Published in final edited form as:

J Alzheimers Dis. 2015 ; 44(4): 1263–1278. doi:10.3233/JAD-142259.

Overexpression of Mutant Amyloid- β Protein Precursor and Presenilin 1 Modulates Enteric Nervous System

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder histologically characterized by amyloid- β (A β) protein accumulation and activation of associated microglia. Although these features are well described in the central nervous system, the process and consequences of A β accumulation in the enteric nervous system have not been extensively studied. We hypothesized that A β also may accumulate in the enteric nervous system and lead to immune cell activation and neuronal dysfunction in the digestive tract not unlike that observed in diseased brain. To test this hypothesis, ileums of the small intestine of thirteen month old A β PP/PS1 and C57BL/6 (wild type) mice were collected and analyzed using immunohistochemistry, western blot analysis, cytokine arrays, and ELISA. A β PP/PS1 mice demonstrated no differences in intestinal motility or water absorption but elevated luminal IgA levels compared to wild type mice. They also had increased protein levels of A β PP and the proteolytic enzyme, BACE, corresponding to an increase in A β _{1–40} in the intestinal lysate as well as an increase in both A β _{1–40} and A β _{1–42} in the stool. This correlated with increased protein markers of proinflammatory and immune cell activation. Histologic analysis localized A β PP within enteric neurons but also intestinal epithelial cells with elevated A β immunoreactivity in the A β PP/PS1 mice. The presence of A β PP, A β , and CD68 immunoreactivity in the intestines of some patients with neuropathologically-confirmed AD are consistent with the findings in this mouse model. These data support the hypothesis that in AD the intestine, much like the brain, may develop proinflammatory and immune changes related to A β PP and A β .

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Authors' disclosures available online (<http://www.jalz.com/disclosures/view.php?id=2612>).

Keywords

Amyloid- β ; cytokines; inflammation; intestine

INTRODUCTION

A hallmark of Alzheimer's disease (AD) brains is the accumulation of amyloid- β (A β) plaques [1, 2]. A β is a peptide cleavage product of amyloid- β protein precursor (A β PP) which aggregates to form extracellular plaques [3]. A β serves as a potent proinflammatory stimulus for microglia lending support to the idea that the reactive microgliosis in AD brains is due to A β stimulation [4–12]. Although this hypothesis has been proposed in the central nervous system, the process of A β accumulation and immune cell activation in the enteric nervous system is not well defined.

Elderly are often afflicted with debilitating intestinal disorders such as constipation, fecal incontinence, and impaired evacuation [13–16] which could be due to a degenerating enteric nervous system. The enteric nervous system is homologous to the central nervous system sharing similar embryology, morphology, and neurochemistry [17]. It heavily innervates the small intestine and controls virtually all gastrointestinal (GI) functions, including motility, secretion, blood flow, mucosal growth, and local immune cell behavior [18]. Indeed, the small intestine is host to multiple cells of the immune system which, through interactions with the enteric neurons, can also evoke alterations in gut function [18]. Therefore, these characteristics along with the knowledge that elderly, including AD patients [19, 20], experience GI dysfunction suggest that A β PP and A β may also accumulate in the enteric nervous system leading to immune cell activation and neuronal dysfunction in the digestive tract not unlike that observed in the diseased brain. Indeed, prior work examining human AD intestines have demonstrated robust enteric neuron A β PP expression [21, 22] and in some cases, plaque-like deposition [23] in the intestines. Similarly transgenic rodent models of AD have demonstrated enteric neuron A β PP immunoreactivity [24] as well as A β staining, neuron loss, and macrophage alteration [25, 26] depending upon the model. Furthermore, there is a precedent for intestinal pathology in other neurodegenerative diseases. For example, enteric biopsies have demonstrated α -synuclein aggregates in patients with Parkinson's disease [27–31].

In spite of demonstrated enteric neuron as well as enterocyte expression of A β PP and A β [21, 22, 32–34], a role for A β PP or its metabolites in regulating gastrointestinal function remains unclear. Our prior work demonstrated that absence of A β PP results in dramatic changes in immune cell behavior in the ileum of mice including altered cytokine profiles and reduced macrophage infiltration. This correlated with impaired intestinal absorption and motility [35]. Based upon this data we hypothesized that increased A β PP expression and A β production would also alter immune cell behavior and intestinal function. Therefore, in this study we compared the small intestine of wild type (C57BL/6) and A β PP/PS1 mice to assess changes in neuronal, immune, and gut function in response to overexpression of mutant A β PP/PS1 and the A β PP metabolite, A β . We also investigated postmortem intestine in

patients with autopsy-confirmed AD to see if changes were consistent with those observed in this AD mouse model.

