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Somatostatin receptor subtype-4 agonist NNC 26-9100 decreases extracellular and intracellular A β ₁₋₄₂ trimers

Karin E. Sandoval¹, Susan A. Farr², William A. Banks³, Albert M. Crider¹, John E. Morley², and Ken A. Witt¹

¹Pharmaceutical Sciences, School of Pharmacy, Southern Illinois University Edwardsville, 200 University Park Drive, Edwardsville, Illinois

²Geriatric Research Education and Clinical Center, Veterans Affairs Medical Center–St Louis, Missouri, and Department of Internal Medicine, Division of Geriatrics, Saint Louis University School of Medicine, Missouri

³Geriatric Research Education and Clinical Center-Veterans Affairs Puget Sound Health Care System, Seattle, Washington and Department of Internal Medicine, Division of Gerontology and Geriatric Medicine, University of Washington School of Medicine, Seattle, Washington

Abstract

Soluble amyloid β -protein (A β) oligomers are primary mediators of synaptic dysfunction associated with the progression of Alzheimer's disease. Such A β oligomers exist dependent on their rates of aggregation and metabolism. Use of selective somatostatin receptor-subtype agonists have been identified as a potential means to mitigate A β accumulation in the brain, via regulation of the enzyme neprilysin. Herein, we first evaluated the impact of the somatostatin receptor subtype-4 agonist 1-[3-[*N*-(5-Bromopyridin-2-yl)-*N*-(3,4-dichlorobenzyl)amino]propyl]-3-[3-(1*H*-imidazol-4-yl)propyl]thiourea (NNC 26-9100) on learning and memory in 12-month SAMP8 mice (i.c.v. injection). NNC 26-9100 (0.2 μ g-dose) was shown to enhance both learning (T-maze) and memory (object recognition) compared to vehicle controls. Cortical and hippocampal tissues were evaluated subsequent to NNC 26-9100 (0.2 μ g) or vehicle administration for changes in neprilysin activity, along with protein expression of amyloid-precursor protein (APP), neprilysin, and A β ₁₋₄₂ oligomers within respective cellular fractions (extracellular, intracellular and membrane). NNC 26-9100 increased neprilysin activity in cortical tissue, with an associated protein expression increase in the extracellular fraction and decreased in the intracellular fraction. A decrease in intracellular APP expression was found with treatment in both cortical and hippocampal tissues. NNC 26-9100 also significantly decreased expression of A β ₁₋₄₂ trimers within both the extracellular and intracellular cortical fractions. No expression changes were found in membrane fractions for any protein. These findings suggest the potential use of selective SSTR4 agonists to mitigate toxic oligomeric forms of A β ₁₋₄₂ in critical regions of the brain identified with learning and memory decline.

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Corresponding Author: Ken A. Witt Ph.D., Department of Pharmaceutical Sciences, School of Pharmacy, Southern Illinois University Edwardsville, Campus Box 2000, Edwardsville, IL., 62026, Phone: 618-650-5144, Fax: 618-650-5145, kwitt@siue.edu.

Disclosure Statement

A use-patent has been obtained for NNC 26-9100 by Southern Illinois University Edwardsville. No other disclosures.

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Keywords

NNC 26-9100; Somatostatin receptor subtype-4; Neprilysin; Amyloid-beta oligomers

1. Introduction

Alzheimer's disease is an age-related neurodegenerative disorder affecting millions of individuals worldwide (Ziegler-Graham et al., 2008). A primary factor in Alzheimer's disease progression is the accumulation of amyloid-beta ($A\beta$) peptide, with the 42-amino acid form ($A\beta_{1-42}$) shown to more readily aggregate (Jarrett et al., 1993). Furthermore, evidence strongly implicates that soluble oligomeric assemblies of $A\beta_{1-42}$ are primary contributors to Alzheimer's disease pathogenesis, as they have been shown to correlate much better than $A\beta$ end-product plaque content with the extent of synaptic loss and severity of cognitive impairment (Mucke et al., 2000; Selkoe, 2008). Therapeutics aimed at degrading $A\beta_{1-42}$ oligomers may delay or even halt Alzheimer's disease progression.

Somatostatin (a.k.a. somatotropin release-inhibiting factor) is a neuropeptide shown to elevate the activity of the enzyme neprilysin within the brain (Saito et al., 2005). Neprilysin is the principle $A\beta$ metabolizing enzyme within the brain (Iwata et al., 2001; Iwata et al., 2000), also shown to be involved in the metabolism of the neurotoxic soluble $A\beta_{1-42}$ oligomers (Huang et al., 2006). With the progression of Alzheimer's disease a decline in somatostatin levels within the brain (~10–20%) occurs, which corresponds with decreased neprilysin activity and increased $A\beta$ content (Hellstrom-Lindahl et al., 2008; Wang et al., 2010). Thus, the use of somatostatin-based therapeutics may serve to enhance neprilysin activity and reduce the toxic oligomeric forms of $A\beta_{1-42}$.

The effects of somatostatin occur through five receptor subtypes, with somatostatin receptor subtype-4 (SSTR4) shown to be heavily concentrated in the cortex and hippocampus (Bruno et al., 1992; Moller et al., 2003). Not only are the cortex and hippocampus areas vital to learning and memory, but are also significantly affected by $A\beta$ accumulation and associated with reduced neprilysin levels in Alzheimer's disease patients (Yasojima et al., 2001a; Yasojima et al., 2001b). The SSTR4 agonist NNC 26-9100 is an enzymatically stable non-peptide drug having a >100-fold selectivity for SSTR4 over other subtypes (Ankersen et al., 1998; Crider et al., 2004). We have previously shown that chronic (i.p.) administration of NNC 26-9100 was able to mitigate the learning and memory decline in senescence accelerated mouse prone-8 (SAMP8) mice and reduce levels of $A\beta_{x-42}$ within the brain (Sandoval et al., 2011). However, the impact of NNC 26-9100 on neprilysin and $A\beta_{1-42}$ oligomers has not been assessed. We hypothesize that NNC 26-9100 acts on SSTR4 to increase neprilysin activity, which reduces the expression of $A\beta_{1-42}$ oligomers, leading to improved learning and memory. Herein, we first evaluated the effects of acute NNC 26-9100 (i.c.v.) treatment on learning (T-maze) and memory (object-recognition) in SAMP8 mice. The dose of NNC 26-9100 shown to enhance learning and memory was then used for subsequent *ex vivo* cortical and hippocampal tissue evaluations. Respective tissues were evaluated for changes in neprilysin activity, as well as protein expression of neprilysin, amyloid-precursor protein (APP), and $A\beta_{1-42}$ oligomers within extracellular, membrane, and intracellular fractions. Delineation of cellular fractions has been identified as key in understanding the impact of $A\beta$ oligomers within the brain (Lesne et al., 2006).

