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Authors

Bilousova, Tina
Taylor, Karen
Emirzian, Ana
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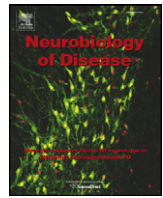
Publication Date

2015-02-01

DOI

10.1016/j.nbd.2014.11.006

Peer reviewed



Parallel age-associated changes in brain and plasma neuronal pentraxin receptor levels in a transgenic APP/PS1 rat model of Alzheimer's disease



Tina Bilousova^{a,b}, Karen Taylor^{a,b}, Ana Emirzian^a, Raymond Gylys^a, Sally A. Frautschy^{a,b}, Gregory M. Cole^{a,b}, Edmond Teng^{a,b,*}

^a Department of Neurology, David Geffen School of Medicine, at UCLA, Los Angeles, CA, United States

^b Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, CA, United States

ARTICLE INFO

Article history:

Received 5 September 2014

Revised 31 October 2014

Accepted 4 November 2014

Available online 13 November 2014

Keywords:

Plasma

Cortex

Hippocampus

Biomarker

Neuronal pentraxin

Transgenic

Alzheimer's disease

Synaptic function

ABSTRACT

Neuronal pentraxin receptor (NPR) is a synaptic protein implicated in AMPA receptor trafficking at excitatory synapses. Since glutamate neurotransmission is disrupted in Alzheimer's disease (AD), NPR levels measured from plasma represent a potential biomarker for synaptic dysfunction associated with AD. We sought to determine the relationship between AD pathology and brain and plasma NPR levels by examining age-associated NPR levels in these compartments in a transgenic APP/PS1 rat model of AD. NPR levels in cortical homogenate were similar in wild-type (Wt) and APP/PS1 rats at 3 months of age (prior to A β plaque deposition), but significantly increased in APP/PS1 rats by 9 and 18–20 months of age (after the onset of plaque deposition). These age-dependent differences were driven by proportional increases in NPR in membrane-associated cortical fractions. Genotype-related differences in NPR expression were also seen in the hippocampus, which exhibits significant A β pathology, but not in the cerebellum, which does not. Plasma analyses revealed increased levels of a 26 kDa NPR fragment in APP/PS1 rats relative to Wt rats by 18–20 months of age, which correlated with the levels of full-length NPR in cortex. Our findings indicate that cerebral accumulation of NPR and A β occurs with similar temporal and regional patterns in the APP/PS1 model, and suggest that a 26 kDa plasma NPR fragment may represent a peripheral biomarker of this process.

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Introduction

Neuronal pentraxin receptor (NPR) is a membrane-associated protein that is almost exclusively produced in the brain (Dodds et al., 1997). NPR is primarily found in excitatory synapses (Cho et al., 2008), and exhibits particularly high levels of expression in the cerebral cortex, hippocampus, and cerebellum (Dodds et al., 1997). It has been implicated in the regulation of glutamate neurotransmission in excitatory neurons. For example, in synapses, NPR binds to other members of the neuronal pentraxin protein family, including neuronal pentraxin 1 (NP1) and neuronal pentraxin 2 (NP2) (Kirkpatrick et al., 2000), in order to recruit and cluster AMPA-type glutamate receptors (Xu et al., 2003; Sia et al., 2007; Koch and Ullian, 2010). Furthermore, a key mechanistic step in the down-regulation of AMPA receptor neurotransmission required for long-term depression (LTD) is the cleavage of NPR by tumor necrosis factor- α converting enzyme (TACE) (Cho et al., 2008). NPR may also mediate the uptake and clearance of synaptic

debris in the process of synaptic remodeling (Bjartmar et al., 2006; Dodds et al., 1997; Kirkpatrick et al., 2000).

The prominent role of NPR in synaptic plasticity raises the possibility that its expression may be altered in neurodegenerative conditions, such as Alzheimer's disease (AD), that are characterized by synaptic dysfunction and loss (Selkoe, 2002). In particular, given the widespread disruption of glutamate neurotransmission seen with AD neuropathology (Revetz et al., 2013), NPR levels in cerebrospinal fluid (CSF) or peripheral blood may represent accessible markers of underlying dysfunction at excitatory synapses.

However, the relationship between AD pathology and CSF NPR levels remains uncertain. Proteomic studies of CSF indicate that NPR levels are altered in sporadic late-onset AD patients relative to controls with normal cognition or other neurological conditions, with one group reporting increased levels in AD (Yin et al., 2009) and others reporting decreased levels in AD (Abdi et al., 2006; Finehout et al., 2007; Hu et al., 2007). These apparently conflicting data reflect the possibility that CSF NPR levels may be modulated by underlying disease severity. One recent study that compared CSF samples from small groups of individuals with normal cognition or mild cognitive impairment (MCI), which often represents incipient AD, found higher NPR levels in MCI, though this result did not reach statistical significance (Wildsmith et al., 2014). However, in the same study, the investigators

* Corresponding author at: Neurobehavior Service (116AF), West Los Angeles VA Healthcare Center, 11301 Wilshire Boulevard, Los Angeles, CA 90073, United States. Fax: +1 310 268 4181.

E-mail address: eteng@ucla.edu (E. Teng).

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also examined longitudinal CSF NPR levels in a larger set of individuals diagnosed with AD. Not only were baseline CSF NPR levels in the AD group numerically lower than in the MCI group, they progressively declined over time, suggesting a potential negative correlation with disease progression at more advanced stages (Wildsmith et al., 2014). Similarly, amongst carriers of mutations in the presenilin 1 (*PSEN1*) or amyloid precursor protein (*APP*) genes, which are associated with autosomal dominant familial AD, elevated CSF NPR levels are seen in pre-symptomatic, but not in cognitively impaired or demented individuals (Ringman et al., 2012). Taken together, these findings raise the possibility that CSF NPR may have utility as an AD biomarker, with higher levels seen at earlier disease stages and lower levels seen at later disease stages.

