Synthesis and Pharmacological in Vitro and in Vivo Profile of 3-Oxo-1,1-diphenyl-tetrahydro-oxazolo[3,4-a]pyrazine-7-carboxylic Acid 4-Fluoro-benzylamide (SHA 68), a Selective Antagonist of the Neuropeptide S Receptor

Naoe Okamura, Stephen A. Habay, Joanne Zeng, A. Richard Chamberlin, and Rainer K. Reinscheid

Departments of Pharmaceutical Sciences (N.O., J.Z., A.R.C., R.K.R.), Pharmacology (N.O., J.Z., R.K.R.), Chemistry (S.A.H., A.R.C.), and Molecular Biology and Biochemistry (R.K.R.), University of California, Irvine, California

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ABSTRACT

Neuropeptide S (NPS) has been shown to modulate arousal, sleep wakefulness, anxiety-like behavior, and feeding after central administration of the peptide agonist to mice or rats. We report here the chemical synthesis and pharmacological characterization of SHA 66 (3-oxo-1,1-diphenyl-tetrahydro-oxazolo[3,4-a]pyrazine-7-carboxylic acid benzylamide) and SHA 68 (3-oxo-1,1-diphenyl-tetrahydro-oxazolo[3,4-a]pyrazine-7-carboxylic acid 4-fluoro-benzylamide), two closely related bicyclic piperazines with antagonistic properties at the NPS receptor (NPSR). The compounds block NPS-induced Ca²⁺ mobilization, and SHA 68 shows displaceable binding to NPSR

Neuropeptide S (NPS) and its receptor, NPSR, are a recently identified transmitter system that modulates a number of brain functions (Okamura and Reinscheid, 2007). NPS is a small peptide of 20 amino acids that occurs in all tetrapod vertebrates but is absent from fish (Reinscheid, 2007). Activation of NPSR produces transient increases in intracellular Ca²⁺ and cAMP and thus increases cellular excitability (Reinscheid et al., 2005). Expression of NPS precursors and receptors is found in specific brain areas that have been associated with arousal, emotional processing, energy, and hormonal homeostasis, as well as learning and memory (Xu et al., 2004, 2007). In the rat, NPS precursor transcripts are in the nanomolar range. The antagonistic activity of SHA 68 seems to be specific because it does not affect signaling at 14 unrelated G protein-coupled receptors. Analysis of pharmacokinetic parameters of SHA 68 demonstrates that the compound reaches pharmacologically relevant levels in plasma and brain after i.p. administration. Furthermore, peripheral administration of SHA 68 in mice (50 mg/kg i.p.) is able to antagonize NPSinduced horizontal and vertical activity as well as stereotypic behavior. Therefore, SHA 68 could be a useful tool to characterize physiological functions and pharmacological parameters of the NPS system in vitro and in vivo.

expressed in only a few brainstem structures; in particular, in a previously uncharacterized nucleus situated between the noradrenergic locus coeruleus and Barrington's nucleus. Besides the pericoerulear region, NPS mRNA is only found in the lateral parabrachial nucleus and the principle sensory 5 nucleus of the rat brainstem. A few scattered cells expressing NPS precursor transcripts are also detected in the amygdala and hypothalamus. In the brainstem, the majority of NPSexpressing neurons coexpress other excitatory transmitters, such as glutamate, acetylcholine, or corticotropin-releasing factor (Xu et al., 2007). NPSR mRNA is found at high levels in hypothalamus, thalamus, amygdala, various cortical regions, and the parahippocampal formation. Central administration of NPS was shown to produce profound arousal that is independent of novelty (Xu et al., 2004). NPS is also able to induce wakefulness by suppressing all stages of sleep, as demonstrated by electroencephalographic recordings in rats. In addition, NPS administration was shown to produce anxDownloaded from jpet.aspetjournals.org at ASPET Journals on March 6,

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ABBREVIATIONS: NPS, neuropeptide S; NPSR, neuropeptide S receptor; SHA 66, 3-oxo-1,1-diphenyl-tetrahydro-oxazolo[3,4-a]pyrazine-7carboxylic acid benzylamide; SHA 68, 3-oxo-1,1-diphenyl-tetrahydro-oxazolo[3,4-a]pyrazine-7-carboxylic acid 4-fluoro-benzylamide; ESI, electrospray ionization; THF, tetrahydrofuran; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin; GPCR, G protein-coupled receptor; PBS, phosphate-buffered saline; PK, pharmacokinetic; BBB, blood-brain barrier; LC/MS/MS, liquid chromatography-coupled tandem mass spectrometry; ANOVA, analysis of variance; HEK, human embryonic kidney; Veh, vehicle; Fmoc, 9*H*-fluoren-9-ylmethoxy carbonyl.

iolytic-like effects across various behavioral paradigms measuring responses to stressful or unfamiliar environments in mice (Xu et al., 2004; Leonard et al., 2005). Other studies reported anorexic effects after central NPS administration (Beck et al., 2005; Smith et al., 2006) and modulation of stress hormone levels (Smith et al., 2006), although a recent study challenged the initial findings on NPS-induced inhibition of feeding behavior (Niimi, 2006).

A functional polymorphism in the human NPSR gene has been identified that encodes an Asn-to-Ile amino acid exchange at position 107 of the receptor protein (A/T: single nucleotide polymorphism database accession no. rs324981). NPSR Ile107 shows a 5- to 10-fold increased agonist sensitivity compared with NPSR Asn107 when measuring second messenger responses, although binding affinity for the natural agonist NPS is unchanged at both receptor isoforms (Reinscheid et al., 2005). This polymorphism had been originally associated with an increased risk of asthma and other allergic diseases (Laitinen et al., 2004), but the role of NPSR in airway function is currently unclear (Allen et al., 2006). We have recently found evidence for a gender-specific association of the NPSR N107I polymorphism with panic disorder (Okamura et al., 2007), and a genome-wide screen for markers of sleep behavior and circadian phenotypes identified the NPSR Ile107 polymorphism strongly associated with mean bedtime in a random population cohort (Gottlieb et al., 2007). These early data indicate that the endogenous NPS system might be involved in modulating sleep or circadian behaviors as well as emotional processing.

