Role of cardiac biomarkers in cognitive impairment and functional decline

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CHAPTER 7

NATRIURETIC PEPTIDES IN POST-MORTEM BRAIN TISSUE AND CEREBROSPINAL FLUID OF NON-DEMENTED HUMANS AND ALZHEIMER'S PATIENTS

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Abstract

Animal studies suggest the involvement of natriuretic peptides (NP) in several brain functions that are known to be disturbed during Alzheimer's disease (AD). However, it remains unclear whether such findings extend to humans. In this study, we aimed to: 1) map the gene expression and localization of NP and their receptors (NPR) in human post-mortem brain tissue; 2) compare the relative amounts of NPR between the brain tissue of AD patients and non-demented controls and 3) compare the relative amounts of NP between the cerebrospinal fluid (CSF) of AD patients and non-demented controls. Using the publicly available Allen Human Brain Atlas dataset, we mapped the gene expression of NP and NPR in healthy humans. Using immunohistochemistry, we visualized the localization of NP and NPR in the frontal cortex of AD patients (n=10, mean age 85.8 ± 6.2 years) and non-demented controls (mean age = 80.2 ± 9.1 years). Using Western blotting and ELISA, we quantified the relative amounts of NP and NPR in the brain tissue and CSF of these AD patients and non-demented controls. Our results showed that NP and NPR genes were ubiquitously expressed throughout the brain in healthy humans. NP and NPR were present in various cellular structures including in neurons, astrocyte-like structures, and cerebral vessels in both AD patients and non-demented controls. Furthermore, we found higher amounts of NPR type-A in the brain of AD patients ($p=0.045$) and lower amounts of NP type-B in the CSF of AD patients ($p=0.029$). In conclusion, this study show the abundance of NP and NPR in the brain of humans suggesting involvement of NP in various brain functions. In addition, our findings suggest alterations of NP levels in the brain of AD patients. The role of NP in the development and progression of AD remains to be elucidated.

Introduction

Natriuretic peptides (NP) refer to a group of peptides that are mostly known for their actions within the cardiovascular system[1]. Three members of this family are atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP)[2]. The biological activities of NP are mediated through activation of three transmembrane receptors including natriuretic peptide receptor A (NPR-A), natriuretic peptide receptor B (NPR-B) and natriuretic peptide receptor C (NPR-C)[3] (Figure 1). While NP have been initially discovered in cardiac myocytes[4], extensive distribution of NP and their receptors in the brain of animal species has been repeatedly reported [5]. It was shown that NP and their receptors are present not only in the neuronal structures, but are also abundant in the glial cells and cerebral vessels[5, 6]. Notably, findings from animal studies suggest that NP are involved in regulation of neuroplasticity[7], blood-brain barrier integrity[8], neuro-inflammation[9, 10] and memory function[11, 12].

The biological roles of NP in the central nervous system (CNS) have been mainly described in rodent and mammalian species[13]. Existing evidence from human subjects indicates that higher plasma levels of NP associate with dementia and accelerated cognitive decline[14]. Although this link was mainly attributed to cardiovascular pathologies[15], recent findings suggest that plasma NP associate with cognitive decline independent of cardiovascular disease[16, 17]. Moreover, higher circulating levels of BNP were linked with lower cerebrospinal fluid (CSF) amyloid beta42 (Aβ42) and total tau/Aβ42 ratios[18]. Interestingly, some studies have reported the presence of NP and their receptors in the brain tissue and CSF of human subjects[19, 20]. Hence, based on the available biological and clinical evidence, we have recently hypothesized that NP may be a potential diagnostic and/or therapeutic markers for Alzheimer's disease (AD)[13].

To substantiate this hypothesis, in this study we aimed to: 1) map the gene expression and localization of NP and their receptors (NPR) in human post-mortem brain tissue, 2) compare the relative amounts of NPR between the brain tissue of AD patients and

non-demented controls and 3) compare the relative amounts of NP between the cerebrospinal fluid (CSF) of AD patients and controls.

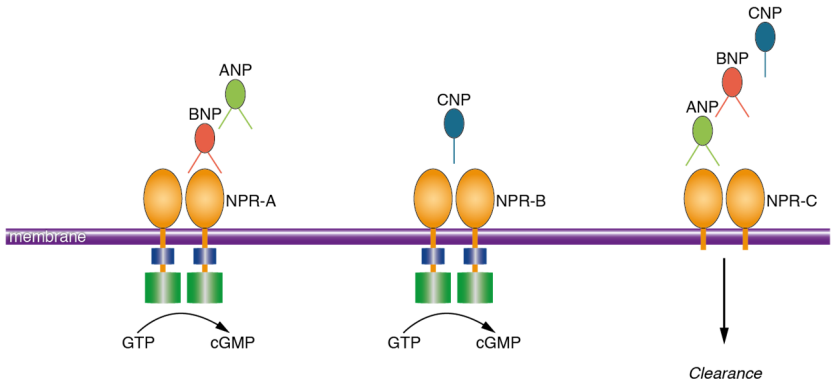


Figure 1. Schematic presentation of the ligand preference of natriuretic peptide receptors. Natriuretic peptides bind to three transmembrane receptors, namely NPR-A, NPR-B and NPR-C. ANP and BNP bind to NPR-A; CNP binds to NPR-B and all the NP (ANP, BNP and CNP) bind to NPR-C. Activation of the NPR increases the intracellular concentration of cyclic guanosine monophosphate (cGMP), being the main mediator of the biological activity of the NP. NPR-C is a clearance receptor.

Methods and Materials

Experimental design

We had access to biomaterials of 17 non-demented controls (mean age = 80 ± 8.5 years, 59% female) and 10 patients with a definitive pathological diagnosis of AD (mean age 85.8 ± 6.2 years, 60% female) (Table 1). The definitive diagnosis of the donors was provided by expert neuropathologists that considered both histopathological results and the clinical diagnosis of the donors. To investigate the localization of NP and NPR in the brain, we performed immunohistochemistry using post-mortem brain tissue of a subset of 13 non-demented controls and 10 AD patients (Table 1). For quantitative assessment of NPR in the brain, we performed Western blotting using a sub-set of donors from whom frozen brain tissue was available (10 controls and 8 AD patients, Table 1). NP in the CSF were detected by enzyme-

linked immunosorbent assay (ELISA) in another subset of the donors that had a frozen CSF sample available (10 controls and 10 AD patients, Table 1). Age and gender were not significantly different between controls and AD patients in any of the subsets (all $p>0.05$).

