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Antinociceptive Effects of Monoamine Reuptake Inhibitors in Assays of Pain-Stimulated and Pain-Depressed Behaviors

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ANTINOCICEPTIVE EFFECTS OF MONOAMINE REUPTAKE INHIBITORS IN ASSAYS OF PAIN-STIMULATED AND PAIN-DEPRESSED BEHAVIOR

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

at Virginia Commonwealth University

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Abstract

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Noxious stimuli can produce pain-related stimulation of some behaviors (e.g. withdrawal responses) and depression of other behaviors (e.g. feeding, locomotion, responding maintained by many types of positive reinforcement). Monoamine reuptake inhibitors are used clinically to treat depression and to manage some types of pain. This study examined the antinociceptive properties of a variety of monoamine reuptake inhibitors selective for SERT, NET and DAT in complementary assays of acute pain-stimulated and pain-depressed behaviors. Intraperitoneal injection of dilute lactic acid (1.8% in a volume of 1ml/kg) was used as a noxious stimulus to stimulate a stretching response and to depress intracranial self-stimulation (ICSS) of the median forebrain bundle. All eight monoamine reuptake inhibitors produced an antinociception-like blockade of acid-stimulated stretching, but only compounds with prominent DA reuptake inhibition (SDRIs RTI-113 and bupropion and the TRI RTI-112) were able to block acid-depressed ICSS, although these effects were produced only at doses that also produced an abuse-related facilitation of control ICSS. Selective or mixed-action inhibitors of 5-HT and NE failed to block acid-induced depression of ICSS. In a separate group of rats, citalopram was also tested using a repeated dosing regimen (10 mg/kg x 3 doses) shown previously to produce antidepressant effects in a forced-swim test in rats. As with acute administration, repeated citalopram decreased acid-stimulated

stretching but failed to block acid-induced depression of ICSS. Taken together, these results suggest that SSRIs, SNRIs and S+NRIs produce relatively non-selective depression of all behavior rather than a selective blockade of sensory sensitivity to noxious stimuli. Conversely, dopamine reuptake may be able to block sensory detection of noxious stimuli. Additionally, these results suggest that assays of pain-depressed behavior can provide new insights on analgesia-related effects of monoamine reuptake inhibitors.

Introduction

Currently, more than 1.5 billion people worldwide suffer from chronic pain of varying degrees (Global Industry Analysts, Inc., 2011), and it is estimated that approximately one-third of Americans will suffer from chronic pain during their lifetime (Harstall et al., 2003). Pain is expensive to treat. The National Institutes of Health has reported that the annual direct costs associated with chronic pain in the United States are over $100 billion, while the indirect costs of pain (absenteeism, unemployment, and lost workplace productivity) are estimated to be $60 billion. For many, persistent pain symptoms are also associated with depression. Studies have reported that the risk of depression increases as a function of worsening pain symptoms and severity (Dworkin et al., 1991, Magni et al., 1993). The combination of pain and depression is associated with poorer quality of life and decreased work function. Research has demonstrated that pain and depression share similar neurochemical etiologies, biological pathways and neurotransmitters, and may respond to similar treatments. Several animal and human studies have concluded that monoamine reuptake inhibitors offer a new and promising class of drugs for the treatment of pain and depressed behaviors. These drugs have the net effect of increasing the neurotransmission of serotonin, norepinephrine and dopamine, which are critical for modulating pain transmission. The research associated with this thesis is concerned with preclinical studies to examine effects of monoamine reuptake inhibitors in assays of pain-related behavioral depression.

Definition and Neurobiology of Pain

Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (IASP, 2011). This definition implies that pain is a subjective experience, involving more than just physical injury. As such, pain is commonly evaluated clinically in humans by verbal reports. However, pain in humans is also associated with nonverbal changes in behavior, and verbal reports are obviously not suitable for assessment of experimental or clinical pain in animals. In order to evaluate pain and analgesia pre-clinically, researchers rely on two general categories of non-verbal behavioral manifestations of pain: (1) pain-stimulated

behaviors and (2) pain-depressed behaviors (Negus et al., 2006; Stevenson et al., 2006). Pain-stimulated behaviors are behaviors that increase in rate, frequency, or intensity in response to the delivery of a painful stimulus (e.g. withdrawal reflexes). In contrast, pain-depressed behaviors are behaviors that decrease in rate, frequency, or intensity in response to a noxious stimulus (e.g. pain-depressed feeding or locomotion). Using these endpoints, pain as well as analgesia can be inferred from behavior in animals.

Pain-related changes in behavior result from a series of signaling events occurring within the peripheral and central nervous systems (Argoff et al., 2011) (Figure 1). Peripheral terminals of nociceptors detect the presence of a noxious stimulus. These nociceptors act as transducers, which convert chemical, mechanical or thermal energy at the site of the stimulus into electrical activity, which is then conducted to the dorsal horn of the spinal cord in the central nervous system. Here, the pain signal is transmitted from the primary afferent to secondary nociceptive neurons in the dorsal horn. Axons of the secondary nociceptive neuron ascend in the anterolateral white matter of the spinal cord and terminate in the thalamus. From there a tertiary nociceptive neuron ascends and terminates in the postcentral gyrus (primary somatosensory cortex, SI). This primary pain sensory system includes branches that target (a) other subcortical regions such as the parabrachial nucleus (PBN), which in turn projects to amygdala and to mesolimbic dopamine neurons in the ventral tegmental area to influence mood and motivation, and (b) other cortical regions such as second somatosensory cortex (SII), anterior cingulate cortex (ACC) and insular cortex (IC) (Price et al., 2000). These cortical targets can also influence limbic regions such as amygdala and nucleus accumbens either directly or via their connections to the prefrontal cortex. Taken together, this neural network provides a mechanism by which noxious stimuli can influence behavior and mood.

Pain can be categorized as acute nociceptive pain, inflammatory pain, and neuropathic pain. **Acute nociceptive pain** serves a vital and adaptive purpose. It serves to detect, localize and limit tissue damage. Acute pain evokes motor withdrawal reactions, which are protective responses that discontinue exposure to the noxious stimulus and terminate the pain. The pain is usually sequestered to the affected area, short in duration and resolved when the underlying problem is treated. Acute pain can result from

injury or sudden illness and can affect skin, subcutaneous tissues, bone, muscle, blood vessels, connective tissue, organs or the linings of the body cavities. Pain associated with surgery, athletic injury and occasional headache are all examples of acute pain. The sensation of acute pain begins with the detection of a noxious stimulus by specialized peripheral nociceptors. Most primary afferent nociceptors respond to a variety of noxious stimuli-extreme hot or cold temperatures, intense pressure (pinching, pinpricks, cuts), increased tissue acidity, or chemical agents released from cells that are damaged or responding to an infectious agent (Fields et al., 2007). Information regarding the noxious stimulus is then transmitted to the central nervous system as described above. **Inflammatory pain** is a type of pain that involves the mechanism of sensitization of nociceptive pathways (Figure 2A). Under inflammatory pain conditions, inflammatory cytokines (e.g. TNFα and IL1β), small molecules (e.g. ATP, bradykinin, prostaglandins) and growth factors (e.g. NGF and BDNF) infiltrate the area of injury, bind to receptors expressed on sensory nerves and sensitize nociceptors. Sensitization leads to increased responsiveness of nociceptors to their normal input, and/or recruitment of responses to normally subthreshold inputs (IASP et al., 2011). Inflammatory mediators have diverse mechanisms and sites of action, including the activation and sensitization of nociceptive terminals; the regulation of primary nociceptive phenotype; and, in spinal cord, the pre-synaptic control of nociceptor transmitter release and the post-synaptic control of neuronal excitability (Meyer et al., 2006). Clinically, sensitization can be inferred indirectly from phenomena such as hyperalgesia or allodynia. Hyperalgesia is defined as increased pain from a stimulus that normally provokes pain, and allodynia is defined as pain due to a stimulus that does not normally provoke pain (IASP et al., 2011). Pain associated with rheumatoid arthritis, inflammatory bowel disease (IBS), and pelvic inflammatory disease (PID) are all examples of inflammatory pain states. **Neuropathic pain** sometimes resembles inflammatory pain because spontaneous pain and hyperalgesia are present at the site of injury. However, the underlying pathology is specifically in nerve tissue. Neuropathic pain is initiated or caused by a pathological lesion or dysfunction in peripheral or central neurons (IASP et al., 2011) (Figure 2B). After peripheral nerve injury, irregular regeneration may occur, resulting in unusual and

spontaneous sensitivity to chemical, thermal and mechanical stimuli (peripheral sensitization). As a result of ongoing spontaneous activity in the periphery, central neurons in the spinal cord (spinothalamic tract neurons) adjust and rewire, causing a heightened responsiveness to afferent impulses, including normally innocuous tactile stimuli (central sensitization). Central sensitization commonly leads to allodynia. A reduction in afferent fiber input decreases the activity of interneurons inhibiting nociceptive neurons and causes hypoactivity of descending inhibitory pain modulating systems. This type of pain is maladalptive because it can occur not only at sites far removed from the original injured area but also at degrees of severity that bear little relationship to the extent of injury. All neuropathic pain is chronic. A wide variety of pathological processes affecting peripheral nerves, sensory ganglia, spinal roots and CNS structures can induce neuropathic pain. These include trauma, vascular and metabolic disorders, bacterial and viral infection, inflammation, autoimmune attack, genetic abnormalities, neurotoxins, etc. (Meyer et al., 2006). Symptoms of neuropathic pain can range from numbness, paresthesias and tingling to shooting, burning, sharp, electric shock-like pain sensations. This is in contrast to most nociceptive pain that is commonly described as aching. Pain associated with multiple sclerosis, spinal cord injury, diabetic neuropathy, HIV-related neuropathies, and cancer-related pain are all examples of neuropathic pain.

Analgesic Drugs

Any patient who experiences pain that impairs functional status or quality of life is a candidate for analgesic drug therapy. Today, opioids are some of the strongest analgesics available to treat pain (Yaksh et al., 2011). Opioid analgesics interact with peripheral and central opioid receptors to produce an overall decreased perception of pain. For acute nociceptive or inflammatory pain, it is routinely recommended that opioids be combined with other analgesic agents, such as nonsteroidal anti-inflammatory drugs (NSAIDs) or acetaminophen in order to minimize the dose requirement of the opioid. According to the World Health Organization Analgesic Ladder (initially targeted for treatment of cancer pain), mild opioid treatment is indicated for persisting or increasing pain only *after* first-line treatment with non-opioids or anti-inflammatory agents has failed. In the presence of severe pain, stronger opioids

should be considered for treatment. In general, neuropathic conditions may be less efficaciously managed by opioids than pain secondary to tissue injury and inflammation (Yaksh et al., 2011). A number of clinical trials evaluating opioids for the treatment of neuropathic pain conditions have demonstrated that opioids partially relieve neuropathic pain symptoms (Watson, CPN et al., 1998, Raja SN et al., 2002, Harati Y et al., 1998, Gimbel JS et al., 2003, Watson CP et al., 2003). These studies also demonstrated that opioids are effective in treating certain qualities of neuropathic pain such as steady pain, paroxysmal pain and allodynic pain. In the case of neuropathic pain, other drug classes such as monoamine reuptake inhibitors may be useful in combination with the opiate, and may act synergistically in some pain states (Yaksh et al., 2011; see below). Despite their clinical efficacy, opioids carry a balance of benefits and burdens. Although the adverse side effects of opioids can limit their clinical utility, they are still prescribed for people in pain. Opioids have the potential to cause respiratory depression, constipation, dependence, tolerance and cognitive disturbances, so careful monitoring is required.

NSAIDs represent an alternative class of effective analgesics. They are potent inhibitors of prostaglandin synthesis because they block cyclo-oxygenase (COX) enzymes that are necessary to produce prostaglandins. NSAIDs have four desirable pharmacological effects: anti-inflammatory, analgesic, antipyretic and anti-thrombotic. NSAIDs are effective in treating acute inflammation associated with postoperative pain (McQuay et al., 2007). There is also good evidence for the efficacy of oral NSAIDs in acute and chronic musculoskeletal pain (Mason 2004, Moore et al., 1998). Most NSAIDs are appropriate for short-term use in inflammatory arthritic conditions such as rheumatoid arthritis and are reported to relieve pain of headache, menstrual cramps, and other mild-to-moderate pain syndromes (Ferrell et al., 2009). They can also be used alone for mild-to-moderate pain or in combination with opioids for severe pain. They have the advantage of being non-habit forming; however, long-term use of NSAIDs can cause a number of adverse effects including gastrointestinal bleeding (Singh et al., 1998), renal failure (Henry et al., 1997), and congestive heart failure (Page et al., 2000). NSAIDs also exhibit a ceiling effect at which increasing the dose results in no further increase in analgesia.

Monoamine Reuptake Inhibitors

As a result of the high incidence of pain and the critical need for analgesics without abuse liability and deleterious side effects, researchers have turned much attention to monoamine reuptake inhibitors as a treatment option. These compounds offer an attractive alternative to opioids for the treatment of pain, because they have a lower abuse potential, and more importantly, monoamine reuptake inhibitors have analgesic effects in chronic neuropathic pain states like fibromyalgia in which NSAID and opioids are not very effective (Perrot et al., 2008). Monoamine reuptake inhibitors are now considered an essential component of the therapeutic strategy for the treatment of many types of persistent pain. Further research is required to understand how and why different types of pain may respond differentially to a given monoamine reuptake inhibitor.

Monoamine reuptake inhibitors have a unique mechanism of action. Inside neurons, neurotransmitters like serotonin, norepinephrine and dopamine are synthesized and packaged into large dense-core vesicles. During an action potential, these vesicles fuse with the inner surface of the presynaptic terminal at the active zone, and through calcium influx and exocytotic mechanisms, neurotransmitters are released into the synaptic cleft. Once neurotransmitters enter the synaptic cleft, they are quickly removed by various mechanisms including diffusion and enzymatic degradation by catechol-O-methyl transferase (COMT) and monoamine oxidase (MAO). Monoamines are removed by reuptake through pre-synaptic membrane-embedded transporter proteins. Transporters rapidly clear neurotransmitter out of the synapse and back into the presynaptic terminal to terminate neurotransmission and replenish neurotransmitter stores. Membrane transporters are also the targets for monoamine reuptake inhibitors. Monoamine reuptake inhibitors bind to transporters and inhibit their activity, forcing increased levels of extracellular monoamines to accumulate, leading to enhanced monoaminergic neurotransmission at spinal and supraspinal levels.

There are multiple subtypes of monoamine reuptake inhibitors with different selectivities for the serotonin (SERT), norepinephrine (NET), and dopamine (DAT) transporters (see Table 1). These subtypes include selective serotonin reuptake inhibitors (SSRIs), selective norepinephrine reuptake

inhibitors (SNRIs), selective dopamine reuptake inhibitors (SDRIs), mixed-action norepinephrine-serotonin reuptake inhibitors (S+NRI) [including the subclass of tricyclic antidepressants (TCAs) named after their chemical structure], and triple reuptake inhibitors (TRIs), which inhibit all three transporters.

Monoamine reuptake inhibitors have been used for decades to treat depression (López-Muñoz et al 2009, Tran et al., 2003, Baldessarini et al., 2005), and more recently, they have emerged as useful medications for the treatment of some types of pain (Sawnyok et al., 2001). Monoaminergic projections largely originate in brainstem and midbrain nuclei, and they project throughout the spinal cord and brain to integrate with ascending pain signals at the spinal and supraspinal level. Monoaminergic systems play an important role in modulating the behavioral expression of inflammatory and neuropathic pain (Ren and Dubnerer 2002) and are targets for pharmacologic management of these conditions. Supraspinal monoaminergic projections are thought to modulate the motivational-affective dimension of pain (Lima and Almeida et al., 2002).

Serotonergic Projections (Figure 3A). Serotonergic cell bodies are located along the midline of the brain stem, in the raphe nuclei. Axons from the serotonergic cell bodies from the Raphe Nuclei in the pons and midbrain (B5-B9 groups) ascend to the forebrain where they play an important role in regulating the responsiveness of cortical neurons involved in mood. Descending serotonergic projections originating in the caudal medulla (B1-B3 groups) travel to the motor and autonomic systems in the spinal cord, while projections originating in the rostral ventral medulla (B4 groups, RVM) project to the dorsal horn to modulate nociception.

Noradrenergic Projections (Figure 3B). Noradrenergic cell bodies are located in two columns (dorsal and ventral) in the medulla. Noradrenergic cell groups located in the locus coeruleus (A6) provide important ascending projections to the cerebral cortex and cerebellum. The locus coeruleus is known to play an important role in maintaining responsiveness to unexpected environmental stimuli, a function that is integral in pain processing. At the level of the pons, noradrenergic neurons (A5/A7 groups) make up the dorsolateral pontine tegmentum (DLPT), which projects mainly to the brainstem and spinal cord and modulate autonomic reflexes and pain sensations.

Dopaminergic Projections (Figure 3C). Ascending dopaminergic projections originate in the substantia nigra (A9 groups) and ventral tegmental area (A10 groups). Dopaminergic projections from the substantia nigra to the striatum (nigrostriatal pathway) are thought to be involved in the initiation of motor responses. The mesocortical pathway connects the ventral tegmentum to the frontal lobes and is involved in the motivational and emotional response to pain. The mesolimbic pathway extends from the ventral tegmental area of the midbrain to areas of the limbic system (nucleus accumbens, amygdala and hippocampus) and is involved in reward and pleasure. All three of these ascending dopaminergic pathways are involved in the inhibitory function of dopaminergic neurons, with the net effect of attenuating nociception, mostly its affective component (Gao et al., 2001). In descending pain pathways, dopamine neurons in the posterior dorsal hypothalamus (A11 groups) project to the spinal cord, and play an important role in sensory and nociceptive processing and sensory integration (Iversen et al., 2009).