MATERIALS AND METHODS

Antibodies and reagents

Anti- β -amyloid precursor protein (A β PP) and anti-occludin antibodies were purchased from Zymed Laboratories (San Francisco, CA). Anti-rabbit (goat), anti-goat (bovine), anti-rat (goat), and anti-mouse (bovine) horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). CD36 and actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Elite Vectastain ABC reagents, Vector VIP, biotinylated anti-rabbit, anti-mouse, and anti-rat antibodies were purchased from Vector Laboratories Inc. (Burlingame, CA, USA). Anti-human and anti-mouse CD68 antibodies were purchased from AbD Serotec (Oxford, UK). BACE antibody was purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). COX-2 antibody was purchased from Cayman Chemical (Ann Arbor, MI, USA). A β clone 4G8 was purchased from Covance (Emeryville, CA, USA). Anti-amyloid beta precursor protein antibody (Y188) was purchased from Abcam (Cambridge, MA, USA). The PHF-1 anti-phosphoSer396/404 mouse monoclonal antibody was a generous gift from Dr. Peter Davies, Albert Einstein College of Medicine.

Mice

A β PP/PS1 (APP^{swe}, PSEN1^{dE9}) double transgenic mice were originally purchased from Jackson Laboratory and have been maintained on a C57BL/6 background. The A β PP/PS1 (B6.CgTg (A β PP^{swe}, PSEN1^{dE9}) 85Dbo/Mmjax) transgenic animals express human A β PP with a mutation that in humans causes familial AD and the amyloid beta (A4) precursor protein (Mo/HuAPP695^{swe}). In addition they express the human *PSEN1* with a mutation that in humans also causes familial AD due to presenilin 1 (PS1-dE9). Five male A β PP/PS1 mice and their C57BL/6 (wild type) littermate controls were provided food and water *ad libitum* and housed in a 12-h light/dark cycle until 13 months of age, then euthanized via CO₂ asphyxiation followed by cervical dislocation and cardiac exsanguination. The investigation conforms to the Guide for the Care and Use of Laboratory Animals (eighth edition, 2010). Animal use was approved by the University of North Dakota IACUC.

Human large intestine

Human large intestine paraffin sections were obtained from the Gift to Life Outpatient Autopsy Planning Program of the Center for Alzheimer's Care, Imaging and Research, at the University of Utah. This Program helps to arrange clinical postmortem examinations at the University of Utah Department of Pathology for patients with neurodegenerative diseases. As part of the autopsy consent, patients and their family members are able to agree to have their autopsy tissue used for educational purposes and in research studies. Intestinal tissue samples obtained during complete body autopsies from eleven patients, eight male and three female, ages 59–92 years at death, were used for histopathological examination in this project. All patients had a clinical diagnosis of probable AD based upon an extensive

clinical examination. At death, all had Braak and Braak staging of IV-VI for neurofibrillary tangles and a CERAD score of frequent neuritic plaques leading to a neuropathological diagnosis of high likelihood that dementia is due to AD using NIA-Reagan criteria [36]. Postmortem interval was 1 to 3 days. None had intestinal pathological abnormalities identified during the clinical postmortem examination.

Western blotting

At 13 months, the ileums of the small intestine of A β PP/PS1 and C57BL/6 mice were collected, rinsed to remove lumen contents, flash frozen, pulverized, and lysed using ice cold radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM Na₃VO₄, 10 mM NaF, 1mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 1% Triton, 0.1% SDS, and 0.5% deoxycholate) with protease inhibitors (AEBSF 1 mM, Aprotinin 0.8 μ M, Leupeptin 21 μ M, Bestatin 36 μ M, Pepstatin A 15 μ M, E-64 14 μ M) and 50 U/mL DNaseI (Amresco Inc., Solon, OH, USA). To remove insoluble material, cell lysates were sonicated and centrifuged (14,000 rpm, 4°C, 10 min). The Bradford method [37] was used to quantify protein concentrations. Proteins were resolved by 7 and 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (PVDF) for western blotting using anti-A β PP, BACE, occludin, COX-2, CD68, CD36, and actin (loading control) antibodies. Antibody binding was detected with enhanced chemiluminescence (GE Healthcare, Piscataway, NJ). In some instances, blots were stripped in 0.2 NaOH, 10 min, 25°C, for reprobing. Western blots were quantified using Adobe Photoshop software (Adobe Systems, San Jose, CA). Optical densities of bands were normalized against their respective loading controls and averaged ($\pm$ SD).