Brain tissue samples

Post-mortem brain tissue from the frontal lobe – middle frontal gyrus – of 10 AD patients and 8 controls were obtained from the Netherlands Brain Bank (NBB, Netherlands Institute for Neuroscience Amsterdam); and for 6 controls tissues were obtained from the Normal Ageing Brain Collection Amsterdam (NABCA). All CSF samples were provided by the NBB. The CSF was collected from the lateral ventricles during autopsy and stored at -80° C for later analysis. All donors gave written informed consent for brain autopsy and for the use of their specimens and medical records for research purposes. According to national ethical guidelines, all samples were coded to maintain the anonymity of donors.

Gene expressions

To investigate the gene expression of NP and NPR in the brain, we used BrainScope[21] (http://brainscope.nl/brainscope). BrainScope is a web-portal for visual analysis of gene expression in the brain of healthy human subjects, based on data from the Allen Human Brain Atlas[22, 23] (http://human.brain-map.org/static/download). The Allen Human Brain Atlas provides the mRNA expression of about 20,000 genes using six healthy adult donors (age range from 24 to 57 years). Gene expression was measured with a customized micro-array chip using ~3,700 samples taken from anatomically annotated regions of the brain. From this data, BrainScope uses 105 expression values per gene corresponding to anatomically annotated brain regions that were sampled for all the six donors. The expression values were averaged to provide a single expression value for each sample across the subjects[21]. Accordingly, we used BrainScope to explore the expression of the following genes: natriuretic peptide A (*NPPA*, encodes ANP protein, Gene ID 4878), natriuretic peptide B (*NPPB*, encodes BNP protein, Gene ID 4879), natriuretic peptide C (*NPPC*, encodes CNP protein, Gene ID 4880), natriuretic peptide receptor 1 (*NPR1*, encodes NPR-A protein, Gene

ID 4881), natriuretic peptide receptor 2 (*NPR2*, encodes NPR-B protein, Gene ID 4882) and natriuretic peptide receptor 3 (*NPR3*, encodes NPR-C protein, Gene ID 4883).

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues were serially cut into 5-µm-thick sections and mounted on coated glass slides (SuperFrost® Plus, VWR). The sections were deparaffinized in xylene and rehydrated through graded ethanol concentrations (100%, 90% and 70%). To block endogenous peroxidase activity, the sections were incubated for 20 minutes in methanol with 0.3% hydrogen peroxide (H_2O_2). This was followed by antigen retrieval by cooking the sections for 20 minutes at 0.76 bar steam pressure in an acidic pH 6 solution (H-3300, Vector labs). The antigen retrieval step was performed depending on the primary antibody (Supplementary Table 1). Next, the sections were washed in phosphate-buffered saline (PBS) and incubated with the primary antibody overnight at room temperature in the blocking buffer (1% bovine serum albumin [BSA] in PBS). The sections were then incubated for 1 hour with a biotin-labelled secondary anti- rabbit or anti- mouse antibody followed by 30 minutes incubation with avidin-biotin complex (ABC, Vector Labs, CA, USA). For the anti-NPR-C primary antibody, the secondary antibody was conjugated to horseradish peroxidase (HRP) instead of biotin. Staining was visualized using 3, 3'-Diaminobenzidine (DAB) which was activated by H_2O_2. Finally, the sections were counterstained with Harris Haematoxylin and coverslipped with Entellan. The sections were scanned using an automatic bright field slide scanner (Philips IntelliSite Ultra-Fast Scanner, Digital pathology slide scanner, Netherlands) for microscopic evaluation.

All sections were evaluated for the presence/absence of NP or NPR staining in the grey matter (GM), white matter (WM) and cerebral vessels. The assessments were performed by two trained observers (SM and IV) who were blinded to the clinical diagnosis of the subjects. Disagreements (n=151 cases, 9.8% of the sections) between the two reviewers were resolved by discussion with the third reviewer (MB).

Table 1. Characteristics of study subjects

Diagnosis	Source	Age	Gender	PMD (h)	IHC	WB	CSF
Control	NBB	84	F	5:36	✓	✓	✓
Control	NBB	70	F	7:35	✓	NA	✓
Control	NBB	64	F	5:40	✓	NA	NA
Control	NBB	83	M	5:15	✓	NA	✓
Control	NBB	91	F	3:47	✓	✓	✓
Control	NBB	73	M	8:00	✓	✓	✓
Control	NBB	72	F	6:50	✓	✓	✓
Control	NBB	89	F	6:30	✓	✓	✓
Control	NBB	88	M	11:10	NA	NA	✓
Control	NBB	82	M	5:20	NA	NA	✓
Control	NBB	73	F	5:30	NA	NA	✓
Control	NABCA	82	M	5:30	✓	NA	NA
Control	NABCA	87	F	8:30	✓	✓	NA
Control	NABCA	72	F	7:15	✓	✓	NA
Control	NABCA	93	M	8:30	✓	✓	NA
Control	NABCA	82	M	7:30	✓	✓	NA
Control	NABCA	73	F	6:30	NA	✓	NA
AD	NBB	88	M	5:30	✓	✓	✓
AD	NBB	85	F	4:05	✓	✓	✓
AD	NBB	86	M	5:10	✓	NA	✓
AD	NBB	81	M	7:50	✓	✓	✓
AD	NBB	88	F	4:40	✓	✓	✓
AD	NBB	73	M	4:45	✓	✓	✓
AD	NBB	96	F	7:55	✓	✓	✓
AD	NBB	82	F	4:35	✓	NA	✓
AD	NBB	90	F	3:55	✓	✓	✓
AD	NBB	89	F	4:30	✓	✓	✓

Abbreviations: AD: Alzheimer's disease; NBB: Netherlands Brain Bank; NABCA: Netherlands Ageing Brain Collection Amsterdam; PMD: post-mortem delay (in hours); IHC: immunohistochemistry; WB: Western blot; NA: not available and CSF: cerebrospinal fluid

Protein isolation and Western blotting

The frozen brain tissues were chopped in GM and WM pieces on dry ice. We were not able to separate the WM of four AD patients due to severe atrophy of the brain tissue. Next, the GM and WM samples were suspended in lysis buffer (50 mM tris pH 7.5 and 1% triton in 10 ml Milli-Q) supplemented with cOmplete Mini Protease Inhibitor Cocktail Tablets (Roche). This was followed by homogenizing the samples in a bullet blender electric homogenizer (Next Advance) for 3 minutes using 0.5 mm stainless steel beads. The homogenized samples

were centrifuged for 30 minutes at full speed before collection of the supernatants. Protein concentration was calculated using the bicinchoninic acid kit (Thermo Fisher Scientific, Waltham, USA) with bovine serum albumin as a standard. The supernatant was further diluted in sample buffer and denatured by boiling for 10 minutes in 95°.