Monoamine reuptake inhibitors have clear clinical applications for the treatment of depression, and their mechanism of action may also be useful to for the treatment of pain, especially depressant effects of pain. These mood and pain-altering effects are thought to occur via enhancement of inhibitory monoaminergic neurotransmission in the brain and spinal cord, areas that are heavily involved in pain circuitry.

Analgesic Effects of Monoamine Reuptake Inhibitors: Clinical Studies

Several studies have investigated the putative antinociceptive and analgesic effects of monoamine reuptake inhibitors. Clinical research has shown that monoamine reuptake inhibitors have analgesic efficacy in treating chronic inflammatory pain, including fibromyalgia (Gendreau et al., 2005, Mease et al., 2009) and arthritis (Lin et al., 2003), and neuropathic pain, including diabetic peripheral neuropathy (Rowbotham et al., 2004, Sindrup et al., 2005, Semenchuk et al., 2000) and postherpetic neuralgia (Kishore-Kumar et al., 1990, Raja et al., 2002). However, there is limited evidence for their clinical efficacy in treating acute pain states (Wallace et al., 2002, Gordon et al., 1994, Dirksen et al., 1998), and monoamine reuptake inhibitors are never used to treat acute pain in humans, except for experimental

research (Mico et al., 2006). The analgesic effects of different categories of monoamine reuptake inhibitors are described below.

Clinical Chronic Pain Studies with Monoamine Reuptake Inhibitors

Selective serotonin reuptake inhibitors (SSRIs) are a class of monoamine reuptake inhibitors that specifically inhibit presynaptic reuptake of serotonin, and are associated with few side effects. Several published reports indicate that SSRIs have analgesic properties (Jung et al., 1997); however, the effects are generally weak. The analgesic effects of SSRIs in pilot, open label studies for the management of irritable bowel syndrome symptoms have been mixed (Masand et al., 2002, Masand et al., 2005); however, anecdotal reports have suggested benefits in irritable bowel syndrome patients with paroxetine (Kirsch et al., 2000), fluvoxamine (Emmanuel et al., 1997), and mirtazapine (Thomas et al., 2000). One of the only *placebo-controlled* studies evaluating an SSRI on visceral perception and irritable bowel syndrome symptoms in *non-depressed* patients reported that fluoxetine administration did not change the thresholds for discomfort/pain during phasic rectal distention in irritable bowel syndrome patients, and did not affect psychological symptoms evaluated on a self-rated questionnaire (Kuiken et al., 2003). Therefore, the observed beneficial effects of SSRIs in clinical practice may depend mainly on its psychotropic action, possibly explaining the lack of effect on symptoms in this selected cohort of patients. Further studies are needed to clarify the impact of possible concomitant psychiatric disease and possible differential effects of SSRIs on objective and subjective pain measures. SSRIs have demonstrated partial analgesic efficacy in neuropathic pain patients (Sindrup et al., 1992, Otto et al., 2008). A clinical study found that citalopram caused a slight relief of the symptoms of chronic diabetic neuropathy, as measured by both observer-and self-rating (Sindrup et al., 1992). Collectively, the limited clinical efficacy of SSRIs in chronic pain states and the reliable analgesic effects demonstrated by dual reuptake inhibitors (see below) suggests balanced inhibition of both serotonin and norepinephrine reuptake yields the best analgesic effects in chronic inflammatory or neuropathic pain conditions.

Currently, little is known about the analgesic effects of **selective norepinephrine reuptake inhibitors** (SNRIs) in chronic pain states. A number of case reports suggest that the SNRI reboxetine may provide relief of chronic back pain and fibromyalgia pain before any significant improvement in actual mood symptoms (Krell et al., 2005). Further placebo-controlled studies with SNRIs for chronic pain are warranted. Related issues of comorbidity of depression and heterogeneity among subtypes of chronic pain must be addressed in these studies as well.

Similarly, little is known about the analgesic effects of **selective dopamine reuptake inhibitors** (SDRIs) in chronic pain. Studies have reported that bupropion administration significantly decreased pain symptoms in patients with chronic low back pain (Semenchuk et al., 2000) peripheral neuropathy (Wolfe GI et al., 2004), and migraine headaches (Goodman JF et al., 1997). A recent series of case studies reported the utility of low-dose dextroamphetamine in the treatment of chronic pelvic pain related to interstitial cystitis and/or chronic idiopathic urticaria (Check et al., 2007, Check et al., 2006, Check et al., 2005). Other published reports suggest that SDRIs are not effective analgesics alone, but may be useful as adjuvant medications to enhance analgesic effects related to opioids and attenuate opioid-induced sedation and cognitive deficits (Evans WO et al., 1967, Ivy AC et al., 1944, Forrest WH et al., 1977). Psychostimulants have been widely used in the treatment of medically ill patients with fatigue, including those with cancer, multiple sclerosis, Parkinson's disease, opioid-induced sedation, and HIV (Breitbart et al., 2001, Holmes et al., 1989, Wagner et al., 2000, Bruera et al., 1989, Sarhill et al., 2001). More clinical trials are needed to assess the analgesic effects of SDRIs alone in chronic pain states.

By far the most potent and efficacious monoamine reuptake inhibitors available for pain management are the class of **serotonin-norepinephrine reuptake inhibitors** (S+NRIs). Numerous clinical studies suggest that dual acting antidepressants with balanced inhibition for both serotonin and norepinephrine uptake are more effective than those characterized by a selective activity on one monoamine (Fishbain et al., 2000). It is hypothesized that these drugs are effective in increasing serotonergic and noradrenergic neurotransmission, which adequately dampens processing of stimuli in pain pathways, leading to substantial pain relief. S+NRIs, as well as many tricyclic antidepressants

(TCAs), share a relatively mixed action at SERT and NET. These two drug classes differ primarily in their selectivity for transporters versus other targets such as acetylcholine or histamine receptors. TCAs have actions at muscarinic, histaminergic and α-adrenergic receptors (Bomholt et al., 2005), while S+NRIs lack affinity at these receptors.

S+NRIs such as duloxetine, milnacipran and venlafaxine have demonstrated strong analgesic efficacy in patients with painful polyneuropathy, fibromyalgia, phantom limb pain and diabetic neuropathy (Wernicke et al., 2006, Kajdasz et al., 2007, Sindrup et al., 2003, Sayar et al., 2003, Vitton et al., 2004, Arnold et al., 2005, Spiegel et al., 2010, Rowbotham et al., 2004). In the past decade alone, the FDA approved duloxetine (Cymbalta) for the treatment of diabetic peripheral neuropathy, fibromyalgia, osteoarthritis, and chronic lower back pain. In a large 27-week, randomized, double-blind, placebo-controlled clinical trial, milnacipran (200 mg/day) significantly reduced pain scores and improved physical functioning in patients with fibromyalgia (Mease et al., 2009). Additional studies have reported that venlafaxine administration was effective in migraine prophylaxis (Ozyalcin et al., 2005, Nascimento et al., 1998) and that duloxetine administration significantly decreased reported pain scores, improved quality of life and improved subjective pain measures in patients with Parkinson's disease (Djaldetti et al., 2007). These results are consistent with a growing body of literature that multiple symptoms of chronic pain conditions, including pain, fatigue, and physical functioning, can be addressed through simultaneous augmentation of norepinephrine and serotonin function. Overall, S+NRIs demonstrate clear clinical efficacy in the treatment of neuropathic pain, but lack efficacy for the treatment of acute clinical pain (see below).

Tricyclic antidepressants (TCAs) non-selectively block the re-uptake of serotonin and/or norepinephrine and are widely used in the clinical treatment of neuropathic pain (Sindrup and Jensen et al., 2000). Today, they are considered the "gold standard" antidepressant for the treatment of persistent neuropathic pain (Bryson et al., 1996, Galer et al., 1995). In low back pain, TCAs have been the most frequently tested antidepressants, and according to the guidelines of the American Pain Society and

American College of Physicians, TCAs are clinically effective for pain relief in low back pain (Chou, et al., 2007). Clinically, TCAs have demonstrated efficacy in the treatment of chronic pain conditions including postherpetic neuralgia (Bowsher et al., 1997, Hempenstall et al., 2005), diabetic neuropathy (Max et al., 1987, Gilron et al., 2009), and fibromyalgia (Heymann et al., 2001, Ginsberg et al., 1996). Results from a randomized, within-patient, cross-over, placebo-controlled trial clearly indicated a better analgesic effect of clomipramine and nortriptyline over placebo in patients with central pain (Panerai et al., 1990). The analgesic effects of tricyclic antidepressants are often seen with a faster onset (one to seven days) and with third/half the dosage used for depression (Lipman et al. 1996). Studies have also confirmed that the actions of tricyclic antidepressants in neuropathic pain are not related to their antidepressant effect. Firstly, treatment with tricyclic antidepressants (and in some studies SSRIs) was shown to significantly reduce painful symptoms associated with diabetic neuropathy, regardless of whether patients had normal or depressed moods (Max et al., 1987, Sindrup et al., 1990). Secondly, analgesic effects of antidepressants occur at lower plasma levels than those required for the antidepressant action (Sindrup et al., 1990). Despite having an effective analgesic profile clinically, TCAs are associated with undesirable side effects due to their actions at multiple receptors, which limits drug compliance in chronic pain patients.

Very recently, **triple monoamine reuptake inhibitors** (TRIs) such as bicifadine were synthesized to inhibit uptake of serotonin, norepinephrine and dopamine. Elevating supraspinal levels of dopamine is proposed to activate mesocorticolimbic dopaminergic pathways, which are central to reward, motivation and the experience of pleasure (Wise et al., 2002). This enhanced dopaminergic neurotransmission is proposed to address the anhedonia, lack of motivation and lack of attention common in pain and depression, which are common symptoms that SSRIs and S+NRIs do not address adequately. For example, early studies with treatment-resistant depressed patients found that when an SSRI was combined with bupropion, patients had higher and faster rates of remission compared to monotherapy (Zisook et al., 2006). Little is known about the clinical efficacy of bicifadine and other novel TRIs. Bicifadine has also undergone several Phase II and III trials for the treatment of chronic low back pain

(http://clinicaltrials.gov/ct2/show/NCT00295711, http://clinicaltrials.gov/ct2/show/NCT00281645). Overall, further clinical investigation is warranted to elucidate the therapeutic potential for TRIs in chronic pain states.

Clinical Acute Pain Studies with Monoamine Reuptake Inhibitors

Monoamine reuptake inhibitors have also been assessed in patients with acute pain; however, their analgesic effects are generally weak. Only limited clinical data exist to suggest that SSRIs demonstrate analgesic efficacy in clinical acute pain states. A study in healthy human volunteers evaluated the effect of a single oral dose of fluvoxamine on subjective measures of pain induced by the application of transcutaneous electrical stimulation to the sural nerve, and found that fluvoxamine significantly increased subjective pain thresholds compared to placebo (Coquoz et al., 1993). A caveat to this study was that fluvoxamine *only* altered the subjective pain threshold, without any influence on the objective measure of antinociceptive effect: the spinal R-III reflex pain threshold. Clinical studies have also demonstrated that fluoxetine (10 mg p.o. daily for 7 days pre-operatively) in combination with the mu-opiate morphine or the kappa-opiate pentazocine reduced the overall analgesic duration of action of opioids in acute postoperative dental pain patients (Gordon et al., 1994).

The clinical literature for analgesic effects of SNRIs in acute pain is also limited. A number of studies in acute postoperative pain patients suggest that when given in combination with opioids, SNRIs potentiate and prolong the analgesic effects of opioids (Max et al., 1992, Levine et al., 1986, Gordon et al., 1993). This literature also suggests that SNRI administration only enhances the analgesic effects of postoperative morphine when it is given in *the pre-operative week* as opposed to post-operative administration. There is no clear explanation for these temporal effects; however, it may reflect the delayed onset for central effects seen with monoamine reuptake inhibitors. The limited available clinical data regarding reboxetine usefulness in pain syndromes (Krell et al., 2005) indicate that SNRIs are mostly ineffective in the treatment of acute pain. Further studies are needed to investigate the noradrenergic

component that contributes to endogenous opioid-mediated analgesia and elucidate the efficacy of these compounds in subtypes of acute pain.

In general, the clinical literature suggests that SDRIs are more useful in reducing fatigue and improving symptoms of alertness and energy in postoperative patients, and less useful for blocking nociceptive pain (Larijani et al., 2004). The most selective SDRIs, including RTI-113, retain exclusive experimental status and are not available clinically. A less selective SDRI, bupropion, has not been evaluated clinically for acute pain. However, compounds like methylphenidate and modafinil, which inhibit DA and NE, have been evaluated, and generally produce weak analgesic effects. One clinical study demonstrated that modafinil failed to produce significant analgesic effects on experimental acute pain in normal healthy volunteers (Taneja et al., 2004). This is in contrast to analgesic effects noted in the literature for other compounds with a similar clinical profile (Cantello et al., 1988). For example, methylphenidate was shown to potentiate the analgesic effects and decrease the sedative effects of narcotics for the treatment of cancer pain in a randomized, double-blind, cross-over study (Bruera et al., 1987). When SDRIs are administered in combination with opioids, they are effective in potentiating the analgesic effects of opioids, and decreasing opioid-related somnolence and cognitive impairments in postoperative pain patients (Forrest et al., 1977). There are far fewer data demonstrating that SDRI administration *alone* is sufficient to treat acute pain symptoms. An early study reported that amphetamine (a DA releaser) had a "pain-threshold-raising" effect on experimentally induced pain (Goetzl et al., 1944). However, a later double-blind study found no analgesic effect of psychostimulants in postoperative pain patients, besides producing a reduction of sedation up to 30 min after operating (Dodson et al., 1980). More clinical trials are needed to elucidate whether SDRIs improve actual pain intensity ratings, in addition to improving sedation and cognitive status.

There are few clinical data to suggest that S+NRI treatment is effective in blocking acute pain in humans. A recent clinical study found that oral venlafaxine administration increased thresholds for pain tolerance and pain summation after electrical nerve stimulation, but did not alter pain intensity or discomfort experienced during the cold pressor test or increase pressure pain thresholds (Enggaard et al.,

2001). The impact of venlafaxine on temporal pain summation in this experiment may indicate a potential analgesic effect for clinical neuropathic pain, which is supported by recent clinical studies (Tasmuth et al., 2002, Rowbotham et al., 2004). One clinical study in postoperative knee replacement surgery patients found that perioperative administration of duloxetine reduced postoperative morphine requirements during the first 48 hours after surgery (Ho et al., 2010). The results from this study suggest that duloxetine and other S+NRIs can be useful adjuvants when used with opioids, non-opioids, and regional analgesic techniques as part of a multimodal approach in postoperative pain management.

Few studies have investigated the analgesic efficacy of TCAs for acute pain. In a dental pain study assessing the analgesic efficacy of amitriptyline buccoadhesive tablets, amitriptyline exhibited analgesia in all study volunteers (Movassaghian et al., 2011). In a 2002 study, desipramine had no significant effect on acute sensory thresholds, pain, secondary hyperalgesia, or flare response induced by intradermal capsaicin (Wallace et al., 2002). One criticism of this study was the disproportionate number of men and women (nine women and three men). Numerous studies have shown that there are sex differences in both pain response and analgesic response (Sarton et al., 2000), which may explain the lack of analgesic effect of desipramine on experimental pain. Overall, TCAs produced weak analgesic effects in acute pain patients.

The analgesic activity of bicifadine and other novel TRIs for acute pain has been evaluated in very few placebo-controlled studies. One clinical study evaluated the analgesic efficacy of bicifadine in postoperative pain patients and found that 150 mg of bicifadine QD demonstrated significant analgesic activity (vs. placebo) (Wang et al., 1982). This study reported minor side effects that did not interfere with the course of therapy. Additionally, there are numerous anecdotal reports suggesting that cocaine and/or amphetamine produce analgesia (http://www.drugs.com/forum/general/cocaine-pajn-relief-24448.html, http://www.idmu.co.uk/amphetpain.htm, http://www.drugs-forum.com/forum/showthread.php?t=24104). Clinical investigations of TRIs regarding dosing and their analgesic activity in other pain modalities such as experimental and/or dental pain are warranted.

In summary, monoamine reuptake inhibitors with actions primarily on 5-HT and NE transport typically do not alleviate acute pain in humans. However, monoamine reuptake inhibitors with prominent actions on DA transport often have more favorable effects on human subjective measures of acute pain.

A key feature of the clinical and pre-clinical literature assessing the efficacy of monoamine reuptake inhibitors in pain is the glaring discordance of effects in acute pain states. As will be discussed further below, monoamine reuptake inhibitors usually demonstrate better antinociceptive effects in pre-clinical assays of acute pain (pain-stimulated behaviors) than in acute clinical pain (e.g. postoperative pain or pain associated with experimental noxious stimuli). Overall, monoamine reuptake inhibitors do not produce reliable analgesia against acute pain in humans, but there is a crucial need for more clinical studies evaluating monoamine reuptake inhibitors in acute pain states.

Taken together, these properties of monoamine reuptake inhibitors suggest that they are useful for the treatment of some types of chronic pain and accompanying depressive symptoms. In general, it appears that S+NRIs and TCAs provide the best analgesic effects for inflammatory/neuropathic pain states, while selective and mixed-action dopamine reuptake inhibitors (SDRIs, N+DRIs, TRIs) may relieve subjective feelings of acute pain and be more useful as adjuvants to reduce opioid sedation and disease-related fatigue. Further research is warranted to clarify the clinical analgesic properties of these compounds.

