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

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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. Using 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 compared with SRI affinity. In addition, 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.

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 (alldynia) and/or painful stimuli (hyperalgesia), as well as ongoing spontaneous pain. Historically, antidepressants, including the norepinephrine (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, postherpetic 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 NE and 5-HT transporters, was the first reuptake inhibitor approved for the treatment of diabetic neuropathic pain (Bymaster et al., 2005; Goldstein et al., 2005).

Whereas 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 described and characterized previously (Gebhart, 1986; Fields et al., 1991; Fields and Basbaum, 1999; Millan, 2002). Norepinephrine and to a lesser extent, 5-HT are major components of the endogenous descending pain inhibitory system from the rostral ventral medulla 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). Consequently, 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 neurogenic 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, 2002).

Materials and Methods

Test Compounds

Desipramine, paroxetine, and ketorolac were purchased from Sigma-Aldrich (St. Louis, MO). Fluoxetine was purchased from Tocris Cookson Inc. (Ellisville, MO). Reboxetine, S,S-reboxetine, duloxetine, and gabapentin were purchased from Organix (Woburn, MA). Three 2-phenyl-2-(1-hydroxy cycloalkyl)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 compound repository (Wyeth, 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

Cell Line and Culture Reagents. [3H]5-HT uptake studies were performed using JAR cells (the human choriocarcinoma cell line) natively expressing the human SERT. JAR cells were incubated for 24 h before the addition of 40 nM stauroporine to enhance the expression of the hSERT. After an additional 24 h, the cells were assayed for the [3H]5-HT uptake (Ramamoorthy et al., 1995). [3H]NE uptake studies were performed using Madin-Darby canine kidney cells natively expressing NET (Pacholczyk et al., 1991). Madin-Darby canine kidney-Net6 cell line, stably transfected with hNET, was assayed for the [3H]5-HT uptake (Ramamoorthy et al., 1995). [3H]NE uptake studies were performed using Madin-Darby canine kidney cells stably expressing NET (Pacholczyk et al., 1991). Madin-Darby canine kidney-Net6 cell line, stably transfected with hNET, were cultured in growth medium containing high-glucose Dulbecco’s modifed Eagle’s medium (catalog number 11995; Invitrogen, Carlsbad, CA), 10% fetal bovine serum (dialyzed, heat-inactivated, Lot FBD11295; U.S. Biotechnologies, Pottstown, PA), and 500 μg/ml G418 (catalog number 10131; Invitrogen). Cells were plated at 300,000 per T75 flask and were split twice weekly. The JAR cell line (human placental choriocarcinoma) was purchased from ATCC (Manassas, VA; catalog number HTB-144). The cells were cultured in growth medium containing RPMI 1640 (catalog number 72400; Invitrogen), 10% fetal bovine serum (catalog number 3000; Irvine Scientific, Santa Ana, CA), 1% sodium pyruvate (catalog number 1136; Invitrogen), and 0.25% glucose. Cells were plated at 250,000 cells per T75 flask and split twice weekly.

Serotonin and Norepinephrine Uptake Assays. All uptake experiments were performed in 96-well plates (catalog number 353947, Optilux; BD Biosciences Discovery Labware, Bedford, MA) in a total volume of 250 μl. Individual wells were supplemented with 200 μl of assay buffer (25 mM HEPES, 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4·7H2O, 2 mg/ml glucose, 0.2 mg/ml ascorbic acid, and 1 μM pargyline, pH 7.4). All test compounds were dissolved in dimethyl sulfoxide, and 25 μl of each test compound was subsequently added to plates in triplicate and incubated at 37°C for 5 min. All compounds were assayed using a nine-point dose-response curve (1 nM-10 μM). Positive controls were run on each plate for both assays. Finally, 25 μl of [3H]5-HT (30 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) or [3H]NE (56.6 Ci/mmol; PerkinElmer) was added to all wells for the SERT and NET assays, respectively, and incubated at 37°C for an additional 5 min. The final concentration of [3H]5-HT and [3H]NE were 12 and 16 nM, respectively. The reaction was terminated by centrifugation (3000 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; PerkinElmer Life and Analytical Sciences), and plates were counted using a Top-Count liquid scintillation counter (PerkinElmer Life and Analytical Sciences, Downer’s Grove, IL).

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 IC50 were performed using a sigmoidal nonlinear regression program (Prism, version 3; GraphPad Software, Inc., San Diego, CA). In this program, maximal uptake is represented by those wells supplemented with assay buffer, and nonspecific 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 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-h light/dark cycle, and food and water were available ad libitum.

L5/L6 Spinal Nerve Ligation 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 described previously (Kim and Chung, 1992). In brief, nerve injury was produced by tight ligation of the left L5 and L6 spinal nerves.