2. Materials and methods

2.1. Chemicals and reagents

The selective SSTR4 agonist 1-[3-[*N*-(5-Bromopyridin-2-yl)-*N*-(3,4-dichlorobenzyl)amino]propyl]-3-[3-(1*H*-imidazol-4-yl)propyl]thiourea (NNC 26-9100) was synthesized, purified, and confirmed via NMR by Dr. A.M. Crider per previously established protocols (Ankersen et al., 1998; Crider et al., 2004). All other chemicals and reagents, unless otherwise stated, were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Animals

Twelve-month old male SAMP8 mice were used for all behavioral and post-treatment (*ex vivo*) assessments. The SAMP8 mouse model has been consistently shown to present primary behavioral, morphological, and neurochemical changes identified with cognitive dementia and Alzheimer's disease (Morley, 2002; Poon et al., 2005). The SAMP8 mouse strain undergoes age-dependent learning and memory deficits associated with increased amounts of APP and soluble A β in brain tissue at 12-months of age (Del Valle et al., 2010; Tomobe and Nomura, 2009). Additionally, at 12-months of age the SAMP8 mice do not express plaques (Morley, 2002; Tomobe and Nomura, 2009), but rather the soluble forms of A β identified with toxic oligomeric activity. Mice were housed in rooms with a 12 hr light/dark cycle (20–22°C) with water and food available *ad libitum*. All experiments were conducted in accordance with the institutional approval of the animal use subcommittee, which subscribes to the NIH Guide for Care and Use of Laboratory Animals. SAMP8 mice were obtained from the breeding colony at the Veterans Affairs Medical Center - VA hospital (St. Louis, MO). The colony is derived from siblings generously provided by Dr. Takeda (Kyoto University, Japan).

2.3. Dosing

Measurement of the effects of NNC 26-9100 on learned acquisition was performed following i.c.v. injection in 12-month-old male SAMP8 mice. Prior to testing, the mice were anesthetized with isoflurane, placed in a stereotaxic instrument, and the scalp was deflected. A unilateral hole was drilled 0.5 mm anterior and 1.0 mm to the right of the bregma. A single i.c.v. (2 μ l) injection of NNC 26-9100 (0.002, 0.02, 0.2, or 2.0 μ g) or vehicle control (20% ethanol/saline) was conducted with a 30 g beveled needle connected to a Hamilton syringe, to a depth of 2 mm. Syringe was kept in place via side-arm attachment. Injections were conducted in a gradual manner over ~1 min and syringe was kept in place for 10 s following to assure no back-flow occurred. The hole was filled in with bone-wax and scalp sealed via Vetbond (3M, St. Paul, MN). Following behavioral evaluations and immediately prior to respective tissue assessments brains were cut along the injection site to assure accuracy. Additional mice were also treated (0.2 μ g dose) in an identical manner and time-frame for the purposes of supplying enough tissues for all respective molecular/biochemical analyses.

2.4. T-maze testing

The T-maze avoidance apparatus testing procedures have been previously described in detail and shown as an effective means to assess learning in SAMP8 mice (Farr et al., 2004; Sandoval et al., 2011). All respective evaluation sets were alphabetically coded, with the individual performing the test blind to treatment. Dosing was performed 24 h prior to T-maze evaluation. Briefly, the T-maze consisted of a black plastic alley with a start box at one end and two goal boxes at the other. The start box was separated from the alley by a plastic guillotine door, which prevented movement down the alley until training began. An electrifiable stainless steel rod floor ran throughout the maze to deliver scrambled foot-

shock. Mice were trained and tested between 07:00 and 15:00 h. Mice were not permitted to explore the maze prior to training. A training trial began when a mouse was placed into the start box. The guillotine door was raised and the buzzer was sounded simultaneously; 5 s later, footshock was applied. The goal box the mouse first entered on the first trial was designated as “incorrect”. Footshock was continued until the mouse entered the other goal box, which on all subsequent trials was designated “correct” for that particular mouse. At the end of each trial, the mouse was removed from the goal box and returned to its home cage. A new trial began by placing the mouse back in the start box, sounding the buzzer, and raising the guillotine door. Footshock was applied 5 s later if the mouse did not leave the start box or had not entered the correct goal box. The number of trials to learned avoidance of the shock by entering the correct goal box within 5 s was determined. The “mean trials to first avoidance” represents acquisition learning.

Training used an intertrial interval of 45 s, and a doorbell type buzzer set at 65 dB as the conditioned stimulus warning of onset of foot shock which was set 0.40 mA (Coulbourn Instruments scrambled grid floor shocker model E13-08). Mice were trained until they made 1 avoidance (acquisition). The acquisition test occurred in single test sessions. Each session lasted from 5 to 15 min. At the end of the acquisition trial, mice were decapitated and the brains snap frozen and stored at -80°C for subsequent analyses.

2.5. Object-recognition testing

Object-recognition is a non-spatial memory recognition task, shown to be effective in SAMP8 mice (Fontan-Lozano et al., 2008). This method has also been used to study aging deficits, early developmental influences, teratological drug exposure and novelty seeking behavior (Bevins and Besheer, 2006). In this task, mice are measured as to the difference in time spent with a familiar (i.e. remembered) object and a novel object. The aim was to test the animal’s memory of the original object by comparing the amount of time spent exploring the novel object against that for the familiar one. All respective evaluation sets were alphabetically coded, with the individual performing test blind to treatment. Mice received a single optimized NNC 26-9100 dose determined from T-maze assessment ($0.2\text{ }\mu\text{g}$), or vehicle control. Dosing was performed 24 h prior to the initial training period and the recognition task was evaluated 24 h after training (i.e. 48 h after dosing).

