國立政治大學心理學研究所碩士論文

慢性疼痛或壓力情境對於類鴉片delta受體的調節與其抗憂鬱功能的改變

Effects of chronic pain or stress on the modulation of delta opioid receptor and its mediated antidepressant-like effect

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中華民國九十九年七月

謝辭

感謝口試委員陳景宗老師、廖瑞銘老師以及柯美全老師的指正與補強，讓本篇論文能夠更完整。感謝指導教授柯美全老師嚴謹及細心的督促我對於論文種種的細節，讓我對於做研究及撰寫學術論文有更深的了解。感謝鄭家珍及蘇品諺不辭勞苦的幫忙一起收集實驗數據，有了你們的幫助，實驗才能加快腳步。感謝映伶學姐提供對於口試方面的技巧；感謝居翰、俊宇學長陪我在研究室聊天紓解壓力；感謝實驗室其他人在各方面的協助。感謝蓁涵的支持與鼓勵，妳充實了我的研究所生活，謝謝妳。感謝羽毛球系隊、校隊的大家在我面臨實驗及論文壓力時，陪我打球舒緩煩悶的心情。感謝我的父母，雖然這一年我很少回家，但總是支持我完成我該做的。最後，要感謝為了實驗犧牲生命的大白鼠們，有了你們的貢獻，本篇論文才能完成。

摘要

憂鬱症是盛行的精神疾病之一。慢性疼痛或是處在長期壓力情境的患者常與憂鬱症產生共病。在動物研究中，類鴉片 delta 受體制效劑能產生抗憂鬱效果，並且在發炎性疼痛的研究也指出類鴉片 delta 受體制效劑能展現抗痛覺過敏的效果。本研究主要利用大白鼠腦室內給予類鴉片 delta 受體制效劑 SNC80 以及三環抗憂鬱劑 amitriptyline，來探討並比較其所產生的抗憂鬱效果在發炎性疼痛或長期壓力情境下與正常情境下的異同。大白鼠強迫游泳試驗被用來比較測試藥物的抗憂鬱效果；佛氏完全佐劑經由皮下注射至大白鼠右後腳掌底板來產生發炎性疼痛；腎上腺皮質酮經由皮下注射且持續 21 天來產生長期性壓力；西方墨點法用來檢驗在發炎性疼痛或長期壓力下，類鴉片 delta 受體蛋白質在大白鼠海馬迴的細胞膜上的改變。另外，拮抗劑實驗則用來確認類鴉片 delta 受體所產生的抗憂鬱效果。實驗結果顯示，大白鼠在正常情境下，SNC80 及 amitriptyline 皆能產生抗憂鬱效果；然而在發炎性疼痛下，SNC80 所產生的抗憂鬱效果有提高的表現，並且類鴉片 delta 受體蛋白質的數量在海馬迴的細胞膜上也隨著疼痛的時間增長而增加，amitriptyline 則跟正常情境下的效果相似。另外，大白鼠在長期性壓力下，SNC80 的抗憂鬱效果則沒有提高的表現，並且類鴉片 delta 受體蛋白質的數量在海馬迴的細胞膜上也未受到改變。本研究透過行為實驗提出類鴉片 delta 受體制效劑的藥理特性，並用分子生物學的方法來對應行為實驗的結果。本研究可做為未來類鴉片 delta 受體制效劑在治療慢性疼痛的憂鬱症患者上，可能發展為抗憂鬱藥的一個證據。

關鍵字：憂鬱症，發炎性疼痛，長期壓力，類鴉片 delta 受體，制效劑

Abstract

Depression is one of the most prevalent mental illnesses all over the world. Patients with chronic pain or stress often have depression. Previous studies have shown that delta opioid receptor (DOR) agonists produced antidepressant-like effects in animal models and that antihyperalgesic effects of DOR agonists can be enhanced in rats under inflammatory pain. The aim of the study was to investigate and compare the antidepressant-like effects of a DOR agonist, SNC80, and a tricyclic antidepressant, amitriptyline, following intracerebroventricular (i.c.v.) administration in rats under different states. The forced swim test was used to determine the antidepressant-like effects of test compounds. Complete Freund's adjuvant was injected subcutaneously into the right hind paw of rats to elicit inflammatory pain. Corticosterone was injected subcutaneously once per day for 21 days to induce chronic stress. The western blot was used to quantify the levels of DOR protein on plasma membrane in the hippocampus of rats under inflammatory pain or chronic stress. In addition, antagonist experiment was conducted to verify the receptor mechanism underlying the antidepressant-like effects of DOR agonist. Results indicated that i.c.v. SNC80 and amitriptyline dose-dependently produced antidepressant-like effects in rats under normal state. More importantly, the potency of SNC80-induced antidepressant-like effects, but not amitriptyline, was enhanced in rats under inflammatory pain. In addition, up-regulation of supraspinal DORs was time-dependently associated with enhanced antidepressant-like effects of SNC80 in rats under inflammatory pain. On the other hand, SNC80 did not produce enhanced antidepressant-like effects, and DOR density was not changed in rats under chronic stress. This study

provides evidence of the DOR agonist's state-dependent effects and suggests that DOR agonists may be more effective as potential antidepressants for patients with depression comorbid with chronic pain.

Key words: depression, inflammatory pain, chronic stress, delta opioid receptor, agonist

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Introduction

Definition of depression

Depression is one of the most prevalent mental illnesses all over the world. According to Diagnostic and Statistical Manual of Mental Disorders, 4th edition, revised (DSM-IV-TR, American Psychiatric Association, 2000), mood disorders can be recognized as major depression and bipolar disorder. Major depression is diagnosed for at least two weeks with depressed mood or loss of pleasure. Furthermore, depressed patients also have insomnia, agitation, poor appetite, loss of energy, or negative self-concept. The World Health Organization has announced that depression is one of three most serious disorders, while the others are cancer and AIDS in the 21st century.

Epidemiology studies showed that one out of six individuals in the United States suffers from depression in their lifetime (Kessler, Chiu, Demler, Merikangas, & Walters, 2005). In Taiwan, based on the statistics in 2008 from the Department of Health, there are 2 hundred thousand patients affected by mood disorders. The first onset of depression is also becoming younger to 24 years old. More importantly, the estimated number of patients may be underestimated as there are people who are not reported nor have slight symptoms but potential to be affected by depression (Sihvola et al., 2007).

In addition, depression often co-exists with other mental illnesses such as anxiety disorder, post traumatic stress disorder, and substance abuse (Regier, Rae, Narrow, Kaebler, & Schatzberg, 1998; Shalev et al., 1998; Conway, Compton, Stinson, & Grant, 2006). Moreover, some physiological disorders such as Parkinson's disease, cancer, and diabetes may also cause depression-like mood state (Cassano & Fava, 2002; Katon & Clechanowski,

2002). In this situation, there are many kinds of treatment for depression. Psychotherapy and pharmacotherapy are the commonly used approaches. However, there are still some patients who took the medication did not show any improvement. Thus, depression is a common but serious problem, and development of effective treatments is necessary.

Etiology of depression

Research on the neurobiology of depression has been studied since 1950s, and the most discussed mechanism of depression is the regulation of monoamines (López-Muñoz & Alamo, 2009). The monoamine hypothesis has been discussed over 50 years. This hypothesis states that people who suffer from depression have decreased monoaminergic activity (Krishnan & Nestler, 2008). The relationship between monoamine and depression was discovered by two unrelated drugs, namely iproniazid and imipramine, developed for non-psychiatric medication. Both drugs could up-regulate central serotonin and norepinephrine transmission, and depressed patients taking such drugs could recover from the illness (Berton & Nestler, 2006). However, the antidepressant effects of these monoaminergic compounds need to take weeks or months to be manifested. In contrast, the synthesis or secret of monoamines is increased right after administration of these compounds. Thus, the monoamine hypothesis is insufficient to explain the mechanism of depression.

Stress often goes along with depression, and the mechanism which the brain reacts to stress is activation of the hypothalamus-pituitary-adrenal (HPA) axis. Under stress condition, neurons in paraventricular nucleus of the hypothalamus secret corticotropin-releasing factor (CRF), and CRF stimulates

anterior pituitary to release adrenocorticotropin hormone (ACTH). ACTH then stimulates the synthesis and release of cortisols from adrenal cortex (Krishnan & Nestler, 2008). Following the continuous secretion of cortisols, the hippocampus plays an inhibitory role to regulate the release of CRF in hypothalamus. Normal individuals increase the cortisols in the adrenal gland to deal with the stress-related situation. When over-expressing cortisols, neurons in hippocampus may be damaged, and the negative feedback of HPA axis is inhibited, causing consistently high cortisols in humans (Parker, Schatzberg, & Lyons, 2003). For example, subjects showed an overwhelming elevation of cortisols which may damage hippocampal neurons or reduce granule cell neurons in hippocampal dentate gyrus under chronic stress (Fuchs & Gould, 2000). Therefore, excessive activation of the HPA axis is often observed in stress-related depressed patients.

Following chronic or severe stress, the damage of hippocampus has been reported with decrements in neurotrophic factors (Monteggia et al., 2004). Thus, the neurotrophic mechanism is also linked to the etiology of depression. The neurotrophic factors regulate neuronal plasticity, survival, and differentiation (Bramham & Messaoudi, 2005; Tongiorgi, Domenici, & Simonato, 2006). Among the nerve growth factor family of neurotrophins, brain-derived neurotrophic factor (BDNF) is the most abundant factor in many brain regions (Kozisek, Middlemas, & Bylund, 2008). Lots of reports demonstrated that BDNF is related to depression. For example, patients who suffer from major depression have decreased serum BDNF levels (Shimizu et al., 2003). Exogenous infusion of BDNF promotes the growth of 5-HT-containing neurons and elevates the secret of norepinephrine in many brain regions in rats (Siuciak, Boylan, Fritsche, Altar, & Lindsay, 1996; Altar,

1999). Local infusion of BDNF in the rat periaqueductal gray, raphe nuclei, and dentate gyrus of hippocampus showed antidepressant-like effects in the forced swim test and learned helplessness model (Siuciak, Lewis, Wiegand, & Lindsay, 1997; Shirayama, Chen, Nakagawa, Russell, & Duman, 2002). Furthermore, knockout of gene encoding BDNF in either mice forebrain or dentate gyrus of hippocampus attenuated antidepressant-like actions of a tricyclic antidepressant (TCA) desipramine or a selective serotonin reuptake inhibitor (SSRI) citalopram in the forced swim test (Monteggia et al., 2007; Adachi, Barrot, Autry, Theobald, & Monteggia, 2008). Thus, these studies have pointed out that BDNF is involved in the pathophysiology of depression and the mechanism of action of antidepressant drugs (Duman & Monteggia, 2006; Castrén, Võikar, & Rantamäki, 2007; Martinowich, Manji, & Lu, 2007).

Medications of depression

In the present, there are lots of antidepressants which can alleviate the symptoms of depression. The first found antidepressants were monoamine oxidase inhibitors (MAOIs). The MAOIs, as implied by the name, block monoamine oxidase from deactivating neurotransmitters, and increase the levels of dopamine, serotonin and norepinephrine in the synapse. The second ones are TCAs. The TCAs, which include amitriptyline, desipramine, imipramine, and nortriptyline, contain three rings on their chemical structure. They were discovered accidently when clinicians were finding cures for schizophrenic patients in early 1950s. The TCAs blocked the reuptake of norepinephrine or serotonin transporters causing increase of neurotransmitters (Gillman, 2007). SSRIs are the latest discovered monoamine-based agents including fluoxetine, paroxetine, sertraline, and citalopram. Because of the

similar therapeutic effect as TCAs and fewer autonomic side effects with less sedation, the SSRIs have become the most widely used drugs for the treatment of depression (Arroll et al., 2005).

Nevertheless, no matter what kind of drugs depressed patients take, there are always some disturbing side effects (Hammen & Watkins, 2008). For example, MAOIs increase blood pressure, stroke, or even death if patients combine the drugs with the food (Rapaport, 2007). Thus, MAOIs are the last choice to treat depression if other antidepressants fail. The common side effects of TCAs include cardiovascular influence such as hypotension. Other side effects include dry mouth, nausea, blurry vision, weight gain and sexual dysfunction. They may be lethal if taking in overdose. SSRIs have some same side effects as TCAs, but have more profound effects such as dry mouth, gastrointestinal problems, and sexual dysfunction (Steffens, Krishnan, & Helms, 1997; Wilson & Mottram, 2004). In general, TCAs are effective in treating pain and depression and SSRIs provide less therapeutic value (Coluzzi & Mattia, 2005; Jann & Slade, 2007). Taken together, these agents are effective in the treatment of depression, but they are associated with a high incidence of autonomic and cardiovascular side effects. When taken in an overdose, they can cause seizures and cardiac arrhythmia (Arroll et al., 2005).

Other studies which focus on dysregulation of HPA axis have developed CRF antagonists to treat depression (Nielsen, 2006; Holsboer, & Ising, 2008). For instance, chronic administration of CRF antagonist R121919 exhibited antidepressant-like effects in the mouse tail suspension test (Nielsen, Carey, & Gold, 2004). In addition, a small clinical trial has studied the ability of the CRF antagonist R121919 to treat depressed patients and have observed improvement in some patients. However, this CRF antagonist needed to be

administered for several weeks before any improvement was seen (Zobel et al., 2000).

