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Identification of the first biased NPS Receptor agonist that retains anxiolytic and memory

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Abstract

The neuropeptide S system has been implicated in a number of centrally mediated behaviors including memory consolidation, anxiolysis, and increased locomotor activity. Characterization of these behaviors has been primarily accomplished using the endogenous 20AA peptide (NPS) that demonstrates relatively equal potency for the calcium mobilization and cAMP second messenger pathways at human and rodent NPS receptors. This study is the first to demonstrate that truncations of the NPS peptide provides small fragments that retain significant potency only at one of two single polymorphism variants known to alter NPSR function (NPSR-107I), yet demonstrate a strong level of bias for the calcium mobilization pathway over the cAMP pathway. We have also determined that the length of the truncated peptide correlates with the degree of bias for the calcium mobilization pathway. A modified tetrapeptide analog (**4**) has greatly attenuated hyperlocomotor stimulation *in vivo* but retains activity in assays that correlate with memory consolidation and anxiolytic activity. Analog **4** also has a bias for the calcium mobilization pathway, at the human and mouse receptor. This suggests that future agonist ligands for the NPS receptor having a bias for calcium mobilization over cAMP production will function as non-stimulatory anxiolytics that augment memory formation.

1. Introduction

Neuropeptide S (NPS) is a 20-amino acid peptide that functions as the cognate ligand for the formerly orphan G protein-coupled receptor GPR154 (now known as the NPS receptor [NPSR]). High expression of the NPSR has been found in the retrosplenial cortex, basolateral amygdala, ventral tegmental area (VTA), substantia nigra, parasubiculum, and

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various regions of the hypothalamus. The observed receptor distribution agrees with genetic association studies linking the NPS receptor with a variety of disease states including anxiety,(Leonard et al., 2008; Vitale et al., 2008; Xu et al., 2004) sleeping disorders, (Gottlieb, O'Connor & Wilk, 2007; Xu et al., 2004) obesity,(Cline, Godlove, Nandar, Bowden & Prall, 2007; Cline, Prall, Smith, Calchary & Siegel, 2008) panic disorder, (Okamura et al., 2007) PTSD,(Jungling et al., 2008; Meis, Bergado-Acosta, Yanagawa, Obata, Stork & Munsch, 2008) and substance abuse.(Badia-Elder, Henderson, Bertholomey, Dodge & Stewart, 2008; Cioccioppo, 2007; Kallupi et al., 2010) Central administration of NPS in mice enhances learning, increases arousal, and produces anxiolytic-like effects. (Okamura et al., 2011; Xu et al., 2004) In addition, NPS stimulates dopamine release in the medial prefrontal cortex,(Jarry et al., 2010) and local intra-VTA microinjections of NPS enhance dopamine release in the nucleus accumbens.(Mochizuki, Kim & Sasaki, 2010)

The three main NPS-mediated behavioral phenotypes may be mechanistically distinct. The hyperlocomotion and anxiolytic-like effects are seen immediately after NPS administration, (Xu et al., 2004) while the memory enhancing effects can be observed several days after administration. Moreover, the memory enhancing effects are still observed when NPS is administered up to an hour after the training session is complete. Further, the anxiolytic-like effects and locomotor effects also seem separable. This is demonstrated using a test (marble burying) for anxiety-like behaviors in which the measure of anxiolytic-like effect is not dependent on action but rather a lack of engaging in a specific motor behavior. In total, these results suggest that the three main phenotypes could be mechanistically distinct and the behavioral phenotypes may be separable based on cell specific signaling in discrete brain regions or through distinct signaling pathways within the same neurons. To directly address this question some researchers have opted for an approach that utilizes the microinjection of NPS into discrete brain regions. However, to investigate the possibility that the NPS behavioral profile is the result of differential signaling, our group has sought to identify new NPSR agonist templates that could be modified for small molecule drug discovery while focusing on scaffolds that preferentially signal through only one or a subset of second messengers. There is some indication that this approach is feasible based on an analog of NPS in which 10 of the C-terminal residues were deleted.(Liao et al., 2016)

In humans, multiple single-nucleotide polymorphisms (SNPs) and a splice variant of the NPSR have been identified. A SNP has been previously described that codes for a single amino acid change (N107I) in the human NPSR (hNPSR), with the hNPSR-107I variant displaying higher agonist efficacy for both calcium mobilization and cAMP accumulation with no change in binding affinity. The hNPSR-N107I polymorphism is located in the first extracellular loop (ECL1) of the receptor. The particular portion of the ECL1 in which the hNPSR-N107I SNP resides displays the lowest level of sequence conservation across a number of peptide GPCRs, but is perfectly conserved among species orthologs of NPSR, and might thus be important for ligand selectivity and/or binding. There is also a splice variant which alters the C-terminal cytoplasmic tail, but this is not known to alter the *in vitro* pharmacological profile of NPSR.(Leonard et al., 2008)

Currently, there are no small molecule NPS agonists reported in either the patent or peer reviewed literature. Because identification of small molecule NPSR agonists has been met

with significant challenges, most of the basic research has been focused on understanding the structure-activity relationships of the endogenous NPS peptide. Initial C-terminal truncation and alanine scanning studies were undertaken by Bernier et al. (Bernier et al., 2006) to determine the smallest fragment that retained potency at the hNPSR-107I and hNPSR-107N variants. (Bernier et al., 2006) C-Terminal truncation of NPS to residue 6 afforded hexapeptide (**1**) with potency in calcium mobilization assays of $EC_{50} = 40.2$ nM and 0.7 nM at the hNPSR-107N and hNPSR-107I variants, respectively. (Tables 1, 2) Further truncation to the pentapeptide (**2**) resulted in a fragment having significantly reduced potencies of >2000 nM and 515 nM for the hNPSR-107N and hNPSR-107I variants, respectively. Compared to the WT NPS peptide which is 5-fold more potent at the hNPSR-107I variant (EC_{50} of 1.6 nM vs. 7.9 nM) the hexapeptide (**1**) reported by Bernier et al. (Bernier et al., 2006) showed a 57-fold selectivity for the hNPSR-107I over the hNPSR-107N. The enhanced potency of truncated peptides at the hNPSR-107I variant coupled with the fact that rodents have an analogous sequence to the hNPSR-107I variant, suggested that truncated peptides may be a reasonable starting point to identify isoform selective agonists that could also be evaluated comparably in rodents.

