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鴉片類止痛藥物耐受性的轉譯研究：從臨床到基礎

Translational Research on Opioid Analgesic Tolerance: from Bedside back to Bench

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本論文係 林至芃 君（學號 D94443001）在國立臺灣大學藥理學研究所完成之博士學位論文，於民國 105 年 7 月 12 日承下列考試委員審查通過及口試及格，特此證明

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（簽名）

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致　謝

終於還是走到了這一步，鬆了一口氣，覺得終於對得起指導老師，對得起為了我而犧牲的老鼠們，對得起一路上幫忙我的人。

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縮寫(Abbreviations)

ANOVA	Analysis of variance
anti-IL6	Neutralizing antibodies against interleukin-6
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
CSF	Cerebrospinal fluid
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DOR	Delta opioid receptor
DRG	Dorsal root ganglion
ECOG	Eastern oncology cooperative group
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal regulated kinase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase.
GFAP	Glial fibrillary acidic protein
Gi	Inhibitory regulative G-protein
GPCR	G-protein coupled receptor
GRK	G protein-coupled receptor kinase
GRO	Growth-related oncogene
IASP	International association for the study of pain
IDDS	Intrathecal drug delivery system
IL	Interleukin
IL-10	interleukin-10
IL-1beta	Interleukin-1 beta

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IL-1ra	Interleukin-1 receptor antagonist
IL-6	Interleukin-6
ILC	Innate lymphoid cell
IOM	Institute of Medicine
JNK	C-jun N-terminal kinase
KOR	Keppa opioid receptor
KOs	Knock-out mice
MAPK	Mitogen-activated protein kinase
MOR	Mu opioid receptor
mRNA	Messenger ribonucleic acid
NK	Natural killer
NMDA	N-methyl-D-aspartate
NTUH	National Taiwan University Hospital
PKA	Protein kinase A
PKC	Protein kinase C
RM	Repeated measures
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SDF-1	Stromal cell-derived factor
SEM	Standard error of the mean
sTNFR	Soluble TNF receptor
TLR4	Toll like receptor 4
TNFα	Tumor necrosis factor alpha
WHO	World health organization

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Abstract in Chinese

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鴉片類藥物是臨床處理中重度疼痛，最有效也最被廣泛使用的藥物。然而長期使用鴉片類藥物止痛卻也會伴隨產生藥物的耐受性。脊椎管內鴉片類藥物投與將藥物直接送至中樞神經產生藥效，是非常有效的止痛方式但卻也更容易產生藥物耐受性。傳統神經科學以神經元為中心的思維下，鴉片類藥物耐受性的致病機轉著重於 Opioid receptor 的 internalization、NMDA receptor 的 upregulation 或 glutamate transporter 的 down regulation。然而這些現象都只能部分解釋耐受性的成因。最近在齧齒類實驗動物上的研究結果顯示神經膠細胞的活化及發炎性物質的過度表現、也就是所謂的神經炎性反應在神經病變疼痛與類鴉片耐受性的致病機轉上有重要的角色。然而相關的人體研究證據則相當稀少。

在本研究的臨床部分，我們首先嘗試建立國內最完整的，有關體外可程控式植入型脊椎內給藥系統的照護流程。包括合適病患的選取、脊椎內嗎啡藥物輸注測試、手術方式的改進、長期追蹤與品質提升計畫。我們紀錄並分析了流程建立初期的病患，其脊椎管內嗎啡的劑量的改變、治療相關併發症的發生率與後續處理及病患日常生活功能的改善程度。藉由給予足夠劑量的脊椎管內嗎啡，病患的疼痛控制與生活品質皆能大幅改善。然而在這當中我們也發現所有接受脊椎內嗎啡輸注療法的病患，其嗎啡的劑量皆快速的增加，遠遠超過病情的演進。

以臨床的照護觀察到的現象為起點，在研究倫理委員會核准後，我們進行了一系列的轉譯醫學研究。我們首先分析了已產生類鴉片藥物耐受性病患的腦脊髓液中發炎相關因子的濃度。包括 TNF-alpha、 CXCL1、CXCL10、CCL2、CX3CL1 及 CXCL12 並與未暴露類鴉片藥物的對照組受試者比較。研究結果發現，已經對類鴉片止痛藥產生耐受性的病患群，其腦脊髓液中的 CXCL1 及 CXCL12 濃度明顯高於未暴露類鴉片藥物的對照組。進一步我們更發現 CXCL1 的濃度與病患所接受的類鴉片止痛藥物劑量成高度正相關。

接著我們建立轉譯動物實驗模式，藉由實驗鼠的閃尾反應，評估嗎啡的止痛效果及相關發炎因子對類鴉片耐受性產生的影響。在實驗大鼠投予嗎啡誘發藥物耐受性後，大鼠脊髓組織之 CXCL1 及 CXCL12 mRNA 皆顯著增加。雖然單獨

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給予椎管內 CXCL1 或 CXCL12 並不會改變老鼠的基礎閃尾行為，然而椎管內給予 CXCL1 或 CXCL12 卻會大幅降低腹腔內給予嗎啡所造成的急性止痛效果。接著我們參照臨床長期椎管內類鴉片輸注用於頑固疼痛的處置，建立植入皮下微幫浦進行長期椎管內嗎啡輸注並誘發大鼠產生藥物耐受性的實驗模式。我們發現耐受性的發生會因同時給予嗎啡與 CXCL1 或 CXCL12 而加速。反之 若被嗎啡輸注時一併給予 CXCL1 或 CXCL12 的中和抗體則會延緩耐受性的發生。針對 CXCL1 訊息傳遞給予其受體 CXCR2 的拮抗劑 antileukinate hexapeptide，或針對 CXCL12 訊息傳遞給予其受體 CXCR4 的拮抗劑 AMD3100，則可更有效的延緩嗎啡耐受性的發生。

綜合以上的實驗結果，我們藉由臨床到實驗動物的轉譯醫學研究模式驗證了趨化激素 CXCL1 與 CXCL12 可能參與鴉片類止痛藥耐受性的形成。阻斷 CXCL1/CXCR2 與 CXCL12/CXCR4 的訊息傳遞路徑則可以延緩藥物耐受性的產生並降低其嚴重度。因此針對 CXCL1/CXCR2 與 CXCL12/CXCR4 的訊息傳遞路徑進行介入將是治療類鴉片止痛藥耐受性的新藥研發之潛力標的。

關鍵詞:

趨化激素、CXCL1、CXCL12、細胞激素、類鴉片止痛藥、脊椎管內嗎啡輸注、耐受性、神經炎性反應

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Abstract in English

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Opioid analgesics remain the most effective and widely used analgesics for the management of moderate to severe pain. However, the efficacy of long-term opioid analgesics is progressively attenuated by tolerance, preventing adequate pain relief under stable opioid dosages for chronic pain patients. Although intrathecal opioid delivery provides very effective analgesia by acting directly on central nervous system, opioid analgesic tolerance is also accelerated. Classical neuron-centered concepts such as internalization of opioid receptors, up-regulation of N-methyl-D-aspartate receptor function, or down-regulation of glutamate transporter activity can only partially explain the phenomenon of tolerance. Recent evidence showing glial activation and upregulated inflammatory mediators in the rodent central nervous system has confirmed the pivotal role of neuroinflammation in neuropathic pain or opioid tolerance, or both. However, human evidence is still sparse.

In clinical part of this study, we developed comprehensive management protocol for totally implantable programmable intrathecal drug delivery system from patient selection, intraspinal morphine trial, surgical procedure to follow up program. Intrathecal morphine dosage adjustment, treatment related complications and patient functional outcomes are recorded regularly and analyzed. By delivering liberal dose of intrathecal morphine, pain severity decreased significantly. Due to much better pain control and improved quality of life, functional performance status also improved.

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Intrathecal morphine delivery by using totally implantable programmable device is an effective alternative method to relieve refractory cancer pain.

Based on our clinical practice, we further conducted subsequent translational research by investigating the intraspinal cytokine and chemokine profiles of opioid-tolerant cancer patients after research ethic committee approval. Cerebrospinal fluid (CSF) samples from opioid-tolerant cancer patients and opioid-naive subjects were compared. The CSF levels of tumor necrosis factor-alpha, CXCL1, CXCL10, CCL2, CX3CL1 and CXCL12 were assayed. CXCL1 and CXCL12 levels in CSF were significantly upregulated in the opioid-tolerant group. Further analysis revealed that CXCL1 level was strongly positively correlated with opioid dosage.

The rat tail flick test was utilized to assess the effects of intrathecal CXCL1 or CXCL12 on morphine-induced acute antinociception and analgesic tolerance. After induction of tolerance by intrathecal morphine infusion, the spinal cord CXCL1 and CXCL12 messenger RNA were significantly upregulated. Although CXCL1 or CXCL12 infusion alone did not affect baseline tail flick latency, the analgesic tolerance was accelerated by intrathecal infusion of CXCL1 or CXCL12 in daily intraperitoneal morphine injection of paradigm. After establishing tolerance by intrathecal continuous infusion of morphine, its development was accelerated by co-administration of CXCL1 or CXCL12. On the contrary, tolerance was attenuated

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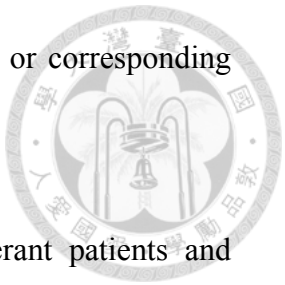

by co-administration of CXCL1 or CXCL12 neutralizing antibody or corresponding receptor antagonists.

CXCL1 and CXCL12 were upregulated in both opioid-tolerant patients and rodents. The onset and extent of opioid tolerance was affected by antagonizing intrathecal CXCL1/CXCR2 and CXCL12/CXCR4 signaling. Therefore, the CXCL1/CXCR2 and CXCL12/CXCR4 signal pathways may be novel drug targets for the treatment of opioid tolerance.

Keywords:

Chemokine, CXCL1, CXCL12, Cytokine, Opioid Analgesics, Intrathecal Morphine, Tolerance, Neuroinflammation, Translational Research

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Chapter 1
Introduction

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1-1. The Role of Opioid Therapy in Pain Management

According to IASP (International association for the study of pain) definition, pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. By definition, acute pain is a sensation results from actual tissue damage related activation of specialized nerve endings located in the nociceptive pathway. Under physiological condition, acute pain serves as a protective mechanism. On the contrary, chronic pain or pathological pain lasts beyond anticipated period of tissue healing or associated with inflammation and/or malfunction of the nervous system. Long lasting pain does not provide protective cue and survival benefit. According to the 2011 Institute of Medicine report, more than 100 million people in the Unites States suffered from chronic pain, and the annual direct and indirect economic burden is as high as $600 billion US dollars (Pizzo and Clark 2012). Adequate pain management thus is an essential part of modern medicine to improve patients' quality of life and maintain patients' psychosocial function. Though current concept of comprehensive pain management incorporate multi-dimension therapy including interventional procedures, rehabilitation and physical/occupational therapy, pharmacotherapy especially opioids are still the main treatments for moderate to severe pain, especially for cancer pain management (Portenoy 2011) and severe acute postoperative or post traumatic pain

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(Wu and Raja 2011). Cancer pain can generally be controlled using adequate analgesics.(Zech *et al,,* 1995) As efficient management of pain ensures patient comfort, studies have reported that early and aggressive management of symptoms, including pain, may even improve patient survival.(Quinten *et al.,* 2009, Temel *et al.,* 2010) Although opioid use for the treatment of chronic non-cancer pain remains controversial both with respect to the efficacy and adverse physical effects and to aberrant behaviors (Stein *et al.,* 2010), opioids use has escalated in recent years and making opioids one of the most commonly prescribed medications (Chapman *et al.,* 2010, Okie 2010). Morphine is most widely used opioid analgesics in clinical setting for decades and is the most important essential opioids in the World Health Organization (WHO) list of medication to be universally provided to relieve suffering in countries with limited medical resources. It is also recommended as a first-line opioid in the WHO Cancer Pain Relief Guidelines. Apart from morphine, various opioids with different intervals, administration routes, and potency are available in the market. Although many guidelines acknowledge transdermal opioids as an alternative to oral opioids (Caraceni *et al.,* 2012, Ripamonti *et al.,* 2012, National Comprehensive Cancer Network 2015), it is still surprising to find that transdermal fentanyl is the most commonly used strong opioids in Taiwan (Lin *et al.,* 2016).

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1-2. Pharmacology of Opioids and Mechanism of Opioid Tolerance

Opioid systems are critical in the modulation of antinociception and pain behavior. Endogenous opioid peptides and their receptors are expressed throughout the nociceptive neural circuitry and critical regions of the central nervous system included in reward and emotion-related brain structures. Just like endogenous opiates such as endorphins, enkephalins and dynorphins, opioid analgesics exert their pharmacological action through binding to opioid receptors. Opioids receptors belong to G-protein coupled receptors (GPCR) which are characterized as seven transmembrane domain (Al-Hasani and Bruchas 2011) (Williams *et al.,* 2013). The possible sites of action of opioids are illustrated in Fig. 1-1.

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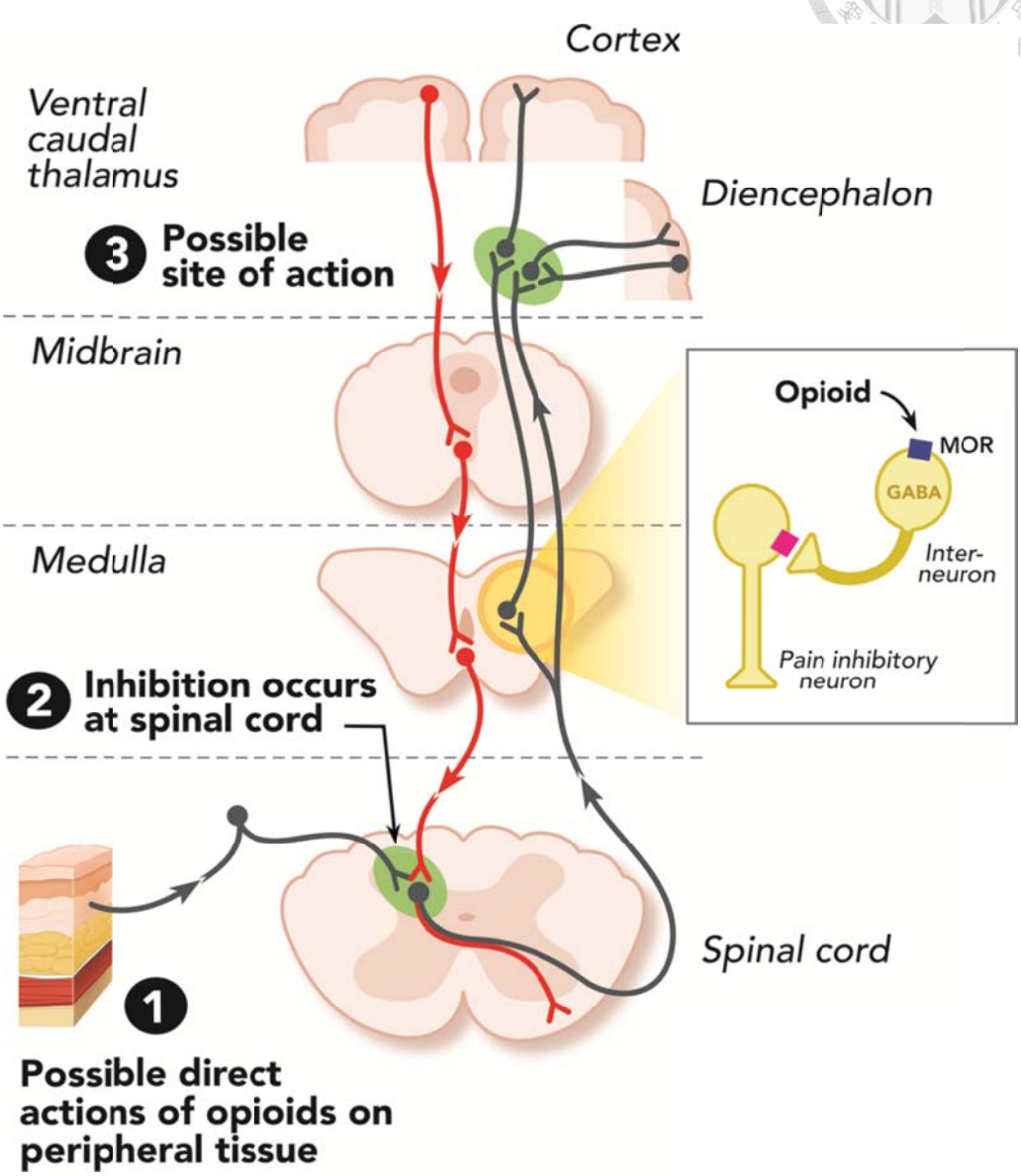
Figure 1-1 Sites of action of opioid analgesics.



The gray pathway shows the sites of action on the pain transmission pathway from periphery to central nervous system. The red pathway shows the actions on pain-modulating neurons in the midbrain and medulla.

Adapted from (Al-Hasani and Bruchas 2011)

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There are four major subtypes of opioid receptors including μ opioid receptor (MOR), δ opioid receptor (DOR), κ opioid receptor (KOR) and newly found nociceptin receptor. Each receptor is involved in different physiological functions. The majority of analgesic effect of either endogenous opiates or opioid medications are from activation of MOR while DOR also mediate some analgesia, antidepressant and physical dependence effect and KOR function as pain relief, sedation, dysphoria and pupil constriction. Activation of MOR is also linked with respiratory depression and supra-spinal analgesia (Al-Hasani and Bruchas 2011). The new class of opioid receptor -nociceptin receptor, participates in appetite, depression and anxiety effect but the role of nociceptin receptor needs further elucidation (Bodnar 2016). Upon binding to opioid receptor, opioids may trigger receptor conformational change and activate inhibitory regulative G-protein (Gi). Stimulation of Gi receptor leads to a decrease of cyclic adenosine monophosphate (cAMP) and the activation of protein kinase A (PKA). Opioids can also inhibit neurotransmitter release by a direct effect on calcium channel to reduce the concentration of calcium in presynapse. The opioid signaling cascade is illustrated in Fig. 1-2.