Characterization of physiological functions modulated by NPS is still at an early stage, and availability of a selective NPSR antagonist is critically important for such research. A series of synthetic compounds with presumed activity at NPSR were published recently in a patent application by Takeda Pharmaceuticals Inc. (Osaka, Japan), but no pharmacological or biological data were presented (Fukatsu et al., 2005). The patent disclosed that structures containing a substituted bicyclic piperazine scaffold possess NPSR antagonistic activity. Two structurally similar compounds were quoted to have affinities below 100 nM, but no primary data were presented to support the claim. We therefore set out to synthesize these compounds and validate their pharmacological properties as potential NPSR antagonists in vitro and in vivo. The availability of synthetic NPSR antagonist compounds will certainly facilitate further research on physiological functions of the NPS system.

Materials and Methods

Chemicals. All chemicals were of analytical grade or higher quality. NPS was synthesized by the Peptide Proteomic Centre, Brain Research Centre, University of British Columbia (Vancouver, BC, Canada), and stock solutions were dissolved in water. [¹²⁵I]Tyr10-NPS was kindly provided as a gift from PerkinElmer Life and Analytical Sciences (Waltham, MA). Other peptides were purchased from Bachem (Torrance, CA) or the American Peptide Co., Inc. (Sunnyvale, CA).

Synthesis and Structure Verification of SHA 66 and SHA 68. ¹H NMR and ¹³C NMR spectra were recorded at ambient temperature at 400 and 100 MHz, respectively, using a Bruker DRX 400 spectrometer (Bruker, Billerica, MA). All spectra were acquired in $CDCl_3$ with chemical shifts reported as δ values in parts per million and are calibrated according to internal CDCl₃ (7.26 ppm) solvent residual peak. The data are reported as follows: multiplicity (br, broad; s, singlet; d, doublet; t, triplet; dd, double doublet; dt, double triplet; m, multiplet), integration, and coupling constants (Hertz). IR spectra were obtained on a PerkinElmer model 1600 series Fouriertransform infrared spectrophotometer and are reported in wave numbers (centimeters⁻¹). High-resolution mass spectra were acquired on a Waters LCT Premier (ESI) spectrometer. Synthesis and characterization of previously unreported compounds are detailed below. Structures are numbered (in bold type) according to the synthesis scheme as depicted in Fig. 1. LogP values for SHA 66 and SHA 68 were calculated with a web-based calculator (http://intro. bio.umb.edu/111-112/OLLM/111F98/newclogp.html).

7-Benzyl-1,1-diphenyl-hexahydro-oxazolo[**3,4-***a*]**pyrazin-3-one (Fig. 1, Structure 6).** To a solution of **4** (1.00 g, 3.74 mmol) in tetrahydrofuran (THF; 12 ml) and N,N,N',N'-tetramethylethylene diamine (1.20 ml, 8.23 mmol) was added *sec*-butyllithium (6.20 ml, 8.23 mmol) drop-wise at -78° C. The mixture was allowed to warm to -30° C over 2 h, at which point the reaction was cooled back to



Fig. 1. Chemical synthesis of SHA 66 and SHA 68. Intermediate compounds are numbered (in bold type) and described under *Materials and Methods*.

-78°C. A solution of benzophenone (1.40 g, 7.48 mmol) in THF (8 ml) was added drop-wise. The flask was allowed to warm to room temperature overnight, and the reaction was quenched by the addition of saturated aqueous $\mathrm{NH_4Cl}$ (10 ml), extracted with ethyl acetate (3 imes30 ml), washed with H_2O , dried (Na_2SO_4), filtered, and concentrated. The residue was purified by column chromatography (10-50%) EtOAc/hexanes) to yield 6 (1.06 g, 76%) as a clear oil that foamed under vacuum. IR (neat): 3027, 2814, 1769, 1449, 1249, 1032, 995, 917, 755, 699 cm⁻¹; ¹H NMR (400 MHz, CDCl₃): δ 7.52–7.55 (m, 2H), 7.23-7.40 (m, 13H), 4.58 (br d, 1H, J = 9.0 Hz), 3.82 (dd, 1H, J = 2.5,13.2 Hz), 3.52 (d, 1H, J = 13.1 Hz), 3.33 (d, 1H, J = 13.1 Hz), 3.12 (br t, 1H, J = 12.1 Hz), 2.71 (br d, 1H, J = 8.5 Hz), 2.58 (dd, 1H, J = 2.6, 11.4 Hz), 1.96 (br t, 1H, J = 8.6 Hz), 1.61 (t, 1H, J = 11.1 Hz); ¹³C NMR (100 MHz, CDCl₃): § 156.1, 142.8, 138.8, 129.1, 128.7, 128.6, 128.5, 128.4, 128.0, 127.5, 126.1, 125.9, 85.4, 63.0, 61.5, 55.9, 50.9, 41.8; HRMS (ESI) calculated for $\mathrm{C}_{25}\mathrm{H}_{24}\mathrm{N}_{2}\mathrm{O}_{2}\mathrm{Na}$ (M+Na) 407.1736, found 407.1732.

