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Resolution of inflammation is altered in Alzheimer's disease

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Abstract

Background—Resolution is the final stage of the inflammatory response, when restoration of tissue occurs. Failure may lead to chronic neuroinflammation, known as part of pathology in Alzheimer's disease (AD) brain.

Methods—Specialized pro-resolving mediators (SPMs), receptors, biosynthetic enzyme, and downstream effectors involved in resolution were analyzed in *post mortem* hippocampal tissue from AD-patients and non-AD subjects. SPMs were analyzed in cerebrospinal fluid (CSF).

Results—Presence of SPMs and SPM receptors was demonstrated in the human brain. Levels of the SPM lipoxin A₄ (LXA₄) were reduced in AD - both in CSF and hippocampus. An enzyme involved in LXA₄ synthesis and two SPM receptors were elevated in AD brains. LXA₄ and RvD1 levels in CSF correlated to mini-mental state examination (MMSE) scores.

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Conflict of Interest Disclosure

C.N.S.'s interests were reviewed and are managed by the Brigham and Women's Hospital and Partners Health Care in accordance with their conflict of interest policies.

Conclusions—The resolution pathway exists in the brain and described alterations strongly suggest its dysfunction in AD. Correlations with MMSE suggest a connection with cognitive function in AD.

Keywords

ChemR23; ELISA; FPRL1; human; immunohistochemistry; lipoxin A₄; LXA₄R; 15-lipoxygenase-2; mild cognitive impairment; resolvin D1; specialized pro-resolving mediators; tau

1. Introduction

Alzheimer's disease (AD) is the most common type of dementia and the number of AD-patients is growing fast worldwide. Pathological hallmarks in AD are the senile plaques, mainly composed of extracellular amyloid beta (A β), and neurofibrillary tangles (NFTs), consisting of intraneuronal hyperphosphorylated tau [1]. Direct evidence of inflammation in the AD brain has been provided by *in vivo* positron emission tomography (PET) studies, showing increased activation of microglia in AD-patients [2]. Moreover, levels of pro-inflammatory cytokines are elevated in *post mortem* brain tissue [3] and serum from AD-patients [4]. *In vitro* studies demonstrate that A β can activate the innate immune cells in the brain [5,6]. Epidemiological studies suggest a neuroprotective role of non-steroidal antiinflammatory drugs (NSAIDs) [7], although prospective clinical trials have generally been ineffective, suggesting a more complex role of inflammation in AD [7].

Inflammation is normally terminated by resolution, with the purpose to promote the healing and return to homeostasis. Resolution results in reduced numbers of immune cells at the site of insult by decreased infiltration and apoptosis, and clearance of apoptotic cells and debris by increased phagocytic activity [8,9]. The resolution is an active process mediated by fatty acid (FA) derivatives named specialized pro-resolving mediators (SPMs). The lipoxin family of SPMs (LXs) was the first uncovered [10], and subsequently characterized [11–13]. Novel SPMs that have since been discovered include the eicosapentaenoic acid (EPA)-derived resolvin E (RvE) series, docosahexaenoic acid (DHA)-derived resolvin D (RvD) series, the protectins that include (PD1)/neuroprotectin D (NPD1), and maresins (MaRs) [8]. The biosynthetic pathways of SPMs can involve oxygenation of their precursors by lipoxygenases (LOXs) or cyclooxygenases [14]. Multi-ligand receptors that recognize SPMs have been discovered [15–19], and upon binding of the SPMs to their receptors, resolution is initiated [20]. However, few studies have hitherto addressed the subject of resolution in the brain. Neuroprotective effects have been shown for NPD1 [21] and LXA₄ [22], and reduced levels of NPD1 were described in AD brains [21]. LXA₄ can also reduce reactive oxygen species (ROS) in activated microglia [23], and inhibit interleukin (IL)-8 expression in astrocytoma cells [24], as well as reduce A β levels and improve cognition in a transgenic mouse model for AD [25]. RvD1 and RvE1 attenuate inflammation-associated pain in mice *via* central and peripheral action [26].

The chronic neuroinflammation in the AD brain indicates that the resolution of inflammation is dysfunctional. To investigate this, we have analyzed *post mortem* brain

tissue and cerebrospinal fluid (CSF) samples from AD-patients and controls with regard to production and transmission of pro-resolving signals.

2. Methods

2.1 Subjects

Human CSF samples were obtained from 15 AD (according to ICD-10 criteria [27], mean age \pm SD = 67.87 ± 10.232), 20 mild cognitive impairment (MCI according to Winblad criteria [28], mean age \pm SD = 65.65 ± 10.373) and 21 subjective cognitive impairment (SCI, no objective cognitive impairment, mean age \pm SD = 57.48 ± 5.409) subjects from the Memory Unit, Geriatric Clinic, Karolinska University Hospital, Huddinge. As part of the diagnostic procedure these subjects were assessed by the mini-mental state examination (MMSE) test, and levels of phosphorylated tau (phosphorylation site threonine 181) was measured by enzyme-linked immunosorbent assay (ELISA) kits (INNOTEST®, Innogenetics, Ghent, Belgium). Brain tissue samples were obtained from 10 AD-patients (9 Braak stage 5–6 definite AD [29], and 1 Braak stage 3–4 probable AD), and 10 non-demented control subjects, all from the Brain Bank at Karolinska Institutet. There was no statistical difference in age or *post mortem* interval (PMI) between the AD and control group (see Table 1). Half of each brain was fixed in formalin and tissue samples embedded in paraffin. The other half was dissected according to region, frozen and stored at -80°C . The study has been approved by the regional ethics committee of Stockholm.

2.2 Tissue processing

Paraffin-embedded tissue blocks of the hippocampus were sectioned into $6\ \mu\text{m}$ thick sections and mounted onto polarized glass slides for morphological analysis. Hippocampal tissues used for biochemical analysis were collected in the coronal plane at the level of lateral geniculate nucleus, which contains the entire hippocampus regions (Cornu Ammonis (CA) 1–4) and dentate gyrus (DG). Prior to analysis, each of the frozen tissue pieces was pulverized with a BioPulverizer (BioSpec Product Inc.), mixed well, divided, and kept at -80°C until further processing.

