased spinal IB4-IR, this is unlikely to underlie changes in δOR function since neonatal capsaicin treatment, which produced a more dramatic decrease in IB4-immunolabeling, returned δOR agonist effects to normal levels. The increase in δOR functional competence could not be attributed to increased δOR biosynthesis since δOR-IR did not change following nerve injury, corroborating our previous western blot data.

While most GPCRs are localized to neuronal plasma membranes where they are available to interact with extracellular ligands, the majority of δORs is retained at intracellular sites. However, the trafficking of δORs from internal stores toward the.
plasma membrane can be initiated by various pro-inflammatory (Cahill et al, 2003b; Morinville et al, 2004; Patwardhan et al, 2005) and pro-nociceptive (Guan et al, 2005, Gendron et al, 2006) stimuli and this cell surface recruitment has correlated with increased δOR functional competence. Using immunogold electron microscopy techniques, we showed that nerve injury indeed increased the cell surface expression of δORs within post-synaptic profiles of the dorsal horn via redistribution of existing receptors. Interestingly, this event was observed bilaterally in the deep dorsal horn, with no change in δOR distribution in the superficial laminae.

The mechanisms underlying nerve injury-induced δOR trafficking remain unclear however we may take cues from other δOR trafficking events. Prolonged treatment with morphine has repeated been shown to induce δOR trafficking to the cell surface in vitro (Cahill et al, 2001b, Hack et al, 2003; Lucido et al, 2005; Gendron et al., 2006; Ma et al, 2006) and in vivo (Cahill et al, 2001b; Morinville et al, 2003; 2004b) and this cell surface recruitment correlates with an increase in δOR agonist effects. Additionally, chronic morphine treatment has also been reported to induce glial hypertrophy and proliferation in the spinal cord (Song and Zhao, 2001; Raghavendra et al, 2004a). The local release of cytokines from activated spinal glia serves to attenuate morphine-induced antinociception (Raghavendra et al, 2004a). Accordingly, repression of the spinal immune response with glial metabolic inhibitors prevents the development of morphine tolerance (Song and Zhao, 2001; Raghavendra et al, 2003b; 2004). However, it is unclear whether such factors also modulate δOR trafficking and functional competence. In a model of central inflammation, δOR function was shown to be enhanced rather than reduced (Cahill,
unpublished observations) suggesting that glial factors may indeed influence δOR-induced effects. Thus, in Chapter 5 we aimed to investigate whether morphine-induced changes in δOR function were linked to changes in spinal glial activity. Indeed, concomitant treatment of rats with morphine and propentofylline, a glial inhibitor, prevented both the activation of spinal glia and the changes in δOR agonist effects observed with morphine alone, suggesting that glial activity contributes to morphine-induced δOR trafficking in vivo. These data support an intimate relationship between glial and opioidergic function and may provide insight into the mechanisms underlying nerve injury-induced δOR trafficking.

6.2 PROPOSED MODEL OF NERVE INJURY-INDUCED δOR CELL SURFACE RECRUITMENT

The mechanisms underlying nerve injury-induced trafficking of δORs to neuronal plasma membranes are unclear; however the data generated in Chapters 2-5 provide a framework from which the following hypothesis is formed.

PROPOSED MODEL: Sciatic nerve injury is known to produce sensitization both peripherally (Chung and Chung, 2002; Devor, 2006b) and centrally (Laird and Bennett, 1993; Devor, 2006a). Hyperexcitable primary afferents are important in driving activity-dependent changes that contribute to the pathogenesis and maintenance of NP pain (Woolf and Salter, 2000). Increased afferent discharge at the central terminals would result in excess glutamate and nociceptive neuropeptides at dorsal horn synapses and potentiation of synaptic strength in pain pathways. In addition to neurons, spinal glia
express many neurotransmitter receptors including those for glutamate (Gallo and Ghiani, 2000; Verkhratsky, 2007), SP (Palma and Maggi, 2000; Rasley et al, 2002), CGRP (Reddington et al, 1995) and ATP (Walz et al, 1993). Spinal microglia and astrocytes become activated following peripheral nerve injury and exhibit changes in both morphology and function. Activated glia perpetuate the excitatory environment in two ways: i) disruption of glutamate homeostasis through impaired transporter-mediated uptake (Tawfik et al, 2008) as well as calcium-mediated release (Araque et al, 1999) of glutamate, and ii) increased synthesis and release of cytokines and inflammatory mediators, arachidonic acid derivatives and reactive oxygen species (Kreutzberg, 1996; Raghavendra et al, 2003). The glial response therefore serves to generate and maintain a ‘sensitized’ state which reinforces the excitability of surrounding neurons and glia in a positive-feedback manner. Increased excitatory neurotransmission invariably leads to increased influx of sodium and calcium ions and intracellular kinase activity in dorsal horn neurons, which increases nociceptive signal propagation and leads to changes in protein expression/function/localization which increase neuronal responsiveness. We hypothesize that compensatory measures are similarly triggered by excitatory activity which serve to counteract the hyperexcitable state and restore neuronal activity to normal levels. The recruitment of δORs from internal stores and insertion into the plasma membrane increases the accessibility of receptors to their endogenous peptide agonists in the extracellular environment and represents a means of inhibitory control during chronic pain states. The importance of enhanced δOR function following nerve injury is evident following pharmacological antagonism (Hao et al, 1998b) or genetic deletion (Nadal et
al, 2006) of δOR, where NP pain is exacerbated while nociceptive pain behaviours in uninjured rats remain unchanged. The mechanisms underlying these trafficking events are not clear but we propose that sustained activation of intracellular messengers and kinases acts to ‘rally up the troops’. Delta OR functional enhancement in response to bradykinin involves PKC activity (Patwardhan et al, 2005) while morphine-induced δOR trafficking was absent in mice lacking β-arrestin-2 (Hack et al, 2005) or calcium calmodulin-dependent protein kinase IV (unpublished observations from our laboratory in collaboration with Dr. Min Zhou). Increased intracellular calcium and kinase activity in dorsal horn neurons may lead to altered function of chaperone and accessory proteins that govern the subcellular trafficking of δORs, such as receptor transporter protein 4, which was recently shown to regulate cell surface trafficking of the µ-δ oligomer in vitro (Décaillot et al, 2008).

