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Author manuscript
Neurochem Res. Author manuscript; available in PMC 2020 November 01.

Published in final edited form as:
Neurochem Res. 2019 November ; 44(11): 2670–2680. doi:10.1007/s11064-019-02890-6.

Somatostatin Receptor Subtype-4 Regulates mRNA Expression of Amyloid-Beta Degrading Enzymes and Microglia Mediators of Phagocytosis in Brains of 3xTg-AD Mice

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Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder resulting in memory and cognitive impairment. The use of somatostatin receptor subtype-4 ($SSTR_4$) agonists have been proposed for AD treatment. This study investigated the effects of selective $SSTR_4$ agonist NNC 26–9100 on mRNA expression of key genes associated with AD pathology (microglia mediators of Aβ phagocytosis, amyloid-beta (Aβ)-degrading enzymes, anti-oxidant enzymes and pro-inflammatory cytokines) in 3xTg-AD mice. Mice were administered NNC 26–9100 (0.2 μg, i.c.v.) or vehicle control, with cortical and subcortical brain tissue collected at 6 h and 24 h post-treatment. At 6 h, NNC 26–9100 treatment decreased cortical expression of *cluster of differentiation-33 (Cd33)* by 25%, while increasing cortical and subcortical *macrophage scavenger receptor-1 (Msr1)* by 1.8 and 2.0-fold, respectively. The *Cd33* downregulation and *Msr1* upregulation support a state of microglia associated Aβ phagocytosis. At 24 h, NNC 26–9100 treatment increased the cortical expression of *Sstr4* (4.9-fold), Aβ-degrading enzymes *neprilysin* (9.3-fold) and *insulin degrading enzyme* (14.8-fold), and the antioxidant *catalase* (3.6-fold). Similar effects at 24 h were found in subcortical tissue with NNC 26–9100 treatment, but did not reach statistical significance. No changes in pro-inflammatory cytokine expression were found. These data demonstrated NNC 26–9100 facilitates transcriptional changes in brain tissue identified with Aβ phagocytosis and clearance, further supporting $SSTR_4$ as a treatment target for AD.

Introduction

Alzheimer's disease (AD) is the most common form of dementia. There are 30–35 million people globally currently living with AD, with a projected increase to 152 million by 2050 [1]. AD is clinically characterized by impairment in multiple cognitive domains, including memory, visuospatial skills, language, and executive function. These impairments result from destruction of the brain's neuronal networks, with amyloid-beta peptide (Aβ), hyperphosphorylated tau, inflammation, and oxidative stress being key contributors to the pathological process [2–4].

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Substantial evidence identifies somatostatin (somatotropin release-inhibiting factor, SRIF) as a major regulator of memory and cognition [5, 6]. An extensive network of SRIF-immunoreactive neuronal fibers exist throughout the central nervous system (CNS). Within the human brain, SRIF-immunoreactivity has been shown in the neocortex, median eminence, amygdala, preoptic area, hippocampus, olfactory regions, mediobasal hypothalamus, and brain stem [6]. These regions are significantly impacted AD, with loss of SRIF neurons representing a pathological feature of AD. SRIF levels were first shown to decrease in the neocortex and hippocampus of AD patients almost four decades ago [7]. Early loss of SRIF-immunoreactive neurons and SRIF mRNA from the hippocampus in AD mouse models supports the assertion that AD pathogenesis involves SRIF mediated processes [8]. Additionally, SRIF is a known regulator of brain neprilysin (NEP) [9], an Aβ-degrading enzyme (AβDE) that regulates Aβ levels in the brain [10]. SRIF deficiency has shown to decrease hippocampal NEP activity, increasing $Aβ_{42}$ in a manner similar to presenilin gene mutations observed with familial AD [9]. Given the significant involvement of SRIF in cognitive function and Aβ-degradation, brain localized SRIF receptors (SSTRs) may be ideal targets for the treatment of AD.

Five distinct SSTRs have been found in humans, all defined as G-protein-coupled receptors shown to be negatively coupled to pertussis-toxin sensitive pathways such as adenylyl cyclase. Of the five, the subtype-4 ($SSTR_4$) shows the greatest promise for AD treatment targeting [11]. $SSTR_4$ has optimal brain localization, being highly expressed in neurons of the neocortex, hippocampus, and olfactory bulb [12]. $SSTR_4$ also has limited peripheral and pituitary expression [13], reducing potential side-effects. $SSTR_4$ brain distribution correlates well between human and rodent, with considerable consistency between mRNA and immunoreactivity determinations [14–17]. Models of cognitive performance and pathological mitigation support $SSTR_4$ regulated cognitive enhancement and disease-modifying capability. We have previously shown administration of selective $SSTR_4$ agonist NNC 26–9100 increased spatial and recognition memory in Senescence Accelerated Mouse-Prone 8 (SAMP8) and APPswe mouse models of AD [18, 19]. Correspondingly, NNC 26–9100 decreased protein expression levels of toxic $Aβ_{40}$ and $Aβ_{42}$ oligomers through a phosphoramidon-sensitive metalloprotease-dependent mechanism, with increased cortical NEP activity [18, 19]. Microglia also express $SSTR_4$ [20], with SRIF shown to enhance microglia phagocytosis and metabolism of Aβ [20, 21]. $SSTR_4$ directed microglia actions may provide another route by which to mitigate AD pathology. While such $SSTR_4$ agonist actions identify viability towards AD treatment, there remains a lack of understanding as to gene expression changes that may be involved.

Herein we evaluated the effects NNC 26–9100 on mRNA expression of high impact genes associated with AD and $SSTR_4$ in brain tissue of 10-month old 3xTg-AD mice. NNC 26–9100 is an enzymatically stable non-peptide $SSTR_4$ agonist (Ki = 6 nM), having >100-fold selectivity for $SSTR_4$ compared to other SRIF receptor subtypes [22]. The 3xTg-AD mice contain three mutations associated with familial AD (APP Swedish, MAPT P301L, and PSEN1 M146V). These mice display age-dependent cognitive decline, immunoreactive microglia, and accumulation of Aβ [23, 24]. Dose and route of NNC 26–9100 used for these evaluations have previously shown to enhance learning and memory, decrease $Aβ_{40}$ and $Aβ_{42}$ oligomer expression, and increase NEP activity in mouse models of AD [18, 19].