Histological stain

At 13 months, the animals were perfused with Ca²⁺/Mg²⁺ free PBS and ileums of the small intestine were collected and immersion fixed for 24 h in 4% paraformaldehyde and cryoprotected through two successive 30% sucrose changes. Ileum samples were serially cryosectioned (10 μ m) for routine histology H & E (hematoxylin and eosin) and Alcian blue stains. In addition, human AD large intestine samples were also H & E and Alcian blue stained. For H & E stain, slides were rinsed in xylene three times for 1 min each, followed by rehydration in 2 changes of absolute alcohol for 2 min, then 95%, 80%, 70% alcohol for 1 min each, followed by rinsing in dH₂O for 1 min. Slides were then stained with Hematoxylin Regulars for 40 s followed by rinsing in running tap water for 10 dips, then differentiated with 3% acid alcohol for 2 dips, followed by rinse in running tap water for 10 dips. Slides were then blued in lithium carbonate (1% sodium bicarbonate) solution for 3–5 dips followed by rinsing again in running tap water before a 1 min rinse in 70% alcohol and then 5–10 s in Eosin. Slides were dehydrated two times each for 1 min in 95% alcohol, 100% alcohol and xylene followed by covering with Permount. For Alcian blue stain, slides were placed in 100% alcohol, 95% alcohol, 70% alcohol, and dH₂O for 1 min each then mordant in 3% aqueous acetic acid for 1 min, 1% Alcian blue in 3% acetic acid pH 2.5 for 10 min followed by rinse in running water for 5–6 min and rinse in dH₂O. Slides were counterstained in Nuclear Fast Red 0.1% (kerectrot) for 1.5 min then rinsed in running water for 10 min before dehydrating in 95% alcohol, 100% alcohol and xylene 2 times each

for 1 min before being covered with Permount. Images were taken using an upright Leica DM1000 microscope and Leica DF320 digital camera system. Figures were made using Adobe Photoshop software.

Immunohistochemistry

Fixed, collected ileum samples were serially cryosectioned (10 μ m) for immunostaining with anti-A β PP (Y188), A β (4G8), CD68, COX-2, occludin, phospho-tau (PHF-1), and CD36 or respective secondary only antibodies. Human AD large intestine slides were first rinsed in xylene three times for 5 min each, followed by rehydration in 2 changes of absolute alcohol for 5 min, then 95%, 80%, 70% alcohol for 5 min each, followed by rinsing in dH₂O for 5 min, and antigen retrieved in boiling Tris-EDTA, pH9, for 20 min. Antibody binding in the intestine was visualized using the Vector VIP chromogen (Vector Laboratories, Burlingame, CA). Images were taken using an upright Leica DM1000 microscope and Leica DF320 digital camera system. Figures were made using Adobe Photoshop software.

Cytokine array

Flash frozen, pulverized collected ileums were lysed according to the manufacturer's protocol (Ray Biotech, Norcross, GA, USA). Insoluble material was removed from cell lysates via centrifugation (14,000 rpm, 4°C, 10 min). The Bradford method [37] was used to quantify protein concentrations. 1 mg per sample was used to probe a 40 cytokine antibody-based membrane array according to the manufacturer's protocol (Ray Biotech, Norcross, GA, USA). Optical density of dots was normalized against their respective positive controls and averaged ($\pm$ SD).

Stool weight

At 13 months, total stool weight was measured by placing the animals in a clean empty cage and collecting all stool produced over a 1 h time period. Stool was then desiccated at 80°C overnight to determine dry weight. Stool water was calculated as the difference between these two measurements.

Enzyme linked immunosorbent assay (ELISA)

At 13 months, prior to collection, animals were placed in an empty cage long enough to obtain one stool pellet, then the animals were perfused with Ca²⁺/Mg²⁺ free PBS and ileum of intestine was collected, flushed with 7mL HBSS to remove luminal contents (stool washing). Stool washing samples were also diluted further by 1:5000 and 300 μ L of the dilute sample was used for commercial IgA ELISA according to manufacturer's protocol (Bethyl Laboratories Inc, Montgomery, TX, USA). Stool pellet and ileums of the small intestine were lysed in RIPA buffer with protease inhibitors and 50 U/mL DNase1. To remove insoluble material, cell lysates were sonicated and centrifuged (14,000 rpm, 4°C, 10 min), the supernatant was removed and used to determine soluble A β concentrations. The pellet was re-suspended in 5M guanidine HCL/50mM Tris HCL, pH 8.0, samples were again sonicated and centrifuged (14,000 rpm, 4°C, 10 min), the supernatant was removed and used to determine fibrillar A β concentrations. The Bradford method [37] was used to quantify protein concentrations. Both the supernatants were then used for commercial high

sensitivity A β ₁₋₄₀ (EZHS40) and A β ₁₋₄₂ (EZHS42) ELISA according to the manufacturer protocol (EMD Millipore, Billerica, MA, USA). Data is the average ($\pm$ SD).