Introduction of delta opioid receptor

Another potential candidate for the treatment of depression is the delta opioid receptor (DOR). The opioid receptors have been identified since 1970s, and receptor binding studies showed that they belong to G-protein coupled receptors (Pert & Snyder, 1973; Pert, Pasternak, & Snyder, 1973). Among opioid receptor subtypes, the DOR was the first to be cloned (Evans, Keith, Morrison, Magendzo, & Edwards, 1992; Kieffer, Befort, Gaveriaux-Ruff, & Hirth, 1992). Both rat and human DORs were cloned around 1990s (Fukuda, Kato, Mori, Nishi, & Takeshima, 1993; Knapp et al., 1994). The DORs were found within the central nervous system as well as peripheral tissues, including gastrointestinal tract, heart, and cells of the immune system (Chang, Porreca, & Woods, 2004; Trescot, Datta, Lee, & Hansen, 2008). Human studies showed that the DORs clearly presented in hippocampus, olfactory bulb, nucleus accumbens, putamen, globus pallidus, and hypothalamus (Simonin et al., 1994). Interestingly, human imaging studies reported volume reductions in frontal cortex and hippocampus, or changes in blood flow in striatum, amygdala, and thalamus in depressed patients (Drevets, 2001; Sheline, 2003). Anatomical studies of rodent also supported that DORs exist in different brain regions, including nucleus accumbens, olfactory bulb, amygdala, striatum, cerebral cortex, hippocampus, and hypothalamus (Mansour, Fox, Akil, & Watson, 1995; Satoh & Minami, 1995). In addition, DORs have been involved in many physiological functions, such as pain, reward, mood, motor integration, and cognitive functions (Meyer & Meyer, 1993; Suzuki, Tsuji, Mori, Misawa, &

Nagase, 1996; Waldhoer, Bartlett, & Whistler, 2004). In particular, a number of studies revealed that DORs are suggested to play essential role in mood regulation (Broom, Jutkiewicz, Rice, Traynor, & Woods, 2002a; Jutkiewicz, 2006).

Three opioid receptor subtypes are different in each other in regulating physiological functions (Gavériaux-Ruff & Kieffer, 2002; Kieffer & Gavériaux-Ruff, 2002). For example, DOR-deficient mice showed hyperlocomotor activity, whereas mu opioid receptor (MOR)-deficient and kappa opioid receptor (KOR)-deficient mice showed no change or slight reduction in locomotion (Sora et al., 1997; Simonin et al., 1998; Filliol et al., 2000). In addition, DOR mutant mice showed no difference in response to acute and inflammatory pain measured by a variety of nociceptive assays (Filliol et al., 2000). In contrast, MOR mutant mice showed decrease nociceptive responses, whereas KOR mutant mice displayed an enhancement of writhes in response to chemical pain (Simonin et al., 1998; Sora, Li, Funada, Kinsey, & Uhl, 1999). In addition to the locomotor activity and nociceptive sensitivity, DOR seems to have other physiological functions. In particular, DOR seems to alter emotional responses. Mice lacking DOR exhibited depression-like behaviors in the forced swim test and anxiety-like behaviors in the elevated plus-maze (Filliol et al., 2000). In addition, mice lacking preproenkekphalin significantly displayed increased anxiety-like responses in fear conditioning paradigm or anxiety-provoking environment (Konig et al., 1996; Ragnauth et al., 2001). These genetic studies indicated that DOR is the main target involved in the regulation of mood.

Antidepressant-like effects of DOR agonists

Pharmacological studies have been conducted extensively to document the role of DOR in animal models of depression (Broom et al., 2002a; Jutkiewicz, 2006). The forced swim test was developed to measure the immobility, swimming, and climbing behaviors of rats when they were exposed to an inescapable water tank (Porsolt, Le Pichon, & Jalfre, 1977). Clinically used antidepressants can decrease the number of immobility in this behavioral assay. Therefore, this procedure has been established to study the potential antidepressant-like effects of experimental compounds (Porsolt, Anton, Blavet, & Jalfre, 1978; Zhang, Shi, Woods, Watson, & Ko, 2007). For instance, mice given enkephalinase inhibitor BL-2401 dose-dependently reduced the time of immobility in forced swim test (Kita et al., 1997). Also, rats who were subjected to inescapable shock showed reduced the escape failure numbers in learned helplessness model when given enkephalinase inhibitor RB101, and the effect was reversed by DOR antagonist naltrindole (Tejedor-Real et al., 1998). Studies using non-peptidic DOR agonists, (+)BW373U86 and SNC80, both produced antidepressant-like effects in the rat forced swim test (Broom et al., 2002b; Torregrossa, Folk, Rice, Watson, & Woods, 2005). The peptidic DOR agonists such as deltorphin II also showed the antidepressant-like effects in the forced swim test (Torregrossa et al., 2006). Furthermore, these behavioral effects could also be blocked by naltrindole, suggesting that activation of DOR indeed mediates antidepressant-like effects in rodents (Broom et al., 2002c; Saitoh et al., 2004).

Importantly, neither MOR agonist morphine nor KOR agonist CI977 could produce antidepressant-like effect, which supports the selective role of DOR participating in the regulation of mood (Broom et al., 2002a). Studies of endogenous peptides demonstrated that only leu-enkephalin and

met-enkephalin significantly decreased immobility in the forced swim test, whereas endomorphin-1 and -2, and dynorphin A did not have the same effect (Zhang et al., 2006). Moreover, the antidepressant-like effects of enkephalins were blocked by naltrindole, and were not affected by MOR or KOR antagonist, which revealed that antidepressant-like effects of enkephalins were mediated through activation of DORs (Zhang et al., 2006). On the other hand, the animal model of olfactory bulbectomy has been conducted in which the behaviors are similar to depressed patients (Kelly, Wrynn, & Leonard, 1997; Song & Leonard, 2005). The olfactory bulbectomized rats showed stress-induced increase in locomotor activity and irritability or hypermotionality to given stimuli (Redmond, Kelly, & Leonard, 1997; Saitoh et al., 2006). These behavioral and neurochemical changes of olfactory bulbectomized animals were attenuated by chronic treatment of antidepressants (Song & Leonard, 2005). Recent studies demonstrated that administration of DOR agonist SNC80 reduced scores of emotional responses and increased the entries and time in the open arm of elevated plus-maze compared with vehicle-treated olfactory bulbectomized rats. These findings indicated that DOR plays a part in this paradigm (Saitoh et al., 2008; Takahashi et al., 2008). Taken together, these studies strongly suggested that DOR activation may be involved in regulating the affective states.

Relationship between antidepressants and BDNF

In addition to gene knockout animals and pharmacological studies, other prevalent studies focus on the effects of DOR modulation of BDNF (Tardito et al., 2006; Kozisek et al., 2008; Krishnan & Nestler, 2008). Increased hippocampal BDNF immunoreactivity has been found in depressed patients

who had been treated with antidepressants (Chen, Dowlatshahi, MacQueen, Wang, & Young, 2001). Other study showed that antidepressant-treated patients reversed the serum BDNF to basal levels compared with normal subjects (Shimizu et al., 2003). Animal studies demonstrated that chronic administration of MAOI phenelzine increased BDNF mRNA expression in the hippocampus and frontal cortex (Dwivedi, Rizavi, & Pandey, 2006). Chronic treatment of TCA amitriptyline also indicated that BDNF immunostaining were elevated in CA regions of hippocampus (Xu, Richardson, & Li, 2003). Moreover, SSRI such as fluoxetine could augment BDNF mRNA expression in hippocampus when given chronically (De Foubert et al., 2004). Thus, up-regulation of BDNF seems to mediate the action of antidepression.

More intriguingly, in situ hybridization showed that single administration of synthetic DOR agonist (+)BW373U86 increased BDNF mRNA expression in CA1 region of hippocampus, frontal cortex, basolateral amygdala, and olfactory cortex in rats, whereas pretreatment of DOR antagonist naltrindole blocked increases of BDNF mRNA expression in (+)BW373U86-treated group (Torregrossa et al., 2004). Peptidic DOR agonists DPDPE and deltorphin II also produced increased BDNF mRNA expression in frontal cortex and CA3 region of hippocampus (Torregrossa et al., 2006). In addition, rats given leu-enkephalin showed increased BDNF mRNA expression in the hippocampus, and this effect was blocked by naltrindole pretreatment (Zhang et al., 2006). Taken together, given that BDNF and clinically used antidepressants are involved in neurocircuits regulating emotion, the evidence from BDNF and experimentally used compounds may indicate that DOR agonists have the therapeutic potential as antidepressants (Torregrossa et al., 2004; Tardito et al., 2006).

Potential limitation of DOR agonists

Although animal studies have suggested the therapeutic potential of DOR agonists as antidepressants, the side-effect profile of DOR agonists may limit its clinical use. For example, high doses of DOR agonists produced convulsions in animals including mice, rats, and monkeys (Comer et al., 1993; Dykstra, Schoenbaum, Yarbrough, McNutt, & Chang, 1993; Broom et al., 2002c). It is suggested that the antidepressant-like effects of DOR agonists might be due to the convulsant action of these drugs. Nevertheless, several studies have revealed that DOR agonists-induced convulsions are not related to the antidepressant-like effects (Broom et al, 2002c; Jutkiewicz, Rice, Traynor, & Woods, 2005). For example, slowing the infusion rate of SNC80 still produced antidepressant-like effects without eliciting convulsions in rats (Jutkiewicz et al., 2005). In addition, central administration of DOR agonists (+)BW373U86, or endogenous DOR-preferring opioid peptides, leu- and met-enkephalin, did not show convulsant activity at doses producing antidepressant-like effects (Zhang et al., 2006). More importantly, Codd et al. (2009) demonstrated that oral, intravenous, or subcutaneous administration of different chemical structures, a DOR agonist JNJ-20788560, did not observe convulsions at pharmacologic doses or at doses in the toxicologic range to animals. Therefore, it is unlikely that convulsion plays a role in the antidepressant-like effects of DOR agonists in animal models of depression.

On the other hand, DOR agonists were reported to produce psychostimulant-like behaviors in rodents, such as hyperlocomotor activity, conditioned place preference, and increased lever pressing in self-administration model (Devine & Wise, 1994; Longoni, Cadoni, Mulas, Di

Chiara, & Spina, 1998; Fraser et al., 2000). Moreover, DOR agonists increased the release of dopamine from nerve terminals or specific brain regions (Longoni et al., 1991; Fusa et al., 2005). However, the interaction of DOR agonists and dopamine systems was still controversial. There are several studies showed that DOR agonists may not mediate psychostimulant-like effects (de Vries, Babovic-Vuksanovic, Elmer, & Shippenberg, 1995). For instance, systemic administration of SNC80 and BW373U86 did not increase dopamine release as measured by *in vivo* microdialysis at doses that stimulated locomotor activity and produced conditioned place preference (Longoni et al., 1998). Furthermore, drug-induced leftward shift in an animal assay of intracranial self-stimulation frequency-rate curve was considered as an indication of abuse liability. Systemic administration of SNC80 did not alter the frequency-rate curve in intracranial self-stimulation, whereas D-amphetamine produced leftward shifts (Do Carmo et al., 2009). More importantly, in the prototypical primate model determining the abuse liability of drugs, monkeys did not self-administer SNC80 (Negus, Gatch, Mello, Zhang, & Rice, 1998). Taken together, these findings indicated that DOR agonists may have relatively low abuse potential.

Functions of DOR agonists under inflammatory pain

Clinical studies have pointed out that patients with chronic pain or neuropathic pain comorbid with affective disorders such as anxiety and depression (Bair, Robinson, Katon, & Kroenke, 2003; Bair, Wu, Damush, Sutherland, & Kroenke, 2008). For example, patients with fibromyalgia exhibited depressive symptoms (Arnoald, 2008). Patients with cancer pain were also burdened with depression and cognitive deterioration (Spoletini,

Caltagirone, Ceci, Gianni, & Spalletta, 2009). Epidemiology studies revealed that patients with cancer pain experienced depressive symptoms with an incidence of 15-50 % (Lydiatt, Moran, & Burke, 2009). Patients with rheumatoid arthritis were highly associated with depression, with mild severity up to 42 % of populations (Bruce, 2008). Previous review indicated that there is 30-60 % co-occurrence rate for pain and depression (Bair et al., 2003). Thus, it will be interesting to study effect of drugs in subjects under pain or depression conditions.

Previous studies have indicated that DOR agonists produce antinociceptive effects in animal models. In particular, the effects of DOR agonists are more prominent in rat under chronic pain (Bie & Pan, 2007; Cahill, Holdridge, & Morinville, 2007). For example, rats receiving complete Freund's adjuvant (CFA) into the plantar surface of the hind paw showed significantly decreased paw withdrawal latency in plantar test (Fraser, Gaudreau, Clarke, Menard, & Perkins, 2000). This effect was attenuated by intracerebroventricular administration of DOR agonists, deltorphin II and SNC80. However, the same dose of DOR agonists were less effective in producing antinociceptive effects against acute noxious stimuli, indicating that DOR agonists have an increased potency in rats under inflammatory pain (Petrillo et al., 2003). Recent study using other DOR agonist JNJ-20788560 increased paw withdrawal latencies in CFA-treated rats which also supports the increased potency of DOR agonists (Codd et al., 2009).

