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Amylin Treatment Reduces Neuroinflammation and Ameliorates Abnormal Patterns of Gene Expression in the Cerebral Cortex of an Alzheimer's Disease Mouse Model

Erming Wang^{a,*}, Haihao Zhu^a, Xiaofan Wang^a, Adam C. Gower^b, Max Wallack^a, Jan Krzysztof Blusztajn^c, Neil Kowall^{d,e}, and Wei Qiao Qiu^{a,e,f,*}

^aDepartment of Pharmacology and Experimental Therapeutics, Boston University School of Medicine, Boston, MA, USA

^bClinical and Translational Science Institute, Boston University School of Medicine, Boston, MA, USA

^cDepartment of Pathology and Laboratory Medicine, Boston University School of Medicine, Boston, MA, USA

^dDepartment of Neurology, Boston University School of Medicine, Boston, MA, USA

^eAlzheimer's Disease Center, Boston University School of Medicine, Boston, MA, USA

^fDepartment of Psychiatry, Boston University School of Medicine, Boston, MA, USA

Abstract

Our recent study has demonstrated that peripheral amylin treatment reduces the amyloid pathology in the brain of Alzheimer's disease (AD) mouse models, and improves their learning and memory. We hypothesized that the beneficial effects of amylin for AD was beyond reducing the amyloids in the brain, and have now directly tested the actions of amylin on other aspects of AD pathogenesis, especially neuroinflammation. A 10-week course of peripheral amylin treatment significantly reduced levels of cerebral inflammation markers, Cd68 and Iba1, in amyloid precursor protein (APP) transgenic mice. Mechanistic studies indicated the protective effect of amylin required interaction with its cognate receptor because silencing the amylin receptor expression blocked the amylin effect on Cd68 in microglia. Using weighted gene co-expression network analysis, we discovered that amylin treatment influenced two gene modules linked with amyloid pathology: 1)

*Correspondence to: Wendy Wei Qiao Qiu, MD, PhD, Department of Psychiatry, Department of Pharmacology and Experimental Therapeutics, Boston University School of Medicine, 72 East Concord Street, R-623, Boston, MA 02118, USA. Tel.: +1 617 638 4336; Fax: +1 617 638 5254; wqiu67@bu.edu and Erming Wang, PhD, Department of Pharmacology and Experimental Therapeutics, Boston University School of Medicine, 72 East Concord Street, R604, Boston, MA 02118, USA. Tel.: +1 617 638 4389; gewang32@bu.edu.

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AUTHORS' CONTRIBUTIONS

W.Q.Q. and E.W. designed the project and wrote the manuscript. H.Z. conducted amylin treatment, immuno staining, and A β burden characterization of Alzheimer's disease mouse models, X.W. performed RNA extraction and probe preparation for microarray, M.W. carried out counting of A β staining, and E.W. performed all the remaining cellular and biochemical experiments. A.G. conducted the initial array data quality control analysis, and E.W. performed all the remaining bioinformatics analysis; J.K.B. and N.K. provided important suggestions on the data interpretation and manuscript writing.

SUPPLEMENTARY MATERIAL

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a module related to proinflammation and transport/vesicle process that included a hub gene of Cd68, and 2) a module related to mitochondria function that included a hub gene of Atp5b. Amylin treatment restored the expression of most genes in the APP cortex toward levels observed in the wild-type (WT) cortex in these two modules including Cd68 and Atp5b. Using a human dataset, we found that the expression levels of Cd68 and Atp5b were significantly correlated with the neurofibrillary tangle burden in the AD brain and with their cognition. These data suggest that amylin acts on the pathological cascade in animal models of AD, and further supports the therapeutic potential of amylin-type peptides for AD.

Keywords

Alzheimer's disease; amylin; hub gene; transcriptome; WGCNA

INTRODUCTION

Alzheimer's disease (AD) is a debilitating neurodegenerative disorder [1, 2]. Pathologically, AD is characterized by amyloid plaques, neurofibrillary tangles (NFTs), and proinflammatory microglia in the brain. Clinically, AD patients usually present with progressive memory decline followed by deterioration of other cognitive domains and activities of daily living. Currently there are no effective treatments for the disease. Several clinical trials targeting single components of AD pathology, such as amyloid- β peptide (A β), have only had modest effects to delay cognitive decline [2]. A drug or a combined therapy targeting multiple key elements in the AD pathological cascade could be a more effective therapeutic strategy.