2.3 Enzyme immunoassay (EIA)

Free FAs (FFAs) were extracted from hippocampal tissue and CSF samples according to the supplier's instructions provided with the LXA₄ EIA assay (Oxford Biomedical research). The brain tissues were collected during a period of 4 years, and stored at -80°C for a similar time period until further processing. The CSF samples were collected during a period of 3 years, and stored at -80°C until further processing. For tissue extraction, hippocampal samples were homogenized in ethanol by a pestle homogenizer, followed by centrifugation at $2700 \times g$ for 15 min. The supernatant was collected and acidified to pH 3.5. These samples as well as acidified CSF samples were extracted through Sep-Pak columns (Waters). Briefly, the columns were equilibrated with methanol and ddH₂O, and acidified samples were loaded immediately. Bound FFAs were eluted with methyl formate, taken to dryness by nitrogen gas, and finally re-suspended by extraction buffer supplied with the LXA₄ EIA kit. This kit was used for analysis of LXA₄, and RvD1 was measured with the

RvD1 EIA kit (Cayman Chemical), following the manufacturer's instructions. Total FFAs were measured by a FFA assay kit (Cayman Chemical).

2.4 Liquid chromatography-tandem mass spectrometry (LC-MS-MS)

Three controls and 3 AD tissues were selected at random from the cohort of samples to carry out full lipid mediator (LM) profiling using LC-MS-MS based lipidomics in order to validate identified SPMs and their presence, as well as the difference between AD and controls, for the LM biosynthetic profiles and SPMs used in further analyses employing specific EIAs. Hippocampal tissue samples were gently homogenized with a glass dounce in methanol, followed by the addition of deuterium-labeled internal standards d_8 -5Hydroxyicosatetraenoic acid (HETE), d_4 -leukotriene B₄ (d_4 -LTB₄), d_5 -LXA₄, and d_4 -prostaglandin E₂ (PGE₂) (500 pg each, Cayman Chemical) to facilitate quantification. Protein precipitation was allowed for 1 h. Samples were centrifuged for 10 min at $850 \times g$ and supernatant taken for C18 (Agilent) extraction as described before [30]. Briefly, using a RapidTrace Automated SPE Workstation (Biotage), columns were equilibrated with methanol and ddH₂O. Sample supernatants were diluted with ddH₂O, acidified (pH ~ 3.5) and immediately loaded onto the C18 column. Columns were washed with ddH₂O and hexane. Products were eluted with methyl formate and taken to dryness under nitrogen. These were then suspended in 50 μ L methanol.

The LC-MS-MS system, QTrap 5500 (ABSciex) was equipped with a Shimadzu LC-20AD binary pump. An Agilent Eclipse Plus C18 column was used with a gradient of methanol/water/acetic acid of 60:40:0.01 (vol:vol:vol) to 100:0:0.01 at 0.5 mL/min flow-rate. To monitor and quantify LMs, a multiple reaction monitoring (MRM) method was developed with signature ion fragments for each molecule. Identification was conducted using published criteria[30] that included retention time and at least 6 diagnostic fragments. Calibration curves were obtained using synthetic and authentic LM mixtures (d_8 -5S-HETE, d_4 -LTB₄, d_5 -LXA₄, d_4 -PGE₂, MaR1, LXA₄, 5,15-diHETE, LTB₄, PGD₂, PGE₂, PGF_{2 α} , thromboxane (TX) B₂, 5-HETE, 12-HETE, 15-HETE) at 2, 10, 80 and 200 pg. Linear calibration curves for each were obtained with r^2 values of 0.98–0.99.

2.5 Cytokine analyses

Frozen hippocampal samples were homogenized as described previously [31]. Briefly, the pulverized tissue was lysed by sonication in homogenization buffer. The homogenates were then centrifuged at $20000 \times g$ for 20 min, and the supernatants collected and stored at -80°C until usage. Human pro-inflammatory 7-plex assay kit (MesoScale Discovery) was used to analyze interferon (IFN)- γ , IL-1 β , IL-12p70, IL-6, IL-8, IL-10 and tumor necrosis factor (TNF)- α , following the manufacturer's instructions.

2.6 Western blot (WB)

The tissue homogenates used for cytokine analysis were also employed for WB analysis, which followed the procedure described previously [31]. Briefly, total amounts of 40 μ g protein per sample were separated on 10% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes (Bio-Rad Laboratories). After blocking, membranes were incubated over night at 4°C with primary antibodies, including LXA₄ receptor (LXA₄R,

1:500, Acris Antibodies), chemokine receptor 23 (ChemR23, 1:200, Cayman Chemical), peroxisome proliferator-activated receptor gamma (PPAR- γ , 1:20, Cayman Chemical), 15-LOX-2 (1:1000, Cayman Chemical), P-tau (clone AT8, 1:1000, Innogenetics), total tau (T-tau, 1:3000, Abcam) and β -actin (1:1000, Cell Signaling). After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare), for 2 h at room temperature (RT). The blots were developed with ECL Prime (GE Healthcare), and visualized using a CCD camera (LAS-3000, Fuji Film). Density of bands was analyzed with Multi Gauge (V 3.0) software (Fuji Film). All samples were run in one blot for each marker and β -actin was used as a loading standard. The level of each marker protein was normalized to β -actin levels in the respective blot.

2.7 Immunohistochemistry

Sections of paraffin-embedded hippocampal tissues were deparaffinized. Endogenous peroxidase was blocked by 1% hydrogen peroxide (H_2O_2). The sections were blocked with 5% normal donkey serum for 30 min at RT, followed by incubation over night at 4°C with primary antibodies, including LXA₄R (1:200, MBL International), ChemR23 (1:300, Cayman Chemical) and 15-LOX-2 (1:200, Cayman Chemical). After washing, biotin-conjugated secondary antibodies (Jackson ImmunoResearch) were applied to the sections for 1.5 h at RT. After washing, the sections were incubated for 30 min at RT with streptavidin-HRP complex (ABC Vectastain kit, Vector Laboratories). The staining was visualized by using 1 mg/ml diaminobenzidine (DAB) solution containing 0.02% H_2O_2 . The sections were then counterstained with cresyl violet, dehydrated and mounted.

Deparaffinized sections were also used for double immunolabeling. For staining with human leukocyte antigen type DR (HLA-DR, Dako), the sections were autoclaved in sodium citrate buffer (pH 6.0) at 120°C for 20 min for antigen retrieval. Endogenous peroxidase and alkaline phosphatase (AP) were blocked by BLOXALL (Vector Laboratories). After washing and serum blocking, sections were incubated over night at 4°C with LXA₄R, ChemR23 or 15-LOX-2 antibodies, mixed with antibodies against glial fibrillary acid protein (GFAP, 1:600, Life Technologies) or HLA-DR (1:25). After washing, the sections were incubated for 1.5 h at RT with biotin-conjugated secondary antibodies mixed with AP-conjugated secondary antibodies (Jackson ImmunoResearch). After washing, the sections were incubated with streptavidin-HRP complex for 30 min at RT. Biotin-peroxidase staining and AP-staining were visualized by Nova Red and Vector Blue, respectively (Vector Laboratories). Stained sections were dehydrated and mounted, and analyzed under a Nikon Eclipse E800 microscope.