3-Oxo-1.1-diphenvl-tetrahvdro-oxazolo[3.4-a]pvrazine-7carboxylic acid 9H-fluoren-9-ylmethyl Ester (Fig. 1, Structure 7). To a solution of 6 (3.48 g, 9.05 mmol) in acetonitrile (45 ml) was added FmocCl (2.58 g, 9.96 mmol) in one portion, and the mixture was brought to reflux at 90°C. After approximately 10 min, a white precipitate formed in the reaction flask. The suspension was allowed to stir at reflux for an additional 5 h, after which the mixture was cooled and vacuum filtered. The precipitate was washed with an additional portion of cold acetonitrile (20 ml). The crude white solid 7 (3.76 g, 80%) was then used without further purification. IR (neat): 1762, 1702, 1449, 1426, 1229, 986, 757, 702 cm $^{-1};\ ^1\mathrm{H}$ NMR (400 MHz, CDCl₃) (spectrum acquired at room temperature): δ 7.74-7.80 (m, 2H), 7.25-7.55 (m, 15H), 7.14-7.16 (m, 1H), 4.50-4.88 (m, 2H), 4.21 (br s, 1.4H), 4.16 (br s, 0.6H), 3.98 (br s, 1H), 3.75 (br s, 1.6H), 3.45 (br s, 0.4H), 2.70–2.92 (m, 2H), 2.13 (t, 1H, J = 12.4 Hz); HRMS (ESI) calculated for $C_{33}H_{28}N_2O_4Na$ (M+Na) 539.1947, found 539.1951.

SHA 66. To a mixture of 7 (2.00 g, 3.87 mmol) and benzyl isocyanate (962 µl, 7.74 mmol) in THF (20 ml) was added 1,8diazabicyclo[5.4.0]undec-7-ene (637 µl, 4.26 mmol) drop-wise. The reaction was stirred for 15 min at room temperature, quenched by addition of saturated aqueous NH₄Cl (10 ml), extracted with ethyl acetate $(3 \times 40 \text{ ml})$, dried (Na_2SO_4) , filtered, and concentrated. The residue was purified by column chromatography (40% EtOAc/hexanes then 10% MeOH/CHCl₃) to give SHA 66 (1.61 g, 98%) as a yellow oil that foamed under vacuum. IR (neat): 3354, 1755, 1633, 1538, 1450, 1263, 1082, 1030, 982, 752, 699; ¹H NMR (400 MHz, $CDCl_3$): δ 7.46–7.48 (m, 2H), 7.15–7.40 (m, 13H), 5.90 (t, 1H, J = 5.5Hz), 4.25–4.41 (m, 3H), 4.05 (dd, 1H, J = 2.7, 13.3 Hz), 3.77 (br d, 1H, J = 11.4 Hz), 3.64 (dd, 1H, J = 2.7, 13.0 Hz), 2.92 (dt, 1H, J = 3.5, 12.7 Hz), 2.67 (dt, 1H, J = 3.4, 13.3 Hz), 2.06 (dd, 1H, J = 11.4, 13.2 Hz); ¹³C NMR (100 MHz, CDCl₃): δ 157.4, 156.0, 141.7, 139.4, 138.2, 128.7, 128.6, 128.5, 128.4, 128.2, 127.4, 127.2, 125.9, 125.7, 85.8,

TABLE 1

Selectivity profile of SHA 68

60.4, 46.5, 44.8, 43.4, 41.2; HRMS (ESI) calculated for $\rm C_{26}H_{25}N_3O_3Na(M+Na)$ 450.1794, found 450.1783.

SHA 68. To a mixture of 7 (2.00 g, 3.87 mmol) and 4-fluorobenzyl isocyanate (986 µl, 7.74 mmol) in THF (20 ml) was added 1,8diazabicyclo[5.4.0]undec-7-ene (636 µl, 4.25 mmol) drop-wise. The reaction was stirred for 15 min at room temperature, guenched by addition of saturated aqueous NH₄Cl (10 ml), extracted with ethyl acetate $(3 \times 40 \text{ ml})$, dried (Na_2SO_4) , filtered, and concentrated. The residue was purified by column chromatography (40% EtOAc/hexanes then 10% MeOH/CHCl₃) to give SHA 68 (1.72 g, 100%) as a clear oil that foamed under vacuum. IR (neat): 3356, 1748, 1633, 1538, 1508, 1449, 1222, 1156, 1032, 981, 832, 753, 701; ¹H NMR (400 MHz, $CDCl_3$): δ 7.49 (br d, 2H, J = 7.2 Hz), 7.22–7.40 (m, 10H), 6.98 (t, 2H, J = 8.6 Hz), 5.07 (t, 1H, J = 5.4 Hz), 4.28-4.42 (m, 3H), 4.03(dd, 1H, J = 2.8, 13.3 Hz), 3.79 (dd, 1H, J = 3.0, 13.0 Hz), 3.67 (br d, Jz)1H, J = 12.1 Hz), 3.04 (dt, 1H, J = 3.6, 12.6 Hz), 2.86 (dt, 1H, J = 3.5, Jz)13.0 Hz), 2.14 (dd, 1H, J = 11.4, 13.1 Hz); ¹³C NMR (100 MHz, CDCl₃): § 157.2, 156.1, 141.9, 138.4, 135.0, 129.5, 129.4, 128.9, 128.8, 128.7, 128.4, 126.0, 125.9, 115.7, 115.5, 85.9, 60.6, 46.6, 44.5, 43.8, 41.4; HRMS (ESI) calculated for $C_{26}H_{24}FN_3O_3Na$ (M+Na) 468.1700, found 468.1700.