Statistical analysis

The data were analyzed by unpaired two-tailed *t*-test with or without Welch correction for unequal variance as required.

RESULTS

A β PP immunoreactivity and protein levels were increased in ileums of A β PP/PS1 mice

Because our prior work has demonstrated dramatic plaque deposition and microgliosis in the brains of this particular strain of AD mouse model at 12–13 months of age, we elected to use 13 month old animals to examine any similar changes in intestines [38]. As a representative section of the GI tract, we again focused our examination on the ileums [39]. H&E staining revealed no robust histological differences between the wild type and A β PP/PS1 mice (Fig. 1). Alcian blue staining also revealed no apparent differences in goblet cells (acid mucosubstances and acetic mucins) between the A β PP/PS1 and wild type mice. A β PP/PS1 mice demonstrated robust neuronal A β PP immunore-activity in both the submucosa and myenteric plexi (Fig. 2A). Interestingly, they also showed increased A β PP immunoreactivity within the columnar epithelium and diffusely within the smooth muscle of the muscularis externa (Fig. 2A). Although typical brain-associated A β -containing plaques were not observed in the A β PP/PS1 mice, diffuse submucosal A β staining was observed in the A β PP/PS1 intestines and not in control mice (Fig. 2A). In order to quantify the staining images, western blot analysis was performed to compare the ileums from A β PP/PS1 and wild type mice. Not only A β PP but also BACE levels were significantly increased in the A β PP/PS1 mice (Fig. 2B & C). For quantitation of A β levels in the ileums, intestinal lysates and stool pellet lysates were collected for A β ₁₋₄₀ and A β ₁₋₄₂ ELISAs. Consistent with our expectations and the observed increase in A β PP and BACE levels in the western blot analysis, there was a quantifiable increase in A β ₁₋₄₀ in the intestinal lysate and an increase in both A β ₁₋₄₀ and A β ₁₋₄₂ in the lysed stool pellet (Fig. 3).

Ileums of A β PP/PS1 mice had increased immunoreactivity and altered levels of several protein epithelial markers

To compare intestinal pathology and immune function, the tissue was immunostained to identify an epithelial tight junction protein, occludin; a common intestine regulatory and proinflammatory protein, cyclooxygenase-2 (COX-2); and the integral membrane and fatty acid transport protein, CD36, to gain an impression of intestinal phenotype. Occludin, COX-2, and CD36 immunoreactivity primarily localized to the enterocyte layer with some areas of robust immunore-activity for COX-2 and CD36 in the A β PP/PS1 intestines compared to the wild type mice and overall decreased occludin immunostaining (Fig. 4A). The staining was followed up by western blot analyses to allow quantitation of the histologic findings. Western blot findings demonstrated increased COX-2 protein levels in A β PP/PS1 mice compared to controls (Fig. 4B). A β PP/PS1 mice also showed elevated CD36 but not occludin protein levels compared to the controls (Fig. 4B). These data supported the idea

that mutant A β PP/PS1 expression regulates some aspects of intestinal behavior suggesting that A β PP may modulate not only immune changes but also digestion and absorption.

Ileums of A β PP/PS1 mice demonstrated increased macrophage immunoreactivity and altered levels of several cytokines

To assess whether immune changes paralleled the increased levels of A β PP and altered epithelial cell phenotype, ileums were next immunostained using anti-CD68 antibodies as a general macrophage marker as a representative of the plethora of immune cells that exist in the intestines. A β PP/PS1 intestines demonstrated robust increased anti-CD68 immunoreactivity compared to control mice (Fig. 5A) suggesting either increased activation or increased numbers in the intestine. Interestingly, this presentation is precisely what is observed in the brain of A β PP/PS1 mice at this age using CD68 to recognize microglia [38]. Based upon the increased COX-2 protein levels and CD68 immunoreactivity in the A β PP/PS1 ileums, it was reasonable to expect that levels of cytokines in the A β PP/PS1 versus control ileums would also differ. To examine this possibility, ileums were used for antibody-based cytokine arrays profiling 40 different cytokines. As expected, the A β PP/PS1 ileums demonstrated significantly increased levels of 6 different factors, IL-12p70, LIX, MCP-1, MCSF, SDF-1, and TIMP-1 compared to control mice (Fig. 6). As an additional assessment of immune phenotype changes, luminal intestinal content was compared between genotypes using a commercially available IgA ELISA to assess plasma cell behavior. A β PP/PS1 intestinal lumens had significantly more secreted IgA compared to the wild type mice again supporting the notion that altered immune phenotypes characterized the mutant mice (Fig. 7A). The combined change in levels of multiple cytokines, proinflammatory and regulatory proteins, and numbers of macrophages suggested that the function of A β PP/PS1 intestines may also differ from control animals. In order to address gross intestinal function, mean stool production and water content were compared between the genotypes. Although this was not an exclusive assessment of small intestine behavior, the A β PP/PS1 animals did not reveal any differences in stool or water content (Fig. 7B). These findings further supported the notion that mutant A β PP/PS1 expression regulates immune cell phenotype in the intestine without overall dramatic intestinal dysfunction.