Serum NPR levels, which are more accessible than CSF NPR levels, have also been shown to be elevated in MCI and AD patients relative to cognitively normal elderly controls (Yin et al., 2009). Unlike the plethora of other putative plasma and serum biomarkers of AD which have been identified through plasma proteomics (Doecke et al., 2012; Hu et al., 2012; O'Bryant et al., 2011; Ray et al., 2007), NPR expression is predominantly restricted to the brain, potentially increasing its specificity for examining underlying neurodegenerative disease. There are relatively few reports regarding proteins of predominantly neuronal origin that are consistently detected in plasma or serum. One notable exception is microtubule-associated protein tau, a component of the axonal cytoskeleton. Although plasma/serum tau levels are elevated in AD (Fiandaca et al., 2014; Zetterberg et al., 2013), they are also elevated after traumatic (Neselius et al., 2013) or hypoxic (Randall et al., 2013) brain injury. Although synaptic proteins, such as SNAP-25, have also been identified in serum (Marshall et al., 2014), their levels have yet to be rigorously investigated in specific disease states. Other synaptic proteins that are specifically expressed in the central nervous system, such as NPR, may have utility as potential biomarkers indicative of synaptic dysfunction that are measurable in plasma and/or serum.

Despite the intriguing results suggesting that NPR levels in CSF or blood could be a useful marker of underlying AD progression, little is known about the effects of AD neuropathology on NPR expression and compartmentalization in the brain. Earlier work demonstrated increased NP1 levels in brain tissue from both human AD patients and a transgenic APP/PS1 mouse model (Abad et al., 2006), suggesting that β -amyloid ($A\beta$) pathology can modulate the expression of other neuronal pentraxins. Therefore, we examined NPR expression in both brain and plasma in a transgenic APP/PS1 rat model of AD. This model exhibits regionally specific age-associated accumulation of $A\beta$ (Flood et al., 2009; Liu et al., 2008; Teng et al., 2011), which allowed us to determine the relationship between $A\beta$ and NPR. Furthermore, since the vast majority of NPR synthesis occurs in the brain, we sought to determine whether NPR levels measured from plasma correlate with those measured in brain.

Methods

Experimental animals

Plasma and brain samples were obtained from transgenic and wild-type Sprague–Dawley rats. The transgenic (Tg) rat model is homozygous for three gene constructs: 1) human APP 695 with the K670N/M671L mutation (rat synapsin-1 promoter); 2) human APP minigene with the K670N/M671L and V717F mutations (platelet derived growth factor β promoter); and 3) human PS-1 with the M146V mutation (rat synapsin-1 promoter) that are expressed on a Sprague–Dawley background (Flood et al., 2009). The time-course of $A\beta$ accumulation in this model has previously been described in detail (Flood et al., 2009; Liu et al., 2008; Teng et al., 2011). Sparse parenchymal $A\beta$ plaques begin to appear between 7 and 9 months of age, with progressive age-associated increases in plaque density seen in cortical and hippocampal regions, but not the cerebellum. Breeding pairs obtained from Cephalon

Inc. (West Chester, PA) were bred and aged at the UCLA School of Medicine vivarium facility. Wild-type (Wt) Sprague–Dawley rats were obtained from outside vendors (Harlan Laboratories, Indianapolis, IN; Charles River, Wilmington, MA). All animals were housed under a 12-hour light/dark cycle and had access to standard rat chow ad libitum. The UCLA Chancellor's Animal Research Committee approved this study and all animal experiments were conducted in compliance with its guidelines.

Behavioral testing

A subset of Tg and Wt rats were tested on the Morris water maze (MWM) (Morris et al., 1982) at 18–19 months of age. Animals were placed in a circular water tank 6 feet in diameter, and were trained for 4 trials per day over 10 days to learn to locate and swim to a hidden escape platform submerged below the surface of the water using distal cues in the periphery of the room. The outcome measure was latency to finding the hidden platform, with a maximum of 90 s permitted for each trial. Trials where the animal failed to find the platform were assigned a latency of 90 s.

Rat brain and plasma processing

One set of Tg and Wt animals was euthanized via carbon dioxide overdose at 3, 9, or 20 months of age. These rats were immediately decapitated, and their brains were removed and dissected into several anatomical regions, including the hippocampus, association cortex (parietal, somatosensory, motor, and visual cortex, denoted as “mixed cortex” below), and cerebellum. The dissected tissue was minced, slowly frozen in 0.32 M sucrose and stored at -80°C . This cryopreserved tissue subsequently underwent gentle mechanical homogenization in ice-cold buffer [0.32 M sucrose, 10 mM TRIS pH 7.5, plus protease inhibitors: pepstatin (4 $\mu\text{g}/\text{ml}$), aprotinin (5 $\mu\text{g}/\text{ml}$), trypsin inhibitor (20 $\mu\text{g}/\text{ml}$), EDTA (2 mM), EGTA (2 mM), PMSF (0.2 mM), leupeptin (4 $\mu\text{g}/\text{ml}$)] as previously described (Arold et al., 2012).

Separate sets of Tg and Wt animals were terminally sedated at 3, 9, and 18–20 months of age with pentobarbital. The chest cavity was opened and 3 ml of blood was collected from the right ventricle into K_2EDTA coated tubes. Blood samples were centrifuged at room temperature at 2000 g for 15 min. The plasma component was aliquoted into polypropylene tubes, snap frozen in liquid nitrogen, and stored at -80°C . Aliquots of a subset of these samples were depleted of highly abundant plasma proteins prior to subsequent analyses using Seppro IgY-R7 spin columns (Sigma-Aldrich; St. Louis, MO), which incorporate avian polyclonal IgY antibodies to remove rat albumin, α 1-antitrypsin, transferrin, fibrinogen, IgG, IgM, and haptoglobin.

Animals were then perfused with a HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer containing protease [leupeptin (1 $\mu\text{g}/\text{ml}$), aprotinin (1 $\mu\text{g}/\text{ml}$), pepstatin (1 $\mu\text{g}/\text{ml}$), and PMSF (50 $\mu\text{g}/\text{ml}$)] and phosphatase [sodium vanadate (1 mM), sodium pyrophosphate (1 mM), and sodium fluoride (50 mM)] inhibitors as previously described (Calon et al., 2004). Brains were removed immediately, dissected as described above, flash frozen in liquid nitrogen, and stored at -80°C . This tissue was subsequently serially extracted into tris-buffered saline (TBS) and lysis fractions (pH for both buffers = 8.0) as previously described (Calon et al., 2004; Yang et al., 2005).