Antinociceptive Effects of Monoamine Reuptake Inhibitors: Preclinical Studies

A number of animal studies involving acute and chronic pain models have concluded that antidepressants have an antinociceptive effect or an antihyperalgesic effect. These effects can vary depending on the route of administration (local vs. systemic), dosing schedule (acute vs. chronic) and pain model/ stimulus employed. However, of particular importance for this thesis project, many preclinical studies have reported antinociceptive effects of monoamine reuptake inhibitors in assays of acute pain. Consequently, there is a discordance in effects of monoamine reuptake inhibitor effects on clinical measures of acute pain (usually ineffective) and preclinical studies of acute nociception (often effective).

These preclinical findings are described in more detail below for different classes of monoamine reuptake inhibitors, and results are summarized in Table 2.

Acute systemic administration of **SSRIs** has been shown to produce antinociceptive effects in the hotplate assay (Ardid et al., 1992, Fasmer at al., 1989) and in acute visceral inflammatory pain models (acetic acid or PPQ-induced abdominal constrictions) (Singh et al., 2001, Leventhal et al., 2007, Korzeniewska-Rybicka et al., 1998, Aoki et al., 2006). Additionally, the SSRIs clomipramine and fluoxetine both increased tail-withdrawal latencies in rhesus monkeys in a warm water tail-withdrawal assay of acute thermal nociception (Gatch et al., 1998). SSRIs have also been reported to attenuate nociceptive behaviors in the formalin test (Bomholt et al., 2005, Mochizucki et al., 2004, Otsuka et al., 2001) and block mechanical allodynia in animals with peripheral nerve injury (Jett et al., 1997, Leventhal et al., 2007, Jesse et al., 2010). Reports on the analgesic efficacy of SSRIs in neuropathic pain models are mixed, ranging from partial to full pain relief. One preclinical study demonstrated antiallodynic effects of paroxetine and fluvoxamine in streptozotocin-induced diabetic rats, but little antiallodynic effect in rats with chronic constriction injury (CCI) (Ikeda et al., 2009). Full antinociception with an SSRI may be limited by the occurrence of troublesome side effects.

SNRIs including reboxetine, maprotilline and nisoxetine have been assessed in preclinical pain assays and have shown the highest efficacy in acute pain models of hotplate, tail flick and writhing assays (Rojas-Corrales et al., 2003, Schreiber et al., 2009, Ardid et al., 1992). In one particular study, wild-type (WT) mice and littermates with gene knockout (KO) of SERT, NET or both transporters were used to investigate the relative contributions of NET and SERT on nociception and the analgesic effects of amitriptyline and morphine. In the study, NET KO mice demonstrated profound baseline hypoalgesia in the hot plate and tail flick assays across a variety of noxious temperatures, as well as a substantial reduction in acetic acid-induced writhing. The effect of NET KO was so great that it impaired the ability to subsequently observe amitriptyline and morphine-induced analgesia in these subjects. The authors concluded that NET has a far greater role than SERT in determining baseline thermal and visceral

nociception in mice, which further emphasizes the importance of noradrenergic neurotransmission in the analgesic effects of monoamine reuptake inhibitors (Hall et al., 2011).

SDRIs have also demonstrated antinociceptive effects in preclinical assays of acute pain. In particular, bupropion administration produced reliable antiallodynic effects in rats with spinal nerve ligation (SNL) or chronic constriction injury (CCI) (Pedersen et al., 2005, Jesse et al., 2010). Other drugs with prominent dopaminergic effects are discussed below under TRIs.

S+NRIs have antinociceptive activity in models of visceral pain (Aoki et al., 2006), acute thermal nociception such as hot plate (Suarez-Roca et al., 2006), and demonstrate antinociceptive effects in formalin- and carrageenan-induced inflammatory pain assays (Yokogawa et al., 2002, Iyengar et al., 2004, Bardin et al., 2010). For example, milnacipran dose-dependently and significantly reduced the number of writhes induced by an injection of acetic acid in male ICR mice (Aoki et al., 2006) and reduced the number of cramps observed in the butyrate/colonic distension assay in rats (a model of irritable bowel syndrome) (Depoortère et al., 2011). Another study evaluating the analgesic effects of monoamine reuptake inhibitors in rats with carrageenan-induced mechanical hypersensitivity and inflammation found that pretreatment with venlafaxine significantly reduced or completely abolished enhanced sensitivity to mechanical stimuli (i.e. von Frey monofilaments) (Aricioğlu et al., 2005). S+NRIs also have been shown to block hypersensitivity associated with mechanical neuropathies (Iyengar et al., 2004, Shin and Eisenbach et al., 2004, Obata et al., 2005, King et al., 2006, Onal et al., 2007, Takeda et al., 2009, Berrocoso et al., 2011), streptozotocin-induced neuropathies (Ikeda et al., 2009) and arthritic pain in animal models (Mico et al., 2011). In this latter study, milnacipran was evaluated in polyarthritic rats using the Randall-Selitto pressure meter. Arthritis was induced by a single intradermal injection of complete Freund's adjuvant in the tail base. Using the Randall-Selitto model, two levels of pressure were applied to both hind paws (a lower one assessing mechanical allodynia and a higher one assessing mechanical hyperalgesia). The pain threshold was determined as the force that induced either a paw withdrawal or vocalization/struggle. Milnacipran dose-dependently increased the vocalization threshold in rats under both low and high pressure levels, indicative of an antinociceptive effect.

Acute peripheral administration of **TCAs** produces antinociceptive effects in models of acute thermal pain (Tura-Tura et al., 1990, Otsuka et al., 2001, Dirksen et al., 1994) as well as antinociceptive and anti-edematogenic effects in models of acute (Aoki et al., 2006-acetic acid writhing, Gray et al., 1998-acetic-acid writhing, Leventhal et al., 2007) and persistent (Hajhashemi et al., 2010- carrageenan paw edema, Bianchi et al., 1995-carrageenan paw edema, Heughan et al., 2002-formalin) inflammatory pain. Some of these studies suggest that TCAs demonstrate stronger antinociceptive effects in pain models employing a chemical stimulus (acetic acid) compared to a thermal stimulus (radiant heat) (Rojas-Corrales et al., 2003), but further investigation into this mechanism is required. Peripheral TCAs also demonstrated antinociceptive effects in animal models of neuropathic pain (Sawynok et al 1999, Mochizucki et al., 2004, Esser et al., 1999, Abdi et al., 1998, Bomholt et al., 2005, Berrocoso et al., 2011). Centrally administered TCAs have also been shown to produce anti-hyperalgesic effects in acute (Korzeniewska-Rybicka et al., 2000) and persistent inflammatory pain models in rats (Sadeghi et al., 2011, Eisenach et al., 1995). Recently it was demonstrated that local administration of tricyclic antidepressants into the paw produce antinociceptive effects in an inflammatory pain assay (formalin test) (Oatway et al., 2003) and peripheral anti-hyperalgesic effects in a neuropathic pain model (spinal nerve ligation) (Sawynok et al., 1999).

Only a few laboratories have examined the antinociceptive effects of novel **TRIs**. For example, pretreatment with cocaine (or amphetamine) produced reliable blockade of abdominal writhing in mice (Frussa-Filho et al., 1996). Similarly, in rhesus monkeys, cocaine administration produced weak but significant antinociceptive effects in a warm water tail-withdrawal assay, and when given in combination with morphine, produced an overall enhancement of morphine analgesia (Gatch et al., 1999). Additionally, studies in rodents have found that cocaine produces weak antinociceptive effects when administered alone and potentiates the antinociceptive effects of mu agonists (Misra et al., 1987, Shimada et al., 1988, Kaupilla and Mercke et al., 1992, Sierra et al., 1992). Administration of the TRI bicifadine was reported to demonstrate antinociceptive effects in assays of acute nociceptive pain (i.e. hot plate, tail flick) (Basile et al., 2007) and in assays of acute inflammatory pain (i.e. kaolin-induced arthritis model,

yeast-inflamed hind paw model, and PPQ-induced abdominal contractions assay. More research is required to evaluate the analgesic effects of TRIs in different pain states.

In summary, all types of monoamine reuptake inhibitors have demonstrated significant, although sometimes weak antinociceptive effects in assays of acute, inflammatory and neuropathic pain.

New Approaches to Preclinical Assessment of Pain and Analgesia

The discordance in monoamine reuptake inhibitor efficacy in preclinical models of acute nociception and clinical studies of acute pain may be related to weaknesses of the procedures used in preclinical research. Preclinical assays of pain have evolved over the years, but most studies of monoamine releasers or other drugs have relied solely on assays measuring **"pain-stimulated behaviors,"** or behaviors that increase in rate, frequency, or intensity in response to the delivery of a painful stimulus (Negus et al. 2006, Stevenson et al. 2006). Preclinical assays of pain-stimulated behaviors include hot plate, tail flick, acetic acid-induced writhing and formalin-induced paw flinching assays, as well as assays that assess thermal or mechanical hypersensitive withdrawal responses associated with inflammation or neuropathy. In these assays, antinociception is inferred from a decrease in pain-stimulated behaviors. However, there are many disadvantages of relying solely on pain-stimulated behaviors to assess candidate analgesics. First, although clinically effective analgesics (e.g. morphine) will block pain-stimulated behaviors, expression of these behaviors can also be blocked by drugs that produce motor impairment rather than a selective decrease in sensitivity to noxious stimuli (resulting in false-positive evidence for analgesia). More importantly, pain-stimulated behaviors are rarely used clinically to diagnose pain or assess analgesic efficacy. It is well documented that pain is also associated with the depression of many behaviors (Von Korff et al., 2005), and there is high co-morbidity between pain and depression (Bair et al., 2003). Furthermore, the efficacy of monoamine reuptake inhibitors to treat depression suggests that these compounds may also be effective in treating the pro-depressant effects of pain. Therefore, we have incorporated more clinically relevant pain assays that measure **"pain-depressed behaviors."** These are behaviors that *decrease* in rate, frequency, or intensity in response to

the delivery of a painful stimulus. Some common examples include pain-related decreases in feeding, locomotion and expression of positively reinforced operant behavior. In these assays, effective analgesics are expected to increase or restore pain-depressed behaviors back to baseline levels. The addition of pain-depressed assays is useful when evaluating candidate analgesics, because these dissociate true analgesic effects from motor depressant effects, and they provide insight into the affective components of pain. Using these two complementary types of preclinical pain assays ensures that our experimental assessment of pain and analgesic drug efficacy includes dependent measures similar to those used in veterinary and human medicine. An optimal profile for an effective analgesic would be a drug that blocks pain-stimulated behaviors and increases/restores pain-depressed behaviors. The effects of monoamine releasers have not been examined in preclinical assays of pain-depressed behavior.

Objectives of this Study

The objective of the present study was to evaluate the antinociceptive properties of a variety of monoamine reuptake inhibitors selective for SERT, NET and DAT in complementary assays of acute pain-stimulated and pain-depressed behaviors. The rationale for studying acute pain is two-fold. First, this study focuses on acute pain as the first step in a larger investigation on effects of monoamine reuptake inhibitors and other drugs on behavioral depression associated with acute and chronic pain states. Further studies in chronic pain models will be designed in part on the basis of results from these acute pain studies. Second, the most salient discrepancies between preclinical and clinical research have occurred in studies of acute pain, for which preclinical studies usually demonstrate significant antinociceptive effects of monoamine reuptake inhibitors, whereas clinical studies show little or no analgesic efficacy of these compounds. Acute assays of pain were used to further elucidate this discrepancy in the literature. For this study, intraperitoneal injection of dilute lactic acid (1.8% in a volume of 1 ml/kg) served as an acute chemical noxious stimulus, and acid-stimulated stretching and acid-depressed intracranial self-stimulation (ICSS) were assessed in rats. Abdominal stretching is a commonly used dependent measure of nociception in assays of pain-stimulated behavior using intraperitoneal administration of acid or other

chemical irritants as the noxious stimulus (Negus et al., 2006). ICSS, by contrast, is commonly used to assess changes in motivated behavior and affect in experimental subjects (Carlezon et al., 2007), and it can also be used to evaluate effects of noxious stimuli and candidate analgesics (Pereira Do Carmo G et al., 2009, Negus et al., 2010). ICSS promotes high levels of stable responding over time and relies on brain reward substrates likely to mediate pro-depressant effects of pain. After exposure to an acute noxious stimulus (1.8% lactic acid), ICSS responding decreases significantly, and acid-induced depression of ICSS can be blocked by clinically effective analgesics including mu opioid receptor agonists and NSAIDs (Pereira Do Carmo, et al., 2009, Negus et al., 2012). Based on previous data evaluating the antinociceptive effects of monoamine reuptake inhibitors in assays of pain-stimulated behavior (Otsuka et al., 2001, Iyengar et al., 2002, Yokogawa et al., 2002, Pedersen et al., 2005, Bomholt et al., 2005, Pelissier et al., 2001, Berrocoso et al., 2011), data from cocaine discrimination studies (Cook et al., 2001), and data evaluating antidepressant efficacy in the forced swim test (Paul et al., 1990), we hypothesized that acute administration of monoamine reuptake inhibitors would exhibit a range of antinociceptive effects in assays of acid-stimulated stretching and acid-depressed ICSS. This hypothesis was tested using drugs that selectively inhibit uptake of serotonin (the SSRI citalopram and TCA clomipramine), norepinephrine (the SNRI nisoxetine and TCA nortryptiline), dopamine (the SDRIs RTI-113 and bupropion), both serotonin and norepinephrine (the S+NRI milnacipran) or all three monoamines (the TRI RTI-112). Specifically, given clinical evidence that pharmacologic or environmental stimulation of dopaminergic systems appears to be more efficacious than stimulation of serotonergic or noradrenergic systems for producing analgesia against acute pain, we hypothesized that only the SDRIs RTI-113 and bupropion and the TRI RTI-112 would block acid-depressed ICSS (See Table 1 below). We also hypothesized that *all* monoamine reuptake inhibitors would demonstrate antinociceptive effects in the assay of acid-stimulated stretching, because reuptake inhibitors of all monoamines have been reported to produce significant antinociception in preclinical assays of other pain-stimulated behaviors. Some literature suggests that monoamine reuptake inhibitors require chronic administration to effectively treat depressive symptoms (Detke et al., 1997). In a separate group of rats, we investigated the effects of

repeated administration of the SSRI citalopram in pain-depressed ICSS, using a repeated dosing regimen shown to be effective in the forced swim test of antidepressant-like activity (Carlezon et al., 2006)

Methods

Subjects

A total of 92 male Sprague-Dawley rats (Harlan, Frederick, MD, USA) weighing 297-334 g at the time of surgery were used for the studies of lactic acid-induced stretching (n=45) and ICSS (n=47). Rats were housed individually and were maintained on a 12-h light/dark cycle with lights on from 6:00 a.m. to 6:00 p.m. Rats had free access to food and water except during testing. Animal maintenance and research were in compliance with National Institutes of Health guidelines on care and use of animal subjects in research, and all animal use protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

Intracranial Self-Stimulation (ICSS)

Surgery

All rats were implanted with a bipolar stainless steel electrode (Plastics One, Roanoke, VA, USA) using stereotaxic surgery. Each bipolar electrode consisted of a cathode (0.25 mm in diameter and covered with polyamide insulation except at the flattened tip) and an anode (0.125 mm in diameter and uninsulated). During surgery, rats were anesthetized with isoflurane gas (2.5–3% in oxygen; Webster Veterinary, Phoenix, AZ, USA). The cathode was implanted in the left medial forebrain bundle at the level of the lateral hypothalamus (2.8 mm posterior to bregma, 1.7 mm lateral from midsagittal suture, and 7.8 mm below dura). The anode was wrapped around one of three skull screws to ground the implant, and the skull screws and electrode were affixed to the skull with orthodontic resin. The animals recovered for at least seven days post surgery prior to commencing ICSS training.

Apparatus

ICSS experiments were conducted in sound-attenuating boxes that contained modular acrylic test chambers (29.2 x 30.5 x 24.1 cm) equipped with a response lever (4.5 cm wide, extended 2.0 cm through

the center of one wall, 3 cm off the floor), stimulus lights (three lights colored red, yellow, and green positioned 7.6 cm directly above the response lever), a 2-W white house light, and an ICSS stimulator (Med Associates, St. Albans, VT, USA). Electrodes were connected to the stimulator via a swivel connector (Model SL2C, Plastics One, Roanoke, VA, USA). The stimulator was controlled by a computer software program that also controlled all the programming parameters and data collection (Med Associates, St. Albans, VT, USA).

Behavioral Procedure

After initial shaping of lever-press responding, rats were trained under a continuous reinforcement schedule of brain stimulation using procedures similar to those described previously (Negus et al., 2010a; Negus et al., 2010b). During initial training sessions lasting 30 to 60 min, the white house light was illuminated, and responding produced electrical stimulation under a continuous schedule of reinforcement. Under this schedule, each lever press resulted in the delivery of a 0.5-s train of square-wave cathodal pulses (0.1-ms pulse duration) and illumination for 0.5-s of the colored stimulus lights over the lever. Responses during the 0.5-s stimulation period did not earn additional stimulation. Initially, the frequency of stimulation was held constant at 126 Hz, and the stimulation intensity for each rat was adjusted gradually to the lowest value that would sustain a high rate of ICSS (≥30 stimulations/min). Frequency manipulations were then introduced, and the terminal schedule consisted of sequential 10-min components. During each component, a descending series of 10 current frequencies was presented, with a 60-s trial at each frequency. The frequency range extended from 158-56 Hz in 0.05 log increments. Each frequency trial began with a 10-s time out, during which the house light was off and responding had no scheduled consequences. During the last 5 s of this time out, 5 non-contingent stimulations were delivered once per second at the frequency available during that trial, and the lever lights were illuminated during each stimulation. This non-contingent stimulation was then followed by a 50-s "response" period, during which the house light was illuminated, and responding produced electrical stimulation under the continuous reinforcement schedule described above. Training continued with

presentation of three sequential components per day, and intensity was again adjusted as necessary until rats reliably responded for at least three and no more than 6 trials of all components for at least two consecutive days. In general, rats were trained in groups of 10-14 for each drug. The first six rats to meet training criteria were then advanced to ICSS testing. As discussed previously (Pereira Do Carmo et al., 2009; Negus et al., 2010b), the remaining rats from each group were assigned to studies of lactic acid-induced stretching using methods described below.

