Differential and Synergistic Effects of Selective Norepinephrine and Serotonin
Reuptake Inhibitors in Rodent Models of Pain

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List of Nonstandard Abbreviations:

norepinephrine reuptake inhibitor = NRI

norepinephrine transporter = NET

para-phenylquinone = PPQ

serotonin reuptake inhibitor =SRI

serotonin transporter = SERT

spinal nerve ligation = SNL

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Abstract

There is increasing recognition that norepinephrine (NE) and serotonin (5-HT) reuptake inhibitors (NRIs and SRIs) are efficacious in treating some types of pain. To date studies have not systematically evaluated the relative activity at the NE and/or 5-HT transporter required for maximal efficacy in rodent pain models. Known selective NE and 5-HT reuptake inhibitors: reboxetine, desipramine, fluoxetine and paroxetine were evaluated in both *in vitro* and *in vivo* assays. Utilizing the spinal nerve ligation model of neuropathic pain, the compounds differentially reversed tactile allodynia. Evaluation of a broader spectrum of reuptake inhibitors in the *para*-phenylquinone (PPQ)-induced abdominal constriction model, a model of acute visceral pain, demonstrated that both the SRIs and the NRIs significantly blocked abdominal constrictions. However, the magnitude of this effect was greater following treatment with compounds having greater affinity for NRI as compared to SRI affinity. Additionally, isobolographic analyses indicated significant synergistic effects for all combinations of desipramine and fluoxetine in the PPQ model of visceral pain. Collectively, the present results support the hypothesis that compounds with greater NRI activity should be more effective for the treatment of pain than compounds having only SRI activity and this hypothesis is also supported by clinical data. These studies also suggest that the potency and effectiveness of NRIs might be enhanced by the presence of 5-HT activity.

Introduction

Both acute and chronic pain are disabling conditions that can result from injury to the nervous system. Pain is often characterized by increased sensitivity to normally non-noxious stimuli (allodynia), and/or painful stimuli (hyperalgesia) as well as ongoing spontaneous pain. Historically, antidepressants including the (NE) and serotonin (5-HT) reuptake inhibitors (NRIs and SRIs) have been used as a first-line therapy for treating pain associated with: diabetic neuropathy, post-herpetic neuralgia, fibromyalgia, irritable bowel syndrome and interstitial cystitis (Sindrup and Jensen, 1999; Collins et al., 2000; Crowell et al., 2004). Recently, duloxetine a mixed SRI and NRI with potency at both transporters was the first reuptake inhibitor approved for the treatment diabetic neuropathic pain (Bymaster et al., 2005; Goldstein et al., 2005).

While the exact pathophysiological mechanism(s) involved in the development and maintenance of chronic pain is not fully understood, a number of neurotransmitters, peptides and channels have been implicated in the modulation of nociceptive processing. The role of the NE and 5-HT systems in nociception has been previously described and characterized (Gebhart, 1986; Fields et al., 1991; Fields and Basbaum, 1999; Millan, 2002). Norepinephrine and to a lesser extent 5-HT are a major components of the endogenous descending pain inhibitory system from the rostral ventral medulla (RVM) to the spinal cord (Zhuo and Gebhart, 1991; Holden et al., 1999). In fact it has been suggested that chronic pain may in part result from altered or reduced levels of endogenous NE

and 5-HT activity at both the spinal and supraspinal levels (Ren and Ruda, 1996; Ren and Dubner, 2002). Consequentially, it is presumed that the NE and 5-HT reuptake inhibitors attenuate pain by preventing presynaptic reuptake of NE/5-HT leading to increased postsynaptic NE/5-HT levels and sustained activation of the descending pain inhibitory pathway (Blakely and Bauman, 2000; Burgess et al., 2002). This ultimately results in attenuation of neuronal hyperexcitability and alleviation of pain.

To date studies have not systematically evaluated the relative activity at the NE and/or 5-HT transporter required for maximal efficacy in rodent pain models. The present series of studies evaluates the potency of NRI/SRI compounds by evaluating *in vitro* functional data of NE and 5-HT uptake and comparing that with efficacy in preclinical pain models. Two approaches were taken, first a series of known selective NE and 5-HT reuptake inhibitors: desipramine, reboxetine, fluoxetine and paroxetine were evaluated in both *in vitro* and *in vivo* assays. As a measure of *in vitro* activity at the transporters functional uptake assays were used to evaluate compounds. Subsequently, the relative potency and efficacy of compounds were evaluated in models of neuropathic and visceral (acute inflammatory) pain. An additional series of compounds with a broader range of *in vitro* potency at the human NE and 5-HT transporters (hNET and hSERT) were also evaluated in the visceral pain model. In the second approach examining the relative contribution of NE and 5-HT to nociception, isobolographic analyses were generated to evaluate potential synergistic effects between NRIs and SRI compounds in the visceral pain model (Tallarida, 2001; Tallarida, 2002).