Microinjection of DOR agonist [D-Ala2,Glu4]deltorphin into the rostral ventromedial medulla of rats produced antihyperalgesic effects in CFA-treated rats (Hurley & Hammond, 2000). More importantly, compared with saline-treated group, [D-Ala2,Glu4]deltorphin-induced antihyperalgesia was

more profound in CFA-treated group. The ED50 dose of [D-Ala2,Glu4]deltorphin of 4-day CFA-treated rats was six fold higher than 2-week CFA-treated rats, indicating the magnitude of enhanced antihyperalgesia paralleled the chronicity of the injury. Another study also revealed that intrathecal administration of [D-Ala2,Glu4]deltorphin produced a dose-dependent thermal antihyperalgesia in both CFA-treated and control rats (Cahill, Morinville, Hoffert, O'Donnell, & Beaudet, 2003). The dose response curve for [D-Ala2,Glu4]deltorphin was significantly shifted to the left by a sixty fold in CFA-treated rats compared with control rats.

In addition to the potencies of DOR agonists against different pain modalities, other studies have indicated that chronic or inflammatory pain could up-regulate DOR densities. Biomedical researches revealed that the level of DOR mRNA was increased in the ipsilateral dorsal horn of rat spinal cord following CFA injection (Cahill et al., 2003). The level of DOR protein in lumbar spinal cord membranes of ipsilateral CFA-injected rats was also increased in the western blot assay (Cahill et al., 2003). The mouse study using immunogold cytochemistry which was used to represent the immunoreactive DOR supported that 72-hour CFA-induced inflammation produced a significantly higher ratio of plasma membrane to intracellular receptors. The membrane density of gold particles had a 75% increase in dendrites of the ipsilateral dorsal horn of spinal cord as compared to control group (Morinville, Cahill, Kieffer, Collier, & Beaudet, 2004). Similarly, the dorsal root ganglion showed a significant increase of internalized fluorescent deltorphin which represent the amount of DOR has the capacity to bind in 72-hour CFA-treated rats (Gendron et al., 2006). Thus, up-regulation of DOR following injection of inflammatory agents may contribute to enhanced

antihyperalgesic effects of DOR agonists.

The pharmacological profiles of potential antidepressant drugs are often studied in animals under normal state. However, it is more complicated that subjects may co-occur with depression under stress condition which can be induced by chronic pain or stress-caused dysfunction of HPA-axis. For example, repeated administration of corticosterone increased the number of immobility in the rat forced swim test (Johnson, Fournier, & Kalynchuk, 2006). Moreover, reduction of BDNF was reported in the stress-induced hippocampal damage observed in depression (Duman & Monteggia, 2006). Previous studies have shown that neurons expressing DOR were located in hippocampus, amygdala, and ventrolateral medulla, and these brain regions are related to the neurobiology of stress (Drolet et al., 2001). However, it is unknown to what degree the DOR density is changed in rats under chronic stress. Based on previous studies, it may be expected that antidepressant-like effects of DOR agonists would be increased in rats under chronic pain. Nevertheless, it would be interesting to study and compare the antidepressant-like effects of DOR agonists in rats under chronic stress versus inflammatory pain. More importantly, it is worth conducting experiments to further determine whether up-regulation of DOR contributes to the enhanced antidepressant-like effects of DOR agonists in rats under either context.

Aim

Based on the findings described above, DOR agonists could produce antinociceptive effects and its antinociceptive effectiveness might be enhanced in the presence of up-regulation of DORs induced by inflammatory pain. However, it is unknown how the antidepressant-like effects of DOR agonists are manifested in subjects under normal versus painful states. Meanwhile, it is unknown to what degree the DOR densities are changed in subjects under inflammatory pain versus stress condition. Thus, the aim of the study is to investigate whether antidepressant-like effects of DOR agonists are enhanced under chronic pain or/and stress conditions and whether enhanced antidepressant-like effects are accompanied by the up-regulation of the DOR.

Methods

Animals

Male Wistar rats (200-250 g) were obtained from Laboratory Animal Center of National Taiwan University and housed individually. All animals were allowed ad libitum access to food and water, and maintained on a 12-hour light / dark cycle with lights on 08:00 AM in a room kept at a temperature around 22±2 ^{0}C. All experiments were conducted following the regulation by the local animal care committee of National Cheng Chi University.

Intracerebroventricular (i.c.v.) surgery

Rats were anesthetized by intraperitoneal injection of Zoletil 50 (Virbac, Carros, France) in a volume of 1 ml/kg. Each rat was prepared with 23-gage stainless steel cannula (Shineteh, Taipei, Taiwan) extending into the right lateral cerebral ventricle (coordinated from bregma, AP: 0.8 mm, ML: 1.5 mm, DV: 4.0 mm, Paxinos & Watson, 2007). After placement, the cannula was fixed to the skull with acrylic dental cement. Animals were allowed 5 to 7 days to recover from surgery. After the experiment, each animal's i.c.v. cannula placement was verified by injecting methylene blue and checking for distribution. Only data from animals with appropriate cannula placement were used and analyzed.

Drug treatment

[(+)-4-[(αR)-α-[(2S,5R)-2,5-dimethyl-4-(2-propenyl)-1-piperazinyl]-(3-methoxyphenyl)methyl]-N,N-diethylbenzamide (SNC80; Tocris, Bristol, UK) was

dissolved in sterile water with 8% of 1N HCl. Amitriptyline hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sterile water. Drugs were intracerebroventricularly administered via the guide cannula in volumes of 10 μL using a 25 μL Hamilton syringe (Hamilton, Reno, NV, USA) attached via a polyethylene PE20 tube (Plastics One, Roanoke, VA, USA) to a 30-gage needle (Shineteh, Taipei, Taiwan). Solution was administered over a period of 60 seconds and the needle was left within the guide cannula for an additional 30 seconds to prevent reflux. For antagonist study, naltrindole hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sterile water, and administered in a volume of 1 ml/kg. Sterile water was administered as vehicle for the control injection. Corticosterone (CORT; Sigma-Aldrich, St. Louis, MO, USA) was suspended in saline with ethanol (Shineteh, Taipei, Taiwan) and polyoxyethlene glycol sorbitan monooleate (Tween-80; Sigma-Aldrich, St. Louis, MO, USA) at a ratio of 8:1:1 with administration in a volume of 1 ml/kg.

Procedures

Forced swim test. The forced swim test was modified from Broom et al. (2002b), and was used to quantify the antidepressant-like effect of test compounds. 15 minutes after drug injection, rats were placed in a clear cylindrical Plexiglas container (46 cm tall x 20 cm diameter) filled with 30-cm depth of 25^{o}C (±1^{o}C) water for a 15-min swim session. Cylinders were cleaned and fresh water added between each test. Behavioral scoring was performed by observers who were blind to the treatment received by each animal. The behaviors were videotaped and scored every 3 seconds for 5-minute periods during the swim. Behaviors were classified as immobility, swimming, and

climbing (Detke, Richels, & Lucki, 1995; Broom et al., 2002b). Immobility was defined as floating in the water without struggling and using only small movements to keep the head above water. Swimming was defined as moving limbs in an active manner. Climbing was defined as making active movements with the forepaws in and out of the water, often directed at the wall of the swim tank (Broom et al., 2002b). The numbers of each behavior scored during each 5-minute period were totaled and averaged within each treatment group. After a period of struggling, rats exhibited immobile posture which was similar to behavioral despair in some patients diagnosed with depression (Porsolt et al., 1977). Only antidepressant drugs, not anxiolytic and antipsychotic drugs, reduced the numbers of immobility (Porsolt et al., 1978; Rupniak, 2003). Thus, the numbers of immobility were used to indicate the antidepressant-like effects of test compounds (Porsolt et al., 1977, 1978; Broom et al., 2002b).

Inflammatory pain model. Chronic inflammation was induced by subcutaneous administration of 100 μl of complete Freund's adjuvant (CFA; Sigma-Aldrich, St. Louis, MO, USA) into the plantar surface of the right hind paw of rats under anesthesia. Control rats received an injection of vehicle in the same volume of 100 μl. Behavioral testing and western blotting were conducted 3 days, 1 week, or 2 weeks after CFA injection. The dose of CFA was selected based on previous studies that produced hyperalgesic effects (Cahill et al., 2003; Gendron et al., 2007).

Chronic stress model. The rats were handled once every day for 7 days in the colony room prior to starting experimental stress manipulations. After the

handling phase, the rats were weight-matched and assigned randomly to the one of the following groups: repeated CORT injection group, or a repeated vehicle (saline with ethanol and Tween80) injection group. The CORT group and vehicle group received a 40 mg/kg injection of CORT and vehicle injection once per day for 21 consecutive days, respectively. All CORT and vehicle injections were subcutaneously injected each day at a volume of 1 ml/kg between 10:00 am and 12:00 pm. The dose of CORT has been reported to elevate plasma CORT levels in rats, with peak levels occurring 4 hours after the injection (Sousa, Madeira, & Paula-Barbosa, 1998). The dose of CORT was selected based on previous studies showing that repeated injection of CORT produced depression-like behavior in the rat FST (Johnson et al., 2006; Marks, Fournier, Kalynchuk, 2009).

Western blot. For quantification the protein level of delta opioid receptor, CFA- and vehicle- injected rats or rats repeated with CORT and vehicle injection (n=5 per group) were killed by decapitation. The hippocampus was placed into eppendorf tubes and quickly frozen to -80°C for later analysis. Next day, the frozen tissue (20-40 mg) was transferred into a pre-cooled container. Membrane proteins were then extracted from tissues. The tissues were grinded with 2 μl of protease inhibitor cocktail set 1 (Cabiochem, Merck, Darmstadt, Germany) and 2 ml of ice-cold Cell Permeabilization Buffer (Fermentas, Ontario, Canada) by using a pestle. The mixture was incubated for 10 minutes at 4°C while rocking continuously. The permeabilized cells were centrifuged at 16000 x g for 15 minutes at 4°C. After rotation, the supernatant which represented cytoplasmic protein extract was carefully removed and

transferred into a new tube. The remaining cell debris pellets were then set on ice. The pellets were extracted by adding 1 ml of ice-cold membrane protein extraction buffer (Fermentas, Ontario, Canada) and 1 µl of protease inhibitor cocktail set 1. The mixtures were incubated for 30 minutes at 4°C in the thermomixer (Shinteh, Taipei, Taiwan) shaking at 1400 rpm. Membrane protein extract was cleared by centrifugation of the mixture at 16000 g for 15 minutes at 4°C. The membrane protein fraction which was in the supernatant was then transferred into a new tube directly or stored at -70°C for later analysis.

After samples were extracted, a standard curve was needed to determine the concentration of samples. The bovine serum albumin (BSA) was added to 6 cuvettes (Medclub, Taoyuan, Taiwan) with following concentrations: 0, 2, 4, 6, 8, and 10 µg/ml. The volumes of BSA of each cuvette were 0, 20, 40, 60, 80, and 100 µl. Each cuvette contained the same volume of 200 µl of the reagent, and the remaining volumes of each cuvette were filled with ddH2O to 1 ml. The S-30 spectrophotometer (Boeco, Hamburg, Germany) was then used to measure the ocular dominance (OD) of BSA at 595 nm. The standard curve was plotted after measurement of BSA. The protein concentration of the sample was then determined by dilution of the sample from 10 mg/ml to 0.1 mg/ml, and tested the OD at volume of 2 µg.

Protein content was determined (Bradford, 1976) and samples were denatured by using Laemmli sample buffer (Bio-Rad, Richmond, CA, USA) and then vortexed for 30 minutes at room temperature. Based on the protein concentration from Bradford assay, samples containing 20 µg of total protein were prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis

(SDS-PAGE). Before running SDS-PAGE, buffers and gels should be made with following steps:

Running buffer		Stacking buffer	
Tris	36.6 g	Tris	12 g
ddH2O	200 ml	ddH2O	200 ml

Tank buffer 10X (1 L)		TBS 10X (pH 8, 1 L)		Transfer buffer 10X	
Tris	30.28 g	Tris	30 g	Tris	30.3 g
glycine	144.13 g	NaCl	80 g	glycine	144 g
SDS	10 g	KCl	2 g	ddH2O	1000 ml

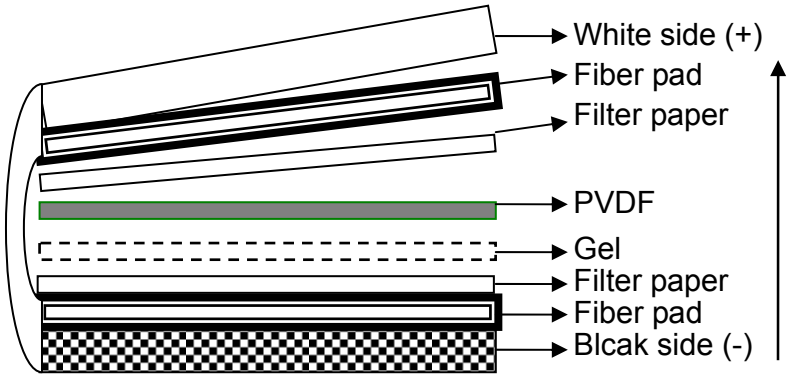
8 % gel

Running gel		Stacking gel	
ddH2O	4.6 ml	ddH2O	2.1 ml
Running buffer	2.5 ml	stacking buffer	833 µl
30 % Acrylamide mixture	2.7 ml	30 % Acrylamide mixture	333 µl
10 % SDS	100 µl	10 % SDS	33.3 µl
10 % Ammonium persulfate (APS)	100 µl	10 % Ammonium persulfate (APS)	33.3 µl
TEMED	7.5 µl	TEMED	4.6 µl

1X tank buffer was poured into the mini-vertical gel electrophoresis unit (Amersham Biosciences, GE Healthcare, UK). 1.5 mm of 8 % Acrylamide gels

(Amersham Biosciences, GE Healthcare, UK) were then placed in the gel holder assembly and immersed into the tank. The inner compartment of the tank should be filled with tank buffer. Samples (15 μl) and sample buffer (0.75 μl β-ME + 14.25 μl sample buffer) were loaded carefully to the well in the gels at a volume of 30 μl. Prestained protein ladder (Fermentas, Ontario, Canada) which were loaded to two wells at volumes of 1 and 3 μl and sample buffer were also loaded to the wells at total volume of 30 μl. The tank was then covered with the lid and plugged to the power source at 60 mA for 2 hours.