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Materials and Methods

Animals, Dosing, and Dissection

3xTg-AD mice (Jackson Labs, Bar Harbor, ME) originated from the laboratory of Dr. LaFerla (University of California, Irvine, CA). Mice were housed in rooms with a 12 h light/dark cycle (20–22°C) with water and food available *ad libitum*. Experimental protocols were approved by the Institutional Animal Care and Use Committee, Southern Illinois University Edwardsville, and adhere to the NIH Guide for Care and Use of Laboratory Animals. Full effort was given to minimize the animal numbers and potential sufferance. At 10-months of age, mice were randomized into 6 h and 24 h, vehicle and NNC 26–9100 treatment groups (n=5/group). This age of 3xTg-AD mice have shown increased intracellular and extracellular Aβ deposition in neocortex and hippocampus [24, 25]. The 24 h time-point was chosen to align with our previous research showing functional effects in mouse models of AD at this time-point following i.c.v. administration at identical dose of NNC 26–9100 used herein [18, 19]. The 6 h time point captures earlier changes in mRNA expression that may be otherwise reset by 24 h. Mice were anesthetized with isoflurane, and a unilateral hole was drilled 0.5 mm posterior and 1.0 mm to the right of the bregma. A 2 μL intracerebroventricular injection of NNC 26–9100 (0.2 μg) or vehicle (20% ethanol/saline) was administered to a depth of 2 mm.

At respective time-points, mice were anesthetized and perfused with 10 mL cold 0.9% NaCl saline via cardiac puncture. Brains were excised and saline washed. Pineal gland, olfactory bulbs, pituitary gland and surface vessels were removed. Cerebellum was excised, along with tissue caudal to midbrain. The corpus callosum was used to demarcate cortical and subcortical tissues. The "subcortical" tissue included the hippocampus (archicortex), caudate putamen, thalamus, globus palladus, basal forebrain, hypothalamus, amygdala, striatum, and midbrain. The "cortical" tissue included the neocortex and the piriform cortex. The fractions were homogenized and stored in RNAlater (ThermoFisher Scientific, Carlsbad, CA) at −20°C until use. Unless otherwise stated all chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

RNA Isolation and cDNA Synthesis

Thawed samples were spun at 4,000 x *g* for 1 min, RNAlater supernatant was removed, Trizol RNA isolation reagent (Life Technologies, Grand Island, NY) was added (100 mg/1mL). Following 200 μL of 100% chloroform and mixing, samples were centrifuged at 4°C for 15 min at 12,000 × *g*. The upper phase was treated with 500 μL 100% isopropanol and centrifuged 4°C for 10 min at 12,000 × *g*. The RNA pellet was washed with 1 mL of 75% ethanol, centrifuged at 4°C for 5 min at 8,000 × *g* and resuspended in 60 μL RNAse-free water. Samples were mixed and incubated on heating block for 5 min at 55°C. Total RNA and purity of samples were quantified via NanoDrop-1000 (ThermoFisher). RNA underwent DNAse treatment via Turbo DNAse-free kit (ThermoFisher) and was the incubated at 37°C for 25 min, followed by DNAse inactivation reagent to remove divalent cations. Reverse transcription of RNA to cDNA was performed via High Capacity Reverse Transcriptase kit (ThermoFisher) per manufacturers instructions, using equal volumes of RNA, 2x reverse transcriptase, and random primers reaction mix at a final volume of 20 μL and run in the

following sequence: 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. Following reverse transcription, samples were stored at −20°C until use.

Reverse transcription Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

Reactions were carried out using PowerUp SYBR Green Master Mix (ThermoFisher) using a QuantStudio3 system (ThermoFisher). All mouse primers (Table1) were obtained from Integrated DNA Technologies (Coralville, IA). The genes investigated include: SRIF (*Sst*), SSTR 1–5 (*Sstr1; Sstr2; Sstr3; Sstr4; Sstr5*), AβDEs (neprilysin: *Nep1*; endothelin converting enzyme-1: *Ece1*; insulin degrading enzyme: *Ide*; angiotensin converting enzyme: *Ace*), inflammatory cytokines (interleukin-1β: *Il-1β*; interleukin-6: *Il-6*; tumor necrosis factor-α: *Tnf-α*; granulocyte-macrophage colony-stimulating factor: *Csf2*), microglia mediators of Aβ clearance (cluster of differentiation 68: *Cd68*; cluster of differentiation 33: *Cd33*; triggering receptor expressed on myeloid cells-2: *Trem2*; cluster of differentiation 36: *Cd36*; macrophage scavenger receptor-1: *Msr1*), and anti-oxidant enzymes (superoxide dismutase: *Sod1*; catalase: *Cat*), with Cytochrome C1 (*Cyc1*) as reference gene. *Cyc1* was chosen as reference given its stability in brain tissue respective to neurodegenerative disease [26], with stability confirmed in our run-up evaluations.

Samples were run in triplicate, with 10 μL of Master Mix, 200–500 nM primer, 8 μL dH_20, and 2 μL cDNA. A negative water control was included in each run. The reaction sequence was 50°C for 2 min, 95°C for 2 min, followed by 45 cycles at 95°C for 15 s and then 60°C for 1 min. Melt curves were performed to verify the absence of primer-dimer formation: 95°C for 15 s, 60°C for 1 min, 95°C for 15 s. No Cq values exceeded 36. Relative mRNA expression for each target was calculated using the $2^{(-\ Ct)}$ method [27].