Western blotting

Western blots were performed on brain homogenate, brain extract, and plasma samples. Protein assays were performed using BCA protein assay kits (Thermo Scientific Pierce; Rockford IL) per the manufacturer's protocol. Equal amounts of protein (20 μg) from each sample were mixed with 1x Novex Tris-glycine SDS sample buffer (Invitrogen; Carlsbad CA) and 100 mM dithiothreitol (DTT; Expedeon, Harston UK), then boiled, resolved by SDS-PAGE on a 10–20% Tris-glycine gradient

gel (Invitrogen; Carlsbad CA) and transferred to a PVDF membrane (Millipore; Billerica MA). To insure equal protein loading and transfer quality, membranes were stained with Ponceau S; only membranes with equal loading were quantified. After blocking with 3% BSA/PBS (1 h at RT), membranes were probed with polyclonal sheep anti-NPR antibody raised against recombinant human NPR [amino acids (aa's) 24–499, which have 87% homology between human and rodent sequences; AF4414, R&D Systems; Minneapolis, MN], polyclonal rabbit anti-TACE antibody (QED Bioscience; San Diego, CA) or polyclonal rabbit anti-phospho-TACE antibody (Thr735, Assay Biotechnology; Sunnyvale, CA) in 0.1% Tween 20/PBS with 0.05% sodium azide (ON, 4 °C). A subset of rat plasma samples was also probed with a polyclonal rabbit anti-NPR antibody targeting the C-terminus (AB15522, Millipore; Billerica MA) or the mid-portion (ab55903¹, Abcam; Cambridge UK) of the protein in the same fashion. Membranes were then washed 3 times in 0.1% Tween 20/PBS and incubated with HRP-conjugated anti-sheep IgG (R&D Systems; Minneapolis, MN) or anti-rabbit IgG (Jackson ImmunoResearch Laboratories; West Grove, PA) in 0.1% Tween 20/PBS (1 h at RT) and washed again. Chemiluminescent signals were detected with Super Signal West Femto substrate (Thermo Scientific Pierce; Rockford, IL) using a BioSpectrum 600 imaging system (UVP; Upland, CA) or on X-ray films (Switzer Medical; Fresno, CA) using a Kodak X-OMAT 2000A processor and quantified using VisionWorks software (UVP; Upland, CA).

A β ELISAs

Total A β levels were measured by a sandwich ELISA as previously described (Lim et al., 2000). The anti-A β antibodies used in this assay are 4G8 (recognizes aa's 17–24 and reacts to both human and rat A β ; Covance; Princeton, NJ), for capture and biotinylated 10G4 (recognizes aa's 5–13 and is presumed to be human specific; Yang et al., 1994) for detection. ELISAs using mixed cortex extracts were loaded with 20 μ g of total protein per well. ELISAs using cerebellar extracts were loaded with up to 80 μ g of total protein per well.

Data analyses

Statistical analyses were performed using SPSS 22 for Macintosh (IBM, Armonk NY). Comparisons of NPR, A β , and TACE expression between Wt and Tg animals were performed using unpaired *t*-tests. Comparisons of NPR expression between TBS and lysis fractions were performed using paired *t*-tests. Correlations between plasma and brain NPR expression were calculated using Pearson's correlation coefficient.

Results

NPR in rat mixed cortex homogenate

Western blotting was performed using AF4414 antibody on mixed cortex homogenate from Wt and Tg rats at 3, 9, and 20 months of age. In Tg rats, A β plaques are absent at 3 months of age, are sparsely present by 9 months of age, and exhibit extensive deposition by 20 months of age (Flood et al., 2009; Liu et al., 2008; Teng et al., 2011). The two full-length NPR species that have previously been identified in brain homogenate are 55 kDa (non-glycosylated) and 65 kDa (glycosylated) in size (Dodds et al., 1997). We quantified the relative abundance of these species in Wt and Tg rats in each age group (Fig. 1). At 3 months of age, similar levels of both the 55 kDa [$t(12) = 0.22, p = 0.83$] and 65 kDa [$t(12) = -1.61, p = 0.13$] NPR species were seen across genotypes. However, higher levels of both NPR species were seen in Tg rats relative

¹ Although this antibody is advertised as N-terminal specific, the synthetic immunogen sequence used to produce it (MADGAWDSPALLVELENA) lies in the mid-region of the rat NPR sequence, starting at amino acid 174 (Abcam, personal communication).

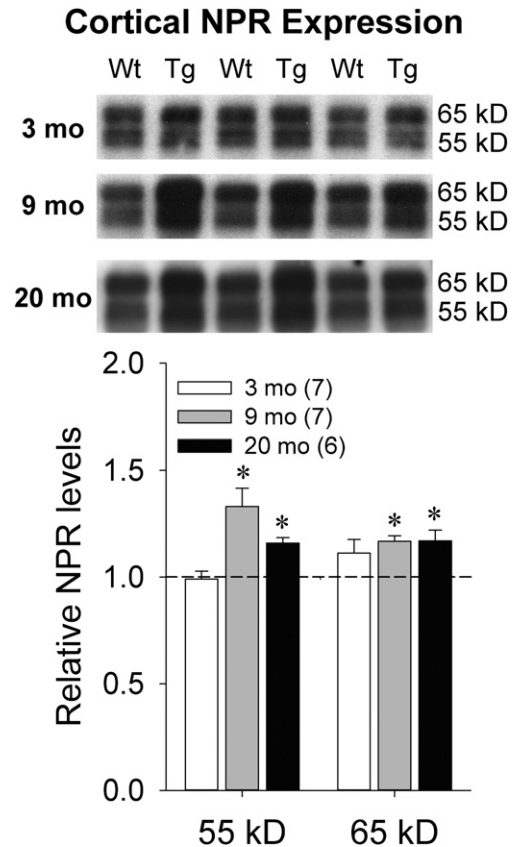


Fig. 1. Representative Western blots and quantification of NPR levels in mixed cortex homogenate from Wt and Tg rats at 3 months, 9 months, and 20 months of age. Dotted line represents normalized optical density of bands from Wt animals at each age (3 months: $n = 5$; 9 and 20 months: $n = 6$). The numbers of Tg animals at each age are shown in parentheses. Error bars represent standard error of the mean (SEM). * $p < 0.05$ vs. Wt.

to Wt rats at both 9 months [55 kDa: $t(10) = -3.02, p = 0.013$; 65 kDa: $t(10) = -3.40, p = 0.007$] and 20 months [55 kDa: $t(10) = -2.97, p = 0.013$; 65 kDa: $t(10) = -2.75, p = 0.002$] of age. These data indicate that increases in cortical NPR levels in Tg relative to Wt animals emerge in an age-dependent fashion that parallels the emergence of A β plaque pathology.