Assessment of Tactile Alldynia. Tactile thresholds were assessed using a series of calibrated von Frey monofilaments (Stoeltting, Wood Dale, IL). Animals were placed in elevated wire cages and allowed 45 to 60 min to acclimate to the testing room. The threshold that produced a 50% likelihood of a withdrawal was determined using the up-down method, as described previously (Chaplan et al., 1994). Tactile thresholds were determined on the day before surgery, and rats with baseline thresholds of <10g force were excluded from studies. Three to 4 weeks after spinal nerve ligation (SNI) surgery, tactile thresholds were reassessed and animals that failed to exhibit subsequent mechanical alldynia (threshold >5g) were excluded from further testing. Subjects were pseudo-randomly divided into test groups (n = 8) so that average baseline and postsurgery sensitivities were similar among groups. For all compound testing, rats...
were administered vehicle or test compound, and tactile thresholds were assessed up to 180 min 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 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 are presented as percentage reversal according to the following equation: percentage reversal = [(50% threshold - vehicle) - (50% threshold - postsurgery)] / (50% threshold - postsurgery) × 100. Maximal effect of 100% reversal represents a return to the mean pre-operative threshold value for subjects in that experimental condition.

### Para-Phenylquinone 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 of para-phenylquinone (PPQ) (dissolved in 4% ethanol in distilled water; Sigma-Aldrich) (Siegmund et al., 1957). All compounds were evaluated for dose- and time-dependent effects (\( n = 10 \)/group). Initial experiments determined the peak activity of each compound by evaluating pretreatment times of 30 to 120 min (data not shown). Once peak activity for each respective compound was determined, dose-response curves were generated. During testing, after PPQ administration, mice were individually placed in a Plexiglas cage, and the total number of abdominal constrictions was recorded for 1-min periods starting at 5 and 10 min after PPQ injection. The nonsteroidal 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, 2002). In brief, 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 of fluoxetine + dose of 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 point is the criterion for establishing a statistical significance. The criterion for establishing a statistical significance was \( p < 0.05 \). Data are presented as percentage blockade compared with vehicle according to the following equation: percentage blockade = [(50% threshold - drug)/(mean vehicle)] × 100.

Maximal effect of 100% reversal represents a return to the mean pre-operative threshold value for subjects in that experimental condition.

**Statistical Evaluation of Additivity from In Vitro and In Vivo Functional Activity.** Differential in vitro functional activity of commercial SRI and NRI compounds was compared using \( IC_{50} \) values determined using nine concentrations in triplicate. Values shown are mean \( \pm S.E.M. (n = 3) \).

**TABLE 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC(_{50}) SERT</th>
<th>IC(_{50}) NET</th>
<th>NET / SERT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paroxetine</td>
<td>3 ± 1</td>
<td>173 ± 25</td>
<td>0.02</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>32 ± 3</td>
<td>1002 ± 170</td>
<td>0.03</td>
</tr>
<tr>
<td>Desipramine</td>
<td>313 ± 47</td>
<td>9 ± 1</td>
<td>35</td>
</tr>
<tr>
<td>Reboxetine</td>
<td>344 ± 44</td>
<td>10 ± 1</td>
<td>35</td>
</tr>
</tbody>
</table>
Acutely administered reboxetine (10, 30, and 100 mg/kg s.c.) significantly dose- and time-dependently reversed tactile allodynia in SNL rats (Fig. 1D). Only the 10 and 30 mg/kg doses of reboxetine significantly reversed tactile allodynia, with peak effects occurring 30 min 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 min, with peak effects occurring 100 min after administration and with an overall reversal of tactile allodynia averaging 55.0 ± 19.2%. Furthermore, 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 after a 60-min 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 ED₃₀ of 23.3 ± 6.1 mg/kg.

Paroxetine (3, 10, and 30 mg/kg s.c.) significantly inhibited PPQ-induced abdominal constrictions, with peak effects observed following a 60-min pretreatment (Fig. 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 ED₃₀ 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 after a 90-min pretreatment (Fig. 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 after a 60-min pretreatment (Fig. 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 4.4 ± 0.8 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 nonsteroidal anti-inflammatory ketorolac (0.3 mg/kg i.p.) was run as a positive control. After a 30-min 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-hydroxy-cycloalkyl)ethylamine derivatives (Tables 1 and 2; Fig. 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 percentage effect of PPQ-induced abdominal constrictions and ED$_{30}$ values. These correlations demonstrated that maximal percentage effect of the PPQ-induced abdominal constrictions and efficacy of each compound (ED$_{30}$) was highly dependent on the potency of the compound at hNET as reflected by $r^2$ values of 0.72 (Fig. 3A) and 0.89, respectively. In contrast, potency at hSERT showed much weaker correlations with maximal percentage effect and ED$_{30}$ values reflected in $r^2$ values of 0.55 (Fig. 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 (Fig. 4). The antinoceptive ED$_{30}$ values at peak activity for desipramine and fluoxetine were 4.4 and 23.3 mg/kg, respectively (Table 3). After the 1:1 combination dosing, the antinoceptive ED$_{30}$ values were increased to 0.53 and 2.64 mg/kg for desipramine and fluoxetine, respectively (Fig. 3A; Table 3). Likewise, 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 (Fig. 4, B and C; Table 3). In summary, all combinations of desipramine and fluoxetine showed additivity in decreasing PPQ-induced abdominal constrictions, with the 1:1 combination dosing being the most potent at reducing abdominal constrictions.