Statistical Analyses

The dependent variable assessed was the Ct [28]. The type I error was set at 0.05 for all analyses, with significance set at $P<0.05$. Independent t-tests conducted between vehicle and NNC 26–9100 treatment groups, respective to tissue and time-point. For t-tests, the data was analyzed for normality of the distribution and that the variances were equal. Equality of variance assessed using a folded F-test. If the variance was not equal between groups, the Satterthwaite approximation was used. If the distribution was non-normal, the Wilcoxon rank sum test was used. 95% confidence intervals constructed for the $2^{(-\ Ct)}$ fold-change determinations. Analyses performed using SAS v9.4 (SAS Institute Inc., Cary, NC).

Results

Cortical 6 h mRNA Expression

NNC 26–9100 treatment examined in cortical tissue at 6 h (Table 2). The mean Ct for *Cd33* significantly increased with NNC 26–9100 treatment, corresponding to a 25% decrease in mRNA expression. The mean Ct for *Msr1* significantly decreased with NNC 26–9100 treatment, corresponding to a 1.8-fold increase in mRNA expression. No other significant differences were found.

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Subcortical 6 h mRNA Expression

NNC 26–9100 treatment examined in subcortical tissue at 6 h (Table 3). The mean Ct for *Msr1* significantly decreased with NNC 26–9100 treatment, corresponding to a 2.0-fold increase in mRNA expression. No other significant differences were found.

Cortical 24 h mRNA Expression

NNC 26–9100 treatment examined in cortical tissue at 24 h (Table 4). The mean Ct for *Sstr4* and *Cat* significantly decreased with NNC 26–9100 treatment, corresponding to a 4.9- and 3.6-fold increase in mRNA expression, respectively. The Ct for both *Nep1* and *Ide* significantly decreased with NNC 26–9100 treatment, corresponding to a 9.3 and 14.8-fold increase in mRNA expression, respectively. While NNC 26–9100 treatment increased *Sst* (4.4-fold), *Ece1* (5.4-fold), and *Ace* (9.3-fold) mRNA expression, they did not reach statistical significance. No other significant differences were found.

Subcortical 24h mRNA Expression

NNC 26–9100 treatment examined in subcortical tissue at 24 h (Table 5). While no statistically significant differences were found between NNC 26–9100 and vehicle treatment, the increases in *Sstr4 (3.3-fold), Sst (4.4-fold), Nep1 (2.4-fold), Ide (4.4-fold), Ece1 (2.8-fold)*, *Ace (3.3-fold),* and *Cat (3.0-fold)* parallel the 24h cortical mRNA expression observations.

Discussion

Herein, we show $SSTR_4$ agonist NNC 26–9100 alters brain tissue mRNA expression of key AD and SRIF associated genes. Overall, cortical tissue mRNA effects were more robust than those effects observed from subcortical tissue. This effect is likely accounted for by the higher level of $SSTR_4$ expression in grey matter tissues [12]. The subcortical regions being more diverse in gray/white matter content. High levels of $SSTR_4$ are expressed throughout the neocortex, with expression decreasing progressively towards more caudal brain regions [14–17]. Moreover, the respective subcortical regions vary significantly in functionality, which may differentially effect gene translation. Nevertheless, mRNA expression changes observed with treatment identify a relatively consistent effect between cortical and subcortical tissues.

The upregulated cortical *Sstr4* mRNA expression at 24 h by NNC 26–9100 is consistent with increased receptor turnover. A similar but non-significant effect was found in subcortical tissue. This parallels CHO-K1 cell evaluations identifying SRIF-induced upregulation of human $SSTR_4$ [29]. While cooperative interaction between $SSTR_4$ and other SSTRs has been shown [30–32], no change in mRNA expression of any other SSTR with NNC 26–9100 treatment was found. This supports the observed expression changes are specific to $SSTR_4$.

Cortical *Nep1* and *Ide* mRNA expression were upregulated by NNC 26–9100 at 24 h, with a similar but non-significant increase observed in subcortical tissue. Each of these zinc-metalloproteases are capable of degrading Aβ [33, 34]. NEP is membrane associated and

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principally expressed in neurons [35]. NEP is capable of degrading Aβ monomers and synthetic oligomers [36, 37]. The observed upregulation of *Nep1*, along with our previous data showing increased cortical NEP activity with NNC 26–9100 [18] further corroborates $SSTR_4$ mediated regulation of NEP in the brain. IDE is primarily cytosolic associated and expressed in neurons and glia [38, 39]. IDE has shown to clear cytoplasmic Aβ in human hippocampal lysates [40]. Notably, SRIF has shown to increase IDE expression and secretion from microglia, with an associated $A\beta_{40}$ decrease [21]. Given the presence of $SSTR_4$ on microglia [20], the upregulation of *Ide* within our evaluation may be microglia derived.

While the effects of NNC 26–9100 on AβDE mRNA expression were consistent between cortical and subcortical homogenates, changes in subcortical tissue did not reach significance. Given NEP is primarily neuronal, it is not unexpected that *Nep1* upregulation would be greater in cortical tissue. IDE may also be impacted by the lower density of microglia in white matter compared to grey matter [41]. Nevertheless, the memory forming hippocampal neurons that were extracted within our subcortical homogenates do express NEP [10], ECE [42], IDE [40], and ACE [43], along with $SSTR_4$ [12].

No changes in mRNA expression were found for *IL-1β*, *IL-6*, *Tnf-α* or *Csf2* in cortical or subcortical, at 6 h or 24 h. Other research has shown $SSTR_4$ agonist use capable of reducing endotoxin-induced granulocyte accumulation and IL-1β synthesis [44]. Additionally, IL-6 and TNF-α have shown to be upregulated under inflammatory conditions in $SSTR_4$ knockout-mice compared to wild-type controls [45]. However, such $SSTR_4$ anti-inflammatory effects were only apparent under heightened inflammatory states, which were not present in our study.