After transferring, the gels were then immersed in methanol for 30 seconds, ddH20 for 5 seconds, and soaked into transfer buffer at 70 rpm for 10 minutes. At the same time, chromatography papers were cut in the same size of gels. The polyvinylidene fluoride (PVDF)-membrane (Immobilon-P, Millipore, MA, USA) was pre-wet by using 100 % methanol for 30 seconds and immersed in ddH20. The PVDF-membrane was rotated in ddH20 for 5 minutes, and then in transfer buffer for 10 minutes. After the electrophoresis, the Mini Trans-Blot electrophoretic transfer cell (Bio-Rad, Richmond, CA, USA) was used for transferring gels to membranes. The gels, membranes, filter papers, and fiber pads should be soaked in transfer buffer. The gel sandwich of holder cassette was assembled by following instructions:



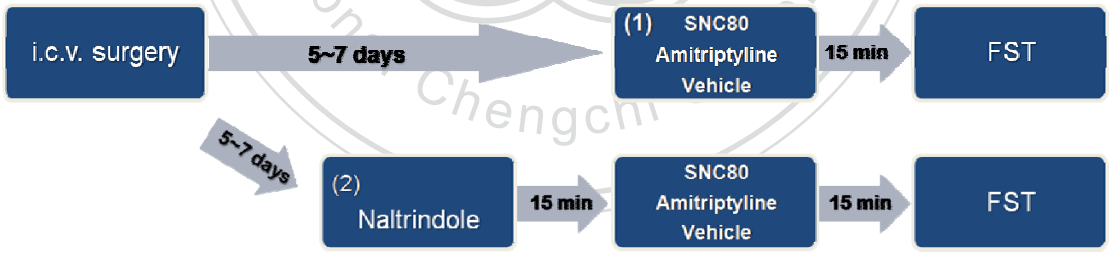
The cassette and the frozen blue cooling unit were then placed in the module. The module was added with transfer buffer and a stir bar to help maintain the buffer temperature and ion distribution during transferring. The proteins were then blotted to a PVDF-membrane at 100 V and 2 A for 1 hour. The membrane was washed by blocking buffer containing 5 % skim milk (5 g skim milk/100 ml 0.05 % TBST (0.05 % Tween-20 in 0.5 M TBS, pH 7.5)) at 40 rpm for 1 hour.

After washing, the membrane was incubated with a rabbit polyclonal antibody for DOR (Santa Cruz, CA, USA) (1:500) in 5 ml of blocking solution and put on a 3D shaker overnight at 4^{o}C. At next day, the membrane was washed in TBST at 70 rpm for 5, 10, and 10 minutes, respectively. Horseradish peroxidase-conjugated anti-rabbit, IgG-HRP (Santa Cruz, CA, USA) was then used as secondary antibody (1:10000) in 5 ml of blocking buffer for 1 hour in room temperature, and washed the membranes in TBST at 70 rpm for 5, 10, and 10 minutes, respectively. After washing in TBST, the proteins were put on a photograph board, and added with immobiln chemiluminiscent HRP blotting substrate (Immobilon-P, Millipore, Bedford, MA, USA).

Blots were digitized with a ChemX 200F bio-image system (Avegene, Taipei County, Taiwan) and image processing was performed on an ASUS-compatible computer. Integrated density measurements of chemiluminiscent bands were performed by using Scion Image software (National Institute of Health, MD, USA). A calibration curve was calculated using the distance traveled by the protein ladder and the molecular weights of chemiluminiscent bands were then estimated by extrapolation.

Experimental design

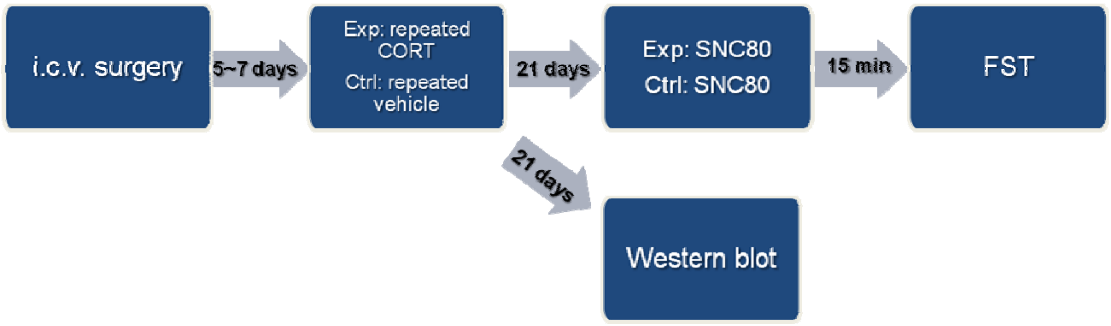
(1) **Antidepressant-like effects of test compounds in rats under normal state.** The experiment 1 was aimed to establish dose-response curves of antidepressant-like effects of test compounds in FST. SNC80 or amitriptyline was injected intracerebroventricularly. Immediately after administration, rats were observed for potential convulsions. The dose range of SNC80 (0, 3, 10, 30, and 60 μg) and amitriptyline (0, 10, 30, and 100 μg) were selected based on their systemic active dose range (Sawynok & Reid, 2001; Broom et al, 2002b; Jutkiewicz et al., 2005). In addition, the antagonist study was conducted to verify the involvement of DOR in i.c.v. SNC80 or amitriptyline-induced antidepressant-like effects. Naltrindole (1 mg/kg, s.c.) was given 15 min before i.c.v. injection of test compounds. The dose of naltrindole was selected based on previous studies showing that naltrindole produced selective DOR antagonism (Broom et al., 2002c).



(2) **Antidepressant-like effects of test compounds in rats under chronic inflammation.** Experiment 2 was aimed to investigate to what degree the antidepressant-like effects of agonists were enhanced under inflammation, and to verify whether there was time-dependent up-regulation of DOR following inflammation. After recovery from surgery, CFA was given to

induce different duration of inflammation, namely 3 days, 1 week, or 2 weeks. After the end of CFA treatment, rats were injected with i.c.v. SNC80 (0, 1, 3, and 10 μg) or amitriptyline (0, 10, 30, and 100 μg) 15 min before the FST. On the other hand, rats (n=5 per group) were injected with CFA or vehicle with same pretreatment time to determine the densities of DOR in the supraspinal regions of rats by using the western blot assay. The quantification of DOR densities following different pretreatment time was used to correlate time-dependent changes of DORs in rats under inflammation.

(3) Antidepressant-like effects of test compounds in rats under chronic stress. The aim of experiment 3 was determine the antidepressant-like effects of agonists under repeated CORT administration. After recovery from surgery, rats were repeatedly given CORT or vehicle for 21 days. After 21-day administration of CORT, rats were divided to test the antidepressant-like effects of SNC80 (0, 3, 10, and 30 μg) 15 min before FST. On the other hand, rats (n=5 per group) were injected with 21-day CORT or vehicle to determine the densities of DOR in the supraspinal regions of rats by using the western blot assay.



Statistical analysis

Behavioral data from the forced swim test were expressed as mean ± S.E.M. The statistical differences between groups were performed by using one-way ANOVA followed by Tukey *post hoc* test to compare differences between groups where $p < .05$ were considered significant. ED50 values of antidepressant-like effects were determined by linear regression analysis of the dose response curves. The numbers of immobility from the control group in experiment 1 were set as 0 percent of antidepressant-like effects and the numbers of immobility from the highest dose of the test compound in experiment 1 were set as 100 percent of antidepressant-like effects. All analyses were performed by using SPSS 16.0 (Chicago, IL, USA) and GraphPad Prism 5 software (San Diego, CA, USA). The figures were drawn by using SigmaPlot 10.0 software (San Jose, CA, USA).

Results

Antidepressant-like effects of SNC80 and amitriptyline in rats under normal state

I.c.v. administration of SNC80 dose-dependently decreased immobility in the FST, indicating an antidepressant-like effect [$F(4, 30) = 24.4$, $p < .05$] (Figure 1A). *Post hoc* comparison showed that SNC80 at 10, 30, and 60 μg produced significant decrease in immobility ($p < .05$). In addition, i.c.v. administration of amitriptyline also decreased immobility in a dose-dependent manner in the FST [$F(3, 24) = 25.3$, $p < .05$] (Figure 1B). *Post hoc* comparisons indicated that amitriptyline at 30 and 100 μg produced significant decrease in immobility ($p < .05$). The ED50 values of SNC80 and amitriptyline in producing antidepressant-like effects were 5.6 and 29.1 μg, respectively.

Figure 1

Effects of naltrindole on the activity of SNC80 and amitriptyline in the FST under normal state

To verify whether DORs mediated the antidepressant-like effects of test compounds, a DOR antagonist naltrindole were administered subcutaneously 15 minutes before i.c.v. administration of SNC80 or amitriptyline. Following the vehicle pretreatment, both SNC80 (30 μg) and amitriptyline (100 μg) significantly decreased immobility compared to control group ($p < .05$) (Figure

2). Naltrindole (1 mg/kg) alone had no effect in the FST. However, pretreatment of naltrindole blocked the antidepressant-like effects of SNC80 (30 μg), while naltrindole had no effect on the changes of the antidepressant-like effects of amitriptyline (100 μg) (Figure 2).

Figure 2

Antidepressant-like effects of SNC80 in rats under inflammatory pain

Figure 3 shows the antidepressant-like effects of SNC80 in rat under inflammatory pain. SNC80 dose-dependently decreased immobility in rats treated with either saline or CFA for 3 days in the FST [$F(4, 30) = 18.4$, $p < .05$; $F(3, 24) = 13.4$, $p<.05$] (Figure 3A, 3B). *Post hoc* comparison revealed that SNC80 at doses of 10 and 30 μg produced significant effects in both groups ($p < .05$). In addition, SNC80 also dose-dependently decreased immobility in rats treated with either saline or CFA for 1 week in the FST [$F(4, 30) = 13.8$, $p < .05$; $F(4, 30) = 17.2$, $p < .05$] (Figure 3C, 3D). *Post hoc* comparison indicated that SNC80 was more potent in 1-week CFA-treated rats. SNC80 at doses of 1 and 3 μg produced significant decreases in CFA-treated rats ($p < .05$). In contrast, the same doses of SNC80 did not produce significant effects in saline-treated rats ($p > .05$). Furthermore, SNC80 also dose-dependently decreased immobility in rats treated with either saline or CFA for 2 weeks in the FST [$F(4, 30) = 9.5$, $p < .05$; $F(4, 30) = 11.6$, $p < .05$] (Figure 3E, 3F). Similarly, SNC80 was more potent in 2-week CFA-treated rats. SNC80 at doses of 1 and 3 μg

was not effective in rats treated with saline ($p > .05$), but both doses were effective in decreasing immobility in rats treated with CFA ($p < .05$).

Figure 3

Dose-response curves of SNC80 in rats under inflammatory pain

Based on the findings of SCN80-induced antidepressant-like effects measured by the FST in rats under normal state, the dose-response curves were analyzed and compared by setting 0 and 100 percent of reduced immobility produced by 0 and 60 μg of SNC80, respectively. Figure 4A shows that the dose-response curve for SNC80 in 3-day CFA-treated rats was close to one that was obtained in 3-day saline-treated rats, indicating that SNC80 have similar potency in producing antidepressant-like effects in both groups. However, the dose-response curve for SNC80 in 1-week CFA-treated rats was shifted to the left compared to one which was determined in 1-week saline-treated rats (Figure 4B). Similarly, the dose-response curve for SNC80 in 2-week CFA-treated rats was also shifted to the left compared to one which was determined in 2-week saline-treated rat (Figure 4C). ED50 values for each dose-response curve are summarized in Table 1.