2.8 Statistics

Analysis of covariance (ANCOVA) followed by Bonferroni test using age as a covariate, was performed on data from CSF samples, as the age-distribution was different among the groups. Data from EIA and WB on hippocampal samples did not follow a normal distribution and were analyzed by non-parametric Mann-Whitney U test. ELISA data from hippocampal samples were analyzed by Mann-Whitney U test or student t-test if following a normal distribution. For correlation analysis, the Spearman's rho test was applied. $P < 0.05$

was considered as statistically significant. All statistical analyses were performed in SPSS software.

3. Results

3.1 Lower levels of LXA₄ in CSF from AD-patients

CSF samples were collected from AD, MCI, and SCI subjects, and analyzed for levels of the two SPMs LXA₄ and RvD1. We here demonstrate measurable levels of these two SPMs in human CSF samples by EIA method, and that the levels of LXA₄ in AD-patients were significantly lower than in the MCI and SCI groups (Fig. 1A). There was no statistical difference between the MCI and SCI groups, even though levels in the MCI group tended to be lower. The levels of RvD1 did not differ significantly between any of the groups (Fig. 1B), although it was noticeable that variability between samples was much greater in MCI and SCI groups than in the AD group. The levels of LXA₄ and RvD1 in CSF were highly correlated with each other (Fig. 1C).

To investigate a potential relevance of the pro-resolving pathway for cognitive function we analyzed the possible correlations between LXA₄ and RvD1 levels in CSF and the scores obtained from the MMSE test. A positive correlation was found between MMSE and LXA₄, as well as between MMSE and RvD1 (Fig. 1D–E), when analyzing SCI, MCI and AD-patients together. A negative correlation was found between levels of P-tau and MMSE (Supplementary Fig. 1A).

3.2 Markers of resolution and inflammation in human hippocampal tissue

3.2.1 Lower LXA₄ levels in AD—In accordance with the data on CSF, the tissue levels of LXA₄ in the hippocampus were significantly lower in AD cases (n = 7) than in non-demented controls (n = 7) (Fig. 1F), whereas no significant difference was observed between the groups with regard to levels of RvD1 (Fig. 1G). In order to investigate possible relationship between resolution and AD pathology, we analyzed the hippocampal levels of T-tau and P-tau to confirm severity of disease, as tau-pathology is a highly relevant biomarker for severity of AD [32,33]. WB analysis showed that the levels of P-tau (Clone AT8) were markedly higher in tissue from AD subjects (Supplementary Fig. 1B) (in agreement with earlier studies [34], as well as the T-tau levels and the P/T-tau ratio (Supplementary Fig. 1C–D).

Based on the above findings, LC-MS-MS was performed on *post mortem* tissue from a subgroup of the cases (3 AD and 3 control cases) for further analysis of LMs. In support of the EIA results, the levels of LXA₄ were higher in control cases, and below the detectable limit in the AD cases (Supplementary Fig. 2). Similarly, the levels of MaR1 were higher in controls, while below limits of detection in the AD cases (Supplementary Fig. 2). This is the first identification of both LXA₄ and MaR1 in the human brain, as well as for LTB₄, which were also lower in AD than in control subjects (Supplementary Fig. 2). Levels of prostaglandins were higher in AD cases than in the control cases (Supplementary Fig. 2), supporting a pro-inflammatory state in the AD cases examined here.

3.2.2 Higher SPM receptor levels in AD—To investigate SPM receptors in the human AD and control brain we performed immunohistochemical studies and WB analysis of LXA₄R (alternative name formyl peptide receptor-like 1, FPRL1), that recognizes LXA₄ [15,16] and RvD1 [17,18], and ChemR23, recognizing RvE1 [19]. Immunohistochemical analysis was performed on paraffin-embedded hippocampal sections. We found the labeling for LXA₄R in both glia and neurons, with a markedly stronger labeling of the glia in AD compared to control brains (Fig. 2A–B). This higher level of LXA₄R staining of glial cells in AD was most evident in CA2–4 and the subgranular zone of DG. LXA₄R-labeled pyramidal neurons were observed in CA2–4, but not in CA1. Occasionally, a very weak staining could be seen in granular cells in DG in a few cases. Double immunostaining with GFAP (astrocyte marker) and HLA-DR (MHC class II marker for activated microglia), revealed the occurrence of LXA₄R in both astrocytes and microglia, respectively (Fig. 2C). WB analysis of LXA₄R in hippocampal tissue (including all of the subjects analyzed by immunohistochemistry), revealed no statistically significant difference between AD (n = 7) and controls (n = 9) (Fig. 2D), despite a marked increase in immunostaining in individual glial cells. There was no significant correlation between LXA₄R and P-tau, T-tau or P/T-tau ratio. Neither LXA₄ nor RvD1 levels were correlated with LXA₄R.

ChemR23 was detected in both neurons and glia in the human hippocampus, with a strong increase in staining intensity in AD (Fig. 3A–B). Labeling for ChemR23 in pyramidal neurons in the CA areas exhibited a weak intensity in CA1, and notably stronger in the CA2–4 areas. The higher levels of ChemR23 immunostaining in AD brains could be seen in the majority of CA2–4 neurons, and in some neurons in the CA1 (Fig. 3A). There was also labeling for ChemR23 in granular cells in the DG (Fig. 3A). Similarly to LXA₄R, ChemR23 immunostaining was also observed in both astrocytes and microglia (Fig. 3B–C), predominantly in CA2–4 and the subgranular zone of DG (Fig. 3A). A marked increase in number and staining intensity for ChemR23 in glia was seen in most AD cases, as compared to control subjects (Fig. 3A). WB analysis of hippocampal tissue showed significantly higher levels of ChemR23 in AD, confirming the immunohistochemical findings (Fig. 3D).

3.2.3 Downstream effectors in resolution—In view of data suggesting involvement of PPAR- γ in neuroprotective effects of SPMs [22,35], the levels of this nuclear receptor/transcription factor were analyzed in the present materials. Markedly higher levels were observed in AD as compared to the controls (Fig. 4A).

As SPMs can regulate cytokine levels, we investigated whether the levels of SPMs correlated with levels of cytokines in the hippocampal tissue samples. Data from the MesoScale assay of pro- and anti-inflammatory cytokines showed significantly lower levels of IL-10 in AD than in control subjects (Fig. 4B), whereas no statistically significant difference was observed for the other cytokines IFN- γ , IL-1 β , IL-6, IL-8, IL-12 and TNF- α (data not shown).