Thus, we propose that the increased afferent discharge from sensitized primary afferents following nerve injury serves to activate and wind-up dorsal horn neurons and recruit spinal astrocytes and microglia which, upon activation, contribute to and maintain a sensitized excitatory state. Increased excitatory neurotransmission produces enhanced intracellular calcium concentrations and increased kinase activity which triggers the cell surface insertion of δORs in order to counteract the hyperexcitable state of dorsal horn neurons. Accordingly, intervention in this cascade by dampening nociceptive afferent drive (with neonatal capsaicin treatment) or the spinal immune reaction (with propentofylline treatment) would attenuate changes in δOR functionality following nerve injury, as was observed in Chapters 3 and 5.
6.3 RATIONALE FOR NERVE INJURY MODEL

Several rodent models of NP pain are available for preclinical investigation (Table 1.6). While these models produce behavioural manifestations which resemble those observed clinically, there are advantages and disadvantages to each. The two most commonly used NP pain models include spinal nerve ligation (SNL) and CCI. The SNL model involves the tight ligation of the L5 or L5 and L6 spinal nerves (Kim and Chung, 1992) and is widely used in preclinical pain research. It produces robust thermal and tactile hypersensitivities and is an important experimental tool in determining the contributions of injured (L5-6) and surrounding uninjured (L4) axons to pathological pain because the injury is specifically localized. However, a complete transection of a segmental spinal nerve is not a common cause of NP pain in the clinical population; traumatic nerve injuries are more likely to occur at peripheral axon sites and nerve dysfunction resulting from metabolic disorders or viral infections does not cause a nerve transection. Furthermore, the sham surgery which serves as a control for the SNL procedure is relatively invasive and involves separation of the paraspinal muscles, removal of the L5 and L6 transverse processes and surrounding facet joints as well as more extensive post-operative care. The CCI model was chosen for the current studies because it is a widely used procedure that is easy to perform, is minimally invasive and the injury is more reflective of common mechanical injuries in humans including nerve compression due to carpal tunnel syndrome, lumbar disk hernation, osteoarthritic bone spurs and the growth of cysts or tumours. The CCI model was originally described by Bennett and Xie (1988) and involves a loose constriction of the common sciatic nerve
with 4 chromic gut ligatures. While the Bennett model produces hypersensitivity and spontaneous pain behaviours reminiscent of clinical NP pain, its shortcomings include variability in suture tightness and spacing between individual ligations and between operators as well as the substantive inflammatory reaction produced by the chromic gut suture itself. In an effort to circumvent these issues, Mosconi and Kruger revised this model using a fixed-diameter polyethylene cuff (1996). This model was chosen for the current studies and affords a uniform nerve constriction with no local immune reaction (Plong, unpublished observations).

**Caveats:** One must be mindful that rodent nerve injury models, like all experimental models, have some shortcomings. These models produce consistent, highly reproducible pain behaviours in nearly all rats afflicted with the injury and they are very useful in assessing neurochemical and pathophysiological changes in a very controlled environment. However, NP pain is not a certain consequence of nerve injury in humans; some patients experience no further complications following tissue repair (Sommer, 2001). Furthermore, NP pain in rodents typically resolves over a period of weeks to months whereas human patients often live with this chronic ailment indefinitely. Nevertheless, rodent models have greatly facilitated the preclinical investigation of the pathophysiology underlying NP pain. Furthermore, they produce characteristic NP pain behaviours such as hyperalgesia, allodynia and spontaneous pain which enable the preclinical investigation of novel therapeutic interventions.
6.4 EMERGING QUESTIONS AND FUTURE DIRECTIONS

The current behavioural, immunohistochemical and molecular techniques serve to validate the δOR as a novel pharmacological target in the management of NP pain. Nevertheless, numerous unanswered questions warrant further exploration of the role of δORs following nerve injury.

6.4.1 All antibodies are not created equally: What impact does δOR antisera selection have on data interpretation?

Our knowledge of OR expression in the central and peripheral nervous systems is based largely on autoradiographic ligand binding assays and immunohistochemical detection. Accordingly, the patterns of OR expression are determined by the selectivity of the ligands and antisera used. Not surprisingly, there is some discord in the literature with respect to the cellular and subcellular localization of δORs (Cahill et al, 2001a; Stone et al, 2004; Chabot-Doré et al, 2008; Mennicken et al, 2003; Morinville et al, 2004). Marked δOR expression has been repeatedly demonstrated in the superficial lamina (I-II) by autoradiographic (Goodman et al, 1980; Moskowitz and Goodman, 1984; Sharif and Hughes, 1989; Besse et al, 1990; 1992) and immunohistochemical (Dado et al, 1993; Arvidsson et al, 1995; Zhang et al, 1998; Robertson et al, 1999; Cahill et al, 2001a; Mennicken et al, 2003; Morinville et al, 2004; Stone et al, 2004) techniques. Reports of δOR expression in the deeper dorsal horn are less congruent, however. Several studies described δOR predominantly in laminae I-II (Goodman et al, 1980; Moskowitz and Goodman, 1984; Dado et al, 1993; Arvidsson et al, 1995; Zhang et al, 1998; Robertson et
al, 1999), while others report more widespread δOR mRNA (Mansour et al, 1994a; Wang and Wessendorf, 2001; Mennicken et al, 2003), binding sites (Stevens et al, 1991a; Gouardères et al, 1993; Mennicken et al, 2003) and immunoreactive labeling (Mailly et al, 1999; Cahill et al, 2001a; Mennicken et al, 2003; Morinville et al, 2004) throughout the spinal gray matter. Furthermore, there is some discrepancy as to which cell populations express δOR. Some groups have reported predominantly presynaptic δOR-IR at the ultrastructural level (Cheng et al, 1995; Zhang et al, 1998) and substantial decreases in fluorescent δOR-IR (Dado et al, 1993) and δOR ligand binding (Besse et al, 1992) following dorsal rhizotomy, while others observed both pre- and postsynaptic ultrastructural labeling (Cheng et al, 1997; Cahill et al, 2001a; b; Morinville et al, 2003) and an increase in δOR fluorescent ligand binding in rhizotomized animals (Morinville et al, 2004). Moreover, Cahill and colleagues analyzed the cellular and subcellular distribution of δOR using two commercially available antisera: one N-terminus-directed and one C terminus-directed (Cahill et al, 2001a). While most of the δOR-IR profiles were dendritic with both antisera, there was significantly greater presynaptic labeling with one antibody compared with the other. The same study showed a substantive difference in the percentage of plasma membrane-associated δORs in dendrites and axons depending on the antibody used. Taken together, these divergent reports of δOR expression may suggest multiple molecular species of the δOR as well as disparate ligand and antisera binding affinities to the putative molecular variants. In support of this, western blot analyses revealed slight differences in the molecular weights of immunoreactive bands using different δOR antisera (Belcheva, 1996; Cvejić and Devi,
1997; George et al, 2000; Cahill et al, 2001a). Slight variations in post-translational modifications and complex formations may be a means of differential regulation of δORs by the cell. It is feasible that even modest molecular differences may dictate interactions with accessory proteins and chaperones as well as trafficking to various intracellular compartments. Moreover, these putative molecular variants may be differentially modulated following nerve injury. Thus, one must be mindful when interpreting the results of seemingly incongruent studies.

6.4.2 How do primary afferent neurons contribute to changes in δOR function?

6.4.2.1 Role of capsaicin-sensitive afferents

The data presented in Chapter 4 revealed an increase in spinal δOR agonist effects in NP rats compared with controls and suggested that this functional change was dependent on capsaicin-sensitive primary afferent neurons. It is unclear, however, which population of neurons is responsible for driving these changes and by what mechanism they do so. In contrast with other reports (Noguchi et al, 2003; Hammond et al, 2004), we observed no change in spinal expression of the neuropeptides, SP and CGRP, following nerve injury. We did observe a significant bilateral reduction in IB4-immunolabeling however this is unlikely to underlie changes in δOR function since neonatal capsaicin treatment, which produced a more dramatic decrease in IB4-immunolabeling, returned δOR agonist effects to normal levels. Furthermore, Morinville and colleagues examined the contribution of primary afferents to spinal changes in δOR subcellular localization (2004). Rhizotomized animals displayed a significant decrease in
ipsilateral spinal IB4-IR while δOR cell surface expression was augmented in these rats, suggesting that the deafferentation itself produced δOR trafficking. This contrasts the current observation that neonatal capsaicin attenuated IB4-IR while decreasing δOR function and, together, these studies suggest that nerve injury produces changes in δOR localization and function independently of changes in lectin-binding afferents.