Materials and Methods

Test compounds

Desipramine, paroxetine and ketorolac were purchased from Sigma Chemical Co. (St. Louis, MO). Fluoxetine was purchased from Tocris (Ellsville, MO). Reboxetine, S,S-reboxetine, duloxetine and gabapentin were purchased from Organix (Woburn, MA). Three 2-Phenyl-2-(1-hydroxycycloalkyl)ethylamine derivatives: (-)-1-(1-(3-bromo-4-methoxyphenyl)-2-(dimethylamino)ethyl)cyclohexanol, hydrochloride (WY-X1), 1-(1-(3-bromo-4-methoxyphenyl)-2-(dimethylamino)ethyl)cyclohexanol, hydrochloride (WY-X2) and 1-(2-(dimethylamino)-1-(3-(trifluoromethyl)phenyl)ethyl)cyclohexanol, hydrochloride (WY-X3) were obtained from the Wyeth compound repository (Princeton, NJ) (Yardley et al., 1990). Doses were calculated as the free base molecular weight of the compound for all *in vivo* testing. All compounds were administered s.c. with the exception of ketorolac (i.p.) and dissolved in sterile water with the exception of gabapentin which was suspended in 2% polysorbate (Tween) 80 and 0.5% methylcellulose.

In vitro experiments

Human 5-HT and NE transporter uptake assays

Cell line and culture reagents: [^3H] 5-HT uptake studies were performed using JAR cells natively expressing the human serotonin transporter (SERT). JAR cells were incubated for 24 hr prior to the addition of 40 nM staurosporine to

enhance the expression of the hSERT. Following an additional 24 hr the cells were assayed for the [3 H] 5-HT uptake (Ramamoorthy et al., 1995). [3 H] NE uptake studies were performed using MDCK cells stably expressing NET (Pacholczyk et al., 1991). MDCK-Net6 cell line, stably transfected with human norepinephrine transporter (hNET) were cultured in growth medium containing high glucose DMEM (Gibco, Cat. No. 11995), 10% FBS (dialyzed, heat-inactivated, US Bio-Technologies, Lot FBD1129HI) and 500 μ g/ml G418 (Gibco, Cat. No. 10131). Cells were plated at 300,000/ T75 flask and cells were split twice weekly. The JAR cell line (human placental choriocarcinoma) was purchased from ATCC (Cat. No. HTB-144). The cells were cultured in growth medium containing RPMI 1640 (Gibco, Cat. No. 72400), 10% FBS (Irvine, Cat. No. 3000), 1% sodium pyruvate (Gibco, Cat. No. 1136) and 0.25% glucose. Cells were plated at 250,000 cells/ T75 flask and split twice weekly.

Serotonin and norepinephrine uptake assays: All uptake experiments were performed in 96-well plates (Falcon Optilux, cat #353947) in a total volume of 250 μ l. Individual wells were supplemented with 200 μ l assay buffer (25 mM Hepes, 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl_2 , 1.2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 mg/ml glucose, 0.2 mg/ml ascorbic acid, 1 μ M pargyline, pH 7.4). All test compounds were dissolved in DMSO and 25 μ l of each test compound was subsequently added to plates in triplicate and incubated at 37° C for 5 minutes. All compounds were assayed using a 9-point dose response curve (1 nM - 10 μ M). Positive controls were run on each plate for both assays. Finally, 25 μ l [3 H] 5-HT (30

Ci/mmol, Perkin Elmer, Boston, MA) or [^3H] NE (56.6 Ci/mmol, Perkin Elmer, Boston, MA) was added to all wells for the SERT and NET assays respectively, and incubated at 37° C for an additional 5 minutes. The final concentration of [^3H] 5-HT and [^3H] NE were 12 and 16 nM, respectively. The reaction was terminated by centrifugation (3,000 rpm for 5 min) and the supernatant was subsequently aspirated. Cells were washed with 50 mM Tris (pH 7.4), centrifuged and aspirated again. Cells were lysed by the addition of 25 μl of 0.25 M NaOH. Wells were supplemented with 100 μl of Microscint-20 (Packard, Perkin Elmer, Boston, MA) and plates counted using a TopCount (Perkin Elmer, Downer's Grove, IL) liquid scintillation counter.

Analysis of Results: For each experiment, a data stream of counts per minute collected from the Packard TopCount was downloaded to a Microsoft Excel statistical application program. Calculations of IC_{50} were performed using a sigmoidal non-linear regression program (Prism Graphpad 3 Software, San Diego, CA). In this program maximum uptake is represented by those wells supplemented with assay buffer and non-specific uptake is determined by wells treated with positive controls (i.e., excess fluoxetine or desipramine).