We used 50 µg of the GM and WM protein lysates per sample. Proteins were separated by SDS-PAGE by running through a 4-20% sodium dodecyl sulphate polyacrylamide gel (Bio-Rad) alongside a proteins size marker (PageRuler^{TM}, Thermo Fisher Scientific). Proteins were transferred to a nitrocellulose membrane using the Trans-blot Turbo Transfer system (Bio-Rad) for 30 minutes at 1.0 A. The transfer of proteins was checked with Ponceau red followed by washing the membrane in Tris-Buffered Saline (TBS). The membrane was blocked in 5% low fat milk in TBS-Tween (mTBST) for 1 hour and incubated with primary antibody (diluted in 5% mTBST) at room temperature for 90 minutes, 2 hours or overnight at -4° (Supplementary table 1). After washing in TBS buffer, the membrane was incubated with secondary antibodies: anti-Rabbit or anti-Mouse IRDye800CW (LI-COR, Lincoln, USA) at 1:5000 for 1.5 hours. For β-actin loading control, the membrane was incubated with mouse β-actin at 1:5000 followed by a secondary anti Mouse IRDye680CW antibody (LI-COR, Lincoln, USA). Target bands were visualized using an Odyssey infrared imaging system (LI-COR). Target bands were visualized using an Odyssey infrared imaging system (LI-COR). The relative densities of the bands were measured using Image Studio Lite software (version 5.0).

CSF analysis using ELISA

The frozen CSF samples were shipped to Johns Hopkins University, Baltimore, USA, for ELISA experiments. ANP, BNP and CNP were measured using commercial ELISAs following the manufacturer's protocol (ALPCO, Salem, NH, USA), except that CSF was concentrated prior to analysis by centifuguation/lyophilisation. For ANP and BNP, samples were reconstituted or diluted in diluent 35 (Mesoscale Diagnostics, Rockville, MD, USA) and for a subset of donors CSF was spiked with a mid-range NP standard and recovery was assessed. A sandwich

pro-ANP ELISA (1-98) was used to quantify ANP where CSF samples taken to dryness were resuspended in a volume to generate a 5-fold concentration. The sensitivity of the assay was 0.05 nmol/L and the dynamic range was 0.63 to 10 nmol/L. The amino terminal BNP fragment (nt-pro-BNP 8-29) was measured by competitive ELISA where CSF samples were also concentrated 5-fold. Under the conditions used in the laboratory, the sensitivity was 34 pmol/L and the dynamic range was between 40 and 1600 pmol/L. Determination of levels of amino-terminal pro-CNP was performed using a sandwich ELISA where CSF was diluted 1:30 with diluent 35 prior to analysis. The sensitivity was 0.7 pmol/L and the dynamic range was 0.1 to 128 pmol/L. When 9 CSF samples were spiked with 1.25 nmol/L pro-ANP, the recovery was 87 ± 9%. Nine samples spiked with 400 pmol/L pro-BNP yielded a recovery of 103 ± 5%, while for 9 samples spiked with 20 pmol/L pro-CNP the recovery was 93 ± 5%.

Statistical analysis

All data represent mean and standard deviations. Depending on the distribution of the outcome, unpaired two-tailed t-test or Mann-Whitney U test were used to compare between AD patients and controls. Statistical analyses were performed using SPSS software and a p-value <0.05 was considered as statistically significant.

Results

Gene expression of NP and NPR in the brain of healthy subjects

Figure 2 shows the mean expression of genes coding for NP and NPR across the brains of six healthy human donors from the Allen Human Brain Atlas. The NP and NPR genes were expressed throughout the CNS, although the different NP had distinctly diverse regional expression levels. In particular, the gene coding for ANP (*NPPA*) had the highest expression in cortical regions, followed by a lower expression in the telencephalic white matter regions (TEWM) and the lowest expression in the subcortical structures. In contrast, the gene coding

for BNP (*NPPB*) had the highest expression in subcortical structures (basal ganglia), followed by a lower expression in the TEWM and very low expression throughout the cortical regions. The gene coding for CNP (*NPPC*) had the highest expression in subcortical structures (thalamic nuclei), followed by a lower expression in the cortical regions and the lowest expression in TEWM. The gene coding for NPR-A (*NPR1*) had the highest expression in subcortical structures (basal ganglia and thalamic nuclei), followed by a lower expression in cortical regions and the lowest expression in the TEWM. The genes coding for NPR-B (*NPR2*) and NPR-C (*NPR3*) had the highest expression in subcortical structures followed with a very low expression in cortical and TEWM regions

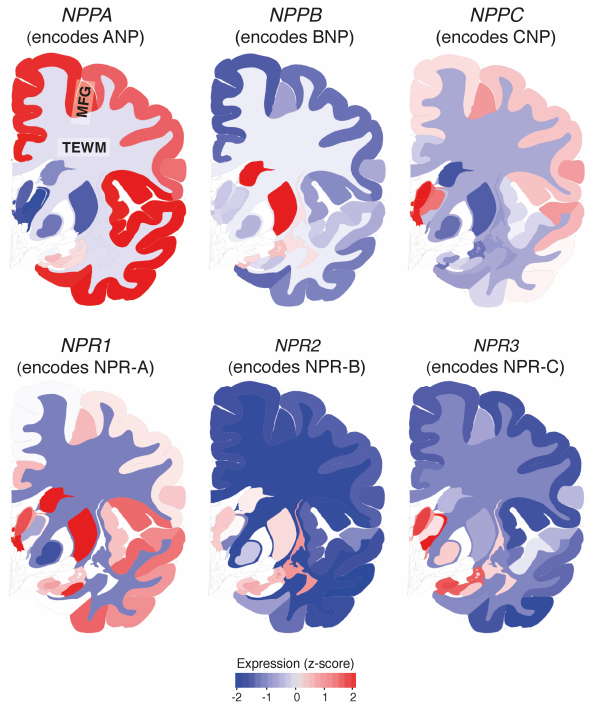


Figure 2. Expression of natriuretic peptides and natriuretic peptide receptors genes in the brain of healthy subjects. The mean mRNA expression of genes coding for natriuretic peptides and natriuretic peptide receptors is shown in a coronal view of the left hemisphere. The Expression of each gene is z-score normalized to the average expression of the gene in the whole brain. Data from the Allen Human Brain Atlas (http://human.brain-map.org/) is visualised using BrainScope (http://w ww.brainscope.nl/brainscope). MFG: middle frontal gyrus; TEWM: telencephalic white matter.