We hypothesize that a separate and, as yet unidentified, subpopulation of capsaicin-sensitive primary afferents may modulate δOR function. Kanai and colleagues reported a significant increase in TRPV1 protein expression in the ipsilateral dorsal horn at days 7 and 14 following CCI (2005). Interestingly, total TRPV1 expression in the DRG has been shown to decrease following nerve injury (Rasband et al, 2001; Schafers et al, 2003) while others report selective up-regulation of TRPV1 in particular DRG cell groups (Hudson et al, 2001; Fukuoka et al, 2002). Preliminary immunohistochemical experiments in our laboratory revealed an increase in spinal TRPV1-IR, and this increase was attenuated in capsaicin-treated NP rats (Fig. 6.1). However, the TRPV1 expression pattern observed was inconsistent with previous reports and we intend to reassess the changes in TRPV1 with an alternative antiserum.

6.4.2.2 Role of glia

Chapter 5 revealed a contributory role of spinal glial activation to changes in δOR agonist activity following prolonged morphine treatment. There is similarly ample evidence supporting a link between glial activation and neuropathic pain states. Once considered mere support cells for CNS neurons, astrocytes and microglia are increasingly recognized as important players in the pathophysiological changes incurred by nerve
Figure 6.1 Fluorescent TRPV1-IR in spinal cords of naïve and day 7 neuropathic (NP) rats treated neonatally with capsaicin (CPS) or vehicle (Veh). Representative photomicrographs of (A) whole spinal cords; (B) ipsilateral (Ipsi) dorsal horns; (C) high-magnification image of TRPV1-IR in vehicle-treated NP. Contra: contralateral.
Peripheral nerve injury has repeatedly been shown to induce the hypertrophy, functional hyperactivity, and migration of astrocytes and microglia in the lumbar spinal cord (Jin et al, 2003; Raghavendra et al, 2002; 2003; Tsuda et al, 2003; 2005; Bursztajin et al, 2004; Zhuang et al, 2005; Narita et al, 2006; Tanga et al, 2006; Zhang and De Koninck, 2006; Zhuang et al, 2006; Moss et al, 2007), while the suppression of glial activation by glial metabolic inhibitors prevents and/or reverses NP pain behaviours in nerve-injured rats (Raghavendra et al, 2003a; 2003b; Taraneh et al, 2006; Tawfik et al, 2007). It is possible that similar glial involvement may underlie, in part, the nerve injury-induced changes in δOR distribution and function reported in Chapters 2-4. In other modalities, δOR trafficking was shown to be dependent on μOR function as trafficking to plasma membranes was absent in μOR null mutant animals. Hence, μOR function is necessary to precipitate δOR plasma membrane trafficking. It is tempting to generalize that perhaps any condition that leads to reduced μOR function will also induce δOR trafficking. This hypothesis proposes that nerve injury induces glial activation, causing the release of pro-nociceptive chemicals which contribute to decreased μOR function and it is perhaps this loss of function that initiates δOR trafficking. Furthermore, if δOR trafficking is dependent on afferent drive and sensitization of post synaptic neurons, then normalization of the pain state by glial inhibitors would neutralize processes that lead to δOR trafficking. Preliminary experiments in our laboratory have indeed shown that chronic treatment with the glial inhibitor, propentofylline, prevents the δOR functional changes observed in saline-treated NP rats, suggesting that activated glia may contribute to δOR trafficking events (Cahill, unpublished observations). Hence,
glial cells in the dorsal horn may help to ‘translate’ increased primary afferent drive following nerve injury into postsynaptic δOR changes. This notion is merely speculative and remains to be addressed experimentally. Interestingly, the microglial (Fig. 6.2) and astrocytic (Fig. 6.3) response in the ipsilateral dorsal horn following nerve injury was attenuated in rats that received neonatal treatment with capsaicin. These data show correlation, not cause, but do support a link between nerve injury, glial activation, and δOR functional changes. It is also noteworthy that the mechanism by which propentofylline inhibits glial activity is unclear. Propentofylline is known to weakly antagonize adenosine A1 receptors and to inhibit adenosine reuptake (Deleo et al, 1988; Borgland et al, 1998). It has similarly been shown to stimulate nerve growth factor release (Nabeshima et al, 1993) and to inhibit cAMP phosphodiesterase activity (Borgland et al, 1998) as well as ischemia-induced release of reactive oxygen intermediates from macrophages and microglia (Banati et al, 1994). It is unclear whether these mechanisms contribute to the observations in Chapter 5.

6.4.2.3 Pre-synaptic vs. post-synaptic

As discussed earlier, the selection of δOR antibody influences the pattern of δOR cellular and subcellular expression observed by immunodetection and, consequently, the effects on which of nerve injury. The ultrastructural immunohistochemical experiments in Chapter 4 were performed using the Chemicon AB1560 δOR antibody (N3-17 amino acid sequence). In agreement with previous studies using this antibody (Cahill et al,
Figure 6.2 Fluorescent OX42-IR in spinal cords of naïve and day 7 neuropathic (NP) rats treated neonatally with capsaicin (CPS) or vehicle (Veh). Representative photomicrographs of (A) whole spinal cords; (B) ipsilateral (Ipsi) dorsal horns. Contra: contralateral.
Figure 6.3 Fluorescent GFAP-IR in spinal cords of naïve and day 7 neuropathic (NP) rats treated neonatally with capsaicin (CPS) or vehicle (Veh). Representative photomicrographs of (A) whole spinal cords; (B) ipsilateral (Ipsi) dorsal horns. Contra: contralateral.
2001a; 2001b; Morinville et al, 2003), while some gold-conjugated δORs were observed in association with presynaptic axon terminals (Fig. 6.4), δOR-IR was primarily localized to intracellular sites within dendritic elements. Accordingly, dendritic δORs were counted and analyzed for the current study. This, of course, by no means implies that δOR changes do not occur in other neuronal profiles including primary afferents, as was reported in small and/or medium DRG neurons in response to agonist exposure, membrane depolarization (Bao et al, 2003; Gendron et al, 2006) or chronic inflammation (Gendron et al, 2006). Indeed, additional ultrastructural analyses are required using different antisera to more adequately study presynaptic events.

The results of Chapter 3 suggest that capsaicin-sensitive primary afferents are required for nerve injury-induced changes in δOR function. We did not observe changes in total δOR protein and cannot conclude from these experiments whether the functional changes occurred pre- or postsynaptically. Indeed, nerve injury could have produced δOR trafficking on capsaicin-sensitive DRG neurons which were abolished following neonatal treatment with the neurotoxin. Alternatively, increased afferent drive from capsaicin-sensitive DRG neurons in NP rats could have precipitated postsynaptic changes in δOR localization and function which were absent in capsaicin-treated NP rats. Additional experiments are required to investigate these possibilities. The utilization of saporin-ligand conjugates has been useful in examining the roles of various DRG and spinal cell populations by selectively destroying their cellular targets (Tarpley et al, 2004; Allen et al, 2006). In our studies, we would first have to determine an appropriate cell population to target (ie. the IB4-saporin used by Tarpley and colleagues would not likely
Figure 6.4  Representative electron photomicrograph depicting δOR-IR axon terminal in ipsilateral lamina V dorsal horn of day 14 neuropathic rat.
yield positive data in our experiments since IB4 changes do not correlate with δOR functional changes).