No mRNA expression changes were found with NNC 26–9100 treatment for *Cd68*, *Trem2* or *Cd36*, which are associated with microglia activation, Aβ-binding, and phagocytosis [46]. However, NNC 26–9100 treatment decreased cortical *Cd33* expression at 6 h. Cd33 is a cell surface protein predominantly expressed on microglia [47], which positively correlates with amyloid plaque burden [48]. Reduction of *Cd33* mRNA expression is most likely beneficial, as *Cd33* has been shown to be elevated in AD patients at both the mRNA and protein level [48]. Furthermore, APP_{Swe}/PS1 $_{E9}$ mice with the *Cd33* gene knocked-out show a marked decrease of insoluble $A\beta_{42}$ and Aβ plaque burden [48]. Conversely, cortical and subcortical *Msr1* (a.k.a. Scara1) expression significantly increased with NNC 26–9100 treatment at 6 h. *Msr1* is a microglia receptor involved in the phagocytosis of fibrillar and soluble Aβ [49, 50]. When *Msr1* was knocked-out in APP_{Swe}/PS1 $_{E9}$ mice a marked increase in soluble Aβ and mortality has been found [50]. Interestingly, both *Nep* and *Ide* are significantly lower in the brains of APP_{Swe}/PS1 $_{E9}$ *Msr1* knock-outs compared to APP_{Swe}/PS1 $_{E9}$ controls [50], identifying a link between microglia Aβ regulation and AβDEs. The *Cd33* downregulation and *Msr1* upregulation at 6 h support a state of microglia associated Aβ phagocytosis occurring prior to the observed AβDE upregulation at 24 h.

While cortical *Cat* expression significantly increased with treatment at 24 h, no change was observed in *Sod1* expression. The corresponding enzymes often act in concert, with superoxide dismutase converting the superoxide radical anion to hydrogen peroxide, and

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catalase breaking down hydrogen peroxide into water and oxygen. Reduced activity of these enzymes has been reported in AD, indicating a compromised oxidative stress defense mechanism [51]. However, variations in superoxide dismutase and catalase have also been noted in evaluations of AD brain tissues. While accumulation of hydrogen peroxide in AD brains without changes in superoxide dismutase was shown by Gsell and colleagues [52], others have shown regional increases in superoxide dismutase and catalase in AD brains [53]. Such disparity may reflecting different disease stages and regional tissue effects. Catalase is also heavily associated with peroxisomes, where it has shown to reduce Aβ induced oxidative stress [54, 55]. Interestingly, IDE is also concentrated in peroxisomes [56], and could benefit from enhanced catalase activity. AβDE oxidation has been suggested to contribute to reduced enzymatic activity with corresponding Aβ deposition [57, 58]. Enhanced IDE oxidation has also been shown in the hippocampus and neocortex of AD patients [57]. As to whether *Cat* upregulation is a direct result of $SSTR_4$ agonist action or secondary to other processes remains to be determined.

These data demonstrated $SSTR_4$ agonist NNC 26–9100 facilitates transcriptional changes in the brain identified with enhanced Aβ phagocytosis and clearance, which aligns with our previous work [18, 19]. The 6 h *Cd33* and *Msr1* expression changes associated with microglia mediated Aβ phagocytosis and the 24 h upregulation of primary Aβ-degrading enzymes *Nep* and *Ide,* as well as the antioxidant *Cat*, identify different time frames of expressional regulation. While $SSTR_4$ has the capacity to activate a number of pathways that influence transcription [59], the specific signaling cascades and potential interdependency of cellular processes involved remains to be determined. This work provides mechanistic insight as to $SSTR_4$ agonist actions within the brain, further supporting $SSTR_4$ as a treatment target for AD.

Acknowledgments

This work was supported the National Institutes of Health, National Institute of Age division, grant no. R01AG047858, and Southern Illinois University Edwardsville Graduate School.

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Table 1.

Genes and corresponding primer pair information.

Gene	Accession no.	Primer sequence (5′–3′)		Bp
*Cyc1**	NM_025567	Sense: Anti-sense:	CACAGTAGCCAGTGAGCAG CCAAAACCATACCCTAACCCT	133
Sst	NM_009215	Sense: Anti-sense:	GGCATCATTCTCTGTCTGGTT AGACTCCGTCAGTTTCTGC	114
Sstr1	NM_009216	Sense: Anti-sense:	AAGGCTCTGGACTGATGAATG CAGGTTTAAAGAACTGGCAAGC	82
Sstr2	NM_009217	Sense: Anti-sense:	GAAGATGAAGACAGCCACTACG TTGTCTCTGCTACCTGTTCATC	130
Sstr3	NM_009218	Sense: Anti-sense:	GAGATGAGGATCAGTCAGCAG AAACCAGACGCAAGCTACC	142
Sstr4	NM_009219	Sense: Anti-sense:	GGTGGCTGTCTTCATCTTGG ATCTATGCGCTCGTGTGTC	90
Sstr5	NM_011425	Sense: Anti-sense:	TGAGGCATTCCAGCTAGGT TTCCACCTACTGAGCTCCA	129
Nep1	NM_008604	Sense: Anti-sense:	CACAGCATTCTCCATATTCCCA GCCTCAGCCGAAACTACAAG	109
Ece1	NM_199307	Sense: Anti-sense:	TGAGTCCACCAGATCCTCTT TTACCACTACTTCCACAAGCTG	102
Ide	NM_031156	Sense: Anti-sense:	CTTCTACTCTGCTCTCCAGGTA TTGGCTATATTGTCTTCAGTGGA	108
Ace	NM_009598	Sense: Anti-sense:	TTCACAGAGGTACACTGCTTG ACAAGTCGATGTTAGAGAAGCC	112
Il-1β	NM_008361	Sense: Anti-sense:	GACCTGTTCTTTGAAGTTGACG CTCTTGTTGATGTGCTGCTG	119
Il-6	NM_031168	Sense: Anti-sense:	TCCTTAGCCACTCCTTCTGT AGCCAGAGTCCTTCAGAGA	146
Tnf-α	NM_013693	Sense: Anti-sense:	AGACCCTCACACTCAGATCA TCTTTGAGATCCATGCCGTTG	145
Csf2	NM_009969	Sense: Anti-sense:	CCTTGAGTTTGGTGAAATTGCC GTCTCTAACGAGTTCTCCTTCA	97
Cd68	NM_009853	Sense: Anti-sense:	TCAGGGTGGAAGAAAGGTAAAG CCATAAGGAAATGAGAGAGACAGG	138
Cd33	NM_021293	Sense: Anti-sense:	TGCAGAACATCACAATGAGAAAC GAAAGGACCATCCAGCTCAA	136
Trem2	NM_001272078	Sense: Anti-sense:	GCTTCAAGGCGTCATAAGTACA GACCTCTCCACCAGTTTCTC	123
Cd36	NM_001159558	Sense: Anti-sense:	CAGTTGCTCCACACATTTCAG TCTCCTTCAACAGTCTCCCT	100
Msr1	NM_031195	Sense: Anti-sense:	CACAAGGAGGTAGAGAGCAATG GCACGTTCAATGACAGCATC	108
Sod1	NM_011434	Sense: Anti-sense:	GTCCTTTCCAGCAGTCACAT GGTTCCACGTCCATCAGTATG	146
Cat	NM_009804	Sense: Anti-sense:	ATCTTCCTGAGCAAGCCTTC CAAGTTGGTTAATGCAGATGGA	112