Patients with clinical and neuropathological AD demonstrated intestinal A β PP, A β , and CD68 immunoreactivity

In order to evaluate whether changes observed in our double transgenic mice also occur in humans with AD, we immunostained tissue from the large intestines of eleven patients with AD (Table 1). Although not all cases demonstrated positive immunostaining for A β PP and the degree of immunostaining was somewhat variable, the A β PP/PS1 mouse model changes we had observed were similar to patterns of immunoreactivity present in human disease. Specifically, AD large intestines demonstrated robust neuronal and columnar epithelial A β PP immunoreactivity (Fig. 8). AD patient intestinal tissue also demonstrated robust CD68 macrophage and intraneuronal A β immunoreactivity (Fig. 8). Interestingly, we also noted that in some cases patient intestines also displayed immunoreactive phospho-tau tangle-like structures in the submucosa and diffusely within the smooth muscle of the muscularis externa. Thus these findings provide evidence that A β PP/PS1 intestines are accurately modeling changes associated with human disease and that that neuronal A β

production/accumulation and associated immune cell activation is occurring in the enteric nervous system in parallel with the central nervous system in AD in humans.

DISCUSSION

We have demonstrated that overexpression of A β PP and possible metabolic cleavage in A β PP/PS1 mice correlates with increased intestinal macrophage immunoreactivity, altered cytokine and immunoglobulin levels, and alteration of levels of diverse proteins in the intestinal epithelium. Immunoreactivity changes observed in the mouse AD model intestine were also consistent with those observed in the human AD intestine. Based upon the quantified increase in cytokine levels as well as secreted IgA, it appears that A β PP has a role in diverse tissue types, such as the intestine, in addition or uniquely from that observed in the brain.

Increased A β deposition in the temporal cortex of twelve month old A β PP/PS1 mice in our previous study as well as others [38, 40] correlated well with the increased levels of A β PP and A β in the intestine. Interestingly, we also observed elevated ileal BACE consistent with changes reported in sporadic AD brains [41–45] suggesting that BACE expression levels correlate with increased A β production in the brain [46]. The deposition of A β in the intestines of these A β PP/PS1 mice was also consistent with our detection of A β immunoreactive plaques in AD intestines as also reported in a prior study [23]. Our findings, as well as prior human and transgenic mouse model intestinal work [21–26, 32–34], suggest the possibility that A β PP and its metabolites may have disease relevance in tissues other than the brain. For example, it is possible that increased A β levels in the intestine may traffic into the vasculature to ultimately contribute to elevated levels in the brain. A β has been shown to colocalize with chylomicron-apoB in enterocytes [33] as well as in cerebral amyloid plaques [47] which has been correlated with increased plasma A β levels [47].

Another prominent feature of AD pathology is inflammatory change [48–52], which was also evident in our A β PP/PS1 ileums demonstrated by the observed increases in the inflammatory marker, COX-2, and the macrophage marker, CD68. This increase in CD68 levels is consistent with elevated numbers of brain macrophages, microglia, that are associated with A β plaque pathology [38, 53–61]. A similar observation was made using another AD transgenic line demonstrating that intestinal macrophages had an altered morphologic phenotype compared to wild type controls [26]. One attractive hypothesis for increased macrophage numbers in the intestines is due to direct stimulation with A β peptide oligomers or fibrils [8, 10, 62–71] as has been hypothesized to occur in the brain. However, the elevated numbers could also be due to the increase in COX-2 levels observed, as COX-2 activity positively regulates intestinal macrophage infiltration in a rodent model of sepsis [72]. Although we have focused on macrophage in the intestines to be able to directly compare to brain microglia, we appreciate the rich immune cell diversity of the intestines and expect that cross-talk and subsequently altered phenotypes may exist for all of these cells. Indeed, the changes in plasma cell secreted IgA seem to indicate this. Although the precise cause(s) of immune cell changes in the intestine that we observed remain unclear, similar to explanations for activated microglia in the brain, these data suggest that immune cell activation or dysfunction in AD may not be limited to brain microglia but includes other

tissue resident or circulating immune cells. Moreover, this supports the growing notion that inflammatory changes in the AD brain may be linked to immune changes in the periphery.

