Neuropeptide FF Receptors Have Opposing Modulatory Effects on Nociception

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ABSTRACT

The role of neuropeptide FF (NPFF) and its analogs in pain modulation is ambiguous. Although NPFF was first characterized as an antiopioid peptide, both antinociceptive and pronociceptive effects have been reported, depending on the route of administration. Currently, two NPFF receptors, termed FF1 and FF2, have been identified and cloned, but their roles in pain modulation remain elusive because of the lack of availability of selective compounds suitable for systemic administration in vivo models. Ligand-binding studies confirm ubiquitous expression of both subtypes in brain tissue, whereas only FF2 receptors are expressed spinally. This disparity in localization has served as the foundation of the hypothesis that FF1 receptors mediate the pronociceptive actions of NPFF. We have identified novel small molecule NPFF receptor agonists and antagonists with varying degrees of FF2/FF1 functional selectivity. Using these pharmacological tools in vivo has allowed us to define the roles of NPFF receptor subtypes as pertains to the modulation of nociception. We demonstrate that selective FF2 agonism does not modulate acute pain but instead ameliorates inflammatory and neuropathic pains. Treatment with a nonselective FF1/FF2 agonist potentiates allodynia in neuropathic rats and increases sensitivity to noxious thermal and to non-noxious mechanical stimuli in normal rats in an FF1 antagonist-reversible manner. Treatment with FF1 antagonists reversed established mechanical allodynia, indicating the possibility of increased NPFF tone through FF1 receptors. In conclusion, we provide evidence for the opposing roles of NPFF receptors and highlight selective FF2 agonism and/or selective FF1 antagonism as potential targets warranting further investigation.

The role of neuropeptide FF (NPFF) and its analogs in pain modulation is ambiguous. Although “classically” NPFF is characterized as an antiopioid peptide, it has been shown to also elicit robust antinociceptive effects depending on the dose and route of administration used (for review, see Yang et al., 2008). For example, intrathecal administration of NPFF or of its stable analog [p-Tyr1,(NMe)Phe3]NPFF (1DMe) either elicits antinociception or potentiates the antinociceptive effect of morphine (Kontinen and Kalso, 1995; Gouardères et al., 1996; Xu et al., 1999). In contrast, intracerebroventricular administration of these agents antagonizes morphine-induced antinociception (Gicquel et al., 1992; Oberling et al., 1993; Dupouy and Zajac, 1995).

To date, two NPFF receptor subtypes, termed FF1 and FF2, have been identified and subsequently cloned (Bonini et al., 2000; Elshourbagy et al., 2000). At present, the specific role of each NPFF receptor subtype, with respect to pain modulation, has not been adequately described. The lack of selective pharmacological tools suitable for systemic administration has made elucidating the in vivo pharmacology of these receptors challenging. Based on results from ligand-binding studies in rodents, it is clear that both NPFF receptors are widely expressed in brain tissue, whereas only the FF2 receptor is expressed spinally (Bonini et al., 2000; Liu et al., 2001; Yang and Iadarola, 2006). The lack of spinal FF1 receptors has led to the hypothesis that the antinociceptive actions of NPFF are mediated via FF2 receptors, whereas the pronociceptive actions of NPFF are mediated via FF1 receptors (Liu et al., 2001). We have identified several novel small molecule, nonpeptidic, ligands with varying degrees of functional selectivity for the NPFF receptor subtypes (Gaubert et al., 2009). The goal of this investigation was to use these pharmacological tools to define the roles of the NPFF receptors as they pertain to pain modulation.

ABBREVIATIONS: NPFF, neuropeptide FF; 1DMe, [p-Tyr1,(NMe)Phe3]NPFF; SNL, spinal nerve ligation; NPAF, neuropeptide AF; BIBP-3226, (R)-N2-(diphenylacetyl)-N-[4-(hydroxyphenyl)-methyl]-argininamide; dPQR, dansyl-Pro-Gln-Arg-NH2; %MPE, percent maximum possible effect; PWT, paw withdrawal threshold; ANOVA, analysis of variance; CI, confidence interval; R-SAT, receptor selection and amplification technology; h, human; CCK, cholecystokinin.
In this study, we present the in vitro and in vivo profiles for a representative set of ligands that have allowed us to unmask the putative roles of the NPF receptor subtypes. Based on our profiling we have identified compounds that, according to in vitro functional assays, are 1) selective agonists for FF2 receptors (i.e., AC-263093), 2) nonselective agonists for NPF receptors (i.e., AC-262616), and 3) selective FF1 receptor antagonists (i.e., AC-262620 and AC-262970). Systemic administration of a nonselective FF1/FF2 receptor agonist resulted in a pronociceptive phenotype as indicated by increased sensitivity to noxious thermal as well as to non-noxious mechanical stimuli. In stark contrast, systemic administration of a selective FF2 receptor agonist resulted in an antinociceptive phenotype as indicated by 1) attenuation of phase II of the formalin test, 2) reversal of carrageenan-induced thermal hyperalgesia, and 3) reversal of mechanical hypersensitivity induced by L5/L6 spinal nerve ligation (SNL). Similar results were obtained with FF1 receptor antagonists.

Taken together, these data suggest that the antinociceptive actions of NPF are driven by activation of FF2 receptors, whereas the pronociceptive and by extension the antipiod actions of NPF are mediated via activation of FF1 receptors. Therefore, selective FF2 receptor agonists and selective FF1 receptor antagonists, alone or in combination, may hold promise for the treatment of various types of chronic pains whether inflammatory or neuropathic in origin.