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Figure 1-2. Summary of opioid receptor signaling

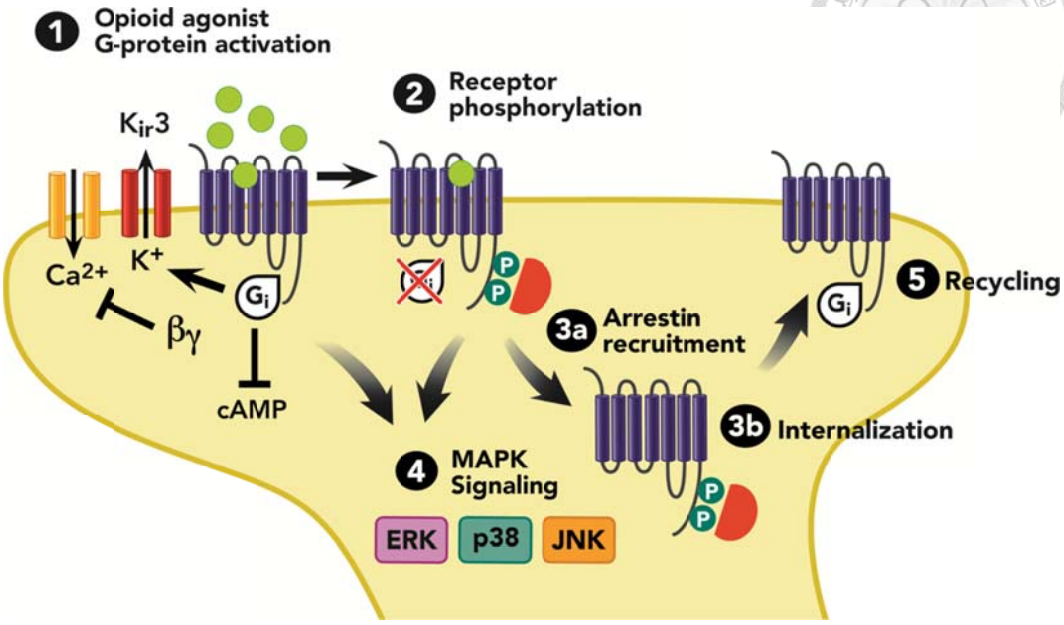

Figure depicts opioid receptor signal transduction and trafficking.

In general, all four opioid receptor subtypes MOR, DOR, KOR and nociceptin receptor share these common pathways. Selective ligands bind to each opioid receptor can direct opioid receptors to favor one or more of these downstream signaling events (biased agonism or ligand-directed signaling).

Arrows refer to activation steps; T lines refer to blockade or inhibition of function.

cAMP: cyclic adenosine monophosphate; ERK: extracellular signal regulated kinase;

JNK: c-jun N-terminal kinase; MAPK: mitogen-activated protein kinase;

P=phosphorylation.

Adapted from (Al-Hasani and Bruchas 2011)

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Clinical use of opioid analgesic is usually limited by opioid related side effects such as respiratory depression, constipation, nausea and vomiting. Potential of addiction (or fear of addiction) also complicates opioid utilization for chronic non-cancer pain. Repetitive administration of opioids for a certain amount of time can cause tolerance. As tolerance develops, a higher dose of opioids is needed to achieve the same level of pain relief, which might further lead to serious side effects and physical dependence. The molecular and cellular mechanism of opioid tolerance has been extensively studied and was illustrated in Fig. 1-3 (Williams *et al.,* 2013). Upon agonist binding to opioid receptor, serial downstream molecular events triggered including receptor phosphorylation by G protein receptor kinase, beta-arresting binding saturated within minutes. By definition, rapid desensitization and re-sensitization reached equilibrium within minutes while short term tolerance manifested by receptor endocytosis happened within one day. On the contrary, long term tolerance involves with multiple regulatory processes that cannot be fully explained by molecular events and is summarized in Fig. 1-4.

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Figure 1-3 Time course of molecular events that leads to long term tolerance

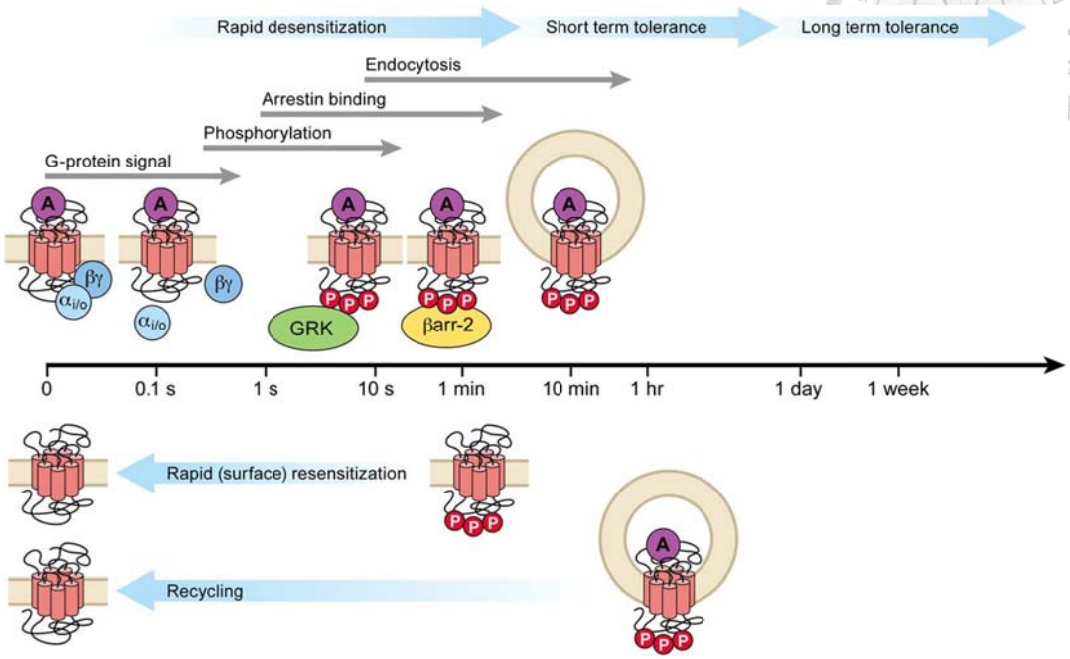

Figure illustrated that molecular events related to rapid desensitization, short term tolerance and long term tolerance. These events can only account for rapid desensitization to short term tolerance which occurred within on day but not long term tolerance.

Adapted from (Williams, Ingram et al., 2013)

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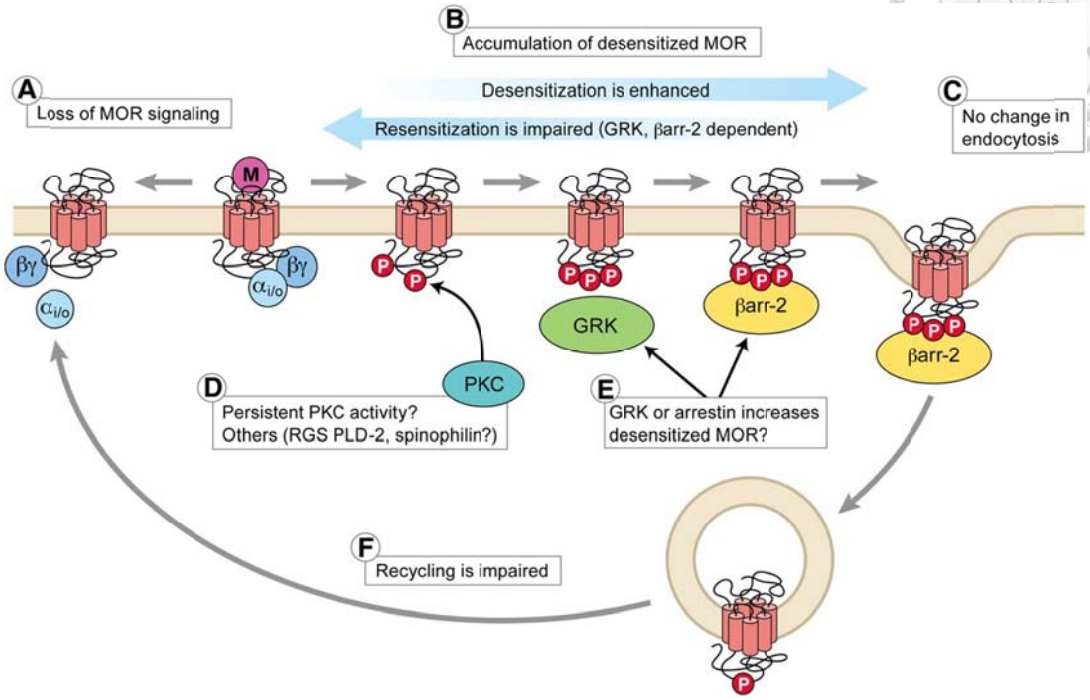
Figure 1-4 Summary of adaptations that contribute to MOR tolerance.



Possible adaptations that might contribute to MOR tolerance after long term opioid exposure are summarized in figure MOR adaptation as follows:

(A) More than 80% loss of functional MOR.

(B) Enhanced rate of MOR desensitization coupled with impaired re-sensitization.

(C) Increased MOR endocytosis

(D) Persistent protein kinase C (PKC) phosphorylation of MOR

(E) Enhanced interactions of G protein-coupled receptor kinase (GRK) and Beta-arresting caused persistent de-sensitization.

(F) Reduced recycling of MOR

Adapted from (Williams, Ingram et al., 2013)

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1-3. Neuroinflammation Counteracts Opioid Analgesia

Since classical neuron-centered concepts such as internalization of opioid receptors (Zuo 2005), up-regulation of N-methyl-D-aspartate receptor function (Shimoyama *et al.,* 2005), or down-regulation of glutamate transporter activity (Mao *et al.,* 2002) can only partially explain the pathogenesis of opioid tolerance, the role of neuroinflammation to counteract opioid analgesia has been proposed in recent years. Neuroinflammation manifested by morphological glial cell proliferation and hypertrophy with pro-inflammatory cytokine/chemokine over-production has been recognized as key contributors to multiple central nervous system diseases including pathological and chronic pain mechanisms (Milligan and Watkins 2009). The first report linking glial activation to opioid tolerance demonstrated that chronic systemic morphine increases astroglial activation showed by increased glial fibrillary acidic protein (GFAP) immunostaining in the spinal cord (Narita *et al.,* 2001). In parallel, co-administration of fluorocitrate (a glial metabolic inhibitor) with morphine significantly attenuates not only glial activation but also morphine tolerance (Song and Zhao 2001). Following studies also showed that chronic morphine treatment activates microglia as well as astrocytes (Raghavendra *et al.,* 2002, Tai *et al.,* 2006). In the spinal cord, along with glial activation, proinflammatory cytokines including Tumor necrosis factor alpha (TNFα), IL-1β and IL-6 are significantly up-regulated

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(Raghavendra *et al.,* 2002, Johnston *et al.,* 2004, Tai *et al.,* 2006). The progressively induced tolerance is temporally well correlated with increasing glial activation and pro-inflammatory cytokine production (Johnston *et al.,* 2004, Raghavendra *et al.,* 2004). More strikingly, morphine tolerance is slowed or reversed by either inhibition of spinal pro-inflammatory cytokines or by knocking out IL-1β signaling (Raghavendra *et al.,* 2002, Johnston *et al.,* 2004, Raghavendra *et al., 2004*, Shavit *et al.,* 2005). Microglia are derived from bone marrow during the perinatal period and are reported to participate in neuropathic and postoperative pain and opioid tolerance (Wen *et al.,* 2011). Intrathecally administered microglial inhibitor minocycline can prevent the development but fails to attenuate established morphine tolerance (Cui *et al.,* 2008). Astrocytes not only metabolically support neurons in the central nervous system (CNS) but also have active roles in multiple pathological conditions such as stroke, seizure, pathological pain, and opioid tolerance (Farina *et al.,* 2007). Astrocytes, as well as microglia, also participate in acute postoperative pain (Obata *et al.,* 2006). Above mentioned evidence demonstrated that glial activation and upregulated inflammatory mediators in the rodent central nervous system has confirmed the pivotal role of neuroinflammation in neuropathic pain or opioid tolerance, or both (Milligan and Watkins 2009, Watkins et al., 2009). In response to chronic morphine and peripheral nerve injury-related pain, astrocytes produce

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proinflammatory cytokines that enhance neuropathic pain behavior and offset the analgesic efficacy of morphine (Raghavendra *et al.,* 2002). By suppressing astroglial activation, both neuropathic pain and morphine tolerance are attenuated (Guo *et al.,* 2007, Lilius *et al.,* 2009). Proinflammatory cytokines (e.g., TNFα) have a pivotal role in neuroinflammation related to nerve injury-induced pain and chronic morphine exposure (Myers *et al.,* 2006, Shen *et al.,* 2012). Gene transfer of tumor necrosis factor soluble receptor can inhibit spinal TNFα production, thus preventing the development of morphine tolerance (Sun *et al.,* 2012). TNFα subsequently induces rapid expression of CCL2 (MCP-1), CXCL10 (IP-10), and CXCL1 (GROα) in primary astroglial cell culture (Gao *et al.,* 2009) and may contribute to the consolidation of morphine tolerance. CCL2 has an important role in the development of neuropathic pain (White *et al.,* 2005, White *et al.,* 2007), but its role in morphine tolerance is based on limited information (Zhao *et al.,* 2012). Series of preclinical researches demonstrated that orchestrated action of different cytokines (including TNF-α, IL-1β, IL-6) and chemokines (including CCL2, CCL21, CX3CL1, CXCL1 and CXCL12) as well as other neuromodulators (including growth factors, neurotransmitters and proteases) powerfully modulate synaptic transmission, lead to central sensitization and enhance chronic pain states. These mediators can further act on glial and immune cells to potentiate neuroinflammation via autocrine and paracrine

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fashion (Ji *et al.,* 2014). Some glial modification compounds also show promising effect on reversing morphine tolerance and are under active studies to validate their therapeutic potential and are summarized in Table 1-1.(Raghavendra *et al.,* 2004, Shavit *et al.,* 2005, Cui *et al.,* 2008, Hutchinson *et al.,* 2009, Hameed *et al.,* 2010)

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Table 1-1 Clinically relevant studies showing when efficacy of opioids is improved in animal models by inhibition of glial activation or proinflammatory cytokine actions

Model	Direction of effect	Intervention
Opioid induced acute analgesia	enhanced	minocycline [28,34], ibudilast (AV411) [31], IL-10 [32], IL-1ra [28,32,36], IL-1 signaling KOs [36], IL-ra over-expressing transgenics [36], classic TLR4 antagonists [45], (+)-naloxone [45], (-)-naloxone [45], ultra-low (-)-opioid antagonists [92], sTNFR [28], anti-IL-6 [28], p38 MAPK inhibitor [28]
Opioid induced analgesia for neuropathic pain	enhanced	propentofylline (SLC022) [93], pentoxifylline [93] IL-1ra + TNF soluble receptors + anti-IL6 [94]
Morphine analgesic tolerance	suppressed	ibudilast (AV411) [64], IL-10 [32], IL-1ra [32], fluorocitrate [95], minocycline [35], pentoxifylline [81], (+)-naloxone [45], propentofylline (SLC022) [33], IL-1 signaling KOs [36], IL-ra overexpressing transgenics [36], IL-10 + IL-1ra [28], IL-1 converting enzyme inhibitor + IL1ra [28]
Morphine withdrawal-induced pain enhancement	suppressed	IL-10 [32], IL-1ra [32], propentofylline (SLC022) [33], (+)-naloxone [45], IL-10 + IL-1ra [28], IL-1 converting enzyme inhibitor + IL1ra [28]
Morphine withdrawal-induced pain enhancement in neuropathic rats	suppressed	IL-1ra + TNF soluble receptors + anti-IL6 [94]
Precipitated opioid withdrawal behaviors	suppressed	ibudilast (AV411) [31], (+)-naloxone [45], minocycline [31]
Spontaneous opioid withdrawal behaviors	suppressed	ibudilast (AV411) [31]
Morphine induced respiratory depression	suppressed	minocycline [34]
Morphine induced dopamine release from brain "reward" area (nuc. Accumbens)	suppressed	(+)-naloxone, ibudilast (AV411) [96]
Morphine induced conditioned place preference	suppressed	minocycline [34], propentofylline (SLC022) [97]
Morphine induced glial activation (IHC)	suppressed	ibudilast (AV411) [31], pentoxifylline [98], propentofylline (SLC022) [99], fluorocitrate [95] minocycline [74]
Morphine induced proinflammatory cytokines & chemokines	suppressed	propentofylline (SLC022) [99], (+)-naloxone [19], ibudilast (AV411) [31]

Adapted from (Watkins *et al.*, 2009)

anti-IL6: neutralizing antibodies against interleukin-6,

IL-1ra: interleukin-1 receptor antagonist,

KOs: knock out mice,

TLR4: toll like receptor 4,

sTNFR: soluble TNF receptor,

IL-10: interleukin-10.

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1-4. The biological function of chemokines of CXCL1 and CXCL12

Chemokines are small secreted chemo-attractant cytokines that can be further divided into four subfamilies based on structure motifs including CXC-, CC-, C- and CX3C- subfamilies. Chemokines of CC subfamily have two consecutive cysteines near the N-terminus (β-chemokine) while members of CXC subfamily have one amino acid between the two cysteines (α-chemokine). CX3C subfamily has only one member which is CX3CL1 (fractalkine). Each subfamily of chemokines exerts their physiological function by binding to chemokine receptors that belong to G-protein coupled receptors. Current evidence shows that single chemokine can activate more than one receptors and one specific chemokine receptor can be activated by more than one chemokine (White *et al.,* 2005). The receptor selectivity and major function of chemokines are summarized in Table 1-2 and 1-3 (Griffith *et al.,* 2014). In addition to the pathological roles of chemokines in the maturation and trafficking of leukocytes during inflammatory process, chemokine signaling has been extensively studied for their neuromodulator function by interfering neurotransmission, neuron-glial cross talk and contribution in the pathogenesis of neuroinflammtory diseases including Alzheimer's disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis and stroke (Ramesh *et al.,* 2013, Reaux-Le Goazigo *et al.,* 2013, Melik Parsadaniantz *et al.,* 2015).