Briefly, mice were habituated to an empty apparatus ($55\times 40\times 40\text{ cm}$) for 5 min a day for 3 days prior to entry of the objects. On the day of training, two different objects were placed in the arena. Selected objects were thoroughly cleansed between trials to ensure the absence of olfactory cues. Mice were placed in the arena and allowed to explore the objects for 5 min. The difference in percent time spent exploring the new object over the familiar object was recorded. The criteria for exploration were based strictly on active exploration, where the mouse had both forelimbs within a circle of 1.5 cm around the object, with its head oriented toward it, or when touching it with its vibrissae.

2.6. Neprilysin activity assay

The dose of NNC 26-9100 shown to enhance learning and memory ($0.2\text{ }\mu\text{g}$) was used for subsequent *ex vivo* cortical and hippocampal tissue evaluations, against vehicle control. Cortical and hippocampal portions of from flash frozen brain tissues were assayed for neprilysin activity (Iwata et al., 2002). Respective portions were homogenized (hippocampi pooled in twos within respective treatment) with a tissue grinder in 5x vol (w/v) of ice-cold 10 mM Tris-HCl buffer (pH 8.0) containing 0.25 M sucrose, protease inhibitor cocktail (Ethylenediaminetetraacetic acid (EDTA)-free CompleteTM, Roche Diagnostics, Indianapolis, IN) and $0.7\text{ }\mu\text{g/ml}$ pepstatin-A. The homogenates were centrifuged at $9,000\times g$ at 4°C for 15 min, and the supernatants were further centrifuged at $200,000\times g$ and for 20

min at 4°C (Sorvall Discover M120 SE MicroUltra Centrifuge, Asheville, NC). The pellets were solubilized on shaker and at 4°C in Tris-HCl buffer containing 1% Triton X-100 (v/v) for 1 h. The solubilized fractions were then centrifuged at $200,000 \times g$ and 4°C for 20 min. The neprilysin-dependent activity was fluorometrically assayed (FluoDia T70, Photon Technology International, Inc., Birmingham, NJ) and determined from the fluorescence intensity (excitation, 360 nm; emission, 450 nm). The assay consisted of 30 µg of isolated protein, 50 µM succinyl-alanine-alanine-phenylalanine-4-methyl-coumarinyl-7-amide (Suc-Ala-Ala-Phe-AMC) (Bachem, Torrance, CA) as substrate, and 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (in diH₂O; pH 6.5) in a total volume of 100 µl. The reaction was initiated by addition of substrate to the assay mixture, and carried out at 37°C for 60 min. The neprilysin activity was determined from the absorbance of the liberated-AMC and from the decrease in the rate of digestion caused by 10 µM thiorphan, a specific inhibitor of neprilysin. Data expressed in pmol/mg/min of liberated AMC. Protein concentrations were determined using standard BCA protein assay kit (Pierce, Rockford, IL).

2.7. Protein extractions

Cortical and hippocampal portions of from flash frozen brain tissues were extracted and isolated into protein fractions based on the protocol by Lesné et al. (Lesné et al., 2006) (hippocampi pooled in twos within respective treatment). Tissues were homogenized (10 strokes with glass Douncer) in 2x vol (w/v) of ice-cold extracellular buffer (50 mM Tris-HCl (pH 7.6), 0.01% NP-40, 150 mM NaCl, 2mM EDTA, 0.1% SDS, 1mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), and Complete protease inhibitor (Roche). Samples were then centrifuged at 3,000 rpm at 4°C for 5 min and extracellular-enriched protein fraction was collected (supernatant). Pellets were then gently homogenized in ice-cold intracellular buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.1% Triton X-100, protease inhibitor) (300 µl: cortical & 150 µl: hippocampi) using a micropipettor. Samples were then centrifuged for 13,000 rpm at 4°C for 90 min and cytoplasmic-enriched protein fraction was collected (supernatant). Pellets were then homogenized in ice-cold membrane buffer (50 mM Tris-HCl [pH 7.4], 150 mM, NaCl, 0.5% Triton X-100, 1 mM EGTA, 3% SDS, 1% deoxycholate, 1 mM AEBSF, and Complete protease inhibitor) (500 µl: cortical & 200 µl: hippocampi) using a micropipettor. Samples were then centrifuged for 13,000 rpm at 4°C for 90 min and membrane-enriched protein fraction was collected (supernatant).

Respective samples within each fraction were then immunodepleted by sequential incubation for 1 h at 4°C on a rotating mixer, with Protein A-Sepharose, followed by Protein G-Sepharose, (Fast-Flow®, GE Healthcare Biosciences, Pittsburgh, PA) (30 µl: cortical & 20 µl: hippocampi). After each incubation-set samples were filtered from Sepharose via 10 µm pore size filter Spin Cups (Pierce). Following clarification, samples were stored at -80°C for subsequent BCA protein analyses for Western blot examination.

2.8. Western blot analysis

Western blot analyses were performed on the respective protein extractions sets for APP (MAB348, clone: cc2211, Millipore, Temecula, CA), neprilysin (ab79423, clone#: EPR2997, Abcam, Cambridge, MA) and Aβ₁₋₄₂ (AB5078P, C-terminus, Millipore). Per distributor testing (Millipore Certificate of Analysis), the Aβ₁₋₄₂ antibody does not react to Aβ₁₋₄₀. For Aβ₁₋₄₂ evaluations, purified Aβ₁₋₄₂ peptide was used as a positive control (Sigma, A9810). For APP and neprilysin expression analyses, samples were heated at 95 °C for 5 min in 2x SDS and then separated using an electrophoretic field using 10% Tris/HCl Criterion gels (Bio-Rad, Hercules, CA) at 175 V for 70 min. The proteins were then transferred to nitrocellulose membranes with 240 mA for 45 min (4°C). The membranes