Materials and Methods

Compounds and Dosing Solutions. The novel small molecule, nonpeptide NPF ligands AC-262616, AC-263093, AC-262620, and AC-262970 were synthesized by ACADIA Pharmaceuticals. Further details pertaining to the chemical properties and structures of these compounds have been disclosed elsewhere (Gaubert et al., 2009). NPF (FLFPQRF-NH₂) and NPAF (AGEGLNSQFWSLAAPQRFNH₂) were purchased from American Peptide Co., Inc. (Sunnyvale, CA) and Bachem Biosciences (King of Prussia, PA), respectively. (R)-N-(2)-(diphenylacetyl)-N-V-(4-hydroxyphenyl)-methyl)-argininamide (BIBP-3226) was purchased from Sigma-Aldrich (St. Louis, MO). The N-terminally modified tripeptide amide and putative NPF antagonist, dansyl-PQRF-NH₂ (dPQR), was synthesized by Phoenix Pharmaceuticals (Burlingame, CA). Morphine sulfate was purchased from Sigma-Aldrich. For intraperitoneal administration compounds were dissolved in dimethyl sulfoxide and brought up to volume in sterile 0.9% saline and administered in a final volume of 1 ml/kg b.wt. Doses are expressed in terms of freebase content.

Receptor Selection and Amplification Technology. NIH-3T3 cells were cotransfected with the mixture of plasmid DNAs containing receptor of interest and β-galactosidase. The plates were incubated at 37°C in a humidified CO₂ incubator for 18 to 24 h, the transfection medium was removed, and cells were harvested and frozen for future use. On the day of the assay, cells were plated onto 96-well tissue culture plates containing various concentrations of ligands. For agonist assays, only agonist was added to the wells. For antagonist assays, antagonist was added to the wells, and the cells were spiked with a fixed concentration of agonist (NPFF and NPAF for FF2 and FF1 receptors, respectively) before addition to the wells. After 5 days of incubation, the cells were assayed for production of β-galactosidase by adding ortho-nitrophenyl-β-galactoside in a 0.5% Nonidet P40 solution to the wells and measuring the absorbance at 420 nm (Burstein et al., 1997).

cAMP Inhibition. HEK-293T cells were transiently cotransfected with DNA for either human FF1 or FF2 receptors and the DNA for EP2 receptor in 10-cm² tissue culture plates. After 48 h, cells were harvested and plated onto 96-well TC plates. Inhibition of cAMP production was measured in the presence of varying concentrations of the agonist of interest after stimulation of cAMP production by prostaglandin E₂ (agonist of the EP2 receptor). For antagonist assays, the ability of each compound to reverse the inhibitory effect of NPFF on cAMP production stimulated by prostaglandin E₂ was measured. cAMP levels were measured using a DiscoveRx Hit Hunter cAMP CL kit following the manufacturer’s instructions.

Binding Assays. HEK-T cells were transfected with either FF1 or FF2 receptors. Cells were harvested 48 h after transfection, and cell homogenates were prepared. The Kd of 125I-NPFF was determined to be 50 and 100 pM for FF2 and FF1 receptors, respectively. Competition binding assays were performed in the presence of 70 pM 125I-NPFF (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and various concentration of the ligand in 96-well plates as described previously (Mollereau et al., 2002). Incubations were terminated by filtration onto GF/B filters followed by three washes with ice-cold buffer. Radioactivity retained on the filters was quantitated by scintillation counting in a Top Count counter (PerkinElmer Life and Analytical Sciences, Waltham, MA). Data were fitted to a nonlinear regression model with an equation for one-site competition using Prism (GraphPad Software Inc.).

Animals. Male, Sprague-Dawley rats (150–300 g; Harlan, Indianapolis, IN) were housed in groups of three in a climate-controlled room on a 12-h light/dark cycle (lights on at 7:00 AM), with food and water available ad libitum. All testing was performed in accordance with the policies and recommendations of the International Association for the Study of Pain and the National Institutes of Health guidelines for the handling and use of laboratory animals and received approval from the Institutional Animal Care and Use Committee of ACADIA Pharmaceuticals.

Formalin Test. Naive rats were injected with 50 μl of a 5.0% formalin solution into the dorsal surface of a hind paw and then placed in individual plastic cages for observation. The number of nociceptive responses (i.e., paw flinches/licks/bites) was counted for a period of 60 min after formalin injection. Vehicle or test compounds were administered 15 min before formalin injection.

Carrageenan-Induced Thermal Hyperalgesia. Naive rats were assessed for their responsiveness to a noxious thermal stimulus. Response latencies were measured using the hot-plate test. Inflammatory pain was produced by injecting 0.1 ml of 2% carrageenan ( Sigma-Aldrich) into the left hind paw. Three hours after carrageenan injection, hot-plate latencies were again obtained. A significant reduction in the hot-plate latency was interpreted as the presence of thermal hyperalgesia. Rats were injected with vehicle or test compound and then tested at various time points after administration. To generate dose-response curves, raw data were converted to percent maximum possible effect (%MPE) by the formula, %MPE = [experimental score – postcarrageenan score]/(naive score – post-carrageenan score) × 100, where the experimental score is the hot-plate latency obtained after compound administration, the postcarrageenan score is the hot-plate latency obtained 3 h after carrageenan treatment, and the naive score is the hot-plate latency obtained before any treatments. Each dose was assessed in separate groups of rats.