6.4.3 How do these data translate?

6.4.3.1 To female rats?

The experiments outlined in Chapter 3 showed that while neonatal capsaicin did not alter thermal nociceptive thresholds in naïve or NP rats, it did attenuate changes in δOR agonist activity following nerve injury. We aimed to duplicate this study in female rats to investigate whether the data was transferrable between sexes. Preliminary results from the female study have yielded vastly different results from the male study. Firstly, the antinociceptive effects of spinal DLT in females peaked at 30 min following injection, as opposed to 20 min for males (Fig. 6.5). Naïve and NP female rats showed similar DLT activity at the 20 min time point however the effects of DLT in naïve females trailed off thereafter, while DLT-antinociception in NP females persisted (Fig. 6.5A, C). Surprisingly, neonatal treatment with capsaicin appeared to decrease thermal nociceptive thresholds in both naïve and NP female rats (Fig. 6.5B). This observation stands in stark contrast with previously published studies which utilized neonatal capsaicin – all of which, to our knowledge, were performed using male rats. Moreover, the effects of intrathecal DLT were augmented in capsaicin-treated female rats (Fig. 6.5A, C, D). Finally, as reported in Chapter 2, CCI produced a time-dependent decrease in mechanical paw withdrawal thresholds, indicating the development of tactile allodynia in the injured hind paw. Allodynia in male rats was evident by post-surgical day 3-4 and
Figure 6.5 Deltorphin (DLT)-mediated antinociception in hot water tail flick test in naïve and neuropathic (NP) female rats treated neonatally with capsaicin (CPS) or vehicle (Veh).  

(A) Time course of tail flick latencies following DLT (10 µg, i.t.);  

(B) Comparison of pre-drug latencies at 50 and 52°C;  

(C) Latency data expressed as percentage of maximum possible effect (% M.P.E.);  

(D) Calculated area under the curve (AUC) for % M.P.E. time course.  

Data represent means ± s.e.m. for n = 7–8 rats per condition.  
Statistical analyses by one-way ANOVA followed by Tukey’s post-hoc MCT.  
Asterisks denote significant difference (**p < 0.01; ***p < 0.001).
persisted throughout the study period, maximizing at day 14. Female rats similarly
developed tactile allodynia during the first week, however mechanical thresholds often
returned to baseline by day 14 (Fig. 6.6). These results are preliminary and further
experiments are planned however they suggest that data obtained in published reports –
most of which were generated using male rodents – are not necessarily transferrable to
females. Future studies in our laboratory will re-examine the behavioural and
pharmacological changes induced by nerve injury in female populations.

6.4.3.2 To humans?

Ultrastructural immunohistochemical and behavioural data suggest that δORs are
localized to intracellular sites and play a relatively minor role in modulating nociception
under basal conditions. These receptors can be recruited to the membrane in response to
various stimuli and physiological stressors (Table 1.3, Appendix A). Rodent studies have
revealed δOR and mRNA expression throughout the spinal gray matter
(Mansour et al, 1994a; Mailly et al, 1999; Cahill et al, 2001a; Wang and Wessendorf,
2001; Mennicken et al, 2003; Morinville et al, 2004) and in DRG cells of all sizes (Wang
and Wessendorf, 2001; Mennicken et al, 2003; Kabli and Cahill, 2007), suggesting that
δORs may perform a variety of roles in the rodent nervous system. In contrast,
autoradiographic ligand binding studies in humans showed predominantly superficial
δOR binding sites in the dorsal horn while in situ hybridization experiments revealed
virtually no δOR mRNA signal in the spinal cord, indicating that the δOR binding sites
were localized to presynaptic primary afferent terminals (Mennicken et al, 2003).
Furthermore, the δOR hybridization signal was greater in human DRGs than in rodent
Figure 6.6 Time course of tactile paw withdrawal thresholds (P.W.T.) in ipsilateral hind paws following chronic constriction injury (CCI) in capsaicin-treated (red) and vehicle-treated (black) neuropathic female rats.
DRGs, and was preferentially localized to small and medium human DRG cells. One could speculate that the rodent data represent a ‘snap shot’ of the δOR early in its evolution. The pattern of expression in humans suggests that δOR may play a more specialized role in nociception than it does in rodents. Indeed, the antinociceptive effects of spinal DADLE in humans were marked (Onofrio and Yaksh, 1983), as compared with the modest effects of δOR agonists in naïve rats. It would be extremely interesting, albeit difficult, to more fully characterize the δOR in human tissue, particularly with respect to its subcellular distribution. In any case, the work of Mennicken and colleagues suggests that δOR agonists may be even more efficacious pharmacological tools than rodent studies would suggest.

6.4.4 The role of δOR complexes in DLT-mediated antinociception

In chapter 4, we used immunogold electron microscopy to directly visualize δOR within dorsal horn dendrites of NP and naïve rats. Our ultrastructural analyses revealed a bilateral shift in distribution of δOR from internal compartments toward the plasma membrane in lamina V neurons. This trafficking event correlated with enhanced δOR-mediated antinociception in a behavioural pain testing paradigm. As a continuation of our neonatal capsaicin study in female rats, we decided to assess δOR cell surface expression in the dorsal horn using a new technique. Recall from section 1.2.2.6 that agonist-GPCR binding and the subsequent interactions with G proteins results in the phosphorylation of the receptor by GRKs. One would hypothesize that nerve injury-induced δOR trafficking would result in augmented agonist-δOR interactions at the cell surface and subsequently an increase in the levels of phosphorylated δOR. Accordingly,
we assessed phosphorylated δOR levels in the dorsal spinal cords of NP and naïve female rats treated neonatally with capsaicin or vehicle. Spinal cord tissue was harvested 10 minutes following injection of DLT (30 µg i.t.). Protein samples were subjected to gel electrophoresis and transferred to nitrocellulose membranes. Membranes were then probed for phospho-δOR (Ser363; Cell Signaling Technology 3641, lot #1; 1:1000 working dilution). Preliminary data revealed enhanced phosphorylated δOR levels in CPS-treated rats compared with vehicle-treated rats, indeed corroborating the behavioural observations in the hot water tail flick test (Fig 6.7). What was perhaps more interesting, however, was the molecular size of the phosphorylated receptor. The phospho-δOR (Ser363) antibody was characterized previously in HEK293 cells stably transfected with, and overexpressing, δOR following incubation with a δOR agonist. The molecular weight was estimated to range from 50 to 65 kDa and likely corresponds with the phosphorylated monomeric δOR. In our study, we observed one immunoreactive band at approximately 166 kDa, suggesting that the δOR molecular species phosphorylated by DLT in vivo is in the form of a complex. Indeed, recent studies have increasingly suggested that opioid receptors form functional complexes with one another (Jordan et al, 2000) and with other GPCRs such as α2 adrenoceptors (Stone et al, 1997; Jordan et al, 2003; Rios et al, 2004; Riedl et al, 2009). It remains unclear whether the molecular δOR species trafficked to neuronal plasma membranes following nerve injury is monomeric or oligomeric and further studies in our laboratory will address this question.
Figure 6.7 Identification of the phosphorylated δOR protein by western blotting. Membranes from dorsal spinal cords of naïve and neuropathic (NP) female rats treated neonatally with capsaicin (CPS) or vehicle (Veh) were isolated 10 minutes following deltorphin injection (DLT; 10 µg, i.t.) or no injection and proteins resolved and electroblotted onto nitrocellulose membranes. Immunoblot analysis revealed one immunoreactive band with estimated molecular weight of 166 kDa. BM: biomarker; NPi: neuropathic ipsilateral; NPC: neuropathic contralateral.
6.4.5 What other ways can pain be measured?