were blocked using 5% nonfat milk-Tris-buffered saline (20 mM Tris base, 137 mM NaCl, pH 7.6) with 0.1% Tween-20 for 4 h and then incubated overnight at 4 °C with primary antibodies (1:2,000–1:4,000 dilution) in PBS-0.5% BSA. The membranes were then washed with 5% nonfat milk-Tris-buffered saline buffer before incubation with the respective secondary antibody at a (1:1,000–1:10,000) dilution (in PBS-0.5% BSA) for 1 h at room temperature. For A β ₁₋₄₂ expression analyses, samples were heated at 95°C for 5 min in 2x SDS and 20x reducing agent (Bio-rad) and loaded into 10% Bis/Tris Criterion XT gels (Bio-Rad). Bands were then separated using an electrophoretic field at 200 V for 35 min in MES running buffer (Bio-Rad). The proteins were then transferred to nitrocellulose membranes with 240 mA at for 30 min (4°C). Following the transfer, the nitrocellulose membranes were boiled for 5 min (sandwiched between filter pads and weighed down in boiling diH₂O) and then blocked in 5% milk-Tris-buffered saline for 4 hours or overnight. After all transfers, gels were then evaluated with GelCode Blue Stain (Pierce, Rockford, IL) to confirm appropriate protein loading. Blots were developed using the enhanced chemiluminescence method (ECL+, Amersham Life Science Products; Springfield, IL; or SuperSignal West Dura or SuperSignal West Femto, Pierce) and protein bands visualized on X-ray film. Optical densities of expressed bands were measured by calibrated densitometer (GS-800, Biorad).

2.9. Statistical analyses

Results were expressed as means with their standard errors. The T-maze acquisition (mean trials to make first avoidance) test scores were analyzed via one-way analysis of variance (ANOVA), groups were compared with Tukey's post-hoc analyses. Student's t-test was used when two groups were evaluated.

3. Results

3.1. Learning and memory

Acquisition learning in SAMP8 mice was conducted via the T-maze foot-shock avoidance test following i.c.v. administration of NNC 26-9100 (0.002, 0.02, 0.2 or 2.0 μ g, with 0 μ g as the vehicle control). NNC 26-9100 at 0.2 μ g showed a significant improvement ($P < 0.01$, one-way ANOVA, $F_{\text{value}} = 6.77$) in learned avoidance, indicated by the lower number of mean trials compared to vehicle control ($n = 8/\text{group}$) (Fig. 1). In a separate group of mice, the optimized dose of NNC 26-9100 (via T-maze determination) was evaluated for its effects on object-recognition, which is a behavioral test of memory ($n = 8/\text{group}$) (Fig. 1 inset). Mice treated with NNC 26-9100 spent significantly more time with the novel object versus the non-novel object when compared to vehicle control ($P < 0.01$, Student's T-test) showing an approximately 2-fold enhancement in recognition memory.

3.2. Neprilysin activity

Effect of NNC 26-9100 on neprilysin activity in both cortical and hippocampal tissues was measured to determine if acute administration altered neprilysin activity (Fig. 2). Mice treated with NNC 26-9100 showed enhanced neprilysin activity ($P < 0.05$, Student's T-test) in cortical tissues compared to vehicle controls ($n = 6/\text{group}$). No significant change was identified within hippocampus with acute NNC 26-9100 treatment when compared to vehicle ($n = 6/\text{group}$).

3.3. Neprilysin protein expression

Neprilysin protein expression evaluations were conducted from extracellular, membrane, and intracellular fractions from cortical (Fig. 3A) and hippocampal (Fig. 3B) tissues ($n = 4-5/\text{group}$) to determine if acute NNC 26-9100 resulted in differential expression changes within different cellular fractions. In the cortical extracellular fraction, expression of

neprilysin was significantly increased in mice treated with NNC 26-9100 compared to vehicle ($p < 0.001$, Student's T-test). In the cortical membrane fraction, no significant changes between mice treated with NNC 26-9100 or vehicle were observed in the expression of neprilysin. In contrast, there was a significant decrease in cortical neprilysin expression within the intracellular fraction in mice treated with NNC 26-9100 compared to vehicle ($P < 0.01$, Student's T-test). No significant expression changes in neprilysin were found between vehicle and NNC 26-9100 treated mice in any of the hippocampal fractions.

3.4. Amyloid precursor protein expression

APP protein expression evaluations were also conducted from extracellular, membrane, and intracellular fractions from cortical (Fig. 4A) and hippocampal (Fig. 4B) tissues ($n = 4-5$ /group). No significant changes in APP expression were found in cortical or hippocampal extracellular or membrane fractions in mice treated with NNC 26-9100 compared to vehicle. However, within the cortical and hippocampal intracellular fraction, expression of APP was significantly decreased in mice treated with NNC 26-9100 compared to vehicle ($P < 0.001$, $P < 0.05$ respectively, Student's T-test).

3.5. Oligomeric A β_{1-42} protein expression

Oligomeric A β_{1-42} protein expression evaluations were conducted from extracellular, membrane, and intracellular fractions isolated from cortical and hippocampal tissues. Within the extracellular fraction, oligomeric bands were found at 56 kDa (dodecamer; a.k.a. A β^* 56), 50 kDa, 40 kDa (nonamer), 25 kDa (hexamer), and 12 kDa (trimer) within the cortex (Fig. 5A) and hippocampus (Fig. 5B). No discernable bands were identifiable below the 12 kDa band, with exception of lane possessing the A β_{1-42} positive control (4 kDa). Within the cortex, no significant changes were found in the expression of the 56, 50, or 40 kDa bands with NNC 26-9100 treatment. While there was not a significant change in expression of the 25 kDa band with NNC 26-9100 treatment, a distinctive double-banding pattern was present. The 12 kDa band showed a significant decrease in expression ($P < 0.01$, Student's T-test) with NNC 26-9100 treatment. While similar trends were observed within the hippocampal tissue, significance was not reached (Fig. 5B).

Within the membrane fraction, A β_{1-42} oligomers within the cortex (Fig. 6A) and hippocampus (Fig. 6B) were observed at 56, 50, and 25 kDa. No significant changes were found within this fraction for any oligomer in either region with NNC 26-9100 treatment when compared to vehicle.