Functional Characterization in Vitro. Generation of stable cell lines expressing human NPSR Asn107, NPSR Ile107, and mouse NPSR has been described before (Xu et al., 2004; Reinscheid et al., 2005). Agonist-induced mobilization of intracellular Ca²⁺ was measured using the fluorometric imaging plate reader technology as described previously (Reinscheid et al., 2005). Antagonist compounds were dissolved as 10 mM stock solutions in dimethyl sulfoxide (DMSO) and then diluted into assay buffer containing 0.1% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) with final DMSO levels at <1% (v/v). Half-maximal inhibitory concentrations (IC_{50}) of antagonists were determined in triplicate by preincubating cells stably expressing NPSR for 10 min with the compounds before challenging with various concentrations of NPS peptide. IC₅₀ values were calculated by nonlinear regression analysis using GraphPad Prism (GraphPad Software Inc., San Diego, CA). Dose ratios for agonist dose responses in the presence of different antagonist concentrations were determined for Schild analysis to obtain $K_{\rm b}$ values for antagonist binding. IC_{50} values at functional agonist EC_{50} were obtained by first running an agonist dose response and then using the calculated agonist EC_{50} on the same batch of cells for antagonist dose responses.

Radioligand binding in cells stably expressing NPSR Ile107 was performed as described before (Xu et al., 2004). SHA 68 was tested in triplicate for displacement of [¹²⁵I]Tyr10-NPS. Nonspecific binding was determined in the presence of 1 μ M NPS. K_i values were calculated with GraphPad Prism.

SHA 68 was tested for selectivity against a number of G proteincoupled receptors (GPCRs), expressed stably, transiently, or endogenously in various cell lines as listed in Table 1. Receptors were

5 1			
Receptor	Cell Line	Expression	Agonist
Vasopressin 1a	HEK 293	Transient	Arg8-vasopressin
Oxytocin	HEK 293	Transient	Oxytocin
Tachykinin 1	U 373 MG	Endogenous	Substance P
μ-Opioid	CHO	Stable	Dermorphin
к-Opioid	СНО	Stable	Dynorphin A
Nociceptin/orphanin FQ	СНО	Stable	Orphanin FQ/nociceptin
Somatostatin 2	HEK 293	Transient	Somatostatin-28
Melanin-concentrating hormone 1	HEK 293	Stable	MCH
Neuropeptide Y 2	HEK 293	Transient	Neuropeptide Y
D2 dopamine	HEK 293	Transient	Dopamine
β2-adrenergic	HEK 293	Endogenous	Isoproterenol
Trace amine 1	HEK 293	Stable	Tyramine
P2Y1	1321 N1	Stable	ADP
P2Y12	CHO	Stable	ADP

CHO, Chinese hamster ovary.

preincubated with 10 μ M SHA 68 for 10 min and then activated with 100 nM of their corresponding agonists (Table 1) to test for both agonistic and antagonistic activity. Receptor activation was monitored as mobilization of intracellular Ca²⁺ using the fluorometric imaging plate reader technology. In cases of G_i-linked receptors (e.g., D2 dopamine receptor or opioid receptors), the corresponding cell lines were cotransfected with a chimeric G protein that allows for coupling of G_i-linked receptors to the phospholipase C pathway, as described before (Saito et al., 1999).

In Vivo Studies. Male C57BL/6 mice (National Cancer Institute, Bethesda, MD; or Charles River Laboratories, Wilmington, MA), 8 to 12 weeks old, were group-housed (four animals per cage) under controlled conditions (temperature, $21 \pm 2^{\circ}$ C; relative humidity, 50-60%; 12-h light/dark cycle; lights on 6:00 AM) with free access to food and water. For i.c.v. drug injections, mice were briefly anesthetized with halothane. NPS was dissolved in phosphate-buffered saline (PBS), pH 7.4, containing 0.1% BSA and injected i.c.v. (total volume, 2 µl) as described before (Xu et al., 2004). SHA 68 was dissolved in PBS containing 10% Cremophor EL (Sigma-Aldrich), and 100 µl was injected i.p.

For pharmacokinetic (PK) studies, mice were weighed and then received i.p. or i.v. injections with the drug dissolved in a vehicle suitable for injection (5% dimethylacetamide, 5% Cremophor EL, and 90% PBS at 0.2 mg/ml for i.v. and 2.5% dimethylacetamide, 2.5% Cremophor EL, and 95% PBS at 0.5 mg/ml for i.p.). A total of 24 animals were used for a total of six time points with four animals per time point. Animals were euthanized, and blood was collected in heparinized tubes. Plasma was isolated by centrifugation, and drug levels were determined by liquid chromatography-coupled tandem mass spectrometry (LC/MS/MS) according to the analytical method described below. Likewise, 12 animals were used for blood-brain barrier (BBB) penetration studies for a total of three time points (0.25, 1, and 2 h) and four animals per time point. At the indicated time intervals, animals were euthanized. Their blood was collected, and plasma was isolated for plasma drug level determination by LC/MS/MS. Brains were harvested, blotted on absorbent paper, and homogenized in a 50:50 (v/v) mixture of saline and ethanol (3 ml/g brain). Drug concentration in brain tissue was determined using the same LC/MS/MS method. All experiments were carried out in accordance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council, 2003) and approved by the local Institutional Animal Care and Use Committees.

Analytical Method. Plasma proteins were precipitated by mixing plasma samples with a stock solution of reserpine in acetonitrile (1 volume of plasma for 3 volumes of acetonitrile). After centrifugation, the supernatant was isolated and analyzed by LC/MS/MS. Homogenized brain samples were treated in a similar fashion with 3 volumes of the acetonitrile solution containing internal standard (reserpine). Precipitated material was removed by centrifugation, and the supernatant was analyzed by LC/MS/MS. Standard curves were prepared by spiking plasma or brain homogenate with a stock solution of test compound dissolved in DMSO. The spiked samples were treated as described above.