3.2.4 Higher levels of enzyme for LXA₄ biosynthesis in AD—15-LOX-2 is an enzyme that is mainly involved in the biosynthesis of LXA₄ [36]. The immunohistochemical analysis of hippocampal tissue revealed labeling for 15-LOX-2 in glial cells (Fig. 5A), but not in neurons. The 15-LOX-2-labeled glia were predominantly seen in the CA2–4 and the

subgranular zone of DG, with markedly fewer immunopositive cells observed in the CA1 (data not shown). Double immunolabeling with GFAP and HLA-DR, respectively, confirmed the localization of 15-LOX-2 in both astrocytes and microglia (Fig. 5C). Compared to controls, the AD cases displayed a higher number and more intensely stained 15-LOX-2-positive glia in CA areas as well as the DG (Fig. 5A). WB analysis also showed higher levels of 15-LOX-2 in AD compared to controls (Fig. 5D).

4. Discussion

By investigating mediators, their precursors and intermediaries, receptors and biosynthetic enzymes, we show that the pathway of resolution exists in the human CNS. We also show that it is altered in the human brain afflicted by AD. The levels of LXA₄ in AD were lower in both *post mortem* hippocampal tissue and CSF. Results from LC-MS-MS analysis of a small sub-group of the cases confirmed lower levels of LXA₄ in the hippocampus of AD cases, and also showed lower levels of MaR1 in AD. Ongoing studies in our laboratory suggest AD-related alterations also in the entorhinal cortex. The importance of the resolution pathway in maintaining normal cognition is suggested by the finding of a positive correlation between MMSE and the levels of both LXA₄ and RvD1 in the CSF. Our data may suggest that resolution can inhibit AD-related cognitive decline, perhaps by interfering with tau-phosphorylation, shown to correlate negatively with MMSE. Increased levels of glial LXA₄R in the AD brain shown in the present study, conceivably result from the pro-inflammatory environment, since inflammation drives expression of LXA₄R [37], which would make the tissue more responsive to pro-resolving signaling. However, LXA₄R also has pro-inflammatory ligands, including A β [38], which together with the decreased levels of LXA₄ shown here, could maintain or exaggerate the alterations in the resolution pathway. Furthermore, we found dramatically increased levels of PPAR- γ in the AD hippocampus, also demonstrated in the temporal cortex [39]. According to the medical journal records, the subjects included in the present studies on the *post mortem* brain tissues, did not receive PPAR- γ agonist medication, indicating that the increased hippocampal levels in AD have an alternative explanation. Interestingly, it has been reported that levels of LXA₄ can be increased by the PPAR- γ agonist rosiglitazone, and that neuroprotective effects of LXA₄ in a stroke model are blocked by a PPAR- γ inhibitor [22]. Combining these data with our results may hypothetically suggest that the increased levels of PPAR- γ in AD hippocampus are a compensatory reaction to the decreased levels of LXA₄.

Previously, an imbalance in the serum levels of IL-10 in relation to pro-inflammatory cytokines has been reported in AD [40], and there is evidence for an association between a polymorphism in the *IL10* promoter and AD [41]. We now show that a deficiency in the levels of IL-10 is present in the human hippocampal region of the AD brain. Functional resolution is characterized by reduced levels of pro-inflammatory cytokines and increased anti-inflammatory cytokines (see [8]). Therefore we hypothesize that although the analysis of pro-inflammatory cytokines did not reveal a significant increase in the AD samples, the decrease in IL-10 levels suggests failure in the resolution of inflammation. Whether or not the decreased levels of IL-10 in AD hippocampus are due to the decrease in LXA₄ needs further investigation, but an LXA₄ analogue has been shown to enhance the levels of anti-inflammatory mediators, including IL-10 [42]. IL-10 is known for its protective effects on

neurons [43], and lower levels may contribute to the pathology by the absence of an inhibitory signal on inflammation, as well as by deprivation of neuroprotection.

The decreased levels of LXA₄ in AD do not seem to be due to lower levels of 15-LOX-2, since this enzyme was increased in the AD hippocampus. In addition, other enzymes involved in the biosynthesis of SPMs, 15-LOX-1 and 5-LOX, have been reported to be elevated in AD hippocampus [44,45]. It is well known that these enzymes produce inflammatory FAs [46], and production of SPMs by these enzymes is dependent on so called “class-switching” for SPMs to be produced [47]. The increased levels of enzymes may represent pro-inflammatory activation, accompanied by a lack of class-switching towards a resolution state.

Our results show the presence of LXA₄R and ChemR23 proteins in pyramidal cells neurons in different regions of the hippocampus, suggesting that SPMs could bind to these neurons and potentially transduce beneficial resolution signals. There was no LXA₄R and little ChemR23 staining observed in the CA1 pyramidal cells (compared to CA2–4), suggesting differential responsiveness to SPMs. We also show the occurrence of these receptors on both astrocytes and microglia. A previous study indicated the transcription of the LXA₄R gene in microglia by *in situ* hybridization histochemistry [38]. The increased levels of LXA₄R and ChemR23 in AD may indicate a compensatory reaction to decreased levels of SPMs, as in the case of decreased LXA₄/increased LXA₄R. It is known that Aβ can bind to LXA₄R [38], and the pro-inflammatory chemerin is a ligand for ChemR23 [48], and hypothetically Aβ and chemerin may compete with binding of LXA₄ or RvE1. In situations with reduced levels of SPMs and higher levels of Aβ and chemerin, ChemR23 and LXA₄R may mediate direct neurotoxic effects due to their neuronal expression.

To summarize, this study demonstrates that the resolution pathway exists in the human brain in terms of mediators, receptors and biosynthetic enzymes, with a differential cellular distribution. We also demonstrate measurable levels of SPMs in human CSF. Similarly to the periphery, the brain thus seems to have the capacity to resolve inflammation, although differences are suggested by the different cellular landscape in the brain. Furthermore, we show that this pathway is dysregulated in AD, in parallel with cognitive dysfunction, which may provide researchers and clinicians with new diagnostic markers. Thus, the current data suggest that measurements of SPMs in CSF have the potential to be utilized for diagnostic purposes in AD. Combining data on SPMs with the well validated CSF markers Aβ and P/Ttau [49], may aid in detection and prediction of disease progression, and perhaps in identifying subtypes of the disease, all of which that can be of value in the design and utilization of personalized treatments. It should be noted that most neurodegenerative disorders are characterised by an inflammatory process in association with the neurodegeneration, and it is therefore not unlikely that *e.g.* Parkinson’s disease may also be associated with a dysfunctional resolution, and possibly result in altered levels of SPMs in the CSF samples. Importantly, treatment with SPMs, or in other ways stimulating the resolution pathway, is suggested as a new promising therapy in neurodegenerative disorders, such as AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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C.N.S. is an inventor on patents [resolvins] assigned to BWH and licensed to Resolvix Pharmaceuticals. C.N.S. was scientific founder of Resolvix Pharmaceuticals and owns equity in the company.