NPR in soluble and membrane-associated brain fractions

Prior work has suggested that cleavage of NPR by tumor necrosis factor- α converting enzyme (TACE) shifts NPR from a membrane-associated compartment to a soluble compartment to modulate synaptic plasticity (Cho et al., 2008). Since Tg rats demonstrate deficits in synaptic plasticity relative to Wt rats (Liu et al., 2008), we subsequently sought to determine whether NPR compartmentalization also shifted with age. At 3 and 9 months of age, examination of the mixed cortex TBS fraction revealed greater expression of both the 55 kDa [3 months: $t(12) = -3.17, p = 0.008$; 9 months: $t(12) = -2.55, p = 0.025$] and 65 kDa [3 months: $t(12) = -2.53, p = 0.026$; 9 months: $t(12) = -3.20, p = 0.008$] NPR species in Tg relative to Wt rats (Fig. 2A). However, in the mixed cortex lysis fraction, similar levels of the 55 kDa [3 months: $t(12) = -0.89, p = 0.39$; 9 months: $t(12) = -1.45, p = 0.17$] and 65 kDa [3 months: $t(12) = -1.71, p = 0.11$; 9 months: $t(12) = -0.91, p = 0.38$] NPR species were seen at both time-points (Fig. 2B). In contrast, at 18–20 months of age, the expression of the 55 kDa species in Tg relative to Wt rats was significantly lower in the TBS fraction [Fig. 2A; $t(12) = 2.28, p = 0.042$], but significantly higher in the lysis fraction [Fig. 2B; $t(12) = -3.77, p = 0.003$]. Likewise, while expression of the 65 kDa species at this age was similar

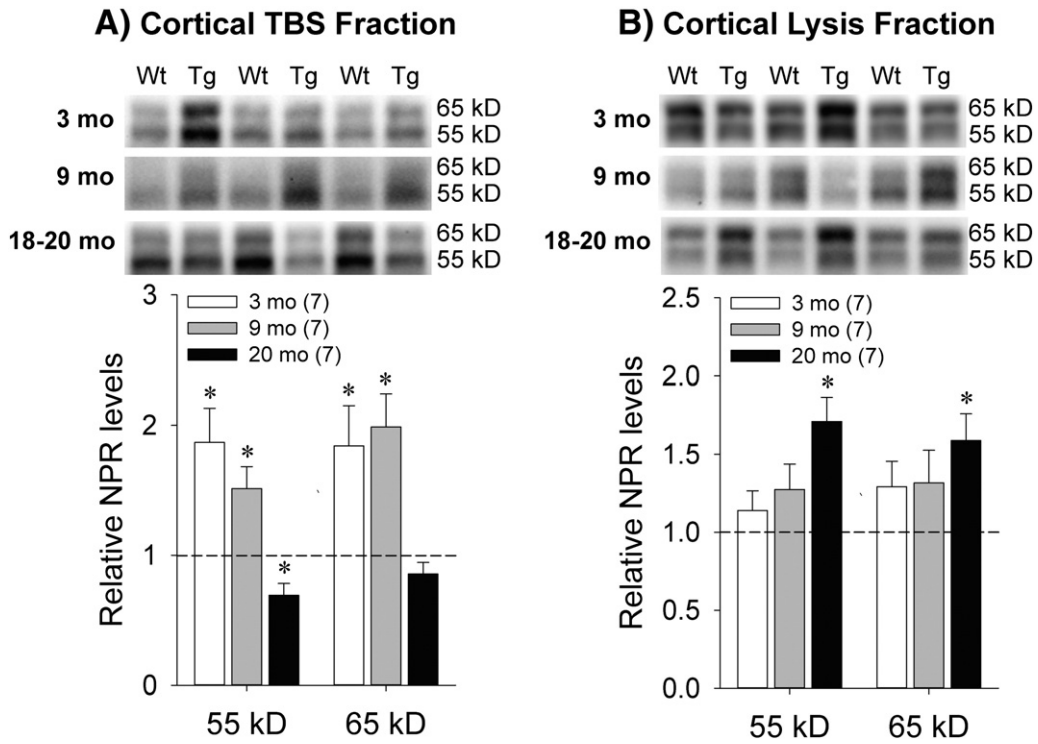


Fig. 2. Representative Western blots and quantification of NPR levels in A) soluble and B) membrane-associated mixed cortex fractions from Wt and Tg rats at 3 months, 9 months, and 18–20 months of age. Dotted line represents normalized optical density of bands from Wt animals at each age ($n = 7$ in all age groups). The numbers of Tg animals at each age are shown in parentheses. Error bars represent standard error of the mean (SEM). * $p < 0.05$ vs. Wt.

across genotypes in the TBS fraction [Fig. 2A; $t(12) = 1.07, p = 0.31$], significantly higher expression was seen in Tg relative to Wt animals in the lysis fraction [Fig. 2B; $t(12) = -3.02, p = 0.011$]. In Fig. 2, optimal exposure times for the TBS blots were longer than those for the lysis blots due to lower NPR concentrations in the TBS fraction. We subsequently quantified the relative expression of the 55 and 65 kDa NPR species at 18–20 months of age in cortical lysis and TBS fractions measured from the same blots and calculated lysis/TBS ratios for each band. In Wt and

Tg animals, higher NPR concentrations were seen in lysis relative to TBS fractions for both the 55 kDa [Wt: ratio = 3.6, SD = 2.0, $t(5) = -4.71, p = 0.005$; Tg: ratio = 9.1, SD = 3.5, $t(7) = -18.85, p < 0.001$] and 65 kDa [Wt: ratio = 5.5, SD = 1.7, $t(5) = -10.03, p < 0.001$; Tg: ratio = 9.4, SD = 4.5, $t(7) = -7.61, p < 0.001$] species. Relative to Wt animals, the lysis/TBS ratios for Tg animals were significantly higher for the 55 kDa band [$t(11) = -3.45, p = 0.005$] and marginally higher for the 65 kDa band [$t(11) = -1.98, p = 0.074$].