Based upon the changes in immune cells, A β PP, and A β observed, we expected changes in other protein markers of intestinal function. Although no gross differences in motility or water absorption were detected, we did make more subtle observations indicating altered function. For example, the increase in COX-2 observed was consistent not only with it being an inflammatory marker, but its prostaglandin products have been documented to negatively regulate intestinal motility [47, 73–76]. Along with COX-2, we also observed a significant increase in CD36 levels as an additional marker for assessing intestinal integrity. However, CD36 is also a fatty acid uptake regulatory protein which suggests that the A β PP/PS1 mice may have altered dietary fatty acid and cholesterol uptake needed for chylomicron formation [77]. The possibility of increased chylomicron formation fits very nicely with the increase in A β observed since, as stated before, A β colocalizes with chylomicron-apoB in enterocytes [33]. Since we did not observe dramatic changes in motility or water absorption, it appears that the enteric system of the mice is not compromised at this age by overexpression of mutant A β PP or its metabolites.

As with lack of A β PP expression in the intestine [78], it is still not clear whether overexpression of A β PP in one or multiple cell types is responsible for the changes in immune cell number, cytokine profile, epithelial cell protein expression, or mucosal secretion observed. Robust immunoreactivity of A β PP was observed in neurons as expected based upon the prion promoter driven expression of the transgenes. However, A β PP/PS1 and control mice as well as the human AD intestine demonstrated epithelial cell A β PP immunoreactivity. Although A β PP metabolism to A β is well characterized in neurons, future work will need to establish whether intestinal epithelial A β PP may also be metabolized to A β . In this case, similar to the brain, multiple A β PP expressing cells may be contributing to A β PP/A β related changes that occur during disease. In addition, the increased intestinal macrophage numbers may also have resulted from increased migratory ability or increased activation of cells in the A β PP/PS1 mice.

The diverse cytokine changes in the A β PP/PS1 intestine provide insight into the complex immunoregulation of the intestines that occurs due to multiple interacting immune cell types. For instance, in inflammatory bowel disease IL-12 production is increased by T-cells [79]. LIX, also known as epithelial-derived neutrophil-activating peptide 78 (ENA-78), expression is increased in mice with dextran sodium sulfate induced colitis [80] and also has been shown to play an important role in neutrophil recruitment from the lamina propria into the epithelial layer of the intestine [81]. In general, monocyte chemoattractant protein-1 (MCP-1) plays an important role in recruitment of monocytes and macrophages to inflamed tissues [82]. Macrophage-colony stimulating factor (M-CSF) influences monocyte/macrophage proliferation, differentiation, and activation and is increased in inflammatory bowel disease (IBD) [83]. The increases in MCP-1 and M-CSF are therefore both consistent with the increased CD68 levels in these A β PP/PS1 mice. Stromal cell-derived factor-1 (SDF-1), also known as CXCL12, is also increased in IBD [84]. Tissue inhibitor of metalloproteinases 1 (TIMP-1) is also increased in IBD [85] especially in ulcerative colitis [86, 87]. The collective increase in these IBD-associated cytokines in A β PP/PS1 intestine

suggests A β PP and/or its metabolites may influence the susceptibility of these mice to gut inflammatory disease and implicates a role in modulating immune cell behavior in conditions other than AD.

Although we observed changes in the AD tissue sections that were remarkably similar to those observed in the A β PP/PS1 mice, it is important to acknowledge that were not, at this time, comparing AD sections to matched non-demented control sections. Therefore, although several clear increases with disease were observed in the A β PP/PS1 mice compared to controls we are not able to make similar conclusions of disease related increase in intestinal pathology from the human tissue analysis. Nevertheless, we did observe AD-related histopathology in the human colon sections similar to what is observed in brains during disease suggesting that the enteric nervous system may also be affected during disease. In the future, we will compare not only matched non-demented control cases but also other type dementia to AD cases across the entire span of small and large intestines during disease course to better define changes occurring in the enteric nervous system.

We have demonstrated that mutant A β PP overexpression results in significant immune changes and increased A β immunoreactivity and levels in the intestines of mice in correlation with findings from human AD intestines and is consistent with immune changes and A β deposition observed in the brain. The complexity of multiple cell type A β PP expression and multiple immune cell types in the intestine suggests that immune-related gastrointestinal changes in these mice and perhaps AD patients may be significantly more complex than those that occur in the brain. Moreover, it is likely that A β PP metabolism or immune changes in either organ influence one another.