The ability to transition from peptides to small molecules is largely dependent on the size of the starting peptide fragment and the conformational flexibility of the side chains present. Considering that truncation of NPS up to residue 5 provided analogs with moderate potency and reasonable selectivity for the hNPSR-107I variant, we investigated if further truncation of the NPS peptide could retain or enhance potency and isoform selectivity. This manuscript details the identification of further truncated NPS peptides with high potency for calcium mobilization at the hNPSR-107I variant. In addition, the tetrapeptide fragments were evaluated for potency and selectivity for mobilization of cAMP. A limited structure-activity study was conducted to identify probes having more drug-like moieties that could be evaluated *in vivo*. Finally, we evaluated a novel tetrapeptide (**4**, Table 1) for *in vivo* function in a variety of animal models of anxiolytic-like activity, locomotor stimulation, and memory consolidation. Behaviors known to be modulated by NPS were selective, but was by no means a complete profile of NPS function. The goal of the present work was to determine, through the use of a subset of NPS-mediated behaviors, which broad classes of NPS-mediated behaviors could be modulated by compound 4.

2. Methods and Materials

Abbreviations

Abbreviations used for amino acids and designation of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* 1972, 247, 977–983. Amino acid symbols denote *L*-configuration unless indicated otherwise. The following additional abbreviations are used: Boc, *tert*-butoxycarbonyl; *t*Bu, *tert*-butyl; DCM, dichloromethane; DIPEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; ELSD, electron light scattering ESI-MS electron spray mass spectroscopy; HOBt, *N*-hydroxybenzotriazole; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; RP-HPLC, reversed-phase high

performance liquid chromatography; TFA, trifluoroacetic acid; Pbf, 2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-sulfonyl; Trt, triphenylmethyl (trityl).

2.1.1 Cell culture—HEK 293T cells stably expressing either the human NPSR 107N (hNPSR-107N) or the NPSR 107I (hNPSR-107I) were created as described.(Clark, Tran, Zeng & Reinscheid, 2010) HEK 293T cells stably expressing the mouse NPSR (mNPSR) were created in a similar manner.(Reinscheid et al., 2005) In addition, stable cell lines overexpressing the hNPSR-107I or hNPSR-107N were created in CHO-Gα16 cells (RD-HGA16, Molecular Devices). CHO-Gα16-hNPSR cells were cultured in Ham's F12 media supplemented with 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, 200 µg/mL hygromycin, and 400 µg/mL geneticin, and were used for Ca⁺² mobilization assay. While HEK 293T hNPSR-107N or hNPSR-107I cells were cultured in DMEM-H media supplemented with 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, and 400 µg/mL hygromycin, and were used in HTRF cAMP assays. The HEK 293 T mNPSR cell line were cultured in DMEM-H media supplemented with 10% FBS, 15 mM HEPES, 100 units/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL hygromycin, and 100 µg/mL geneticin and were used for both Ca⁺² mobilization and cAMP accumulation assays. Media was purchased from HyClone (GE Life Sciences); FBS, penicillin/streptomycin, hygromycin, and geneticin were purchased from Invitrogen (Thermo Scientific).

2.1.2 Ca⁺² Mobilization Assay—The day before the assay, cells were plated into 96-well black-walled assay plates at 25,000 cells/well for CHO-Gα16 hNPSR-107I/N and 35,000 cells/well for HEK mNPSR in the appropriate growth medium without selection antibiotics. Cells were incubated overnight at 37 °C, 5% CO₂. FLIPR Calcium 5 assay kit (Molecular Devices) was used according to the manufacturer's instructions with minor modifications. Briefly, reconstituted dye was diluted 1:40 in pre-warmed (37 °C) assay buffer (HBSS, 20 mM HEPES, 2.5 mM probenecid, pH 7.4 at 37 °C). Growth medium was removed, and the cells were gently washed with prewarmed assay buffer. Diluted Calcium 5 dye was added to the cells and the plate was incubated for 45 minutes at 37 °C, 5% CO₂. Serial dilutions of test compounds and NPS were prepared at 10× the desired final concentration in 0.25% BSA/1% DMSO/assay buffer, aliquoted into 96-well polypropylene plates, and warmed to 37 °C. Cells were pretreated in a final concentration of 1% DMSO for 15 minutes at 37 °C. Assay plates were read with a FlexStation II instrument (excitation at 485 nm, detection at 525 nm, Molecular Devices). Calcium-mediated changes in fluorescence were monitored every 1.5 seconds over a 60 seconds' time period. Test compound or NPS was added during the read and the peak kinetic reduction (SoftMax, Molecular Devices) in relative fluorescent units (RFU) was measured. Data were fit to a three-parameter logistic curve to generate EC₅₀ values (GraphPad Prism, 6.0, GraphPad Software, Inc., San Diego, CA). HBSS and HEPES were purchased from Gibco (Thermo Scientific); probenecid, BSA, and DMSO were purchased from Sigma-Aldrich.

2.1.3 cAMP Accumulation Assay—Experiments were performed using the LANCE Ultra cAMP kit (Perkin Elmer) with cells in suspension according to the manufacturer's instructions with minor modifications. Briefly, cells were harvested using versene (Gibco, Thermo Scientific), spun at 100×g for 5 minutes and resuspended in assay buffer (HBSS, 5

mM HEPES, 0.5 mM IBMX (Sigma-Aldrich), 0.1% BSA, pH 7.4) at 8,000 cells/well for hNPSR-107N, 4,000 cells/well for hNPSR-107I, and 20,000 cells/well for mNPSR. Serial dilutions of test compounds and NPS were prepared at 2× the desired final concentration in assay buffer. Cells were transferred to 96-well ½ area white polystyrene plates, and NPS or test compounds were added at the indicated concentrations. Plates were incubated for 30 minutes (hNPSR-107N or hNPSR-107I) or 1 hour (mNPSR) at room temperature. After addition of Eu-tracer cAMP and *ULight*-anti-cAMP, plates were incubated for 1 hour at room temperature in the dark. Data was collected on a FlexStation III instrument (excitation at 340 nm, emission at 615 and 665 nm, Molecular Devices) or a CLARIOstar (excitation at 340 nm, emission at 620 and 665 nm, BMG LABTECH). TR-FRET data in relative fluorescent units (RFU) was converted to fmol cAMP through interpolation using a cAMP standard curve and data were fit using a three-parameter non-linear regression to generate EC₅₀ values (GraphPad Prism, 6.0).