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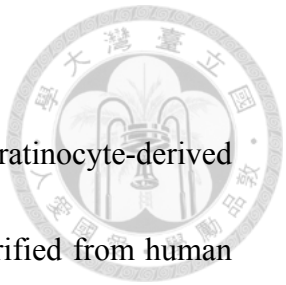

CXCL1 (also known as growth-related oncogene (GRO) or keratinocyte-derived chemokine) is a chemokine in the CXC family. CXCL1 is first purified from human malignant melanoma cells and is reported to play an important role in inflammation and cancer (Richmond *et al.,* 1985, Verbeke *et al.,* 2012). Among the three isoforms of GRO (GROα/CXCL1, GROβ/CXCL2, and GROγ/CXCL3), GROα/CXCL1 possesses the highest affinity to their shared receptor, CXCR2 (Haskill et al., 1990, Hammond *et al.,* 1996). CXCL1 is reported to attract neutrophils, stimulate endothelial cell-mediated angiogenesis, and activate macrophages or T cells (Eck *et al.,* 2003, Wang *et al.,* 2006, Verbeke *et al.,* 2012). An animal study shows that CXCL1 also plays a crucial role in neuroinflammation. One study shows that CXCL1 (when activated via NF-κB signaling) can recruit neutrophils to sites of inflammation in traumatic spinal cord injury (Kang *et al.,* 2011). Upregulation of CXCL1 is also involved in brain injury (Johnson *et al.,* 2011, Lee *et al.,* 2012). Long-term opioid administration activates spinal cord glial cells and neuroinflammation, which is considered to be one of the mechanisms leading to morphine tolerance (Johnston *et al.*, 2004, Hutchinson *et al.,* 2008). CXCL1 is involved in neutrophil chemotaxis and degranulation at the early phase of inflammation in peripheral tissue. In the nervous system, CXCL1 can also modulate neuronal excitability (Wang *et al.,* 2008, Yang *et*

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al., 2009)]. A recent study shows that CXCL1 is upregulated in the spinal nerve ligation model of neuropathic pain and is colocalized with spinal astrocyte markers. Intraspinally-applied lentiviral vectors of CXCL1 short hairpin RNA can persistently attenuate neuropathic pain behavior. This evidence implies the involvement of CXCL1/CXCR2 in nerve injury-induced neuropathic pain (Zhang *et al.,* 2013).

The role of CXCL12/CXCR4 signaling in the development and maintenance of pathological pain has been extensively studied in different animal models including chronic constriction injury of the sciatic nerve (Dubovy *et al.,* 2010), partial sciatic nerve ligation (Luo *et al.,* 2014), HIV-associated sensory neuropathy (Bhangoo *et al.,* 2009), diabetic neuropathy (Menichella *et al.,* 2014) and bone cancer (Shen *et al.*, 2014, Hu *et al.,* 2015). Opioids can trigger neuroinflammation through direct and indirect activation of microglia and astrocytes. Protein and mRNA study shows that microglia and astrocytes express opioid receptors and opioid can trigger downstream signaling (Horvath and DeLeo 2009). Opioids can also activate glial cells through non-classical, non-stereoselective mechanism by binding to Toll-like receptor 4 (TLR4) and trigger downstream MyD88 and TRIF-dependent intracellular signaling pathways including cell motility and survival/apoptosis related phosphatidylinositol 3-Kinase pathway and proinflammatory mediator production related NF*k*B and

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mitogen activated protein kinase pathways (Watkins *et al.,* 2009). Recent study has demonstrated that CXCL12 expression in dorsal root ganglion (DRG) neuron is increased after repeated morphine exposure (Wilson *et al.*, 2011). The primary sensory neuron may release CXCL12 from its central terminal to spinal cord dorsal horn (Reaux-Le Goazigo *et al.,* 2012). CXCL12/CXCR4 signaling thus might mediate morphine-induced tactile allodynia, and might offset the analgesic potency in rodent models and be involved in the pathogenesis of the clinically important phenomenon of opioid induced hyperalgesia (Wilson *et al.,* 2011, Rivat *et al.,* 2014). CXCR4 is co-expressed with opioid receptors in different areas of the rodent peripheral and central nervous systems, including dorsal root ganglion (Wilson *et al.,* 2011), dorsal horn (Reaux-Le Goazigo *et al.,* 2012), periaqueductal gray (Szabo **et al.,** 2002) and brain (Heinisch *et al.,* 2011). The cross talk between chemokines and the opioid system provides new perspectives for optimizing analgesic therapies (Melik Parsadaniantz *et al.,* 2015).

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Table 1-2 Summary table for Chemokines and corresponding immune function

Chemokine	Other names	Receptor	Key/main immune function[b]
CXCL1	GROα, MGSA, mouse KC	CXCR2	Neutrophil trafficking
CXCL2	GROβ, MIP-2α, mouse MIP2	CXCR2	
CXCL3	GROγ, MIP-2β	CXCR2	
CXCL4	PF4	?	Procoagulant
CXCL5	ENA-78, mouse LIX	CXCR2	Neutrophil trafficking
CXCL6	GCP-2 (no mouse)	CXCR1, CXCR2	
CXCL7	NAP-2	CXCR2	
CXCL8	IL-8 (no mouse)	CXCR1, CXCR2	
CXCL9	Mig	CXCR3	Th1 response; Th1, CD8, NK trafficking
CXCL10	IP-10	CXCR3	
CXCL11	I-TAC	CXCR3	
CXCL12	SDF-1	CXCR4	Bone marrow homing
CXCL13	BLC, BCA-1	CXCR5	B cell and Tfh positioning LN
CXCL14	BRAK	?	Macrophage skin homing (human)
Cxcl15	Lungkine (mouse only)	?	?
CXCL16		CXCR6	NKT and ILC migration and survival
CCL1	I-309, mouse TCA3	CCR8	Th2 cell and Treg trafficking
CCL2	MCP-1, mouse JE	CCR2	Inflammatory monocyte trafficking
CCL3	MIP-1α	CCR1, CCR5	Macrophage and NK cell migration; T cell–DC interactions
CCL4	MIP-1β	CCR5	
CCL5	RANTES	CCR1, CCR3, CCR5	
CCL6	C-10, MRP-1 (mouse only)	Unknown	?
CCL7	MCP-3, mouse Fic or MARC	CCR2, CCR3	Monocyte mobilization
CCL8	MCP-2	CCR1, CCR2, CCR3, CCR5	Th2 response; skin homing (mouse)

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Chemokine	Other names	Receptor	Key/main immune function[b]
		(human); CCR8 (mouse)	
CCL9/10	MIP-1γ, MRP-2 (mouse only)	Unknown	?
CCL11	Eotaxin-1	CCR3	Eosinophil and basophil migration
CCL12	MCP-5 (mouse only)	CCR2	Inflammatory monocyte trafficking
CCL13	MCP-4 (no mouse)	CCR2, CCR3, CCR5	Th2 responses
CCL14	HCC-1 (no mouse)	CCR1	?
CCL15	Leukotactin-1, HCC-2, MIP-5 (no mouse)	CCR1, CCR3	?
CCL16	HCC-4, NCC-4, LEC (no mouse)	CCR1, CCR2, CCR5	?
CCL17	TARC	CCR4	Th2 responses, Th2 cell migration, Treg, lung and skin homing
CCL18	PARC, DC-CK1 (no mouse)	CCR8	Th2 response; marker AAM, skin homing
CCL19	ELC, MIP-3β	CCR7	T cell and DC homing to LN
CCL20	MIP-3α, LARC	CCR6	Th17 responses; B cell and DC homing to gut-associated lymphoid tissue
CCL21	SLC, 6CKine	CCR6, CCR7	T cell and DC homing to LN
CCL22	MDC	CCR4	Th2 response, Th2 cell migration, Treg migration
CCL23	MPIF-1, MIP-3 (no mouse)	Unknown	?
CCL24	Eotaxin-2, MPIF-2	CCR3	Eosinophil and basophil migration
CCL25	TECK	CCR9	T cell homing to gut; thymocyte migration
CCL26	Eotaxin-3	CCR3, CX3CR1	Eosinophil and basophil migration
CCL27	CTAK	CCR10	T cell homing to skin
CCL28	MEC	CCR3, CCR10	T cell and IgA plasma cell homing to mucosa

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Chemokine	Other names	Receptor	Key/main immune function[b]
XCL1	Lymphotactin α, SCM-1α	XCR1	Cross-presentation by $CD8^+$ DCs
XCL2	Lymphotactin β, SCM-1β (no mouse)	XCR1	
CX3CL1	Fractalkine	CX3CR1	NK, monocyte, and T cell migration

Abbreviations: AAM, alternatively activated macrophages; DC, dendritic cell; ILC, innate lymphoid cell; LN, lymph node; NK, natural killer; Tfh, T follicular helper cell; Th, T helper cell; Treg, regulatory T cell.

Adapted from (Griffith *et al.*, 2014)

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Table 1-3 Summary of chemokine receptor and corresponding immune function

Receptor	Immune cell expression	Key immune function
G protein–coupled receptors		
CXCR1	Neutrophil > monocyte, NK, mast cell, basophil, $CD8^+$ T_{EFF}	Neutrophil trafficking
CXCR2	Neutrophil > monocyte, NK, mast cell, basophil, $CD8^+$ T	B cell lymphopoiesis, neutrophil egress from bone marrow, neutrophil trafficking
CXCR3	Th1, $CD8^+$ T_{CM} and T_{EM}, NK, NKT, pDC, B cell, Treg, Tfh	Th1-type adaptive immunity
CXCR4	Most (if not all) leukocytes	Hematopoiesis, organogenesis, bone marrow homing
CXCR5	B cell, Tfh, Tfr, $CD8^+$ T_{EM}	B and T cell trafficking in lymphoid tissue to B cell zone/follicles
CXCR6	Th1, Th17, γδ T, ILC, NKT, NK, plasma cell	ILC function, adaptive immunity
CCR1	Monocyte, macrophage, neutrophil, Th1, basophil, DC	Innate immunity, adaptive immunity
CCR2	Monocyte, macrophage, Th1, iDC, basophil, NK	Monocyte trafficking, Th1-type adaptive immunity
CCR3	Eosinophil > basophil, mast cell	Th2-type adaptive immunity, eosinophil distribution and trafficking
CCR4	Th2, skin- and lung-homing T, Treg > Th17, $CD8^+$ T, monocyte, B cell, iDC	Homing of T cells to skin and lung, Th2-type immune response
CCR5	Monocyte, macrophage, Th1, NK, Treg, $CD8^+$ T, DC, neutrophil	Type 1 adaptive immunity
CCR6	Th17 > iDC, γδ T, NKT, NK, Treg, Tfh	iDC trafficking; GALT development, Th17 adaptive immune responses
CCR7	naive T, T_{CM}, T_{RCM}, mDC, B cell	mDC, B cell, and T cell trafficking in lymphoid tissue to T cell zone, egress of DC and T cells from tissue
CCR8	Th2, Treg, skin T_{RM}, γδ T, monocyte, macrophage	Immune surveillance in skin, type 2 adaptive immunity, thymopoiesis
CCR9	Gut-homing T, thymocytes, B, DC, pDC	Homing of T cells to gut, GALT development and function,

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Receptor	Immune cell expression	Key immune function
		thymopoiesis
CCR10	Skin-homing T cell, IgA-plasmablasts	Humoral immunity at mucosal sites, immune surveillance in skin
XCR1	Cross-presenting $CD8^+$ DC, thymic DC	Antigen cross-presentation by $CD8^+$ DCs
CX3CR1	Resident monocyte, macrophage, microglia, Th1, $CD8^+$ T_{EM}, NK, γδ T cell, DC	Patrolling monocytes in innate immunity, microglial cell and NK cell migration, type 1 adaptive immunity
Atypical (nonsignaling) receptors		
ACKR1 (DARC; Duffy)	RBC, LEC	Chemokine transcytosis, chemokine scavenging
ACKR2 (D6)	LEC, DC, B cell	Chemokine scavenging
ACKR3 (CXCR7)	Stromal cells, B cell	Shaping chemokine gradients for CXCR4
ACKR4 (CCRL1; CCX-CKR)	Thymic epithelium	Chemokine scavenging

Abbreviations: DC, dendritic cell; GALT, gut-associated lymphoid tissue; iDC, immature dendritic cell; ILC, innate lymphoid cell; LEC, lymphatic endothelium; NK, natural killer; NKT, natural killer T; RBC, red blood cell; TCM, central memory T cell; TEFF, effector T cell; TEM, effector-memory T cell; Tfh, T follicular helper cell; Tfr, follicular regulatory T cell; Th, T helper; TRCM, recirculating memory T cell; Treg, regulatory T cell; TRM, resident-memory T cell

Adapted from (Griffith *et al.*, 2014)

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Chapter 2
Materials and Methods

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2-1 IDDS Clinical Study

Patient selection for intraspinal morphine trial

Refractory cancer pain is defined as failure to achieve adequate analgesia despite maximal opioids escalation and rotation, and development of analgesic-related toxicity or intolerant to opioid side effects. We followed the protocol for refractory cancer pain management at NTUH (figure 2-1.1 ITM flow chart). Patients suffering from wide-spread pain or failed to respond to neuroablative procedures were indicated for intraspinal (epidural or intrathecal) morphine therapy. These patients were considered eligible for permanent IDDS implantation if their life expectancy was greater than 3months. We excluded patient with bleeding tendency, active infection and brain metastasis.

Before permanent IDDS implantation, all patients were admitted to hospital and initiated an intraspinal morphine trial for 7 days. The intraspinal morphine can be delivered by daily intrathecal injection, continuous epidural infusion or continuous intrathecal infusion. The relative potency for intravenous: epidural: intrathecal morphine is 1: 10: 100. It is well-known that under equipotent dose, the opioids related side effects, especially nausea / vomiting and constipation, are markedly decreased when delivered more centrally (Myers *et al.*, 2010). The intraspinal morphine dosage was adjusted according to each patient's pain intensity and side

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effects. Patients who achieved greater than 50% pain reduction were considered eligible to permanent IDDS implantation.

Implantation techniques for permanent IDDS

We applied standardized surgical procedure for every patient. After induction of general anesthesia, patient was put in lateral decubitus position on the operating table with the pre-planned side of implantation upward. Intra-operative fluoroscopy was mandatory to confirm access to the L3-4 intrathecal space and the catheter tip to the optimal position according to patient disease status. Intrathecal catheterization was performed by paramedian approach with gentle oblique angle to optimize cerebrospinal fluid flow and decrease the risk of catheter kink or fracture. The catheter was then fixed on dorsal lumbar fascia by special anchorage device to accommodate possible vigorous movement after patient's general condition improved. The intrathecal catheter was then tunneled to lower abdomen subcutaneous pocket where we implanted the programmable pump.

Patients follow up after IDDS implantation

After operation, IDDS was set according to the intraspinal morphine trial result. Extra dose of analgesics might be necessary for acute postoperative wound pain. Patients were discharged 7 to 10 days after healing of surgical wounds and stabilization of

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intrathecal morphine dose. Patient then continued their previous treatment plan of either systemic chemotherapy or supportive care. Patients were hospitalized whenever clinically indicated.

Each patient's pain severity was measured by numeric rating scale from 0 to 10. Pain scores and equipotent morphine dosages were recorded at every visit and further analyzed at following time points: before pain specialist consultation, screen for eligibility of IDDS (before intraspinal morphine trial), after stable dose of intraspinal morphine trial, 14 days after IDDS implantation, and optimal condition during regular follow-up. Patient's functional status was recorded by Eastern oncology cooperative group (ECOG) performance status before and 14 days after implantation.

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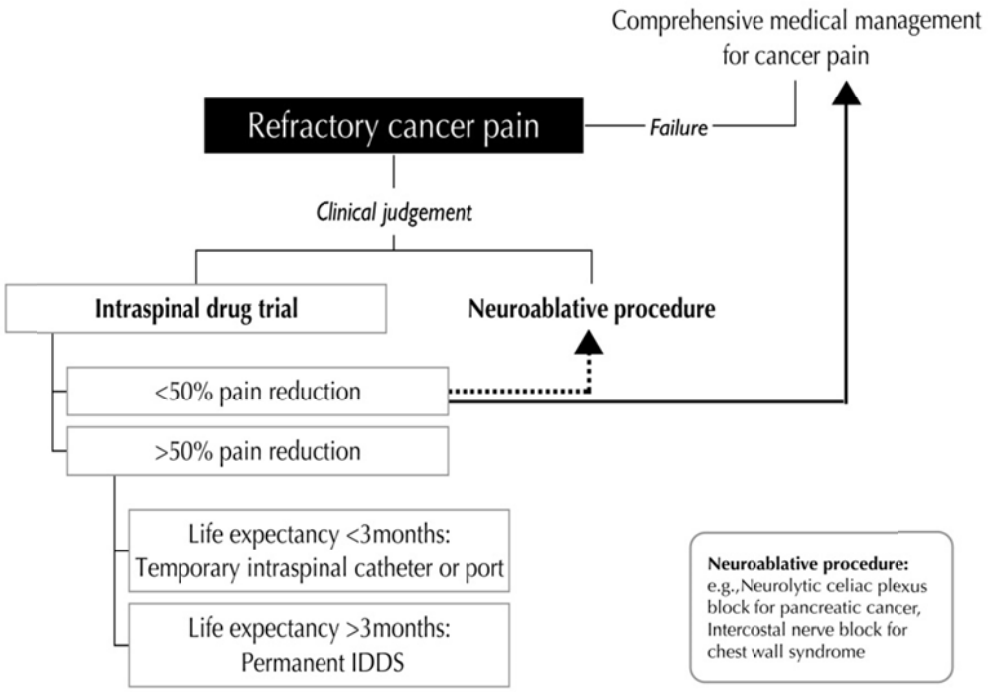
Figure 2-1 Summary flow chart for refractory cancer pain management



For patients failed to achieve adequate analgesia under non-invasive management, clinicians can choose neuroablative procedures for focal pain relief or intraspinal opioid trial for more diffuse pain base on clinical scenario. For those patients who can get more than 50% pain relief after intraspinal opioid trial, clinician will further perform temporary intraspinal catheter or port implantation if patient's expected survival is less than 3 months. For those who are expected to survive more than 3 months, permanent intraspinal drug delivery system surgery would be a reasonable choice.

IDDS: intraspinal drug delivery system

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2-2 Translational Human Research

Setting and Consent Process for Translational Human Study

After obtaining Institutional Research Ethics Committee (National Taiwan University Hospital Research Ethics Committee, Taipei, Taiwan) approval, this prospective case-control study was conducted at a tertiary medical center in accordance with the Helsinki Declaration and the International Association for the Study of Pain's Guidelines for Pain Research in Animals and Humans. Participant recruitment and sample collections were carried out from June 2010 to Aug 2014. All participants were informed by the investigators about the aims of the study and that the study would not affect any of their ongoing therapies. Informed consents were obtained before the collection of CSF samples.

Definition Opioid Tolerant Patients

An opioid tolerant patient was defined as a patient regularly taking strong opioids for pain management for more than one month. Daily treatments included intravenous morphine at a dosage greater than 100 mg, or other strong opioids given at an equipotent dose by other routes of administration, e.g., transdermal fentanyl and orally- or intraspinally-delivered opioids. We recruited advanced stage cancer patients suffered from cancer related pain under strong opioids and optimal adjuvant drugs. In

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general, we followed National Comprehensive Cancer Network guideline to titrate opioid dosage and to manage breakthrough pain. The goal of pain management was to keep each patient has no background pain (or only mild pain, i.e. numeric rating scale less than 3). The breakthrough pain was managed by proper dose of immediate release oral morphine when at home or intravenous morphine when hospitalized. The duration of regular opioids usage was longer than one month. Patients with evidence of central nervous system involvement were excluded. CSF samples (3 ml) were collected immediately after intrathecal catheterization or immediately before a scheduled refilling of an implanted intrathecal pump. We made certain that the pain score is less than 3 at the time of CSF sampling.