Figure 4

Table of ED50 values of SNC80 on antidepressant-like effects in rats under inflammatory pain

Table 1 compares ED50 values of i.c.v. SNC80 which were determined in rats under different experimental conditions. ED50 values for SNC80 in rats treated with either saline or CFA for 3 days were estimated to be 6.3 and 5.4 μg, respectively. The dose ratio between both groups was 1.2. Thus, the potency of SNC80 in producing antidepressant-like effects in CFA-treated rats was similar to that in saline-treated rats. The ED50 values for SNC80 in rats treated with either saline or CFA for 1 week were estimated to be 5.6 and 0.9 μg, respectively. The dose ratio between both groups was 6.5. Thus, SNC80 was approximately 6-fold more potent in producing antidepressant-like effects in 1-week CFA-treated rats compared to saline-treated rats. In addition, the ED50 values for SNC80 in rats treated with either saline or CFA for 2 weeks were estimated to be 5.2 and 0.7 μg, respectively. The dose ratio between both groups was 7.3. Thus, SNC80 was approximately 7-fold more potent in producing antidepressant-like effects in 2-week CFA-treated rats compared to saline-treated rats.

Table 1

Antidepressant-like effects of amitriptyline in rats under inflammatory pain

Like SNC80, amitriptyline dose-dependently decreased immobility in rats treated with either saline or CFA for 3 days [$F(3, 24) = 19.4$, $p < .05$; $F(3, 24) = 27.9$, $p < .05$] (Figure 5). *Post hoc* comparison indicated that amitriptyline at doses of 30 and 100 μg significantly decreased the immobility in both groups ($p < .05$).

ED50 values of amitriptyline in producing antidepressant-like effects in rats treated with either saline or CFA for 1 week were 23.4 and 26.1 μg, respectively (Table 1). The dose ratio between these groups was 0.9. Thus, the potency of amitriptyline in producing antidepressant-like effects in CFA-treated rats was similar to that in saline-treated rats.

Figure 5

Table 1

Identification of DOR densities in hippocampus of rats under inflammatory pain

To investigate whether the enhanced antidepressant-like effects of SNC80 under inflammatory pain are correlated with changes in protein levels of DORs, the western blot was used to measure the total DOR proteins in membranes prepared from hippocampus of saline-treated and CFA-treated rats. The immunoreactive bands at estimated molecular weight of 125 kDa were

observed in rats treated with either saline or CFA for 3 days, 1 week, and 2 weeks (Fig 6A, 6C, 6E). To quantify the difference of immunoreative bands, the following data were normalized as DOR/β-actin ratios. Figure 6B shows that no difference of DOR proteins was detected in both sides of hippocampus in rats treated with either saline or CFA for 3 days [$F(3, 16) = 0.5$, $p > .05$]. However, a significant difference of DOR protein density was detected in membranes prepared from the hippocampus in 1-week CFA-treated rats compared to saline-treated rats [$F(3, 16) = 10.7$, $p < .05$] (Figure 6D). *Post hoc* comparison indicated that proteins from the ipsilateral side of hippocampus of CFA-treated rats were significantly higher than saline-treated rats ($p < .05$). Similarly, a significant difference of DOR density was also detected in membranes prepared from the hippocampus in 2-week CFA-treated rats compared to saline-treated rats [$F(3, 16) = 3.4$, $p < .05$] (Figure 6F). *Post hoc* comparison indicated that DOR proteins from the ipsilateral side of hippocampus of CFA-treated rats were significantly higher compared to saline-treated rats ($p < .05$).

Figure 6

Mean body weight of rats under chronic stress

To induce chronic stress model, the rats were started to subcutaneous administration of saline or corticosterone for 21 consecutive days. Figure 7 shows the mean body weights of rats in two groups during 21-day injection

phase of the experiment. Saline-treated rats gradually increased their body weights over the injection phase. However, corticosterone-treated rats did not increase the body weight over the 21-day injection phase compared to saline-treated rats. The statistical analysis revealed a significant main effect of day [$F(3, 264) = 170.8$, $p < .05$], a significant main effect of group [$F(1, 88) = 101.7$, $p < .05$], and a significant day x group interaction [$F(3, 264) = 325.9$, $p < .05$]. *Post hoc* comparison revealed that the corticosterone-treated rats weighted significantly less than the saline-treated rats by day 7 of the experiment and continued to do so until day 21 ($p < .05$).

Figure 7

Antidepressant-like effects of SNC80 in rats under chronic stress

I.c.v. administration of SNC80 dose-dependently decreased immobility in the FST in rats treated with saline for consecutive 21 days [$F(3, 27) = 11.7$, $p < .05$] (Figure 8A). *Post hoc* comparison showed that SNC80 at 10 and 30 μg produced significant decrease in immobility ($p < .05$). ED50 values of SNC80 in producing antidepressant-like effects in saline-treated rats were 5.4 μg. On the other hand, SNC80 also dose-dependently decreased immobility in the FST in rats treated with CORT for consecutive 21 days [$F(4, 30) = 14.8$, $p < .05$]. However, the effects of SNC80 only partially decreased the immobility in rats treated with CORT compared to saline groups. *Post hoc* comparison showed that SNC80 at 10, 30, and 60 μg produced significant decrease in

immobility ($p < .05$). ED50 values of SNC80 in producing antidepressant-like effects in CORT-treated rats were 5.2 μg.

Figure 8

Identification of DOR densities in hippocampus of rats under chronic stress

To investigate whether the DOR densities in hippocampus were changed during chronic stress, the western blot was used to measure the total DOR proteins in membranes prepared from hippocampus of rats receiving consecutive administration of saline or CORT for 21 days. The immunoreactive bands at estimated molecular weight of 125 kDa were observed in rats treated with either saline or CORT for 21 days (Fig 9A). To quantify the difference of immunoreative bands, the following data were normalized as DOR/β-actin ratios. Figure 9B indicates that there was no difference of DOR proteins in both sides of hippocampus either in CORT-treated or saline-treated rats [$F(3, 16) = 0.7$, $p > .05$].

Figure 9

Discussion

Based on previous findings show that antinociceptive effects of DOR agonists were enhanced and the DOR density was up-regulated in rats under inflammatory pain, the present study was proposed to investigate the antidepressant-like effects of a DOR agonist, SNC80, in rats under different contexts, namely normal state, inflammatory pain, and chronic stress. Meanwhile, the western blot was conducted to determine whether the DOR density in the hippocampus of rats was changed accordingly.

EXP1: Antidepressant-like effects of SNC80 in rats under normal state

The FST has been validated by using clinically used antidepressants to decrease the numbers of immobility (Porsolt et al., 1977). Therefore, the FST has been used extensively in studying novel experimental compounds that have potential antidepressant-like effects (Jutkiewicz, 2006). The present study showed that i.c.v. administration of SNC80 dose-dependently decreased immobility in the FST. This finding is consistent with previous studies by showing antidepressant-like effects of i.c.v. (+)BW373U86, a selective non-peptidic DOR agonist, in the FST (Zhang et al., 2006). Neither MOR agonist morphine nor KOR agonist CI-977 decreased immobility in the FST (Broom et al., 2002a). Thus, only DOR agonists produced antidepressant-like effects in this behavioral assay.

In addition, previous findings indicated that systemic administration of SNC80 still produced antidepressant-like effects even the increased locomotor activity had returned to baseline 3 hours after administration (Broom et al.,

2002a). In the present study, there were no change of locomotor activity and other side effects after i.c.v. administration of SNC80 at the doses that produced antidepressant-like effects.

The antagonist study showed that antidepressant-like effects of i.c.v. SNC80 were blocked by a selective DOR antagonist, naltrindole. In contrast, naltrindole did not block the antidepressant-like effects of amitriptyline. Furthermore, systemic administration of naltrindole, but not MOR and KOR antagonists, naltrexone and nor-BNI, reversed the antidepressant-like effects of an enkephalinase inhibitor, RB101 (Jutkiewicz et al., 2006). These results strongly indicate that activation of central DOR is involved in antidepressant-like effects elicited by these compounds.

EXP2: Antidepressant-like effects of SNC80 in rats under inflammatory pain

In the experiment 2, antidepressant-like effects of i.c.v. SNC80 were determined in rats under inflammatory pain. Although the present study did not evaluate the effects of intraplantar injection of CFA or saline on paw withdrawal latency or paw diameter in rats, a previous study indicated that CFA increased paw diameter last for 2 weeks (Hurley & Hammond, 2000). Meanwhile, CFA significantly decreased the paw withdrawal latency compared to vehicle group for 2 weeks, indicating a prolonged inflammatory pain (Schepers, Mahoney, & Shippenberg, 2008; Hamity, White, & Hammond, 2010).

Central administration of SNC80 dose-dependently decreased the immobility in the FST in rats treated with CFA for 3 days, 1 week, and 2 weeks. More intriguingly, i.c.v. SNC80-induced antidepressant-like effects were

enhanced in 1-week and 2-week CFA-treated rats. SNC80's dose ratios for 1-week and 2-week CFA-treated rats were 6.5 and 7.3, as compared to saline-treated rats, respectively. More importantly, the dose response curves were shifted leftward in both groups, indicating that SNC80 was more potent in producing antidepressant-like effects in CFA-treated rats under prolonged inflammation.

Previous studies have shown that antinociceptive effects of DOR agonists were more prominent in chronic pain (Fraser et al., 2000; Petrillo et al., 2003). Microinjection of a DOR agonist [D-Ala2,Glu4]deltorphin produced more profound antihyperalgesia in CFA-treated rats as compared to saline-treated rats (Hurley & Hammond, 2000). The ED50 values of [D-Ala2,Glu4]deltorphin were decreased following treatment of CFA for 4 hours, 4 days, and 2 weeks. In addition, locomotor activity or other sedative effects was not affected following CFA treatment or administration of SNC80 (Fraser et al., 2000). Taken together, these studies demonstrate that DOR agonists are more potent in producing both antihyperalgesic effects and antidepressant-like effects in rats under inflammatory pain.

The antidepressant-like effects of SNC80 in the FST were similar in rats treated with either saline or CFA for 3 days. In contrast, a previous study showed that [D-Ala2,Glu4]deltorphin produced a significantly leftward shift in the dose response curve for antinociception in 3-day CFA-treated rats compared to control rats (Cahill et al., 2003). This difference between two studies may be due to different administration routes or behavioral endpoints. For example, intrathecal administration of [D-Ala2,Glu4]deltorphin produced an increased potency greater than 50-fold in rats treated with CFA compared to

saline (Cahill et al., 2003). Microinjection of [D-Ala2,Glu4]deltorphin into the rostral ventromedial medulla in CFA-treated rats increased the potency up to 6-fold compared to saline-treated rats (Hurley & Hammond., 2000). Both areas are highly relevant to the processing of pain modulation. Thus, the behavioral effects of DOR agonists may be influenced by different administration routes in rats under inflammatory pain. Meanwhile, the present results from the western blot showed that no changes of DOR protein in hippocampus were found in rats treated with CFA or saline for 3 days, and it may provide an explanation for similar potency of SNC80 in both conditions.

On the other hand, i.c.v. amitriptyline produced antidepressant-like effects in rats treated with either saline or CFA for 1 week. The ED50 values and dose response curves were similar in both groups. Thus, antidepressant-like effects of i.c.v. amitriptyline were not enhanced in rats under inflammatory pain.

Damage of hippocampus has been reported with the reductions in neurotrophic factors (Monteggia et al., 2004). In addition, depressed patients have been reported with decreased serum BDNF levels (Shimizu et al., 2003). Systemic administration of (+)BW373U86 increased BDNF mRNA expression in frontal cortex, hippocampus, olfactory cortex in rats (Torregrossa et al., 2004). Furthermore, central administration of DOR preferring peptides, leu- and met-enkephalin, augmented BDNF mRNA expression in the hippocampus of rats, and this effect could be reversed by pretreatment with naltrindole (Zhang et al., 2006). Therefore, the hippocampus may play an important role in mood regulation.

Both human and animal studies have shown that DORs present in many brain regions. For example, DORs were presented in hippocampus and

olfactory bulb in human brain (Simonin et al., 1994). Rodent studies also reported that DORs exist in hippocampus, olfactory bulb, and hypothalamus (Mansour et al., 1995). Intraplantar injection of CFA increased DOR densities in rat spinal cord, which were corresponded with the enhanced antihyperalgesic effects of DOR agonists (Cahill et al., 2003). However, changes of DOR in specific brain regions in rats under inflammatory pain were not investigated. Given that hippocampus was an essential region for mood regulation, and the DORs exist in hippocampus, it would be interesting to determine whether CFA-induced inflammatory pain could lead to up-regulation of DOR proteins in the hippocampus; and whether such changes could correlate with enhanced antidepressant-like effects of SNC80 in the FST.

The present findings showed that DOR proteins were up-regulated at the ipsilateral side of hippocampus in rats treated with CFA for 1 week and 2 weeks as compared to their control groups. Such changes were correlated with the enhanced potency of i.c.v. SNC80 in rats under CFA treatment for either 1 or 2 weeks.

In contrast, no changes of DOR protein were observed in rats treated with either saline or CFA for 3 days. However, previous studies have demonstrated that DOR proteins were increased in the ipsilateral side of dorsal lumbar spinal cord in rats treated with CFA for 3 days as compared to the control group (Cahill et al., 2003). The difference between both studies may be due to the examination of DOR proteins in different regions. The spinal dorsal horn has been considered as the primary processing center for nociceptive information, which contains abundant opioid receptors (Bie & Pan, 2007). The spinal cord transmits pain stimuli to the brain through ascending pathways and sends

responses to peripheral areas through descending pathways. Thus, the DOR density in the spinal cord may be up-regulated more directly in rats under CFA-induced inflammatory pain.