2.1.4 Calculation of Bias—Bias was calculated for each peptide's response in each assay through the calculation of Log(max/EC₅₀) values (where max is the maximal response to that agonist and EC₅₀ the concentration of agonist producing 50% maximal response to the agonist). This is a surrogate for system independent estimates of efficacy and affinity from the Black/Leff operational model for agonism (Black & Leff, 1983) and function as single estimates of the power of each agonist to induce a particular response (Kenakin, 2013; Kenakin & Christopoulos, 2013). The maximal responses to the agonists were calculated as a fraction of the maximal window for response in the assay, i.e. no response can be greater than unity. Log(max/EC₅₀) values were determined for activation of both signaling pathways and the effects of differences in assay sensitivity canceled through comparison of Log(max/EC₅₀) values for each agonist as a fraction of that found for a references standard, in this case NPS; this furnished Log(max/EC₅₀) values for each signaling pathway. These values were then used to calculate Log(max/EC₅₀) values between pathways to estimate the logarithm of biased signaling. Bias was calculated as $10^{\text{Log(max/EC}_{50})}$ (Kenakin, Watson, Muniz-Medina, Christopoulos & Novick, 2012) This method requires that concentration-response curves with slopes not significantly different from unity as they are in the data presented. In our experiences, biased values calculated in this manner exceeding 2–3 most likely produce a significant change in efficacy quality and signaling.

2.2 Behavioral Testing

2.2.1 Animals—Male mice between 8 and 12 weeks of age were used for all tests. C57/Bl6 were obtained from the NCI colony maintained by Charles River. All experiments were approved by the Institutional Animal Care and Use Committee of the State University of New York at Buffalo (PMY09073N) and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory animals.

2.2.2 Drug Administration—RTI-118 was synthesized as described previously, (Hassler et al., 2014) and delivered intraperitoneal in saline. NPS was purchased from AnaSpec (Fremont, CA) and compound **4** was synthesized as described above. For intracerebroventricular (ICV) drug injections, mice were briefly anesthetized with isoflurane. NPS or compound **4** was dissolved in artificial cerebral spinal fluid (aCSF, pH

7.4) containing 0.1% bovine serum albumin and injected into the lateral ventricle (total volume: 2 μ L) as described before.(Xu et al., 2004) Briefly, mice are lightly anesthetized by isoflurane vapor and then injected using a Hamilton syringe with a sharpened needle tip of suitable length to reach the animal's lateral ventricle. The tissue damage inflicted by this method is smaller than that produced by implanting cannulae. After the injection the animals are given sufficient time to recover from the anesthesia before testing (typically 5–10 minutes). Injection sites are verified post-mortem and those that land AP -2 ± 0.5 mm, ML $+2 \pm 0.5$ mm (from Bregma) are deemed successful and the remainder will be removed from the analysis (a total of 347 mice were used with 263 having proper placement of the ICV injection).

2.2.3 Locomotor Stimulation—Open field testing was conducted in clear plexiglass cages measuring 40 $\times$ 40 cm interfaced by a grid array of infrared beams connected to a computer system which tracked and quantified the animals location and movements (OMNITECH Instruments, Columbus, OH, USA). Testing was conducted in a room novel to the mice.

Mice were moved to the testing room and immediately placed in the testing cages, where they remained undisturbed for 1 hour. Each mouse was removed and administered test compounds, placed back into the test arena and once recovered from the anesthesia (minimum five minutes) the test boxes were started. Testing was conducted during the light phase.

2.2.4 Passive Avoidance—Shuttle boxes (MedAssociates) were configured so that one compartment was clear plexiglass with an open top, and the other side had black sides and a top. A guillotine style door separated the two compartments. Infrared beams connected to a computer system recorded the time spent in both compartments and caculated the time it took for the mice to enter the dark compartment. Before training or testing mice were housed in the testing room for at least one hour before conducting experiments.

On the training day, the boxes were programed so that a light was illuminated on the light side (start side), and once the mouse entered the dark side the light extinguished. The experimenter then closed the door separating the compartments and initiated a shock delivered through the metal bar floor (0.5 mA, 1 second). Mice were left in the dark side for 30 seconds post-shock, then removed and placed in their home cage. Twenty minutes after the end of the training session mice were injected with test compounds. Forty-eights hours later mice were tested for their latency to enter the dark side. The boxes perfomed just as they did on training day accept no shock was delivered.

2.2.5 Light-Dark Box—Custom built plexiglass boxes with two equal compartments (in cm 19L $\times$ 15W $\times$ 30H) separated by an opening sufficient for the free movement of a mouse were used. One compartment had transparent walls, floor, and no lid. The other compartment had black walls, floor and lid. The box was placed in a testing room with indirect light (measured to be 100 Lux in the clear side). Mice were injected with test compounds, then were allowed to recover for a minimum of 5 minutes before being placed in the light compartment. The activity of the mice was recorded using a camcorder

(Panasonic HC-V100M). Subsequently, video recordings were scored by an observer blind to the condition for the total time spent on the light side and number of transitions between the compartments.

2.2.6 Marble Burying—Marbles (18) were arranged evenly in home cages with corncob bedding 5 cm deep. Mice were placed into the cages twenty minutes after administration of test compounds. Filter top lids were placed on top of the cages. After 30 minutes, mice were removed and birds-eye-view photographs of the cages were taken. From these photos an experimenter who was blind to the condition then counted how many marbles were buried (at least two-thirds buried).

2.2.7 Statistical Analysis—Data was graphed and analyzed with GraphPad Prism Software (v6.0). One way ANOVA with Dunnett's Multiple Comparison Test was used when comparing to vehicle and ANOVA with Tukey Test was used when comparing to all conditions.