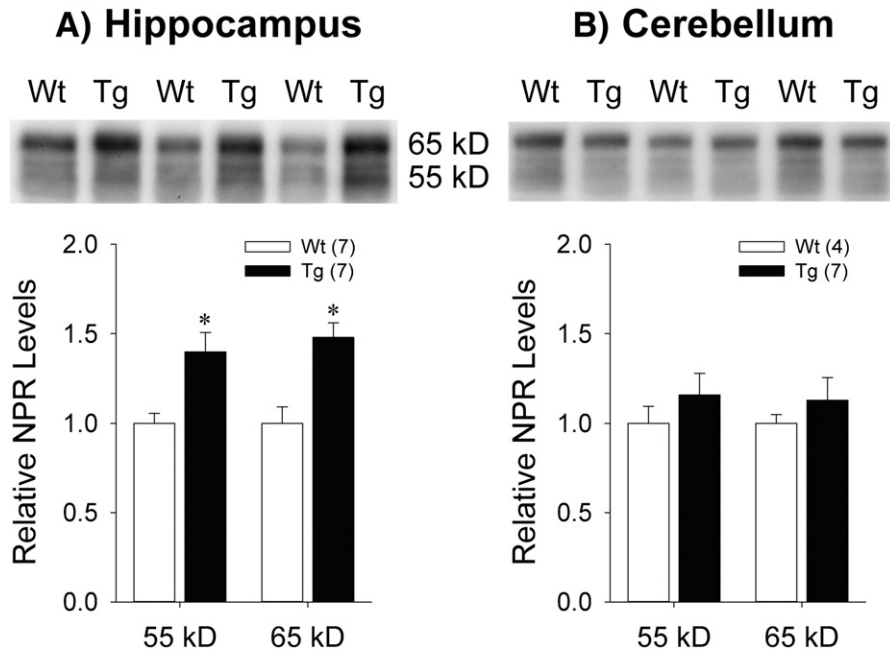


Fig. 3. Representative Western blots and quantification of NPR levels in membrane-associated A) hippocampal and B) cerebellar fractions from Wt and Tg rats at 18–20 months of age. Error bars represent standard error of the mean (SEM). * $p < 0.05$ vs. Wt.

Given the age-related accumulation of NPR in the membrane-associated compartment seen in cortical fractions from Tg rats, we also used the AF4414 antibody to examine genotype effects on NPR expression at 18–20 months of age in two other neuroanatomical regions that also demonstrate high levels of NPR expression (Dodds et al., 1997) but have differential expression of A β pathology (Teng et al., 2011): hippocampus (which exhibits marked age-associated A β plaque deposition) and cerebellum (which does not demonstrate any age-associated A β plaque deposition). In 18–20 month old hippocampal lysis fractions, the expression of both the 55 kDa [$t(12) = -3.37, p = 0.006$] and 65 kDa [$t(12) = -3.88, p = 0.002$] NPR species were significantly increased in Tg relative to Wt rats (Fig. 3A). In contrast, Western blots for NPR using 18–20 month old cerebellar lysis fractions showed no differences between Tg and Wt rats in the expression of either the 55 kDa [$t(9) = -0.92, p = 0.38$] or 65 kDa [$t(9) = -0.77, p = 0.46$] NPR species (Fig. 3B).

Soluble A β levels in Tg and Wt brain

We further sought to clarify whether the regional differences in NPR expression in Tg animals were related to soluble A β levels in the TBS fraction as measured by ELISA. In mixed cortex TBS fractions from 18 to 20 month old animals, there was the expected marked elevation of total A β levels in Tg relative to Wt animals [Fig. 4; $t(12) = -3.14, p = 0.008$]. However, in cerebellar TBS fractions from the same animals, total A β levels remained undetectable in both genotypes, even after increasing the sample loading from 20 to 80 μ g of total protein per well (data not shown). These results suggest that increased expression of NPR species in membrane-associated fractions occurs only in regions where increased A β accumulation is seen. However, soluble A β levels in mixed cortex in these animals did not directly correlate with expression of the full-length 55 kDa [$r(14) = 0.27, p = 0.35$] or 65 kDa [$r(14) = 0.38, p = 0.19$] NPR species in the membrane-associated fraction.

Brain NPR levels and behavioral performance

Previous work with this Tg rat model has demonstrated age-associated deficits on the MWM (Liu et al., 2008), which is particularly sensitive to hippocampal dysfunction (Morris et al., 1982). A subset of Wt and Tg rats were tested on the MWM at 18–20 months of age. Tg animals exhibited significantly longer latencies to finding the hidden

platform across training days 6 through 10 than Wt animals [Fig. 5A; $t(12) = -3.55, p = 0.004$]. These animals were euthanized shortly after completing MWM testing. Subsequent analyses indicated that significantly poorer MWM performance was seen in animals with higher levels of the 65 kDa full-length NPR species in membrane-associated hippocampal fractions [Fig. 5B; $r(14) = 0.836, p < 0.001$], suggesting that regional NPR accumulation may contribute to the behavioral deficits seen in Tg rats.

Phospho-TACE levels in Tg and Wt brain

The age-associated increase in NPR localization in the membrane-associated compartment in Tg animals may be due to reductions in TACE activity that are mediated by A β accumulation. Previous work in hippocampal neurons cultured from Tg2576 mice suggests that A β accumulation increases phosphoinositide-dependent kinase-1 (PDK-1) activity and decreases TACE activity via Thr735 phosphorylation (Pietri et al., 2013). Other reports indicate that inactive TACE is maintained in a dimeric state (Xu et al., 2012). Therefore, we performed Western blots to assess total and phosphorylated TACE levels in membrane-associated mixed cortex fractions from rats at 3 months and 20 months of age (Fig. 6). At 3 months of age, when Tg rats express similar levels of membrane-associated NPR and increased levels of soluble NPR relative to Wt rats, total TACE levels were similar between Wt and Tg rats in the monomeric (~75 kDa) band [$t(12) = 0.26, p = 0.80$]. Monomeric phospho-TACE expression was also similar between Wt and Tg animals at this age [$t(12) = 1.55, p = 0.15$], but Tg animals had marginally lower expression of dimeric (~160 kDa) phospho-TACE [$t(12) = 1.91, p = 0.08$] relative to Wt animals. At 20 months of age, similar total TACE levels were again seen in the Wt and Tg groups in the monomeric band [$t(11) = -0.42, p = 0.68$]. While monomeric phospho-TACE expression was again similar between Wt and Tg animals [$t(12) = -1.47, p = 0.17$], significantly higher expression of dimeric phospho-TACE was seen in Tg animals [$t(12) = -7.32, p < 0.001$]. These results are consistent with greater TACE inactivation in older Tg animals and represent a potential A β -mediated mechanism for the age-associated accumulation and compartmental shifts seen with NPR in this model.