ACKNOWLEDGMENTS

We are grateful to Dr. John Watt for helpful suggestions during tissue sectioning and Hongyan Wang for her technical expertise in histological staining. We thank Dr. Mark Musch for helpful suggestions regarding intestinal protein analyses. This work was supported by NIH 1P20RR17699, NIH 1R01AG026330, NSF REU Site Grant 0851869, and NIH 1R01AG045819.

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Fig. 1.

Histology of the small intestine was altered in A β PP/PS1 mice compared to wild type controls. Ileum samples were collected from C57BL/6 (wild type) and A β PP/PS1 mice, fixed in 4% paraformaldehyde, serially sectioned at 10 μ M, and stained using routine histology H&E (hematoxylin and eosin) and Alcian blue. Arrows indicate the location of the region of interest shown as an enlarged inset to the right of each panel. Representative images from 5 animals per condition are shown.

Fig. 2.

Small intestine immunoreactivity for AβPP and 4G8 increased corresponding to increased AβPP and BACE protein levels in the small intestine of AβPP/PS1 mice compared to wild type controls. Ileum samples were collected from C57BL/6 (wild type) and AβPP/PS1 mice, fixed in 4% paraformaldehyde, serially sectioned, and immunostained. Tissue sections were immunostained using anti-AβPP (Y188) and 4G8 (Aβ) antibodies and antibody binding was visualized using Vector VIP as the chromogen. Representative images from 5 animals per condition are shown (A). Ileum of the small intestine was collected from AβPP/PS1 and C57BL/6 (wild type) mice. The tissue was lysed, resolved by 10% SDS-PAGE and western

blotted using anti-A β PP, BACE, or actin (loading control) antibodies. Antibody binding was visualized by chemiluminescence. Blots from all animals are shown (B). Optical densities of the western blotted ileum proteins were normalized against actin loading control and averaged ($\pm$ SD) from 5 animals per each condition and graphed (C) * p < 0.05, *** p < 0.001.

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Fig. 3.

Small intestine and stool pellet have increased Aβ in AβPP/PS1 mice compared to wild type controls. Ileum of the small intestine and a stool pellet were collected from AβPP/PS1 and C57BL/6 (wild type) mice. Ileum lysates and lysed stool pellet were also used for Aβ₁₋₄₀ and Aβ₁₋₄₂ ELISAs (D) **p* < 0.05.

Fig. 4. Small intestine immunoreactivity and protein levels for COX-2 and CD36 increased in A β PP/PS1 mice compared to wild type controls with decreased occludin immunoreactivity and protein levels. Ileum samples were collected from C57BL/6 (wild type) and A β PP/PS1 mice, fixed in 4% paraformaldehyde, serially sectioned, and immunostained. Tissue sections were immunostained using anti-occludin, COX-2, and CD36 antibodies and antibody binding was visualized using Vector VIP as the chromogen. Representative images from 5 animals per condition are shown (A). Ileum of the small intestine was collected from A β PP/PS1 and C57BL/6 (wild type) mice. The tissue was lysed, resolved by 10% SDS-

PAGE and western blotted using anti- COX-2, CD36, occludin, or actin (loading control) antibodies. Antibody binding was visualized by chemiluminescence. Blots from all animals are shown (B). Optical densities of the western blotted ileum proteins were normalized against actin loading control and averaged ($\pm$ SD) from 5 animals per each condition and graphed (C). * $p < 0.05$, ** $p < 0.01$.

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Fig. 5.

Small intestine immunoreactivity and protein levels for CD68 increased in AβPP/PS1 mice compared to wild type controls. Ileum samples were collected from C57BL/6 (wild type) and AβPP/PS1 mice, fixed in 4% paraformaldehyde, serially sectioned, and immunostained. Tissue sections were immunostained using anti-CD68 antibodies and antibody binding was visualized using Vector VIP as the chromogen. Representative images from 5 animals per condition are shown (A). Ileum of the small intestine was collected from AβPP/PS1 and C57BL/6 (wild type) mice. The tissue was lysed, resolved by 10% SDS-PAGE and western blotted using anti- CD68 or actin (loading control) antibodies. Antibody binding was visualized by chemiluminescence. Blots from all animals are shown (B). Optical densities of the western blotted ileum proteins were normalized against actin loading control and averaged ($\pm$ SD) from 5 animals per each condition and graphed (C). * $p < 0.05$.