2.3.1 Peptide Synthesis—All standard reagents were commercially available. Compounds were purified by HPLC on an Agilent-Varian HPLC system equipped with Prostar 210 dual pumps, a Prostar 335 Diode UV detector and a SEDEX75 (SEDERE, Olivet, France) ELSD detector. The HPLC solvent system was binary, water containing 0.1% trifluoroacetic acid (TFA) and solvent B (acetonitrile containing 5% water and 0.1% TFA). A semi-preparative Synergi Hydro[®] RP 80A C18 column (4 μ m 250 $\times$ 21.2 mm column; Phenomenex) was used to purify final compounds at 15 mL/min using a linear gradient from 5% to 50% B over 20 minutes, Absorbance was monitored at 280 nm. The purity of final compounds was determined using an analytical Synergi Hydro[®] RP 80A C18 (4 μ m 250 $\times$ 4.60 mm column; Phenomenex) with a linear gradient of 5–95% solvent B over 20 minutes at a flow rate of 1 mL/min or a Gemini-NX C18 (5 μ m 250 $\times$ 4.6 mm; Phenomenex) with a linear gradient of 5–45% solvent B over 20 minutes at a flow rate of 1 mL/min. Absorbance was monitored at 220 nm. The molecular ion of final compounds was determined using a PE Sciex API 150 EX LC/MS system from Perkin Elmer (San Jose, California). ¹H NMR spectra were recorded at 300 MHz on a Bruker Avance 300 Spectrospin instrument and are reported as follows: chemical shift δ in ppm (multiplicity, coupling constant (Hz), and integration. The following abbreviations were used to explain multiplicities: s = singlet, d = doublet; m = multiplet, br = broad, dd = doublet of doublets.

2.3.2 Synthesis Materials—N α -Fmoc-protected amino acids, HBTU and HOBt were purchased from AAPPTec (Louisville, KY) and from Chem-Impex International Inc. (Wood Dale, IL). Trityl resin was purchased from AnaSpec (Fremont, CA). Rink resin was purchased from Chem-Impex (Wood Dale, IL). Peptide synthesis solvents, reagents, as well as CH₃CN for HPLC were acquired from commercial sources and used without further purification. The synthesis of NPS analogues was performed on solid-phase resin method in a stepwise fashion via peptide synthesizer. N α -Fmoc-AA1-OH (AA1: Asn(Trt), Gly, Val) was coupled to Rink resin (0.52 meq/g) via peptide coupling or coupled to Trityl resin (0.1 g, 0.9 meq/g) with DIEA (2 M in NMP) for 90 minutes. The following protected amino acids were then added stepwise N α -Fmoc-AA2-OH (AA2: Fmoc-Gly-OH, N α -Fmoc-

Asn(Trt)-OH, Na-Fmoc-Arg(Pbf)-OH, Na-Fmoc-Lys(Ne-Boc)-OH, Na-Fmoc-Phe-OH, or Na-Fmoc-Ser(*t*Bu)-OH. Each coupling reaction was accomplished using a 3-fold excess of amino acid with HBTU and HOBt (1.5 mL, 0.5 M each in DMF) in the presence of DIEA (0.8 mL, 2 M in NMP). The Na-Fmoc protecting groups were removed by treating the protected peptide resin with a 20% solution of piperidine in DMF, (1 × 5 min, 1 × 10 minutes). The peptide resin was washed three times with DMF and the next coupling step was initiated in a stepwise manner. All reactions were performed under an N₂ atmosphere. The peptide resin was washed with DMF (3×) and the deprotection protocol was repeated after each coupling step. The N-terminal Fmoc group was removed as described above, the resin washed with DMF (3×) and DCM (3×), and the peptide was released from the resin with TFA / DCM (1/1, 5 mL) over 0.5 h. The resin was removed by filtration and the crude peptide was recovered by precipitation with cold anhydrous ethyl ether to give a white powder which was used crude or purified by semi-preparative RP-HPLC using a gradient of CH₃CN in 0.5% aqueous TFA (from 5 to 20% in 20 minutes) at a flow rate of 15.0 mL/min. The product was obtained as the TFA salt by lyophilization of the appropriate HPLC fractions. Analytical RP-HPLC indicated a purity > 99% and molecular weights were confirmed by ESI-MS. The peptides eluted near 40 to 48% acetonitrile. The analytical data of the compounds synthesized in this paper are given in Table S1 of the supporting information.

3. Results

3.1.1 Calcium Mobilization

Previous NPS truncation studies demonstrated that the shortest peptide fragment to retain agonist potency at the hNPSR-107N NPSR variant was the amidated hexapeptide (**1**) having an EC₅₀ of 40.2 nM (Table 2). (Bernier et al., 2006) Further truncation to the amidated pentapeptide (**2**) caused a complete loss of potency at the hNPSR-107N variant and a 735-fold reduction in potency at the hNPSR-107I variant. (Bernier et al., 2006) In order to gain additional insight into the structure-activity relationships of the NPS peptide our group synthesized C-terminal amidated tetrapeptide (**3**) and tested it at both the hNPSR-107N and hNPSR-107I variant. At the hNPSR-107N variant, **3** possessed agonist activity albeit with low potency (EC₅₀ = 998 nM) compared to full length NPS (Table 2). At the hNPSR-107I variant, the tetrapeptide **3** was significantly more potent with an EC₅₀ = 2.43 nM, only 4-fold less potent than full length NPS (Table 1). The fact that **3** was potent and maximally efficacious, coupled with data indicating a critical role of Asn4 in agonist potency, suggested that the tetrapeptide was the smallest fragment required for activity at the hNPSR-107I variant. In general, the analogous C-terminal carboxylic acids were inactive or significantly less potent than the amidated peptides (See Supplementary Data).

In order to fully characterize the potency and efficacy of truncated peptides for use *in vivo*, we performed a species comparison using a mouse NPSR (mNPSR) expressing cell line. A similar trend in potency for calcium mobilization was observed for the truncated peptides at the mNPSR as that seen with the hNPSR-107I variant (Table 3). Both the C-terminal amidated hexa- (**1**) and tetra- (**3**) peptides possessed moderate potency with EC₅₀'s = 2.03 and 66.6 nM, respectively, while the amidated pentapeptide had a substantially decreased

potency ($EC_{50} = 1014$). This data is in line with what is observed for the hNPSR-107I variant suggesting that the mouse variant is also selectively activated by the tetrapeptide **3** versus peptides of longer length.