Definition of Opioid Naïve Control Subjects

An opioid naïve patient was defined as an individual that had not taken opioids within 3 months of the CSF sampling. An opioid naïve control subjects must have no chronic pain or ongoing acute pain at the time of CSF sampling. Patients scheduled for surgical removal (under spinal anesthesia) of implants used to treat lower extremity bone fractures were recruited. Lumbar puncture was performed at the L3-4 or L4-5 interlaminar space with a 27G spinal needle, without traumatic tapping or repeated puncture attempts. CSF was collected immediately before injection of bupivacaine for

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spinal anesthesia.

CSF Sample Processing and Cytokine/Chemokine Quantification

All CSF samples were centrifuged immediately after collection at 3,000 rpm for 5 minutes at 4°C and aliquots were stored at –80°C until assayed. The Procarta cytokine profiling kit (Panomics-Affymatrix, Santa Clara, CA, USA) was used to quantify TNFα, CXCL1, CXCL10, CXCL12, CCL2, and CX3CL1 in CSF according to the manufacturer's protocol. Briefly, a 96-well filter plate was pre-wet with reading buffer. The reagents (in the order of addition to the plate) were as follows: pre-mixed antibody beads prior to buffer removal by vacuum filtration; CSF samples with incubation on a shaker at 600 rpm for 60 minutes at room temperature and then washing; pre-mixed detection antibodies with incubation on a shaker at 600 rpm for 30 minutes at room temperature; streptavidin phycoerythrin with incubation on a shaker at 600 rpm for 30 minutes at room temperature and then washing, and finally reading buffer. The plate was read by a Luminex (Austin, TX) instrument and the data were analyzed by the designated Luminex acquisition software.

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2-3 Translational Rat Study

Chemicals and Reagents

Morphine hydrochloride was purchased from the National Bureau of Controlled Drugs, National Health Administration (Taipei, Taiwan). CXCL1, CXCL12, CXCL1-neutralizing antibody (CXCL1-Ab), CXCL12-neutralizing antibody (CXCL12-Ab) and CXCR4 blocker AMD3100 were purchased from R&D Systems (Minneapolis, MN, USA). CXCR2 blocker Antileukinate hexapeptide (RRWWCR, with an acetylated N terminus and amidated C terminus) was purchased from Yao-Hong Biotechnology (Taipei, Taiwan).

Generation of Fab Fragments of CXCL1-Ab and CXCL12-Ab

To exclude the possible interaction of Fc fragment with Fc receptors on glia cells, Fab fragments of antibody were prepared by Professor Chuang WJ's Lab (in NCKU). Fab fragments were prepared with Immobilized Papain (Pierce; Rockford, IL) according to the manufacturer's protocol. Briefly, the antibodies were dialyzed against 20 mM sodium phosphate buffer at pH 7.0 containing 10 mM EDTA. The digestion buffer (20 mM sodium phosphate, 20 mM cysteine-HCl, 10 mM EDTA at pH 7.0) was freshly prepared before the digestion reaction. Immobilized papain slurry was prewashed with the digestion buffer. The dialyzed antibodies were mixed with immobilized

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papain slurry in a 1:10 v/v ratio and incubated at 37°C for 6 h. The digestion was stopped by adding 10 mM Tris-HCl at pH7.5 and the immobilized enzyme was separated from IgG fragments by centrifugation. The Fab fragments were then separated from undigested IgG and Fc fragments using an immobilized protein A resin (Pharmacia). IgG fragment mixture was dialyzed against phosphate buffer saline and incubated with immobilized protein A at room temperature for 30 min. After centrifugation, the supernatant, which contains the Fab fragments, was collected. The immobilized protein A resin was washed and supernatant was combined to Fab fraction. The Fab fraction was ready for downstream experiments.

Experimental Animals

All experiments were performed in accordance with the International Association for the Study of Pain and the National Institutes of Health Guidelines on Laboratory Animal Welfare and the recommendations of National Taiwan University Animal Care and Use Committee. Adult male Sprague-Dawley rats (n=3~7 per protocol; weight, 250~275 g) were purchased from BioLASCO Taiwan Co., Ltd (I-Lan, Taiwan). Rats were housed individually and maintained in a controlled environment (12 h light/dark cycle) with food and water freely available. The rats were randomly allocated to different experimental conditions. The behavior test (Tail-flick response)

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was performed in a blinded manner.

Intrathecal Catheterization and Osmotic Pump Implantation

Intrathecal catheters (polyethylene PE10 tubing, 5 cm; Becton Dickinson, Franklin Lakes, NJ) were inserted into the upper thoracic spine by laminotomy under anesthesia with chlorohydrate through the dura mater into the subarachnoid space. The catheter was advanced caudally so that the tip rested on the lumbar enlargement. The rostral end of the catheter was firmly fixed to the thoracic spine and hidden in the interscapular soft tissue. Rats showing signs of motor dysfunction (e.g., paralysis) were excluded from the study. After recovery for 7 days, the catheter was connected to primed Alzet osmotic minipumps (Durect Corp., Cupertino, CA, USA) for the delivery of drugs for 5 days.

Evaluation of the Tail Flick Response and Antinociceptive Effect of Morphine

The analgesic effect of morphine was evaluated using the tail flick assay. Using a tail flick analgesia apparatus (Columbus, OH, USA), the tail flick latency was measured with 0.1 s precision. A 15-s cut-off time was used to prevent permanent tissue damage. Three measurements were made per rat per time point, on the distal half of the tail. The same thermal intensity was set for all animals, which resulted in a baseline tail

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flick latency of around 2 to 3 seconds. To assess the morphine antinociceptive effect, the percentage of the maximal possible antinociceptive effect (% MPE) was calculated by comparing the latency before [baseline (BL)] and after drug injection (TL), using the equation: % MPE = [(TL-BL)/(cutoff time-BL)]×100.

Morphine Tolerance Paradigms

Two paradigms were used to assess the effects of chemokine signaling on morphine tolerance. The first was the daily intraperitoneal morphine injection paradigm. After establishing intrathecal saline or chemokine continuous infusion by osmotic minipumps for 24 hours, morphine (10 mg/kg) was injected intraperitoneally. Morphine antinociceptive effects were assessed at 15, 30, 45, 60, 75, and 90 minutes and %MPEs were calculated. The same procedure was done for 5 consecutive days. The area under the curve (AUC) for time-response was considered an index of the antinociceptive effect at each dose of intraperitoneal morphine.

The second paradigm was intrathecal continuous infusion of morphine. After establishing intrathecal continuous infusion of morphine (15 μg/hr) using osmotic minipumps (with or without tested factors, antinociceptive effects of morphine were assessed at 16, 24, 48, 72, 96, and 120 hours. The study design for CXCL1 is shown in Figure 2-2. The study design for CXCL12 is shown in Figure 2-3.

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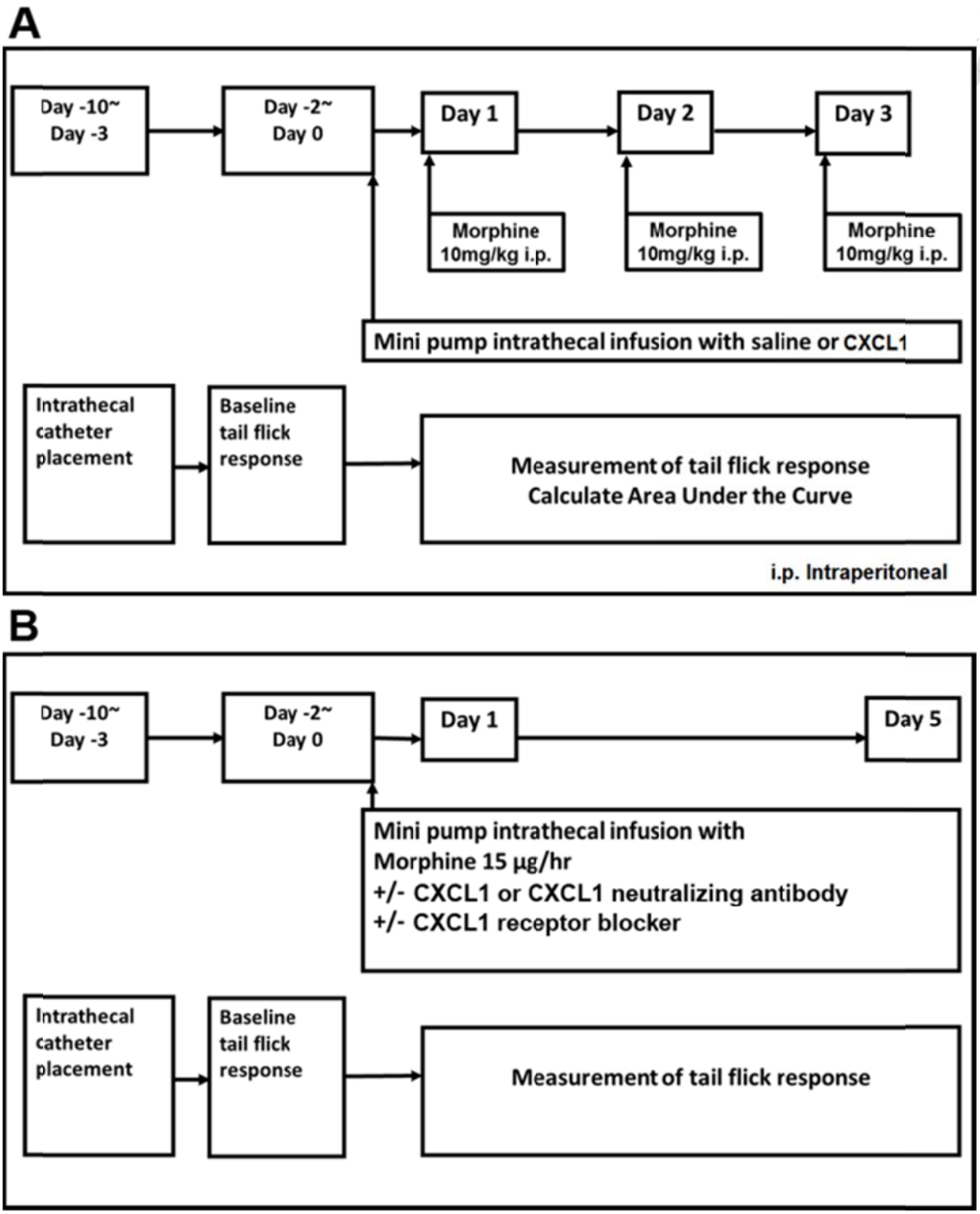
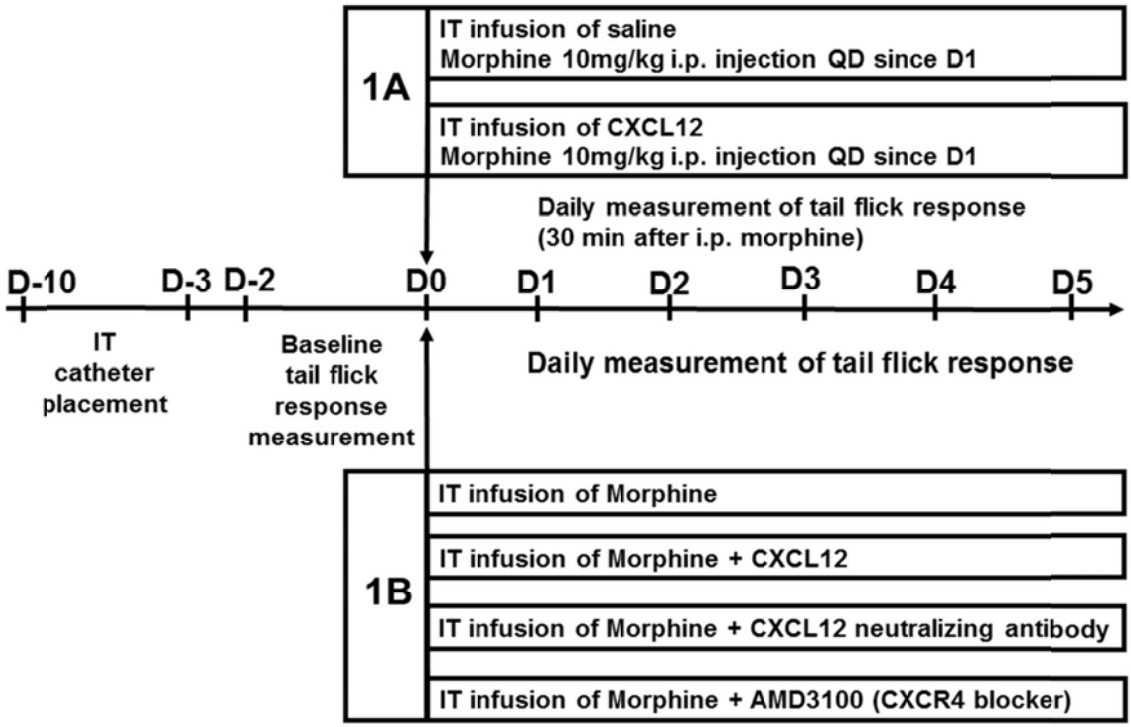
Figure 2-2 Animal study protocol for CXCL1



doi:10.6342/NTU201600959

Figure 2-3 Animal study protocol for CXCL12 study



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Total RNA Extraction and Real-Time Quantitative PCR

Rats were sacrificed after continuous intrathecal of infusion of morphine or saline by osmotic minipump for 2 and 5 days. The spinal cord L4-L5 dorsal horn regions were identified and isolated for the total RNA extraction. Single-strand complementary DNA was synthesized using SSIII reverse transcription reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. All cDNA samples were stored at -20°C. Real-time PCR was performed using a StepOnePlus™ Real-Time PCR system (Invitrogen). Relative mRNA levels were calculated according to the $2^{-\otimes\otimes CT}$ method. All ΔCt values were normalized to Glyceraldehyde 3-phosphate dehydrogenase. Oligonucleotide primers were used as follows:

Rat CXCL1:

forward 5'- AGA ACA TCC AGA GTT TGA AGG TGA-3' and

reverse 5'-GTG GCT ATG ACT TCG GTT TGG-3',

Rat CXCL12:

forward 5′- GCCGATTCTTTGAGAGCCATGT-3′ and

reverse 5′- GCACACTTGTCTGTTGTTGCTT-3′

Rat GAPDH:

forward 5'- GGC AAG TTC AAT GGC ACA GT -3' and

reverse 5'- TGG TGA AGA CGC CAG TAG ACT C -3'

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2-4 Statistical Analysis

The CSF cytokine / chemokine concentrations are presented as medians (25th; 75th percentiles) or means ± standard error of the mean (SEM). Comparisons between the opioid naïve control subjects and opioid tolerant patients were done by using the Mann–Whitney test or t-test when appropriate. Linear regression was used to illustrate the possible relationship between daily morphine equivalent dose and the selected chemokine. The difference was considered statistical significant when $p < 0.05$.

As for animal studies, results are presented as means ± SEM. For rat studies, we selected a minimal sample size of 3 to detect 3 folds upregulation of mRNA level (type 1 error=5% and power=0.8) between morphine infused and saline infused rats. We selected a minimal sample size of 5 to detect 30% difference of %MPE (type 1 error=5% and power=0.8). Rat mRNA expression was compared by t-test on Day-2 and Day-5, respectively. The time dependent data were tested using two-way ANOVA with repeated-measures. The two factors were treatment and time. After assessing that the treatment-by-time interaction was statistically significant, we then compared groups at each time point. Posttests were done by t-test with Bonferroni correction for p-value to compare %MPE difference of treatment group and morphine only control group at each time points. The differences of %MPE between different time points were not tested. The differences were considered statistically significant when

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adjusted $p < 0.05$.

The data were analyzed using GraphPad Prism, version 6.0 for Windows (GraphPad Software, Inc., San Diego, CA).

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Chapter 3
Development and Validation of Intraspinal Opioid Delivery System Services for Refractory Cancer Pain Management

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According to current consensuses (Burton *et al.,* 2004, Myers *et al.,* 2010), IDDS can be a good alternative for selected cancer pain patients, such as those with refractory cancer pain that cannot be relieved by conventional routes or suffered from intolerable side effects. However, the experience of using IDDS to manage refractory cancer pain is very limited in Taiwan. This part of study is our preliminary report on development and validation of IDDS clinical service in our institute. We presented the detailed patient selection criteria, intraspinal morphine trial procedures, surgical techniques of final pump placement, treatment related complications and drug adjustment strategies. Based on these experiences, our institute can regularly provide both temporary and long term intrathecal opioid analgesia to manage refractory cancer pain.

Results

From January 2007 to January 2010, 6 refractory cancer pain patients received IDDS. The characteristics of these patients were summarized in table 3-1. Four patients had inadequate pain control despite maximal drug escalation and rotation. Two patients were intolerant to opioids adverse effects with intractable nausea and vomiting.

The intraspinal morphine trial procedures were not consistent in our study population. For the first case, we tried daily lumbar puncture by 27 G spinal needle to deliver intrathecal morphine. This procedure had some drawbacks. Patient needed repeated

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transfer to operation room for consecutive 7 days. Analgesic effect was not stable and the duration of the single shot intrathecal morphine lasted only for 18-22 hr; patient suffered from intractable pain during the drug windows. Afterward, we switched to continuous epidural morphine infusion program before implanting IDDS. Epidural catheter was inserted in the operation room under fluoroscopy guide. After confirming proper catheter position and fixation, dosage adjustment could be easily managed in the ward. Since intrathecal space is an immune-privileged site and infection was always a concern especially in cancer patients, we were very cautious with externalized catheter. Only one patient shifted to temporary intrathecal catheter to provide adequate analgesia. His initial intrathecal dose was too high for effective epidural route.

The patient's pain scores were 10 (9-10) at pain specialist consultation, 9 (8-10) after medication adjustment including opioids and adjuvant agents. After the intraspinal morphine trial, the pain scores decreased to 3.5 (2-4) which was statistically significant and was illustrated in Fig. 3-1. The two patients who suffered from severe nausea and vomiting related to opioids could tolerate the intraspinal morphine trial well and easily escalated dose to adequate analgesia. All the 6 patients were satisfied with the analgesic efficacy and received IDDS according to trial result, the pain score was stabe from the immediate post-implantation period to follow-up visits (Fig. 3-1).