Recently, we and others have shown that chronic peripheral treatment with human amylin or its clinical analog, pramlintide, reduces amyloid burden in the brain and improve learning and memory in AD mouse models [3, 4]. Amylin is a 37 amino acid, gut-brain axis hormone secreted by the pancreas that easily crosses the blood-brain barrier [5, 6] and mediates important brain functions, including appetite inhibition [7], cerebrovascular structure relaxation [8, 9], and potential neural regeneration [10]. Amylin also plays a role in regulating glucose metabolism [7] and modulating inflammation [11]. The mechanism behind the beneficial effects of amylin treatment for AD has yet to be elucidated. Since amylin binds to the amylin receptor, leading to a cascade of cellular reactions [12], we hypothesized that amylin-like peptides not only reduce brain A β but also affect the entire pathological cascade responsible for AD through binding to amylin receptor.

Although it is unclear yet how amylin treatment influences gene expressions in the AD brain, transcriptome-wide gene expression profiles have been used to find that the gene expression patterns are similar between diabetes and AD brains in both humans and mouse models [13]. This genetic tool was also used to define the transcriptome-genome relationship in healthy and AD [14], and to identify disease-related molecular signatures for drug discovery [15]. The advent of numerous gene ontology and pathway analysis algorithms greatly facilitated the functional annotation of discovered genes and offset the effects caused by the FDRs (false discovery rates) inherent in multiple comparisons of

differential gene expression in AD [16]. Gene network analysis methods, such as the weighted gene coexpression network analysis (WGCNA), were designed to further uncover the genetic connectivity from a systems biology perspective [17, 18]. WGCNA is also a useful bioinformatics tool to understand transcriptional networks in cells and tissues influenced by cytokines or hormones such as progranulin [19].

In the present study, we investigated amylin effects on other aspects of AD pathology, especially neuroinflammation marked by Cd68 expression in microglia. Microarrays with WGCNA analysis was deployed to investigate transcriptome-wide gene expression alterations in the cerebral cortex of an AD mouse model in response to chronic treatment of amylin. Our study demonstrates that amylin probably targets the whole pathological cascade of AD and have the potential to be an effective therapeutic for AD.

MATERIALS AND METHODS

Mouse treatments, RNA extraction, and microarray assays

An AD model, 5XFAD mice (APP mice), and the relevant wild type (WT) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). 5XFAD mice were treated by intraperitoneal (i.p.) injection of human peptide amylin from AnaSpec (Fremont, CA, USA) (APP-amylin) or 1x phosphate-buffered saline (PBS) solution (APP-saline), while WT mice were treated i.p. with 1 × PBS solution (WT-saline), on daily basis for 10 weeks as described by Zhu et al. [5]. Upon completion of the treatments, the mice were sacrificed, and total RNA was extracted from each mouse cortex using an RNA extraction solution, QIAzol lysis reagents, according to the manufacturer's instructions (Qiagen, CA, USA). The extracted RNA was purified by with RNeasy columns (Qiagen, CA, USA). RNA quality was monitored with a Bioanalyzer and processed for analysis by Affymetrix Mouse Gene 1.0 ST chips (Affymetrix, CA, USA). A total of nine microarray profiles, three independent arrays per treatment, were obtained.

Characterization of amylin effects on neuroinflammation in AD

Immunohistochemistry was used to characterize the mouse brain pathology. Mouse brains were removed, post-fixed in 4% paraformaldehyde for 4 h, treated with 30% sucrose (in PBS) for 24 h and then embedded in Tissue-Tek optimal cutting temperature compound. Serial coronal cryosections (8–10 µm) were cut and stored at 20°C. After air drying and washing with PBS, quenching of endogenous peroxidase activity was performed by incubating the sections for 30 min in 0.3% H₂O₂ in methanol followed by PBS washes.

Slides were preincubated in blocking solution (5% [vol/vol] goat serum [Sigma, St. Louis, MO, USA] and 0.3% [vol/vol] Triton-X 100 in PBS) for 1 h at room temperature, followed by incubation in Mouse on Mouse Blocking Reagent (Vector Labs, Inc., Burlingame, CA, USA, MKB-2213) for 1 h. Primary antibodies against different components were incubated individually with the slides overnight. Antibodies against the microglial markers CD68 (mouse anti-CD68 antibody [KP1], 1:300, ab955, Abcam) and Iba1 (rabbit anti Iba1, 1:500, WAKO) were used for microglial immunostaining. For Aβ, mouse mAb 6E10 (SIG-39320, 1:300, Covance, Princeton, NJ, USA) was used. The secondary antibodies used were

biotinylated mouse antibodies (Vector Labs, Inc.; 1:500) for immunohistochemistry. Immunobinding of primary antibodies was detected by biotin-conjugated secondary antibodies and a Vectastain ABC kit (Vector Labs, Inc.) using DAB (3, 3'-diaminobenzidine; Vector Labs, Inc.) as a substrate for peroxidase and counter-staining with hematoxylin. The end products were visualized as eight-bit RBG images using NIS Elements BR 3.2 software (Software/NIS-Elements-Br-Microscope-Imaging-Software) at a total magnification of $\times 40$.