Abbreviations

| | |
|-----------------------------------|------------------------------------------------|
| Aβ | amyloid beta |
| AD | Alzheimer's disease |
| ANCOVA | Analysis of covariance |
| CA | Cornu Ammonis |
| ChemR23 | chemokine receptor 23 |
| CSF | cerebrospinal fluid |
| DAB | diaminobenzidine |
| DG | dentate gyrus |
| DHA | docosahexaenoic acid |
| EIA | enzyme immunoassay |
| ELISA | enzyme-linked immunosorbent assay |
| EPA | eicosapentaenoic acid |
| FFAs | free fatty acids |
| GFAP | glial fibrillary acid protein |
| H₂O₂ | hydrogen peroxide |
| HETE | hydroxyeicosatetraenoic acid |
| HLA-DR | human leukocyte antigen type DR |
| HRP | horseradish peroxidase |
| IL | interleukin |
| IFN | interferon |
| LC-MS-MS | liquid chromatography-tandem mass spectrometry |

| | |
|---------------------------------|--------------------------------------------------|
| LM | lipid mediator |
| LOXs | lipoxygenases |
| LTB4 | leukotriene B4 |
| LXA₄ | lipoxin A4 |
| LXA₄R | LXA ₄ receptor |
| LXs | lipoxins |
| MaRs | maresins |
| MMSE | mini-mental state examination |
| MRM | multiple reaction monitoring |
| NFTs | neurofibrillary tangles |
| NPD1 | neuroprotectin D1 |
| NSAIDs | non-steroidal anti-inflammatory drugs |
| P-tau | phosphorylated tau |
| PD1 | protectin D1 |
| PET | positron emission tomography |
| PGE2 | prostaglandin E2 |
| PMI | <i>post mortem</i> interval |
| PPAR-γ | peroxisome proliferator-activated receptor-gamma |
| RvD | resolvin D |
| RvE | resolvin E |
| ROS | reactive oxygen species |
| RT | room temperature |
| SPMs | specialized pro-resolving mediators |
| T-tau | total tau |
| TNF | tumor necrosis factor |
| TX | thromboxane |
| WB | western blot |

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Research in context

1. Systematic review: The role of inflammation in Alzheimer's disease (AD) has been studied for decades, but still remains unclear. The concept of programmed resolution of inflammation may shed light on inflammation-associated diseases, including AD. We reviewed PubMed and internet for resolution-related studies. Many reports concern resolution in the peripheral immune system and a few investigate the brain, whereas the AD-brain is virtually unexplored.
2. Interpretation: Our study provides the first systematic report that the resolution pathway - biosynthetic enzymes, specialized pro-resolving mediators (SPMs) and their receptors - exists in human brain. We demonstrate alterations in ADpatients, with strong correlation to cognitive function (assessed by MMSE). We demonstrate that SPMs are measurable in CSF, providing incentive for exploring potential as novel diagnostic markers.
3. Future directions: Our findings open a new gate to resolve AD, and build a basis for resolution-related hypotheses in AD and other inflammatory brain disorders. Future work includes exploring diagnostic potential and resolution as a viable therapeutic target in AD, including role in disease development, and if stimulation of resolution can modify AD.