	Wild Type	AβPP/PS1
BLC	1.00±0.12	1.08±0.15
CD30L	0.87±0.08	0.88±0.03
Eotaxin	0.98±0.12	1.01±0.07
Eotaxin-2	1.10±0.13	1.14±0.08
Fas Ligand	0.83±0.08	0.84±0.05
Fractalkine	0.92±0.09	0.94±0.11
GCSF	0.73±0.06	0.83±0.29
GM-CSF	1.02±0.08	1.01±0.05
IFN gamma	0.93±0.05	0.94±0.05
IL-1 alpha	1.23±0.27	1.35±0.07
IL-1 beta	0.92±0.19	0.82±0.05
IL-10	0.80±0.06	0.78±0.04
IL-12p40p70	0.87±0.11	0.94±0.15
IL-12p70	1.11±0.19	1.30±0.22*
IL-13	1.04±0.15	1.03±0.06
IL-17	0.86±0.08	0.85±0.04
IL-2	0.84±0.06	0.86±0.06
IL-3	0.79±0.03	0.81±0.04
IL-4	1.10±0.26	1.24±0.07
IL-6	0.77±0.13	0.87±0.27
IL-9	0.95±0.13	0.89±0.03
I-TAC	0.85±0.06	0.87±0.04
KC	0.80±0.21	0.83±0.19
Leptin	0.83±0.03	0.89±0.08
LIX	1.05±0.06	1.14±0.06*
Lymphotactin	0.87±0.08	0.94±0.06
MCP-1	0.78±0.06	0.91±0.17*
MCSF	1.30±0.16	1.49±0.07*
MIG	0.83±0.05	0.85±0.04
MIP-1alpha	0.92±0.07	0.94±0.09
MIP-1gamma	0.96±0.06	1.03±0.14
RANTES	0.90±0.17	0.94±0.07
SDF-1	0.99±0.04	1.12±0.09*
sTNFRI	1.09±0.13	1.10±0.14
sTNFRII	1.08±0.13	1.19±0.16
TCA-3	1.12±0.07	1.19±0.05
TECK	0.88±0.11	0.95±0.08
TIMP-1	0.80±0.04	0.87±0.05*
TIMP-2	0.85±0.05	0.89±0.06
TNF alpha	0.79±0.07	0.79±0.05

Fig. 6.

Small intestine of AβPP/PS1 mice demonstrated altered levels of multiple cytokines compared wild type controls. Ileum from C57BL/6 (wild type) and AβPP/PS1 mice were collected, lysed, and used in an antibody cytokine array. AβPP/PS1 mice demonstrated increased IL-12p70, LIX, MCP-1, MCSF, SDF-1, and TIMP-1 compared to wild type mice. A representative array for one animal from each genotype is shown. Optical densities of normalized dot intensities from 5 animals in each group are displayed as mean (±SD). * $p < 0.05$.

Fig. 7.

AβPP/PS1 mice demonstrated increased luminal IgA but no differences in intestinal motility or water absorption compared to wild type mice. Small intestine was washed with 7 mL of HBSS to remove intestinal contents. Samples were diluted 1:5000 and 300 μL of sample was used for IgA ELISA (A). Data from 7 animals in each condition are displayed as mean ($\pm$ SD) ** $p < 0.01$. To compare intestinal absorption and motility, water and stool production were quantified from C57BL/6 (wild type) control and AβPP/PS1 mice (B). To assess stool production and water absorption, wet and dry stool weights and the differences (water content) were collected over 1hr. Data from 5 animals in each group are displayed as mean ($\pm$ SD).

Fig. 8.

AD intestines demonstrated AβPP, CD68, Aβ, and phospho-tau immunoreactivity. Large intestine tissue sections from eleven AD patient large intestines were visualized via H&E, Alcian blue, or antibody immunostaining using anti-AβPP (Y188), CD68, 4G8 (Aβ), and PHF-1 (phospho-tau) antibodies and antibody binding was visualized using Vector VIP as the chromogen. Examples of robust immunostaining are shown.

Table 1

Case #	Age @ death	Gender	Post mortem interval	BRAAK stage	A β (4GB)	A β PP (Y188)	PHF-1	CD68
1	92	F	2 days	VI	++	–	–	+++
2	76	M	2 days	VI	+++	+++	–	+++
3	73	M	1 day	V	+	++	–	+++
4	84	F	1 day	VI	+	–	–	++
5	82	M	1 day	Vi	++	++	+	++
6	87	M	1 day	VI	+++	++	–	+
7	69	M	1 day	VI	+++	+++	+	+++
8	72	M	3 days	IV	+++	–	–	+++
9	59	M	2 days	VI	+++	+	–	++
10	72	F	2 days	V	+++	–	+	+++
11	84	M	2 days	V-VI	+++	–	++	+++

– no detectable immunostaining, +scare immunostaining, ++ occasional immunostaining, +++ robust immunostaining.