Bp: length of the amplicon in base pair. Cyc1* served as reference gene [26].

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Table 2.

Cortical 6 h mRNA expression.

	Gene		Vehicle 6-hr	Treated 6-hr	P-value
Somatostatin & Somatostatin Receptors	***Sst***	**Ct**	0.000 ± 0.314	−0.654 ± 0.379	P=0.2207
		$2^{(-\ \ Ct)}$	1.000 (0.546 – 1.831)	1.574 (0.759 – 3.265)	
	Sstr1	**Ct**	0.000 ± 0.101	−0.229 ± 0.089	P=0.1265
		$2^{(-\ \ Ct)}$	1.000 (0.824 – 1.214)	1.172 (0.988 – 1.389)	
	Sstr2	**Ct**	0.000 ± 0.283	0.514 ± 0.108	P=0.1281
		$2^{(-\ \ Ct)}$	1.000 (0.580 – 1.723)	0.700 (0.569 – 0.863)	
	Sstr3	**Ct**	0.000 ± 0.168	−0.029 ± 0.064	P=0.8767
		$2^{(-A\ \ Ct)}$	1.000 (0.723 – 1.383)	1.020 (0.902 – 1.154)	
	Sstr4	**Ct**	0.000 ± 0.810	0.077 ± 0.309	P=0.9317
		$2^{(-\ \ Ct)}$	1.000 (0.211 – 4.749)	0.948 (0.523 – 1.718)	
	Sstr5	**Ct**	0.000 ± 0.290	1.026 ± 0.395	P=0.0695
		$2^{(-\ \ Ct)}$	1.000 (0.572 – 1.748)	0.491 (0.230 – 1.049)	
Aβ-degrading Enzymes	***Nep1***	**Ct**	0.000 ± 1.265	0.325 ± 0.162	P=0.8108
		$2^{(-\ \ Ct)}$	1.000 (0.088 – 11.405)	0.798 (0.585 – 1.089)	
	Ece1	**Ct**	0.000 ± 1.100	0.718 ± 0.370	P=1.000^{np}
		$2^{(-\ \ Ct)}$	1.000 (0.120 – 8.311)	0.608 (0.298 – 1.238)	
	Ide	**Ct**	0.000 ± 0.978	−0.085 ± 0.446	P=0.9392
		$2^{(-\ \ Ct)}$	1.000 (0.152 – 6.566)	1.060 (0.449 – 2.503)	
	Ace	**Ct**	0.000 ± 0.464	−0.517 ± 0.434	P=0.4392
		$2^{(-\ \ Ct)}$	1.000 (0.409 – 2.445)	1.431 (0.621 – 3.297)	
Inflammatory Cytokines	***IL-1β***	**Ct**	0.000 ± 0.681	−0.531 ± 0.607	P = 0.5762
		$2^{(-\ \ Ct)}$	1.000 (0.270 – 3.706)	1.445 (0.450 – 4.646)	
	IL-6	**Ct**	0.000 ± 0.715	−0.929 ± 0.408	P=0.3235^{np}
		$2^{(-\ \ Ct)}$	1.000 (0.253 – 3.957)	1.904 (0.868 – 4.174)	
	Tnf-α	**Ct**	0.000 ± 0.364	−0.110 ± 0.363	P = 0.8354
		$2^{(-\ \ Ct)}$	1.000 (0.496 – 2.016)	1.080 (0.537 – 2.171)	
	Csf2	**Ct**	0.000 ± 0.364	−0.253 ± 0.560	P=0.7144
		$2^{(-\ \ Ct)}$	1.000 (0.496 – 2.015)	1.192 (0.406 – 3.503)	
Microglia Mediators of Aβ Clearance	***Cd68***	**Ct**	0.000 ± 0.169	0.324 ± 0.104	P=0.1412
		$2^{(-\ \ Ct)}$	1.000 (0.722 – 1.385)	0.799 (0.654 – 0.976)	
	Cd33	**Ct**	0.000 ± 0.099	0.420 ± 0.136	***P=0.0369**
		$2^{(-\ \ Ct)}$	1.000 (0.827 – 1.209)	0.747 (0.575 – 0.971)	
	Trem2	**Ct**	0.000 ± 0.329	−0.015 ± 0.104	P=0.4250^{np}
		$2^{(-\ \ Ct)}$	1.000 (0.531 – 1.884)	1.011 (0.827 – 1.235)	