Assessment of Thermal Hyperalgesia. This test was performed by placing rats in a Plexiglas enclosure on a thermostatically controlled metal plate maintained at 52°C. The time elapsed until the rat showed an obvious nociceptive response (i.e., licking/stomping/hind paw) was measured. Animals were tested before and at various time points after compound administration. A cutoff time of 40 s was used to prevent tissue damage.

L5/L6 Spinal Nerve Ligation Surgery. SNL injury was produced as described previously (Kim and Chung, 1992) in six separate cohorts of rats, five of which had n = 24 and the sixth had n = 30...
subjects. Anesthesia was induced with 2% isoflurane in O₂ at 2 l/min and maintained with 1.5% isoflurane in O₂. The dorsal vertebral column from L4 to S2 was exposed, and the L5 and L6 spinal nerves were identified and carefully isolated. The L5 and L6 spinal nerves were tightly ligated distal to the dorsal root ganglion with 6-0 silk suture. The incision was closed, and the animals were observed for uneventful recoveries. Rats were allowed a period of at least 2 weeks before test compound evaluation.

Assessment of Mechanical Hypersensitivity. Paw withdrawal thresholds (PWTs) of the left hind paws (i.e., ipsilateral to SNL) of the rats were determined in response to probing with eight calibrated von Frey filaments (Stoelting Inc., Wood Dale, IL) in logarithmically spaced increments ranging from 0.41 to 15 g (4–150 mN). Each filament was applied perpendicularly to the plantar surface of the paw of rats kept in suspended wire-mesh cages. PWT was determined in grams by sequentially increasing and decreasing the stimulus intensity and estimated using a Dixon nonparametric test. A significant reduction in PWT from the pre-SNL baseline value indicated the presence of mechanical hypersensitivity. Rats were allowed to recover for a minimum of 2 weeks before test compound assessment. Any rat that exhibited motor deficiency or a lack of subsequent increased sensitivity to innocuous mechanical stimulation (PWT ≤4.3 g) was excluded from additional testing (12 of a possible 186). To generate dose-response curves, raw data were converted to %MPE by the formula, %MPE = [(PWT after drug − PWT for vehicle-control)/(15 − PWT for vehicle-control)] × 100. Each dose was assessed in separate groups of rats.

Statistical Analyses. Statistical analyses were conducted using Prism 5 (GraphPad Software Inc.). The effects of test compounds were analyzed by performing two-way ANOVAs. Planned comparisons of means (each group versus vehicle) were performed using a Bonferroni post hoc test, provided a significant main effect was assessed. Any rat that exhibited motor deficiency or a lack of subsequent increased sensitivity to innocuous mechanical stimulation (PWT ≤4.3 g) was excluded from additional testing (12 of a possible 186). Statistical analyses were conducted using Prism 5 (GraphPad Software Inc.). The effects of test compounds were analyzed by performing two-way ANOVAs. Planned comparisons of means (each group versus vehicle) were performed using a Bonferroni post hoc test, provided a significant main effect was detected. Significance was set at the level of $p < 0.05$. The dose that elicited 50% efficacy (ED$_{50}$) and the corresponding 95% confidence interval (95% CI) were determined using linear regression analysis. These calculations were performed with the pharmacological statistics package FlashCalc (Dr. Michael H. Ossipov, University of Arizona, Tucson, AZ).

Results

In Vitro Characterization of Nonpeptidic Small Molecule NPFF Agonists. The in vitro receptor profiles of several novel, nonpeptidic, small molecule NPFF agonists were determined using recombinant human FF1 and FF2 receptors. Two functional assay formats, R-SAT and cAMP assays, in addition to binding assays using $^{125}$I-NPFF were evaluated (Fig. 1; Table 1). As internal reference standards, the peptides NPAF and NPFF were used for FF1 and FF2 receptors, respectively. The pEC$_{50}$ values obtained for these reference peptides in the R-SAT and cAMP assays were consistent with those reported elsewhere using other functional assays (Kotani et al., 2001; Liu et al., 2001). In the R-SAT assay, test compound AC-263093 demonstrated full agonist activity (~100% efficacy) at FF2 receptors while having a potency value that is ~3-fold less than that of the reference peptide NPFF (EC$_{50} = 5.9$ ± 0 versus EC$_{50} = 6.5$ ± 0.3, respectively). However, this compound only demonstrated minimal agonist activity (~12% relative efficacy) at FF1 receptors. Finally, AC-262616 showed full agonist activity at FF2 receptors while demonstrating partial agonist activity at FF1 receptors (Fig. 1, A and B). In both cases AC-262616 was more potent than the reference peptides NPFF (3-fold) and NPAF (>6-fold) for FF2 and FF1 receptors, respectively. Thus, based on our functional assays, compound AC-263093 is a highly selective full agonist of FF2 receptors with little, if any, intrinsic efficacy at FF1 receptors in recombinant systems. However, AC-262616 has agonist activity at both FF1 and FF2 receptors with minimal selectivity.
The agonist activity and functional selectivity of the test compounds were confirmed in the cAMP assay. The selective FF2 agonist, AC-263093, was fully efficacious at FF2 receptors and only showed weak agonist activity at FF1 receptors (23% relative efficacy), confirming that this compound is functionally selective for FF2 over FF1 receptors. AC-262616, which displayed agonist activity at both NPFF receptors in the R-SAT assay, behaved similarly in the cAMP assay. Moreover, as in R-SAT, AC-262616 demonstrated slightly higher potency at FF1 over FF2 receptors in the cAMP assay (Fig. 1, C and D).