Localization of NP and NPR in the frontal lobe

In the cortex of control subjects, the immunohistochemistry study showed positive staining of all NP and NPR proteins in the neuronal structures. ANP, BNP and CNP positive stainings were observed in the cytoplasmic body and neuronal processes of pyramidal neurons (layers II-VI) (Figure 3A, 3B and 3C, respectively). BNP-positive staining was mainly localized to Nissl bodies that were characterized as granular bodies within the cytoplasm of neurons (Figure 3B). In addition, the CNP-positive staining was observed in networks of short and long fibers throughout the cortex (Supplementary Figure 4A). NPR-A, NPR-B and NPR-C positive stainings were also localized to the cytoplasmic body and neuronal processes of pyramidal neurons (layers II-VI) (Figure 5A, 5B and 5C, respectively). Furthermore, a strong NPR-A positive staining was observed in the Nissl body of neurons throughout the cortex. These Nissl bodies were characterized as large granular bodies in the cytoplasm with a stronger staining pattern compared to the cytosol of neurons (Supplementary Figure 4B). In addition to the cytoplasm, the NPR-B-positive neurons also showed a prominent staining in the nucleolus of neurons (Supplementary Figure 4C). We observed no staining in the negative control sections using only the secondary antibody (Supplementary Figure 1).

In the WM, we observed ANP-positive astrocytes-like structures that consisted of short packed processes (Figure 3D). These astrocytes-like structures were spread throughout the WM, occasionally associated with blood vessels and their cell bodies were not always distinct. Some ANP-positive astrocytes-like structures were also observed throughout the cortical layers. Similarly, we observed NPR-positive astrocyte-like staining in the WM of some control subjects (Figure 5D, 5E and 5F). We did not observe BNP or CNP-positive astrocyte-like structures in the WM or GM (Figure 3E and 3F). Other glial cells were not stained for NP or NPR.

In the cerebral vessels, ANP-positive staining was observed in the leptomeningeal and parenchymal vessels, and was localized to the endothelium and smooth muscle layers (Figure 3G). BNP-positive staining was only weakly present in the endothelium of some

leptomeningeal vessels (Figure 3H), while CNP staining was not detected in the cerebral vessels at all (Figure 3I). NPR-A and NPR-B stainings were positive in the endothelium and smooth muscle layers of leptomeningeal and parenchymal vessels (Figure 5G and 5H), while NPR-C staining was less prominent in the cerebral vessels (Figure 5I). Supplementary Figure 2 and 3 show the localization of the NP and NPR in the frontal lobe of AD patients. We observed a similar pattern of NP and NPR staining in the frontal lobe of AD patients and control subjects.

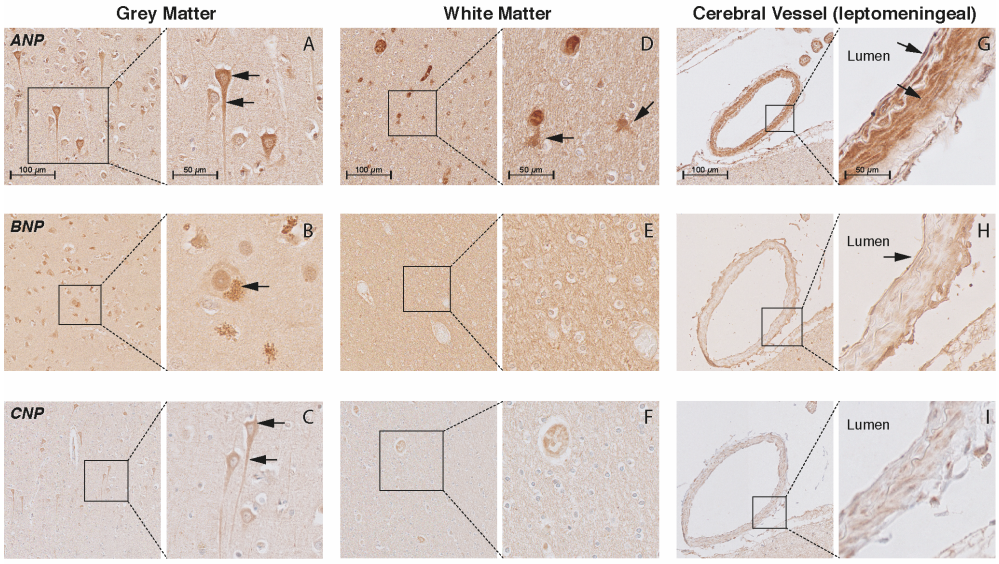


Figure 3. Localization of ANP, BNP and CNP in the frontal lobe of non-demented subject. NP immunohistochemistry in the frontal lobe (middle frontal gyrus) of non-demented control subjects. A, B and C) ANP, BNP and CNP-positive neurons. Arrows point to cytoplasm (ANP and CNP), neuronal processes (ANP and CNP) and Nissl bodies (BNP); D) ANP-positive astrocyte-like cells in the white matter; E and F) negative BNP and CNP staining in the white matter; G) ANP-positive endothelium and smooth muscles; H) BNP-positive endothelium and I) negative CNP staining in the leptomeningeal vessels.

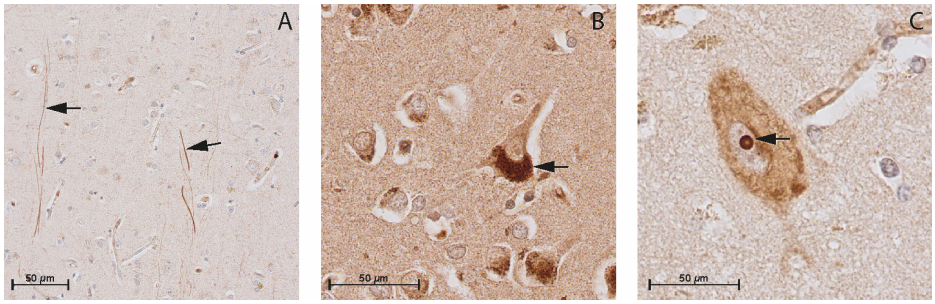


Figure 4. Immunohistochemistry of CNP, NPR-B and NPR-A in the grey matter. A) Immunohistochemistry of CNP in the grey matter. Arrows point to CNP-positive fibres in the grey matter; B) Immunohistochemistry of NPR-A in the grey matter. Arrow point to the granular bodies resembling Nissl bodies in the cytoplasm of neuron and C) Immunohistochemistry of NPR-B in the grey matter. Arrow point to the nucleolus of the neuron.