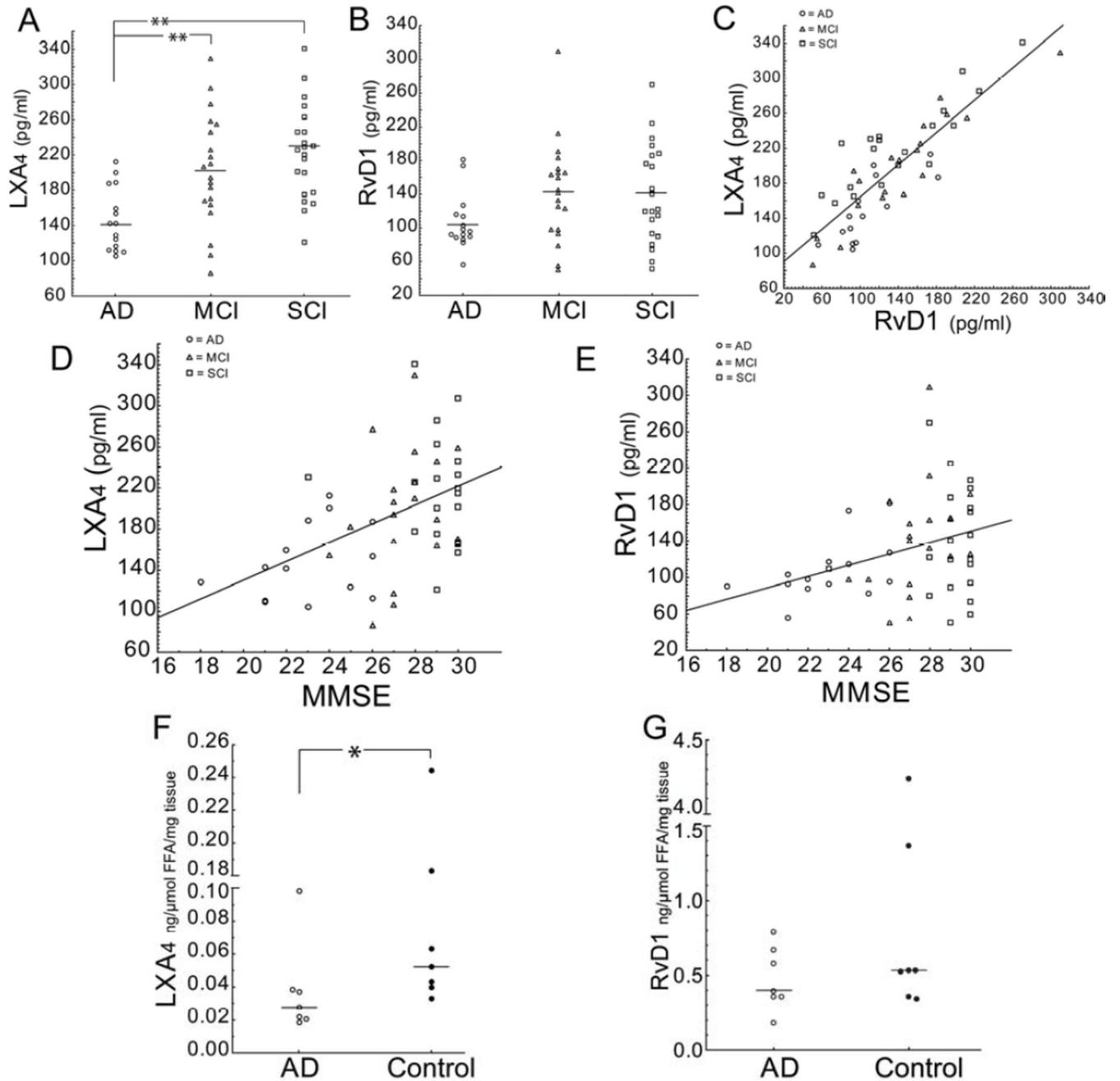


Fig. 1. LXA₄ and RvD1 in human CSF and hippocampus

(A and B) Lower CSF levels of LXA₄ in AD (n = 15) as compared to MCI (n = 20; ANCOVA corrected for age, Bonferroni *post hoc*, $p = 0.006$) and SCI (n = 21; age as covariate by ANCOVA corrected to age, Bonferroni *post hoc*, $p < 0.001$) groups, whereas RvD1 levels do not differ between the groups. (C) Levels of LXA₄ and RvD1 in the CSF are positively correlated (Spearman's rho test, $r = 0.843$, $p < 1 \times 10^{-6}$). (D and E) Levels of LXA₄ and RvD1 in the CSF are correlated to MMSE score in a positive manner (Spearman's rho test, $r = 0.475$, $p < 0.0005$, and $r = 0.343$, $p < 0.05$ respectively). (F) Lower tissue levels of LXA₄ in the hippocampus of AD-patients (n = 7) than in control subjects (n

= 7, Mann-Whitney U test, $z = -2.236$, $p = 0.026$). (G) No difference found between AD-patients ($n = 7$) and control subjects with regard to hippocampal levels of RvD1 ($n = 7$, Mann-Whitney U test, $z = -0.575$, $p = 0.62$). Horizontal bar indicates mean (A and B) or median (F). * $p < 0.05$, ** $p < 0.01$.

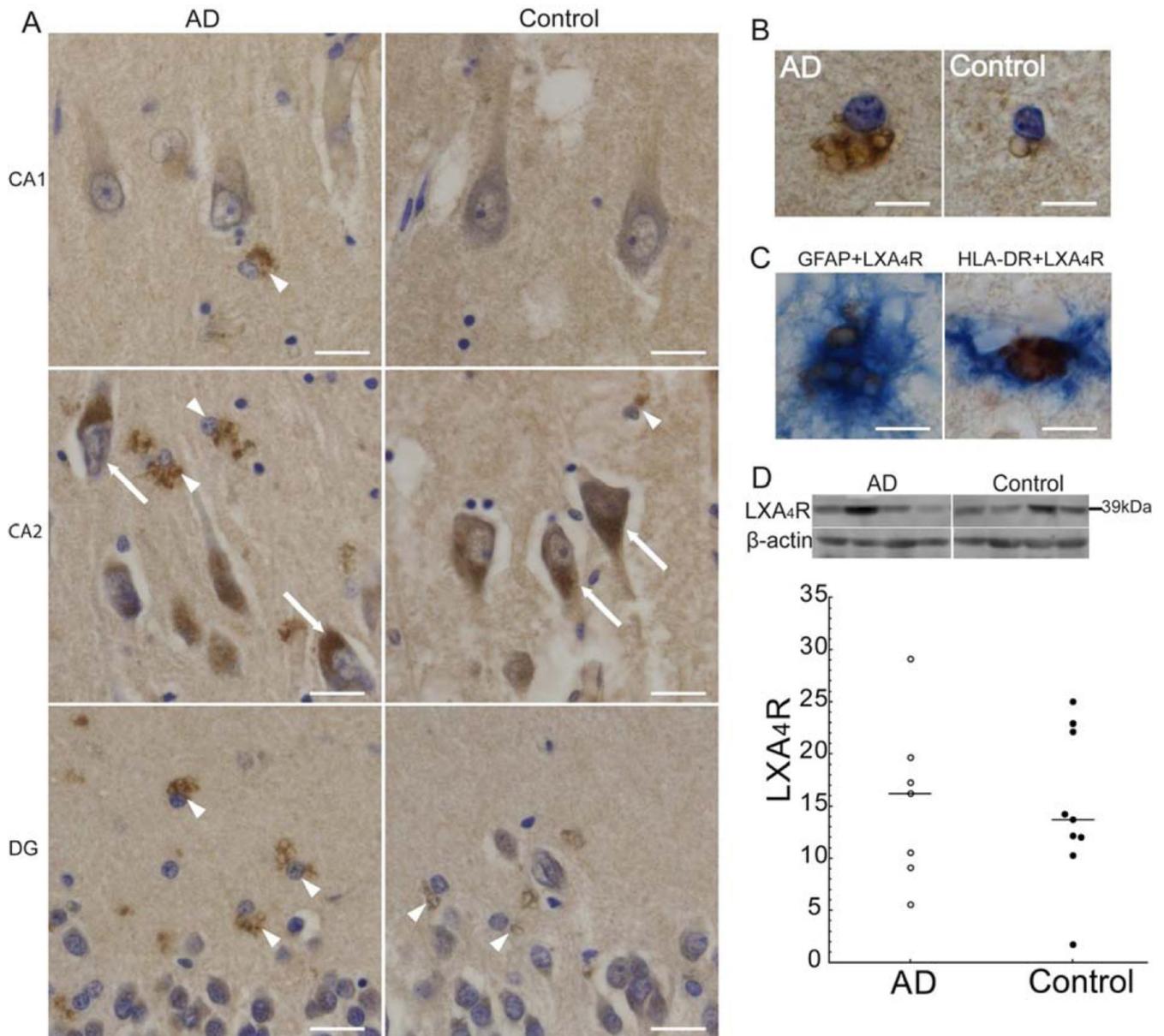


Fig. 2. LXA₄R in human hippocampus

(A) Immunohistochemistry of LXA₄R in AD and control shows neuronal (arrow) and glial (arrowhead) staining; no labeling is observed in CA1 pyramidal neurons, neither in AD nor control; neuronal staining in CA2 is similar in AD and control; glial labeling is stronger in AD, in CA1–CA4 as well as in the dentate gyrus; bar = 20 μm. (B) Strongly labeled glial cells containing multiple large vesicles can be seen in AD. The glia in controls have weaker staining and fewer vesicles; bar = 8 μm. (C) Double labeling of cells with LXA₄R (red) and GFAP (blue) or HLA-DR (blue), shows localization of LXA₄R in both astrocytes and microglia; bar = 8 μm. (D) WB data show no statistically significant difference in LXA₄R levels between AD (n = 7) and control (n = 9) cases (Mann-Whitney U test, $z = -0.053$, $p = 0.958$; Horizontal bar indicates median).