Samples were analyzed on a Finnigan TSQ Quantum Ultra (Thermo Electron Corporation, Waltham, MA) mass spectrometer, equipped with a Turbo-Ionspray interface, in positive-ion mode (source temperature, 400°C) and with an Agilent 1100 Series LC system comprising autosampler, pump, and column oven (Agilent Technologies, Santa Clara, CA). A Kromasil 100-5 C4 (30×4.6 mm) column was used for the separation (3-min gradient using 0.1% formic acid in water as solvent A and 0.1% formic acid in acetonitrile as solvent B). Transitions m/z 446.2/167.0 and 609.7/195.0 were monitored for SHA-68 and the internal standard (reserpine), respectively.

Chromatograms were automatically integrated using Finnigan Xcalibur Software. A 1/x2-weighted least-squares linear regression was applied to calibration standards (nanograms per milliliter in plasma or brain sample) using peak area ratio of analyte to internal standard. Finally, concentrations for analyte in plasma and brain samples were interpolated. Plasma and brain concentrations (nanograms per milliliter and nanograms per gram, respectively) were back-calculated from the interpolated concentrations and the dilution factors.

NPS-Induced Hyperlocomotion. Locomotion of mice was monitored in an automated activity system equipped with IR sensors for both horizontal and vertical activity measurements (Versamax; AccuScan Instruments, Inc., Columbus, OH). Male C57BL/6 mice were allowed to habituate to the recording chamber for 2 h. Vehicle or SHA 68 (in PBS, 10% Cremophor EL) were injected i.p. 10 min before central administration of either NPS (1 nmol in 2 µl of PBS, 0.1% BSA) or vehicle (PBS, 0.1% BSA). Recording of locomotor activity began 5 min after the i.c.v. injections and continued for 90 min. Horizontal activity represents IR beam breaks in the x and y dimensions, whereas vertical activity was recorded by IR sensors located 10 cm above the chamber floor (z dimension). Stereotypic behavior is defined as repetitive breaks of a single beam that is not followed by a consecutive beam break of an adjacent sensor. The automated system does not allow for differentiation of individual stereotypic behaviors, such as grooming or sniffing.

Statistical Analysis. PK data for SHA 68 (plasma concentration versus time) were obtained at 1 mg/kg i.v. and 2.5 mg/kg i.p. PK curves were analyzed using a noncompartmental approach (noncompartmental analysis, model 200, extravascular input, for i.p. injection, and NCA model 201, i.v. bolus input, for i.v. dosing) with linear trapezoidal interpolation and uniform weighting in WinNonlin version 5.1.1 (Pharsight, Mountain View, CA).

Cumulative horizontal, vertical, or stereotypic activities for the complete 90-min session were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test, where appropriate. Selected data sets of cumulative activity data were also compared by unpaired Student's t test. The time course of horizontal, vertical, or stereotypic activity over the 90-min session was divided in 5-min intervals and analyzed by two-way ANOVA with time and drug treatment as variables. Values of p < 0.05 were considered significant.

Results

Synthesis and Structure Verification. The piperazine derivatives SHA 66 and SHA 68 were both synthesized (Fig. 1) in five steps from cheap, commercially available piperazine hexahydrate 1 in 54 and 55% overall yields, respectively. Monobenzylation of 1 was accomplished in the presence of one equivalent of piperazinium dihydrochloride monohydrate 2 (synthesized from 1) to yield 3 in 90% yield after recrystallization (two crops) (Cymerman-Craig, 1959; Cymerman-Craig and Young, 1962, 1973). Standard Boc protection of 3 gave the differentially protected 4 in quantitative yield (Berkheij et al., 2005). Intermediate 4 was then treated with sec-butyllithium/N,N,N',N'-tetramethylethylene diamine, at -78°C, followed by trapping of the intermediate anion with benzophenone. The formation of 6 occurred smoothly, in good vield (76%), with only a small amount of the dialkylated side product (<10%) observed. After several unsuccessful attempts to selectively remove the benzyl-protecting group of 6 via hydrogenolysis, an alternate strategy was employed. Fortunately, amine debenzylation could be accomplished by addition of FmocCl to an acetonitrile solution of 6 at room temperature. After approximately 10 min, 7 precipitated from the solution and could be filtered and used without further purification. The yield of the reaction could be increased to 80% by heating to 90°C for 5 h, after which time, no further conversion was observed. Compounds **SHA 66** and **SHA 68** were then obtained in almost quantitative yield from a mixture of **7** and the requisite isocyanate (**8** or **9**) by treatment with 1,8-diazabicyclo[5.4.0]undec-7-ene, to remove the Fmoc-protecting group, followed by addition of the secondary amine to the isocyanate. The structures of **SHA 66** and **SHA 68** were fully characterized by IR, ¹H and ¹³C NMR, and mass spectrometry. Both compounds were insoluble in water or aqueous buffers, and calculated logP values of 4.21 for SHA 66 and 4.35 for SHA 68, respectively, supported this observation. Therefore, stock solutions of the compounds were prepared in 100% DMSO and diluted appropriately thereafter.