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	Gene		Vehicle 6-hr	Treated 6-hr	P-value
	Cd36	**Ct**	0.000 ± 0.200	−0.378 ± 0.370	P=0.3949
		$2^{(-\ \ Ct)}$	1.000 (0.680 – 1.470)	1.300 (0.638 – 2.648)	
	Msr1	**Ct**	0.000 ± 0.301	−0.872 ± 0.166	* **P=0.0347**
		$2^{(-\ \ Ct)}$	1.000 (0.561– 1.784)	1.830 (1.331 – 2.518)	
Anti-oxidants	***Sod1***	**Ct**	0.000 ± 0.081	0.054 ± 0.030	P=0.5507
		$2^{(-\ \ Ct)}$	1.000 (0.856 – 1.168)	0.964 (0.909 – 1.021)	
	Cat	**Ct**	0.000 ± 0.468	−0.243 ± 0.332	P=0.6823
		$2^{(-\ \ Ct)}$	1.000 (0.407 – 2.459)	1.184 (0.625 – 2.242)	

Data shown as mean Ct ± SEM, with t-test used to identify the difference between vehicle and NNC 26–9100 treatment groups, significance set at *P<0.05. Fold difference relative to vehicle shown as $2^{(-\ \ Ct)}$ with 95% confidence interval.

[np] denotes use of Wilcoxon rank sum test.

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Table 3.

Subcortical 6 h mRNA expression.

	Gene		Vehicle 6-hr	Treated 6-hr	P-value
Somatostatin & Somatostatin Receptors	***Sst***	**Ct**	0.000 ± 0.616	0.498 ± 0.459	P=0.5346
		$2^{(- \quad Ct)}$	1.000 (0.306 – 3.271)	0.708 (0.293 – 1.711)	
	Sstr1	**Ct**	0.000 ± 0.101	−0.006 ± 0.130	P=0.9713
		$2^{(- \quad Ct)}$	1.000 (0.823 – 1.215)	1.004 (0.782 – 1.289	
	Sstr2	**Ct**	0.000 ± 0.184	0.157 ± 0.188	P=0.5666
		$2^{(- \quad Ct)}$	1.000 (0.702 – 1.424)	0.897 (0.625 – 1.287)	
	Sstr3	**Ct**	0.000 ± 0.178	0.242 ± 0.133	P=0.3076
		$2^{(- \quad Ct)}$	1.000 (0.710 – 1.409)	0.846 (0.655 – 1.092)	
	Sstr4	**Ct**	0.000 ± 0.622	0.275 ± 0.436	P=0.7267
		$2^{(- \quad Ct)}$	1.000 (0.302 – 3.311)	0.826 (0.357 – 1.911)	
	Sstr5	**Ct**	0.000 ± 0.184	−0.093 ± 0.393	P=0.8355
		$2^{(- \quad Ct)}$	1.000 (0.702 – 1.424)	1.067 (0.501 – 2.271)	
Aβ-degrading Enzymes	***Nep1***	**Ct**	0.000 ± 0.840	0.651 ± 0.497	P=0.5233
		$2^{(- \quad Ct)}$	1.000 (0.199 – 5.035)	0.637 (0.245 – 1.656)	
	Ece1	**Ct**	0.000 ± 1.071	0.966 ± 0.426	P=0.8392^{np}
		$2^{(- \quad Ct)}$	1.000 (0.127 – 7.858)	0.512 (0.225 – 1.163)	
	Ide	**Ct**	0.000 ± 0.732	0.936 ± 0.428	P=0.3019
		$2^{(- \quad Ct)}$	1.000 (0.244 – 4.090)	0.523 (0.229 – 1.192)	
	Ace	**Ct**	0.000 ± 0.945	0.504 ± 0.499	P=0.6498
		$2^{(- \quad Ct)}$	1.000 (0.162 – 6.163)	0.705 (0.270 – 1.841)	
Inflammatory Cytokines	***IL-1β***	**Ct**	0.000 ± 0.523	0.764 ± 0.469	P=0.3086
		$2^{(- \quad Ct)}$	1.000 (0.365 – 2.738)	0.589 (0.239 – 1.452)	
	IL-6	**Ct**	0.000 ± 0.372	−1.075 ± 0.598	P=0.1657
		$2^{(- \quad Ct)}$	1.000 (0.489 – 2.046)	2.106 (0.666 – 6.663)	
	Tnf-a	**Ct**	0.000 ± 0.440	−0.817 ± 0.459	P=0.2349
		$2^{(- \quad Ct)}$	1.000 (0.429 – 2.332)	1.761 (0.728 – 4.262)	
	Csf2	**Ct**	0.000 ± 0.355	−0.842 ± 0.375	P=0.1418
		$2^{(- \quad Ct)}$	1.000 (0.505 – 1.981)	1.793 (0.871 – 3.691)	
Microglia Mediators of Aβ Clearance	***Cd68***	**Ct**	0.000 ± 0.086	−0.151 ± 0.213	P=0.5309
		$2^{(- \quad Ct)}$	1.000 (0.847 – 1.180)	1.110 (0.736 – 1.674)	
	Cd33	**Ct**	0.000 ± 0.116	0.052 ± 0.130	P=0.7709
		$2^{(- \quad Ct)}$	1.000 (0.800 –1.250)	0.964 (0.751 – 1.238)	
	Trem2	**Ct**	0.000 ± 0.161	0.031 ± 0.157	P=0.8953
		$2^{(- \quad Ct)}$	1.000 (0.733 – 1.364)	0.979(0.724 – 1.325)	
	Cd36	**Ct**	0.000 ± 0.149	0.106 ± 0.227	P=0.7068

	Gene		Vehicle 6-hr	Treated 6-hr	P-value
		$2^{(-\ \ Ct)}$	1.000 (0.751 – 1.332)	0.929 (0.600 – 1.439)	
	Msr1	**Ct**	0.000 ± 0.257	−1.019 ± 0.185	***P=0.0124**
		$2^{(-\ \ Ct)}$	1.000 (0.609 – 1.641)	2.026 (1.419 – 2.892)	
Anti-oxidants	***Sod1***	**Ct**	0.000 ± 0.038	−0.013 ± 0.034	P=0.8042
		$2^{(-\ \ Ct)}$	1.000 (0.930 – 1.076)	1.009 (0.945 – 1.078)	
	Cat	**Ct**	0.000 ± 0.837	0.934 ± 0.456	P=0.8392[np]
		$2^{(-\ \ Ct)}$	1.000 (0.200 – 5.011)	0.523 (0.218 – 1.259)	

Data shown as mean Ct ± SEM, with t-test used to identify the difference between vehicle and NNC 26–9100 treatment groups, significance set at *P<0.05. Fold difference relative to vehicle shown as $2^{(-\ \ Ct)}$ with 95% confidence interval.