Once training was completed, ICSS testing began. A test session consisted of six sequential components. The first component of each session was considered to be a "warm up" component, and data from this component were discarded. Data from the second and third components were used to calculate baseline parameters of frequency-rate curves for that session (see "Data analysis"). After the third component, rats were taken out of the ICSS chambers, administered drug and placed back into their home cages. After the designated pretreatment time elapsed, 1.8% lactic acid or its vehicle (bacteriostatic water) was administered IP in a volume of 1 ml/kg, and rats were immediately placed back into their ICSS chambers for three test components. This 30 min test session was chosen to match the session length for stretching studies (see below), and because our previous studies demonstrated that lactic acid produced sustained decrease in ICSS for up to 90 min (Pereira Do Carmo et al. 2009). Eight different monoamine reuptake inhibitors were evaluated, and data from the literature on their selectivities for the serotonin transporter (SERT), dopamine transporter (DAT) and norepinephrine transporter (NET) are shown in Table 1. Thus, test drugs included the SSRIs citalopram (3.2-32 mg/kg) and clomipramine (3.2-32 mg/kg), the SNRIs nisoxetine (1-10 mg/kg) and nortriptyline (1-10 mg/kg), the SDRIs RTI-113 (0.32-3.2 mg/kg) and bupropion (3.2-32 mg/kg), the S+NRI milnacipran (0.32-3.2 mg/kg), and the TRI RTI-112 (0.1-1 mg/kg). Each monoamine reuptake inhibitor or its vehicle (bacteriostatic water) was administered 30 min (before lactic acid or its vehicle, except for RTI-113 and RTI-112, which were administered 10 min before acid or vehicle. Pretreatment times were based on previously published behavioral studies in rats (Paul et al., 1990, Cook et al., 2001, Otsuka et al., 2001, Iyengar et al., 2002, Yokogawa et al., 2002, Pedersen et al., 2005, Bomholt et al., 2005, Pelissier et al., 2001, Berrocoso et al., 2011). Test drug doses

were delivered in a modified Latin-square order across rats, so that each week, a rat was tested with a given dose of test drug in combination with lactic acid vehicle on one test day and with 1.8% lactic acid on another test day. Test sessions were typically conducted on Tuesdays and Fridays, and 30-min training sessions were conducted on Mondays, Wednesdays, and Thursdays.

Repeated dosing with monoamine reuptake inhibitors is sometimes required to demonstrate efficacy in preclinical assays of antidepressant-like effects (e.g. the forced-swim test in rats) (Detke et al., 1997, Vazquez-Palacios et al., 2004, Cryan et al., 2005). Consequently, the SSRI citalopram was also tested using a dosing regimen of repeated treatment shown to be effective in the modified forced-swim test of antidepressant-like drug effects (Carlezon et al. 2006). Specifically, at least one week after completion of acute dosing, rats were tested with three repeated injections of citalopram (10 mg/kg, i.p.) at 23, 19, and 1 h before receiving acid administration and ICSS testing.

Data analysis

The primary dependent variable was reinforcement rate in stimulations per minute during each frequency trial. To normalize these data, raw reinforcement rates from each trial were converted to percent maximum control rate (%MCR), with the MCR defined as the mean of the maximal rates observed during the second and third "baseline" components for that session. Thus, %MCR values for each trial were calculated as (response rate during a frequency trial ÷ maximum control rate) x 100. For each ICSS experiment, data from the second and third baseline components were averaged to yield a baseline frequency-rate curve, and data from the three test components were averaged to yield a test frequency-rate curve. Baseline and test curves were then averaged across rats to yield mean baseline and test curves for each manipulation. For statistical analysis, results were compared by two-way analysis of variance (ANOVA), with treatment and ICSS frequency as the two factors. A significant ANOVA was followed by a Holman-Sidak post hoc test, and the criterion for significance was set at $p<0.05$.

To provide an additional summary of ICSS performance, the total number of stimulations obtained at all frequencies was summed for each test component and averaged across the three test

components of each experimental session in each rat. Data for total stimulations per component were then expressed as a percentage of the baseline number of stimulations per component in each rat and averaged across rats.

Assay of lactic acid-stimulated stretching

Behavioral procedure

Test sessions were conducted once per week. Test drugs were administered IP prior to treatment with 1.8% lactic acid (IP in a volume of 1 ml/kg). Immediately after acid injection, rats were placed into acrylic test chambers (31.0cm x 20.1cm x 20.0cm) for 30 min observation periods. A stretch was operationally defined as a contraction of the abdomen followed by extension of the hind limbs, and the number of stretches during the observation period was counted. Dose effect curves were determined for citalopram (3.2-32 mg/kg, 30 min pretreatment), clomipramine (3.2-32 mg/kg, 30 min pretreatment), nisoxetine (0.32-3.2 mg/kg, 30 min pretreatment), nortriptyline (0.32-10 mg/kg, 30 min pretreatment), RTI-113 (0.32-3.2, 10 min pretreatment), bupropion (3.2-32 mg/kg, 30 min pretreatment), milnacipran (0.1-3.2 mg/kg, 30 min pretreatment) and RTI-112 (0.032-1 mg/kg, 10 min pretreatment). Test drugs were delivered in a Latin-square order across rats. Each week, a rat was tested with a given drug dose in combination with 1.8% lactic acid. At the conclusion of these acute dosing studies, repeated dosing studies were conducted with citalopram (10 mg/kg) administered 23, 19, and 1 h before acid administration.

Data Analysis

Test drug effects on lactic acid-stimulated stretching were evaluated by one-way ANOVA. A significant ANOVA was followed by the Dunnett's post hoc test, and the criterion for significance was set at $p< 0.05$.

Drugs

Lactic acid, citalopram HBr, clomipramine HCl, nisoxetine HCl, nortriptyline HCl and bupropion HCl were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Milnacipran HCl was purchased from Tocris Bioscience (Minneapolis, MN, USA). RTI-113 [3β-(4-chlorophenyl) tropane-2β-carboxylic acid phenyl ester hydrochloride] and RTI-112 [3β-(3-methyl-4-chlorophenyl) tropane-2β-carboxylic acid methyl ester hydrochloride] were synthesized at Research Triangle Institute and generously provided by Dr. Ivy Carroll. All solutions were prepared in sterile water for IP injection.

Results

Effects of monoamine uptake inhibitors in the assay of acid-stimulated stretching

Across all 45 rats used for studies of acid-stimulated stretching, IP administration of 1.8% lactic acid (1.0 ml/kg) after drug vehicle pretreatments elicited a mean±SEM of 21.24 ± 1.39 stretches. The absolute number of stretches elicited by acid alone in each group is shown in the figures (open bars). Figure 4 shows that all eight monoamine uptake inhibitors produced a dose-dependent decrease in acid-stimulated stretching. Table 3 shows the lowest dose of each compound to significantly decrease stretching.

Effects of monoamine uptake inhibitors in the assay of acid-depressed ICSS

Acid-induced depression of ICSS. Figure 5 shows effects of the same noxious stimulus (IP injection of 1.8% lactic acid) on ICSS. During each test session, a "baseline" frequency-rate curve was determined before experimental treatments to permit determination of the Maximum Control Rate (MCR) for that session. Over the course of the entire study, the mean±SEM MCR was 54.03±0.57 stimulations per trial, and MCR values in each group are shown in Table 4. Reinforcement rates during each frequency trial of a session were then expressed as a percentage of that session's MCR, and the average frequency-rate curve for all studies with drug vehicle + acid vehicle is shown in Fig. 5. Maximum reinforcement rates were usually observed at the highest stimulation frequencies (2.15-2.2 log Hz), and responding generally decreased in a frequency-dependent manner at lower frequencies. Administration of 1.8% lactic acid depressed ICSS, producing a rightward shift in the frequency-rate curve. Figure 5 also shows summary data for the total number of stimulations delivered across all 10 frequencies during each component. The overall mean±SEM baseline number of stimulations per component for all rats in the study was 206.13±6.49, and the mean±SEM baseline number of stimulations per component in each group is shown in Table 4. Total ICSS after treatment with drug vehicle + acid vehicle was nearly

identical to baseline predrug ICSS, but acid treatment decreased the number of stimulations per component. This acid-induced depression of ICSS provided a measure of pain-related behavioral depression, and drugs were evaluated for their ability to block acid-induced depression of ICSS.

Effects of selective serotonin reuptake inhibitors. Figure 6 shows that citalopram and clomipramine failed to block acid-induced depression of ICSS, and consequently failed to produce antinociception in this assay. When citalopram was administered as a pretreatment to acid vehicle, citalopram produced a downward shift in the ICSS frequency-rate curve (Fig. 6A). A low dose of 3.2 mg/kg citalopram had no effect on ICSS. However, 10 and 32 mg/kg citalopram significantly decreased rates of reinforcement at the two highest frequencies (2.15 and 2.2 log Hz). When citalopram was administered as a pretreatment to 1.8% lactic acid, it exacerbated acid-induced depression of ICSS (Fig. 6B) with significant effects by 10 and 32 mg/kg citalopram at the two highest frequencies (2.15-2.2 log Hz). The lowest doses of citalopram and all other drugs to significantly alter ICSS in the absence or presence of acid treatment are shown in Table 3. Overall, acute citalopram produced a depression of ICSS whether it was administered before lactic acid vehicle or 1.8% lactic acid (Fig. 6C).

When clomipramine was administered as a pretreatment to acid vehicle, it produced a downward and rightward shift in the ICSS frequency-rate curve (Fig. 6D). All doses of clomipramine (3.2, 10, and 32 mg/kg) produced significant decrease in ICSS at the highest stimulation frequencies (2.0-2.2 log Hz). Similarly, when clomipramine was administered as a pretreatment to 1.8% lactic acid, it exacerbated acid-induced depression of ICSS. Only the highest dose of clomipramine (32 mg/kg) produced significant decreases in rates of reinforcement at the highest frequency (2.2 log Hz) (Fig. 6E). Overall, acute clomipramine produced a depression of ICSS whether it was administered before lactic acid vehicle or 1.8% lactic acid (Fig. 6F).

Effect of selective norepinephrine reuptake inhibitors. Figure 7 shows that nisoxetine and nortriptyline also failed to block acid-induced depression of ICSS. When nisoxetine was administered as a pretreatment to acid vehicle (Fig. 7A), it produced a rightward shift of the frequency rate curve that was

significant at all doses tested (1-10 mg/kg) at frequencies ranging from 1.95-2.15 log Hz. As a pretreatment to acid, nisoxetine further depressed ICSS responding (Figure 7B). Higher doses of 3.2 and 10 mg/kg caused significant decreases in ICSS at frequencies of 2.15-2.2 log Hz. Overall, acute nisoxetine depressed ICSS responding in the absence or presence of acid (Fig. 7C).

When nortriptyline was administered as a pretreatment to acid vehicle, it produced a downward and rightward shift in the ICSS frequency rate curve (Fig. 7D). All doses of nortriptyline (1, 3.2, 10 mg/kg) produced a significant decrease in rates of reinforcement at frequencies ranging from 1.9-2.2 log Hz. Similarly, when nortriptyline was administered as a pretreatment to lactic acid, it produced a downward and rightward shift in the ICSS frequency rate curve (Fig. 7E) with significant decreases at the lowest and highest doses (1 and 10mg/kg) at a range of high frequencies (2.05-2.2 log Hz). Overall, acute nortriptyline depressed ICSS responding in the absence or presence of acid (Fig. 7F).

Effect of selective dopamine reuptake inhibitors. Figure 8 shows that, in contrast to the SSRIs and SNRIs, the SDRIs RTI-113 and bupropion dose-dependently and completely blocked acid-induced depression of ICSS at or near doses that also facilitated control ICSS in the absence of the noxious acid stimulus. When administered as a pretreatment to acid vehicle, RTI-113 produced a dose-dependent leftward shift in the ICSS frequency-rate curve (Fig. 8A). A low dose of 0.32 mg/kg RTI-113 had no effect on basal ICSS. However, 1 and 3.2 mg/kg RTI-113 dose-dependently increased rates of reinforcement, with significant effects at the highest dose tested. Similarly, when administered as a pretreatment to 1.8% lactic acid, RTI-113 increased ICSS responding and ameliorated acid-induced depression of ICSS (Fig. 8B). Significant increases in ICSS responding were observed after pretreatment with 1 and 3.2 mg/kg RTI-113. Overall, acute RTI-113 produced non-selective facilitation of ICSS in the absence or presence of acid (Fig. 8C).

Pretreatment with bupropion also non-selectively increased ICSS responding in the absence (Fig. 8D) or presence of acid (Fig. 8E). High doses of 10 and 32 mg/kg bupropion significantly increased rates of reinforcement under both conditions, and these effects of bupropion are summarized in Fig. 8F.

Overall, acute treatment with either SDRI was sufficient to produce non-selective facilitation of ICSS and block acid-induced depression of ICSS.

Effect of mixed-action monoamine uptake inhibitors. Figure 9 shows the effect of the S+NRI milnacipran and the TRI RTI-112 on control and acid-depressed ICSS. Pretreatment with milnacipran did not affect control ICSS (Fig. 9A). In the presence of lactic acid, milnacipran trended towards a restoration of acid-depressed ICSS (Fig. 9B), but this effect did not reach statistical significance. Overall, acute administration of milnacipran trended towards a selective blockade of acid-depressed ICSS, but the effect did not reach statistical significance (Fig. 9C).

Effects of the TRI RTI-112 were similar to effects of the SDRIs discussed above. When RTI-112 was administered as a pretreatment to acid vehicle, it produced a dose-dependent leftward shift in the ICSS frequency-rate curve (Fig. 9D). Significant increases in ICSS responding were observed after pretreatment with all doses of RTI-112 at lower frequencies (1.75-1.95 log Hz). When RTI-112 was administered as a pretreatment to lactic acid, significant leftward shifts in the frequency-rate curve were seen only with 0.32 and 1 mg/kg RTI-112 at the lower range of frequencies (1.75-2.05 log Hz) (Fig. 9E). Overall, all acute doses of RTI-112 were sufficient to produce a facilitation in ICSS responding under basal conditions, but only the two highest doses of RTI-112 (0.32 and 1 mg/kg) were able to significantly block acid-induced depression of ICSS (Fig. 9F).

Effects of repeated citalopram in the assay of lactic acid-depressed ICSS. Citalopram was re-tested using a repeated-dosing regimen shown previously to produce antidepressant effects in a forced-swim test in rats (Carlezon et al. 2006). As with acute administration, repeated citalopram (10 mg/kg x 3 doses) significantly decreased acid-stimulated stretching. Intraperitoneal administration of 1.8% lactic acid (1.0 ml/kg) elicited a mean±SEM of 13.17 ± 2.40 and 5.67±2.09 stretches after treatment with citalopram vehicle and repeated citalopram, respectively, and this effect was significant ($t(5)=3.16$, $p=0.025$). However, when repeated citalopram was administered as a pretreatment to lactic acid in the

ICSS procedure, it failed to block acid-induced depression of ICSS (Fig.10). Thus, as with acute citalopram, repeated citalopram produced antinociception in the assay of acid-stimulated stretching but not in the assay of acid-depressed ICSS.

Discussion

The main finding of this study was that all eight monoamine reuptake inhibitors produced an antinociception-like blockade of acid-stimulated stretching, but only compounds with prominent DA reuptake inhibition (SDRIs RTI-113 and bupropion and the TRI RTI-112) were able to block acid-depressed ICSS, although these effects were produced only at doses that also produced an abuse-related facilitation of control ICSS. Nonetheless, these findings are consistent with clinical findings that SDRIs or TRIs can relieve affective components of acute pain. Selective or mixed-action inhibitors of 5-HT and NE failed to block acid-induced depression of ICSS. These results are consistent with poor efficacy of SSRIs, SNRIs and S+NRIs to treat acute pain in humans. These results suggest that assays of pain-depressed behavior can provide new insights on analgesia-related effects of monoamine uptake inhibitors.

Effects of Monoamine Reuptake Inhibitors in Acid-Stimulated Stretching

The effects of the monoamine reuptake inhibitors in the present assay of lactic acid-stimulated stretching are consistent with the effects of these compounds reported previously in other assays measuring acute pain-stimulated behaviors, including hot plate, tail flick, acid-induced stretching, formalin, and mechanical allodynia elicited by carageenan, CFA, or peripheral nerve injury (Leventhal et al., 2007, Rojas-Corrales et al., 2003, Ardid et al., 1992, Pedersen et al., 2005, Yokagawa et al., 2002, Aoki et al., 2006, Aricioğlu et al., 2005, Heughan et al., 2002, Mochizucki et al., 2004, Frussa-Filho et al., 1996, Basile et al., 2007).