In vivo experiments

Animal maintenance and research were conducted in accordance with the National Research Council's policies and guidelines for the handling and use of laboratory animals outlined in the *Guide for the Care and Use of Laboratory*

Animals (National Research Council, 1996). Research protocols were approved by the Wyeth Institutional Animal Care and Use Committee according to the guideline of the Office of Laboratory Animal Welfare. All assays were performed in a randomized manner by individuals blinded to the experimental condition.

Subjects: For the neuropathic studies male Sprague-Dawley rats (125 – 150 g, Harlan; Indianapolis, IN) were housed individually. For the visceral pain studies male CD-1 mice (20 – 25 g, Charles River; Kingston/Stoneridge, NY) were housed in groups of five. For all studies animals were housed on bedding in rooms in a climate-controlled room on a 12-hour light/dark cycle and food and water were available ad libitum.

L5/L6 Spinal nerve ligation (SNL) model of neuropathic pain

Surgery: Rats were anesthetized with 3.5% halothane in O₂ at 1 L/min and maintained with 1.5% halothane in O₂ during surgery. Surgery was performed as previously described (Kim and Chung, 1992). Briefly, nerve injury was produced by tight ligation of the left L5 and L6 spinal nerves.

Assessment of tactile allodynia: Tactile thresholds were assessed using a series of calibrated von Frey monofilaments (Stoelting; Wood Dale, IL). Animals were placed in elevated wire cages and allowed 45-60 minutes to acclimate to the testing room. The threshold that produced a 50% likelihood of a withdrawal was determined using the up-down method, as previously described (Chaplan et al., 1994). Tactile thresholds were determined on the day prior to surgery and rats with baseline thresholds < 10g force were excluded from

studies. Three to four weeks after SNL surgery tactile thresholds were reassessed and animals that failed to exhibit subsequent mechanical allodynia (threshold ≥ 5 g) were excluded from further testing. Subjects were pseudo-randomly divided into test groups (n=8) so that average baseline and post-surgery sensitivities were similar among groups. For all compound testing, rats were administered vehicle or test compound and tactile thresholds were assessed up to 180 minutes after dosing. For each study gabapentin (100 mg/kg, s.c.) was run as a positive control.

Analysis of results: Statistical analysis was done using a repeated measures analysis of variance (ANOVA) using a customized SAS-excel application (SAS Institute, Cary, NC). Significant main effects were analyzed further by subsequent least significant difference analysis. The criterion for significant differences was $p < 0.05$. Data is presented as percent reversal according to the following equation: percent reversal = $[(50\% \text{ threshold}^{\text{drug + post surgery}}) - (50\% \text{ threshold}^{\text{post surgery}}) / (50\% \text{ threshold}^{\text{pre surgery}}) - (50\% \text{ threshold}^{\text{post surgery}})] \times 100$. Maximal effect of 100 % reversal represents a return to the mean pre-operative threshold value for subjects in that experimental condition.

Para-phenylquinone (PPQ) model of visceral pain

Assessment of PPQ-induced constrictions (writhing): The ability of compounds to attenuate acute visceral (abdominal) pain was assessed following an i.p. injection of 2 mg PPQ (dissolved in 4% ethanol in distilled water, Sigma-Aldrich,

St. Louis, MO) (Siegmund et al., 1957). All compounds were evaluated for dose- and time dependent effects ($n = 10/\text{group}$). Initial experiments determined the peak activity of each compound by evaluating pretreatment times of 30-120 minutes (data not shown). Once peak activity for each respective compound was determined dose-response curves were generated. During testing, following PPQ administration, mice were individually placed in a Plexiglas cage and the total number of abdominal constrictions was recorded for one-minute periods, starting at 5 and 10 minutes after PPQ injection. The non-steroidal anti-inflammatory agent ketorolac (1 mg/kg) was run as a positive control.

Isobolographic analysis

Statistical evaluation of interactions between SRI and NRI compounds was accomplished using the graded dose-response method (Tallarida, 2001; Tallarida, 2002). Briefly, ED_{30} values of each compound alone were calculated by linear regression when at least three data points were available on the linear portion of the dose-effect curve. A combination of the two drugs was administered in a constant dose ratio based on ED_{30} values for each compound. Different contributions of fluoxetine:desipramine activity were evaluated: 1:1, 3:1 and 1:3. For drug combinations (i.e., dose fluoxetine + dose desipramine), experimental values from fixed ratio designed studies were also analyzed using linear regression, and the ED_{30} values for each combination that produced a 30% decrease (blockade) in PPQ-induced abdominal constrictions were also analyzed using linear regression. ED_{30} values were chosen because that effect level was common to all compounds and an estimated ED_{50} value would introduce

additional variability to the already complex study of synergistic and additive effects.