[np]denotes use of Wilcoxon rank sum test.

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Table 4.

Cortical 24 h mRNA expression.

	Gene		Vehicle 24-hr	Treated 24-hr	P-value
Somatostatin & Somatostatin Receptors	***Sst***	**Ct**	0.000 ± 1.385	−2.164 ± 1.015	P=0.1777^{np}
		$2^{(- Ct)}$	1.000 (0.070 – 14.382)	4.483 (0.636 – 31.595)	
	Sstr1	**Ct**	0.000 ± 0.070	−0.141 ± 0.192	P=0.5109
		$2^{(- Ct)}$	1.000 (0.874 – 1.144)	1.102 (0.762 – 1.595)	
	Sstr2	**Ct**	0.000 ± 0.110	0.180 ± 0.187	P=0.4316
		$2^{(- Ct)}$	1.000 (0.810 – 1.235)	0.883 (0.616 – 1.266)	
	Sstr3	**Ct**	0.000 ± 0.150	−0.109 ± 0.209	P=0.6838
		$2^{(- Ct)}$	1.000 (0.750 – 1.334)	1.078 (0.721 – 1.612)	
	Sstr4	**Ct**	0.000 ± 0.712	−2.299 ± 0.480	***P=0.0280**
		$2^{(- Ct)}$	1.000 (0.254 – 3.933)	4.920 (1.955 – 12.383)	
	Sstr5	**Ct**	0.000 ± 0.432	0.264 ± 0.313	P=0.6338
		$2^{(- Ct)}$	1.000 (0.435 – 2.298)	0.833 (0.456 – 1.521)	
Aβ-degrading Enzymes	***Nep1***	**Ct**	0.000 ± 0.834	−3.217 ± 0.285	***P=0.0065**
		$2^{(- Ct)}$	1.000 (0.201 – 4.975)	9.301 (5.375 – 16.096)	
	Ece1	**Ct**	0.000 ± 0.575	−2.426 ± 1.025	P=0.0729
		$2^{(- Ct)}$	1.000 (0.330 – 3.026)	5.375 (0.748 – 38.631)	
	Ide	**Ct**	0.000 ± 1.700	−3.889 ± 0.568	***P=0.0472**^{np}
		$2^{(- Ct)}$	1.000 (0.038 – 26.358)	14.819 (4.964 – 44.234)	
	Ace	**Ct**	0.000 ± 1.547	−3.222 ± 0.508	P=0.0832
		$2^{(- Ct)}$	1.000 (0.051 – 19.636)	9.328 (3.510 – 24.789)	
Inflammatory Cytokines	***IL-1β***	**Ct**	0.000 ± 0.352	−0.024 ± 0.514	P=1.000^{np}
		$2^{(- Ct)}$	1.000 (0.508 – 1.969)	1.017 (0.378 – 2.733)	
	IL-6	**Ct**	0.000 ± 0.510	0.064 ± 0.358	P=1.000^{np}
		$2^{(- Ct)}$	1.000 (0.375 – 2.668)	0.956 (0.480 – 1.907)	
	Tnf-a	**Ct**	0.000 ± 0.159	0.274 ± 0.586	P=0.6717
		$2^{(- Ct)}$	1.000 (0.736 – 1.358)	0.827 (0.268 – 2.552)	
	Csf2	**Ct**	0.000 ± 0.396	0.478 ± 0.800	P=0.6073
		$2^{(- Ct)}$	1.000 (0.466 – 2.144)	0.718 (0.154 – 3.349)	
Microglia Mediators of Aβ Clearance	***Cd68***	**Ct**	0.000 ± 0.155	−0.195 ± 0.155	P=0.4004
		$2^{(- Ct)}$	1.000 (0.742 – 1.347)	1.144 (0.849 – 1.543)	
	Cd33	**Ct**	0.000 ± 0.096	0.097 ± 0.104	P=0.5152
		$2^{(- Ct)}$	1.000 (0.831 – 1.204)	0.935 (0.765 – 1.143)	
	Trem2	**Ct**	0.000 ± 0.092	−0.033 ± 0.167	P=0.8687
		$2^{(- Ct)}$	1.000 (0.838 – 1.193)	1.023 (0.742 – 1.410)	

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	Gene		**Vehicle 24-hr**	**Treated 24-hr**	**P-value**
	Cd36	**Ct**	0.000 ± 0.410	0.549 ± 0.379	P=0.3544
		$2^{(- \quad Ct)}$	1.000 (0.454 – 2.201)	0.684 (0.330 – 1.417)	
	Msr1	**Ct**	0.000 ± 0.507	0.454 ± 0.301	P=0.4636
		$2^{(- \quad Ct)}$	1.000 (0.377 – 2.653)	0.730 (0.409 – 1.303)	
Anti-oxidants	***Sod1***	**Ct**	0.000 ± 0.047	0.054 ± 0.064	P=0.5183
		$2^{(- \quad Ct)}$	1.000 (0.913 – 1.095)	0.964 (0.853 – 1.089)	
	Cat	**Ct**	0.000 ± 0.553	−1.843 ± 0.409	* **P=0.0279**
		$2^{(- \quad Ct)}$	1.000 (0.345 – 2.896)	3.589 (1.632 – 7.890)	

Data shown as mean Ct ± SEM, with t-test used to identify the difference between vehicle and NNC 26–9100 treatment groups, significance set at *P<0.05. Fold difference relative to vehicle shown as $2^{(- \quad Ct)}$ with 95% confidence interval.

np denotes use of Wilcoxon rank sum test.