Monoamine Reuptake Inhibitor Effects on Control-ICSS

We investigated the effects of eight monoamine reuptake inhibitors on ICSS, in the presence and in the absence of a noxious stimulus. In the absence of a noxious stimulus, citalopram and clomipramine

produced significant decreases in ICSS responding. These findings are consistent with the effects of SSRIs reported previously in ICSS (Lee et al., 1998, Katz and Carroll et al., 1977) in which acute fluoxetine administration produced elevations in ICSS reward thresholds and decreases in rates of responding for ICSS in both mice and rats. Previous investigations examining the interaction of 5-HT on the mesolimbic DA system employing ICSS have resulted in a complex picture. Acute administration of fluoxetine (Cazala et al., 1980) and the 5-HT precursor 5-hydroxytryptophan (5-HTP) (Bose et al., 1974), results in decreases in rates of responding to rewarding brain stimulation, suggesting an inhibitory role for serotonin. Similarly, Fletcher and colleagues (Fletcher et al., 1995) reported a lowering of ICSS thresholds after producing an inhibition in 5-HT cell firing via injections of the 5-HT1A agonist 8-OH-DPAT into the median raphe nucleus. However, other studies using fluoxetine have resulted in no change (Andreev et al., 1979, Matthews et al., 1996), or increased rates of responding following direct perfusion of 5-HT into the brains of rats self-stimulating (Redgrave et al., 1976). However, those studies, which measure the rate of response as the dependent variable, may have confounded results due to the inhibitory function of 5-HT on locomotor activity and possibly operant responding in rodents (Gerson et al., 1980). For example, systemic injections of fluoxetine and 5-HTP (E.L. Rodriguez Echandia et al., 1983) decrease rat locomotor activity and various 5-HT agonists have been shown to attenuate the hyperlocomotor activity produced by amphetamine (Hollister et al., 1976, Layer et al., 1992). The differences in results could also be attributed to the time of pretreatment with fluoxetine. The discrepant results reported by Andreev and colleagues, in which fluoxetine failed to produce changes in rate of responding in rats, may be due to their procedure of testing the animals 4 h after administration of fluoxetine. Through microdialysis it has been shown that acute intraperitoneal injections of fluoxetine significantly increase extracellular levels of serotonin in the nucleus accumbens (Guan et al., 1988) and the striatum and hippocampus (Kreiss et al., 1995) of rats for approximately 2 hours. Based on these and other studies (Otsuka et al., 2001, Iyengar et al., 2002, Yokogawa et al., 2002, Pedersen et al., 2005, Bomholt et al., 2005, Pelissier et al., 2001, Berrocoso et al., 2011), we tested our animals 30 min after administration of intraperitoneal injections of citalopram and clomipramine for a period of 30 min. Our

results in conjunction with published literature further support the hypothesis that serotonin produces an inhibitory effect on the mesolimbic dopaminergic reward system. Furthermore, these results suggest that the antidepressant effects of fluoxetine are not the direct result of excitation of brain reward systems, at least in the same manner as abused substances, like cocaine. It is well established that drugs that lower brain stimulation reward thresholds are highly abused, whereas drugs for which there is no abuse potential either have no effect or raise the threshold for rewarding brain stimulation (Kornetsky et al., 1990, Kornetsky et al., 1979). Our findings in rats, as well as self-administration studies of SSRIs in primates (Howell et al., 1995) strongly suggest that SSRIs do not possess abuse potential similar to other drugs of abuse such as cocaine (Esposito et al., 1978).

Similarly, the SNRIs, nisoxetine and nortriptyline also produced a depression in both control and acid-depressed ICSS. To our knowledge, this is the first experiment to evaluate nisoxetine and nortriptyline in ICSS. In our study, SNRI-induced depression of control ICSS is consistent with findings from other labs assessing the abuse liability of SNRIs. In primates, nisoxetine failed to maintain self-administration responding (Woolverton et al., 1987), and therefore, did not function as a reinforcer. Additionally, a drug discrimination study reported that DAT inhibitors did not substitute for nisoxetine, when nisoxetine was trained as the discriminative stimulus (Dekeyne et al., 2001), implying that the internal state induced by DA reuptake inhibitors was not similar to the internal state elicited by nisoxetine. However, nisoxetine has been shown to substitute for *d*-amphetamine in mice, pigeons and rhesus monkeys trained to discriminate *d*-amphetamine (Snoddy et al., 1983, Woolverton et al., 1984). The failure of DAT inhibitors to generalize to nisoxetine demonstrates that nisoxetine does not have a DA component. However, nisoxetine substituting for *d*-amphetamine suggests that *d*-amphetamine has a NE component. Overall, our study and others suggest that SNRIs may have little or no abuse potential.

The SDRIs, RTI-113 and bupropion, as well as the TRI, RTI-112 demonstrated a non-selective facilitation in ICSS responding, with or without the presence of a noxious stimulus. Existing evidence suggests that DA reuptake inhibitors and cocaine produce an abuse-related facilitation of basal ICSS (Kling-Petersen et al., 1994, Tomasiewicz et al., 2008). A strong positive correlation exists between

elevated levels of dopamine and enhanced central reward mechanisms of the mesolimbic dopamine system that originates in cells of the ventral tegmental area and project to the nucleus accumbens (Koob et al., 1992, Kornetsky et al., 1979). Therefore, our results are consistent with previous literature, and further support the high abuse liability of SDRIs.

The S+NRI milnacipran did not affect control ICSS in our study. In another study, venlafaxine administration failed to produce conditioned place preference in rats (Tzschentke et al., 2006), which is consistent with the lack of effect of milnacipran in control ICSS. Studies have also demonstrated that chronic, but not acute treatment with the tricyclic antidepressant desipramine increased rates of responding (Fibiger et al., 1981) and lowered thresholds for rewarding brain stimulation in rats (Valentino et al., 1991). Additionally, tricyclic antidepressants selective for NET/SERT, such as desipramine and imipramine produce minimal reinforcement in monkey self-administration studies (Gasior et al., 2005).

Effects of monoamine reuptake inhibitors in acid-depressed ICSS

These are the first studies to examine effects of monoamine reuptake inhibitors in assays of pain-depressed behavior. SSRIs, SNRIs and S+NRI failed to produce antinociception in the assay of acid-depressed ICSS at doses that did produce antinociception in the assay of acid-stimulated stretching. Rather, in both assays, these drugs produced a decrease in behavior. Moreover, these drugs also decreased control ICSS in the absence of a noxious stimulus. These findings suggest that acute administration of SSRIs, SNRIs and S+NRIs produce relatively non-selective depression of all behavior rather than a selective blockade of sensory sensitivity to noxious stimuli. Conversely, SDRIs and TRIs blocked both acid-stimulated stretching and acid-induced depression of ICSS, suggesting that blockade of dopamine reuptake may be able to block sensory detection of noxious stimuli. These findings are consistent with previous evidence that cocaine and amphetamine produce analgesia in animals and humans (Franklin et al., 1999, Yang et al., 1982). Part of the analgesic effect of cocaine can be attributed to blockade of sensory nerves, since cocaine can act as a local anesthetic (Bahar et al., 1984), which may also explain the analgesic effects of RTI-112 in our study. Reward system activation may offer an

alternative explanation for the present antinociceptive results with SDRIs and TRIs. The relationship between reward processing and pain relief is supported by prior research showing analgesic benefits from pharmacological manipulation of key reward systems (Altier et al., 1998, Taylor et al., 2003, Wood et al., 2006). In fact, a recent clinical study reported that when patients viewed pictures of romantic partners (causing activation of mesolimbic DA brain areas), they experienced reductions in acute pain (Younger et al., 2010). However, doses of SDRIs that blocked pain-related behaviors also produced an abuse-related facilitation of control ICSS in the absence of the noxious stimulus, suggesting that use of these SDRIs to treat pain would be complicated by abuse liability.

These results support clinical evidence for efficacy of DAT inhibitors to alleviate the pro-depressant effects of pain. In general, deployment of SDRIs as analgesics has been limited in part by high abuse liability. However, it may be possible to develop novel dopamine reuptake inhibitors that retain analgesic effects but have reduced abuse liability. For example, bicifadine is a TRI with slightly greater potency to block reuptake of 5-HT and NE than DA. This drug produced antinociception in many preclinical assays of pain-stimulated behavior (Basile et al., 2007), but in preclinical assays of abuse liability, it produced weaker abuse-related effects than some other TRIs (e.g. cocaine) with more prominent DA reuptake effects (Nicholson et al., 2009). This is consistent with other literature suggesting that blockade of serotonergic reuptake can oppose and limit abuse-related effects of DA reuptake inhibitors and releasers (Howell et al., 2007, Czoty et al., 2002). Future studies will be required to assess the degree to which proportion of DA vs. other monoamine effects might influence efficacy to produce antinociception in assays of pain-depressed behavior vs. abuse-related effects.

The present results agree with the low clinical utility of 5-HT/NE compounds for treatment of acute pain. These compounds may be more useful for treatment of depressant effects of chronic pain, and results from this study will provide a basis for design and interpretation of future studies on chronic pain. From a drug development viewpoint, the best pharmacotherapeutic profile for a single effective monoamine reuptake inhibitor in the treatment of acute pain is an SDRI (or TRI) that retains analgesic effects but is associated with a reduced abuse liability. Bicifadine seems to fit this profile well. For

example, bicifadine was associated with weaker stimulus cues, lower rates of motor activation, and lower rates of self-administration compared to well-known psychostimulants, cocaine and *d*-amphetamine, and the NET/DAT inhibitor, bupropion in monkeys (Nicholson et al., 2009). This study also demonstrated through rat microdialysis that bicifadine-induced elevations in accumbens DA levels were consistently lower than either bupropion or *d*-amphetamine. This low efficacy of bicifadine in elevating accumbens DA suggests its weak reinforcing properties are due to the low potency of bicifadine as a [^{3}H]DA uptake inhibitor relative to its ability to inhibit [^{3}H]NE and [^{3}H]5-HT uptake (Basile et al., 2007). Moreover, coadministration of drugs that increase 5-HT levels decrease the reinforcing efficacy of cocaine and other psychomotor stimulants (Wee et al., 2005, Howell et al., 2007), possibly because of 5-HT-induced decreases in DA release in the nucleus accumbens and striatum (Czoty et al., 2002, Fink et al., 2007). Thus, future analgesic drug development should target other novel triple reuptake inhibitors that have greater potency in blocking 5-HT uptake than DA uptake to minimize abuse potential in pain patients, yet retain analgesic effects.

Future directions

In future studies, we propose to study drug effects in preclinical assays of chronic, pain-related depression of behavior. We are not sure yet if we can produce chronic behavioral depression with conventional assays of chronic inflammatory pain (e.g. models of arthritis) or chronic neuropathic pain (e.g. nerve injury models like SNL or CCI). These assays are under development in our lab. We also propose to re-evaluate the effects of the SDRI, RTI-113, in a pain-depressed feeding assay, in an attempt to tease apart true antinociceptive effects from a non-selective facilitation in behavior. We predict that under control conditions, RTI-113 will not affect feeding in rats. However, under lactic acid conditions, RTI-113 will produce a selective restoration of pain-depressed feeding only. Finally, we plan to evaluate bicifadine in addition to RTI-112, in acid-stimulated stretching and acid-depressed ICSS because its potencies at SERT, NET and DAT are more reflective of a true triple reuptake inhibitor.

Table 1 Drug/ Class	Selectivity (Rat brain tissue)
Selective serotonin reuptake inhibitors (SSRIs)	
Citalopram	SERT>> NET ≥ DAT[c]
Fluvoxamine	SERT>> NET > DAT[a]
Sertraline	SERT>> DAT ≥ NET[a]
Fluoxetine	SERT> NET ≥ DAT[c]
Norepinephrine Reuptake Inhibitors (SNRIs)	
Nisoxetine	NET >> SERT ≥ DAT[e]
Reboxetine	NET> SERT > DAT[d]
Dopamine Reuptake Inhibitors (SDRIs)	
RTI-113	DAT > NET ≥ SERT[f]
Bupropion	DAT ≥ NET ≥ SERT[a]
Serotonin-Norepinephrine Reuptake Inhibitors (S+NRIs)	
Milnacipran	SERT ≥ NET>> DAT[h]
Venlafaxine	SERT ≥ NET ≥ DAT[a]
Duloxetine	SERT ≥ NET[i]
Triple Reuptake Inhibitors (TRIs)	
RTI-112	NET≥ DAT ≥ SERT[f]
Bicifadine	NET ≥ SERT ≥ DAT[j*]
Cocaine	DAT≥ SERT > NET[g]
Tricyclic Antidepressants (TCAs)	
Clomipramine	SERT> NET> DAT[d]
Imipramine	SERT≥ NET >> DAT[b]
Amitriptyline	NET≥ SERT>> DAT[a]
Desipramine	NET> SERT> DAT[c]
Nortriptyline	NET> SERT >> DAT[b]

a (Sanchez C et al., 1999) b (Owens MJ et al., 1997) c (Rothman RB et al., 2001) d (Millan MJ et al., 2001) e (Davids E et al., 2002)
f (Kuhar et al., 1999) g (Carrol FI et al., 1995) h (Mochizuki et al., 2002) I (Beique JC et al., 1998) j (Basile et al., 2007)
* Inhibition assay using recombinant human transporter
≥ potency difference is 0-10 fold
> potency difference is 10-100 fold
>> potency difference is more than 100-fold

Table 2	Acute thermal		Acute chemical		Inflammatory		Neuropathic	
	Hot plate	Tail Flick	Acid/PPQ stretching	Formalin (2nd phase lick/flinch)	Carrageenan (mechanical allodynia)	CFA	SNL (mechanical allodynia)	CCI
SSRIs								
- Citalopram	+[t]	-[a]	+[p]	+[a]				-[a]
- Fluvoxamine			+[j]	+[d]				-[f]
- Sertraline	-[i]			+[i]	+[h]			
- Fluoxetine	-[b]	-[b]	+[k]		-[h]	+[m]	+[r]	-[f]
SNRIs								
- Nisoxetine	+[b]	+[b]		+[u]				
- Reboxetine	+[q]	+[s]	+[r]	+[s]			+[r]	-[s]
SDRIs								
- RTI-113								
- Bupropion				-[s]				+[s]
S+NRIs								
- Milnacipran			+[j]	+[d]		+[o]	+[d]	
- Venlafaxine		+[s]		+[s]	+[h]		+[e]	-[s]
- Duloxetine	+[a]	+[e]		+[a]	+[h]	+[m]	+[e]	
TRIs								
- RTI-112								
- Bicifadine		+[l]	+[l]	+[l]		+[l]	+[l]	
- Cocaine	+[v]			+[v]				
TCAs								
- Clomipramine	+[c]	-[c]	+[c]	+[c]	-[p]			
- Imipramine	+[c]	-[c]	+[c]	+[c]				+[f]
- Amitriptyline	+[c]	+[c]	+[c]	+[a]		-[n]	+[g]	+[f]
- Desipramine	+[i]	+[c]	+[c]	+[c]	+[h]		+[r]	
- Nortriptyline	+[c]	-[c]	+[c]	+[c]				

+ Effective in producing antinociceptive effect in assay

- Ineffective in producing antinociceptive effect in assay

a (Bomholt et al., 2005) b (Hall et al., 2011) c (Rojas-Corrales et al., 2003) d (Mochizucki et al., 2004) e (Iyengar et al., 2004) f (Garcia et al., 2010) g (Sung et al., 2004) h (Jones et al., 2006) i (Otsuka et al., 2001) j (Aoki et al., 2006) k (Singh et al., 2001) l (Basile et al., 2007) m (Boyce-Rustay et al., 2010) n (Matson et al., 2007) o (Mico et al., 2011) p (Ardid et al., 1992) q (Schreiber et al., 2009) r (Leventhal et al., 2007) s (Pedersen et al., 2005) t (Fasmer et al., 1989) u (Yokogawa et al., 2002) v (Lin et al., 1989)

Table 3. Lowest dose of each compound to produce a significant change in acid-stimulated stretching, control ICSS in the absence of the acid noxious stimulus, or acid-depressed ICSS. The valence of effect is also shown, with downward arrows to indicate a decrease in stretching or ICSS (i) or upward arrows to indicate an increase in ICSS (h).

	Acid-Stimulated Stretching	**Control ICSS**	**Acid-Depressed ICSS**
Citalopram	32 ↓	10 ↓	10 ↓
Clomipramine	10 ↓	3.2 ↓	32 ↓
Nisoxetine	0.32 ↓	1.0 ↓	3.2 ↓
Nortriptyline	3.2 ↓	1.0 ↓	1.0 ↓
RTI-113	3.2 ↓	3.2 ↑	1.0 ↑
Bupropion	10 ↓	10 ↑	10 ↑
Milnacipran	1.0 ↓	>3.2	>3.2
RTI-112	0.032 ↓	0.1 ↑	0.32 ↑

Table 4. Mean±SEM maximum control response rates (MCR) and total stimulations per component for rats used to test each monoamine reuptake inhibitor.