The selective FF2 agonist, AC-263093, retained its selectivity in the binding assay (2 fold for FF2 over FF1), whereas AC-262616 displayed equal or increased affinity for the FF1 receptor (Table 1). Furthermore, AC-263093 showed little to no binding across a broad screening panel \((n = 50)\) of other receptors, channels, and enzymes \((IC_{50} \geq 10 \text{ M}; \text{CEREP, Poitiers, France})\).

### In Vitro Characterization of Nonpeptidic Small Molecule FF1 Antagonists

The in vitro receptor profiles of several novel, nonpeptidic, small molecule NPFF antagonists were determined using recombinant human FF1 and FF2 receptors. Two functional assay formats, R-SAT and cAMP assays, as well as binding assays using \(^{125}\text{I-NPFF}\) were used for this characterization (Fig. 2; Table 2). As internal reference standards, the peptides BIBP-3226 and dPQR were included for comparison in both functional assays (Table 2). The p\(K_i\) values obtained for BIBP-3226 in the R-SAT and cAMP assays were consistent with those reported elsewhere (Mollereau et al., 2002). The lack of stability of dPQR precluded us from characterizing it in R-SAT; however, the p\(K_i\) value obtained in the cAMP for-
TABLE 2
In vitro activity of NPFF receptor antagonists at human FF1 and FF2 receptors as measured by R-SAT, cAMP, and binding assays

<table>
<thead>
<tr>
<th>Compound</th>
<th>R-SAT Antagonist pKᵦ</th>
<th>cAMP Antagonist pKᵦ</th>
<th>Binding Kᵦ (nM)</th>
<th>R-SAT Antagonist pEC₅₀ (%Eff)</th>
<th>Binding Kᵦ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIBP-3226</td>
<td>7.6 ± 0.6</td>
<td>7.5 ± 0.4</td>
<td>N.T.</td>
<td>7.2 ± 0.0 (66 ± 0)</td>
<td>N.T.</td>
</tr>
<tr>
<td>dPQR</td>
<td>N.D.</td>
<td>4.4 ± 0.4</td>
<td>N.T.</td>
<td>N.D.</td>
<td>N.T.</td>
</tr>
<tr>
<td>AC-262620</td>
<td>8.1 ± 0.3</td>
<td>7.7 ± 0.4</td>
<td>16.4 ± 10.1</td>
<td>5.9 ± 0.5 (92 ± 25)</td>
<td>320.3 ± 84.8</td>
</tr>
<tr>
<td>AC-262970</td>
<td>8.2 ± 0.3</td>
<td>7.4 ± 0.3</td>
<td>14.3 ± 3.0</td>
<td>6.7 ± 0.3 (96 ± 38)</td>
<td>17.3 ± 0.20.5</td>
</tr>
</tbody>
</table>

N.T., not tested.

* dPQR is not stable enough for R-SAT assays and thus was not determined (N.D.).

mat was concordant with previous binding studies (Prokai et al., 2001).

Test compounds AC-262970 and AC-262620 demonstrated potent antagonist activity at FF1 receptors in both R-SAT and cAMP assay formats. Although showing similar binding affinity for FF2 and FF1 receptors (Kᵦ values of 17.3 ± 20.5 and 14.3 ± 5.0 nM, respectively) (Fig. 2C), AC-262970 was a selective antagonist of FF1 receptors. AC-262970 antagonized the effect of NPFF (R-SAT) and NPFF (cAMP) at FF1 receptors, achieving Kᵦ values of 8.2 ± 0.3 and 7.4 ± 0.4, respectively (Fig. 2, A and B). AC-262970 behaved as a full agonist at FF2 receptors with a pEC₅₀ value of 6.7 ± 0.3, representing a 30-fold lower activity at FF2 receptors based on functional activity in the R-SAT assay (Table 2). Likewise, AC-262620 showed functional selectivity as an antagonist at the FF1 receptor. This compound antagonized the effect of NPFF (R-SAT) and NPFF (cAMP) at FF1 receptors, achieving pKᵦ values of 8.1 ± 0.3 and 7.7 ± 0.4, respectively (Fig. 2, D and E). AC-262620 behaved as a full agonist at FF2 receptors with a pEC₅₀ value of 5.9 ± 0.5, representing >150-fold lower activity at FF2 receptors based on functional activity in the R-SAT assay (Table 2). However, unlike AC-262970, selectivity of AC-262620 for FF1 receptors was further demonstrated by 20-fold higher binding affinity for FF1 receptors compared with FF2 receptors, having Kᵦ values of 16.4 ± 10.1 and 320.3 ± 84.8 nM, respectively (Fig. 2F). Furthermore, AC-262970 showed little to no binding to a broad screening panel (n = 50) of other receptors, channels, and enzymes (IC₅₀ > 10 μM).