Pain is a multi-faceted, highly subjective experience in humans. The relationship between a peripheral stimulus and the perception of that stimulus is not a linear one; it is dependent on a plethora of variables including the presence of other somatic stimuli and psychological factors such as arousal, attention and expectation, which in turn are influenced by contextual cues that determine the relative significance of the stimulus in order to generate an appropriate response to it (Fields et al, 2006). Accordingly, direct measurements of pain in animals are difficult and such information must be inferred from objective measurements of thresholds and latencies to evoked responses by specific stimuli. Unevoked or spontaneous pain is assessed by recording the characteristic nocifensive behaviours displayed during a defined observation period. While useful, these controlled experimental environments are not always representative of the day-to-day experiences of the clinical NP pain population. We therefore look to newer paradigms to evaluate pain in rodents. Our laboratory is currently working to validate several new techniques as reliable indices of NP pain including measurements of cardiovascular function, motor behaviour and conditioned place aversion. A pilot project with Dr. Michael Adams of this Department aims to assess changes in cardiovascular measures such as heart rate and blood pressure in NP and sham-operated rats implanted with radiotelemetric transducers. These rats can be monitored 24 hours per day in their home cages with minimal handling by investigators and may reveal patterns of cardiovascular activity which correlate with developing NP pain symptoms following nerve injury. Moreover, it may be possible to infer ongoing pain – and the ability of δOR
agonists to reverse it – in NP rats by such patterns. We have also assessed changes in motor behaviour following nerve injury using a voluntary running wheel apparatus in which the rat can choose to roam freely on the cage floor or run in the wheel. Wheel running is generally a stimulating and rewarding exercise for caged rodents and thus a decrease in voluntary wheel running activity in nerve injured animals may be a novel measure of NP pain-induced impairment. Finally, we are currently working on a conditioned place aversion paradigm to assess whether patterns of aversive behaviour may be associated with nerve injury. The latter two studies are particularly interesting because they are analogous with the adverse impact on patient functionality and day-to-day life. More importantly, the ability of pharmacological agents to improve these experimental measures in rodents may indicate highly clinically relevant therapeutic benefits. The impact of NP pain on overall quality of life in humans is dependent on more than the intensity of the pain, but also the functional impairments that interfere with daily activities, hobbies, work and relationships. In addition, we aim to incorporate video-based locomotor analysis technology to quantify pain-induced gait adaptations in nerve-injured rats.
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Appendix A:

Trafficking of δ-opioid receptors and other G-protein-coupled receptors: implications for pain and analgesia

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A cell can regulate how it interacts with its external environment by controlling the number of plasma membrane receptors that are accessible for ligand stimulation. G-protein-coupled receptors (GPCRs) are the largest superfamily of cell surface receptors and have a significant role in physiological and pathological processes. Much research effort is now focused on understanding how GPCRs are delivered to the cell surface to enhance the number of ‘bioavailable’ receptors accessible for activation. Knowing how such processes are triggered or modified following induction of various pathological states will inevitably identify new therapeutic strategies for treating various diseases, including chronic pain. Here, we highlight recent advances in this field, and provide examples of the importance of such trafficking events in pain.

G-protein-coupled receptors and pain

G-protein-coupled receptors (GPCRs) have a significant role in normal physiological processes and can contribute to pathological states when such processes are disrupted [1,2]. Indeed, drugs that either directly or indirectly modulate GPCR function have proved to be effective therapeutics for the treatment of many disease states, and as many as 50% of marketed drugs target GPCRs [2]. GPCRs have also been implicated in either the suppression or generation of states symptomatic of chronic pathological pain including hyperalgesia (exaggerated response to a normally painful stimulus), allodynia (pain in response to a normally innocuous stimulus) and paroxysmal or spontaneous pain (Table 1). Chronic pain is thought to affect 17–31% of the population in North America – Canadian Pain Coalition (http://www.canadianpaincoalition.ca/). In addition to the physical and psychological consequences and the deleterious effects on quality of life of a sufferer, chronic pain has a tremendous economic impact and is associated with costs estimated to be over US$150 billion annually in the USA through healthcare expenses, disability and other expenditures. Considering the impact that chronic pain has on our society, a crucial need exists for the development of more effective pharmacotherapies due to the vast degree of unmet medical needs in this area.

Some GPCRs, such as cannabinoid (CB) and opioid receptors, have validated therapeutic value for pain management (Table 1), and continued exploitation of these receptor families has yielded more selective, potent analgesics with favorable side-effect profiles (for recent review, see Ref. [3]). Various institutions have mandated the identification and characterization of orphan GPCRs to discover novel receptor targets that have potential for treating chronic pain. This strategy led to the discovery of sensory neuron-specific receptors (SNSRs) [4], which seem to have discrete, appropriate anatomical localization and physiological properties consistent with a role in pain processing and thus are a feasible target for drug development to treat chronic pain. Nevertheless, we need not rely solely on the discovery or deorphanization of GPCRs for novel pain targets, because modifying the cell surface density of a specific GPCR can result in altered functional responses. Investigation of such events, and ways in which to exploit them to modulate cellular responses, is at an early stage. Certainly, one of the most intriguing prospects offered by controlling or regulating cell surface receptor density could be the treatment of pain.

Trafficking of GPCRs

The density of GPCRs at the plasma membrane is dynamic and is regulated by several processes that seek to adjust cellular responsiveness to external stimuli. Much of the research to date on the trafficking of GPCRs has concentrated on the events elicited after the application of agonist. Following agonist binding and the induced conformational change in the receptor, the ‘activated’ receptor is phosphorylated by G protein-receptor kinases recruited from the cytosol (reviewed in Refs [5–8]). This phosphorylation event and the ensuing recruitment of one or more guanine nucleotide-binding regulatory proteins (GPRPs) (reviewed in Refs [5–8]) results in rapid ‘desensitization’ of the receptor (reviewed in Refs [5,6,8–10]). The subsequent internalization of the ligand–receptor complex (also known as receptor-mediated endocytosis) reduces the density of receptors at the cell surface but does not necessarily lead to a decrease in the overall number of receptors (receptor downregulation). The internalized receptor can be recycled back to the cell surface or can be directed to the lysosomes for receptor degradation leading to ‘long-term desensitization’ of a receptor
(Figure 1). Several processes are thus implicated in regulating receptor density after the application of an agonist.