	Group Average Maximum Control Rate (MCR)	**Group Average Total Stimulations Per Component**
Citalopram	49.63± 5.69	161.40±24.38
Clomipramine	53.43±4.61	138.35±39.03
Nisoxetine	54.58±2.92	158.58±26.28
Nortriptyline	48.84±3.89	157.48±25.86
RTI-113	56.10±4.25	271.72±62.74
Bupropion	54.23±4.18	212.62±30.95
Milnacipran	61.03±4.99	224.39±36.44
RTI-112	55.97±4.32	320.99±23.36

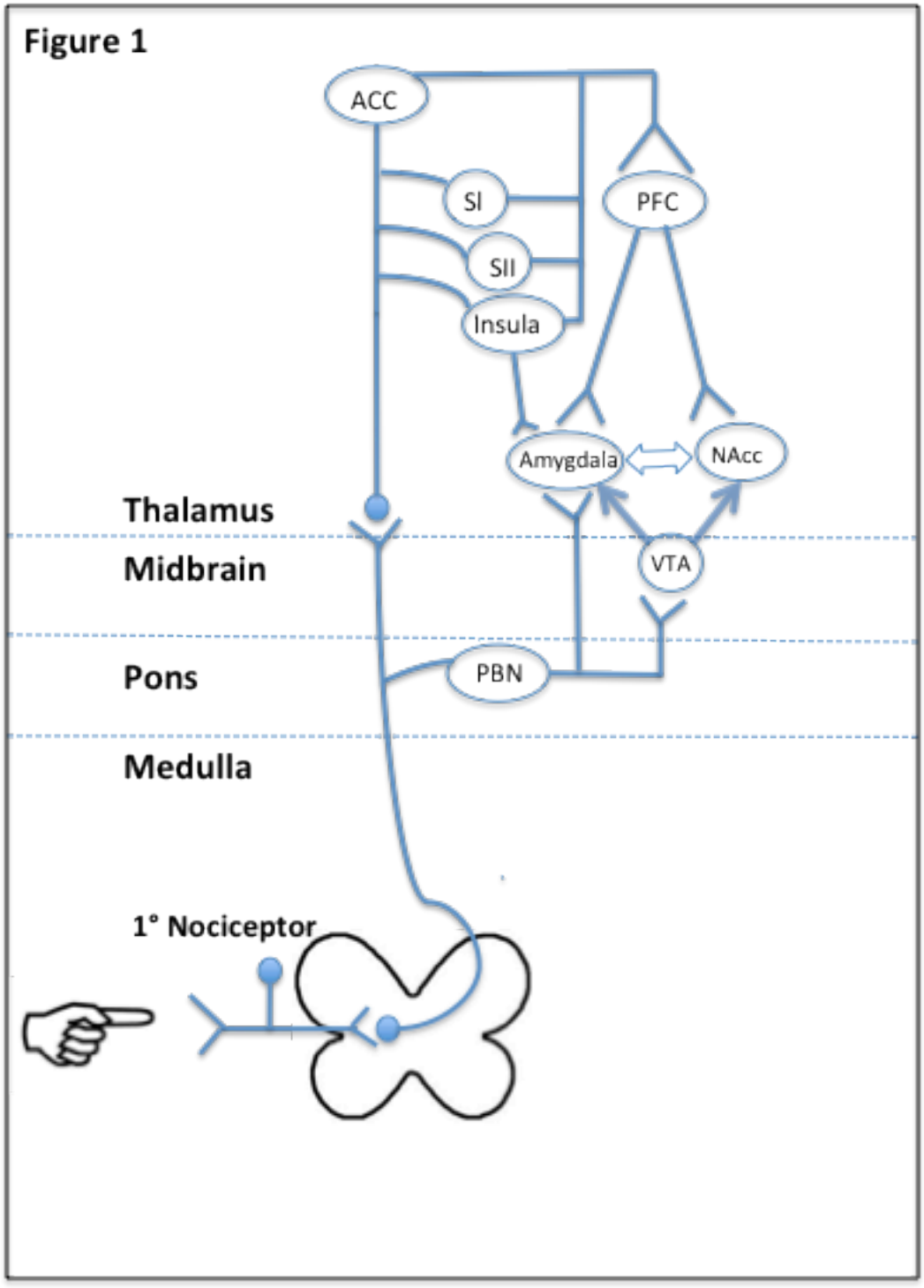

Figure 2A

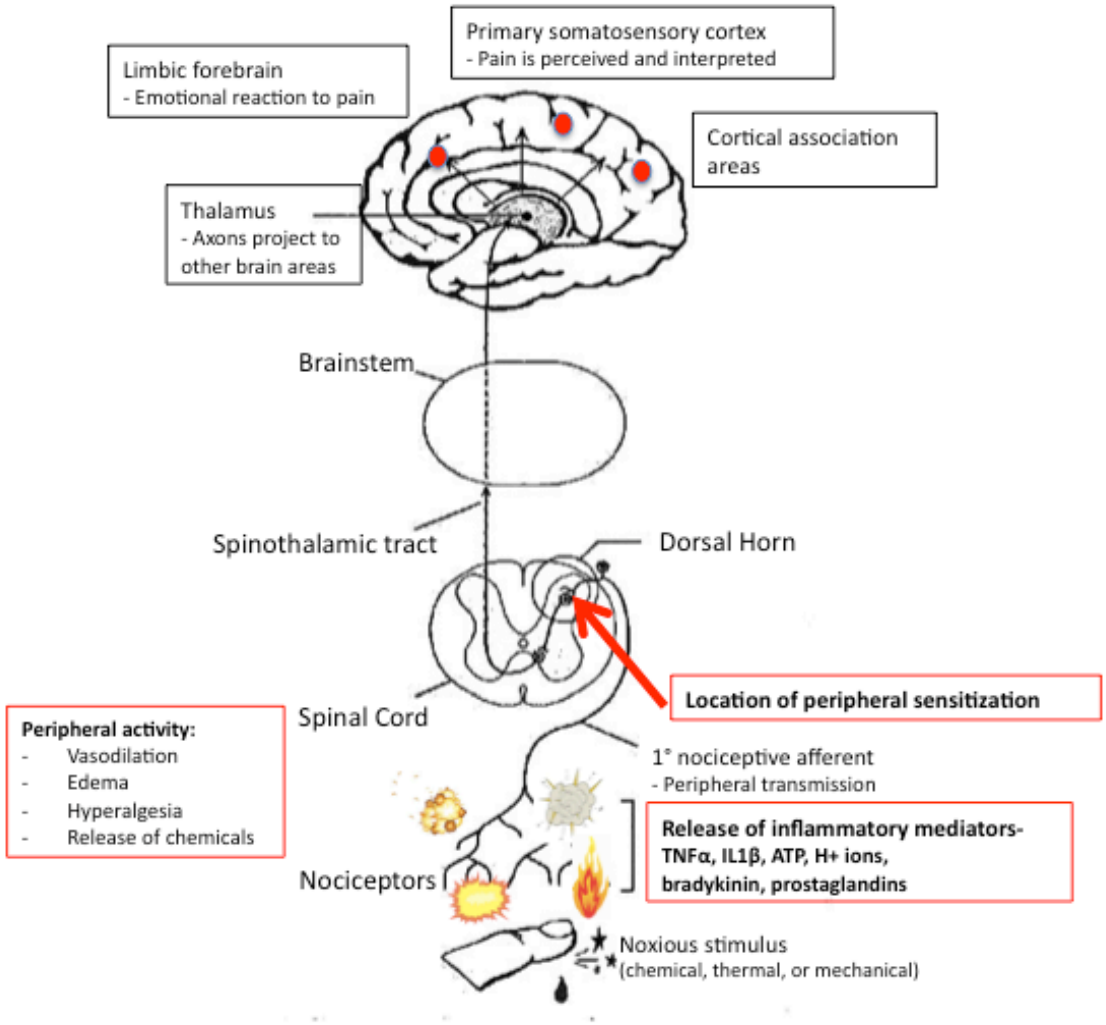

Figure 2 Mechanisms of inflammatory pain

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Figure 2B

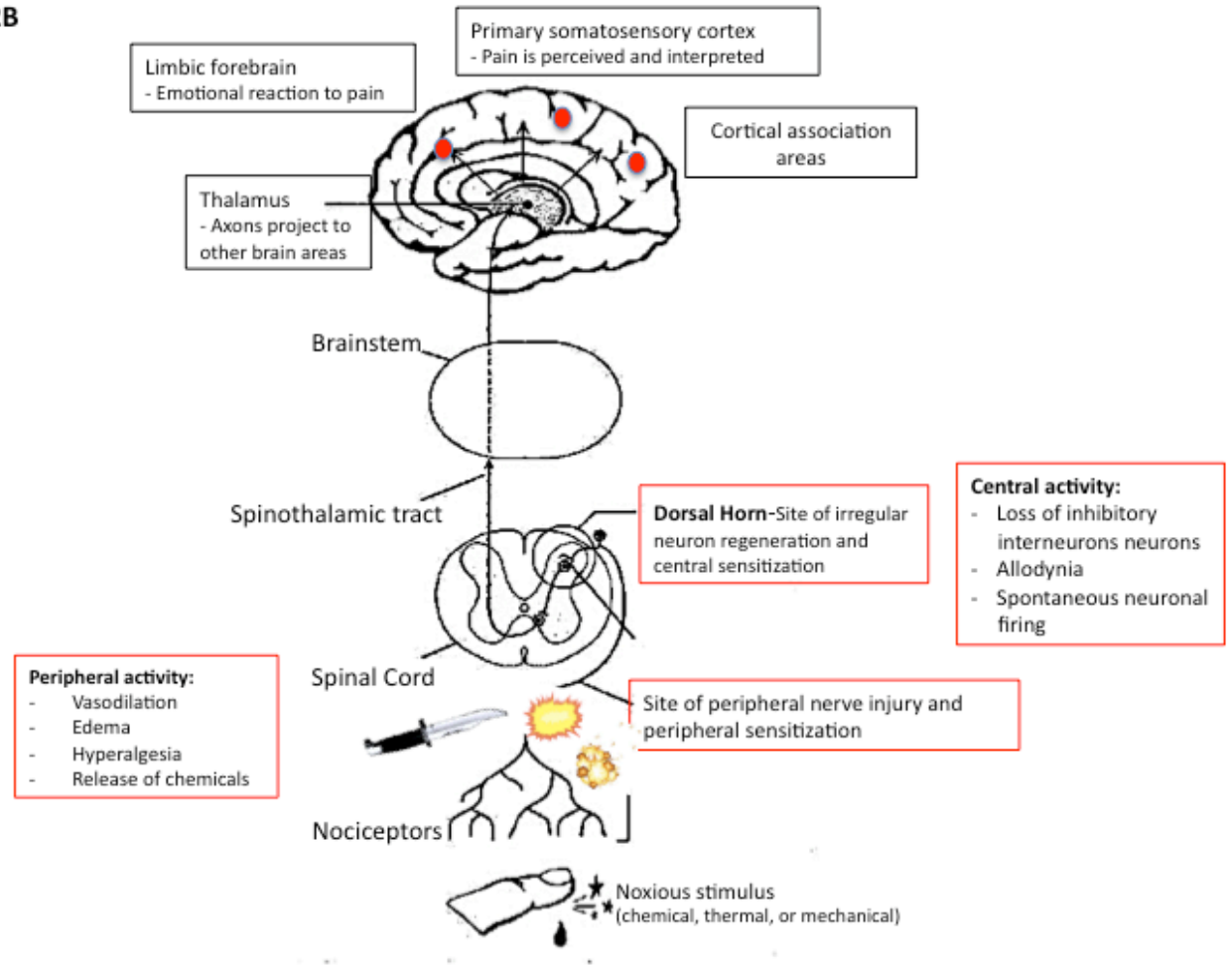

Figure 2 Mechanisms of neuropathic pain

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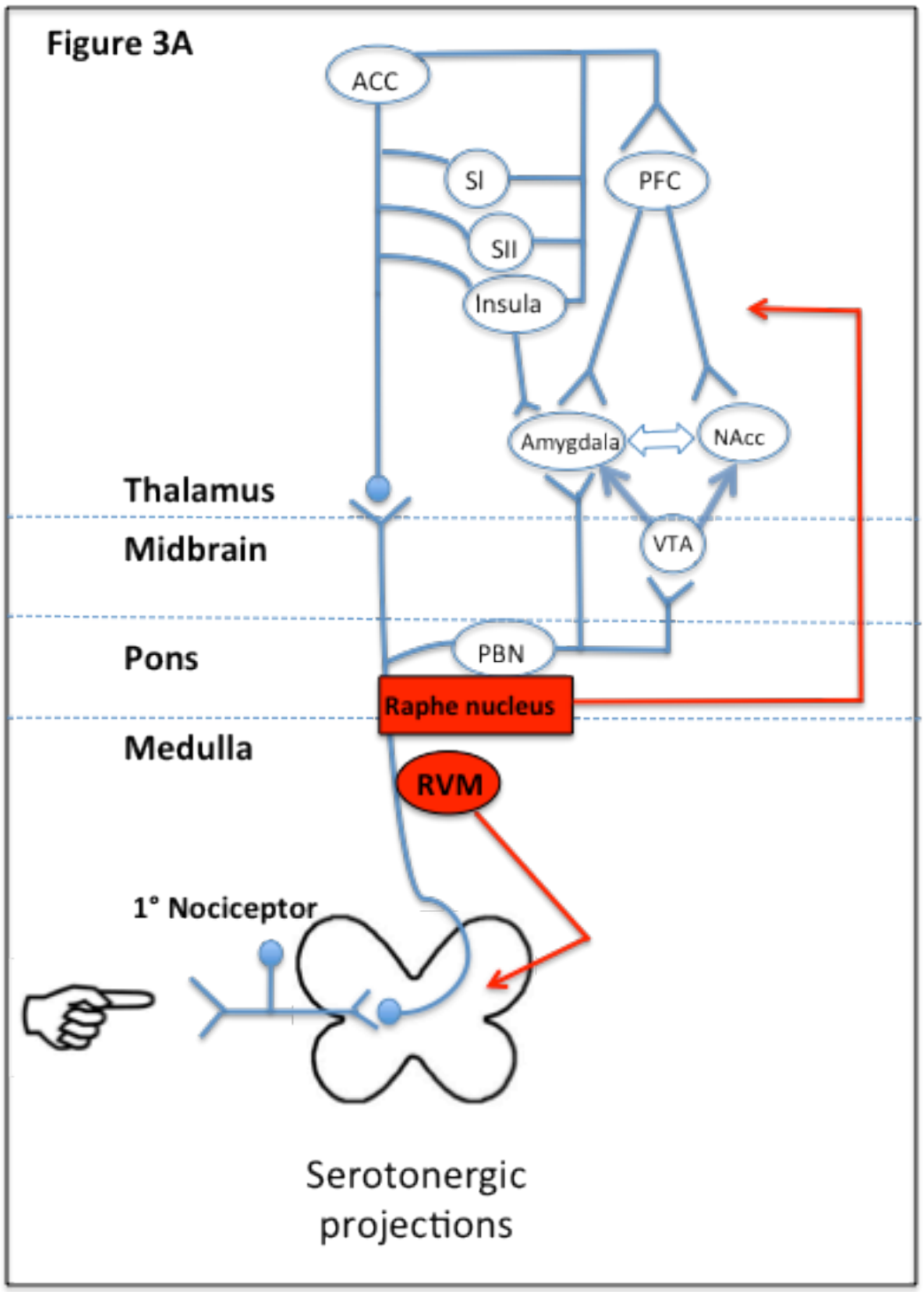