A β_{1-42} oligomers within the intracellular fractions of the cortex (Fig. 7A, Student's T-test) and hippocampus (Fig. 7B) were observed at 56, 50, 25, and 12 kDa. NNC 26-9100 produced no significant changes in expression of the 56, 50, or 25 kDa bands in either tissue set when compared to vehicle. However, NNC 26-9100 significantly decreased the 12 kDa band ($P < 0.01$, Student's T-test) within the cortex compared to vehicle controls. In contrast, there was no significant decrease in expression of the 12 kDa band within the hippocampus.

4. Discussion

Somatostatin decline in cortical and hippocampal tissues with Alzheimer's disease progression has been hypothesized to be a critical component in the loss of cognitive function (Epelbaum et al., 2009). The use of selective somatostatin subtype agonists may prove a viable means to mitigate the disease. We have previously shown that chronic treatment with the selective SSTR4 agonist NNC 26-9100 enhanced learning and memory in 12-month SAMP8 mice, with an associated reduction in A β_{x-42} (Sandoval et al., 2011). We theorized that NNC 26-9100 was reducing soluble A β by increasing neprilysin activity, the principle A β metabolizing enzyme. Moreover, whether NNC 26-9100 impacts toxic A β_{1-42}

oligomers levels has not been elucidated. Herein, we investigated the single dose (i.c.v.) administration of NNC 26-9100, with *ex vivo* assessment of neprilysin, APP, and toxic A β ₁₋₄₂ oligomers alterations within cortical and hippocampal tissues of 12-month SAMP8 mice. To ensure viability, the dose of NNC 26-9100 chosen for the tissue evaluations was based on enhancement of learned acquisition (T-maze behavioral testing).

The first portion of the examination evaluated dose-response impact of NNC 26-9100. SAMP8 mice given the 0.2 μ g dose of NNC 26-9100, but not the respective 0.002, 0.02 or 2 μ g doses, showed an ~2-fold improvement in acquisition learning using the T-maze paradigm over vehicle controls. This identified a dose specific effect for NNC 26-9100. Such dose-specific mediated learning responses are common for substances impacting learning and memory (Farr et al., 2006; Farr et al., 1999; Jaeger et al., 2002). While low doses may not be sufficient to produce a learning and/or memory effect, doses that are too high may also impede learning and memory processes. Moreover, the 0.2 μ g dose of NNC 26-9100 was also shown to enhance memory recognition in a separate group of mice, via the object-recognition test. Object recognition is unique in that it allows for memory evaluation without stressors that may influence the results, and is associated with cortex-dependent declarative memory (Winters et al., 2008). Object recognition impairment has also been shown with increased levels of A β (Mouri et al., 2007; Simon et al., 2009). Thus, we identified that the 0.2 μ g dose (i.c.v.) of SSTR4 agonist NNC 26-9100 directly enhanced learning and memory in the SAMP8 mouse model.

While several A β -degrading enzymes have been identified within the brain (Miners et al., 2008), neprilysin has been shown to be a primary mediator of A β ₁₋₄₂ catabolism (Howell et al., 1995; Iwata et al., 2000) with its decline associated with Alzheimer's disease progression (Hersh and Rodgers, 2008; Wang et al., 2010). To determine the impact of NNC 26-9100 on neprilysin we evaluated both activity, as well as its expression within respective cellular fractions. NNC 26-9100 treatment significantly enhanced cortical neprilysin activity, with a similar trend in the hippocampus. While previous evaluations have identified somatostatin to significantly elevate neprilysin activity (Saito et al., 2005), this is the first data to show that a selective SSTR4 agonist can enhance the activity of neprilysin. Initial evaluations of neprilysin expression from radio-immuno assay (RIPA)-buffer extracted tissues (non-fractionated) did not show any significant alterations with treatment (data not shown); however, there were identifiable expression alterations between cellular fractions. A significant increase in neprilysin expression was found in the extracellular fraction with NNC 26-9100 treatment, while a corresponding decrease was found in the intracellular fraction. While a similar trend was shown in hippocampal tissue, it lacked statistical significance. Our data suggests that with NNC 26-9100 treatment may induce a shift in neprilysin expression from the intracellular fraction to the extracellular fraction.

The level of APP within the brain is consistently shown to increase in Alzheimer's disease. Alterations in processing, trafficking, and proteolysis of APP directly impact generation of A β (Chow et al., 2010). Initial evaluations of APP expression from RIPA-buffer extracted tissues (non-fractionated) did not show any significant protein expression alterations with treatment (data not shown). However, when APP expression was evaluated within different cellular fractions, NNC 26-9100 treatment resulted in a decreased intracellular expression in both cortical and hippocampal tissues. The impact of NNC 26-9100 on APP may or may not be independent of direct actions via neprilysin. An earlier study showed neprilysin did not impact APP metabolism (Howell et al., 1995), indicating the decreased intracellular expression of both proteins would be unrelated. Yet, more recently APP processing has been shown to regulate neprilysin expression through APP intracellular domain (AICD). One study found that generation of AICD by β - and γ -secretase increased the expression of neprilysin (Belyaev et al., 2010). In contrast, when β - or γ -secretase (but not α -secretase)

were blocked, a decreased neprilysin expression was shown. As our data shows an intracellular decrease in neprilysin and APP expression with treatment, there is the potential that NNC 26-9100 may also impact the activity of β - or γ -secretase. Yet, if our agonist increased β or γ secretase activity, we would expect to see an increase and not a reduction in $A\beta_{1-42}$. Nevertheless, to date no studies have assessed the impact of somatostatin or respective receptor subtypes on β - or γ -secretase activity.

The final portion of our evaluation assessed NNC 26-9100 treatment effect on $A\beta_{1-42}$ oligomer expression within respective cellular fractions. Although low-n $A\beta$ oligomers are hypothesized to be primary contributors to Alzheimer's disease, their respective impact on the disease remains unclear. This is in part due to the lack of delineation of $A\beta$ oligomer alterations within the context of their cellular localization. While extracellular alterations in $A\beta$ content have long been affiliated with downstream plaque formation, emerging evidence strongly implicates intracellular accumulation is an early marker of Alzheimer's disease that directly contributes to neurodegeneration (Gimenez-Llort et al., 2007).