In Vitro Pharmacological Activity. As shown in Fig. 2, A to D, SHA 66 and SHA 68 potently antagonized NPSR

activation by the endogenous agonist NPS in HEK cells stably expressing human NPSR Asn107 or NPSR Ile107, respectively. Schild analysis revealed that SHA 66 inhibited NPS-induced Ca²⁺ mobilization with pA₂ values of 7.662 at NPSR Asn107 and 7.486 at NPSR Ile107, respectively (Fig. 2E; Table 2). The fluorinated analog SHA 68 displayed pA₂ values of 7.771 at NPSR Asn107 and 7.554 at NPSR Ile107, respectively. Because the slope of the Schild plot for both compounds at both receptor isoforms was close to 1.0 (Table 2) and therefore indicated that they are competitive antagonists, $K_{\rm b}$ values were extrapolated from the linear regression curves (Table 2). Schild analysis also indicated that both compounds have approximately 1.5-fold higher affinity at NPSR Asn107 than NPSR Ile107. At functional agonist EC₅₀ concentrations, SHA 66 displayed IC₅₀ values of 26.1 and



Fig. 2. Pharmacological activity of SHA 66 (A and B) and SHA 68 (C and D) at NPSR Asn107 (NPSR-N¹⁰⁷) and NPSR Ile107 (NPSR-I¹⁰⁷). Both compounds were tested in the presence of increasing concentrations of NPS to generate dose ratios for Schild analysis by measuring mobilization of intracellular Ca²⁺ in HEK 293 cells stably expressing the different NPSR isoforms. Concentrations of antagonist compounds are given in the figure legend of B. Data are shown as means \pm S.E.M. E, Schild analysis of dose ratios followed by linear regression analysis. F, displacement of radioligand binding by SHA 68 in cells stably expressing NPSR Ile107. [¹²⁵I]Tyr10-NPS (40 pmol) was used as a tracer, and incubations were done in triplicate. Data are shown as means \pm S.E.M.

TABLE 2

Pharmacological characterization of SHA 66 and SHA 68

	SH	SHA 66		SHA 68	
	NPSR Asn107	NPSR Ile107	NPSR Asn107	NPSR Ile107	
pA_2 (95% CI) K_b Schild slope IC_{50} (at functional agonist EC_{50}) K_c (binding)	$\begin{array}{c} 7.662 \ (7.997 - 7.426) \\ 21.7 \ nM \\ 1.007 \ \pm \ 0.06363 \\ 26.1 \ \pm \ 8.1 \ nM \end{array}$	$\begin{array}{c} 7.486 \; (7.886 - 7.225) \\ 32.6 \; nM \\ 1.041 \pm 0.07110 \\ 28.8 \pm 12.2 \; nM \end{array}$	$\begin{array}{c} 7.771 \; (8.167 - 7.502) \\ 16.9 \; nM \\ 1.066 \pm 0.08553 \\ 22.0 \pm 4.4 \; nM \end{array}$	$\begin{array}{c} 7.554 \ (7.823 - 7.354) \\ 27.9 \ \mathrm{nM} \\ 0.9817 \pm 0.05494 \\ 23.8 \pm 9.4 \ \mathrm{nM} \\ 47.7 \ \mathrm{nM} \end{array}$	

 pA_2 , negative logarithm of antagonist concentration shifting the agonist EC_{50} by a factor of 2; K_b , dissociation constant of antagonist binding extrapolated from Schild analysis; Schild slope, slope from linear regression curve of dose ratios; IC₅₀, half maximal concentration of antagonist required to block agonist response at functional agonist EC_{50} (EC_{50} in this experiment: NPSR Asn107 = 15.6 nM; NPSR Ile107 = 4.3 nM); K_i , equilibrium dissociation constant determined by radioligand binding; CI, confidence interval.

28.8 nM at NPSR Asn107 and NPSR Ile107, respectively, whereas SHA 68 yielded IC₅₀ values of 22.0 and 23.8 nM at the two NPSR isoforms, respectively (Table 2). Cells stably expressing mouse NPSR showed similar inhibition of NPS-induced Ca²⁺ mobilization by SHA 66 and SHA 68 with IC₅₀ values of 60.8 \pm 13.8 and 48.7 \pm 14.7 nM against a single agonist concentration of 12.5 nM NPS, respectively (data not shown). In radioligand binding experiments, SHA 68 competed for labeled NPS binding at NPSR Ile107 with a K_i value of 47.7 nM (95% confidence interval, 39.01–58.31 nM), which is reasonably close to the calculated K_b of 27.9 nM for SHA 68 at NPSR Ile107 (Fig. 2F; Table 2). Nonspecific binding was approximately 14% of total binding under these conditions.

Selectivity Profile of SHA 68. Fourteen different GPCRs were tested to establish a selectivity profile for SHA 68 (Table 1). Each receptor was expressed in a suitable cellular environment, endogenously, transiently, or as a stable clone, and activated with its endogenous ligand at 100 nM. SHA 68 was tested at a concentration of 10 μ M. No agonistic or antagonistic activity of SHA 68 was observed at any of the GPCRs tested, indicating that the compound appears to be selective for NPSR. In particular, no activity of SHA 68 was detected at vasopressin or oxytocin receptors, which are sequentially closest to NPSR, albeit at only 25 to 29% amino acid identity.

Pharmacokinetic Profile of SHA 68. SHA 66 and SHA 68 display very similar pharmacological profiles in vitro, but SHA 68 appears to have slightly higher potency at mouse NPSR than SHA 66. Therefore, we selected SHA 68 for further in vivo experiments in mice. Due to the lipophilic nature of SHA 68 (calculated $\log P = 4.35$), we first analyzed the pharmacokinetic profile of the compound in mice. SHA 68 was injected into male C57BL/6 mice at 1 and 2.5 mg/kg for i.v. bolus and i.p. administration, respectively. Plasma samples were collected from four mice at six different time points. and concentration of drug in plasma was determined by a standard LC/MS/MS protocol. Dose-normalized PK curves (plasma concentration, C_p, as a function of time) are presented in Fig. 3A. Noncompartmental analysis of the data (WinNonlin; Pharsight) was used to generate PK parameters that are summarized in Table 3.