Quantitative comparison of NP and NPR between AD and control subjects

For quantitative comparisons in brain tissue, we performed Western blots using GM and WM samples isolated from each donor. Using an antibody against NPR-A, we observed specific bands of ~150 kDa in both AD patients and controls. The NPR-A specific bands were highly pronounced in the GM while no or very weak bands were present in the WM (Supplementary Figure 4). Bands reacting with anti-NPR-C antibody were observed at ~60 kDa in both AD patients and controls; and in GM and WM samples (Supplementary Figure 4). We were not able to detect any NPR-B specific signals despite the use of different antibodies. Figure 6 shows quantification of Western blot bands in the GM of AD patients and controls. We found significantly higher amounts of NPR-A in the GM of AD patients compared to controls ($t(16)= -2.18$, $p=0.045$, independent sample t-test). NPR-C signals appeared to be higher in the GM of controls, although the differences between AD patients and controls were not statistically different (Mann-Whitney $U=18.0$, $p=0.051$ two-tailed). In the WM, none of the Western blot bands was significantly different between AD patients and controls (data not shown).

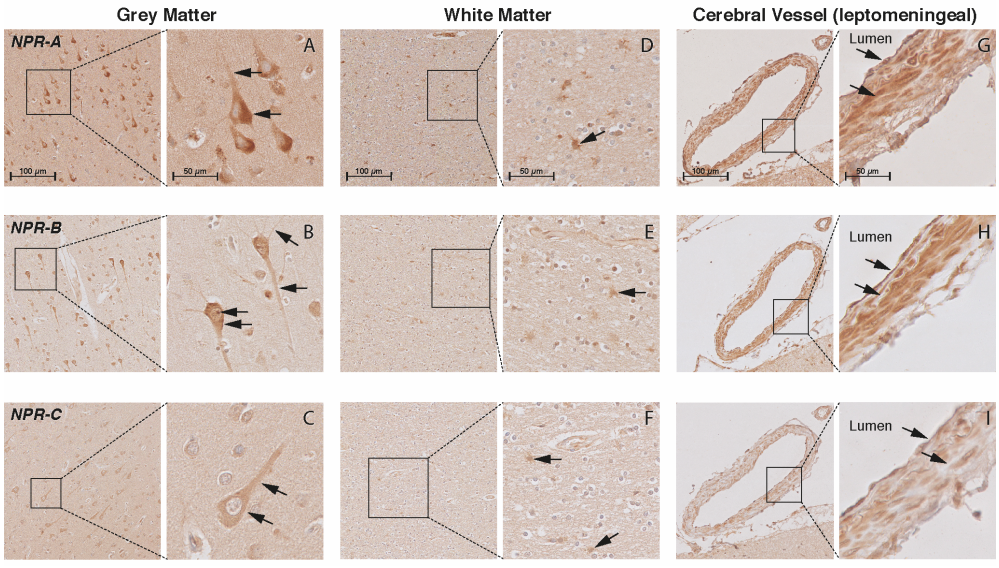


Figure 5. Localization of NPR-A, NPR-B and NPR-C in the frontal lobe of non-demented subject. NPR immunohistochemistry in the frontal lobe (middle frontal gyrus) of non-demented control subjects. A, B and C) NPR-A, NPR-B and NPR-C-positive neurons. Arrows point to cytoplasm (all NPR), neuronal processes (all NPR) and Nissl bodies (NPR-A); D, E and F) NPR-A, NPR-B and NPR-C-positive astrocyte-like cells in the white matter; G, H and I) NPR-A, NPR-B and NPR-C-positive endothelium and smooth muscles in leptomeningeal vessels.

When comparing between GM and WM, NPR-A-specific signals were significantly higher in GM compared to WM ($p < 0.001$), while NPR-C-specific signals were not different between GM and WM. Comparison of the gene expression data (Figure 2) with our Western blot findings (Figure 6) showed similar patterns: the gene coding for NPR-A was highly expressed in the cortex (expression z-score > 0) compared to the TEWM (expression z-score <-1). On the other hand, the gene coding for NPR-C had low mRNA expression in both GM and WM (expression z-score <0 in GM and WM).

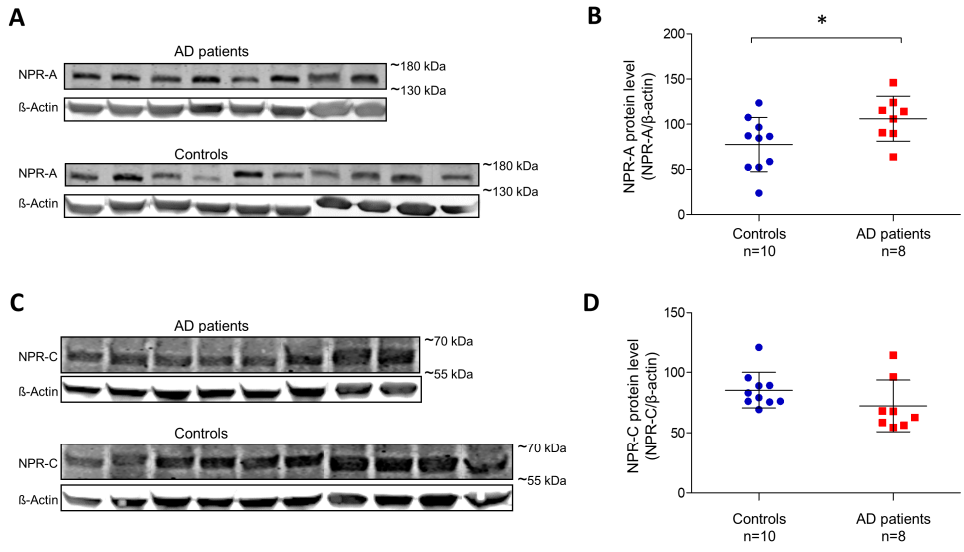


Figure 6. Relative amounts of NPR-A and NPR-C in the grey matter of non-demented controls and Alzheimer's disease patients. A) NPR-A and β-Actin Western blot bands in AD patients and non-demented controls; B) quantification of NPR-A Western blots between AD patients and controls; C) NPR-C and β-Actin Western blot bands in AD patients and non-demented controls; D) quantification of NPR-C Western blots between AD patients and controls. Bars show the mean (standard deviation) of the normalized signals. Analyses were performed using t-test for NPR-A and Mann Whitney U test for NPR-C. *show p-value <0.05. Abbreviations: NPR-A: natriuretic peptide receptor A; NPR-C: natriuretic peptide receptor C and AD: Alzheimer's disease.