Analysis of results: To determine statistical significance of a compound compared to vehicle treatment a one-way ANOVA was performed on raw data (total number of abdominal constrictions) using a customized SAS-excel application (SAS Institute, Cary, NC). The criterion for significant differences was $p < 0.05$. Data is presented as percent blockade compared to vehicle according to the following equation: percent blockade = $[(\text{mean vehicle} - \text{drug}) / (\text{mean vehicle})] \times 100$. The ED₃₀ (effective dose estimated to a 30% reduction in the number of PPQ-induced abdominal stretching) was generated for each drug or combination of drugs using a customized SAS-excel (SAS Institute, Cary, NC) application. All statistical correlations were performed using Sigma plot analysis (San Jose, CA).

For the isobolographic analyses the least significant difference (LSD) test was used to determine significance of the difference between the theoretical additive ED₃₀ value and the experimentally derived ED₃₀ value of the dosing combinations. The criterion for establishing a statistical significance was $p < 0.05$. An experimental ED₃₀ value significantly lower than the theoretical additive ED₃₀ value was considered to indicate a supra-additive or synergistic interaction between fluoxetine and desipramine. In addition to statistical analysis, interactions between the two drugs were assessed using a graphical approach (Tallarida, 2001; Tallarida, 2002)). Graphically, mean ED₃₀ values (95% confidence limits) for each drug administered either alone or as part of a

combination were plotted as a function of the ED_{30} value of the other drug in the combination. This data presentation format is known as an isobologram, and the line is an isobologram that connects the data points for each drug alone shows predicted data points for drug mixtures assuming additivity. Points that fall above the line of additivity (away from the origin) are suggestive of subadditivity, whereas points that fall below the line (toward the origin) are suggestive of supra-additivity (i.e., synergistic).

Results

Activity of fluoxetine, paroxetine, desipramine and reboxetine in human 5-HT and NE transporter uptake assays

The compounds tested inhibited uptake of [^3H]5-HT by the hSERT by differing degrees as expected (Table 1). Compounds also differentially inhibited the uptake of [^3H]NE by the hNET (Table 1). Based on IC_{50} values, the relative rank order of potency for these compounds in the hSERT uptake assay were paroxetine>fluoxetine>desipramine>reboxetine and in the hNET uptake assay were desipramine=reboxetine>paroxetine>fluoxetine.

Effects of fluoxetine, paroxetine, desipramine and reboxetine in a neuropathic pain model

Compounds were evaluated for their ability to reverse tactile allodynia in SNL rats. Before surgery, the tactile sensitivities were 15g (Figure 1; points above 'Pre'). Following surgery, the rats were significantly more sensitive to tactile stimuli (Figure 1; points above 'BL'). Acutely administered fluoxetine (10, 30 and 56 mg/kg, s.c.) significantly reversed tactile allodynia in SNL rats (Figure 1A). Only the highest dose (56 mg/kg) significantly reversed tactile allodynia, with peak effects occurring between 100 and 180 minutes after administration and producing a modest 14.6 ± 7.9 % reversal. Higher doses of fluoxetine could not be tested due confounding effects of sedation.

Acutely administered paroxetine (3, 10 and 30 mg/kg, s.c.) significantly and time-dependently reversed tactile allodynia in SNL rats (Figure 1B). All doses of paroxetine significantly reversed mechanical allodynia, with peak effects occurring 100 minutes after administration. Only modest effects were observed after the 3 or 10 mg/kg doses. In contrast, the highest dose (30 mg/kg) of paroxetine produced a maximal 59.5 ± 17.9 % reversal.

Acutely administered desipramine (30, 56 and 100 mg/kg, s.c.) significantly and time-dependently reversed tactile allodynia in SNL rats (Figure 1C). Desipramine had a steep dose-response curve with only the highest dose (100 mg/kg) significantly reversing tactile allodynia, with peak effects occurring 30 minutes after administration and producing a maximal 35.1 ± 11.1 % reversal.

Acutely administered reboxetine (10, 30 and 100 mg/kg, s.c.) significantly dose- and time-dependently reversed tactile allodynia in SNL rats (Figure 1D). Only the 10 and 30 mg/kg doses of reboxetine significantly reversed tactile allodynia, with peak effects occurring 30 minutes after administration. The highest dose (100 mg/kg) of reboxetine produced a maximal 51.0 ± 16.4 % reversal.

The anticonvulsant gabapentin (100 mg/kg, s.c.) was run as a positive control. In all studies gabapentin significantly reversed tactile allodynia over 180 minutes, with peak effects occurring 100 minutes after administration and with an overall reversal of tactile allodynia averaging 55.0 ± 19.2 %.

Additionally, no confounding sedative/neuromuscular effects were observed for any of the compounds evaluated during the reported peak activity of each compound as measured by an accelerating rotarod (data not shown).