3.1.2 cAMP Studies

Additional functional characterization of the truncated peptides was undertaken utilizing a cAMP accumulation assay. In calcium mobilization, a roughly 10-fold decrease in potency for NPS and related compounds has been observed at the hNPSR-107N versus the hNPSR-107I variants and this has also been reported when cAMP is used as the functional readout.(Reinscheid et al., 2005) Consistent with this aspect in our studies, NPS displayed an EC_{50} of 4.19 nM at the hNPSR-107I variant versus an EC_{50} of 48.9 nM at the hNPSR-107N variant using cAMP accumulation (Tables 1 and 2). Similar to the calcium mobilization studies at the hNPSR-107I variant, the amidated hexapeptide **1** demonstrated a comparable potency to NPS, while the amidated pentapeptide **2** was much less potent. Interestingly, the amidated tetrapeptide **3** demonstrated a relative decrease in potency for cAMP accumulation ($EC_{50} = 129$) compared to calcium mobilization at the hNPSR-107I variant. As expected, due to the mNPSR possessing an analogous isoleucine residue at position 107, the mNPSR cAMP data was more closely aligned with what is observed for the hNPSR-107I variant, regarding relative potency for NPS and the test compounds compared to the hNPSR-107N variant.

Due to the apparent decrease in relative potency between cAMP accumulation and calcium mobilization with some of the truncated peptides, we further analyzed the functional data for indications of potential ligand bias. In our's and others' hands the full length NPS peptide has a lower EC_{50} in calcium mobilization assays than cAMP accumulation.(Reinscheid et al., 2005) Generally, this has been credited to inherent differences in the assays. However, clear evidence for ligand bias was revealed when we calculated agonist bias from $\text{Log}(\text{max}/EC_{50})$ values for calcium versus cAMP in all three receptor lines.(Ehlert, 2008) Importantly, at the hNPSR-107I variant, selectivity for the calcium pathway over the cAMP pathway correlates inversely with the length of the peptide (Figure 1). In this cell line, the amidated hexapeptide **1** displayed no bias for a signaling pathway but the amidated pentapeptide **2** had a bias of 2.06, and the amidated tetrapeptide **3** had a bias of almost 8 for the calcium versus the cAMP pathway (Table 1, Figure 1). In the mNPSR cell line, the tetrapeptide had a bias of 14.61 for the calcium versus the cAMP pathway (Table 3).

3.2 Arg3 Modifications of the Tetrapeptide

One of the primary objectives of our program was to identify molecular templates that could be transitioned to small molecule agonists through chemical modification. Considering the tetrapeptide contained an arginine that could potentially be a significant impediment to blood-brain barrier permeability we initially focused on removal of the positively charged Arg3. An abbreviated series of analogs was synthesized that altered the Arg3 guanidine to determine if Arg3 could be replaced with moieties that would provide favorable drug-like properties and to also begin to see if the tetrapeptide was interacting at the NPS receptor in a manner similar to the full length NPS peptide. Arg3 was converted to Lys3 (**4**), N,N-dimethylLys3 (**5**), N-trifluoroacetyl Lys3 (**6**), and Aoc3 (**7**) and evaluated for calcium

mobilization at the hNPSR-107I variant (Table 1). Substitution of Lys3 (**4**, $EC_{50} = 3.02$ nM) for Arg3 (**3**, $EC_{50} = 2.43$ nM) had little impact on potency or efficacy suggesting that the guanidine was not required for agonist activity. Dialkylating the amine nitrogen of Lys3 afforded **5** ($EC_{50} = 43.5$ nM), which was 18-fold less potent than the parent tetrapeptide **3**, suggesting either steric bulk, amine basicity, or a reduction in hydrogen bonding capacity was responsible for the reduced potency. Tetrapeptide **6** substituted trifluoroacetyl lysine for lysine (**3**) and was synthesized to test if an acidic amide proton could substitute for a basic amine. Compound **6** ($EC_{50} = 563$ nM) retained little activity compared to the parent tetrapeptide. Finally compound **7** ($EC_{50} = 435$ nM) was prepared to remove the amine functionality completely and was 180-fold less potent compared to the parent peptide **3**, indicating a basic functionality is critical to high potency for calcium mobilization. The SAR data is in general agreement with SAR data previously obtained for the full length NPS and indicates that further structural modifications can be made to enhance metabolic stability and drug-like properties. (Bernier et al., 2006; Camarda et al., 2008)

In order to evaluate the potential signalling bias and suitability for *in vivo* studies of the most promising analog, compound **4** was evaluated for cAMP accumulation in the hNPSR-107I variant and at the mNPSR (Table 3). At the hNPSR-107I, compound **4** had an EC_{50} of 117 nM for cAMP versus an EC_{50} of 3.02 nM for calcium mobilization. At the mNPSR, compound **4** had an EC_{50} of 100 nM for calcium versus an EC_{50} of 911 nM for cAMP accumulation. Again the method of comparing $\text{Log}(\text{max}/EC_{50})$ values was used, and a bias of 6.44 and 9.53 was calculated for the hNPSR-107I and mNPSR, respectively (Tables 1 and 3). This is in line with what was observed for the amidated tetrapeptide **3**. This finding suggested that **4** would be a useful tool to investigate the consequences of signalling bias *in vivo*.

3.3 Behavioral Results

The central administration of NPS into mice produces three robust behavioral effects: hyperlocomotion, enhanced memory consolidation, and anxiolytic-like behaviors. We hypothesized that the use of a biased ligand would increase our understanding of the specific signaling cascades that are involved in NPS-mediated behaviors. Compound **4** lacks an arginine group and if effective could serve as a template for a more robust medicinal chemistry effort focused on brain penetrant small molecule biased agonists. Therefore, we chose compound **4** to evaluate *in vivo* because it displayed biased signaling and it has the most drug-like structural motif.