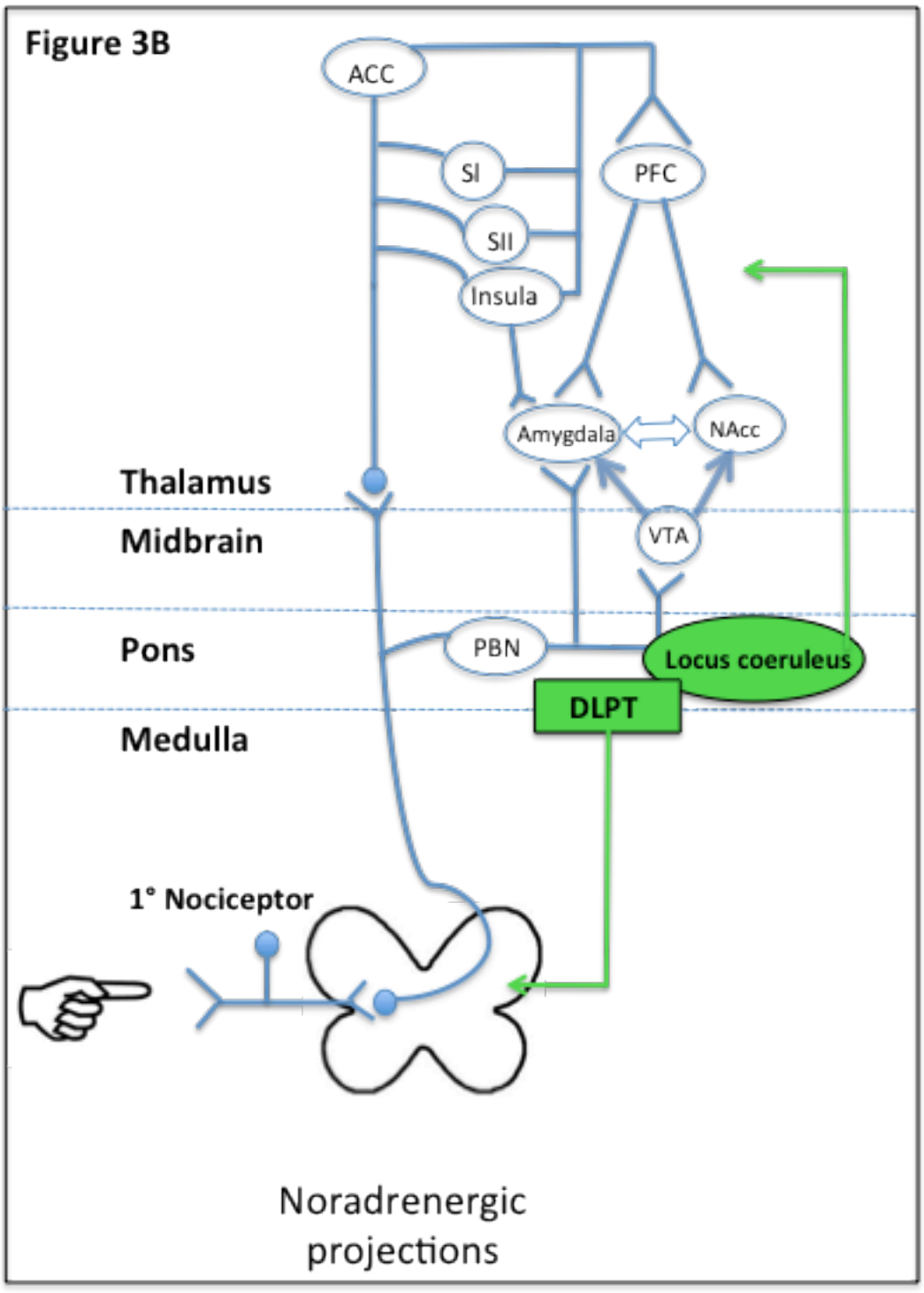

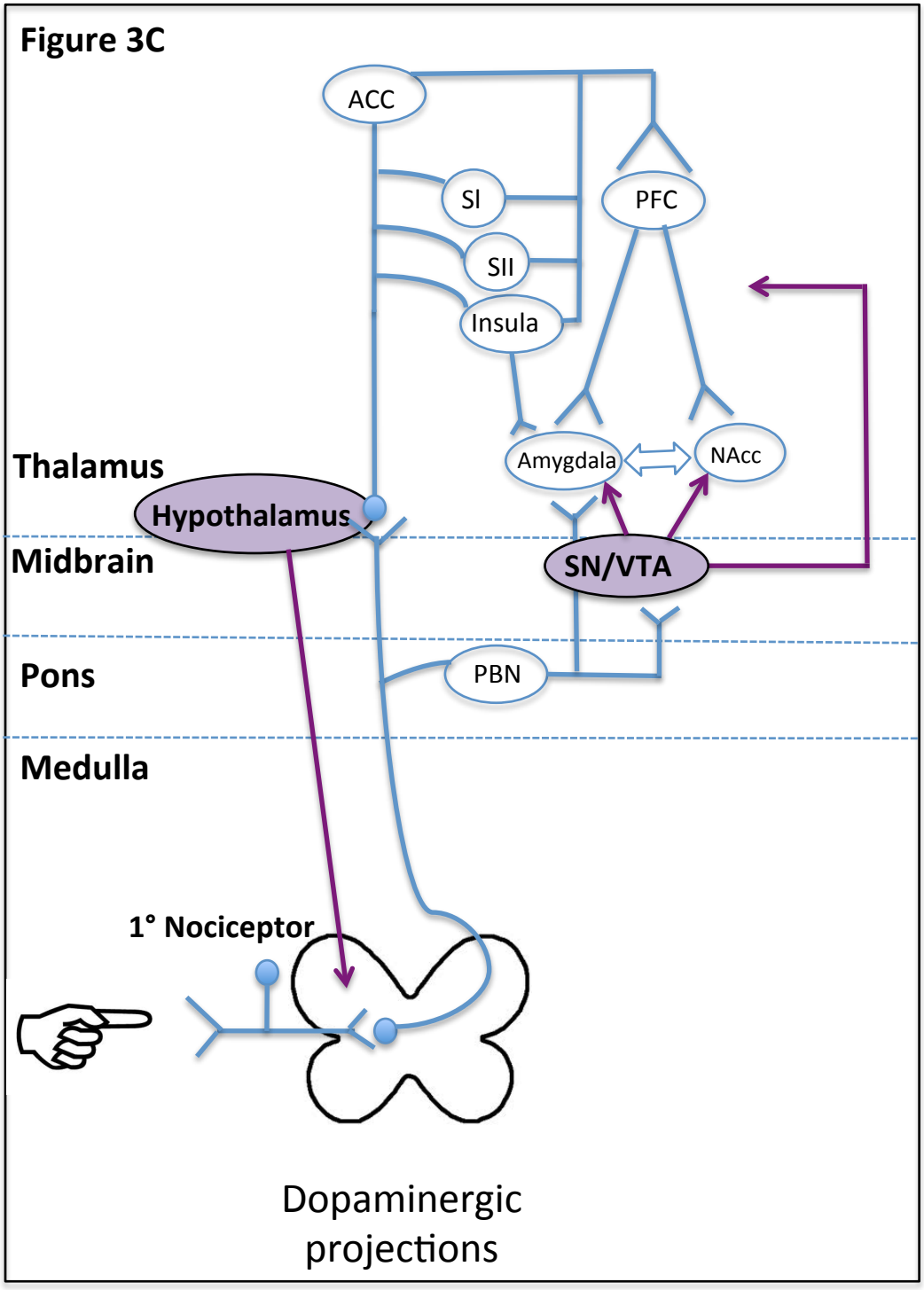

Figure 4

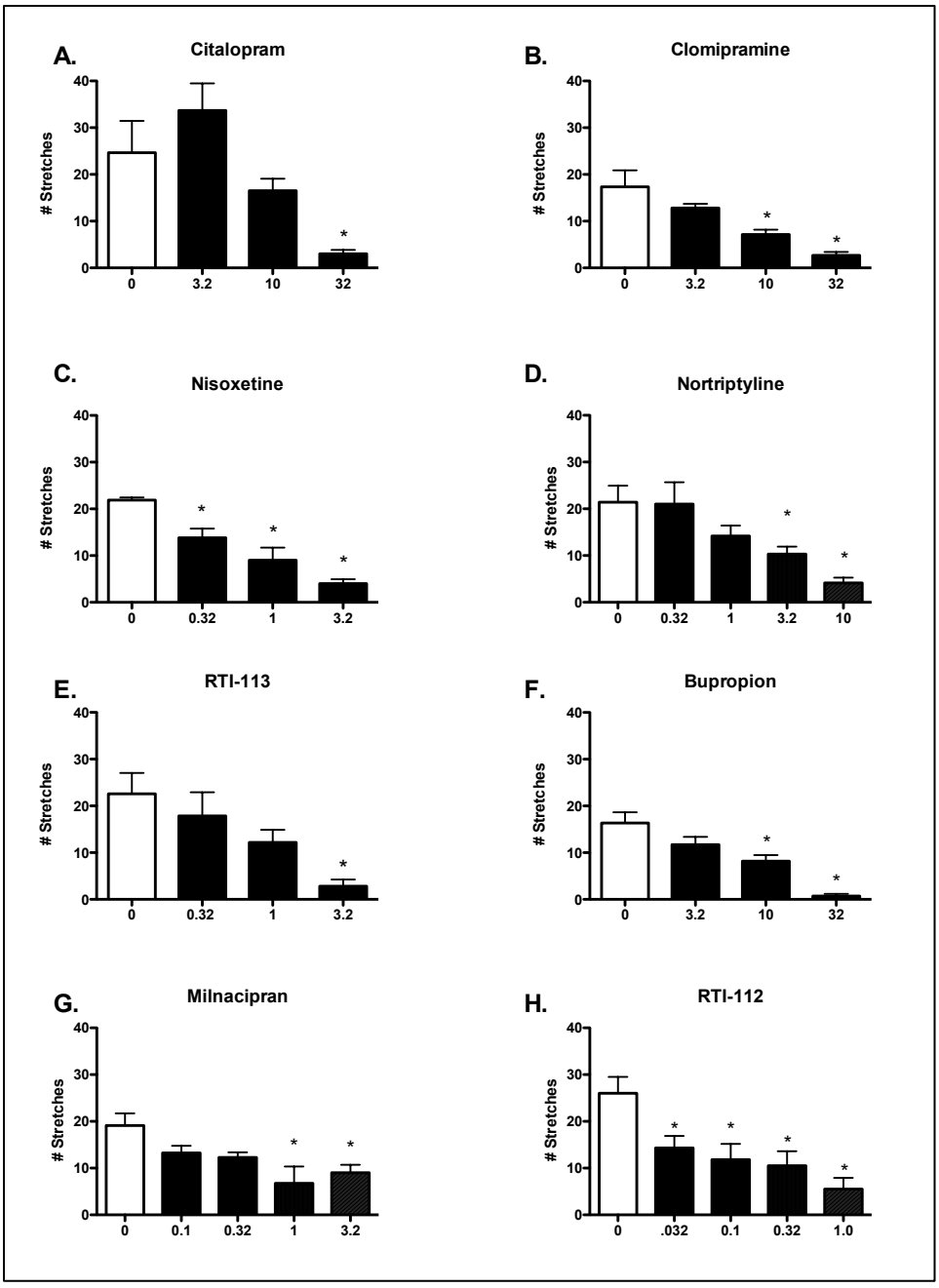

Figure 4. Effects of monoamine reuptake inhibitors in the assay of acid-stimulated stretching. Abscissae: Dose in mg/kg. Ordinates: Number of acid-stimulated stretches. All bars show mean data from 5-6 rats. Error bars show SEM data. Asterisks (*) indicate a significant difference from vehicle treatment (Dunnett's post hoc test; $p<0.05$). All monoamine uptake inhibitors decreased acid-stimulated stretching. ANOVA results are as follows. (A) Citalopram [$F(3,15)= 9.973$, $p=0.0007$], (B) Clomipramine [$F(3,15)= 10.60$, $p=0.0005$], (C) Nisoxetine [$F(3,12)= 28.50$, $p<0.0001$], (D) Nortriptyline [$F(4,20)= 6.301$, $p=0.0019$], (E) RTI-113 [$F(3,15)= 7.938$, $p=0.0021$], (F) Bupropion [$F(3,15)= 13.93$, $p=0.0001$], (G) Milnacipran [$F(4,12)= 4.071$, $p=0.0260$], (H) RTI-112 [$F(4,20)= 7.589$, $p=0.0007$].

Figure 5

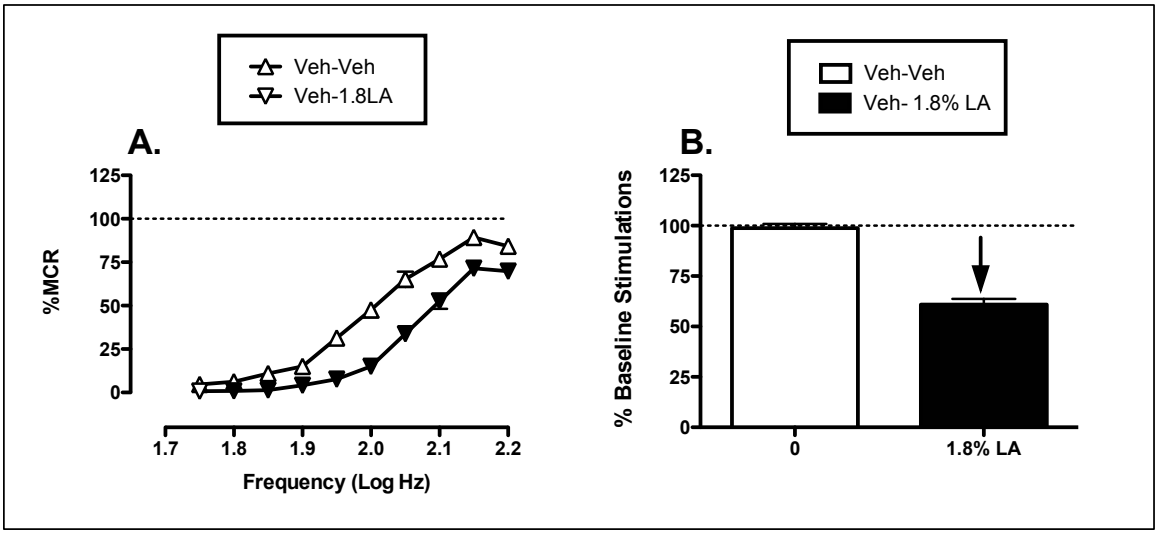

Figure 5. Acid-induced depression of ICSS. Left panel (A) compares effects of pretreatment with Vehicle + Vehicle and Vehicle + 1.8% lactic acid on full frequency-rate curves. Abscissa: Frequency of electrical brain stimulation in log Hz. Ordinate: Percent maximum control response rate (%MCR). Filled symbols indicate a significant difference from Veh-Veh (Holm-Sidak post hoc test, $p<0.05$). Right panel (B) shows summary data for lactic acid effects on the total number of stimulations per component Abscissa: Concentration of lactic acid. Ordinate: Percent baseline number of stimulations per component. The downward arrow indicates that lactic acid produced a significant decrease in ICSS at one or more frequencies in the full frequency-rate curve. Statistical results for two-way ANOVA of full frequency-rate curves are as follows: (A) Significant main effect of frequency [$F(9,414)= 238.257$, $p<0.001$] and treatment [$F(1,46)= 224.646$, $p<0.001$]; the interaction was also significant [$F(9,414)=16.634$, $p<0.001$].

Figure 6

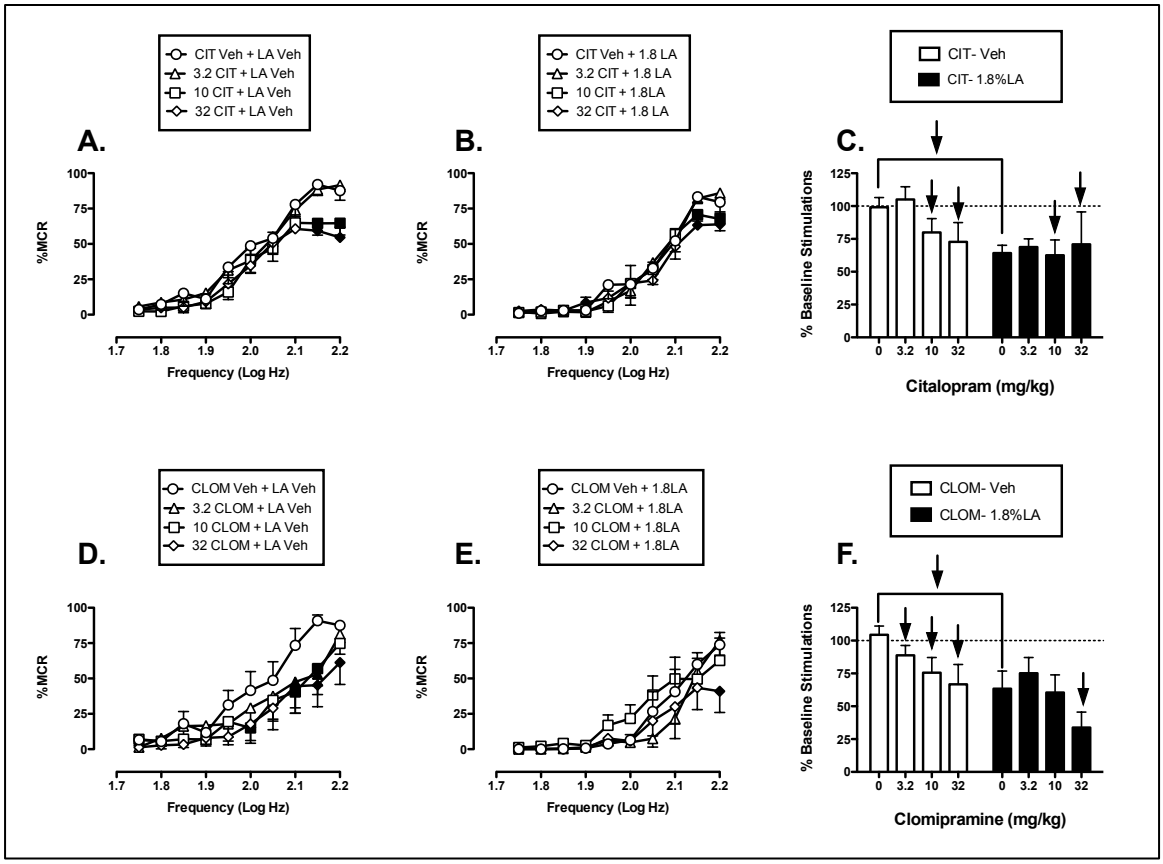

Figure 6. Effects of citalopram (A-C) and clomipramine (D-F) on control and acid-depressed ICSS. Left and center panels show drug effects on full frequency-rate curves when drugs were administered as a pretreatment to vehicle (Left panels A, D) or 1.8% lactic acid (center panels B, E). Abscissae: Frequency of electrical brain stimulation in log Hz. Ordinates: Percent maximum control response rate (%MCR). Filled symbols indicate a significant difference from Veh-Veh (A, D) or Veh-LA (B, E) (Holm-Sidak post hoc test, $p<0.05$). Right panels (C,F) show summary data for drug effects on the total number of stimulations per component when drugs were administered as a pretreatment to vehicle (open bars) or acid (filled bars). Abscissae: Dose of drug in mg/kg. Ordinate: Percent baseline number of stimulations per component. Upward/downward arrows indicate that the drug dose produced a significant increase/decrease in ICSS at one or more frequencies in the full frequency-rate curve. Statistical results for two-way ANOVA of full frequency-rate curves are as follows: (A) Significant main effect of frequency [$F(9,36)=60.71$, $p<0.001$] and dose [$F(3,12)=3.49$, $p=0.050$], but the interaction was not significant [$F(27,108)=1.57$, $p=0.054$]. (B) Significant main effect of frequency [$F(9,36)=40.84$, $p<0.001$] but not dose [$F(3,12)=0.7$, $p=0.573$]; the interaction was significant [$F(27,108)=1.64$, $p=0.040$]. (D) Significant main effect of frequency [$F(9,45)=17.94$, $p<.001$] and dose [$F(3,15)=4.33$, $p=0.022$], but the interaction was not significant [$F(27,135)=1.51$, $p=0.065$]. (E) Significant main effect of frequency [$F(9,45)=17.23$, $p<0.001$] but not of dose [$F(3,15)=1.96$, $p=0.164$]; the interaction was significant [$F(27,135)=2.04$, $p=0.004$].