NPR levels in Tg and Wt rat plasma samples

A prior report suggested that elevated NPR levels were seen in serum samples from human subjects meeting criteria for AD or mild cognitive impairment relative to cognitively normal aged controls (Yin et al., 2009). Therefore, we performed Western blots using AF4414 antibody to determine whether age-associated increases in relative NPR expression in Tg rat cortex were paralleled by similar changes in NPR expression in the plasma. The dominant NPR species seen in plasma was the 55 kDa species, but its expression did not differ by genotype at 3, 9, or 18–20 months of age (data not shown). The 65 kDa NPR species was not seen in plasma. However, an immunoreactive band at 26 kDa demonstrated age- and genotype-associated changes (Fig. 7). This 26 kDa plasma NPR species showed similar expression in Wt and Tg animals at 3 months [$t(12) = 0.15, p = 0.89$] and 9 months [$t(12) = 0.15, p = 0.89$] of age. However, by 18–20 months of age, plasma expression of this 26 kDa fragment was significantly higher in Tg animals [$t(12) = -2.70, p = 0.019$]. We repeated Western blots with plasma samples from 18 to 20 month animals after spin column depletion of high abundance proteins, which include immunoglobulins. Expression of the 26 kDa NPR species in the depleted samples remained significantly higher in Tg versus Wt animals [$t(12) = -4.70, p = 0.001$]. The 26 kDa NPR species was not detected in Western blots using antibodies targeting the C-terminus or mid-portions of the protein (data not shown), suggesting that it represents a region of the protein that lies closer to the N-terminus and neighboring transmembrane domain. This NPR fragment was only inconsistently seen by Western blot in

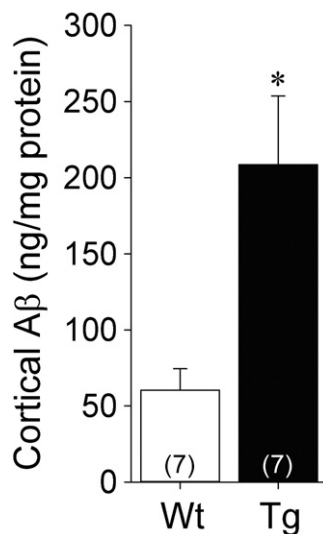


Fig. 4. Total A β levels in soluble mixed cortex fractions from Wt and Tg rats at 18–20 months of age. The numbers of animals in each group are shown in parentheses. Error bars represent standard error of the mean (SEM). * $p < 0.05$ vs. Wt.

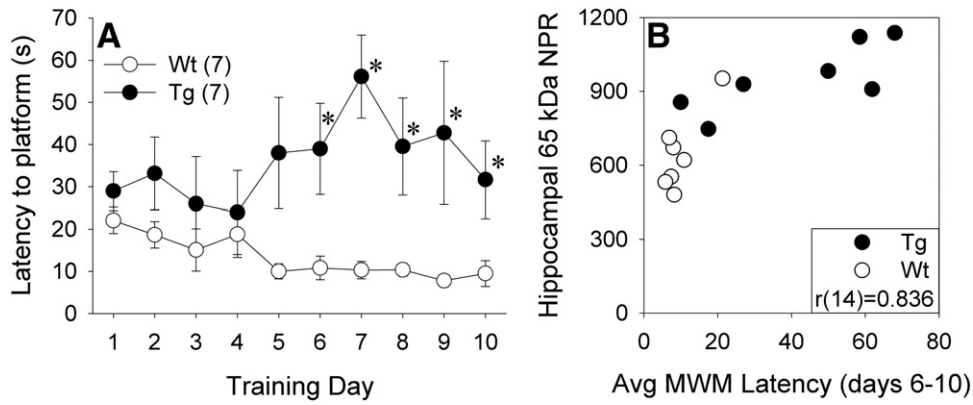


Fig. 5. A) Morris water maze (MWM) platform latency in 18–20 month old Wt and Tg rats across training days. Error bars represent standard error of the mean (SEM). * $p < 0.05$ vs. Wt. B) Scatterplot of average MWM platform latency across training days 6–10 versus membrane-associated NPR levels in hippocampal fractions from 18 to 20 month old Wt and Tg rats. Hippocampal NPR levels are expressed in arbitrary units of optical density.

mixed cortex homogenate, TBS, or lysis fractions. In brain samples from 18 to 20 month animals, much longer exposure times were needed to visualize this 26 kDa band relative to the aforementioned 55 kDa and 65 kDa full length bands, and the expression of this band (when visualized) did not consistently exhibit genotype effects.

Correlations between the brain and plasma NPR expression

Correlational analyses were performed in order to determine whether plasma levels of the 26 kDa NPR species reflected cortical levels of full-length NPR. Significant correlations were seen between plasma expression of the 26 kDa NPR species and mixed cortex lysis fraction expression of the 55 kDa [$r(11) = 0.633, p = 0.037$] and

65 kDa [$r(11) = 0.657, p = 0.028$] NPR species (Fig. 8) which supports a possible precursor–product relationship. However, plasma expression of the 26 kDa NPR species did not correlate with mixed cortex TBS fraction expression of either the 55 kDa [$r(11) = -0.027, p = 0.94$] or 65 kDa [$r(11) = 0.259, p = 0.44$] NPR species.

Discussion

Our data demonstrate that increasing cortical and hippocampal NPR levels parallel the age-associated deposition of cerebral A β pathology that has previously been characterized in this transgenic APP/PS1 rat model of AD (Flood et al., 2009; Liu et al., 2008; Teng et al., 2011). The accumulation of NPR in older Tg animals is seen in