Effects of fluoxetine, paroxetine, desipramine and reboxetine in a visceral pain model

Compounds were evaluated for their ability to reduce the number of PPQ-induced abdominal constrictions. Fluoxetine (3, 10 and 30 mg/kg, s.c.) significantly inhibited PPQ-induced abdominal constrictions with peak effects observed following a 60 minute pretreatment. At peak activity the 3 and 10 mg/kg doses of fluoxetine failed to block PPQ-induced abdominal constrictions only the highest dose (30 mg/kg) of fluoxetine significantly reversed PPQ-induced abdominal constrictions producing a 32.7 ± 4.4 % blockade and an overall ED_{30} of 23.3 ± 6 mg/kg. (Figure 2A).

Paroxetine (3, 10 and 30 mg/kg, s.c.) significantly inhibited PPQ-induced abdominal constrictions with peak effects observed following a 60 minute pretreatment (Figure 2B). At peak activity the 3 and 10 mg/kg doses of paroxetine failed to block PPQ-induced abdominal constrictions only the highest dose (30 mg/kg) of paroxetine significantly reversed PPQ-induced abdominal constrictions producing a 37.5 ± 16.5 % blockade and an overall ED_{30} of 16 ± 2.6 mg/kg.

Desipramine (1, 3, 10, 17.8 and 30 mg/kg, s.c.) significantly inhibited PPQ-induced abdominal constrictions with peak effects observed following a 90 minute pretreatment (Figure 2C). At peak activity the 3, 10, 17.8 and 30 mg/kg doses of desipramine significantly reversed PPQ-induced abdominal constrictions producing 28.0 ± 5.2 %, 34.7 ± 7.2 %, 54.7 ± 1.6 % and 63.3 ± 3.5 % blockades, respectively and an overall ED₃₀ of 4.4 ± 0.7 mg/kg.

Reboxetine (0.3, 1, 3, 10 and 30 mg/kg, s.c.) significantly inhibited PPQ-induced abdominal constrictions with peak effects observed following a 60 minute pretreatment (Figure 2D). At peak activity the 0.3 mg/kg dose of reboxetine failed to block PPQ-induced abdominal constrictions, whereas the 1, 3, 10 and 30 mg/kg doses of reboxetine significantly reversed PPQ-induced abdominal constrictions producing 28.0 ± 7.7 %, 65.9 ± 9.4 %, 72.9 ± 9.6 % and 90.6 ± 2.9 % blockades, respectively and an overall ED₃₀ of 1.1 ± 0.3 mg/kg.

The rank order of potency in the PPQ assay based on ED₃₀ values (i.e., dose that produced a 30% blockade) is as follows reboxetine (1.15 ± 0.3 mg/kg)>desipramine (4.4 ± 0.8 mg/kg)>paroxetine (16.0 ± 2.6 mg/kg)>fluoxetine (23.3 ± 6.1 mg/kg).

The non-steroidal anti-inflammatory ketorolac (0.3 mg/kg, i.p.) was run as a positive control. Following a 30 minute pretreatment ketorolac significantly inhibited PPQ-induced abdominal constrictions with a blockade of 85.8 ± 4.5 %.

Correlation of in vitro and in vivo activity in the PPQ model of visceral pain model

In addition to fluoxetine, paroxetine, desipramine and reboxetine, additional compounds with a broader range of activity in the functional hSERT and hNET assays were evaluated in the PPQ model: S,S-reboxetine, duloxetine and three 2-Phenyl-2-(1-hydroxycycloalkyl)ethylamine derivatives. (Table 1, 2 and Figure 3). All compounds significantly blocked PPQ-induced abdominal constrictions (data not shown). Correlative analyses among these six compounds as well as fluoxetine, paroxetine, desipramine and reboxetine and their in vitro potency at the transporters were generated for two measures of in vivo activity: maximal percent effect of PPQ-induced abdominal constrictions and ED₃₀ values. These correlations demonstrated that maximal percent effect of the PPQ-induced abdominal constrictions and efficacy of each compound (ED₃₀) was highly dependent on the potency of the compound at hNET as reflected by r^2 values of 0.72 (Figure 3A) and 0.89, respectively. In contrast, potency at hSERT showed much weaker correlations with maximal percent effect and ED₃₀ values reflected in r^2 values of 0.55 (Figure 3B) and 0.36, respectively.