Events modulating the intracellular trafficking or routing of receptors to the plasma membrane before agonist stimulation can also have profound consequences on receptor function and cellular responsiveness (Figure 2). GPCRs must undergo a continual process of maturation, where proteins are exocytosed from the endoplasmic reticulum (ER) to the plasma membrane by greatly

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**Table 1. GPCRs and pain**

<table>
<thead>
<tr>
<th>GPCRs targeted by clinically available analgesic drugs</th>
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<tbody>
<tr>
<td>Opioid receptors</td>
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<tr>
<td>Cannabinoid receptors</td>
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<tr>
<td>GABA&lt;sub&gt;B&lt;/sub&gt; receptors</td>
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<tr>
<td>α&lt;sub&gt;2&lt;/sub&gt;-Adrenoceptors</td>
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**GPCRs implicated in pain**

<table>
<thead>
<tr>
<th>Adenosine receptors</th>
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<tr>
<td>Calcitonin-gene-related peptide receptor</td>
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<tr>
<td>Cholecystokinin receptors</td>
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<td>Galanin receptors</td>
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<tr>
<td>GPR10 (prolactin-releasing peptide receptor)</td>
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<tr>
<td>5-Hydroxytryptamine receptors</td>
</tr>
<tr>
<td>Melanocortin receptors</td>
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<tr>
<td>Muscarinic receptors</td>
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<tr>
<td>Neurexinin U receptors</td>
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<tr>
<td>Neuropeptide Y receptors</td>
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<tr>
<td>Nociceptin receptor (opioid-like receptor 1)</td>
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<tr>
<td>Oxytoxin receptors</td>
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<tr>
<td>Prokineticin receptors</td>
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<tr>
<td>Prostaglandin receptors</td>
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<tr>
<td>Somatostatin receptors</td>
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<tr>
<td>Bradykinin receptors</td>
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<tr>
<td>Chemokine receptors</td>
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<tr>
<td>Dopamine receptors</td>
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<tr>
<td>G-protein-coupled receptor 7 (GPR7, neuropeptide B receptor)</td>
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<tr>
<td>Histamine receptors</td>
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<tr>
<td>Kinin receptors</td>
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<tr>
<td>Metabotropic glutamate receptors</td>
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<td>Neurokinin receptors</td>
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<td>Neuropeptide FF receptors</td>
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<td>Neurotensin receptors</td>
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<td>Orexin receptors</td>
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<tr>
<td>Parathyroid hormone receptor 2</td>
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<tr>
<td>Proteinase-activated receptors</td>
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<tr>
<td>Sensory neuron-specific receptors (Mas-related gene receptors)</td>
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<td>Vasoactive intestinal peptide receptors</td>
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</table>

*Table lists GPCRs with published findings related to modulation of pain or nociception. This list is not exhaustive and shows that although GPCRs have an important role in the transmission and processing of painful stimuli, few GPCRs have so far been exploited therapeutically.
conserved mechanisms (reviewed in Refs [11,12]). Only successfully folded proteins are exported from the ER to the Golgi complex where they can undergo posttranslational modifications such as glycosylation. Upon exiting from the trans Golgi network, proteins are sorted to the constitutive or the regulated vesicular pathway. In the constitutive pathway, vesicles containing proteins are constantly exported to the plasma membrane, whereas in the regulated pathway, vesicles are exported to the plasma membrane in response to a particular signal. Although the information on this topic is scarce, GPCRs are generally believed to be exported from the trans Golgi network to the plasma membrane through the constitutive pathway, although exceptions have been reported.

Formation and trafficking of functional receptors leading to cell surface expression and activity have also been demonstrated to occur by means of multiple regulatory proteins (for recent review, see Refs [13–16]). Chaperone molecules, such as the receptor-activity-modifying proteins (RAMPs; for review, see Ref. [17]) have been implicated in the proper folding or exocytosis (or both) of some GPCRs to the cell membrane. Chemicals have also been reported to rescue intracellularly retained mutant proteins; for example, 4-phenylbutyric acid led to the secretion of the intracellularly trapped α1-antitrypsin both in vitro and in vivo [18]. In contrast to the nonspecific actions of chemical chaperones, cell-permeable opioid ligands (‘pharmacological chaperones’) promoted the maturation of immature δ-opioid (DOP) receptors present in the ER in HEK293S cells, leading to enhanced DOP receptor plasma membrane density [19]. In fact, pharmacological chaperones might account for the paradoxical augmentation of opioid-induced analgesia and attenuation of morphine tolerance by ultra-low doses of opioid receptor antagonists [20], whereby the opioid antagonists act as chaperones for the maturation of DOP receptors to retain morphine-induced analgesia. [Such a hypothesis assumes that DOP receptor trafficking modulates mechanisms responsible for μ-opioid (MOP) receptor desensitization or tolerance.]

Recently, it has been reported that some GPCRs are localized within intracellular compartments and seem to be fully functional, but are awaiting a certain stimulus to be targeted to the cell surface. In vitro studies have proposed that homologous (the same receptor) or heterologous (different receptors) cell surface recruitment could be one of the mechanisms responsible for regulating plasma membrane receptor density. In one example of homologous recruitment, stimulation with dopamine D1 agonists for 1–15 min led to targeting of intracellular D1 receptors to the cell surface of renal epithelial cells [21]. Heterologous recruitment has also been reported where atrial natriuretic peptide induced the trafficking of D1 receptors to plasma membranes in a renal epithelial cell line and in kidney cells [22]. Additionally, neuropeptide Y causes recruitment of cell surface α-adrenoceptors to a renal epithelial cell line [22]. Thus, agonist treatment of one receptor can potentially affect the cell surface expression of either the same protein, or proteins from the same or different receptor classes.

The focus of the current review is to summarize, in the context of pain, research aimed at assessing the events modulating the density of GPCRs at the plasma membrane before the application of a ligand. Other comprehensive review articles on the regulation of GPCR trafficking, including receptor maturation processes, are available [7,23]. DOP receptors will be used as a model system because much research aimed at investigating GPCR trafficking to the cell surface before agonist application in the context of pain and analgesia has studied this receptor. Examples from other GPCRs will also be discussed, with an emphasis on findings with potential applications to relieve pain.

**A case in point: modifying DOP receptor cell surface density to improve analgesic potency**

Substantial interest has existed for several decades in developing selective DOP receptor ligands for the treatment of chronic pain because DOP receptor ligands are believed to have a much lower abuse potential than MOP receptor agonists such as morphine [24–26] in addition to reduced respiratory [27–29], cognitive [30,31] and gastrointestinal [32,33] impairments. Preclinical studies have demonstrated that δ-selective agonists elicit antinociception in various persistent and chronic pain models including inflammatory [34–38], neuropathic [26,39,40] and cancer [41] pains. Furthermore, spinal administration of
DOP receptor agonists in acute (tail-flick and hot-plate) was accompanied by increased antinociceptive potency of change in the subcellular distribution of DOP receptors stimulation of MOP receptors produced targeting of DOP receptors to the plasma membrane probably accounts for the observed augmented functional competence of DOP receptors rather than a change in protein synthesis. Box 1 highlights mechanisms involved in the translocation of DOP receptors and ensuing functional consequences subsequent to chronic morphine treatment (Table 2).

Translocation of DOP receptors from intracellular compartments to neuronal plasma membranes could account for the enhanced antinociceptive effectiveness and intracellular signaling of δ-selective agonists in chronic pain states. Indeed, chronic inflammatory pain induced by intraplantar injection of complete Freund’s adjuvant (CFA) induced an increase in the cell surface expression of DOP receptors in postsynaptic [38,51] and presynaptic [54] sites in the dorsal spinal cord ipsilateral to the site of injury. The enhanced translocation of DOP receptors correlated with a leftward shift in the dose-dependent reversal of thermal hyperalgesia following spinal administration of a selective DOP receptor agonist [38]. Thus, events that alter DOP receptor subcellular localization have profound consequences for receptor function, and have implications for pain management.