Previous studies have identified the 56 kDa and 40 kDa $A\beta_{1-42}$ oligomers correlated positively with the onset of impaired spatial memory in APP^{swe} (Tg2576) mice (Lesne et al., 2006). We observed no change in the 56 or 40 kDa $A\beta$ oligomeric bands with acute single-dose NNC 26-9100 treatment, despite elevated neprilysin activity and expression. Other studies have demonstrated similar findings, as neprilysin overexpressing transgenic mice cross-bred with human-APP transgenic mice did not result in the reduction in the 56 kDa oligomeric form (Meilandt et al., 2009).

A distinctive expression change was found with the 25 kDa band of $A\beta_{1-42}$. The 25 kDa band showed a consistent "split" within the extracellular cortical fraction with NNC 26-9100 treatment. This may represent a post-translational modification (i.e. phosphorylation) or degradation. The effect was not observed in the hippocampal extracellular fraction, nor was any significant change shown in respective membrane or intracellular fractions with treatment. Interestingly, a recent evaluation showed SAMP8 mice treated with an antibody against $A\beta_{1-42}$ oligomers in the molecular weight range of 16.5-25 kDa had enhanced learning and memory (Zhang et al., 2011). Thus, while there is limited data with regards to the hexamer form, this low-weight oligomer form may play a significant role in neuronal toxicity.

The 12 kDa trimer has been one of the more heavily evaluated $A\beta_{1-42}$ oligomers and has been strongly implicated as an inhibitor of long-term potentiation (Selkoe, 2008; Townsend et al., 2006). When APP transgenic mice (APP23) were cross-bred with neprilysin-deficient mice, this resulted in an increase in $A\beta_{1-42}$ oligomeric trimers, and correlated with exacerbated cognitive impairments (Huang et al., 2006). Our data identifies a clear decrease in the cortical trimer band with NNC 26-9100 treatment, within both the extracellular and intracellular fractions, with a similar trend in the hippocampal fractions. Moreover, the decrease in cortical $A\beta_{1-42}$ trimer expression corresponded with increased neprilysin activity and expression in the extracellular cortical tissue. Yet, within the respective intracellular fractions, the decreased $A\beta_{1-42}$ trimer expression does not correspond with neprilysin expression, as neprilysin expression was significantly decreased. However, it may be possible that despite decreased intracellular neprilysin expression the overall increase in neprilysin activity may be sufficient to degrade intracellular $A\beta_{1-42}$ trimers.

5. Conclusion

Current evidence supports the hypothesis that neurotoxic $A\beta_{1-42}$ oligomers play a seminal role in Alzheimer's disease. Therapeutic approaches aimed at the reduction of toxic $A\beta$ oligomers have the potential to mitigate Alzheimer's disease. Our data shows that i.c.v.

administration of NNC 26-9100 increased neprilysin activity with a corresponding decrease in cortical A β ₁₋₄₂ trimers, intracellularly and extracellularly, at doses identified to improve learning and memory. While further examination is required to determine the relationship between the behavioral outcomes and the alterations in A β ₁₋₄₂ oligomers, we have shown that an SSTR4 agonist is capable of mitigating A β ₁₋₄₂ oligomeric trimer expression within the brain. Taken together, agonists directed SSTR4 may provide a viable avenue of therapy in the treatment of Alzheimer's disease.

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References

- Ankersen M, Crider AM, Liu S, Ho B, Andersen HS, Stidsen CE. Discovery of a Novel Non-Peptide Somatostatin Agonist with SST4 Selectivity. *Journal of the American Chemical Society*. 1998; 120:1368–1373.
- Belyaev ND, Kellett KA, Beckett C, Makova NZ, Revett TJ, Nalivaeva NN, Hooper NM, Turner AJ. The transcriptionally active amyloid precursor protein (APP) intracellular domain is preferentially produced from the 695 isoform of APP in a {beta}-secretase-dependent pathway. *J Biol Chem*. 2010; 285:41443–41454. [PubMed: 20961856]
- Bevins RA, Besheer J. Object recognition in rats and mice: a one-trial non-matching-to-sample learning task to study 'recognition memory'. *Nature protocols*. 2006; 1:1306–1311.
- Bruno JF, Xu Y, Song J, Berelowitz M. Molecular cloning and functional expression of a brain-specific somatostatin receptor. *Proc Natl Acad Sci U S A*. 1992; 89:11151–11155. [PubMed: 1360663]
- Chow VW, Mattson MP, Wong PC, Gleichmann M. An overview of APP processing enzymes and products. *Neuromolecular medicine*. 2010; 12:1–12. [PubMed: 20232515]
- Crider AM, Liu S, Li T, Mahajan S, Ankersen M, Stidsen CE. Somatostatin receptor subtype 4 (sst4) ligands: Synthesis and evaluation of indol-3-yl- and 2-pyridyl-thioureas. *Letters in Drug Design & Discovery*. 2004; 1:84–87.
- Del Valle J, Duran-Vilaregut J, Manich G, Casadesus G, Smith MA, Camins A, Pallas M, Pelegri C, Vilaplana J. Early amyloid accumulation in the hippocampus of SAMP8 mice. *J Alzheimers Dis*. 2010; 19:1303–1315. [PubMed: 20061609]
- Epelbaum J, Guillou JL, Gastambide F, Hoyer D, Duron E, Viollet C. Somatostatin, Alzheimer's disease and cognition: an old story coming of age? *Prog Neurobiol*. 2009; 89:153–161. [PubMed: 19595735]
- Farr SA, Banks WA, Morley JE. Effects of leptin on memory processing. *Peptides*. 2006; 27:1420–1425. [PubMed: 16293343]
- Farr SA, Banks WA, Uezu K, Gaskin FS, Morley JE. DHEAS improves learning and memory in aged SAMP8 mice but not in diabetic mice. *Life Sci*. 2004; 75:2775–2785. [PubMed: 15464829]
- Farr SA, Uezu K, Flood JF, Morley JE. Septo-hippocampal drug interactions in post-trial memory processing. *Brain Res*. 1999; 847:221–230. [PubMed: 10575091]
- Fontan-Lozano A, Romero-Granados R, Troncoso J, Munera A, Delgado-Garcia JM, Carrion AM. Histone deacetylase inhibitors improve learning consolidation in young and in KA-induced-neurodegeneration and SAMP-8-mutant mice. *Molecular and cellular neurosciences*. 2008; 39:193–201. [PubMed: 18638560]
- Gimenez-Llort L, Blazquez G, Canete T, Johansson B, Oddo S, Tobena A, LaFerla FM, Fernandez-Teruel A. Modeling behavioral and neuronal symptoms of Alzheimer's disease in mice: a role for intraneuronal amyloid. *Neurosci Biobehav Rev*. 2007; 31:125–147. [PubMed: 17055579]
- Hellstrom-Lindahl E, Ravid R, Nordberg A. Age-dependent decline of neprilysin in Alzheimer's disease and normal brain: inverse correlation with A beta levels. *Neurobiol Aging*. 2008; 29:210–221. [PubMed: 17098332]