Effects of the Selective FF2 Receptor Agonist AC-263093 on Thermal Hyperalgesia Produced by Carrageenan. At 3 h after intraplantar carrageenan administration, rats demonstrated significant reductions in hot-plate latency; values obtained before carrageenan and after carrageenan were 10.7 ± 0.1 and 6.8 ± 0.4 s, respectively (p < 0.001, Bonferroni post-test). Administration of AC-263093 intraperitoneally produced a significant dose- and time-dependent attenuation of thermal hypersensitivity produced by carrageenan treatment (Fig. 3B). The two-way ANOVA yielded F₃,₅,₁₂₀ = 28.4, p < 0.0001 and F₆,₁₂₀ = 55.5, p < 0.0001 for factors associated with dose and time, respectively. Furthermore, the dose × time interaction was statistically significant, F₁₈,₁₂₀ = 5.4, p < 0.0001. AC-263093 at doses of 1 and 3 mg/kg was without effect. In contrast, both the 3 and 10 mg/kg doses of AC-263093 produced significant attenuation of carrageenan-induced thermal hypersensitivity, and the levels of efficacy were maintained for 2 to 3 h (p < 0.05, Bonferroni post-tests). The effect of 10 mg/kg AC-263093 was absent by 3 h as indicated by a return of hot-plate latency to pretreatment levels (p > 0.05, Bonferroni post-tests). The calculated ED₅₀ value for intraperitoneal AC-263093 was 2.4 mg/kg (1.6–3.6 (95% CI)) after 30 min post-treatment. No significant effects of 10 mg/kg AC-263093 were noted in non-inflamed rats (p > 0.05, Bonferroni post-tests), suggesting that systemic administration of a selective FF2 agonist is unlikely to alter basal sensory thresholds (Fig. 3C).

Effects of the Selective FF2 Receptor Agonist AC-263093 on SNL-Induced Mechanical Hypersensitivity. After L5/L6 SNL rats show significant reductions in PWTs, values obtained before SNL and after SNL were 15 ± 0 and 2.7 ± 0.2 g, respectively (p < 0.05, Bonferroni post-test). AC-263093 produced a dose-dependent reversal of SNL-induced mechanical hypersensitivity, and the levels of efficacy were maintained for 2 to 3 h (p < 0.05, Bonferroni post-tests). The calculated ED₅₀ value for intraperitoneal AC-263093 was 2.4 mg/kg (1.6–3.6 (95% CI)) after 30 min post-treatment. No significant effects of 10 mg/kg AC-263093 were noted in non-inflamed rats (p > 0.05, Bonferroni post-tests), suggesting that systemic administration of a selective FF2 agonist is unlikely to alter basal sensory thresholds (Fig. 3C).
produced significant attenuation of SNL-induced mechanical hypersensitivity and the levels of efficacy were maintained for 1 to 2 h \( (p < 0.001, \text{Bonferroni post-tests}) \). The effect of 30 mg/kg AC-263093 was absent by 3 h as indicated by a return of PWTs to pretreatment levels \( (p > 0.05, \text{Bonferroni post-tests}) \). The calculated ED_{50} value for intraperitoneal AC-263093 was 6.2 mg/kg \([4.7–8.1 \text{ (95\% CI)}]\) after 30 min post-treatment.

**Effects of the Nonselective NPFF Receptor Agonist AC-262616 on SNL-Induced Mechanical Hypersensitivity.** After L5/L6 SNL rats show significant reductions in PWTs, values obtained before SNL and after SNL were 15 ± 0 and 2.7 ± 0.1 g, respectively \( (p < 0.05, \text{paired } t \text{ test}) \). AC-262616 produced a dose-dependent potentiation of SNL-induced mechanical hypersensitivity after intraperitoneal administration (Fig. 4B). A two-way repeated-measures ANOVA of the data revealed significant main effects for both dose and time, \( F_{3,160} = 7.9, p = 0.001 \) and \( F_{8,160} = 740.2, p < 0.0001 \), respectively, as well as a significant dose × time interaction, \( F_{(24,160)} = 2.5, p = 0.0004 \). AC-262616 at a dose of 1 mg/kg was without effect as the PWTs were not significantly different from those values obtained for the vehicle-treated controls \( (p > 0.05, \text{Bonferroni post-tests}) \). In contrast, both the 3 and 10 mg/kg doses of AC-262616 produced a significant potentiation of SNL-induced mechanical hypersensitivity, and this pronociceptive effect peaked 1 h post-treatment and was maintained for an additional 0.5 to 1.5 h \( (p < 0.01, \text{Bonferroni post-tests}) \). To further characterize the pronociceptive actions of AC-262616, we administered a dose of 10 mg/kg i.p. AC-262616 to sham-operated SNL and to naive rats and monitored PWTs and hot-plate latencies, respectively.

Administration of AC-262616 (10 mg/kg i.p.) to sham-operated SNL rats produced a marked and significant reduction in PWTs (Fig. 4C). A two-way repeated-measures ANOVA of these data revealed significant main effects for both treatment and time, \( F_{1,10} = 46.9, p < 0.0001 \) and \( F_{8,90} = 22.1, p < 0.0001 \), respectively, as well as a significant treatment × time interaction, \( F_{8,90} = 19.5, p < 0.0001 \). The onset of action for AC-262616 was approximately 1 h and the pronociceptive action persisted at least 2 h \( (p < 0.01, \text{Bonferroni post-tests}) \). Likewise, intraperitoneal administration of AC-262616 (10 mg/kg) to naive rats significantly decreased hot-plate latencies (Fig. 4D). A two-way repeated-measures ANOVA of these data revealed significant main effects for both treatment and time, \( F_{1,10} = 6.3, p = 0.031 \) and \( F_{5,50} = 9.9, p < 0.0001 \), respectively, as well as a significant treatment × time interaction, \( F_{5,50} = 8.8, p < 0.0001 \). The onset of action for AC-262616 was approximately 90 min, and the pronociceptive action persisted at least 30 min \( (p < 0.001, \text{Bonferroni post-tests}) \). These data confirm the pronociceptive actions of AC-262616 across two different sensory modalities.