Bioavailability of the compound administered i.p., calculated as the ratio of dose-normalized area under the curve between i.p. and i.v. dosing, is almost quantitative. Elimination $t_{1/2}$ is shorter for i.p. than for i.v. route of administration. However, this observation is driven by the last time points of the PK curve, which are the most susceptible to experimental errors. Mean residence times, on the other hand, are similar between the two injection routes. Volume of distribution at steady state was calculated to be 2.5 l/kg, showing extensive distribution of the compound outside the vasculature and the extracellular space.

BBB penetration of SHA 68 was tested by i.p. administration of 5 and 50 mg/kg in male C57BL/6 mice. Animals (four per group) were euthanized at 0.25, 1, and 2 h postdose. Graphs of plasma and brain concentrations of SHA 68, in nanograms per milliliter and nanograms per gram, respectively, as well as their ratio, are presented in Fig. 3B. Although the brain/plasma ratio increases with progressing time at the 5 mg/kg dose, both brain and plasma concentrations of SHA 68 drop dramatically over the same period of time, as expected from PK data (decrease factor ~ 50 for plasma and ~ 8 for brain concentration ratio between 0.25 and 2 h). On the contrary, at the 50 mg/kg dose, plasma and brain concentrations of SHA 68 are more stable over time (decrease factor ~ 5 for plasma and ~ 3 for brain concentration ratio between 0.25 and 2 h). This is probably due to a slowdown in elimination of the compound as the dose increases. Conversion to molar concentrations indicated that at a dose of 50 mg/kg i.p., pharmacologically relevant concentrations of SHA 68 at or above its $K_{\rm b}$ value can be reached for at least 1 h in both plasma and brain (plasma, 87.99 ± 21.99 μ M at 15 min, 49.63 ± 10.98 μ M at 60 min; brain, 6.33 ± 0.33 μ M at 15 min, 6.06 \pm 0.23 μ M at 60 min; data are averages \pm S.E.M., n = 5).

In Vivo Activity of SHA 68. NPS is known to induce hyperlocomotion in mice after central administration. To test whether this central effect of NPS can be antagonized by SHA 68, we recorded motor activity in mice that had received injections with vehicle or SHA 68 (5 and 50 mg/kg i.p.) and subsequently received central administrations of either vehicle or 1 nmol NPS i.c.v. As shown in Fig. 4A, injection of NPS + vehicle increased horizontal activity that lasted for at least 90 min. Injection of NPS + SHA 68 (50 mg/kg) reduced horizontal activity to approximately 50% of the activity recorded from mice receiving NPS + vehicle injections, demonstrating that this dose of SHA 68 was effective in at least partially blocking NPS-induced horizontal activity. Statistical analysis revealed significant main effects of drug treatment ($F_{4.68} = 211.52, p <$ 0.0001) and time ($F_{17,68} = 19.12; p < 0.0001$) with interaction (F = 1.47; p = 0.0087). Analysis of cumulative activity data revealed that SHA 68 alone (Veh + SHA 68, 50 mg/kg) was able to reduce basal horizontal activity compared with mice receiving vehicle injections (p = 0.022, unpaired Student's t test; Fig. 4D). However, this effect is not statistically significant across the five treatment groups of the experiment by one-way ANOVA. At the highest dose (50 mg/kg), SHA 68 was able to block approximately 50% of 1 nmol NPS-induced motor activa-



Fig. 3. Pharmacokinetic analysis of SHA 68 in mice. A, dose-normalized plasma concentrations [in (nanograms per milliliter)/(milligrams per kilogram)] as a function of time (hours) for i.v. administration of SHA 68 at 1 mg/kg dose (open squares) and i.p. administration at 2.5 mg/kg (triangles) dose. Plasma and brain concentrations of SHA 68 administered i.p. at 5 (B) and 50 mg/kg (C), 0.25, 1, and 2 h postdose. D, brain/plasma ratio (milliliter per gram) calculated from B and C as a function of dose and time. Animal numbers were n = 5 at all time points. Data are shown as means \pm S.E.M.

TABLE 3

Pharmacokinetic parameters of SHA 68

PK parameters obtained by noncompartmental analysis of PK curves (see text) for i.v. administration of SHA 68 at 1 mg/kg and i.p. dosing at 2.5 mg/kg. Data were calculated as described under *Materials and Methods*.

	i.v.	i.p.
AUC_last (h/ng/ml)	231	548
AUC_inf (h/ng/ml)	233	549
CL (l/h/kg)	4.29	4.56
V _{ss} (l/kg)	2.53	
MRT (h)	0.54	0.52
$t_{1/2}$ (h)	0.74	0.43
Bioavailability	94%)

AUC_last, area under the curve between time zero and the last measured time point; AUC_inf, area under the curve extrapolated to infinity; CL, clearance; $V_{\rm ss}$, volume of distribution at steady state; MRT, mean residence time.

tion. In contrast, a 10-fold lower dose of SHA 68 (5 mg/kg) did not produce significant effects in blocking NPS-induced horizontal activity.