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The course equipotent morphine dosage escalation was illustrated in figure 3-2. Two patients suffered from pocket seroma and one patient also had back wound seroma. During mean 5±4.1 months follow-up, no other complication was noted such as central nervous system infection, nerve roots or spinal cord injury. Four patients had functional improvement at 14 days after IDDS implantation, while the other two are the same (Fig. 3-3). All 6 patients felt significant improvement of their quality of life with better pain control.

Discussion:

Our results in these 6 refractory cancer pain patients showed that IDDS improved pain control, performance status and quality of life. The pain scores significantly decreased from 10 to 3.5, although concomitantly daily intravenous morphine equivalent dose was rapidly increased under intrathecal drug administration indicating development of tolerance. During intraspinal morphine trial period, the equipotent morphine dose nearly doubled from the opioid dosage before the trial. With IDDS, we can deliver morphine directly to receptors in spinal cord dorsal horn and brain. This improves efficacy and reduces those common side effects such as nausea/vomiting and especially constipation (Myers *et al.,* 2010). Functional status improved in 4 of our patients after better pain control. Prolonged refractory pain status could lead to

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physical exhaustion and functional decline. Therefore, early intervention is essential to improve performance status among cancer patients. We cannot generalize the results of this study to patients with refractory pain due to the small sample size.

IDDS related complications have been reported (Fluckiger *et al.*, 2008) and up to 1% IDDS related central nerves system infection that necessitates pump and catheter removal. Minor complications such as local seroma around pump pocket site are not uncommon. In our experience, no surgical related infection or acute complication was noted. However, two patients suffered from post-operative pocket seroma; one of them combined with back wound seroma. One of the possible causes is the low serum albumin level (Stearns *et al.*, 2005), as observed in our patients (3.3 g/dl and 3.1g/dl respectively). Further studies are needed on the efficacy of albumin supplements prior to the procedure for reducing seroma. The back wound seroma might also be secondary to persistent cerebrospinal fluid (CSF) leakage (Belverud *et al.*, 2008). This explanation is less likely in our study because our patients did not show any other symptoms related to persistent CSF leakage such as positional headache. In addition, their seroma resolved spontaneously after short-term use of abdominal binder compression. Another complication with IDDS implantation is epidural or intrathecal bleeding/hematoma accumulation which is suspected if patients complained of rapid increase of focal back pain associated with progressive neurologic deficit (Belverud,

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et al., 2008). Magnetic resource imaging (MRI) study is necessary to confirm the diagnosis. None of the patients suffered from similar symptoms. However, one patient had to undergo regular MRI exam for monitoring his cancer status. IDDS pump will shut down when exposed to magnetic field greater than 1.5 Tesla, and resume its preprogrammed setting after leaving the magnetic field. After MRI examination, IDDS worked well and MR image quality was affected only at the pump area.

Morphine remains to be the gold standard for spinal administered analgesia and the only opioid approved by the FDA for intrathecal delivery. In this study, two patients required very high dose of morphine daily for adequate pain control: 16 mg and 21 mg intrathecal morphine (equivalent to 1600 mg and 2100 mg intravenously) respectively. However, delivering high concentration (>25 mg/ml), high daily dose (>10mg/day) morphine intrathecally may increase risk of intrathecal granuloma formation (Hassenbusch *et al.,* 2002). According to recent guidelines from the 2007 Polyanalgesic Consensus Conference (Timothy *et al.,* 2007), morphine may be shifted to other first-line medication (including hydromorphone and ziconotide) or second-line medication. Unfortunately, these drugs are not available in Taiwan.

Morphine delivered by IDDS can be adjusted easily at both outpatient clinic and inpatient setting by hand-held programmer. Dosage titration is guided according to patient's pain level and site of care as morphine adjustment of hospitalized patients

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can be managed more aggressively. Generally, if pain score is 5-6, dose can be increased by 25%-50% daily. If pain score is 7-10, 50%-100% dose escalation might be mandatory and patient should be closely monitored for possible drug toxicities in the initial 12-24 hours. As cancer progressed, patient's morphine-equivalent daily dosage is tailored to reach adequate pain control without hesitation. In our practice, IDDS pump drug refill is arranged in an ambulatory surgery setting for complete sterile environment. Each refill lasts from 2 weeks to 3 months depending on the daily dose requirement.

This is a preliminary report on the effect of intrathecal morphine delivery on patients with refractory cancer pain. Although this study has small sample size, it supports the use of intrathecal morphine delivery with totally implantable programmable pumps to ameliorate cancer pain. Further evaluation is necessary to validate the efficacy of intrathecal morphine delivery, but it can be an alternative for cancer patients with refractory pain.

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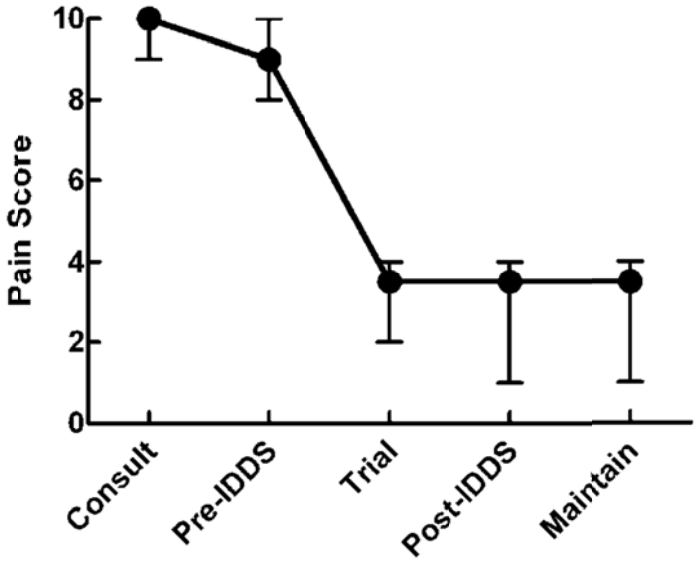
Figure 3-1 Pain scores of different time points



The evolutional change of pain scores at five time points including pain specialist consultation (Consult), before intraspinal morphine trial (Pre-IDDS), during intraspinal morphine trial (Trial), 14 days after IDDS implantation (Post-IDDS) and maintenance phase (Maintain);

Pain scores were presented by median and range.

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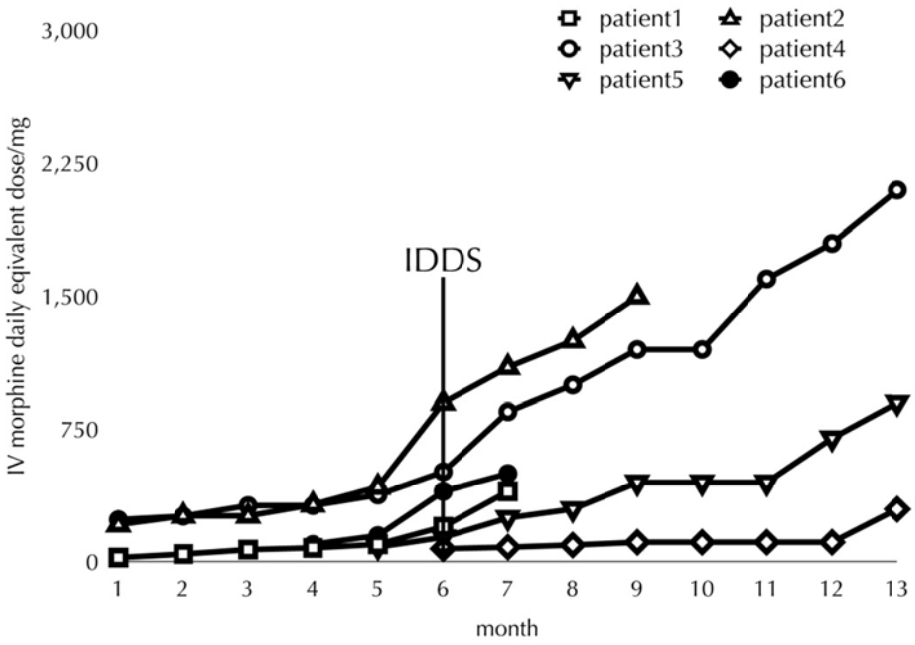
Figure 3-2 Analgesics dosage adjustment before and after IDDS



The summary figure illustrates daily morphine equivalent dose before and after IDDS implantation.

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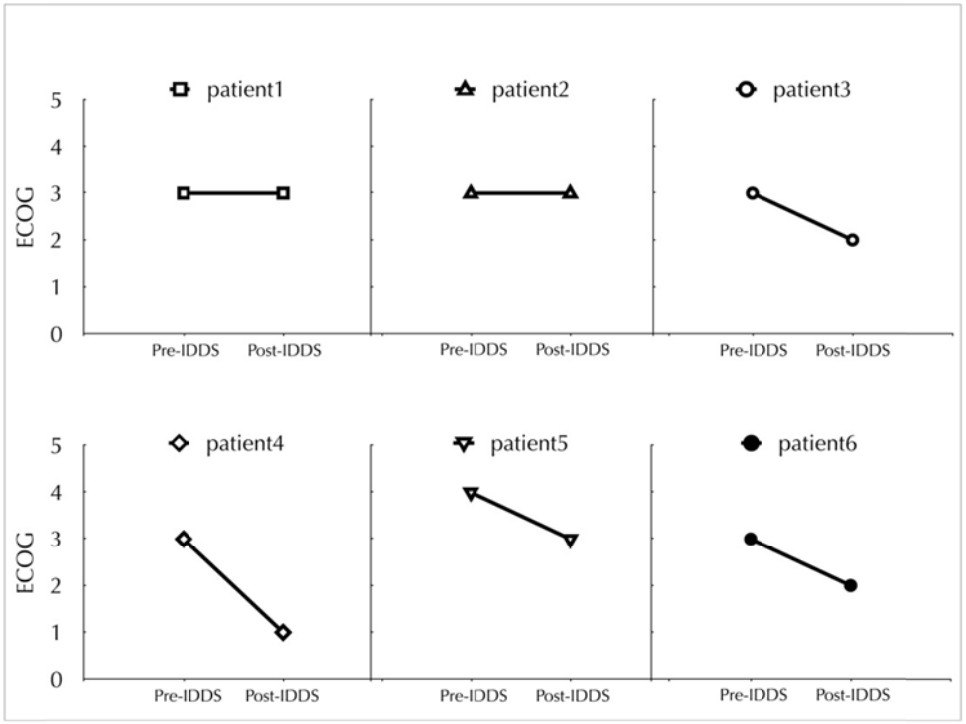
Figure 3-3: Functional status change before and after IDDS



The summary figure illustrated patients' physical function status before and after IDDS implantation.

ECOG: Eastern Cooperative Oncology Group functional status

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Table 3-1 Patient demographics

	Gender/age	Primary cancer diagnosis	Major Pain character	ECOG* before IDDS	ECOG* after IDDS
P't 1	M/58	Hepatocellular carcinoma	Somatic	3	3
P't 2	M/56	Rectal cancer	Visceral	3	3
P't 3	M/58	Rectal cancer	Neuropathic	3	2
P't 4	F/25	Buttock synovial sarcoma	Neuropathic	3	1
P't 5	F/70	Cholangiocarcinoma	Somatic	4	3
P't 6	M/48	Sigmoid colon cancer	Visceral	3	2

*ECOG: Eastern oncology cooperative group performance status, 0 fully active and 4 completely disabled

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Chapter 4
Role of Spinal CXCL1 (GROα) in Opioid Tolerance: A Human-to-Rodent Translational Study

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Although evidence in animals shows that neuroinflammation participates in the pathogenesis of morphine tolerance, there is no human evidence to support. In this reverse translational study, we explored the possibility that TNFα, CXCL1, CXCL10, CCL2, and CX3CL1 play roles in human opioid tolerance. We found that CXCL1 levels were significantly higher in the CSF of a group of 30 opioid tolerant patients compared to a control group of 10 age-matched opioid naïve patients. The CSF level of CXCL1 was positively correlated to opioid dosage. Lastly, we conducted proof-of-concept animal studies to confirm the relationship between CXCL1 and morphine tolerance.

Results

Cytokine and Chemokine Analysis in Opioid-tolerant Patients

Patient Demographics

Thirty patients with lung carcinoma (n=6), colorectal carcinoma (n=12), pancreatic carcinoma (n=6), hepatobiliary carcinoma (n=2), breast carcinoma (n=2) and sarcoma (n=2) and ongoing cancer-related pain controlled by strong opioids were recruited as the opioid tolerant group. All the recruited patients were stage 4 but no CNS involvement and had their cancer pain well controlled at the time of CSF sampling. Ten age-matched opioid naïve patients were also recruited as control subjects. The age,

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gender, and cancer diagnosis are summarized in Table 4-1.

Cytokine / Chemokine Measurement in CSF Samples

The median CSF concentration of CXCL1 was significantly higher among opioid tolerant patients than among opioid naïve patients (18.8 pg/ml vs. 13.2 pg/ml; p=0.02). There was no significant between-group difference in CSF concentrations of TNFα, CXCL10, CCL2, and CX3CL1. The concentration distributions for CXCL1, CXCL10, CCL2, and CX3CL1 were shown in Fig. 4-1. Re-analysis of this relationship after logarithmic transformation of the concentration values (Fig. 4-2A) identified two populations within the opioid tolerant patients with different CSF CXCL1 levels. Interestingly, CSF CXCL1 concentration was positively correlated with daily morphine equivalent dose (r^2=0.49, p<0.01) (Fig. 4-2B).

Effects of CXCL1/CXCR2 signaling on rat morphine tolerance

Effects of intrathecal morphine, CXCL1, CXCL1-Ab and hexapeptide on baseline tail flick latency

To examine whether intrathecal CXCL1 (1.2 ng/hr), CXCL1-Ab (3.6 ng/hr), and CXCR2 antagonist-Antileukine hexapeptide (5 μg/hr) affected baseline thermal response, these substances were administered via intrathecal continuous infusion

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using osmotic pump. The tail flick responses (presented in seconds) were examined at 0, 4, 16, 24, 48, 72, 96 and 120 hr. We found that administration with tested dose of CXCL1, CXCL1-Ab and antileukine hexapeptide did not affect the tail-flick latency compared with saline group. Morphine analgesic effect reached maximal at 16 hour then gradually declined (Fig. 4-3).

Increase of CXCL1 mRNA expression following intrathecal continuous morphine infusion

Continuous intrathecal infusion of morphine (15 μg/hr) or saline was administered using osmotic pump for 48 hours. The rat spinal cord L4-L5 dorsal horn region was identified and isolated for the expression of CXCL1 mRNA by real-time PCR. It was found that intrathecal infusion with morphine increased CXCL1 mRNA levels to 32.5 ± 11.9-fold of saline control (n=4 for each treatment) (Fig. 4-4).

Effects of Exogenous CXCL1 on Morphine Antinociception and Development of Tolerance in Rats

Based on our human study finding which suggested that CXCL1 had a potential role in the development of opioid tolerance, we tested whether this phenomenon could be verified experimentally in an animal model. Continuous intrathecal infusion of

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CXCL1 (1.2 ng/hr) was administered using an osmotic pump for 24 hours before the first dose of intraperitoneal morphine. Exogenous CXCL1 significantly decreased the antinociceptive efficacy of morphine (Fig. 4-5). On Day-1, the analgesic efficacy expressed by AUC of 10 mg intraperitoneal morphine in CXCL1-treated rats was only 66% of the AUC in saline-infused control rats. On Day-2, the AUC was 45% for CXCL1-treated rats which was significantly lower than saline-infused control rats (86%, compared with Day-1). On Day-3, the AUC was 15% for CXCL1-treated rats while saline-infused control rats still retained 50% efficacy. Therefore, intrathecally delivered CXCL1 decreased morphine analgesic efficacy and accelerated the development of morphine tolerance.

Modulating Morphine Tolerance by Intervening CXCL1/CXCR2 Signaling

Since intrathecal exogenous CXCL1 infusion accelerated the development of tolerance to intraperitoneally administered morphine, we then co-infused morphine with CXCL1, CXCL1-Ab, or CXCL1 receptor (CXCR2) antagonist intrathecally using osmotic minipumps to mimic intrathecal morphine infusion in clinical setting. As shown in Fig. 4-6, analgesic efficacy peaked after 16 hr of intrathecal continuous infusion of morphine (15 μg/hr), then declined gradually. MPE decreased to 43.8±7.1%, 18.8±2.5% and 7.1±4.4% at 24, 48, and 72 hr, respectively.

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Co-administration of morphine with CXCL1 further accelerated the development of morphine tolerance ($p=0.02$). The MPE in CXCL1 plus morphine co-infusion rapidly declined to 4.8 ± 2.7% at 24hr, which was significantly lower than morphine alone infusion ($p<0.001$). On the other hand, co-infusion of CXCL1-neutralizing antibody partially preserved morphine analgesic efficacy ($p=0.02$). Post hoc tests showed the significantly higher MPE among CXCL1-Ab plus morphine co-infusion than morphine alone infusion at 48hr (58.1 ± 8.0% vs 18.8± 2.5%, $p<0.001$), 72hr (34.6±2.9% vs 7.1± 4.4%, $p<0.05$) and 96 hr (30.0±2.7% vs 2.0±2.5%, $p<0.05$) (Fig. 4-6A). The analgesic efficacy of intrathecal morphine was also preserved by co-administration with CXCR2 antagonist-antileukinate hexapeptide at 24 (92.1± 6.4%, $p<0.001$), 48 (52.1±7.7, $p<0.001$), 72 (32.7±4.4, $p<0.05$) and 96 (24.2±3.4, $p<0.05$) hr, respectively (Fig. 4-6B).

Discussion

Herein, we documented evidence that CXCL1 might be implicated in the pathogenesis of opioid tolerance in both humans and rodents.

While the involvement of CXCL1 in neuroinflammation has already been demonstrated, the relationship between morphine tolerance and CXCL1 is unknown at the start of our studies. Our study in humans found a significant increase in CSF

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CXCL1 in opioid tolerant cancer patients and a strong positive correlation between CSF CXCL1 level and daily opioid dosage. CXCL1 has been detected in humans in a variety of neurological diseases (Zwijnenburg *et al.,* 2003, Franciotta *et al.,* 2006, Pranzatelli *et al.,* 2013)]. For example, CXCL1 is markedly upregulated in bacterial meningitis but not in aseptic meningitis and healthy controls (Zwijnenburg *et al.*, 2003), and upregulated in neuroinflammatory diseases such as multiple sclerosis, acute disseminated encephalomyelitis (Franciotta *et al.,* 2006), and opsoclonus-myoclonus syndrome (Pranzatelli *et al.,* 2013). Of note, the CSF CXCL1 level in our opioid naïve control group was also comparable with levels reported in healthy control subjects of the above-mentioned neurological disease studies. Our findings suggested that increase in CXCL1 may be related to opioid tolerance, since our opioid tolerant patients had neither neurological comorbidity nor cancer with central nervous system involvement.