- Hersh LB, Rodgers DW. Neprilysin and amyloid beta peptide degradation. *Current Alzheimer research*. 2008; 5:225–231. [PubMed: 18393807]
- Howell S, Nalbantoglu J, Crine P. Neutral endopeptidase can hydrolyze beta-amyloid(1-40) but shows no effect on beta-amyloid precursor protein metabolism. *Peptides*. 1995; 16:647–652. [PubMed: 7479298]
- Huang SM, Mouri A, Kokubo H, Nakajima R, Suemoto T, Higuchi M, Staufenbiel M, Noda Y, Yamaguchi H, Nabeshima T, Saido TC, Iwata N. Neprilysin-sensitive synapse-associated amyloid-beta peptide oligomers impair neuronal plasticity and cognitive function. *J Biol Chem*. 2006; 281:17941–17951. [PubMed: 16636059]
- Iwata N, Takaki Y, Fukami S, Tsubuki S, Saido TC. Region-specific reduction of A beta-degrading endopeptidase, neprilysin, in mouse hippocampus upon aging. *J Neurosci Res*. 2002; 70:493–500. [PubMed: 12391610]
- Iwata N, Tsubuki S, Takaki Y, Shirotani K, Lu B, Gerard NP, Gerard C, Hama E, Lee HJ, Saido TC. Metabolic regulation of brain Abeta by neprilysin. *Science*. 2001; 292:1550–1552. [PubMed: 11375493]
- Iwata N, Tsubuki S, Takaki Y, Watanabe K, Sekiguchi M, Hosoki E, Kawashima-Morishima M, Lee HJ, Hama E, Sekine-Aizawa Y, Saido TC. Identification of the major Abeta1-42-degrading catabolic pathway in brain parenchyma: suppression leads to biochemical and pathological deposition. *Nat Med*. 2000; 6:143–150. [PubMed: 10655101]
- Jaeger LB, Farr SA, Banks WA, Morley JE. Effects of orexin-A on memory processing. *Peptides*. 2002; 23:1683–1688. [PubMed: 12217429]
- Jarrett JT, Berger EP, Lansbury PT Jr. The carboxy terminus of the beta amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. *Biochemistry*. 1993; 32:4693–4697. [PubMed: 8490014]
- Lesne S, Koh MT, Kotilinek L, Kaye R, Glabe CG, Yang A, Gallagher M, Ashe KH. A specific amyloid-beta protein assembly in the brain impairs memory. *Nature*. 2006; 440:352–357. [PubMed: 16541076]
- Meilandt WJ, Cisse M, Ho K, Wu T, Esposito LA, Searce-Levie K, Cheng IH, Yu GQ, Mucke L. Neprilysin overexpression inhibits plaque formation but fails to reduce pathogenic Abeta oligomers and associated cognitive deficits in human amyloid precursor protein transgenic mice. *J Neurosci*. 2009; 29:1977–1986. [PubMed: 19228952]
- Miners JS, Baig S, Palmer J, Palmer LE, Kehoe PG, Love S. Abeta-degrading enzymes in Alzheimer's disease. *Brain pathology (Zurich, Switzerland)*. 2008; 18:240–252.
- Moller LN, Stidsen CE, Hartmann B, Holst JJ. Somatostatin receptors. *Biochim Biophys Acta*. 2003; 1616:1–84. [PubMed: 14507421]
- Morley JE. The SAMP8 mouse: a model of Alzheimer disease? *Biogerontology*. 2002; 3:57–60. [PubMed: 12014843]
- Mouri A, Noda Y, Hara H, Mizoguchi H, Tabira T, Nabeshima T. Oral vaccination with a viral vector containing Abeta cDNA attenuates age-related Abeta accumulation and memory deficits without causing inflammation in a mouse Alzheimer model. *FASEB J*. 2007; 21:2135–2148. [PubMed: 17341681]
- Mucke L, Masliah E, Yu GQ, Mallory M, Rockenstein EM, Tatsuno G, Hu K, Kholodenko D, Johnson-Wood K, McConlogue L. High-level neuronal expression of abeta 1-42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. *J Neurosci*. 2000; 20:4050–4058. [PubMed: 10818140]
- Poon HF, Farr SA, Banks WA, Pierce WM, Klein JB, Morley JE, Butterfield DA. Proteomic identification of less oxidized brain proteins in aged senescence-accelerated mice following administration of antisense oligonucleotide directed at the Abeta region of amyloid precursor protein. *Brain Res Mol Brain Res*. 2005; 138:8–16. [PubMed: 15932783]
- Saito T, Iwata N, Tsubuki S, Takaki Y, Takano J, Huang SM, Suemoto T, Higuchi M, Saido TC. Somatostatin regulates brain amyloid beta peptide Abeta42 through modulation of proteolytic degradation. *Nat Med*. 2005; 11:434–439. [PubMed: 15778722]