On the other hand, up-regulation of DOR proteins in dorsal lumbar spinal cord could account for the enhanced antihyperalgesia after intrathecal administration of [D-Ala2,Glu4]deltorphin in 3-day CFA-treated rats (Cahill et al., 2003). The present findings in the FST showed that antirepressant-like effects of i.c.v. SNC80 were similar in rats treated with either saline or CFA for 3 days, which may support the unchanged DOR density of hippocampus in both groups.

The modest antinociceptive effect of DOR agonists has been shown in the acute-phase nociceptive paradigm (Fraser et al., 2000). The reason why DOR agonists produce modest antinociceptive effects can be attributed to the predominately intracellular localization of DOR (Cahill et al., 2007). In addition, anatomy studies have indicated both MOR and KOR, not DOR, are main opioid receptor subtypes in the ascending pain pathway, as well as descending pain pathway (Mansour et al., 1995). Therefore, the DOR may not play a dominant role in alleviating acute nociception. However, a previous study showed that prolonged inflammation significantly increased DOR density trafficking to plasma membrane in small and medium dorsal root ganglion neurons in rats treated with CFA (Gendron et al., 2006). In addition, other inflammatory factors such as bradykinin, substance P, and ATP can also induce DOR trafficking to plasma membrane in cultured sensory neurons *in vitro* (Bao et al., 2003; Guan et al., 2005; Patwardhan et al., 2005).

On the other hand, MORs and KORs distribute in several brain regions

such as hippocampus, amygdale, and olfactory bulb, which may be involved in many physiological functions (Mansour et al., 1995; Berrocoso, Sánchez-Blázquez, Garzón, & Mico, 2009), but MOR and KOR agonists did not produce antidepressant-like effects (Broom et al., 2002b). Whether CFA could up-regulate MOR or KOR densities in rat hippocampus under inflammatory pain were still unknown. Future experiments may explore the levels of MOR and KOR proteins in rat hippocampus under inflammatory pain by using western blot.

To date, there is no study investigating the membrane trafficking of DOR in the specific brain region of rats under chronic pain, the present study may provide a correlation between behavioral effects of i.c.v. SNC80 and the DOR density in plasma membrane in the hippocampus of rats under inflammatory pain. These results may suggest that up-regulation of DOR in hippocampus may account for the enhanced antidepressant-like effects of i.c.v. SNC80 in rats under inflammatory pain.

EXP3: Antidepressant-like effects of SNC80 in rats under chronic stress

Depression and stress sometimes co-exist as mentioned in the introduction. However, antidepressant-like effects of DOR agonists in rats under chronic stress are still unknown. Several models of chronic stress have been used previously. However, various stressors may cause different levels of CORT, which in turn could lead to different interpretations of results. In addition, tolerance developed to elevated CORT levels after repeated restraint stress treatment compared to acute restraint stress treatment (Galea et al., 1997). One way to avoid this problem is to administer CORT into rats. Repeated

stress or hyperactivation of the HPA-axis have been linked with development of depression. Thus, repeated CORT administration can be considered as a chronic stress model.

The present findings showed that the mean body weight of CORT-treated rats were significantly less than saline-treated rats over the injection phase. This finding was consistent with other studies indicating that subcutaneous administration of CORT also gained less weight over the 21-day injection period than the vehicle-treated rats (Gregus et al., 2005; Johnson et al., 2006). Moreover, the locomotor activity did not change between both groups, indicating that repeated administration of CORT did not affect the spontaneous activity even their body weight were less than control rats (Marks et al., 2009). Furthermore, the basal serum levels of CORT were significantly increased after 21-day repeated administration of CORT, while acute injection of CORT did not reveal any difference of serum levels of CORT in rats treated with either saline or CORT (Johnson et al., 2006).

The present study showed that i.c.v. SNC80 dose-dependently decreased immobility in rats treated with saline for consecutive 21 days. The ED50 value of SNC80 under this condition was similar to that in experiment 1. I.c.v. SNC80 also produced antidepressant-like effects in rats treated with repeated CORT. However, CORT-treated group did not show depression-like behaviors compared to the vehicle group, which was different from previous studies showing that repeated CORT increased immobility slightly in the FST (Gregus et al., 2005; Johnson et al., 2006). The discrepancy between these studies may be due to the different quantifications and testing time periods. Previous studies evaluated the repeated CORT-induced depression-like behaviors by

scoring the immobile time for 10 minutes; whereas the present study evaluated the behaviors by scoring the numbers of immobility every 3 seconds for 15 minutes.

In addition, the numbers of immobility were only partially decreased in CORT-treated rats as compared to saline-treated rats. Previous studies indicated that chronic CORT up-regulated the 5-HT2A receptor and potentiated behavioral responses to the 5-HT2A receptor agonist (Kuroda, Mikuni, Ogawa, & Takahashi, 1992; Gorzalka, Brotto, & Hong, 1999). Subcutaneous administration of melatonin, a putative 5-HT2A receptor antagonist, reduced the immobility in the FST (Hill, Brotto, Lee, & Gorzalka, 2003). However, combined melatonin and CORT reversed the reduced immobility of melatonin. In addition, the relationship between DOR and serotonin was studied in an olfactory bulbectomized rat model (Satoh et al., 2008). Chronic treatment of SNC80 decreased the time spending on the open arm of a plus-maze, indicating antidepressant-like effects. Moreover, reduced concentration of 5-HT in hippocampus in olfactory bulbectomized rats was returned to normal following SNC80 treatment. Thus, DOR may interact with serotonin in some paradigms. However, the signaling cascades of the DOR under chronic stress are unknown. Future experiments need to further clarify the functions of DOR agonists under chronic stress.

The HPA axis is an important circuit to deal with stress-related conditions. Hippocampus is considered as an important brain region for regulating hormones and stress (Duman & Monteggia, 2006). Previous studies have demonstrated that hyper-elevation of cortisols may damage hippocampal neurons under chronic stress, which inhibit the negative feedback of the HPA

axis (Parker et al., 2003). Thus, excessive activation of the HPA axis is considered as a symptom observed in stress-related depression.

Hippocampus may be involved in regulation of mood under chronic stress. However, the present study showed that DOR protein density in hippocampus was not altered in rats treated with either saline or CORT for consecutive 21 days. Although anatomy studies have demonstrated that DOR existed in specific brain regions including hippocampus (Mansour et al., 1995). Animals that had been treated with antidepressants could reverse the CORT-induced decrease of BDNF mRNA expression in hippocampus (Dwivedi et al., 2006). The evidence from previous studies only indirectly point out the correlation of hippocampus and DOR. Whether the DOR protein in hippocampus is changed under chronic stress was unclear. Based on the present results, it seems that DOR in hippocampus may not be the main target to regulate the behavioral effects of i.c.v. SNC80 in rats under chronic stress. Future experiments need to clarify the functions of DOR in hippocampus under chronic stress.

Conclusion

In summary, the present study showed that central administration of SNC80 produced antidepressant-like effects. This study is the first to demonstrate that antidepressant-like effects of SNC80 are enhanced in rats under inflammatory pain. The Western blot indicates that the DOR density is increased in the ipsilateral side of hippocampus in rats treated with CFA, which corresponds with the enhanced antidepressant-like potency of SNC80 in rats under the same context. Although i.c.v. SNC80 only partially decreases the immobility in rats under chronic stress, the DOR density is not changed in

hippocampus compared to control groups. Future studies are required to investigate the functions and signaling cascades of DOR in rats under chronic stress.

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Figure description

Figure 1: Effects of i.c.v. administration of SNC80 and amitriptyline on behaviors in the rat FST. SNC80 and amitriptyline were administered 15 min before the FST. Results from the FST are displayed for the total 15-min swim session. Data are represented as mean ± SEM (n=7). Asterisks represent significant differences from the vehicle condition (*p < .05).

Figure 2: Effects of DOR antagonist on i.c.v. administration of SNC80 and amitriptyline (AMT) on behaviors in the rat FST. Sterile water (Veh) or Naltrindole (NTI) was injected 1 mg/kg subcutaneously 15 min before injection of SNC80 or amitriptyline. SNC80 (30 μg) and amitriptyline (100 μg) were administered 15 min before the FST. Results from the FST are displayed for the total 15-min swim session. Data are represented as mean ± SEM (n=7). Asterisks represent significant differences from the vehicle condition (*p < .05).

Figure 3: Effects of i.c.v. administration of SNC80 on behaviors in rats under inflammatory pain in the FST. 100 μl of Complete Freund's adjuvant (CFA) was injected subcuatneously into the right hind paw of rats to elicit inflammatory pain. SNC80 was administered 15 min before the FST. Results from the FST are displayed for the total 15-min swim session. Data are represented as mean ± SEM (n=7). Asterisks represent significant differences from the vehicle condition (*p < .05).

Figure 4: Dose-response curves of i.c.v. administration of SNC80 in rats under

inflammatory pain in the FST. Doses of SNC80 are plotted on a logarithmic scale. Each symbol is represented as mean ± SEM (n=7).

Figure 5: Effects of i.c.v. administration of amitriptyline on behaviors in rats under inflammatory pain in the FST. 100 μl of Complete Freund's adjuvant (CFA) was injected subcuatneously into the right hind paw of rats to elicit inflammatory pain. Amitriptyline was administered 15 min before the FST. Results from the FST are displayed for the total 15-min swim session. Data are represented as mean ± SEM (n=7). Asterisks represent significant differences from the vehicle condition (*$p < .05$).

Figure 6: Identification of DOR proteins by Western blot. (A) Immunoblot analysis of rats treated with either saline or CFA for 3 days. The molecular weights of immunoreactive bands for DOR proteins were estimated to be 125 kDa. (B) Comparison of integrated density values of DOR protein in rats treated with either saline or CFA for 3 days. (C) Immunoblot analysis of rats treated with either saline or CFA for 1 week. (D) Comparison of integrated density values of DOR protein in rats treated with either saline or CFA for 1 week. (E) Immunoblot analysis of rats treated with either saline or CFA for 2 weeks. (F) Comparison of integrated density values of DOR protein in rats treated with either saline or CFA for 2 weeks. Quantification of integrated density values of immunoreactive bands was converted to ratios of DOR to β-actin using Scion Image (NIH). Data are represented as mean ± SEM (n = 5). Asterisks represent significant differences from the ipsilateral side of hippocampus of saline-treated rats. (*$p < .05$).

Figure 7: The mean body weights of rats in each group during the 21-day repeated injection sessions of the experiment. Saline or corticosterone (40 mg/kg) was injected subcutaneously to vehicle and corticosterone group, respectively. Data are represented as mean ± SEM (vehicle group, n=39; corticosterone group, n=51). Asterisks represent significant differences from the vehicle condition (*$p < .05$).

Figure 8: Effects of i.c.v. administration of SNC80 on behaviors in rats under chronic stress in the FST. SNC80 was administered 15 min before the FST. Results from the FST are displayed for the total 15-min swim session. Data are represented as mean ± SEM (n=7). Asterisks represent significant differences from the vehicle condition (*$p < .05$).

Figure 9: Identification of DOR proteins by Western blot. (A) Immunoblot analysis of rats treated with either saline or corticosterone for 21 days. (B) Comparison of integrated density values of DOR protein in rats treated with either saline or corticosterone for 21 days. Quantification of integrated density values of immunoreactive bands was converted to ratios of DOR to β-actin using Scion Image (NIH). Data are represented as mean ± SEM (n = 5). Asterisks represent significant differences from the ipsilateral side of hippocampus of saline-treated rats. (*$p < .05$).

Table 1: ED50 values of SNC80 on antidepressant-like effects in rats under inflammatory pain in the FST. ED50 values were determined by nonlinear regression analysis of the dose-response curves presented in Figure 4.