The lessons learned from trafficking of DOP receptors to the plasma membrane before agonist application might not be directly applicable to other GPCRs. However, they do suggest that, in general, trafficking of GPCRs to the plasma membrane might be a regulated process that could be exploited pharmacologically, as was illustrated above with MOP receptor agonist treatments and DOP receptor cell surface recruitment.

**Mechanisms underlying trafficking events of GPCRs involved in pain**

**DOP receptor**

In addition to mechanisms cited earlier, enhanced plasma membrane expression of DOP receptors was also shown to occur in cultured dorsal root ganglion (DRG) neurons following brief depolarization by capsaicin, elevated extra-cellular potassium or ATP [57,58]. These latter studies have demonstrated that such activity-dependent trafficking events were mediated through a regulated pathway rather than the constitutive pathway because DOP receptors were inserted into large dense-core vesicles for transport to neuronal plasma membranes (for review, see Ref. [59]). Although such results have not been consistently reported [54,60,61], activity-dependent translocation of DOP receptors in DRG neurons following in vivo administration of capsaicin or induction of chronic inflammation has been demonstrated [54]. In addition, the population of DRG neurons exhibiting cell surface DOP receptor targeting was dependent on the type of stimulus, suggesting that modality-specific activity regulates receptor trafficking [54]. Indeed, there exist multiple pathways for regulated receptor translocation, in addition to evidence for receptor trafficking to distinct membrane compartments [62]. Figure 3 illustrates the various mechanisms proposed to trigger DOP receptor trafficking to neuronal plasma membranes.

The activity-dependent translocation of GPCRs, such as DOP receptors, raises the question of whether neuronal responsiveness is dynamically regulated by electrical activity and what advantage it poses to GPCR responsiveness. One provocative possibility is that activity-dependent...
control of agonist responsiveness at GPCRs might be part of a mechanism that controls or modulates synaptic plasticity, which is fundamental to the generation of various pain states. Using the DOP receptor as an example, we know that these receptors are localized to intracellular and plasma membranes that extend along the soma, axon, terminals and dendrites in various neuronal types within the peripheral and central nervous systems. Translocation to augment cell surface expression can be induced by various stimuli, including brief depolarization or noxious stimulation (Table 2), raising the question of whether DOP receptors could have an important role in modulating activity-dependent plasticity and thereby dampening or reversing mechanisms maintaining chronic pain states. A putative role for DOP receptors in activity-dependent synaptic plasticity has been reported in the hippocampus [63], but evidence of such effects remains absent in regions important for pain transmission. A more simplistic generalized view is that stimulus-evoked translocation of GPCRs to neuronal plasma membranes is an inherent mechanism that has evolved to control the transmission of nociceptive information to higher brain centers. However, whether regulation of GPCR trafficking is responsible for the modulation of synaptic events associated with various pain states has not been directly addressed.

### Other GPCRs

For other receptors, association with several accessory proteins seems to be necessary for proper delivery to the plasma membrane and for functional activity (for recent reviews, see Refs [13–15]). For instance, in the case of GABAr receptors, heteromeric assembly between GABA receptor subunits was shown to be necessary for cell surface expression and receptor recognition characteristics in addition to coupling to intracellular signaling cascades [64–67]. GABAr receptors are known to control neuronal excitability and modulate synaptic neurotransmission; they have an important role in many physiological processes.

### Table 2. In vitro and in vivo trafficking of DOP receptors

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Cell population</th>
<th>Mechanism</th>
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<td><strong>In vitro</strong></td>
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<td>DOP receptor agonist</td>
<td>DRG culture neurons and PC12 cells</td>
<td>(\Delta\text{pH} ) through Ca(^{2+}) influx and release of Ins(1,4,5)P(_3)-sensitive intracellular stores (blocked by NTI)</td>
<td>[57]</td>
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<td>Pharmacological chaperone possibly through receptor palmitoylation</td>
<td>[19,95]</td>
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<td>GABA-containing neurons in PAG</td>
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<tr>
<td></td>
<td>Nucleus accumbens, dorsal neostriatum, but not frontal cortex</td>
<td>?</td>
<td>[53]</td>
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<td>DRG neurons</td>
<td>?</td>
<td>[54]</td>
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<tr>
<td></td>
<td>GABA-containing neurons in NRM</td>
<td>?</td>
<td>[55]</td>
</tr>
<tr>
<td></td>
<td>Cortical culture neurons</td>
<td>MOP receptor activation</td>
<td>[49]</td>
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<tr>
<td>K(^+)</td>
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<td>[57]</td>
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<tr>
<td>K(^+) (NGF?)</td>
<td>PC12 cells</td>
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<td>Forced swim test</td>
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*This table summarizes stimuli shown to induce trafficking of DOP receptors from intracellular compartments to neuronal plasma membranes in various cell types including neurons and transfected systems. The mechanisms identified in the trafficking event for individual studies are indicated.

*Abbreviations: CTAP, \(\alpha\)-Phc-Cys-Tyr-\(\alpha\)-Trp-Arg-Thr-Pen-Thr-NH\(_2\); DRG, dorsal root ganglion; NGF, nerve growth factor; NRM, nucleus raphe magnus; NTI, naltrindole; PAG, periaqueductal gray; Pen, penicillamine; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol myristate acetate.

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activities and have been implicated in a variety of neurodegenerative and pathophysiological disorders including chronic pain.

Homer proteins participate in the regulation of metabotropic glutamate (mGlu) receptors. Because mGlu receptors can modulate nociceptive processing at various levels of the nervous system (spinal and supraspinal) and are crucially involved in both peripheral and central sensitization associated with prolonged and chronic morphine treatment [71], Homer proteins contain a PDZ-like domain that specifically binds to mGlu receptors, and these proteins are rapidly induced by excitatory synaptic activity in neurons [69]. Such proteins have been found to regulate the retention (Homer 1b) or maturation (Homer 1a) of mGlu receptors to be inserted into the plasma membrane [70] and are required for clustering (Homer 1c) of the mGlu receptors at the cell surface in neuronal dendrites [71]. Additionally, Homer 1a was previously shown to attenuate constitutive (agonist-independent) activity of type I mGlu receptors [72], demonstrating that this protein modulates not only trafficking, but also signaling. Conversely, long-form Homer proteins are not only involved in GPCR trafficking but are also important in the coupling of type I mGlu receptors to intracellular mitogen-activated protein kinases (MAPKs) [73], which are important messengers linking synaptic activity to nuclear transcriptional control of plasticity-related genes including those involved in chronic pain [74]. A recent study identified that Homer 1a operates in a negative feedback loop to regulate the excitability of the pain pathway in an activity-dependent manner in a model of chronic inflammatory pain [75]. In this study, preventing the activity-induced upregulation of Homer 1a exacerbated inflammatory pain, most probably as a result of the role of Homer 1a in uncoupling glutamate receptors (metabotropic and ionotropic) from intracellular signaling cascades, which in turn resulted in counteracting spinal cord sensitization. Thus, modulating the activity of Homer proteins, in turn resulting in alterations in mGlu receptor function, could be a new therapeutic avenue to alleviate chronic pain.