SHA 68 displayed similar effects on vertical activity, i.e., rearing and climbing, in mice. As shown in Fig. 4B, central NPS administration increased vertical activity, and this effect was at least partially blocked by coadministration of 50 mg/kg SHA 68, whereas the lower dose of the antagonist had no effect. Statistical analysis by two-way ANOVA revealed significant main effects of drug treatment ($F_{4,68} = 53.24, p < 0.0001$) and time ($F_{17,68} = 1.98; p = 0.0102$) without interaction. Analysis of cumulative vertical activity showed that SHA 68 also reduced basal vertical activity compared with Veh + Veh-treated animals (unpaired Student's *t* test; *p* =

0.0114), although this effect was not significant across the five treatment groups (Fig. 4E). Furthermore, central NPS administration was found to significantly increase stereotypic behavior (defined as repetitive movements recorded by breaks of a single IR beam), as shown in Fig. 4C. Statistical analysis by two-way ANOVA revealed significant main effects of drug treatment ($F_{4,68} = 135.65, p < 0.0001$) and time ($F_{17,68} = 12.75; p < 0.0001$) without interaction. NPS increased the cumulative duration of stereotypic behavior approximately 3-fold compared with Veh + Veh-treated animals, and SHA 68 (50 mg/kg) blocked this effect by approximately 50% (Fig. 4F).

Together, these data demonstrate that peripheral administration of SHA 68 is able to, at least partially, antagonize behavioral effects produced by central administration of NPS. Thus, SHA 68 behaves as an NPSR antagonist in vivo.

Discussion

Analysis of physiological functions of any neurotransmitter system depends on the availability of selective, high-affinity agonists and antagonists. So far, the recently discovered NPS system has only been studied by using the endogenous peptide agonist NPS. To our knowledge, the present study is therefore the first report of compounds with antagonistic activity at the NPS receptor.

The data presented in this study demonstrate that SHA 66 and SHA 68 are potent antagonists at NPSR in vitro, and the selectivity profile of SHA 68 indicates that this compound



Fig. 4. Effect of SHA 68 on motor activity in mice. Mice had been habituated for 2 h before injection. SHA 68 was dissolved in 10% Cremophor EL (vehicle) and injected (i.p.) 10 min before NPS or vehicle (PBS, 0.1% BSA) were injected centrally (i.c.v.). Group sizes: Veh + Veh, n = 12; Veh + SHA 68 (50 mg/kg), n = 12; NPS + Veh, n = 13; NPS + SHA 68 (50 mg/kg), n = 15; NPS + SHA 68 (5 mg/kg), n = 22. Time course of horizontal activity (A), vertical activity (B), and stereotypic behavior (C) over 90 min. Cumulative horizontal activity (D), cumulative vertical activity (E), and cumulative stereotypy time (F) during the 90-min observation period. ***, p < 0.001, Veh + Veh versus NPS + Veh; #, p < 0.05; ###, p < 0.001 NPS + Veh versus NPS + SHA 68 (50 mg/kg), one-way ANOVA with Bonferroni post hoc test.

might be selective for NPSR, although the panel of 14 GPCRs used in the screening is certainly not exhaustive. The two closely related bicyclic piperazines SHA 66 and SHA 68 display almost identical affinities, indicating that the fluorination does not affect receptor binding. The pharmacological profiles of the two compounds do however reveal a slightly higher affinity for the NPSR Asn107 variant over NPSR Ile107. These observations indicate that the amino acid in position 107 of NPSR might interfere with binding of the two antagonist compounds. This is of particular interest because we have previously shown that the polymorphism at position 107 of NPSR (Asn versus Ile) does not affect agonist binding affinity but has in turn profound effects on agonist efficacy by shifting the EC_{50} 5 to 10-fold (Reinscheid et al., 2005). Therefore, the Ile107 side chain might stabilize a receptor conformation that is promoting agonist activation, whereas the Asn107 side chain might facilitate antagonist binding. Using the bicyclic piperazine scaffold, it should therefore be possible to develop compounds that display enhanced selectivity toward one of the two isoforms of the human NPS receptor. Such compounds could not only be useful in understanding the structural role of this critical amino acid within the receptor protein but might lead to individualized drugs that target specific genotypes of NPSR. We have recently shown that the NPSR Asn107 isoform is underrepresented in male panic disorder patients (Okamura et al., 2007); thus, specifically targeting NPSR Ile107 with selective NPSR antagonists might be a viable concept for developing novel therapeutics to treat panic disorder. In addition, specific NPSR genotypes were repeatedly found to be associated with asthma or other immunological disorders, although the functional role of NPSR in these pathologies remains unclear (Laitinen et al., 2004; Feng et al., 2006; D'Amato et al., 2007; Malerba et al., 2007).

The present study also provides evidence that SHA 68 is able to enter the brain and block NPS-induced behavioral responses in vivo. However, the pharmacokinetic profile of SHA 68 indicates that the compound has only limited BBB penetration. This might explain our observation that even at a relatively high dose of 50 mg/kg, SHA 68 was able to block only approximately 50% of the motor-activating effect produced by NPS. It is obvious that compounds with improved pharmacokinetic profiles are needed to extend these studies, and the chemical scaffold of SHA 66 and SHA 68 may be useful as a lead structure to design new compounds with improved potency and pharmacokinetic properties. It should also be mentioned that the automated behavioral observation system used in this study does not allow for detection of more complex behaviors that are often associated with arousal, such as increased grooming or sniffing. These behaviors and other small repetitive movements are detected and summarized as stereotypic activity. Differentiation of locomotion into horizontal, vertical, and stereotypic activity has been chosen to illustrate the arousing properties of NPS and the attenuation of these effects by SHA 68 coadministration.

In summary, we present evidence that two closely related bicyclic piperazines are potent and selective antagonists at NPSR in vitro and are able to antagonize NPS-induced effects in vivo. These molecules should therefore be useful in the characterization of physiological functions of the NPS system and might lead to the development of novel therapeutic compounds.

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Address correspondence to: Dr. Rainer K. Reinscheid, Department of Pharmaceutical Sciences, University of California Irvine, 360 Med Surge II, Irvine, CA 92697-4625. E-mail: rreinsch@uci.edu