Parallel to evidence in humans, we also found a rapid and significant upregulation of CXCL1 mRNA in the rat spinal cord after the induction of tolerance by intrathecal morphine infusion for 48 hours. Although CXCL1 alone infused intrathecally did not affect tail flick latency throughout the study period for 5 days, exogenous CXCL1 can markedly decrease morphine antinociceptive efficacy and accelerate the development of morphine tolerance. By using intrathecal co-infusion technique, we found that

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morphine analgesic efficacy dropped to nearly undetectable within 24 hours among CXCL1 co-infused rats. On the contrary, by blocking CXCL1/CXCR2 signaling with co-infused CXCL1 neutralizing antibody or receptor antagonist, morphine analgesic efficacy could be at least partially preserved. Thus, morphine tolerance might be attenuated by CXCL1/CXCR2 signaling interventions. Though the antileukinate hexapeptide (a potent inhibitor of CXCR2) has been reported to suppress inflammatory injury in acute pancreatitis or lung injury (Lomas-Neira *et al.,* 2004, Bhatia and Hegde 2007), it has never been reported to suppress the development of morphine tolerance.

Although CXCL1 has been implicated in both pain (Wang *et al.,* 2008, Zhang *et al.,* 2013) and cancer progression (Dhawan and Richmond 2002, Verbeke *et al.,* 2011), we recruited only opioid tolerant cancer patients, not chronic non-cancer pain patients, in our human study. Opioid dose was typically titrated to effect, but was generally greater in patients with more advanced disease. Based on our study design, it is therefore difficult to interpret whether the upregulation of CXCL1 is related to cancer disease progression per se or related to long term opioid use that causes tolerance. In this study, we tried to minimize confounding effect from cancer progression by recruiting relatively homogenous patients. They were all stage 4 cancer patients with distant metastasis but none of them had CNS involvement. Thus, we could rule out

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the possibility that changes of CSF CXCL1 were resulted from CNS metastasis. Although all the participants were in similar disease status, their opioid dosage range was very wide. We found a strong positive correlation between CSF CXCL1 level and daily opioid dosage, which further implied that upregulated CXCL1 might be related to long-term use of high dose opioids. Although we recruited only patients with advanced stage cancer with relatively stable dosage of opioids and disease status, the underlying cancer diagnosis would still be an inevitable confounding covariate. We could not recruit chronic non-cancer pain patients as study subjects because in our society, non-steroidal anti-inflammatory drugs and weak opioids are widely used to control most neuropathic pain and chronic musculoskeletal pain. However, strong opioid use for chronic non-cancer pain in our society is very limited and it is difficult to recruit enough non-cancer patients using high dose opioids (Lin *et al.*, 2010). Furthermore, chronic pain per se would also be another inevitable confounding co-variate. In human research setting, it is unethical to conduct study by inducing opioid tolerance in healthy subjects without pain. Therefore we conducted subsequent translational animal studies to illustrate that not only CXCL1 was upregulated in morphine tolerant rat but also exogenous CXCL1 decreased morphine analgesic efficacy and blocking CXCL1/CXCR2 signaling will restore morphine analgesic efficacy.

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In our human study, the subjects were prospectively recruited through a convenience sample. The number of participants in each group was designed to exceed the lower bound of large sample interference for clinical research, 30 patients. However, only subjects in the opioid tolerant group met a sample size of 30. We barely recruited 10 age-compatible subjects in the opioid naive group for the following reasons:

1. Most surgeries of the removal of implant for healed fracture were done under intravenous general anesthesia, not spinal anesthesia, in our institute.

2. Most of our citizens believe lumbar puncture with CSF sampling is bad for their spine health and will cause low back pain.

Zhang et al., have recently noted in a spinal nerve ligation model that CXCL1 upregulation occurred primarily in reactive astrocytes and paralleled neuropathic pain behaviors such as mechanical allodynia and heat hyperalgesia (Zhang *et al.,* 2013). Knockdown of CXCL1 mRNA by intrathecal short hairpin RNA lentiviral vector is shown to persistently attenuate spinal nerve ligation-induced pain hypersensitivity. Since peripheral nerve injury and long-term opioid exposure both turn on neuroinflammation manifested by sustained astrocyte activation (Raghavendra *et al.,* 2002, Johnston *et al.,* 2004), it would be reasonable to hypothesize that modulating CXCL1-CXCR2 signaling could be a promising therapeutic approach to attenuate opioid tolerance. Directly suppressing astrocyte activation using commercially

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available Ibudilast (a phosphodiesterase inhibitor used for asthma) restores the antinociceptive effect of morphine in opioid tolerant lab animals (Lilius et al., 2009). This finding further illustrates the potential for control of neuropathic pain and opioid tolerance by novel drugs targeting astrocytes.

To our surprise, we could not find a difference in CSF TNFα level between opioid naïve and tolerant subjects despite abundant lab animal data suggesting a difference (Shen *et al.,* 2012). Although the assay was very sensitive, the level of CSF TNFα in both the naïve and tolerant groups was very low (and even below detection limits in some patients), and was comparable to the level reported in patients with lumbar stenosis-related radicular pain and complex regional pain syndrome (Alexander et al., 2005, Ohtori *et al.,* 2011). This finding implies that, just as in the pathogenesis of nerve injury-induced neuropathic pain, the pathogenesis of opioid tolerance might involve TNFα at the initial stage but not the well-established stage as in our patient group (Myers *et al.,* 2006). Although the involvement of CCL2 and CX3CL1 in neuropathic pain-associated neuroinflammation has been shown in lab animals (Milligan *et al.,* 2008, Abbadie *et al.,* 2009), we could not detect a statistically significant difference in CSF levels of CCL2 and CX3CL1 between age compatible opioid naïve and tolerant human subjects.

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In conclusion, our investigation of the levels of various cytokines and chemokines in the CSF of opioid-tolerant cancer patients suggests that CXCL1 may be involved in the pathogenesis of opioid tolerance. Our animal studies showed that blockade of CXCL1/CXCR2 signaling can inhibit the development of morphine tolerance. Therefore, CXCL1/CXCR2 may be a new target for developing drugs that attenuate morphine tolerance and may be especially useful for treating patients requiring high dose opioids.

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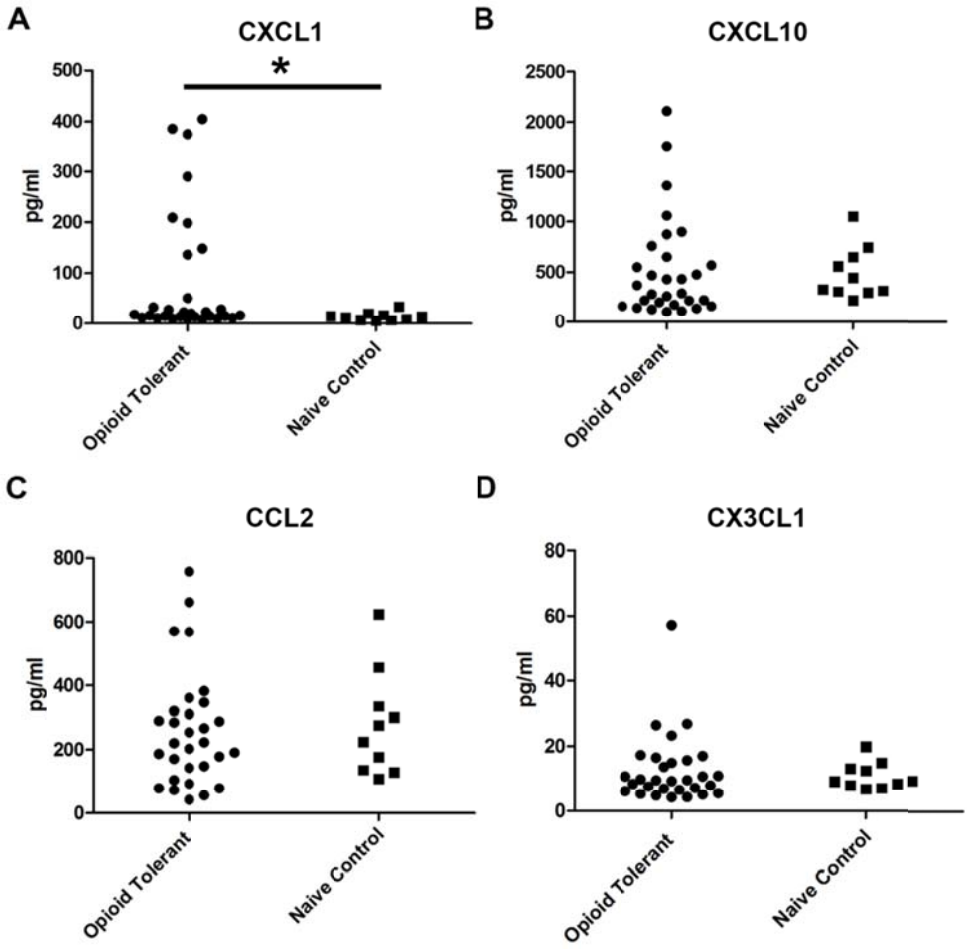
Figure 4-1 CXCL1 concentration is higher in cerebrospinal fluid of opioid tolerant patients than in naïve control subjects



Cerebrospinal fluid samples were collected from 30 opioid tolerant patients and 10 naïve control subjects. Note that CXCL1 was significantly increased in morphine-tolerant patients **(A)**. However, levels of CXCL10 **(B)**, CCL2 **(C)** and CX3CL1 **(D)** were not significantly different between opioid-tolerant patients and naïve controls. *, $p < 0.05$ as compared with naïve controls

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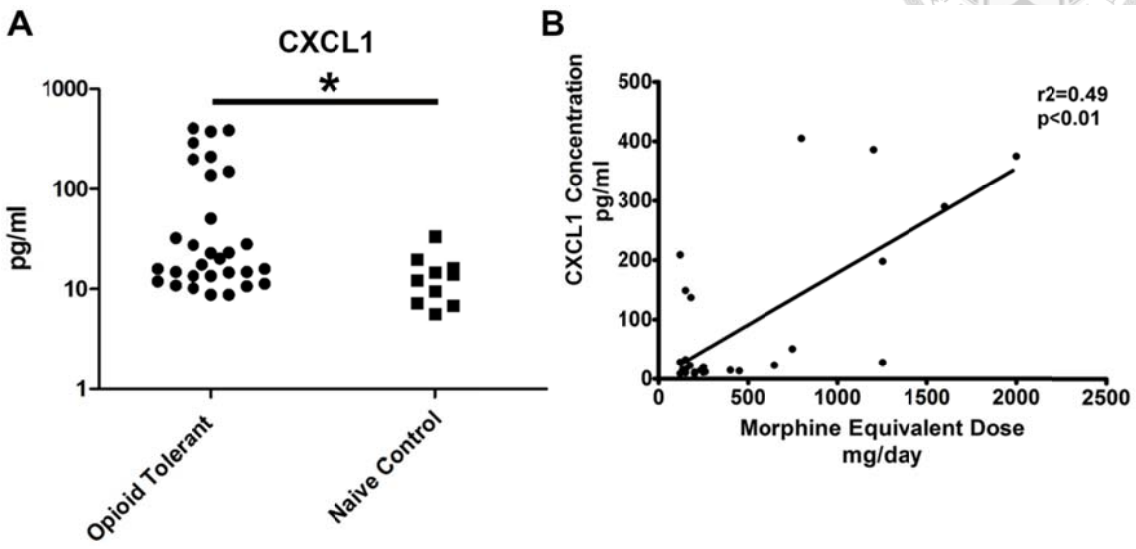
Figure 4-2 CXCL1 is positively correlated with daily morphine equivalent dose



(A) Log scale was used to re-analyze the differences in cerebrospinal fluid concentration between opioid tolerant patients and naïve controls.

(B) Among opioid-tolerant patients, cerebrospinal fluid CXCL1 level was shown to be positively correlated with the daily morphine equivalent dose.

*, $p < 0.05$ as compared with naïve controls

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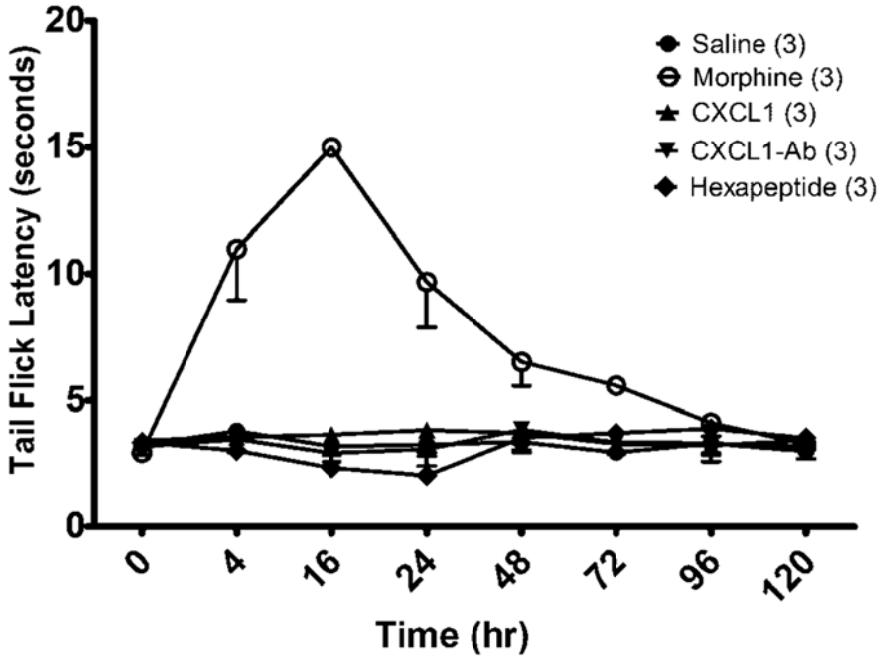
Figure 4-3 CXCL1, CXCL1-Ab or Hexapeptide alone does not affect rat tail flick latency throughout 120 hours infusion.



CXCL1 (1.2 ng/hr), CXCL1-Ab (3.6 ng/hr), hexapeptide (5 μg/hr), morphine (15 μg/hr) and saline control were individually infused intrathecally via osmotic minipump. Tail-flick latency responses (sec) were observed at 0, 4, 16, 24, 48, 72, 96 and 120 hour. Morphine analgesic efficacy peaked at 16 hr and gradually declined. None of the other infused substances affected tail-flick latency.

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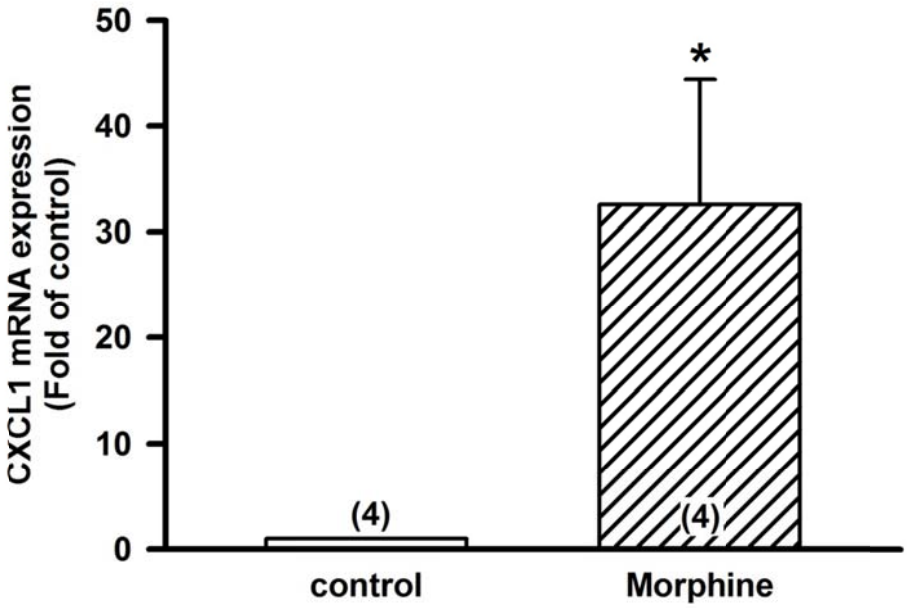
Figure 4-4 Intrathecal morphine infusion increases the expression of CXCL1 in rat



Morphine was administered via osmotic pump at an infusion rate of 15 μg/hr. After 48 hr, spinal L4-L5 dorsal horn was isolated for real-time polymerase chain reaction analysis. Note that treatment with morphine increased the expression of CXCL1 in spinal cord dorsal horn.

Data were presented as mean ± S.E.M.

*, $p<0.05$ compared with control (saline infusion).

mRNA = messenger RNA

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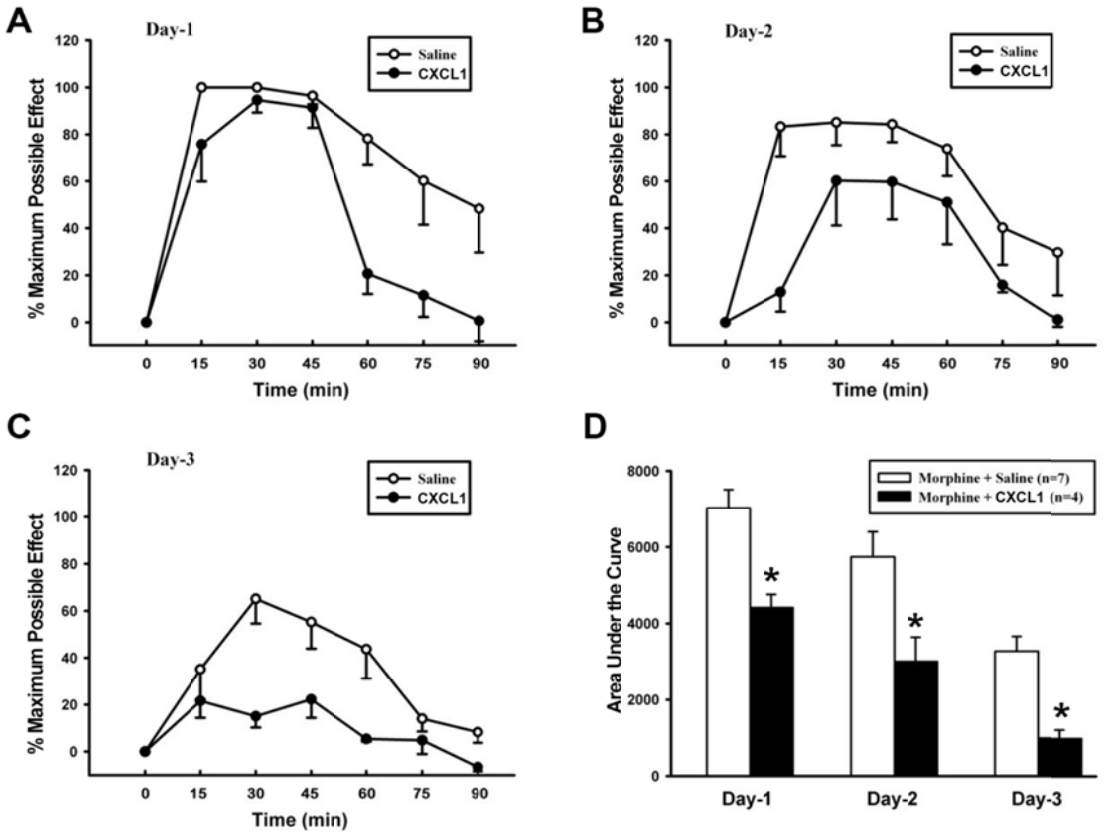
Figure 4-5 Exogenous CXCL1 decreases morphine analgesic efficacy and accelerates development of morphine tolerance in daily intraperitoneal morphine paradigm.