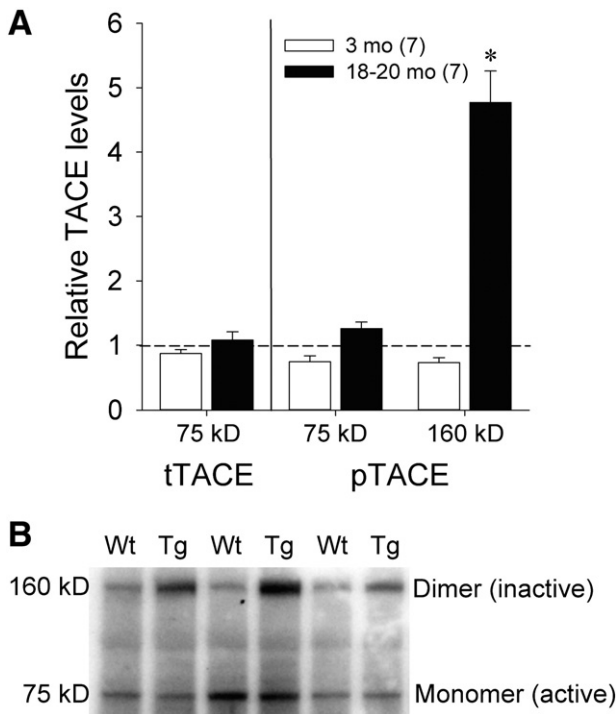


Fig. 6. A) Quantification of Western blots for total and phospho-TACE levels in membrane-associated mixed cortex fractions in 3 and 18–20 month old Wt and Tg rats. Dotted line represents normalized optical density of bands from Wt animals at each age ($n = 7$ for both age groups). The numbers of Tg animals at each age are shown in parentheses. Error bars represent standard error of the mean (SEM). * $p < 0.05$ vs. Wt. B) Representative Western blots for monomeric (~75 kDa) and dimeric (~160 kDa) phospho-TACE levels in 18–20 month old Wt and Tg rats.

Plasma 26 kDa NPR Expression

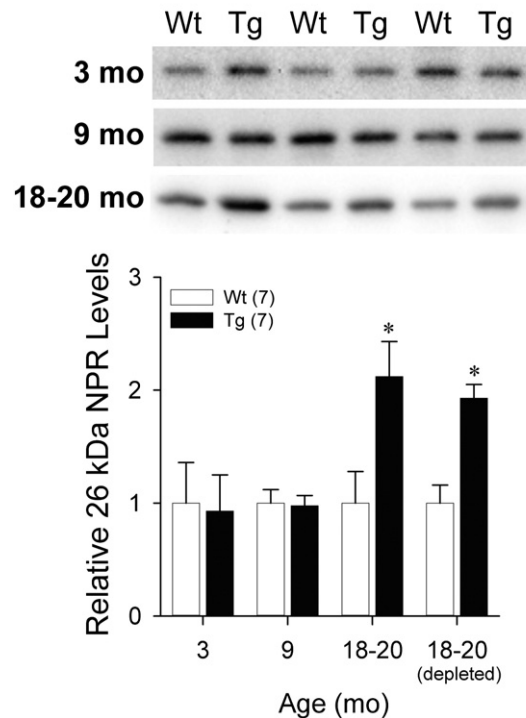


Fig. 7. Representative Western blots and quantification of the 26 kDa NPR species in plasma from Wt and Tg rats at 18–20 months of age. Depleted samples underwent removal of high abundance plasma proteins prior to Western blotting. Error bars represent standard error of the mean (SEM). * $p < 0.05$ vs. Wt.

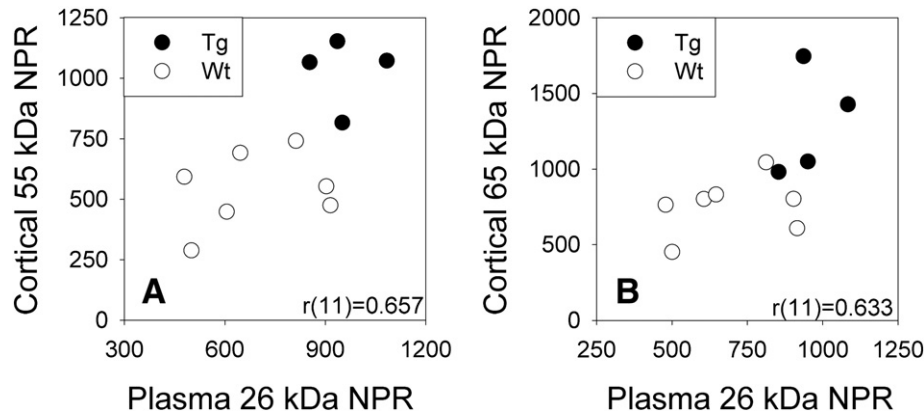


Fig. 8. Scatterplots of plasma expression of 26 kDa NPR species versus membrane-associated mixed cortex expression of full-length A) 55 kDa and B) 65 kDa NPR species in 18–20 month old Wt and Tg rats. Plasma and cortical NPR levels are expressed in arbitrary units of optical density.

the cortex and hippocampus, which both express high levels of both soluble A β and A β plaques, suggesting that NPR accumulation may be mediated by A β pathology. This hypothesis is supported by our results demonstrating elevated NPR levels in cortical homogenate (which includes both soluble and membrane-associated fractions) from Tg animals by 9 months of age, when sparse A β plaques are just starting to deposit in this model (Flood et al., 2009; Liu et al., 2008). Conversely, genotype effects on NPR expression were not observed in the cerebellum, where soluble A β levels were undetectable and A β plaques are largely absent (Teng et al., 2011). Nevertheless, since we did not find significant linear correlations between cortical A β and NPR levels, additional downstream mechanisms, such as oxidative damage or neuroinflammation, may be mediating A β regulation of NPR levels in the brain.

In its full-length forms, NPR has a short transmembrane domain near its N-terminus (Cho et al., 2008), and it is thus unsurprising that a greater proportion of cortical NPR is seen in the membrane-associated fraction. However, this finding was most pronounced in 18–20 month old Tg animals (i.e. those with the most advanced A β pathology), which demonstrated higher expression of membrane-associated NPR than age-matched Wt animals. This result may be mechanistically related to A β -related TACE inactivation (Pietri et al., 2013), since TACE-mediated cleavage of NPR is required for its release from the plasma membrane (Cho et al., 2008). TACE dimers in the cell membrane represent inactive TACE (Xu et al., 2012), and at older ages, Tg rats exhibit higher levels of phospho-TACE dimers than Wt rats. Furthermore, since NPR cleavage and release from the membrane are also needed for LTD (Cho et al., 2008), the accumulation of NPR in membrane-associated cortical fractions may contribute to the deficits in synaptic plasticity that have been previously reported in this model (Liu et al., 2008).