Isobolographic analysis of evaluating combined activity of fluoxetine and desipramine in the PPQ model of visceral pain model

Interaction studies were performed by generating isobolographic analyses using fixed dose combinations of desipramine and fluoxetine (Figure 4). The antinociceptive ED₃₀ values at peak activity for desipramine and fluoxetine were 4.4 and 23.3 mg/kg, respectively (Table 3). Following the 1:1 combination dosing the antinociceptive ED₃₀ values were increased to 0.53 and 2.64 mg/kg for

desipramine and fluoxetine, respectively, relative to each compound administered alone (Figure 3A and Table 3). Similarly, both the 3:1 (1.96 and 3.5 mg/kg) and 1:3 (0.6 and 9.26 mg/kg) combinations of desipramine and fluoxetine increased the relative potency of each compound, but to a lesser degree than the 1:1 combination (Figure 4B, 4C and Table 3). In summary, all combinations of desipramine and fluoxetine produced statistically significant synergistic effects in the PPQ model of visceral pain.

Discussion

Previous studies have shown that both 5-HT and NE may contribute to antinociception, 5-HT in spinal pathways and NE in supraspinal descending pathways (Millan, 2002, Ren and Dubner, 2002, Suzuki et al., 2004). Furthermore, multiple studies have also suggested that the actions of 5-HT and NE in these pathways may produce synergistic effects (Zhou and Gebhart, 1991). In order to examine the contribution of 5-HT and NE to antinociception, the present series of experiments initially evaluated representative SRI and NRI compounds for *in vitro* potency at both transporters using functional uptake assays. Having defined *in vitro* activity of each compound, additional non-commercial compounds were evaluated and isobolographic analyses were used to examine the relative contributions of 5-HT and NE to antinociception in an model of acute inflammatory (visceral) pain model (Tallarida, 2001; Tallarida, 2002). In the functional uptake studies, the rank order potency at SERT was paroxetine > fluoxetine > desipramine > reboxetine and at NET was desipramine = reboxetine > paroxetine > fluoxetine. The present findings demonstrate that these compounds have differential activity in preclinical models of pain relative to their activity at the SERT and NET transporters and that the contribution of NE in mediating antinociception may be greater than that of 5-HT.

In the SNL model of neuropathic pain, both NRIs desipramine and reboxetine reversed tactile allodynia and the overall magnitude of the effect was equivalent to that of the anticonvulsant gabapentin. However, discrepant effects were

observed with the SRIs paroxetine and fluoxetine, such that the activity of paroxetine was similar to that observed with the NRIs. In contrast, fluoxetine had minimal activity in the SNL model. This unexpected finding with paroxetine, which is reported to be a selective SRI *in vitro*, may in part be due to findings demonstrating that paroxetine increases levels of NE *in vivo* (Owens et al., 2000; Gilmor et al., 2002). Therefore, the observed efficacy with paroxetine may not be due to increased level of 5-HT, but also concomitant increased levels of NE that are contributing to the activity in the SNL model. Literature reports evaluating NE and 5-HT and reuptake inhibitors have been mixed. In studies evaluating tactile allodynia, both fluoxetine and desipramine were inactive in the SNL model, but desipramine significantly reversed mechanical allodynia in a pinprick test (Jett et al., 1997; LaBuda and Little, 2005). It has also been reported that fluoxetine is modestly active in a test of tactile allodynia, but reboxetine was inactive (Pedersen et al., 2005). Additionally, it was reported that only reboxetine and not fluoxetine was active in a test of thermal hyperalgesia (Pedersen et al., 2005). In studies using an intrathecal route of administration, reboxetine was active in modifying tactile allodynia and paroxetine was inactive (Obata et al., 2005b). However, none of the previous studies evaluating selective reuptake inhibitors in the SNL model are directly comparable to the present findings due to different dosing regimes and routes of administration (Jett et al., 1997; Owens et al., 2000; Bomholt et al., 2005; LaBuda and Little, 2005; Obata et al., 2005a; Pedersen et al., 2005). Overall, the effects observed in current studies although utilizing a limited number of compounds, replicate what is observed clinically for

treating neuropathic pain conditions. Specifically, these studies suggest that compounds with greater affinity for NRI are more effective in addressing pain, and compounds with greater affinity for SRI have limited efficacy (Max et al., 1992; Collins et al., 2000).

In the inflammatory assay, the PPQ model of acute visceral pain, both the SRIs and the NRIs dose- and time-dependently blocked PPQ-induced abdominal constrictions, however, similar to results with the SNL model, the magnitude of this effect was greater following treatment with the NRIs compared to the SRI compounds. Further, the maximal effect observed with both desipramine and reboxetine was similar in magnitude to that observed with the positive control, the NSAID, ketorolac. In contrast to the effects observed in the SNL model, both compounds with more potent SRI affinities had similar levels of activity and were less efficacious than the NRI compounds. Specifically, in the PPQ model the magnitude of effect for fluoxetine was greater than that observed in the neuropathic model (33% vs. 15%, respectively) suggesting that SRI activity may be more beneficial for treating inflammatory and or visceral pain conditions. The present data is similar to previous literature reports evaluating these compounds in rodent visceral pain models (i.e., acetic acid). One study reported that the NRI maprotiline was more active than the SRI citalopram following both acute (1 day) and chronic (21 day) administration in a rat acetic acid model (Korzeniewska-Rybicka and Plaznik, 1998). It has also been reported that fluoxetine (Singh et al., 2001) and desipramine (Spiegel et al., 1983) dose-dependently blocked acetic acid-induced abdominal constrictions in rats and mice, respectively.