Quantitative comparison of NP in the CSF between AD patients and controls

ANP, BNP and CNP levels in the CSF ranged from 56.48 to 192.36 pmol/L, 44.12 to 127.39 pmol/L and 125.25 to 1180.13 pmol/L, respectively. Figure 7 shows the comparison of ANP, BNP and CNP levels between AD patients and controls in post-mortem CSF. We observed significantly lower amounts of BNP in the CSF of AD patients compared to controls (Mann-Whitney U = 21.0, p=0.029 two-tailed). Similarly, ANP and CNP levels appeared to be lower in the CSF of AD patients although these differences were not statistically significant (Figure 7).

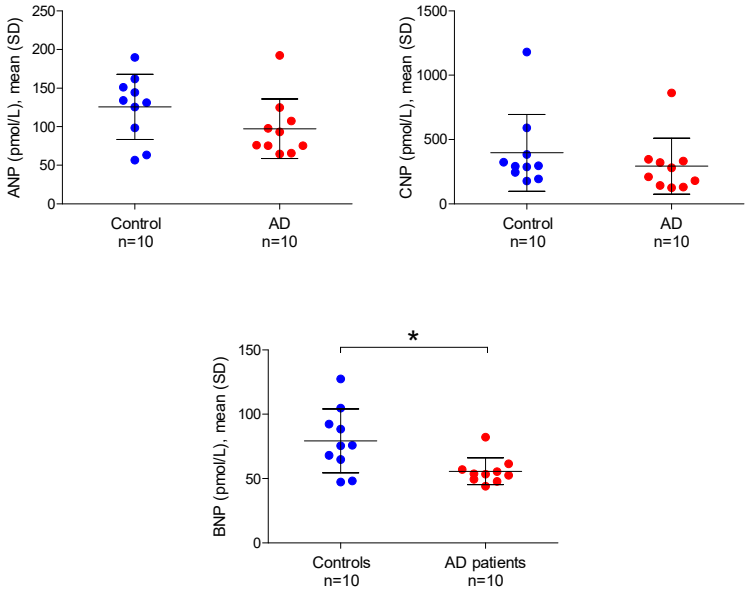


Figure 7. ANP, BNP and CNP in the post-mortem cerebrospinal fluid of non-demented controls and Alzheimer's disease patients. Bars represent the mean (standard deviation) of each NP in the cerebrospinal fluid of AD patients and controls. Analyses were performed using Mann Witney U test. *shows p-value <0.05. Abbreviations: CSF: cerebrospinal fluid; AD: Alzheimer's disease; ANP: atrial natriuretic peptide; BNP: brain natriuretic peptide; CNP: C-type natriuretic peptide and SD: standard deviation.

Discussion

In this study, we first showed that the genes coding for NP and NPR are ubiquitously expressed across the brain of healthy humans. We detected NP and NPR proteins in neuronal structures, cerebral vessels, and in structures resembling astrocytes in the frontal lobe of non-demented individuals and patients with AD. When comparing between AD patients and non-demented controls, NPR-A levels were higher in the brain tissue of AD patients while BNP levels were lower in the CSF of AD patients.

Previous animal studies have detected the expression and localization of NP and NPR in numerous structures of the CNS[13]. For example, ANP was detected in neuronal structures of the hypothalamus, telencephalon and cerebellum [5], and in the astrocytes of human and canine cortex[24, 25]. Similarly, BNP and CNP were found to be widely distributed in the neuronal structures of the cerebral cortex, hypothalamus, spinal cord and retina[5, 13]. Furthermore, the receptors for ANP and BNP were detected on the luminal membrane of cerebral vessels *in vitro* and *in vivo*[26, 27]. Despite the wealth of evidence from animal studies, limited data on the distribution and expression of NP and NPR in the brain of human subjects is available. In this study, we extended the evidence from animal species to human subjects by providing a detailed mapping of NP and NPR in the frontal lobe of human subjects. As in animal species, in humans the expression of NP and NPR genes seems to be complementary in various brain regions[28]. This suggest that the NP are not only produced in cardiac myocytes, but are also locally produced in the CNS of humans[5]. Furthermore, we found that NP and NPR are present in various cellular structures of the frontal lobe, suggesting a wide range of NP functions in the human brain. In particular, the presence of NP and NPR in both neuronal and cerebral vessels might indicate a potential role of NP in neurovascular functioning[29]. In line with this, recent *in vitro* studies have shown that administration of ANP and BNP resulted in a significant dose-dependent relaxation of the middle cerebral artery and basilar artery[30]. On the other hand, NP might also be involved in synaptic transmission and processing of information. In support of this, animal and *in vivo* studies have demonstrated that NP regulate the release and re-uptake of neurotransmitters such as noradrenalin, dopamine and glycine[13]. Furthermore, we could also detect ANP and NPR in structures resembling astrocytes in the WM. This is consistent with previous studies showing the presence of ANP and NPR in astrocyte of the cortex[24], suggesting a potential role of astrocytes in physiological functions of NP in the brain[13]. Collectively, our qualitative and gene expression data are consistent with previous animal studies indicating NP as neuropeptides that might regulate several functions in the brain[31]. Nevertheless, future research is needed to further disentangle the putative role(s) of centrally acting NP in the brain of human subjects.