Figure 3. Proposed model of stimulus-induced DOP receptor insertion into the neuronal plasma membranes based on currently available data. DOP receptors are transported from the rough endoplasmic reticulum (1) of DRG, spinal cord dorsal horn, or periaqueductal gray (PAG) neurons through the trans Golgi network (2) and targeted to neuronal plasma membranes by either constitutive or regulated pathways (3). In the Golgi complex, DOP receptors undergo posttranslational modifications, such as glycosylation, to yield mature, functional receptors. Maturation of the protein is enhanced by permeation of DOP receptor ligands (4) that act as chaperones to enable enhanced transport to the cell surface. Trafficking of DOP receptors to neuronal plasma membranes following prolonged or chronic morphine treatment probably does not involve maturation of the protein, but alternative mechanisms that have yet to be identified. However, stimulation of MOP receptors (5) and recruitment of β-arrestin proteins are necessary for the morphine-induced effect, although it is unknown whether the MOP receptors are present on the same or adjacent cells. It has been proposed that stimulation of MOP receptors might contribute to the formation of MOP-DOP receptor heterodimer complexes, which might, in turn, regulate cellular signaling and synaptic targeting. Mature DOP receptors are packaged into vesicles, such as LDCVs (3), for intracellular storage and transport by the regulated pathway. Functional DOP receptors are trafficked to and inserted into the neuronal plasma membrane by vesicular exocytosis (6). Membrane insertion of DOP receptors can be induced by increases in intracellular calcium (7) produced by application of extracellular stressors such as high potassium, ATP or capsaicin through activation of ligand-gated ion channels (LGIC). DOP receptor stimulation by agonist (8) similarly induces DOP receptor targeting through rises in intracellular calcium by release of inositol (1,4,5)-trisphosphate (IP3)-sensitive calcium stores or direct opening of ion channels. Bradykinin 1 (BK-1) receptor activation also causes targeting of DOP receptors to the cell surface through a protein kinase C (PKC)-independent pathway (9).
A RAMP protein was shown to be required for the transport of calcitonin receptor-like (CRL) receptors to the plasma membrane, but the RAMP protein associated with the receptor dictated the pharmacological profile: thus, RAMP1 association was necessary for a mature calcitonin gene-related peptide (CGRP) receptor, but RAMP2 produced an adrenomedullin receptor [76]. Furthermore, it was recently reported that Apg8L, a GABA<sub>A</sub> receptor-associated protein-like 1 belonging to a family of microtubule-associated proteins, was necessary for the cell surface trafficking of κ-opioid (KOP) receptors [77]. As can be seen from these examples, several GPCRs implicated in pain processing are regulated through the actions of accessory or chaperone proteins. These accessory or chaperone proteins could represent an alternative target for modulating pain. For example, strategies aimed at reducing the expression of RAMP1 or impairing the coupling of RAMP1 with CRL receptor would be predicted to lead to a decrease in the cell surface expression of the CGRP receptor and hence diminish the effects of its pronociceptive peptide ligand.

**GPCR trafficking and implications for other disease states**

Various disease states are now recognized as emanating from improper intracellular routing or misfolding of proteins (for review, see Ref. [78]). For example, the majority of patients afflicted with congenital nephrogenic diabetes insipidus possess mutations of a vasopressin receptor that result in the retention of misfolded receptors in the endoplasmic reticulum [13]. This inadequate trafficking of receptors to neuronal plasma membranes consequently prevents arginine vasopressin from being able to elicit its antidiuretic effects. Likewise, retinitis pigmentosa might result from improper intracellular trafficking and localization of rhodopsin receptors (reviewed in Ref. [79]). Thus, aberrations in protein trafficking might underlie the pathophysiology of various diseases and could represent potential sites for pharmacological intervention.

Estimates of the prevalence of mood disorders in patients with chronic pain indicate that a substantial proportion of these patients display debilitating depression. On the basis of a large-scale, population-based survey of pain and depression in the USA, Magni and colleagues found that 18% of people suffering from chronic pain could also be classified as depressed [80]. Moreover, another study reported that the prevalence of clinical depression in patients with chronic pain is as high as 30–54% [81]. Although comorbidity does not necessarily indicate commonality of underlying mechanisms, antidepressant drugs have been proven to be efficacious in alleviating neuropathic pain symptoms [82]. Interestingly, in addition to their analgesic effects in chronic pain, DOP receptors have also been implicated in mood disorders. DOP receptor-null mutant animals exhibit depressive-like behaviors, suggesting that an endogenous tone at this receptor site regulates mood [83]. Additionally, DOP receptor agonists and endogenous opioid peptides produce antidepressant effects in animal models of depression and anxiety [84–91]. Subjecting rats to a cold water swim test (which is similar to the forced swim test used in anxiety paradigms) has been shown to elicit trafficking of DOP receptors to neuronal plasma membranes [92]. In this latter study, under homeostatic conditions the DOP receptors were associated with large dense-core vesicles within GABA-containing neurons localized in the ventrolateral periaqueductal gray, whereas the stress stimulus produced an increase in plasma membrane-bound receptors. Hence, activity-dependent initiation of a regulated vesicular pathway was responsible for DOP receptor trafficking. Further studies will be required to determine whether regulating DOP receptor trafficking could be a viable treatment strategy for treating mood disorders such as depression.

Interestingly, a member of the s100 EF-hand protein family (p11) was shown recently to be necessary for the cell surface expression of 5-hydroxytryptamine (5-HT)<sub>1B</sub> receptors [93]. In this latter study, coexpression of p11 with 5-HT<sub>1B</sub> receptors enhanced the ability of this GPCR to counteract forskolin-stimulated cAMP formation. This discovery has relevant clinical implications with respect to neuropsychiatric disorders such as obsessive compulsive disorder, depression, anxiety and aggression. Indeed, p11 expression was reduced in patients who suffered from unipolar depression, and antidepressant agents increased p11 expression [93]. It is tempting to speculate that the elevation of p11 by antidepressant drugs and consequential increase in functional, bioavailable 5-HT<sub>1B</sub> receptors accounts for at least part of the clinical efficacy of such drugs.

**Concluding remarks**

Taken together, alterations in the subcellular distribution of GPCRs can have dramatic physiological and potentially pathological consequences for cellular function. We have only just begun to investigate such events and ways in which to exploit them to modulate cellular responses. Certainly, a potential application of controlling or regulating cell surface receptor density could be the treatment of pain. Using the DOP receptor as an example, it is clear that modulating the number of cell surface receptors has tremendous potential for treatment of pain and other disease states such as mood disorders. However, we must be mindful that this is an evolving area of research and it is not yet known whether what we have learned from the DOP receptor can be extrapolated to other GPCRs. Additionally, many GPCRs seem to have various mechanisms, whether through oligomerization, heteromerization, chaperones or accessory proteins, for regulating export to the plasma membrane, casting doubt on the general belief that GPCRs are constitutively delivered to the plasma membrane.

It is predicted that extensive investigation of trafficking events for various GPCRs will be required before we can identify whether commonalities can be extrapolated to GPCRs within receptor classes or families. Nevertheless, as we elucidate how GPCRs are regulated in various pathological states, the potential for intervention to harness trafficking events could prove to be a valuable opportunity that enables better diagnostics and novel strategies for optimizing therapeutic action. It could be timely to explore the regulation of GPCR cell surface trafficking because these mechanisms have major potential in achieving desired clinical endpoints for various diseases.
References
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