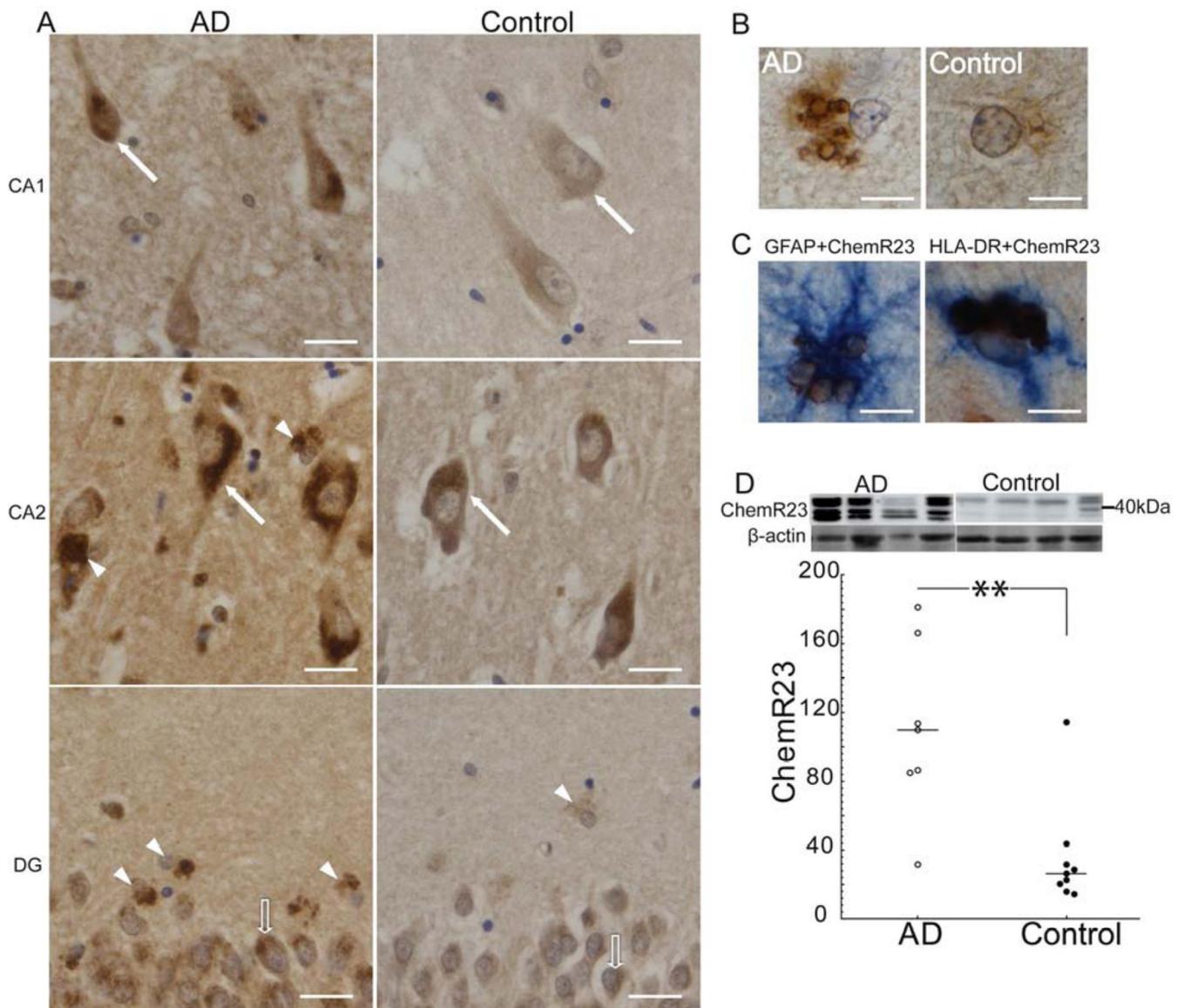


Fig. 3. ChemR23 in human hippocampus

(A) Immunohistochemistry of ChemR23 in AD and control shows staining in pyramidal cells (solid arrows) and glia (arrow heads), and in dentate gyrus (DG) granular cells (unfilled arrows); in AD ChemR23-labeled neurons in CA1 with strong staining are more numerous than in control; ChemR23 labeling in CA2 neurons and glial cells is stronger in AD than in control. Similarly, the staining of granular cells and glia in the DG is stronger in AD; bar = 20 μ m. (B) Strongly labeled glial cells containing multiple large vesicles can be seen in AD. The glia in controls have weaker staining and fewer vesicles; bar = 8 μ m. (C) Double labeling of cells with ChemR23 (red) and GFAP (blue) or HLA-DR (blue) shows localization of ChemR23 in both astrocytes and microglia; bar = 8 μ m. (D) WB data show higher levels of ChemR23 in AD (n = 7) than in controls (n = 9) (Mann-Whitney U test, z = -2.593, p = 0.008; Horizontal bar indicates median; **p < 0.01).