Given the involvement of NP in several of the pathological pathways that are also disturbed during the course of AD, we have recently hypothesized NP as potential markers for diagnosis and/or treatment of AD[13]. We postulated that decreased action of NP in the brain might impair the structural and/or functional integrity of the brain and predispose subjects to a higher risk of cognitive decline[13]. In line with this hypothesis, we found lower levels of BNP in the CSF of AD patients, coupled with higher amounts of NPR-A in the brain tissue of AD patients. This may suggest impaired function of NP in the brain of AD patients, which could in turn accelerate neuro-inflammation, oxidative stress and neurodegeneration. One explanation for reduced levels of BNP in the CSF could be attributed to their elevated levels in the systemic circulation. In fact, systemic and central NP might act in a feedback loop such that increased NP in the plasma inhibits production and/or biological activity of NP in the brain[13, 32]. In line with this, previous research has shown that higher plasma BNP in ovine sheep associated with decreased BNP levels in the hypothalamus[32]. Interestingly, recent findings in humans suggest that higher plasma BNP associates with lower CSF Aβ42 and higher t-tau/Aβ42 ratios[18]. Collectively, our pilot results point towards a potential role of NP in the pathology of AD. Nevertheless, larger scale studies are needed to replicate our findings and explore the potential association of centrally acting NP with markers of neurodegeneration.

We used frontal lobe to detect NP and NPR in the brain tissue of AD patients and non-demented controls. Frontal lobe involvement is a well-described feature of AD pathology [33-35]. Furthermore, atrophy of the middle frontal gyrus has been demonstrated in patients with mild cognitive impairment[36]. It is worth mentioning that previous research have detected NP and NPR in other brain regions such as thalamus and hypothalamus[28]. In animal models, the highest concentration of NP and NPR were reported in various nuclei of hypothalamus[28]. Consistent with this, we observed high expression of NP and NPR in the subcortical structures and hypothalamus of healthy humans. In this study, future studies focusing on other brain regions are needed to further disentangle the localization and function of NP in different brain regions of humans.

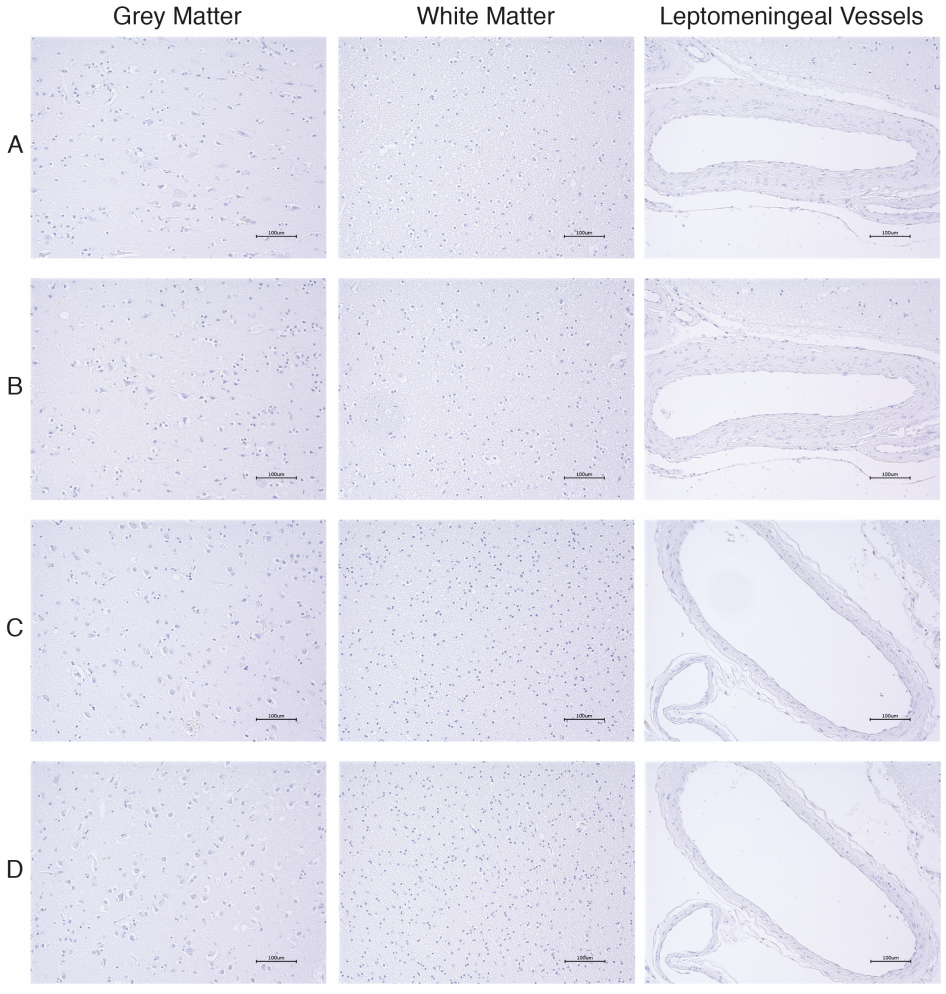
In summary, we showed the widespread presence of all NP and their receptors in the brain of humans. In line with our hypothesis, we observed higher amounts of NPR-A in the brain tissue and lower levels of BNP in the CSF of AD patients. These findings further highlight that NP may be potential markers for AD. Given the widespread distribution of NP in different structures of the human brain, future research should determine the specific function of NP in the brain of humans, in health and disease.

Supplementary Material

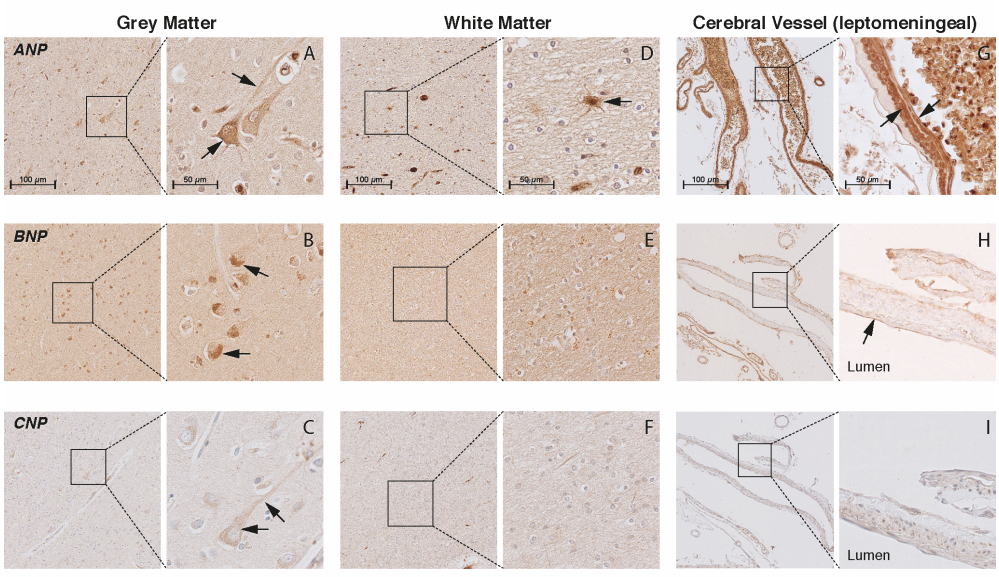
Supplementary Table 1. List of antibodies in this study