Overall these findings suggest that compounds with selective activity at NET or SERT are active in models of visceral pain. However, in the present studies utilizing reuptake inhibitors with a broad range of functional activity, the potency at hNET is more strongly correlated with measures of in vivo activity (i.e., percent maximal effect and ED₃₀ values) compared to activity at hSERT. Interestingly, the combination dosing of fluoxetine and desipramine in the PPQ model produced significant synergistic effects such that the potency of each compound appeared to be increased (i.e. lower ED₃₀ values). Three different ratio combinations of fluoxetine and desipramine (SRI:NRI combinations) were evaluated and while the 1:1 combination clearly achieved synergy, the 3:1 and 1:3 combination both produced significant effects but approach the confidence limits of the line of additivity. In an isobologram analysis, activity conclusions are based on the results from administration of two individual compounds and exactly how this will translate into efficacy from 2 activities in a single molecule remains unknown. It is surprising in the present study that paroxetine, which has activity at both transporters, only had a moderate effect in the PPQ model. However, it is possible that when one compound with mixed activity has greater affinity for one transporter over the other, that activity takes precedence or drives the response. In the example of paroxetine, the higher affinity is for the 5-HT rather than the NE transporter. If this is indeed the case, and paroxetine is maximally functional at the 5-HT transporter and the NE transporter is the major contributor to pain inhibition as the correlative studies suggest, then it follows that there may be less

pain reversal using a single molecule with two activities than would be expected using two compounds with high affinity at each transporter.

In the PPQ model that is more inflammatory in nature both the compounds with 5-HT were active. In contrast to pathways involved in neuropathic pain, the role of 5-HT in gastrointestinal (GI)-tract functioning that is critical to visceral pain is well characterized. A large portion of the 5-HT in the body resides in the gut and 5-HT plays a role in normal gut functioning as well as in brain-gut communication (see review: (Baker, 2005)). Additionally, SERTs are located within the GI tract and SERT knock out mice exhibit GI symptoms (e.g., diarrhea) (Baker, 2005). Thus the active role 5-HT plays in the gut may account for the synergistic activity observed in the isobolographic analyses. Taken together the present findings in the PPQ model suggest a greater role for NE activity in the treatment of pain; however the potency and effectiveness of NRIs in visceral pain and possibly neuropathic pain can be enhanced by the presence of 5-HT activity.

In summary, because it is hypothesized that nociceptive information is processed and integrated, at the peripheral, spinal and supraspinal levels, two models of pain were chosen to evaluate the SRI and NRI compounds, which represent a broad level of pain processing and modulation. The SNL model mimics pain that is chronic and neuropathic in nature, the result of central sensitization (Kim and Chung, 1992). In contrast, the PPQ model is more representative of acute inflammatory (visceral) pain. The overall greater activity of the NRI versus SRI compounds in both pain models suggest that this class of compounds will provide a broader range clinically in treating distinct types of pain. It is also

important to note that for all studies the compounds were administered subcutaneously to minimize any potential differences in pharmacokinetics and/or brain penetration that may also account for differences in exposure and/or efficacy.

Recently, duloxetine a mixed SRI and NRI with potency at both transporters was the first reuptake inhibitor approved for the treatment diabetic neuropathy (Bymaster et al., 2005; Goldstein et al., 2005). Published preclinical data evaluating this compound in both neuropathic and inflammatory models of pain have demonstrated activity (Iyengar et al., 2004; Jones et al., 2005). However, since the compound has potent activity at both transporters and increases levels of both NE and 5-HT, it is difficult to distinguish which activity is contributing to the reported efficacy *in vivo* (Iyengar et al., 2004; Jones et al., 2005). These studies do however support the present findings that potency at the NET results in efficacy in preclinical models of pain.

Collectively, the present results suggest that compounds with affinity for both NRI and SRI may be beneficial for the treatment of neuropathic pain, whereas, compounds with greater affinity for NRI may be more beneficial for the treatment of visceral pain and that the potency and effectiveness of NRIs can be supra-additive in the presence of 5-HT activity. This data also supports clinical data suggesting that compounds with greater NRI versus SRI activity would be more effective for the treatment of pain than compounds with only SRI activity (Sindrup and Jensen, 1999; Collins et al., 2000).

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Figure Legends.