CXCL1 was intrathecally administered via an osmotic pump at an infusion rate of 1.2 ng/hr. Morphine was intraperitoneally injected at 10 mg $kg^{-1}day^{-1}$. The time-course of analgesic action of acute morphine treatment was evaluated by assessment of the latency of the tail flick response and the calculated % maximum possible effects on Day 1 through Day 3 **(A~C)**. The area under the curve was summarized in **(D)**.

Note that intrathecal administration of CXCL1 accelerated tolerance induced by

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intraperitoneal injection of morphine in rats.

*, $p < 0.05$ as compared with morphine+saline control group at different time points tested by Bonferroni posttests.

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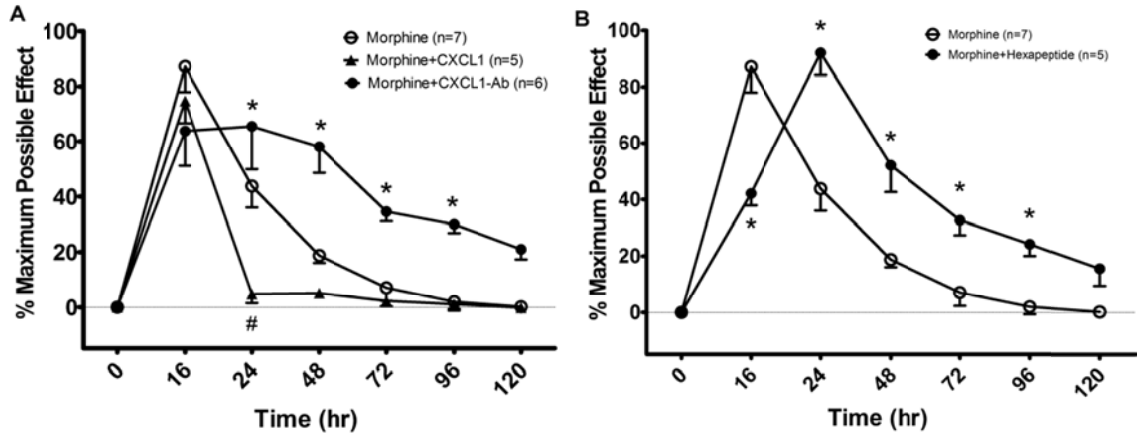
Figure 4-6 The effect of intervening CXCL1/CXCR2 signaling on morphine tolerance



All the drugs were administered intrathecally using an osmotic pump. The infusion rate was as follows: morphine 15 μg/hr, CXCL1 1.2 ng/hr, CXCL1-Ab 3.6 ng/hr, antileukinate hexapeptide (CXCR2 receptor blocker) 5 μg/hr. Note that exogenous CXCL1 markedly accelerated the development of morphine tolerance **(A)**, whereas, CXCL1 neutralizing antibody **(A)** or antileukinate hexapeptide **(B)** inhibited the induction of morphine tolerance and partially restored morphine analgesic efficacy.

*, $p < 0.05$ as compared with morphine group at different time points tested by Bonferroni posttests.

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Table 4-1 Subject Characteristics

	Opioid Tolerant	Naïve Control
Age	52.8 (13)	51.5 (13.5)
Gender		
Male	20	7
Female	10	3
Diagnosis	Lung Cancer (6)	Spinal Anesthesia (10)
	Colorectal Cancer (12)	
	Pancreatic Cancer (6)	
	Hepatobiliary Cancer (2)	
	Breast Cancer (2)	
	Sarcoma (2)	

Cerebrospinal fluid was collected from opioid-naïve control subject during scheduled spinal anesthesia before injecting local anesthetics.

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Chapter 5
CXCL12/CXCR4 Signaling Contributes to the Pathogenesis of Opioid Tolerance: A Translational Study

Anesthesia & Analgesia (2016, In Press)

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In this translational study, we explored the contribution of CXCL12 in the pathogenesis of opioid tolerance. First, we analyzed human cerebrospinal fluid samples to determine whether CXCL12 is upregulated among opioid tolerant patients. In light of those results, we performed a translational study using two clinically relevant animal models, a once daily around-the-clock intraperitoneal (i.p.) morphine injection paradigm and an intrathecal minipump continuous morphine infusion paradigm, to explore if centrally delivered CXCL12 interferes with the time course of opioid tolerance.

Results

Patient Demographics

From Sep 2012 to Aug 2014, 27 patients with colorectal carcinoma (n = 9), pancreatic carcinoma (n = 8), lung carcinoma (n = 5), hepatobiliary carcinoma (n = 2), breast carcinoma (n = 2) and sarcoma (n = 1), having ongoing cancer-related pain managed by long-term strong opioids, were recruited into the opioid tolerant group. All the recruited patients were in the advanced stage but did not show CNS involvement, and their cancer pain was well controlled at the time of the CSF sampling. Ten age-matched opioid naïve patients were recruited as naïve control subjects. The characteristics of the enrolled subjects are summarized in Table 5-1.

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The CSF Concentration of CXCL12 is Significantly Increased Among Opioid Tolerant Patients

Compared with opioid naïve control subjects, the mean CXCL12 CSF concentration was significantly increased among opioid tolerant patients. (naïve control vs opioid tolerant: 755 ± 33 pg/mL vs. 892 ± 34 pg/mL; p = 0.03). (Fig 5-1)

Intrathecal Morphine Infusion Increases CXCL12 mRNA expression in Rat Spinal Cords

A continuous intrathecal infusion of morphine (15 g/h) or saline was administered for 2 and 5 days using an osmotic pump. The rat spinal cord L4-L5 dorsal horn region was identified and isolated for real-time PCR analysis of CXCL12 mRNA expression. We found that intrathecal morphine infusions upregulated CXCL12 mRNA expression to 3.2 ± 0.7 folds compared to the saline control on Day-2 (n =4 in each group, p=0.016) (Fig 5-2. A) and 3.4 ± 0.3 (Fig. 5-2B) folds on Day-5 (n=5 in each group, p=0.003)

Intrathecal Administration of CXCL12 Accelerates Morphine Tolerance

Continuous intrathecal infusion of CXCL12 (3.6 ng/h) was administered using an osmotic pump for 24 h before the first i.p. morphine injection. Exogenous CXCL12 did not decrease morphine analgesic efficacy on Day-1. However, exogenous CXCL12 significantly accelerated the onset of tolerance in daily i.p. morphine

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injection paradigm (Fig 5-3, n=6 for both groups). On Day-2, the %MPE for 10 mg of i.p. morphine in CXCL12-infused rats was only 49.5 ± 9.2% while in saline-infused control rats it remained 88.1 ± 6.2% (p=0.0003). On Day-3, the MPE was 26.6 ± 10.2% for CXCL12-infused rats, which was still significantly lower than saline-infused control rats (72.3 ± 6.4%, p<0.0001). On Day-4 and thereafter, saline-infused control rats developed significant tolerance to i.p. morphine. On these days, the difference in %MPE between CXCL12-infused and saline-infused rats were no longer statistically significant. The time course of the analgesic effect of i.p. morphine was evaluated through Day-1 to Day-5 (Fig. 5-3).

Morphine Tolerance Can be Modulated by Targeting CXCL12/CXCR4 Signaling

Since intrathecal exogenous CXCL12 infusion accelerated tolerance development in daily i.p. morphine paradigm, we co-infused morphine with CXCL12, CXCL12 neutralizing antibody or CXCL12 receptor (CXCR4) antagonist AMD3100, intrathecally using osmotic pumps to mimic the intrathecal morphine infusion in clinical scenarios. As shown in Fig. 5-4, the analgesic efficacy of 15 μg/h intrathecal morphine infusion declined gradually. %MPEs for morphine only infusion group (n=6) were 43.4 ± 6.4%, 17.5 ± 2.4%, 4.4 ± 1.5%, 3.6 ± 1.3% and 1.7 ± 1.9% on Day-1 to

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Day-5, respectively. Co-administration of morphine with CXCL12 further accelerated the development of morphine tolerance. The MPE in rats receiving CXCL12/morphine co-infusion (n=5) rapidly declined to 9.4 ± 7.1% on Day-1 ($p<0.0001$), which was significantly lower than morphine only infusion group. On the other hand, co-infusion with CXCL12-Ab plus morphine (n=5) delayed the induction of morphine tolerance. Posttests showed significantly higher %MPEs with CXCL12-Ab/morphine co-infusion than morphine alone on Day-1 (72.5 ± 11.6%, $p<0.0001$) and Day-2 (47.6 ± 11.3%, $p<0.0001$).

Another set of experiments showed that the analgesic efficacy of intrathecal morphine was persistently preserved by the co-administration of AMD3100 (Fig. 5-5). The %MPEs in rats receiving morphine co-infusion with AMD3100 vs. morphine infusion only (n=6 in both groups) were as follows: Day-1, 65.8±12.3% vs 46.9±8.4%, $p=0.28$; Day-2, 59.1 ± 9.6% vs. 19.4 ± 3.1%, $p=0.0005$; Day-3, 47.8 ± 11.4% vs. 9.4 ± 4.9%, $p=0.0007$; Day-4, 33.0 ± 4.6% vs. 3.1 ± 2.9%, $p=0.01$; and Day-5, 27.9 ± 4.1% vs. 0.9 ± 1.6%, $p=0.03$ (Fig 5-5).

Discussion

CXCL12, also commonly known as stromal cell-derived factor 1 (SDF-1), belongs to the CXC subfamily of chemokine. Our CSF study provides human evidence of significantly upregulated CXCL12 levels among opioid tolerant patients, which is

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complemented by experimental results on lab animals. These findings imply that CXCL12/CXCR4 signaling might contribute to the pathogenesis of opioid tolerance in both rodents and humans.

Although the CXCL12 levels of our opioid naïve subjects are comparable with previous research (Fischer et al., 2009), it may be argued that they are still relatively high, and that the difference between the naïve and tolerant patient groups, though statistically significant, is not vast. Since CXCL12/CXCR4 is widely distributed in the central nervous system (Reaux-Le Goazigo et al., 2012, Reaux-Le Goazigo et al., 2013) and is involved in multiple essential physiological functions, including plasticity processes during development and multiple normal and pathological conditions, maintaining a certain signaling level is vital (Reaux-Le Goazigo et al., 2013, Guyon 2014). Elevated CXCL12 levels have been linked to cancers with central nervous system involvement (Groves et al., 2009). In the current study, we recruited opioid tolerant patients without image study evidence of central nervous system metastatic lesions to eliminate this potential confounding factor. In light of our aforementioned clinical observations, we designed translational animal experiments.

In our rodent study, after intrathecal infusion of morphine for as short as 2 days, the

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CXCL12 mRNA was significantly upregulated in the spinal cord dorsal horn tissue and could last throughout the study period for 5 days when opioid tolerance was well established and morphine analgesic effects were markedly reduced as shown in Fig 5A and 5B. These evidences indicated that centrally delivered morphine can induce rapid and robust upregulation of CXCL12/CXCR4 signaling. Although the time course of opioid tolerance experiments was much faster (within 5 days) than in clinical setting, both lab animal (Kissin et al., 1991) and human studies (Chia et al., 1999) provided evidence that opioid tolerance might be initiated shortly after opioid exposure and may persist for a long time. Continuous intrathecal administration of low dose CXCL12 accelerated morphine tolerance but did not affect acute morphine antinociception. After a daily 10 mg morphine i.p. injection, the analgesic potency in the control rats gradually declined to 88.1% on Day-2 and 72.3% on Day-3. Whereas when CXCL12 intrathecal infusion was administered for 24 h before the first bolus of i.p. morphine, the analgesic potency rapidly declined to 49.5% on Day-2 and 26.6% on Day-3. We then utilized an even lower dose of CXCL12 to test the hypothesis that upregulated CXCL12 could accelerate tolerance and counteract the morphine analgesic effect in a more clinically important intrathecal morphine infusion model. Intrathecal continuous infusion of morphine in rats caused rapid analgesic tolerance within 24 h, as previously reported (Lin et al., 2015). In these control rats, the

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analgesic effect was 43.4% on Day-1 and declined thereafter. Co-infusion of morphine with CXCL12 (1.2 ng/h) significantly accelerated tolerance induction, while co-infusion with CXCL12-Ab or AMD3100 inhibited the development of tolerance. Previous studies have shown that a single injection of CXCL12 (up to 100 ng) directly into the periaqueductal grey matter decreases the analgesic response of selective Mu opioid receptor agonist-DAMGO (Szabo et al., 2002), morphine, and Delta opioid receptor agonist-DPDPE (Chen et al., 2007), without affecting the basal tail flick response. In the present study, we utilized much lower CXCL12 doses compared to aforementioned researches. The acute antinociception effect of morphine was not affected. However, tolerance development was accelerated by the very low doses of exogenous CXCL12. The CXCR4 antagonist, AMD3100, has been reported to partially reverse established neuropathic pain (Dubovy et al., 2010), as well as morphine-induced tactile hyperalgesia when delivered peripherally, but the effect only last for hours (Wilson et al., 2011). Since intrathecal administration AMD3100 does not influence motor function (Luo et al., 2014) and can directly block CXCR4 downstream signaling, we concluded from the current study that continuous infusion of AMD3100 can persistently suppress opioid tolerance. Therefore, inhibition of CXCL12/CXCR4 signaling could be a drug target for prevention of opioid tolerance.

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There are several limitations to our study. In our human study we recruited only opioid tolerant cancer patients. It is indeed very difficult to interpret whether the up-regulation of CXCL12 is related to cancer or to opioid-induced neuroinflammation. We tried to minimize this confounding factor by recruiting patients that had no CNS involvement. Theoretically, chronic non-cancer pain patients might be a better target population for chronic pain, or opioid tolerance research, because of their longer survival period and better physical status. However, in our society, NSAIDs and weak opioids are used to control most neuropathic pain and chronic musculoskeletal pain. In our practice, strong opioid use for chronic non-cancer pain is very limited and it is difficult to recruit enough non-cancer patients using high dose opioids (Cheng et al., 2015). On the other hand, chronic pain per se might also induce neuroinflammation. This is another inevitable confounding covariate (Grace et al., 2014, Ji et al., 2014). It is unethical to conduct human studies in which long-term opioid tolerance is induced in healthy volunteers without chronic pain. Therefore, well designed translational animal studies are conducted to study the role of CXCL12 in opioid tolerance.

Conclusions

The CXCL12/CXCR4 pathway contributes to the pathogenesis of opioid tolerance. Our study indicates that intervening with CXCL12/CXCR4 signaling has therapeutic potential for opioid tolerance.

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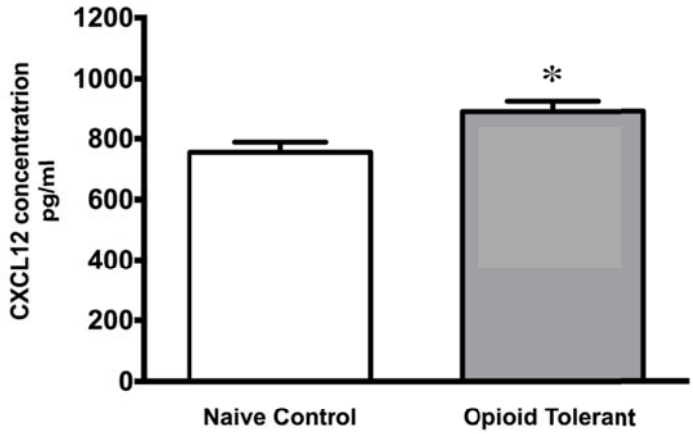
Figure 5-1 CXCL12 Concentration is Higher in the Cerebrospinal Fluid of Opioid Tolerant Patients Than in Naïve Control Subjects



Cerebrospinal fluid samples were collected from 27 opioid tolerant patients and 10 naïve control subjects. The CXCL12 concentration was significantly increased in opioid-tolerant patients (naïve control vs opioid tolerant 755 ± 33 pg/mL vs.892 ± 34 pg/mL ; $p = 0.03$).

Data are presented as means ± SEM

*, $p < 0.05$ compared with naïve control group by t-test.