**Effects of the FF1 Receptor Antagonist dPQR on the Pronociceptive Effects of AC-262616.** To address whether the pronociceptive effects of AC-262616 were driven by FF1 receptors, we attempted to pharmacologically block the effects of AC-262616 with dPQR, a putative NPFF receptor antagonist (Fig. 5A). After intraperitoneal administration of AC-262616 (10 mg/kg) to naive rats, there was a significant decrease in PWTs in response to von Frey filaments; values

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**Fig. 3.** Effects of NPFF receptor agonists on various types of pain. A, data are expressed as average number of flinches over a 5-min period. Animals were administered vehicle (○), morphine 10 mg/kg (●), or AC-263093 10 mg/kg (□) intraperitoneally, and the number of flinches was tabulated across a 1-h testing period. B, data are expressed as average hot-plate latency in seconds. Carrageenan-treated rats received intraperitoneal vehicle (○) or the selective FF2 receptor agonist AC-263093 at doses of 1 (●), 3 (□), and 10 mg/kg (□), and hot-plate latencies were obtained at various time points across a 3-h testing period. C, data are expressed as average hot-plate latency in seconds. Noninflamed rats received intraperitoneal vehicle (○) or 10 mg/kg AC-263093 (●), and hot-plate latencies were obtained at various time points across a 3-h testing period. D, data are expressed as average hot-plate latency in seconds. Naive rats received intraperitoneal vehicle (○) or 10 mg/kg AC-262616 (●) and hot-plate latencies were obtained at various time points across a 3-h testing period. ***, p < 0.001; **, p < 0.01; *, p < 0.05, versus vehicle controls (two-way repeated-measures ANOVA followed by Bonferroni post-tests). n = 6 rats/group.
obtained before treatment (i.e., naive) and after treatment (i.e., baseline) were 14.7 ± 0.2 and 4.4 ± 0.4 g, respectively (p ≤ 0.05, paired t test). Immediately after baseline testing, rats received either vehicle or dPQR (30 mg/kg) intraperitoneally and then were tested across a 2.5-h period. A two-way repeated-measures ANOVA of these data revealed significant main effects for both treatment and time, F(2,120) = 64.7, p < 0.0001 and F(5,120) = 51.1, p < 0.0001, respectively, as well as, a significant treatment × time interaction, F(10,120) = 23.9, p < 0.0001. AC-262616-treated rats that received intraperitoneal vehicle treatment continued to demonstrate significantly lower PWTs relative to vehicle-treated controls (p ≤ 0.001, Bonferroni post-tests). In contrast, AC-262616-treated rats that received 30 mg/kg i.p. dPQR showed a significant reversal of mechanical hypersensitivity. The effect of 30 mg/kg dPQR was almost complete, but short-lived as the reversal was maintained for approximately 0.5 h (p < 0.001).

Effects of the FF1 Receptor Antagonist dPQR on SNL-Induced Mechanical Hypersensitivity. After L5/L6 SNL rats show significant reductions in PWTs; values obtained before SNL and after SNL were 15 ± 0 and 2.4 ± 0.2 g, respectively (p ≤ 0.05, paired t test). Administration of intraperitoneal dPQR produced a dose-dependent reversal of SNL-induced mechanical hypersensitivity (Fig. 5B). A two-way repeated-measures ANOVA of these data revealed significant main effects for both dose and time, F(3,160) = 17.4, p < 0.0001 and F(8,160) = 141.6, p < 0.0001, respectively, as well as a significant dose × time interaction, F(24,160) = 6.5, p < 0.0001. A dose of 3 mg/kg dPQR produced a small increase in PWTs, which peaked at 15 min and declined over time; this effect was not significantly different from those values obtained for the vehicle-treated controls (p > 0.05, Bonferroni post-tests). In contrast, both the 10 and 30 mg/kg doses of dPQR produced significant attenuation of SNL-induced mechanical hypersensitivity, and the levels of efficacy were maintained for 1 h (p < 0.05, Bonferroni post-tests). The effects of 10 and 30 mg/kg dPQR were absent by 1 h as indicated by a return of PWTs to pretreatment levels (p > 0.05, Bonferroni post-tests). The short-lived effects of dPQR are consistent with its published pharmacokinetic parameters (Prokai et al., 2000). The calculated ED[50] value for dPQR administered intraperitoneally was 9.5 mg/kg [7.0–12.7 (95% CI)] after 15 min post-treatment.

Effects of the NPPF Receptor Antagonists AC-262970 and AC-262620 on SNL-Induced Mechanical Hypersensitivity. After L5/L6 SNL rats show significant reductions in PWTs; values obtained before SNL and after SNL were 15 ± 0 and 2.4 ± 0.2 g, respectively (p ≤ 0.05, paired t test). AC-262970 produced a dose-dependent reversal of SNL-induced mechanical hypersensitivity after intraperitoneal administration (Fig. 6A). A two-way repeated-measures ANOVA of these data revealed significant main effects for both dose and time, F(3,90) = 12.8, p = 0.0001 and F(5,90) = 203.3, p < 0.0001, respectively, as well as a significant dose × time interaction, F(15,90) = 6.4, p < 0.0001. AC-262970 at a dose of 1 mg/kg produced a small increase in PWTs, which peaked at 30 min and declined over time; this effect was not significantly different from those values obtained for the
vehicle-treated controls ($p > 0.05$, Bonferroni post-tests). In contrast, both the 3 and 10 mg/kg doses of AC-262970 produced significant attenuation of SNL-induced mechanical hypersensitivity, and the levels of efficacy were maintained for up to 1 h ($p < 0.05$, Bonferroni post-tests). The effect of 3 and 10 mg/kg AC-262970 were absent by 2 h as indicated by a return of PWTs to pretreatment levels ($p > 0.05$, Bonferroni post-tests).