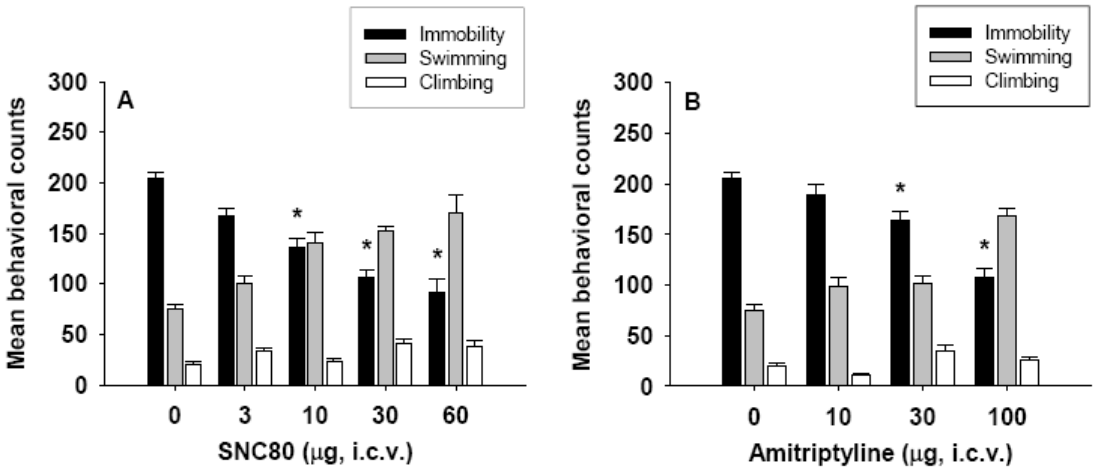
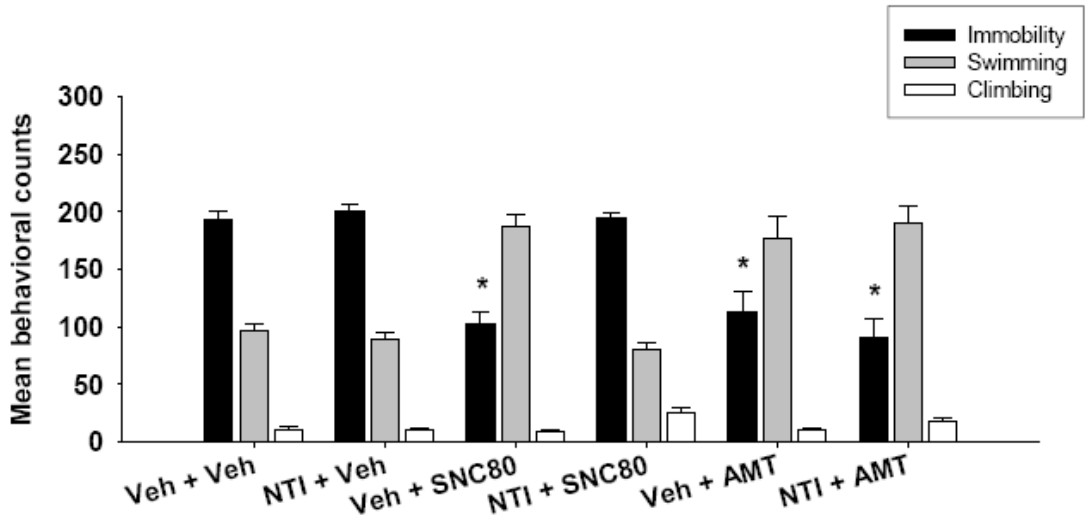

A
Immobility
Swimming
Climbing
Mean behavioral counts
300
250
200
150
100
50
0
0
3
10
30
60
SNC80 (μg, i.c.v.)
B
Immobility
Swimming
Climbing
Mean behavioral counts
300
250
200
150
100
50
0
0
10
30
100
Amitriptyline (μg, i.c.v.)

Figure 1


Immobility
Swimming
Climbing
Mean behavioral counts
300
250
200
150
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50
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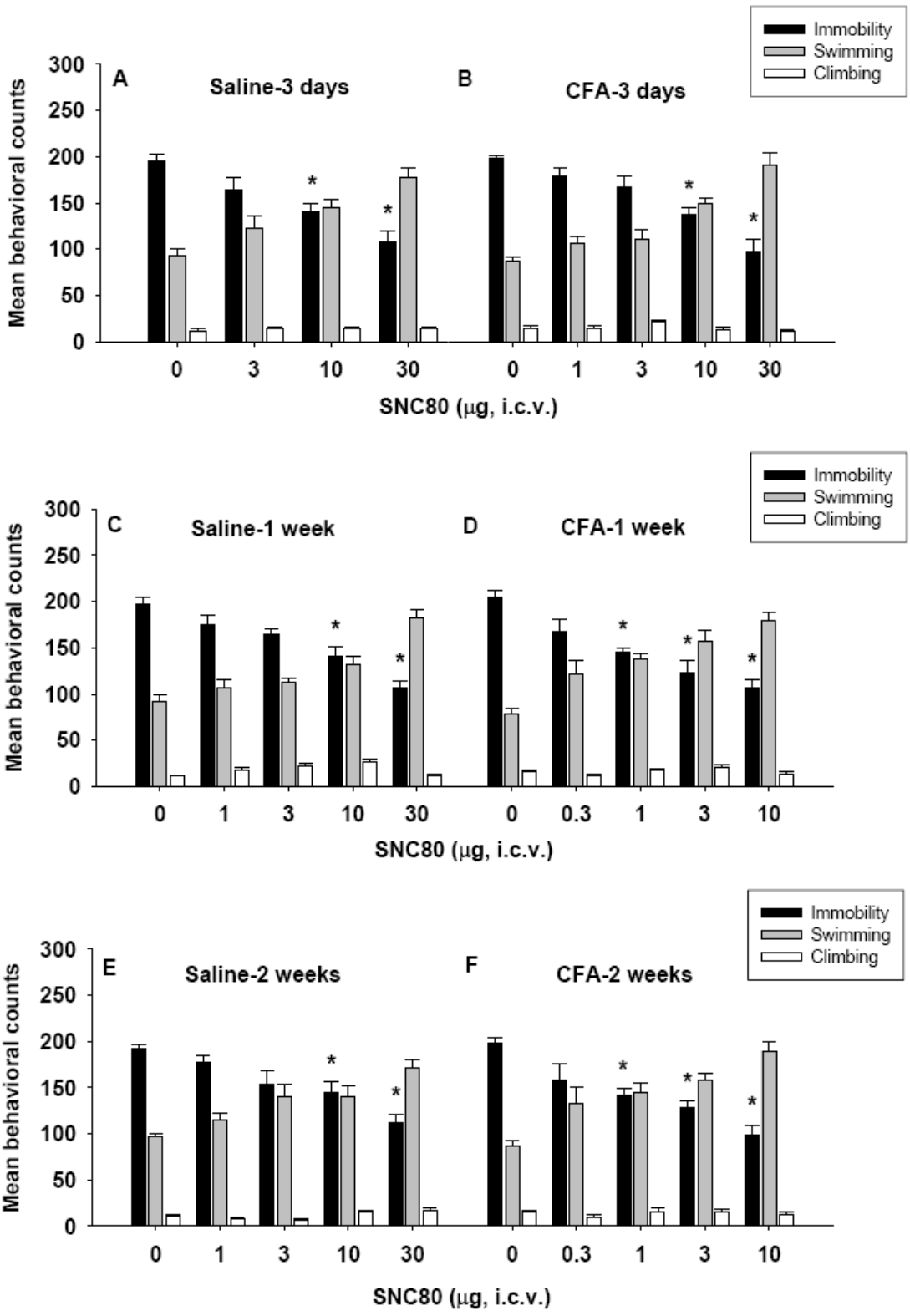
Veh + Veh
NTI + Veh
Veh + SNC80
NTI + SNC80
Veh + AMT
NTI + AMT

Figure 2


Immobility
Swimming
Climbing
A
Saline-3 days
B
CFA-3 days
Mean behavioral counts
300
250
200
150
100
50
0
0
3
10
30
0
1
3
10
30
SNC80 (μg, i.c.v.)
C
Saline-1 week
D
CFA-1 week
0
1
3
10
30
0
0.3
1
3
10
SNC80 (μg, i.c.v.)
E
Saline-2 weeks
F
CFA-2 weeks
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1
3
10
30
0
0.3
1
3
10
SNC80 (μg, i.c.v.)

Figure 3

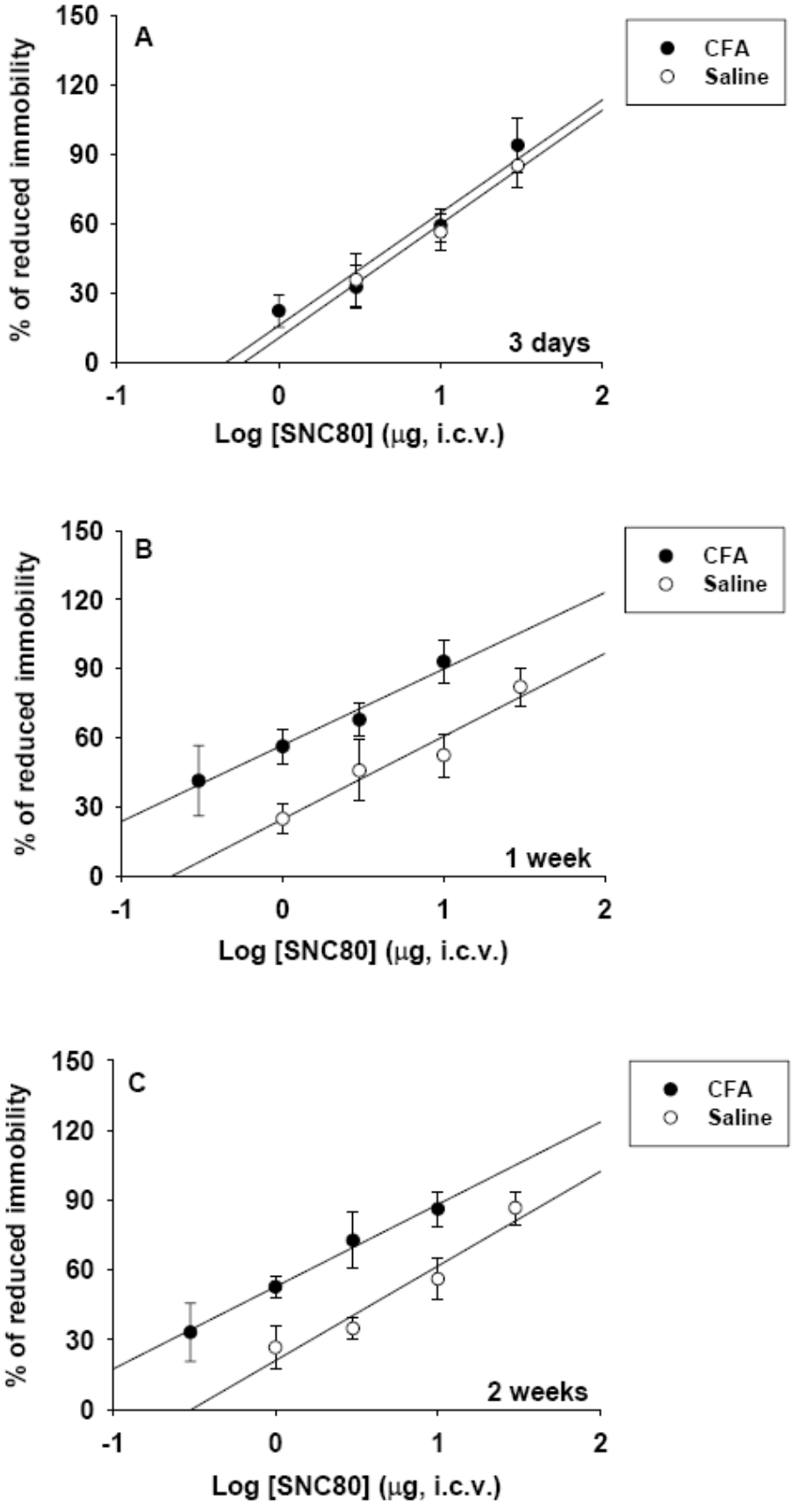
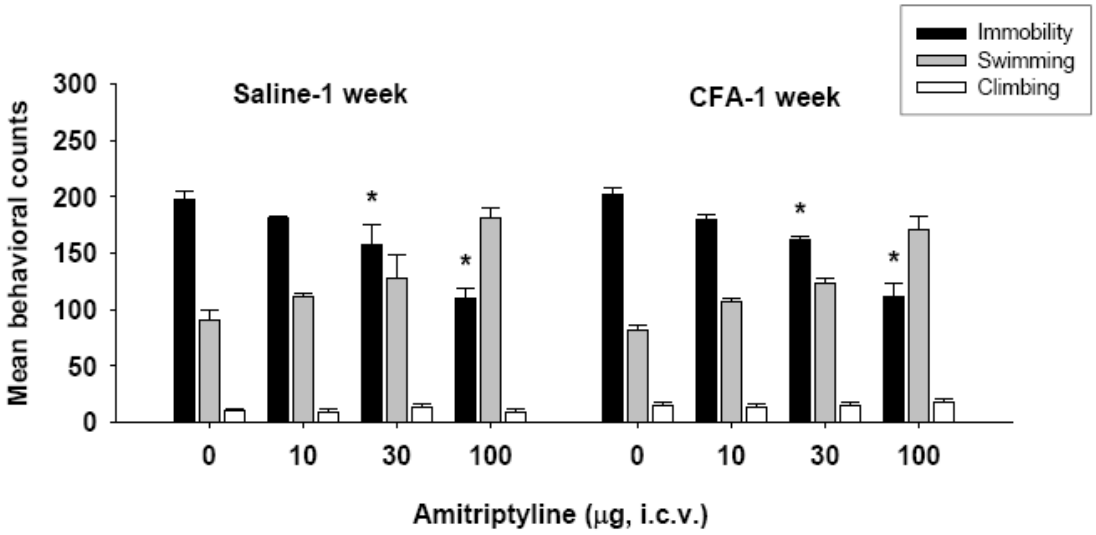

A
CFA
Saline
% of reduced immobility
150
120
90
60
30
0
-1
0
1
2
3 days
Log [SNC80] (μg, i.c.v.)
B
CFA
Saline
% of reduced immobility
1 week
Log [SNC80] (μg, i.c.v.)
C
CFA
Saline
% of reduced immobility
2 weeks
Log [SNC80] (μg, i.c.v.)