**TABLE 2**
In vitro and in vivo activity of NRI and SRI compounds
IC$_{50}$ values were determined using nine concentrations in triplicate. Values shown are mean ± S.E.M. (n = 3).

<table>
<thead>
<tr>
<th>Compound</th>
<th>SERT IC$_{50}$</th>
<th>NET IC$_{50}$</th>
<th>Maximal % Effect</th>
<th>ED$_{30}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC$_{50}$</td>
<td>nM</td>
<td>mg/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S,S-Reboxetine</td>
<td>2000 ± 178</td>
<td>1 ± 0.03</td>
<td>93</td>
<td>0.68</td>
</tr>
<tr>
<td>Duloxetine</td>
<td>15 ± 1.6</td>
<td>20 ± 2.8</td>
<td>63</td>
<td>2.54</td>
</tr>
<tr>
<td>WY-X1</td>
<td>30 ± 3.2</td>
<td>13 ± 3.4</td>
<td>65</td>
<td>1.8</td>
</tr>
<tr>
<td>WY-X2</td>
<td>21 ± 2.1</td>
<td>31 ± 3.3</td>
<td>68</td>
<td>2.77</td>
</tr>
<tr>
<td>WY-X3</td>
<td>534 ± 28</td>
<td>32 ± 3.3</td>
<td>89</td>
<td>2.81</td>
</tr>
</tbody>
</table>
mine 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). 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 noncommercial compounds were evaluated, and isobolographic analyses were used to examine the relative contributions of 5-HT and NE to antinociception in a model of acute inflammatory (visceral) pain model (Tallarida, 2001, 2002). In the functional uptake studies, the rank order potency at SERT was paroxetine > fluoxetine > desipramine > reboxetine, and at NET, it was desipramine = reboxetine > paroxetine > fluoxetine. The present findings demonstrate that these compounds have differential activity in preclinical

Fig. 3. Correlation of in vitro potency at hNET and hSERT with percentage maximal effect in the PPQ model of visceral pain. A and B, activity in the hNET functional assay (A) and the activity in hSERT functional assay (B) where all IC50 data are in the nanomolar range are shown. Data are expressed as percentage blockade relative to vehicle-treated mice. Each point represents the mean data with n = 10/group.

Fig. 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 (A), 1:3 (B), and 3:1 (C) of fluoxetine and desipramine were evaluated. Closed black circles correspond to the ED30 values (i.e., dose that 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. * indicates statistical significance (p < 0.05).
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 that reboxetine was inactive (Pedersen et al., 2005). Furthermore, 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 is 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 using 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 that 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 with the SRI compounds. Furthermore, the maximal effect observed with both desipramine and reboxetine was similar in magnitude to that observed with the positive control, the nonsteroidal anti-inflammatory drug 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 versus 15%, respectively), suggesting that SRI activity may be more beneficial for treating inflammatory and or visceral pain conditions. The present data are 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 days) administration in a rat acetic acid model (Korzynska-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 finding suggest that compounds with selective activity at NET or SERT are active in models of visceral pain. However, in the present studies using 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., percentage maximal effect and ED$_{30}$ values) compared with 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 seemed to be increased (i.e., lower ED$_{30}$ values). Three different ratio combinations of fluoxetine and desipramine (SRI: NRI combinations) were evaluated, and although the 1:1 combination clearly achieved synergy, the 3:1 and 1:3 combination both produced significant effects but approached 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 two 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. 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, which 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 in Baker, 2005). Furthermore, SERTs are located within the GI tract, and SERT knockout mice exhibit GI symptoms (e.g., diarrhea) (Baker, 2005). Thus, the active role 5-HT that 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; how-

TABLE 3

Summary of ED$_{30}$ values (milligram/kilogram subcutaneously) from isobolographic studies

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Desipramine ED$_{30}$</th>
<th>Fluoxetine ED$_{30}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dose mg/kg</td>
<td></td>
</tr>
<tr>
<td>Alone</td>
<td>4.4 ± 0.7</td>
<td>23.3 ± 6</td>
</tr>
<tr>
<td>1:1</td>
<td>0.5 ± 0.1</td>
<td>2.6 ± 0.4</td>
</tr>
<tr>
<td>1:3</td>
<td>0.6 ± 0.1</td>
<td>9.3 ± 1.6</td>
</tr>
<tr>
<td>3:1</td>
<td>2.0 ± 0.2</td>
<td>3.5 ± 0.6</td>
</tr>
</tbody>
</table>
ever, 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 SNP 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 suggests 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 of 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, because 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). However, these studies do 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. These data also support 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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References


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