**Discussion**

In the present investigation, we report on the activity of a set of novel, small molecule, NPFF receptor ligands that are exemplified by 1) AC-263093, a selective FF2 receptor agonist, which is fully efficacious in vitro in both R-SAT and cAMP functional assays at hFF2 receptors (relative to NPFF) while virtually devoid of intrinsic efficacy at hFF1 receptors (relative to NPAF), 2) AC-262616, a pan FF1/FF2 receptor agonist, which is fully efficacious at both NPFF receptor subtypes in an cAMP assay, and 3) AC-262970 and AC-262620, selective FF1 antagonists, which bind hFF1 receptors with low nanomolar affinity and display functional selectivity values of 30- and 150-fold, respectively, for FF1 over FF2 receptors. Using these molecules we have, for the first time, been able to elucidate the pharmacology of NPFF receptors after systemic administration. Our results provide the first direct in vivo evidence for the opposing roles of NPFF receptor subtypes as pertains to the modulation of nociception. Using these compounds, we have been able to directly show that FF1 receptor activation is pronociceptive, whereas FF2 receptor activation is antinociceptive.

Intrathecal administration of NPFF elicits acute antinociception and enhances the efficacy of spinal opioids, whereas intracerebroventricular administration of NPFF antagonizes the effects of opioids and results in pronociceptive effects (for review, see Yang et al., 2008). Receptor localization studies have established that whereas NPFF receptors are expressed ubiquitously in the brain, only the FF2 receptor has been detected at the level of the spinal cord (Bonini et al., 2000; Liu et al., 2001; Yang and Iadarola, 2006). The apparent lack of FF1 receptor expression spinally has led to the idea that the pronociceptive actions of NPFF are mediated via FF1 receptors (Liu et al., 2001). However, this hypothesis has never been adequately addressed in vivo because of a lack of highly selective nonpeptidic NPFF ligands suitable for systemic administration. Using our novel small molecule, nonpeptidic NPFF receptor ligands as pharmacological tools, we have clearly demonstrated that NPFF receptors act in an opposing manner to modulate nociceptive input.

We were unable to detect any significant antinociceptive effects of AC-263093, a selective FF2 selective agonist, after intraperitoneal administration in the hot-plate test. Although it remains plausible that NPFF agonists can potentiate the antinociceptive actions of opioids (Kontinen and Kalso, 1995; Gouardères et al., 1996; Xu et al., 1999), depending on the route of administration, this possibility was beyond the scope of the present investigation. Given the lack of activity of AC-263093 on acute sensory thresholds, we also profiled this compound in the formalin and carrageenan models. Like morphine, AC-263093 markedly attenuated flinching behavior, although this effect was specific to the second
phase, suggesting that AC-263093 may be active in models of spinal sensitization. Consistent with this interpretation, AC-263093 dose dependently alleviated thermal hyperalgesia produced by intraplantar carrageenan, an effect similar to that previously reported for 1DMe after intrathecal administration (Xu et al., 1999). Increases in NPFF immunoreactivity have been reported in the spinal cord dorsal horn after carrageenan treatment (Kontinen et al., 1997). Morphine pretreatment completely blocks the increase in spinal NPFF immunoreactivity (Kontinen et al., 1997), suggesting that enhanced release of NPFF may promote thermal hyperalgesia. NPFF binding sites are not significantly altered by either neonatal capsaicin treatment or by dorsal rhizotomy receptors, suggesting that these receptors are almost exclusively expressed postsynaptically in the spinal cord (Lombard et al., 1995). It is possible that spinal FF2 receptors may act as autoreceptors to negatively regulate NPFF release. A reduction in endogenous NPFF release could drive the antihyperalgesic effects of selective FF2 agonists as well as provide a mechanism by which the antinociceptive activity of intrathecal morphine may be enhanced by these agents.

In addition, systemic administration of AC-263093 dose dependently attenuated mechanical hypersensitivity after SNL, without altering the response thresholds of sham-operated rats, consistent with previous studies using microinjections in discrete brainstem regions (Xu et al., 1999; Altier et al., 2000; Wei et al., 2001). In stark contrast to the effects observed with AC-263093, intraperitoneal administration of the nonselective FF1/FF2 receptor agonist AC-262616 potentiated the severity of the SNL-induced hypersensitivity. Furthermore, intraperitoneal administration of AC-262616 produced marked increases in sensitivity to both noxious thermal and to non-noxious mechanical stimuli in both naive and sham-operated rats, respectively. Given that efficacy observed in the SNL model is correlated with the degree of FF2 functional selectivity, we hypothesized that the pronociceptive actions were a direct result of supraspinal FF1 receptor activation.

To date, the only well characterized and commercially available NPFF receptor antagonist is BIBP-3226 (Mollereau et al., 2002; Fang et al., 2006). Although intracerebroventricular administration of BIBP-3226 has been shown to block the effects produced by NPFF and NPVF (Fang et al., 2006), BIBP-3226 remains a very potent and selective neuropeptide Y1 receptor antagonist (Doods et al., 1995). As a result, efficacy measured in vivo would be confounded as NPY1 antagonists effectively reverse SNL-induced mechanical hypersensitivity in their own right (Ossipov et al., 2002). Therefore, we opted for dPQR, a putative NPFF receptor antagonist (Prokai et al., 2001), which crosses the blood-brain barrier (Prokai et al., 2000), for our initial in vivo assessments. Systemic administration of dPQR reversed the pronociceptive actions of AC-262616, confirming biological activity of this FF1 receptor antagonist.