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Table 5.

Subcortical 24 h mRNA expression.

	Gene		Vehicle 24-hr	Treated 24-hr	P-value
Somatostatin & Somatostatin Receptors	***Sst***	**Ct**	0.000 ± 1.114	−2.150 ± 0.864	P=0.1656
		$2^{(- Ct)}$	1.000 (0.117 – 8.528)	4.439 (0.842 – 23.391)	
	Sstr1	**Ct**	0.000 ± 0.082	0.110 ± 0.078	P=0.3573
		$2^{(- Ct)}$	1.000 (0.855 – 1.170)	0.926 (0.797 – 1.076)	
	Sstr2	**Ct**	0.000 ± 0.205	0.167 ± 0.103	P=0.4864
		$2^{(- Ct)}$	1.000 (0.674 – 1.483)	0.891 (0.730 – 1.087)	
	Sstr3	**Ct**	0.000 ± 0.053	0.134 ± 0.120	P=0.3356
		$2^{(- Ct)}$	1.000 (0.904 – 1.107)	0.911 (0.724 – 1.147)	
	Sstr4	**Ct**	0.000 ± 0.766	−1.714 ± 0.613	P=0.1190
		$2^{(- Ct)}$	1.000 (0.229 – 4.369)	3.280 (1.008 – 10.675)	
	Sstr5	**Ct**	0.000 ± 0.295	−0.022 ± 0.182	P=0.9502
		$2^{(- Ct)}$	1.000 (0.567 – 1.764)	1.016 (0.716 – 1.441)	
Aβ-degrading Enzymes	***Nep1***	**Ct**	0.000 ± 0.894	−1.244 ± 0.687	P=0.2980
		$2^{(- Ct)}$	1.000 (0.139 – 7.186)	2.368 (0.631 – 8.884)	
	Ece1	**Ct**	0.000 ± 0.520	−1.124 ± 0.676	P=0.2238
		$2^{(- Ct)}$	1.000 (0.368 – 2.719)	2.180 (0.594 – 8.003)	
	Ide	**Ct**	0.000 ± 0.908	−1.990 ± 0.772	P=0.1336
		$2^{(- Ct)}$	1.000 (0.174 – 5.743)	3.973 (0.889 – 17.567)	
	Ace	**Ct**	0.000 ± 0.911	−1.898 ± 0.612	P=0.1161
		$2^{(- Ct)}$	1.000 (0.134 – 7.457)	3.278 (1.148 – 12.105)	
Inflammatory Cytokines	***IL-1β***	**Ct**	0.000 ± 0.374	0.662 ± 0.299	P=0.2038
		$2^{(- Ct)}$	1.000 (0.487 – 2.052)	0.632 (0.335 – 1.124)	
	IL-6	**Ct**	0.000 ± 0.514	0.146 ± 0.217	P=0.7998
		$2^{(- Ct)}$	1.000 (0.372 – 2.689)	0.904 (0.595 – 1.373)	
	Tnf-a	**Ct**	0.000 ± 0.434	0.112 ± 0.277	P=0.8335
		$2^{(- Ct)}$	1.000 (0.434 – 2.304)	0.925 (0.543 – 1.576)	
	Csf2	**Ct**	0.000 ± 0.178	−0.104 ± 0.257	P=0.7480
		$2^{(- Ct)}$	1.000 (0.709 – 1.410)	1.075 (0.656 – 1.762)	
Microglia Mediators of Aβ Clearance	***Cd68***	**Ct**	0.000 ± 0.223	−0.403 ± 0.229	P=0.2433
		$2^{(- Ct)}$	1.000 (0.651 – 1.537)	1.322 (0.851 – 2.054)	
	Cd33	**Ct**	0.000 ± 0.114	−0.145 ± 0.154	P=0.4250^{np}
		$2^{(- Ct)}$	1.000 (0.804 – 1.244)	1.105 (0.822 – 1.486)	
	Trem2	**Ct**	0.000 ± 0.088	−0.262 ± 0.137	P=0.1472
		$2^{(- Ct)}$	1.000 (0.844 – 1.185)	1.199 (0.921 – 1.562)	
	Cd36	**Ct**	0.000 ± 0.474	0.177 ± 0.458	P=0.8392^{np}

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	Gene		Vehicle 24-hr	Treated 24-hr	P-value
		$2^{(- \quad Ct)}$	1.000 (0.402 – 2.488)	0.885 (0.367 – 2.134)	
	Msr1	**Ct**	0.000 ± 0.619	0.143 ± 0.319	P=0.8419
		$2^{(- \quad Ct)}$	1.000 (0.304 – 3.290)	0.905 (0.490 – 1.673)	
Anti-oxidants	***Sod1***	**Ct**	0.000 ± 0.071	0.026 ± 0.072	P=0.8018
		$2^{(- \quad Ct)}$	1.000 (0.873 – 1.146)	0.982 (0.855 – 1.128)	
	Cat	**Ct**	0.000 ± 0.712	−1.569 ± 0.670	P=0.1290^{np}
		$2^{(- \quad Ct)}$	1.000 (0.254 – 3.940)	2.968 (0.818 – 10.772)	

Data shown as mean Ct ± SEM, with t-test used to identify the difference between vehicle and NNC 26–9100 treatment groups, significance set at *P<0.05. Fold difference relative to vehicle shown as $2^{(- \quad Ct)}$ with 95% confidence interval.

^{np}denotes use of Wilcoxon rank sum test.