Age-associated TACE inactivation in Tg rats also provides a potential explanation for why these animals have lower NPR expression in soluble cortical fractions at 18–20 months of age relative to Wt controls. However, Tg rats also exhibit increased NPR expression in soluble cortical fractions relative to Wt rats at younger ages, when soluble A β levels are already elevated, but plaque deposition has not yet (3 months) or just begun (9 months) to occur (Flood et al., 2009; Liu et al., 2008). These results are consistent with the hypothesis that at early stages of AD progression, there is a compensatory increase in synaptic plasticity, including at glutamatergic synapses, which may represent an ultimately ineffective attempt to counter A β -related synaptotoxicity (Bell et al., 2007; Williams et al., 2009). Alternatively, greater levels of soluble NPR in younger Tg animals could be a consequence of relatively increased LTD, since LTD-associated cleavage of NPR by TACE (which does not yet appear to be inactivated at younger ages) would yield such soluble species and increasing levels of soluble A β appear to

facilitate LTD in hippocampal slice preparations (Cheng et al., 2009; Shankar et al., 2008). However, other reports suggest that LTD may actually be more difficult to induce in transgenic mouse models of AD than in wild-type controls (Song et al., 2014; Tamagnini et al., 2012), which would oppose this hypothesis.

Beyond demonstrating a potential brain basis for AD-related changes in CSF NPR levels, our data also demonstrate that a 26 kDa plasma NPR immunoreactive species exhibits age- and genotype-associated changes in expression that parallel changes in full-length brain NPR expression and thus may represent a viable peripheral marker of brain NPR levels. This NPR fragment appears to be derived from the N-terminus of the full-length protein, but is present in relatively low abundance in the cortical samples. It may correspond with a proteolytically processed N-terminal fragment of similar size that has previously been detected in mouse forebrain lysate (Cho et al., 2008). NPR fragments of this approximate size have also been detected via two-dimensional gel electrophoresis from human CSF samples (Finehout et al., 2007; Yin et al., 2009). The relatively higher abundance of this fragment in plasma samples could mean that a greater degree of proteolytic breakdown may occur peripherally rather than in the brain. However, the protease(s) responsible for generating this 26 kDa fragment have not been conclusively identified (Cho et al., 2008). The significant correlation between the expression of the 26 kDa plasma NPR species and expression of the membrane-associated, but not soluble full-length cortical NPR species would appear to argue against the simple diffusion of soluble NPR across the blood–brain barrier. Instead, NPR may reach the periphery in a membrane-bound form. Although exosomes, which can cross the blood–brain barrier and represent a potential clearance mechanism for degraded proteins (Kalani et al., 2014) might be considered for this role, recent proteomic characterization of CSF suggests relative depletion rather than enrichment of NPR in extracellular vesicular fractions (Chiasserini et al., 2014).

Prior human CSF proteomic studies in AD suggest that NPR levels increase at early stages of the disease but decrease with subsequent disease progression (Ringman et al., 2012; Wildsmith et al., 2014). Therefore, our results demonstrating the greatest relative elevations in brain and plasma NPR levels in the oldest cohorts of APP/PS1 rats, which have extensive and widespread A β plaque pathology (Flood et al., 2009; Liu et al., 2008; Teng et al., 2011), might appear to contrast with these human CSF results. However, others have suggested that transgenic rodent models of cerebral amyloidosis, even at advanced ages, most closely model the earliest stages of AD, when cognition is intact or only mildly impaired and synaptic and neuronal losses remain relative modest (Ashe, 2005). Therefore, it seems likely that the elevated NPR levels seen in this transgenic rat model are analogous to those seen in patients at the presymptomatic (Ringman et al., 2012) or MCI (Wildsmith et al., 2014; Yin et al., 2009) stages of AD, and subsequent

reductions in NPR expression, which may be associated with more advanced AD (Abdi et al., 2006; Finehout et al., 2007; Hu et al., 2007; Wildsmith et al., 2014) may only be apparent in more aggressive animal models.

When taken together, our data indicate that corresponding increases in NPR expression in brain and plasma compartments can be detected with increasing disease progression in a transgenic APP/PS1 rat model, suggesting the potential utility of plasma NPR levels as a peripheral biomarker of underlying synaptic dysfunction. Previous work has indicated that CSF NPR levels are increased in AD but not in Parkinson's disease (PD) (Yin et al., 2009). Although our results are consistent with those findings, since they are derived from a model that overexpresses A β , the prominent role of the neuronal pentraxins in synaptic function raises the possibility that NPR expression may also be altered by other conditions characterized by synaptic disruption. Indeed, relative decreases have been reported in NPR RNA levels in rat brain after cerebral ischemia (Kim et al., 2002) and in a mesencephalic dopaminergic neuronal culture model of PD (Anantharam et al., 2007). Furthermore, decreased CSF NPR levels have been reported in multiple sclerosis (Kroksveen et al., 2012). Although a relative upregulation of NPR RNA in cardiac muscle has been reported after myocardial infarction (Andersson et al., 2006), the expression of NPR RNA in brain is far higher than that seen in other organs (Dodds et al., 1997). These data indicate that plasma NPR levels are likely to be primarily driven by brain NPR levels, but we cannot entirely exclude the possibility that a small proportion of plasma NPR levels may be derived from other sources.

There are two other factors that may limit the interpretation of our results. Although both the Tg and Wt animals are Sprague–Dawley rats, the Tg animals were obtained from a homozygous in-bred colony while the Wt animals were obtained from commercial outbred colonies. Therefore, we cannot completely rule out the possibility that other genetic variables beyond the APP/PS1 transgenes may have contributed to our findings. A β is the predominant pathology expressed by the Tg rats, though they also exhibit some hyperphosphorylated tau accumulation in dystrophic neurites (Liu et al., 2008). As such, a portion of the alterations in NPR expression seen in clinical AD populations may be driven by more prominent tau-related pathology. This question warrants further investigation in transgenic rodent models that overexpress tau.

Further translational studies of plasma NPR expression in clinical populations, particularly those that compare participants with either normal cognition or MCI that have positive or negative A β PET imaging, may provide additional insight into the relationship between peripheral NPR levels and cerebral A β pathology.

Acknowledgments

This work was supported by the National Institute on Aging (K08 AG34628 [to ET; jointly sponsored by NIA, AFAR, the John A. Hartford Foundation, the Atlantic Philanthropies, the Starr Foundation and an anonymous donor] and RC1 AG035878 [to GMC]), the National Center for Research Resources and the National Center for Advancing Translational Sciences (UL1 TR000124), and the UCLA Clinical and Translational Science Institute.

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