The behaviors evaluated were selected to cover the spectrum of known functions of the NPS-system. Initially equivalent doses of NPS and **4** (1 nmole) were tested in habituated mice for the typical NPS-mediated hyperlocomotion. Previously, 1 nmole of NPS administered intracerebroventricularly (ICV) was found to produce the maximal response (2 μL of 5 mM (1 nmole) administered into an estimated brain volume of 2 mL results in ~ 5 μM final concentration in the brain). Compound **4** (1 nmol) failed to produce a significant increase in locomotor activity when directly compared to NPS (produced significant effect, $p < 0.001$, ($F_{3,21}$)) despite the estimated brain concentration being five times the EC_{50} in the cAMP assay in mNPSR expressing cells (Figure 2). As a follow-up, was run without an NPS

control and found to indeed increase locomotor activity albeit at a tenth of the maximal effect of NPS (Figure 3, 10 nmole $p < 0.001$, 1 nmole $p < 0.01$ ($F_{4,41}$)). Moreover, even at a dose of 10 nmole (estimated brain concentration of 50 μM) or 50 times the EC_{50} of **4** in the cAMP assay using mNPSR expressing cells, there was no further increase in locomotor activity (Figure 3). The lack of effect of the higher dose suggests that **4** is already producing a maximal effect, at least in locomotor activity, at the 1 nmole dose. The effects of **4** are NPSR specific as the induced locomotor effects can be blocked by the pretreatment with a potent and highly selective NPSR antagonist (**RTI-118**, $p < 0.05$, (Hassler et al., 2014)).

In the light-dark box paradigm, the 1 nmole dose of **4** produces anxiolytic-like effects (as compared to vehicle, $p < 0.05$), however the effects are significantly less than that of NPS (Figure 4A, NPS vs aCSF $p < 0.001$, NPS vs compound **4** $p < 0.05$ ($F_{2,41}$)). In assessing the number of entries into the light compartment it is seen that **4** does not increase this measure as robustly as NPS (Figure 4B, NPS vs compound **4** $p < 0.01$, ($F_{2,41}$)). This suggests that the apparent anxiolytic-like effects of NPS in this assay may be due in part to the hyperlocomotion it induces or the dose used is not maximally effective. To further support this hypothesis we used an assay in which hyperlocomotion would have lessened confounding effects; not produce false positives. In marble burying the measure of an anxiolytic-like effect is not dependent on action but rather on a lack of engaging in a specific motor behavior. In this assay NPS and **4** produce similar effects (Figure 5A, $p < 0.01$, ($F_{2,36}$)). Again, the effects of **4** can be blocked by the pretreatment of an NPSR antagonist (**RTI-118**), thus demonstrating pharmacological specificity (Figure 5B, $p < 0.01$, ($F_{3,33}$)).

In the passive avoidance paradigm, administration of **4**, 20 minutes after the training session, produced dose-dependent increases in the latencies to enter the compartment previously paired with the mild shock, in mice tested 48 hours after training (Figure 6, compound **4** 1 nmole $p < 0.05$, ($F_{5,66}$)). Compound **4** and NPS appear to have the same maximal effects, with the 10 nmole dose of **4** not producing further increases in latency ($p < 0.01$).

The behavioral results combined with the *in vitro* bias of **4** leads us to hypothesize that NPSR-mediated arousal is driven by the pathways associated with cAMP production, while the anxiolytic-like and memory enhancing effects are due to those pathways associated with calcium mobilization. However, as discussed below future experiments to be performed to specifically address this hypothesis. In any case, these results demonstrate that **4** is a valuable tool compound that can be used to dissect the neural and signaling pathways responsible for NPSR-mediated behaviors.

4. Discussion

Our results show that the minimal active domain of NPS at the hNPSR-107I variant is amino acids 1 through 4 as opposed to 1 through 6 as previously reported. (Bernier et al., 2006) This truncated form of NPS is a full agonist for both calcium mobilization and cAMP production. SAR studies also indicate that Arg3 of the tetrapeptide is not critical for receptor activation or potency and can be replaced with a Lys, but displays reduced potency when the basic amine functionality is altered. These results are in line with SAR studies undertaken on

the full length peptide and suggest that the truncated peptides may be interacting with the hNPSR-107I variant in a manner analogous to the full length peptide.

The biased agonist **4** produces anxiolytic-like and learning effects similar to NPS. However, **4** is not able to produce increases in locomotor activity to the same degree as NPS. We hypothesize that the arousal properties of NPS may be primarily mediated or require the simultaneous activation of NPSR-mediated increases in cAMP while other behaviors are reliant on receptor-mediated calcium increases (Figure 7). However, an exhaustive profile of all NPS-mediated behavioral effects was not completed. It is possible that the use of other measures of learning (e.g., novel object recognition test) or anxiety-like behaviors (e.g., stress-induced hyperthermia test) would support alternative hypotheses.

Intraventricular administration of NPS produces robust hyperlocomotion in animals habituated to their environment (Figure 2). (Xu et al., 2004) Compound **4** also significantly increased locomotion, but even at 10 times the dose compound **4** produces only ~1/10 the increase in locomotion as that of NPS (Figure 3). These results mirror the *in vitro* pharmacological profile of **4** in NPSR-mediated cAMP production and likely points to the hyperlocomotor effects being due to NPSR-mediated increases in cAMP. This may fit with findings by Dr. Boeck's group which demonstrated that the inhibition of either the adenosine(2A)-receptor or ecto-nucleotidases also block NPS-mediated hyperlocomotion. (Boeck et al., 2010; Pacheco et al., 2011) In these studies it was proposed that extracellular adenosine is permissive in the effects of NPS. Alternatively, it could be that NPS-mediate both intracellular and subsequently extracellular increases in cAMP, which would result in adenosine(2A)-receptor activation. The testing of this hypothesis would require the inhibition of multidrug resistance protein channels in NPSR expressing neurons or in structures known to express NPSR and are thought to play a role in NPS-mediated hyperlocomotion (e.g., hypothalamus). (Paneda et al., 2009)