ID	Company	Description	Host	Antigen recognized	AR
ab14356	Abcam	Anti-NPRA	Rb (pc)	aa 294-308 of h-NPRA	+
ab55724	Abcam	Anti-NPRB	Ms (mc)	aa 131-231 of h-NPRB	+
ab37617	Abcam	Anti-NPRC	Rb (pc)	aa 67-97 (N terminal) h-NPRC	+
ab19646	Abcam	Anti-BNP	Rb (pc)	h-BNP	+
ab91250	Abcam	Anti-ANP	Rb (pc)	aa 30-56 of h-ANP	–
HPA035362	Sigma	Anti-NPPC	Rb (pc)	aa 29-126 of h-CNP	+

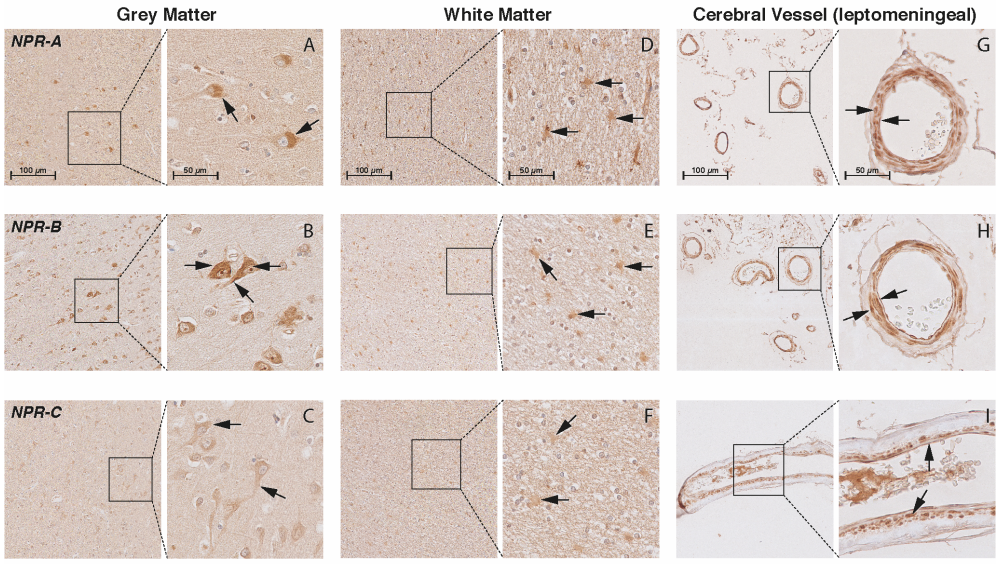
Abbreviations: Rb: rabbit; Ms: mouse; pc: polyclonal; mc: monoclonal; AR: antigen retrieval; h: human and aa: amino acid.



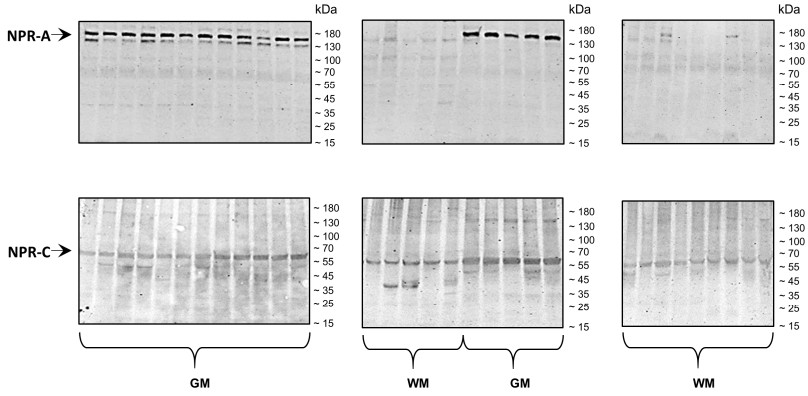
Supplementary Figure 2. Negative controls treated only with secondary antibodies. A) Frontal cortex of a control subject incubated only with secondary goat anti rabbit antibody and B) secondary rabbit anti mouse antibody. C) Frontal cortex of an AD patient incubated only with secondary goat anti rabbit antibody and D) secondary rabbit anti mouse antibody



Supplementary Figure 2. Localization of ANP, BNP and CNP in the frontal lobe of AD subjects. NP immunohistochemistry in the frontal lobe (middle frontal gyrus) of Alzheimer's disease patients. A, B and C) ANP, BNP and CNP-positive neurons. Arrows point to cytoplasm (ANP and CNP), neuronal processes (ANP, and CNP) and Nissle bodies (BNP); D) ANP-positive astrocyte-like cells in the white matter; E and F) negative BNP and CNP staining in the white matter; G) ANP-positive endothelium and smooth muscles; H) BNP-positive endothelium and I) negative CNP staining in the leptomeningeal vessels.



Supplementary Figure 3. Localization of NPR-A, NPR-B and NPR-C in the frontal lobe of AD subjects. NPR immunohistochemistry in the frontal lobe (middle frontal gyrus) of Alzheimer's disease patients. A, B and C) NPR-A, NPR-B and NPR-C-positive neurons. Arrows point to cytoplasm (all NPR), neuronal processes (all NPR) and Nissl bodies (NPR-A); D, E and F) NPR-A, NPR-B and NPR-C-positive astrocyte-like cells in the white matter; G, H and I) NPR-A, NPR-B and NPR-C-positive endothelium and smooth muscles in leptomeningeal vessels



Supplementary Figure 4. Western blots of natriuretic peptide receptors. Each subject has one band corresponding to grey matter and one band corresponding to white matter. All images were scanned using the Image Studio software with the same resolution and image quality. Abbreviations: NPR-A: natriuretic peptide receptor A; NPR-C: natriuretic peptide receptor C; GM: grey matter and WM: white matter.

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