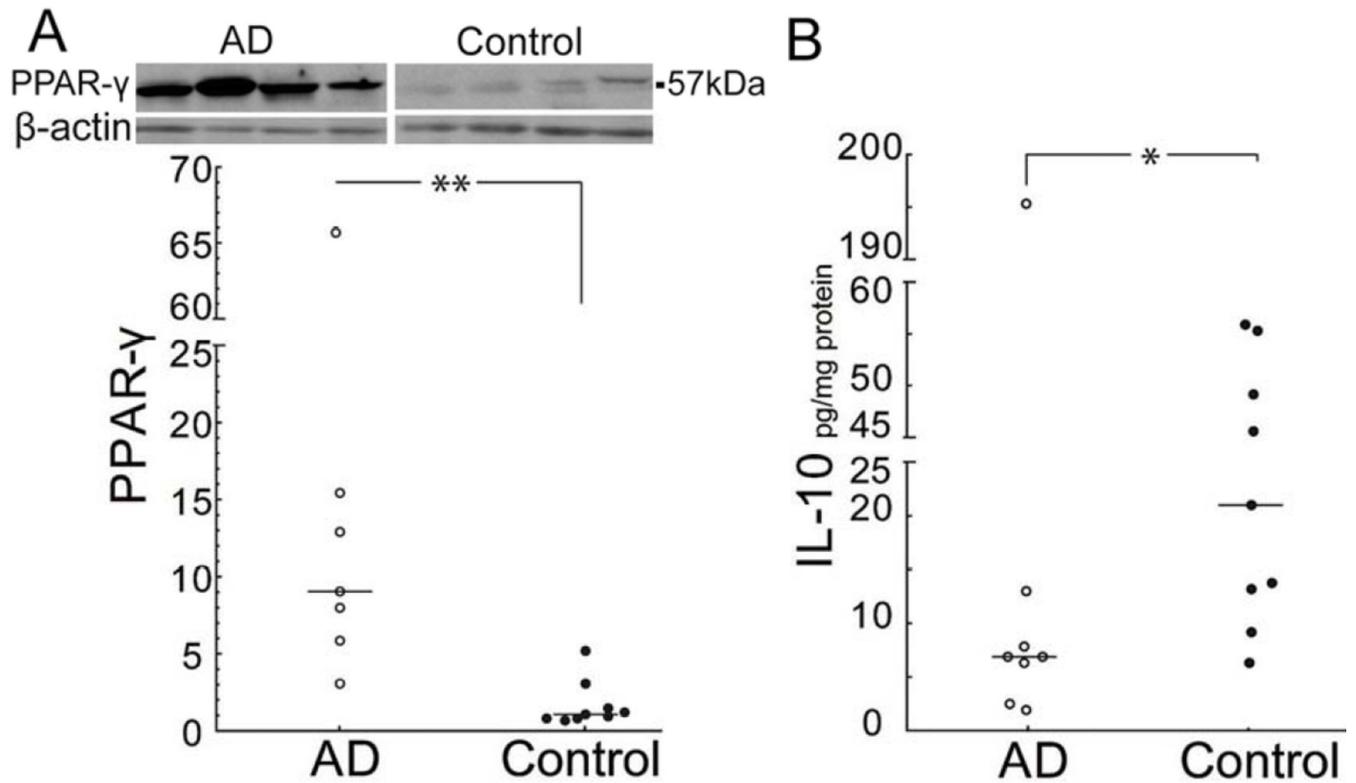


Fig. 4. PPAR- γ and IL-10 in human hippocampus

(A) The levels of PPAR- γ are higher in AD (n = 7) than in controls (n = 9) (Mann-Whitney U test, $z = -3.228$, $p < 0.001$). (B) Levels of IL-10 are lower in AD (n = 8) than in controls (n = 9) (Mann-Whitney U test, $z = -2.117$, $p = 0.036$). Horizontal bar indicates median (A, D); * $p < 0.05$, ** $p < 0.01$.

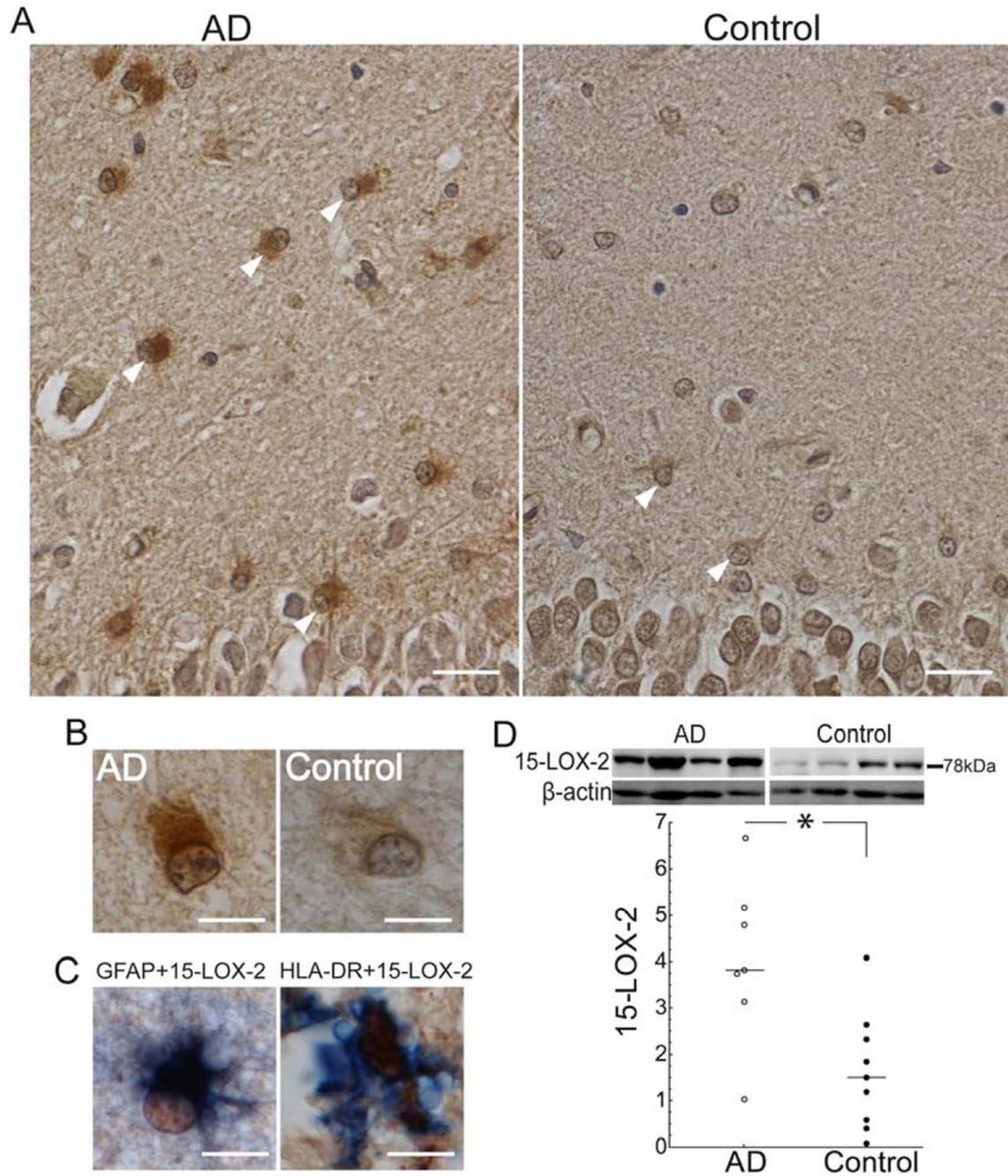


Fig. 5. 15-LOX-2 in human hippocampus

(A) Immunohistochemistry of AD and control shows glial staining (arrow) with higher intensity and higher numbers of labeled cells in AD; bar = 20 μ m. (B) Close-up view of 15-LOX-2; bar = 8 μ m. (C) Double labeling of cells with 15-LOX-2 (red) and GFAP (blue) or HLA-DR (blue) show localization of 15-LOX-2 in both astrocytes and microglia; bar = 8 μ m. (D) Western blot data show higher levels of 15-LOX-2 in AD (n = 7) than in controls (n

= 9) (Mann-Whitney U test, $z = -2.382$, $p = 0.016$; Horizontal bar indicates median; * $p < 0.05$).

Table 1

Subject information for hippocampal tissue samples.

| | control (n=10) | AD (n=10) |
|---------------------|-----------------------|-------------------|
| Age (mean \pm SD) | 80.40 \pm 6.275 | 80.20 \pm 7.021 |
| Gender (male) | 6(4) | 8(2) |
| PMI (mean \pm SD) | 17.40 \pm 12.131 | 23 \pm 12.266 |

PMI = *post mortem* interval (hours)