It has been previously argued that NPS-mediated anxiolytic-like effects are separable from the effects on locomotion. It was reasoned that although hyperlocomotion can lead to false positives in the light-dark box paradigm this is thought to be less likely to be the case for marble burying. NPS-mediate anxiolytic-like effects in both of these paradigms, as well as in the elevated plus maze paradigm despite the hyperlocomotion phenotype. (Xu et al., 2004) Alternatively, hyperlocomotion could produce a false positive in marble burying because the mice could be generally distracted and ignore the marbles during the test period. However, data presented here suggest that the locomotor and anxiolytic-like effects are indeed separable. Although **4** significantly increased locomotion, this increase is a fraction of that mediated by NPS (Figure 3). The lower capacity to mediate hyperlocomotion does not abolish the anxiolytic-like effects of **4** in the light-dark box (Figure 4) or marble burying (Figure 5) paradigms. Albeit the time spent on the light-side induced by **4** is significantly lower than an equivalent dose of NPS. In addition, compound **4** does not increase the number of light-side entries, which is generally viewed as a measure of locomotion. This not only matches what we found for exploration of a familiar environment (Figure 2) but may suggest that at least part of the described anxiolytic-like effect of NPS in the light-dark box is actually due to NPS-mediated hyperlocomotion. These findings further support the notion that the hyperlocomotor effects of NPSR activation are separable from the anxiolytic-like

effects. Moreover, because **4** is biased towards calcium mobilization we hypothesize that the anxiolytic-like effects are due to NPSR-mediated increases in calcium.

Centrally administered NPS improves memory as measured by novel object recognition and passive avoidance, and enhances extinction of fear conditioning.(Jungling et al., 2008; Okamura et al., 2011; Slaterry et al., 2015) In the passive avoidance paradigm **4** increased the latency to enter the compartment associated with a mild electrical shock (Figure 6). The effects in passive avoidance, like that of NPS, are thought to be mediated through strengthening the consolidation of the memory. This has been hypothesized because the effects are seen when **4** is administered 20 minutes after training, and the animals are tested in a drug-free state 48-hours post-training. Moreover, NPS-mediated effects are seen four days after a single treatment in both passive avoidance and novel object recognition. (Okamura et al., 2011) Whether the effects of **4** are as long-lasting and have the same time dependency as NPS still needs to be explored. Like the anxiolytic-like effects, we hypothesize that the memory enhancing effects are likely due to NPSR-mediated increases in calcium, which would easily be interpretable within the framework of the canonical NMDA-calcium-LTP pathway.(Nicoll & Roche, 2013) The memory effects seen with NPS/**4** are likely not due to anxiolytic-like effects because the peptides are administered 20 minutes after the training session. Further, it is more plausible that the anxiolytic-like effects would diminish the salience of the training session resulting in decreased latencies in passive avoidance paradigms. In this light, it suggests that the non-cAMP-mediated behavioral effects of NPS/**4** could be further mechanistically separable.

It may be too simplistic to think that in neurons that express NPSR increased cAMP would lead to apparent arousal and increases in intracellular calcium lead to enhanced learning and diminished anxiety-like behavior. There could be subpopulations of NPSR expressing neurons that do not possess the capacity to produce calcium signaling mediated by NPSR activation. Although we show here that a biased ligand can separate the behavioral effects, it may have more to do with an anatomical separation of responsiveness, and **4** is simply able to reveal this nuance. It also has to be considered that the arousal effects of NPS may require the simultaneous increase in cAMP and calcium within the same neuron. Again, there could be subpopulations of NPSR expressing neurons that mediate this effect. These hypothetical subpopulations could reside in distinct anatomical structures, which would allow the microinfusion of **4** into these brain regions to address this possibility.

It also needs to be considered that we have not completed an exhaustive pharmacological profile, and so it is possible that the inability of **4** to produce robust increases in locomotion may lie in a failure to activate another pathway which we have yet to evaluate. Likewise, the anxiolytic-like and learning effects could be due to an alternative pathway for which **4** remains a full agonist (e.g., β -arrestin, ERK). Until the neuron selective blockade of NPSR-mediated cAMP production or calcium mobilization can be achieved, our current working hypothesis is that the hyperlocomotor effects are due to NPSR-mediated increases in cAMP.

Regardless of the exact mechanism of **4** to separate the hyperlocomotive effects from that of the anxiolytic-like and learning effects, compound **4** shows it is pharmacologically possible to make this separation. This is of key importance, as those with anxiety disorders may not

benefit from increased arousal. Although there are situations that this combination would be desirable (first responders), heighten arousal and the accompanying increased social and environmental interactions may act counter to the desired anxiolytic effects.

Overall this manuscript details the identification and characterization of the first calcium mobilization biased agonist of the NPSR that has a profoundly decreased effect on the locomotor component of the NPS-system. Compounds identified from this effort show an even greater level of bias for the hNPSR compared to the mouse variant suggesting that further modification of these templates may ultimately lead to drugs with similar effects on the hNPS-system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

NPS peptide truncation affords bias ligands at the hNPS-107I isoform

Figure 2. NPS-mediated hyperlocomotion

Mice were habituated to a novel environment for 1 hour, they were then administered vehicle or test compound. Mice were returned to the apparatus and locomotion was recorded. Mice receiving NPS (1 nmole) moved significantly more than mice injected with aCSF (***) $p < 0.001$; one way ANOVA with Dunnett's Multiple Comparison Test). Neither dose of compound **4** produced significant locomotion, likely because of the very large NPS-mediated response masking the effect in the statistical analysis. [aCSF, $n = 5$; compound **4** 0.1 nmole, $n = 8$; compound **4** 1 nmole, $n = 8$; NPS 1 nmole, $n = 4$]

Fig. 3. Compound 4 mediates hyperlocomotion

New groups of mice underwent the same procedure as in Figure 1. Compound **4** mediates a significant increase in locomotion as compared to vehicle treated, and this effect is blocked by pretreatment with a selective NPSR antagonist (**RTI-118**, 50 mg/kg i.p.). (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$; one way ANOVA with Dunnett's Multiple Comparison Test) [aCSF, $n = 10$; compound **4** 1 nmole, $n = 11$; compound **4** 10 nmole, $n = 10$; compound **4** 10 nmole + **RTI-118** 50 mg/kg, $n = 8$; **RTI-118** 50 mg/kg, $n = 7$]

Figure 4. Compound 4 increases time spent on light side

Mice were administered vehicle or test compound 5–10 minutes before placement into the Light-Dark Box apparatus. Time spent on the light-side and the number of entries into the light-side was quantified. (A) Both NPS and **4** significantly increased the time spent on the light-side (as compared to aCSF treated). However, NPS treated animals also spent significantly more time on the light-side than the compound **4** treated animals. (B) Neither NPS nor **4** significantly increased the number of entries into the light-side. However, NPS treated animals had significantly more entries than compound **4** treated animals. The number

of entries is a measure of activity, and so this data is consistent to that of Figure 2. (** $p < 0.01$, * $p < 0.05$; one way ANOVA with Tukey Test). [aCSF, $n = 14$; compound **4** 1 nmole, $n = 18$; NPS 1 nmole, $n = 12$].