Figure 7

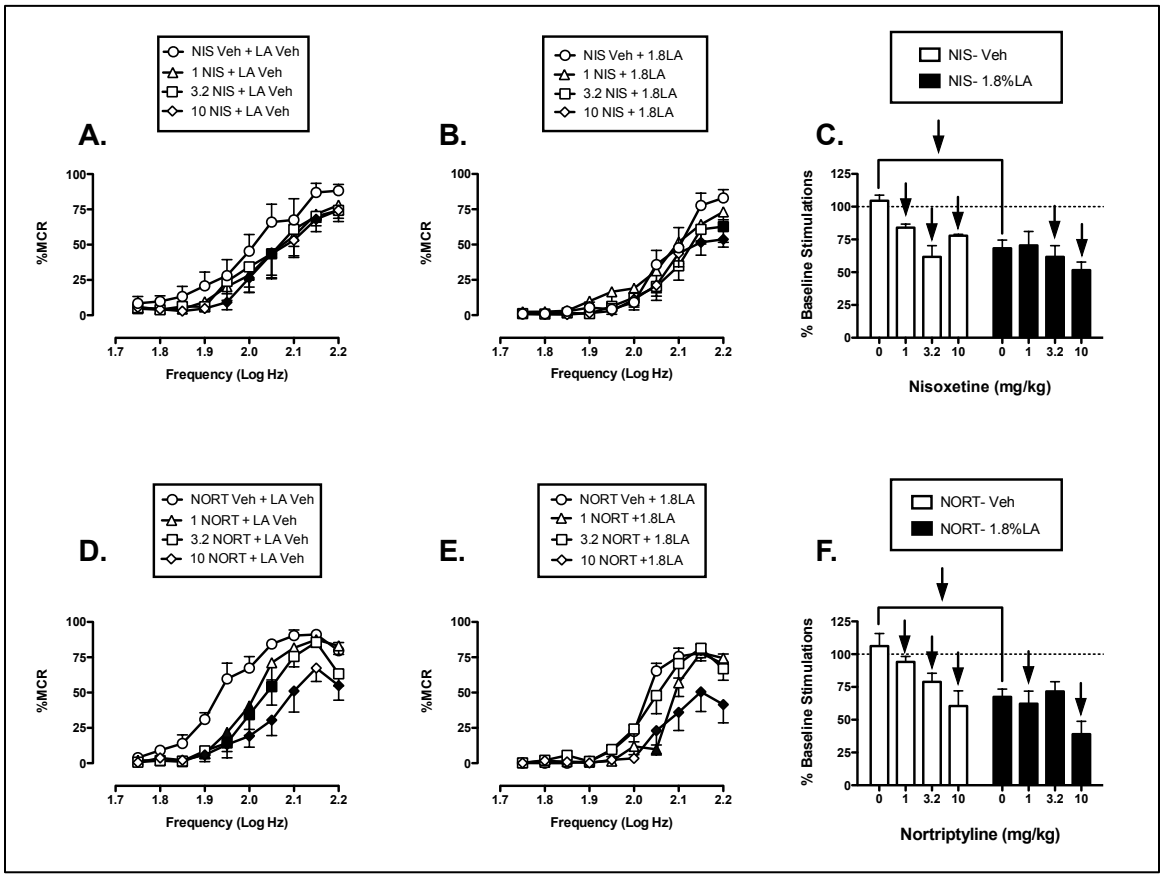

Figure 7. Effects of nisoxetine (A-C) and nortriptyline (D-F) on control and acid-depressed ICSS. Left and center panels show drug effects on full frequency-rate curves when drugs were administered as a pretreatment to vehicle (Left panels A, D) or 1.8% lactic acid (center panels B, E). Abscissae: Frequency of electrical brain stimulation in log Hz. Ordinates: Percent maximum control response rate (%MCR). Filled symbols indicate a significant difference from Veh-Veh (A, D) or Veh-LA (B, E) (Holm-Sidak post hoc test, $p<0.05$). Right panels (C,F) show summary data for drug effects on the total number of stimulations per component when drugs were administered as a pretreatment to vehicle (open bars) or acid (filled bars). Abscissae: Dose of drug in mg/kg. Ordinate: Percent baseline number of stimulations per component. Upward/downward arrows indicate that the drug dose produced a significant increase/decrease in ICSS at one or more frequencies in the full frequency-rate curve. Statistical results for two-way ANOVA of full frequency-rate curves are as follows: (A) Significant main effect of frequency [$F(9,45)=21.3$, $p<0.001$] and dose [$F(3,15)=10.18$, $p<0.001$]; the interaction was not significant [$F(27,135)=0.68$, $p=0.875$]. (B) Significant main effects of frequency [$F(9,45)=26.94$, $p<0.001$] but not dose [$F(3,15)=2.25$, $p=0.124$]; the interaction was significant [$F(27,135)=2.07$, $p=0.003$. (D) Significant main effect of frequency [$F(9,45)=51.82$, $p<0.001$] and dose [$F(3,15)=9.58$, $p<0.001$]; the interaction was significant [$F(27,135)=3.27$, $p<0.001$]. (E) Significant main effect of frequency [$F(9,45)=87.57$,$p<0.001$] and dose [$F(3,15)=5.79$, $p=0.008$]; the interaction was significant [$F(27,135)=4.48$, $p<0.001$].

Figure 8

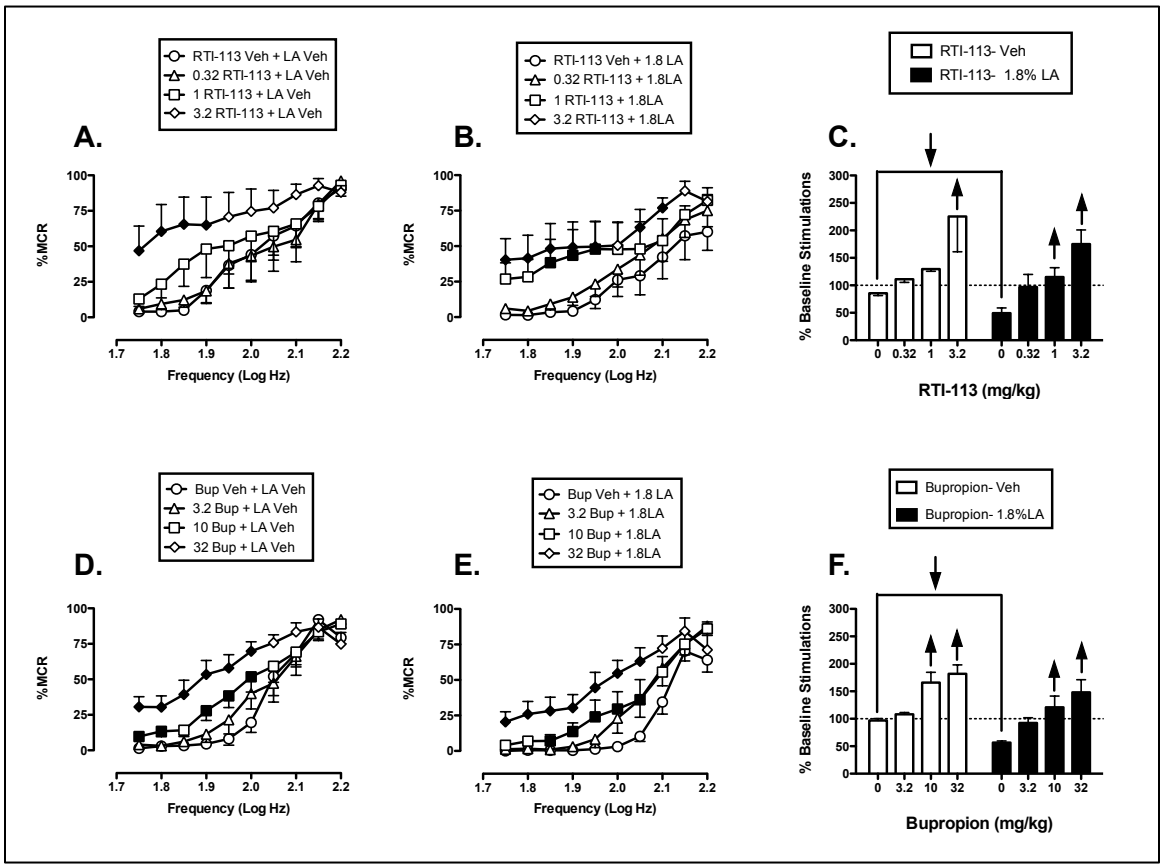

Figure 8. Effects of RTI-113 (A-C) and bupropion (D-F) on control and acid-depressed ICSS. Left and center panels show drug effects on full frequency-rate curves when drugs were administered as a pretreatment to vehicle (Left panels A, D) or 1.8% lactic acid (center panels B, E). Abscissae: Frequency of electrical brain stimulation in log Hz. Ordinates: Percent maximum control response rate (%MCR). Filled symbols indicate a significant difference from Veh-Veh (A, D) or Veh-LA (B, E) (Holm-Sidak post hoc test, $p<0.05$). Right panels (C,F) show summary data for drug effects on the total number of stimulations per component when drugs were administered as a pretreatment to vehicle (open bars) or acid (filled bars). Abscissae: Dose of drug in mg/kg. Ordinate: Percent baseline number of stimulations per component. Upward/downward arrows indicate that the drug dose produced a significant increase/decrease in ICSS at one or more frequencies in the full frequency-rate curve. Statistical results for two-way ANOVA of full frequency-rate curves are as follows: (A) Significant main effect of frequency [$F(9,45)=14.35$, $p<0.001$] and dose [$F(3,15)=5.27$, $p=0.011$]; the interaction was significant [$F(27,135)=2.27$, $p=0.001$]. (B) Significant main effects of frequency [$F(9,45)=16.99$, $p<0.001$] and dose [$F(3,15)=10.42$, $p<0.001$]; the interaction was not significant [$F(27,135)=0.69$, $p=0.0871$]. (D) Significant main effect of frequency [$F(9,63)=47.70$, $p<0.001$] and dose [$F(3,21)=23.04$, $p<0.001$]; the interaction was significant [$F(27,189)=4.9$, $p<0.001$]. (E) Significant main effect of frequency [$F(9,63)=54.97$, $p<0.001$] and dose [$F(3,21)=11.12$, $p<0.001$]; the interaction was significant [$F(27,189)=2.63$, $p<0.001$].

Figure 9

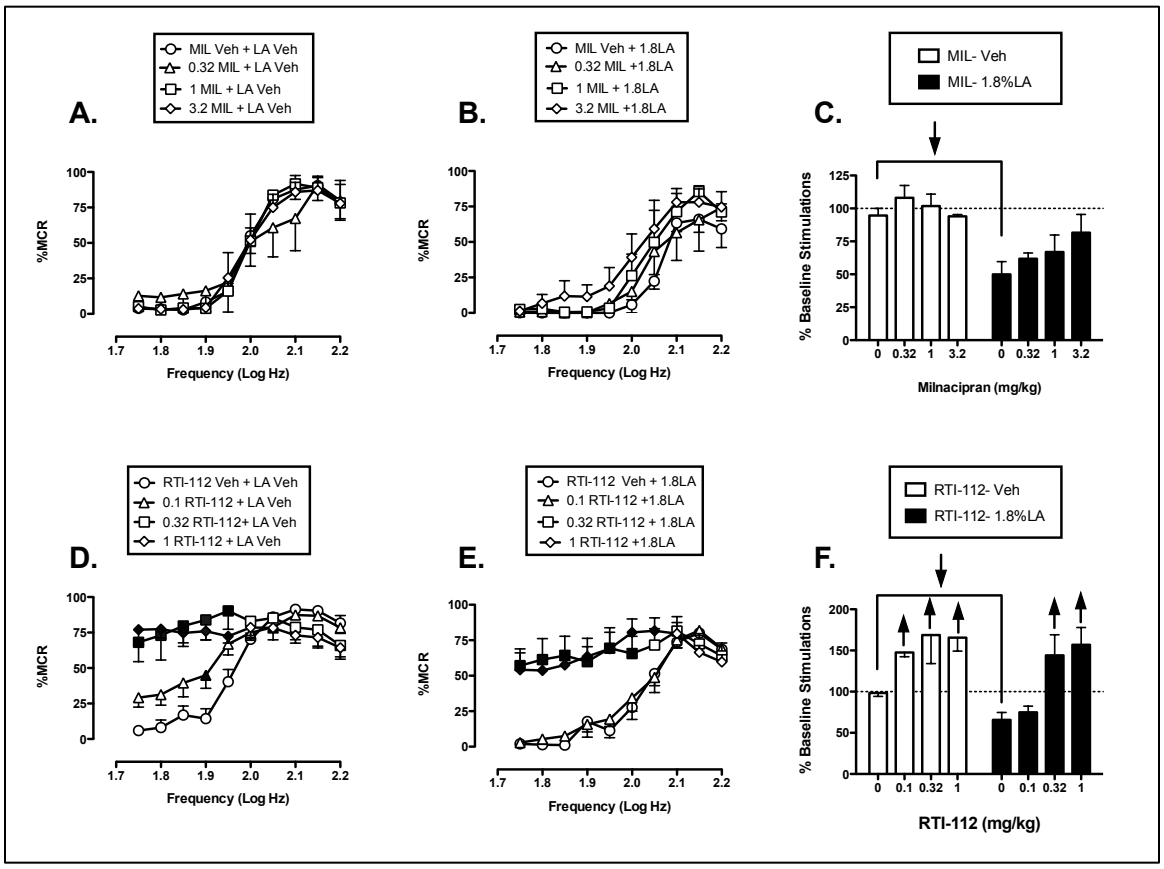

Figure 9. Effects of milnacipran (A-C) and RTI-112 (D-F) on control and acid-depressed ICSS. Left and center panels show drug effects on full frequency-rate curves when drugs were administered as a pretreatment to vehicle (Left panels A, D) or 1.8% lactic acid (center panels B, E). Abscissae: Frequency of electrical brain stimulation in log Hz. Ordinates: Percent maximum control response rate (%MCR). Filled symbols indicate a significant difference from Veh-Veh (A, D) or Veh-LA (B, E) (Holm-Sidak post hoc test, $p<0.05$). Right panels (C,F) show summary data for drug effects on the total number of stimulations per component when drugs were administered as a pretreatment to vehicle (open bars) or acid (filled bars). Abscissae: Dose of drug in mg/kg. Ordinate: Percent baseline number of stimulations per component. Upward/downward arrows indicate that the drug dose produced a significant increase/decrease in ICSS at one or more frequencies in the full frequency-rate curve. Statistical results for two-way ANOVA of full frequency-rate curves are as follows: (A) Significant main effect of frequency [$F(9,27)=32.231$, $p<0.001$], but not of dose [$F(3,9)=0.00693$, $p=0.999$]; the interaction was not significant [$F(27,81)=0.693$, $p=0.858$]. (B) Significant main effect of frequency [$F(9,27)=29.911$, $p<0.001$] but not of dose [$F(3,9)=1.436$, $p=0.296$]; the interaction was not significant [$F(27,81)=0.829$, $p=0.702$]. (D) Significant main effect of frequency [$F(9,45)=27.022$, $p<0.001$] and dose [$F(3,15)=5.403$, $p=0.010$]; the interaction was significant [$F(27,135)=11.075$, $p<0.001$]. (E) Significant main effect of frequency [$F(9,45)=36.328$, $p<0.001$] and dose [$F(3,15)=12.033$, $p<0.001$]; the interaction was significant [$F(27,135)=7.585$, $p<0.001$].

Figure 10

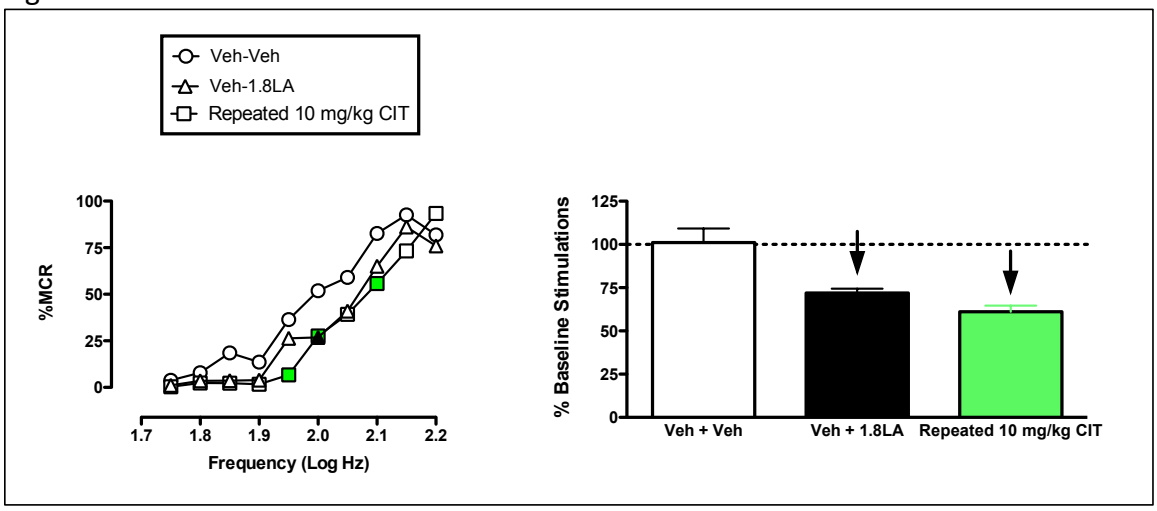

Figure 10. Effects of repeated administration of citalopram. Left panel (A) compares effects of pretreatment with Veh + Veh, Veh+ LA and repeated CIT + LA on full frequency-rate curves. Abscissa: Frequency of electrical brain stimulation in log Hz. Ordinate: Percent maximum control response rate (%MCR). Filled black symbol indicates a significant difference from Veh-Veh (Holm-Sidak post hoc test, $p<0.05$). Right panel (B) shows summary data for the same treatments on the total number of stimulations per component. Abscissa: Treatment. Ordinate: Percent baseline number of stimulations per component. Downward arrows indicate a significant decrease in ICSS at one or more frequencies in the full frequency-rate curve relative to Veh+Veh.

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