To determine whether endogenous NPFF systems play a role in the maintenance of mechanical hypersensitivity after SNL, we administered dPQR to neuropathic rats. Systemic administration of dPQR dose dependently alleviated allodynia with a duration of action consistent with its published pharmacokinetic profile (Prokai et al., 2001). These initial proof-of-concept data provided the impetus for the discovery of FF1 receptor antagonists. Like dPQR, both
AC-262970 and AC-262620, two highly potent FF1 antagonists, with low nanomolar affinity for FF1 receptors, attenuated SNL-induced mechanical hypersensitivity. It should be noted that these antagonists also elicited full agonist activity at FF2 receptors with potencies in the range of AC-263093. Therefore, one possible interpretation is that the FF2 agonist activity of AC-262970 and AC-262620 is sufficient to attenuate SNL-induced hypersensitivity. However, this possibility is unlikely given that 1) administration of AC-262616 produced mechanical and thermal hypersensitivity in naive rats, 2) dPQR is devoid of FF2 agonist activity, and 3) AC-262620 is >150-fold selective in the functional R-SAT assay and is 20-fold selective for FF1 receptors in binding assays. Taken together, these data support the concept that after peripheral nerve injury, FF1 receptor tone may be enhanced.

As mentioned previously, we are unaware of any reports confirming the presence of FF1 receptors in the spinal cord of rats; therefore, the pronociceptive actions of FF1 receptor activation would most likely originate in the brain. Because NPVF preferentially activates FF1 receptors, this endogenous peptide may be the source of pronociceptive drive, as in vitro studies have confirmed that it possesses antiopioid activity (Liu et al., 2001; Kersanté et al., 2006). The ability of AC-262616 to significantly decrease baseline sensory thresholds suggests the possibility that FF1 receptor activation, in addition to promoting morphine antinociceptive tolerance, may also contribute to opioid-induced paradoxical pain. This idea is supported by the observation that intracerebroventricular administration of an anti-NPFF antiserum completely restores the efficacy of intracerebroventricular morphine in tolerant rats (Lake et al., 1991). In addition, administration of a pan FF1/FF2 receptor antagonist, RF9, prevents heroin-induced hyperalgesia and effectively blocks the development of antinociceptive tolerance after repeated heroin treatment (Simonin et al., 2006).

Mechanistically, opioid-induced paradoxical pain and neuropathic pain share common features, including the activation of descending pain facilitatory pathways (Porreca et al., 2001; Burgess et al., 2002; Gardell et al., 2002). The activation of descending pain facilitation by antiopioid peptides is not without precedence. For example, microinjection of cholecystokinin (CCK) into the rostral ventromedial medulla produces mechanical hypersensitivity in naive rats (Xie et al., 2005), an effect similar to that observed after administration of AC-262616. Furthermore, CCK2 receptor antagonists restore the antinociceptive potency of morphine in tolerant rats, reverse opioid-induced paradoxical pain (Xie et al., 2005), and diminish mechanical allodynia in SNL rats (Kovelowski et al., 2000). Given the similarities between the aforementioned effects with CCK and those reported herein with our FF1 receptor ligands, together with those reported elsewhere (Simonin et al., 2006), there is a possibility that supraspinal FF1 receptor activation may drive descending pain facilitatory pathways, although further work in this area is warranted.

In addition to the activation of descending pain facilitatory systems, opioid-induced paradoxical pain and neuropathic pain share another common feature, the up-regulation of spinal dynorphin (Gardell et al., 2002, 2003). Although dynorphin is an endogenous κ opioid receptor agonist, enhanced levels of this peptide promote neuropathic pain (Wang et al., 2001; Burgess et al., 2002), as well as opioid antinociceptive tolerance and opioid-induced paradoxical pain (Gardell et al., 2002). Critically, manipulations that inhibit the pronociceptive actions of spinal dynorphin alleviate neuropathic pain (Wang et al., 2001; Burgess et al., 2002; Gardell et al., 2003, 2004) and reverse antinociceptive tolerance and the accompanying abnormal pain (Vanderah et al., 2000; Gardell et al., 2002). This result is highly relevant because a recent study has shown that 1DMe suppresses spinal dynorphin release in anesthetized rats (Ballet et al., 2002). Because FF1 receptors are not localized spinally, the effects of 1DMe must therefore be driven by FF2 receptors and thus represents a mechanism by which selective FF2 agonists may alleviate neuropathic pain and restore the antinociceptive potency of opioids. Moreover, the suppression of dynorphin release also would have beneficial effects in chronic inflammatory pain as this type of pain is associated with both descending pain facilitation as well as spinal dynorphin up-regulation (Dubner and Ruda, 1992). Of course, this proposed mechanism of selective FF2 agonism does not preclude potential supraspinal effects, although further work in this area is warranted.

In conclusion, we have demonstrated the opposing roles of FF1 and FF2 receptors as they pertain to the modulation of nociception. We have shown that FF2 agonism is efficacious in various pain models, whereas FF1 agonism is pronociceptive. Selective FF2 agonists, FF1 antagonists, or bifunctional ligands may represent novel approaches for the treatment of chronic pain whether of inflammatory or neuropathic in origin. However, identification of the selective tools necessary to validate these targets is only the first step.

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