Figure 4


Immobility
Swimming
Climbing
Saline-1 week
CFA-1 week
Mean behavioral counts
300
250
200
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50
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0
10
30
100
0
10
30
100
Amitriptyline (μg, i.c.v.)

Figure 5

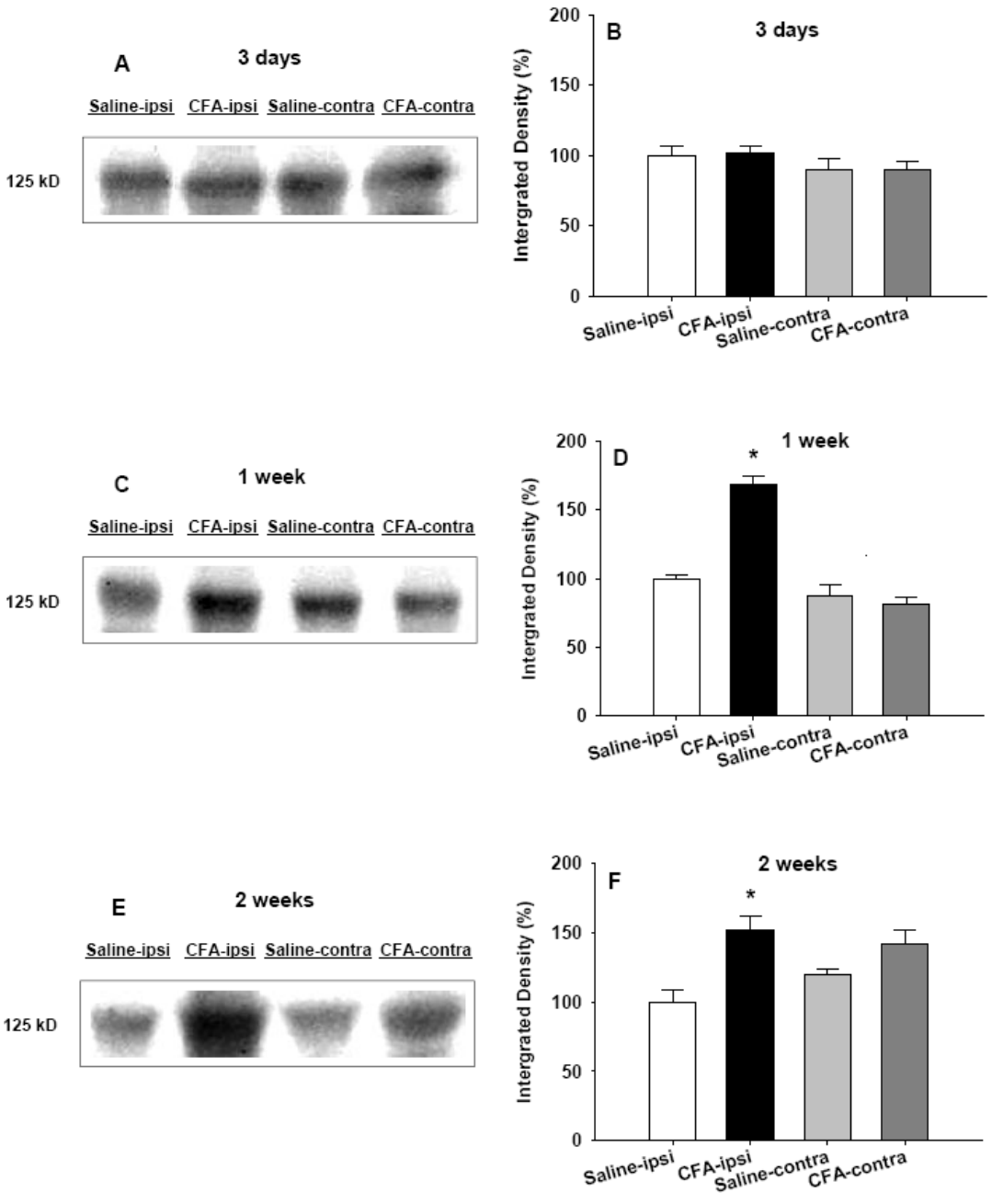
A
3 days
Saline-ipsi
CFA-ipsi
Saline-contra
CFA-contra
125 kD
B
3 days
Intergrated Density (%)
200
150
100
50
0
Saline-ipsi
CFA-ipsi
Saline-contra
CFA-contra
C
1 week
Saline-ipsi
CFA-ipsi
Saline-contra
CFA-contra
125 kD
D
1 week
*
Intergrated Density (%)
200
150
100
50
0
Saline-ipsi
CFA-ipsi
Saline-contra
CFA-contra
E
2 weeks
Saline-ipsi
CFA-ipsi
Saline-contra
CFA-contra
125 kD
F
2 weeks
*
Intergrated Density (%)
200
150
100
50
0
Saline-ipsi
CFA-ipsi
Saline-contra
CFA-contra

Figure 6

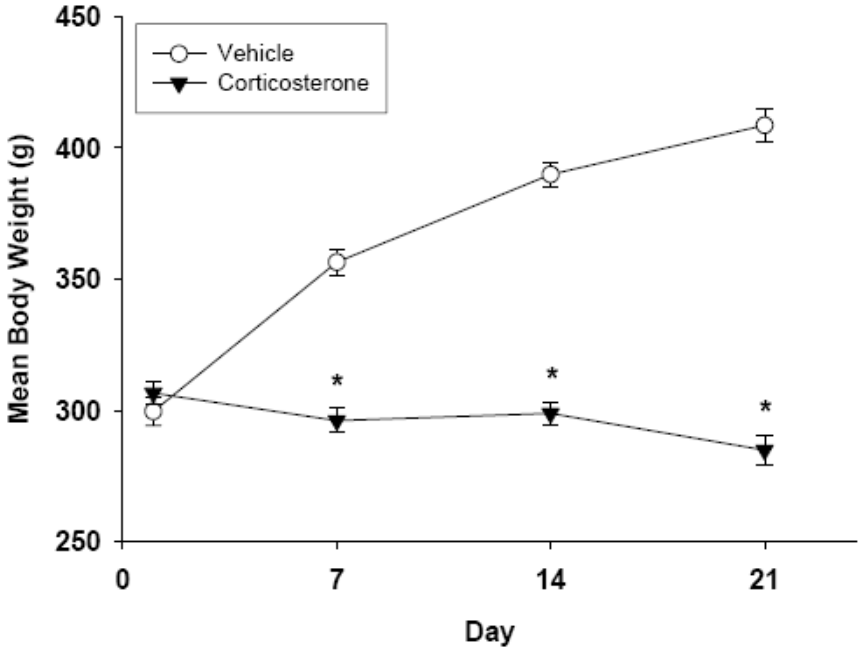
Vehicle
Corticosterone
Mean Body Weight (g)
450
400
350
300
250
0
7
14
21
Day
*
*
*

Figure 7

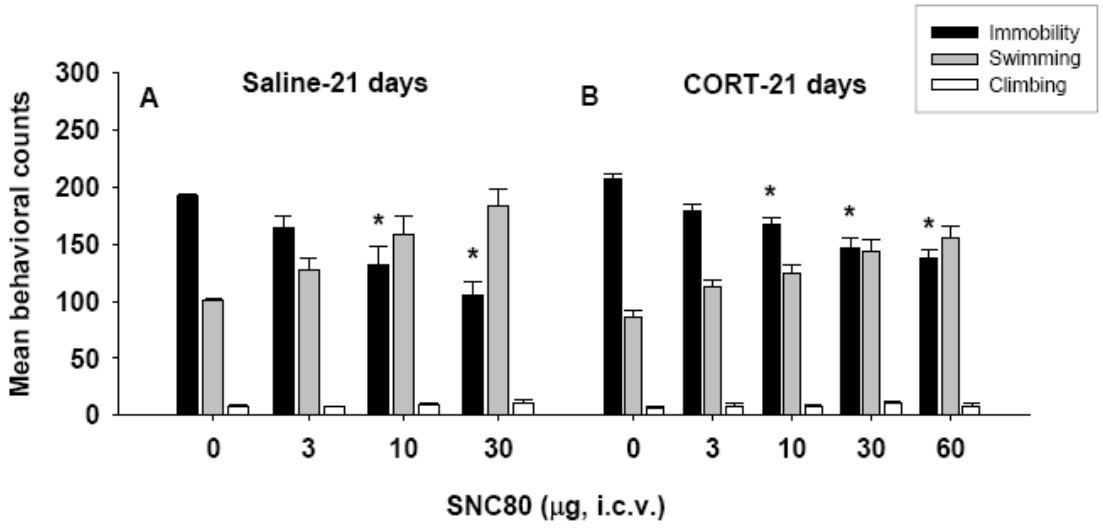
Immobility
Swimming
Climbing
A
Saline-21 days
B
CORT-21 days
Mean behavioral counts
300
250
200
150
100
50
0
0
3
10
30
0
3
10
30
60
SNC80 (μg, i.c.v.)

Figure 8

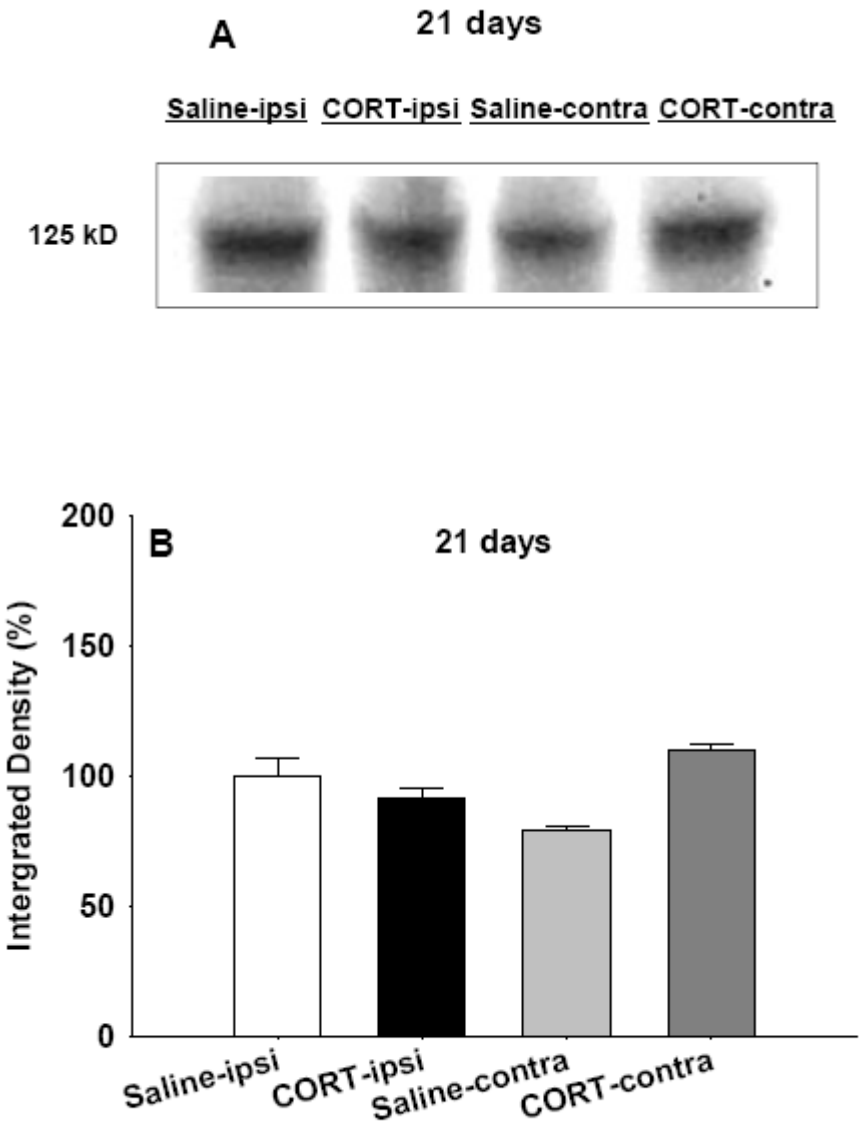
A
21 days
Saline-ipsi
CORT-ipsi
Saline-contra
CORT-contra
125 kD
B
21 days
Intergrated Density (%)
200
150
100
50
0
Saline-ipsi
CORT-ipsi
Saline-contra
CORT-contra

Figure 9

SNC80						
Treatment	ED50 (μg)	95% CL	Treatment	ED50 (μg)	95% CL	Dose Ratio
Saline 3 days	**6.3**	3.6 to 10.9	CFA 3 days	**5.4**	3.3 to 8.7	**1.2**
Saline 1 week	**5.6**	3.7 to 8.6	CFA 1 week	**0.9**	0.5 to 1.5	**6.5**
Saline 2 weeks	**5.2**	3.1 to 9.0	CFA 2 weeks	**0.7**	0.4 to 1.3	**7.3**

Amitriptyline						
Treatment	ED50 (μg)	95% CL	Treatment	ED50 (μg)	95% CL	Dose Ratio
Saline 1 week	**23.4**	13.4 to 40.7	CFA 1 week	**26.1**	15.7 to 43.3	**0.9**

Table 1