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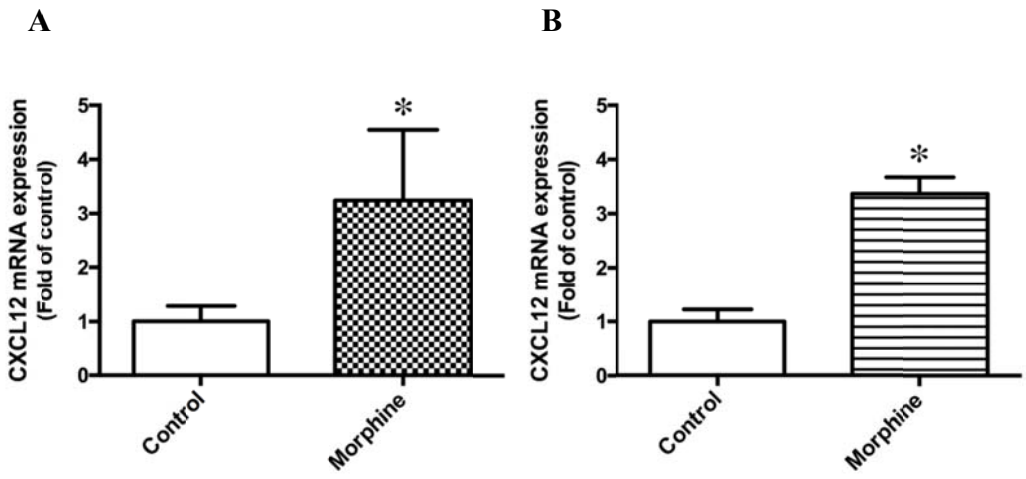
Figure 5-2 Intrathecal Morphine Infusion Increases the Expression of CXCL12 in Rat Spinal Cords



Morphine was intrathecally administered via osmotic pump at an infusion rate of 15 μg/h. The L4-L5 spinal cord dorsal horn was isolated for real-time polymerase chain reaction analysis on Day-2 (A) and Day-5 (B), respectively. Chronic intrathecal morphine infusion increased the expression of CXCL12 mRNA by 3.2 ± 0.7 folds in the lumbar spinal cord dorsal horn on Day-2 (n=4, * p=0.016) (A) and 3.4 ± 0.3 folds on Day-5 (n=5, * p=0.003) (B), respectively.

The CXCL2 mRNA level was compared by t-test.

Data are presented as means ± SEM.

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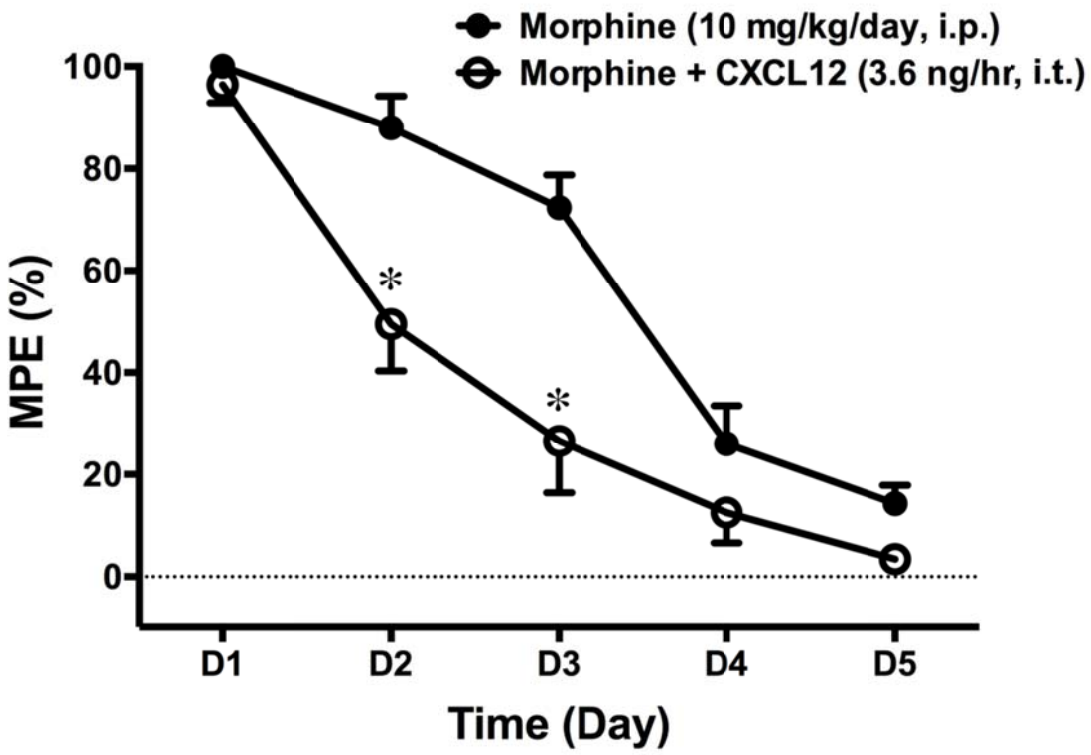
Figure 5-3 Intrathecal Administration of Exogenous CXCL12 Accelerates the Development of Morphine Tolerance Induced by Daily Intraperitoneal Injection of Morphine



CXCL12 was intrathecally administered via an osmotic pump at an infusion rate of 3.6 ng/h for 24 h before the first dose of morphine. Morphine was intraperitoneally injected at a fixed dose of 10 mg•kg-1•day-1. The time-course of morphine's analgesic effect was evaluated by assessing the latency of the tail flick response 30 min after morphine injection. The calculated % maximum possible effects (%MPE) were recorded on Day-1 ~ Day-5. Intrathecal administration of CXCL12 accelerated the onset of tolerance to intraperitoneal injections of morphine in rats (morphine +

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CXCL12 co-treatment vs. morphine control groups: 49.5 ± 9.2% vs. 88.1 ± 6.2% on Day-2, p=0.0003; 26.6 ± 10.2% vs. 72.3 ± 6.4% on Day-3, p<0.0001)

Data are presented as means ± SEM.

Statistical analysis was done by 2-way ANOVA with repeated measures followed by t test for each time points with Bonferroni correction of the p values (n = 6 for each group)

The asterisk (*) denote statistical significance compared with morphine control group at different time points and tested using the Bonferroni posttests.

Maximal possible antinociceptive effect (%MPE) was calculated by comparing the latency before [baseline (BL)] and after drug injection (TL), using the equation:

% MPE = [(TL-BL)/(cutoff time-BL)]×100.

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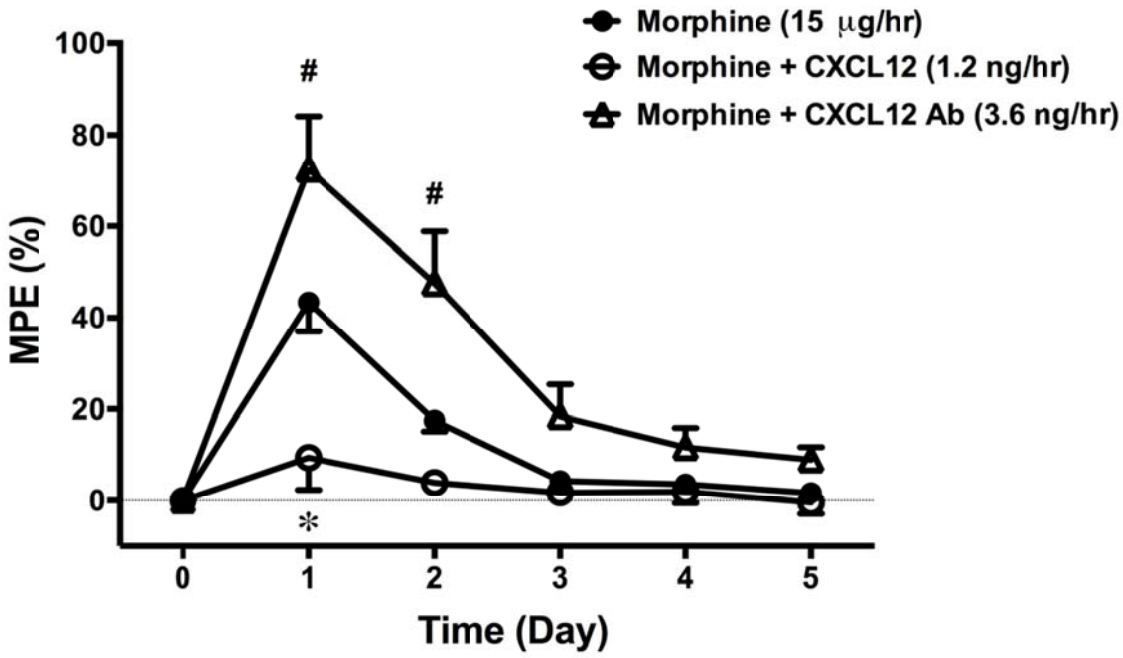
Figure 5-4 Effect of Intervening with CXCL12/CXCR4 Signaling on Morphine Tolerance



All the drugs were administered intrathecally using an osmotic pump. The infusion rate was as follows: morphine 15 μg/h, CXCL12 1.2 ng/h and CXCL12-Ab 3.6 ng/h. Exogenous CXCL12 markedly accelerated the development of morphine tolerance (9.4 ± 7.1% in morphine + CXCL12 group vs. 43.4 ± 6.4% in morphine control group at Day-1, $p<0.0001$), whereas CXCL12 neutralizing antibody inhibited morphine tolerance. (72.5 ± 11.6% in morphine + CXCL12 Ab group vs. 43.4 ± 6.4% in morphine group on Day-1, $p<0.0001$; 47.6 ± 11.3% vs. 17.5 ± 2.4% on Day-2, $p<0.0001$)

Data are presented as means ± SEM.

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Each point represents the mean ± SEM for 6 animals in morphine control group and 5 animals in morphine +CXCL12 and morphine + CXCL12 Ab group.

Statistical analysis was done by 2-way ANOVA with repeated measure followed by t test at each time points with Bonferroni correction of the p values to compare with morphine control group.

*, # represent statistical significance compared with morphine control group at different time points and tested using the Bonferroni posttests.

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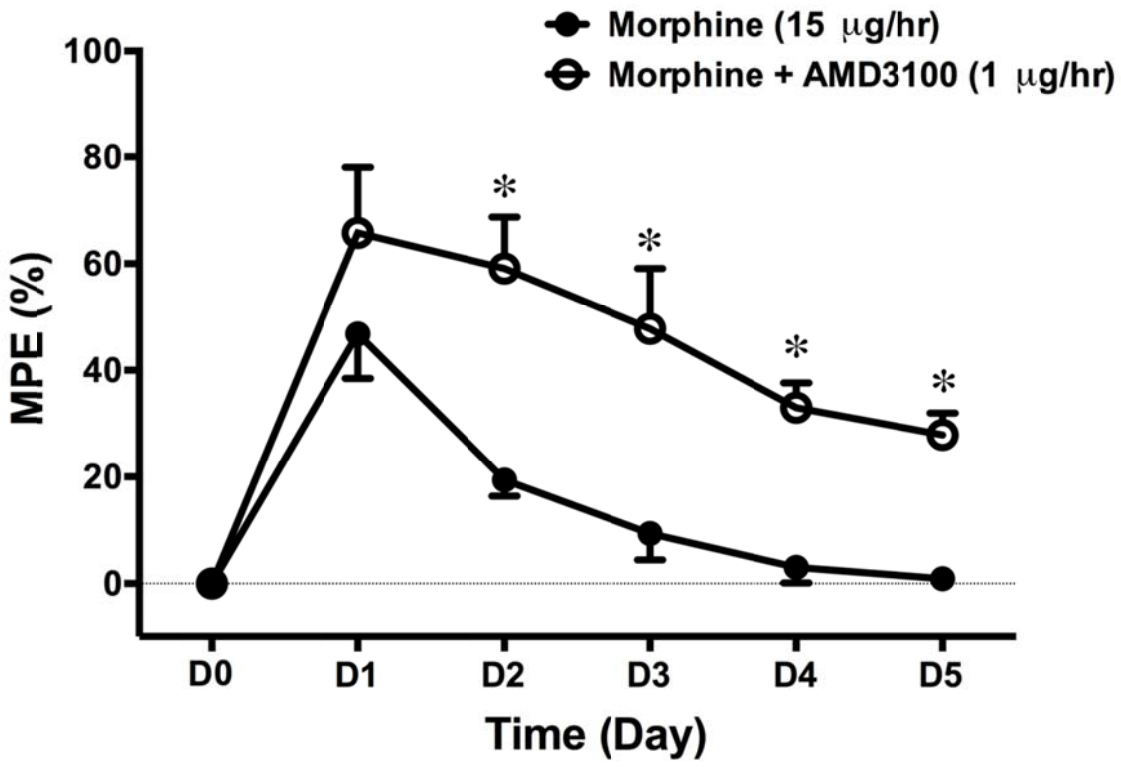
Figure 5-5 Effect of CXCL12/CXCR4 Antagonist on Morphine Tolerance



All the drugs were administered intrathecally using an osmotic pump. The infusion rate was as follows: morphine 15 μg/h and AMD3100 (CXCR4 receptor blocker) 1 μg/h.

Co-infusion of AMD3100 (1 μg/h) persistently inhibited morphine tolerance.

(morphine + AMD3100 group vs morphine control group; 65.8±12.3% vs 46.9±8.4% on Day-1 p=0.28; 59.1 ± 9.6% vs. 19.4 ± 3.1% on Day-2, p=0.0005; 47.8 ± 11.4% vs. 9.4 ± 4.9% on Day-3, p=0.0007; 33.0 ± 4.6% vs. 3.1 ± 2.9% on Day-4, p=0.01 and 27.9 ± 4.1% vs. 0.9 ± 1.6% on Day-5, p=0.03)

Data are presented as means ± SEM (n =6 for the both groups)

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Statistical analysis was done by 2-way ANOVA with repeated measure followed by t test for each time points with Bonferroni correction of p values

* represents statistical significance as compared with the morphine group, tested using Bonferroni posttests.

Maximal possible antinociceptive effect (%MPE) was calculated by comparing the latency before [baseline (BL)] and after drug injection (TL), using the equation:

% MPE = [(TL-BL)/(cutoff time-BL)]×100.

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Chapter 6
Conclusion and Prospect

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Chronic unremitting pain is not only a disease entity but also a major socioeconomic burden to our society. Opioid therapy remains the most effective and widely accepted treatment strategy for the management of moderate to severe pain, especially cancer related severe pain.

From clinical perspective, we have developed comprehensive intraspinal morphine infusion therapy protocol to provide our patients high quality, easy-to-care pain management during our early phase study period. As clinicians and educators, we further share our experience to colleagues and medical personnel in Taiwan through continuous medical education activities and seminars in terms of proper patient selection criteria, intraspinal trial infusion protocol, pump implantation surgery details, complication management and long term follow-up programs. In parallel to more effective analgesia, we are still bothered by the problems from opioid tolerance that complicate our patient care. Opioid analgesic tolerance, by definition, the efficacy of long-term opioid is progressively attenuated thus dosage escalation is needed to provide same level of pain relief for chronic pain patients. Either frequent dosage adjustment or increased severity of opioid related side effects will significantly impair patients' satisfaction and quality of life.

In our "reverse" translational studies, we discovered that CXCL1 and CXCL12 were significantly up-regulated among opioid-tolerant patients and positively correlated

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with daily opioid dosage. Then we translated these clinical findings to animal model study. In our translational rat experiment, after induction of opioid tolerance by intrathecal morphine infusion that mimicking intrathecal morphine infusion therapy in clinical scenario, the lumbar spinal cord CXCL1 and CXCL12 messenger RNA was significantly upregulated as in opioid tolerant humans. Although exogenous very low dose CXCL1 and CXCL12 infusion alone did not affect baseline behavior, the analgesic efficacy of intraperitoneal injection of morphine dropped significantly. After establishing tolerance by intrathecal continuous infusion of morphine, opioid tolerance development was markedly accelerated by co-administration of exogenous CXCL1 and CXCL12. On the contrary, tolerance was attenuated by co-administration of ligand neutralizing antibodies or receptor antagonists.

Based our current results, we will keep working on the following projects:

A. To further elucidate the mechanism underlying how opioid triggers CXCL1 and CXCL12 upregulation. Is it involved in non-stereoselective TLR4 activation prosposed by or specific chemokine over production is the downstream product of MOR activation and through what molecular mechanisms?

B. Since CXCL1 and CXCL12 are upregulated in both opioid-tolerant patients and rodents and in parallel the onset and extent of opioid tolerance was affected by intervening intrathecal CXCL1/CXCR2 and CXCL12/CXCR4 signaling pathway.

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Therefore, the CXCL1/CXCR2 and CXCL12/CXCR4 signal pathways may be novel drug targets for the potential treatment of opioid tolerance. If possible, we will collaborate with other labs to further screen and validate small compounds of CXCR2 and CXCR4 blockers and hopefully can introduce them into early phase clinical trial.

Based on our current study paradigm, we first discover potential biomarkers of opioid tolerance and do following "reverse" translational studies in lab animal. During these years we have already collected many human CSF samples thus we can keep on screening potential molecular targets not only chemokines but also other cytokines or soluble factors that may or even may not be involved in neuroinflammation process. For example, recently, our lab has discovered leukemia inhibitory factor (LIF) was also upregulated among opioid tolerant patients and animals. However, in translational lab animal research, we found that exogenous LIF can potentiate morphine acute antinociceptive effects and attenuate tolerance development (Tu *et al.,* in press). These findings are completely in the opposite direction of our results that implies during opioid tolerance and neurinflammation, there should be other physiological adaptations to counterbalance the proinflammatory cascades. These counterbalance factors would be also important for future drug development.

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From lab animal research in recent decades, neuropathic pain and opioid tolerance share a lot of common pathogenic mechanisms, for example, dysregulated neuroinflammation. Since neuropathic pain is extremely difficult to treat and poorly responds to all currently available therapeutic approaches including strong opioids and neuromodulatory agents such as tricyclic antidepressants and calcium channel modulators, for example, pregabalin. New treatment for neuropathic pain is urgently needed. In the following year, we will follow the same study paradigm to facilitate exploration of biomarkers and pathogenesis then translate into lab animal research. First, we can screen our current available CSF sample bank for patients with predominantly neuropathic pain features such as burning, tingling, electric shooting pain patters with compatible image evidence such as tumor external compression or direct invasion of nerve trunk or plexus. While concurrent use of strong opioids in the severe pain patient population is inevitable, careful study design is paramount. Following our current study result, theoretically, chemo/cytokine perturbation might come from opioid tolerance or neuropathic pain or both. We thus can compare their CSF sample retrieved during different time points and compare with healthy volunteers. If one specific biomarker is obviously different from healthy control but stay the same level between different opioid dosages, then the biomarker is for neuropathic pain. If the biomarker is

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different from healthy control but also different between opioid dosage, then the biomarker is for opioid tolerance. Since most of current neuropathic pain model might not mimic clinical scenario, for example, spared nerve injury neuropathic pain model, 2 branches of sciatic nerve have to be sectioned and test the behavior on sural nerve territory. Most of the nerve injury related neuropathic pain in clinical setting the nerve injury site is usually very vague. On the contrary, most of cancer related neuropathic pain has direct image evidence of site of nerve injury. Therefore, we will develop neuropathic cancer pain model by implanting exnograft to the lumbar or sacral plexus area and do following researches. This project would be our labs mid to long term project.

In conclusion, our research provided both human and lab animal evidence to show that CXCL1/CXCR2 and CXCL12/CXCR4 signaling pathway may be involved in the pathogenesis of opioid tolerance. Hopefully we can provide a new insight of further research and new drug discovery strategy to combat opioid tolerance and neuropathic pain.

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