Figure 1: Differential effects of fluoxetine (panel A), paroxetine (panel B), desipramine (panel C) and reboxetine (panel D) (s.c.) in the rat SNL model of neuropathic pain. Data above 'Pre' indicates sensitivity before nerve injury, and data above 'BL' indicates hypersensitivity following nerve injury. Each point represents the mean data ($\pm$ SEM). Asterisk indicates significant ($p < 0.05$) differences from the 'BL' value, plus indicates significant ($p < 0.05$) differences from the 'Vehicle' value.

Figure 2: Differential effects of fluoxetine (panel A), paroxetine (panel B), desipramine (panel C) and reboxetine (panel D) (s.c.) in the mouse PPQ model of acute visceral pain. Data is expressed as percent blockade relative to vehicle-treated mice. Each point represents the mean data ($\pm$ SEM). Asterisk indicates significant ($p < 0.05$) differences from the vehicle-treated mice (data not shown).

Figure 3: Correlation of in vitro potency at hNET and hSERT with percent maximal effect in the PPQ model of visceral pain. Panel A; activity in the hNET functional assay and Panel B is the activity in hSERT functional assay where all IC_{50} data is in the nM range. Data is expressed as percent blockade relative to vehicle-treated mice. Each point represents the mean data with $n = 10$ /group.

Figure 4: Combination dosing of desipramine and fluoxetine is synergistic in the mouse PPQ model of visceral pain. Isobolograms were generated with different

dosing ratios of desipramine and fluoxetine (s.c.). Combinations of 1:1 (panel A), 1:3 (panel B) and 3:1 (panel C) of fluoxetine and desipramine were evaluated. Closed black circles correspond to the ED₃₀ values (i.e., dose which produced a 30% blockade of PPQ-induced abdominal stretching) of each respective drug alone and the black solid line depicts the theoretically calculated line of additivity with its 95% confidence limits (black hatched lines). The gray crosses correspond to the experimentally derived ED₃₀ value and the length of line corresponds to the 95% confidence limits. **Inset** represents actual data and dosing combinations from which isobolograms were generated. Asterisk indicates statistical significance ($p < 0.05$).

Table 1. Differential *in vitro* functional activity of commercial SRI and NRI

compounds.

IC₅₀ values were determined using 9 concentrations in triplicate. Values shown are mean $\pm$ SEM (n=3).

Compound	SERT IC ₅₀ (nM)	NET IC ₅₀ (nM)	Fold Separation NET / SERT
paroxetine	3 $\pm$ 1	173 $\pm$ 25	0.02
fluoxetine	32 $\pm$ 3	1002 $\pm$ 170	0.03
desipramine	313 $\pm$ 47	9 $\pm$ 1	35
reboxetine	344 $\pm$ 44	10 $\pm$ 1	35

Table 2. In vitro and in vivo activity of NRI and SRI compounds.

IC₅₀ values were determined using 9 concentrations in triplicate. Values shown are mean $\pm$ SEM (n=3).

Compound	SERT IC ₅₀ (nM)	NET IC ₅₀ (nM)	Maximal % Effect	ED ₃₀ (mg/kg)
S,S-reboxetine	2000 $\pm$ 178	1 $\pm$ 0.03	93	0.68
duloxetine	15 $\pm$ 1.6	20 $\pm$ 2.8	63	2.54
WY-X1*	30 $\pm$ 3.2	13 $\pm$ 3.4	65	1.8
WY-X2*	21 $\pm$ 2.1	31 $\pm$ 3.3	68	2.77
WY-X3*	534 $\pm$ 28	32 $\pm$ 3.3	89	2.81

Asterisk denotes: WY-X1 = (-)-1-(1-(3-bromo-4-methoxyphenyl)-2-(dimethylamino)ethyl)cyclohexanol, hydrochloride, WY-X2 = 1-(1-(3-bromo-4-methoxyphenyl)-2-(dimethylamino)ethyl)cyclohexanol, hydrochloride and WY-X3 = 1-(2-(dimethylamino)-1-(3-(trifluoromethyl)phenyl)ethyl)cyclohexanol, hydrochloride

Table 3. Summary of ED₃₀ values (mg/kg, s.c.) from isobolographic studies.

Values shown are ED₃₀ values $\pm$ SEM

Desipramine: Fluoxetine Ratio	Desipramine ED₃₀ (dose, mg/kg)	Fluoxetine ED₃₀ (dose, mg/kg)
alone	4.4 $\pm$ 0.7	23.3 $\pm$ 6
1:1	0.5 $\pm$ 0.1	2.6 $\pm$ 0.4
1:3	0.6 $\pm$ 0.1	9.3 $\pm$ 1.6
3:1	2.0 $\pm$ 0.2	3.5 $\pm$ 0.6

Figure 1.

Figure 2.

Figure 3.

Figure 4.

A. 1:1 Desipramine/Fluoxetine

B. 1:3 Desipramine/Fluoxetine

C. 3:1 Desipramine/Fluoxetine