Figure 5. Compound 4 decreases marble burying behavior

(A) Mice were administered vehicle or test compound twenty minutes before being placed into the test chambers. Mice were allowed to explore a novel home cage with bedding and 18 marbles. After 30 minutes the mice were removed and the number of marbles buried was quantified. Both NPS and **4** significantly decreased the number of marbles buried (as compared to aCSF treated). [aCSF, $n = 13$; compound **4** 1 nmole, $n = 13$; NPS 1 nmole, $n = 13$]. (B) Mice were administered saline or **RTI-118** (20 mg/kg) fifteen minutes before being administered aCSF or compound **4** (0.5 nmole). Then 30 minutes later the mice underwent

the same procedure as in Figure 5A. When pretreated with saline, compound **4** significantly decreased the number of marbles buried but this was blocked by the pretreatment with **RTI-118** (as compared to saline/aCSF treated). [saline/aCSF, n = 12; **RTI-118**/aCSF, n = 8; saline/compound **4** 0.5 nmole, n = 8; **RTI-118**/compound **4** 0.5 nmole, n = 9] (** p < 0.01; one way ANOVA with Dunnett's Multiple Comparison Test).

Figure 6. Compound 4 increases the latency to enter a compartment associated with an aversive stimulus

Mice were trained to associate the dark compartment with a mild electrical shock, and then 20 minutes later were administered vehicle or test compound; 48 hours later mice were tested for latency to enter the dark compartment. NPS and **4** significantly increased the latency time, as compared to aCSF injected (** $p < 0.01$, * $p < 0.05$; one way ANOVA with Dunnett's Multiple Comparison Test). [aCSF, $n = 12$; compound **4** 0.01 nmole, $n = 9$; compound **4** 0.1 nmole, $n = 8$; compound **4** 1 nmole, $n = 12$; compound **4** 10 nmole, $n = 18$; NPS 1 nmole, $n = 13$]

Figure 7.
Proposed mechanism through which NPS and compound 4 produce disparate behavioral effects.

Table 1

Functional assessment of truncated NPS Peptides at the hNPSR-107I

		Calcium Mobilization						cAMP Accumulation			
hNPSR 107I Cell Line											
		EC ₅₀ (nM)	Max	Log(max/EC ₅₀)	log(max/EC ₅₀)	Log(max/EC ₅₀)	BIAS [†]	log(max /EC ₅₀)	Log (max/EC) 50	Max	EC ₅₀ (nM)
NPS		0.59 ± 0.08	0.96	9.21	0.00	0	1.00	0.00	8.38	1	4.19 ± 0.37
1. SerPheArgAsnGlyVal-NH ₂		0.7 ± 0.2 *	0.96	9.14	-0.07	-0.40	0.40	0.33	8.71	1	1.95 ± 0.28
2. SerPheArgAsnGly-NH ₂		515 ± 51.2 *	0.96	6.27	-2.94	0.31	2.06	-3.25	5.13	0.41	3056 ± 640
3. SerPheArgAsn-NH ₂		2.43 ± 0.42	1	8.61	-0.60	0.89	7.85	-1.49	6.89	1	129 ± 18
4. SerPheLysAsn-NH2		3.02 ± 0.74	1	8.52	-0.69	0.81	6.44	-1.50	6.88	0.89	117 ± 22
5. SerPheDimethylLysAsn-NH2		43.5 ± 16									
6. SerPheLysCOCF3Asn-NH2		563 ± 332									
7. SerPheAOCAsn-NH2		436 ± 46									

[†] Bias Toward Calcium

* Data from Bernier et. al.

Table 2

Functional assessment of truncated NPS Peptides at the hNPSR-107N

	Calcium Mobilization						cAMP Accumulation			
hNPSR 107N Cell Line										
	EC ₅₀ (nM)	Max	Log(max/EC ₅₀)	log(max/EC ₅₀)	Log(max /EC ₅₀)	BIAS ^I	log(max/EC) 50	Log(max/EC) 50	Max	EC ₅₀ (nM)
NPS	2.00 ± 0.19	0.97	8.69	0.00	0.00	1.00	0.00	7.30	0.97	48.9 ± 2.9
1. SerPheArgAsnGlyVal-NH ₂	40.2 ± 11.6 [*]	0.97	7.38	-1.31	-0.36	0.43	-0.94	6.36	0.97	427
2. SerPheArgAsnGly-NH ₂	>2000 [*]	0.97								>10,000
3. SerPheArgAsn-NH ₂	998 ± 104	1	6.00	-2.69	2.69					>10,000

^I Bias Toward Calcium^{*} Data from Bernier et. al.

Table 3

Functional assessment of truncated NPS Peptides at the mNPSR

	Calcium Mobilization	mNPSR Cell Line										cAMP Accumulation
		EC ₅₀ (nM)	Max	Log(max/EC ₅₀)	log(max/EC ₅₀)	Log(max /EC ₅₀)	BIAS ¹	log(max/EC ₅₀)	Log(max/EC ₅₀)	Max	EC ₅₀ (nM)	
NPS		5.55 ± 0.51	1	8.26	0.00	0	1.00	0.00	8.35	0.82	3.67 ± 0.35	
1. SerPheArgAsnGlyVal-NH ₂		2.03 ± 0.3	0.98	8.68	0.42	0.90	7.96	-0.48	7.87	1	13.4 ± 4.8	
2. SerPheArgAsnGly-NH ₂		1014 ± 206	0.64	5.80	-2.46						>10,000	
3. SerPheArgAsn-NH ₂		66.6 ± 5.7	0.86	7.11	-1.15	1.16	14.61	-2.31	6.04	0.87	800 ± 359	
4. SerPheLysAsn-NH2		100 ± 5.3	0.89	6.95	-1.31	0.98	9.53	-2.29	6.04	1	911 ± 86	

¹ Bias Toward Calcium