- Sandoval KE, Farr SA, Banks WA, Niehoff ML, Morley JE, Crider AM, Witt KA. Chronic peripheral administration of somatostatin receptor subtype-4 agonist NNC 26-9100 enhances learning and memory in SAMP8 mice. *Eur J Pharmacol.* 2011; 654:53–59. [PubMed: 21185826]
- Selkoe DJ. Soluble oligomers of the amyloid beta-protein impair synaptic plasticity and behavior. *Behav Brain Res.* 2008; 192:106–113. [PubMed: 18359102]
- Simon AM, Schiapparelli L, Salazar-Colocho P, Cuadrado-Tejedor M, Escibano L, Lopez de Maturana R, Del Rio J, Perez-Mediavilla A, Frechilla D. Overexpression of wild-type human APP in mice causes cognitive deficits and pathological features unrelated to Abeta levels. *Neurobiol Dis.* 2009; 33:369–378. [PubMed: 19101630]
- Tomobe K, Nomura Y. Neurochemistry, neuropathology, and heredity in SAMP8: a mouse model of senescence. *Neurochemical research.* 2009; 34:660–669. [PubMed: 19247832]
- Townsend M, Shankar GM, Mehta T, Walsh DM, Selkoe DJ. Effects of secreted oligomers of amyloid beta-protein on hippocampal synaptic plasticity: a potent role for trimers. *The Journal of physiology.* 2006; 572:477–492. [PubMed: 16469784]
- Wang S, Wang R, Chen L, Bennett DA, Dickson DW, Wang DS. Expression and functional profiling of neprilysin, insulin-degrading enzyme, and endothelin-converting enzyme in prospectively studied elderly and Alzheimer's brain. *J Neurochem.* 2010; 115:47–57. [PubMed: 20663017]
- Winters BD, Saksida LM, Bussey TJ. Object recognition memory: neurobiological mechanisms of encoding, consolidation and retrieval. *Neurosci Biobehav Rev.* 2008; 32:1055–1070. [PubMed: 18499253]
- Yasojima K, Akiyama H, McGeer EG, McGeer PL. Reduced neprilysin in high plaque areas of Alzheimer brain: a possible relationship to deficient degradation of beta-amyloid peptide. *Neurosci Lett.* 2001a; 297:97–100. [PubMed: 11121879]
- Yasojima K, McGeer EG, McGeer PL. Relationship between beta amyloid peptide generating molecules and neprilysin in Alzheimer disease and normal brain. *Brain Res.* 2001b; 919:115–121. [PubMed: 11689168]
- Zhang Y, He JS, Wang X, Wang J, Bao FX, Pang SY, Yin F, Hu HG, Peng XL, Sun WM, Zheng YP, Hou LL, Hong T. Administration of amyloid-beta42 oligomer-specific monoclonal antibody improved memory performance in SAMP8 mice. *J Alzheimers Dis.* 2011; 23:551–561. [PubMed: 21297277]
- Ziegler-Graham K, Brookmeyer R, Johnson E, Arrighi HM. Worldwide variation in the doubling time of Alzheimer's disease incidence rates. *Alzheimers Dement.* 2008; 4:316–323. [PubMed: 18790458]

Figure 1.

Acquisition learning of SAMP8 mice evaluated via T-maze task following i.c.v. injection of NNC 26-9100 (0.002, 0.02, 0.2 or 2.0 µg) or vehicle ($n = 8/\text{group}$). Inset shows memory recognition of SAMP8 mice evaluated via object-recognition testing following i.c.v. injection of 0.2 µg NNC 26-9100 or vehicle ($n = 8/\text{group}$). ** $P < 0.01$ as compared to vehicle (0 µg), values are the mean $\pm$ S.E.M. T-maze evaluated via one-way ANOVA ($F_{\text{value}} = 6.77$) followed by Tukey's post-hoc analysis, and object-recognition evaluated via Student's t-test.

Figure 2.

Cortical and hippocampal portions of from flash frozen brain tissues were assayed for neprilysin activity ($n = 6/\text{group}$). Activity determined from flourometric absorbance of liberated AMC against $10 \mu\text{M}$ thiorphan. Data expressed in pmol/mg/min of liberated AMC.

* $P < 0.05$ as compared to vehicle (Student's T-test), values are mean $\pm$ S.E.M.

Figure 3.

(A) Cortical and (B) hippocampal tissues analyzed via Western blot for neprilysin expression changes in extracellular, intracellular, and membrane enriched cellular fractions. Protein expression of NNC 26-9100 treated animals evaluated with optical densities normalized to respective vehicles ($n = 4-5/\text{group}$). Representative blots shown to right of bar graph. ** $P < 0.01$ and *** $P < 0.001$ (Student's T-test), values are mean $\pm$ S.E.M.

Figure 4.

(A) Cortical and (B) hippocampal tissues analyzed via Western blot for APP expression changes in extracellular, intracellular, and membrane enriched cellular fractions. Protein expression of NNC 26-9100 treated animals evaluated with optical densities normalized to respective vehicles ($n = 4-5/\text{group}$). Representative blots shown to right of bar graph. * $P < 0.05$ and *** $P < 0.001$ (Student's T-test), values are mean $\pm$ S.E.M.

Figure 5.

(A) Cortical and (B) hippocampal tissue extracellular fractions analyzed via Western blot for alterations in A β_{1-42} protein expression. Oligomeric A β_{1-42} forms identified at 56, 50, 40, 25, and 12 kDa. Protein expression of NNC 26-9100 treated animals evaluated with optical densities normalized to respective vehicles ($n = 4-5/\text{group}$). Representative blots shown to right of bar graph. $** P < 0.01$ (Student's T-test), values are mean $\pm$ S.E.M. ¥ indicates delineated split of the 25 kDa band with NNC 26-9100 treatment.

Figure 6.

(A) Cortical and (B) hippocampal tissue membrane fractions analyzed via Western blot for alterations in $A\beta_{1-42}$ protein expression. Oligomeric $A\beta_{1-42}$ forms identified at 56, 50, and 25 kDa. Protein expression of NNC 26-9100 treated animals evaluated with optical densities normalized to respective vehicles ($n = 4-5/\text{group}$). Representative blots shown to right of bar graph. Values are mean $\pm$ S.E.M.

Figure 7.

(A) Cortical and (B) hippocampal tissue intracellular fractions analyzed via Western blot for alterations in A β_{1-42} protein expression. Oligomeric A β_{1-42} forms identified at 56, 50, 25, and 12 kDa. Protein expression of NNC 26-9100 treated animals evaluated with optical densities normalized to respective vehicles ($n = 4-5$ /group). Representative blots shown to right of bar graph. ** $P < 0.01$ (Student's T-test), values are mean $\pm$ S.E.M.