Microglia cell line BV-2 cells were cultured with RP1640 medium, containing 10% FBS in 5% CO₂ at 37°C. BV-2 cells (1×10^5) were seeded into 6-well plates and stimulated with 400 ng/mL LPS (Sigma, MO, USA) followed by the addition of different concentrations of human amylin and incubation for different periods of time as indicated. After the treatment, cells were harvested and washed with 1x PBS solution, and total cellular protein was released by boiling the cells in 80 μ l SDS-PAGE loading buffer (Bio-Rad, CA, USA) for 5 min. Proteins from one-fifth of the total mixture were separated on 4–12% gradient SDS-PAGE gels (Life Technologies, NY, USA), transferred to PVDF membranes (Bio-Rad, CA, USA) and detected via western blots using the appropriate antibodies against Cd68 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and Iba-1 (Wako, Richmond, VA, USA). siRNAs that specifically target receptor activity modifying proteins 1 (Ramp1) or Ramp3 and a mock siRNA control were introduced into BV-2 cells using the transfection reagent siPORT™ AMINE, following the manufacturer's instructions (Life Technologies, NY, USA). The siRNAs IDs were s78669 and s232521 for Ramp1 (Life Technologies, NY, USA) and s80082 and s80083 for Ramp3 (Life Technology, NY, USA). Reverse transcription polymerase chain reaction (RT-PCR) was performed using the method as described by Wang et al. [20].

Mouse microarray data processing

The microarray assays and initial array data quality control analyses were performed by the Boston University Microarray Resource Core Facility. Briefly, the technical quality of the arrays was assessed by RLE and NUSE using the *affyPLM* Bioconductor package (version 1.34.0). The RLE and NUSE values for all arrays were well below the limits for RLE (0.1) and NUSE (1.05). Variations among mice subjected to different treatments were further assessed with one-way analysis of variance (ANOVA) using the *f.pvalue* function in the *sva* package (version 3.4.0). The results indicated that robust variations in gene expression were caused by the different treatments.

The expression levels of probe sets in each chip were normalized with a robust multichip average (RMA) algorithm [21] via the “oligo” R package obtained from Bioconductor. The raw data were deposited into NCBI (<http://www.ncbi.nlm.nih.gov/geo/>) under the entry number, GSE 77373.

After preprocessing, a total of 35,556 probe sets were identified from each individual microarray. Control probe sets and non-annotated probe sets in the Affymetrix database (<http://www.affymetrix.com>) were discarded. Overall, 22,594 probes were assigned ‘gene symbol’ annotations and identified as “genes”. Student's two-sample *t*-test was performed on each of these genes to identify those with significant differential expression patterns in

the APP-amylin versus APP-saline treatments. To gain an independent verification, gene expression changes of representative amylin-influenced genes (AIGs) were validated by RT-PCR and immunostaining.

Human AD microarray data processing

To estimate the possible clinical relevance of our findings on mice to AD in humans, published microarray data from an AD analysis (GSE1297) [16] was used as a reference, hereafter referred to as “the human Alzheimer’s disease study”. The human AD study consisted of microarray experiments assessing differential gene expression in the CA1 region of the hippocampus of 31 individual subjects. The severity of AD in each of the 31 individual subjects was determined by the Mini-Mental State Examination (MMSE) score and the NFT burden value. This resulted in the classification of the individuals into four groups: 9 normal controls, 7 incipient AD, 8 moderate AD, and 7 severe AD cases [16]. The raw microarray data were imported from the Gene Expression Omnibus Database. The expression levels of the probe sets and genes in each chip were normalized with a robust multichip average (RMA) algorithm [21] via the “affy” R package obtained from the Bioconductor.

Gene enrichment analysis

Gene enrichment analyses were performed using the DAVID algorithm (the Database for Annotation, Visualization and Integrated Discovery) [22, 23]. An enriched GO term typically implies that the experimental conditions or treatments produce significant effects on the overall expression of genes in a pathway.

WGCNA

WGCNA was used to functionally group genes into modules (gene clusters) of biologically related genes. These groupings are based on the direct correlation of individual gene pairs and their overall shared relation to other genes in the network [18]. The WGCNA was conducted essentially as previously described [17] using the code provided by Langfelder et al. [18]. WGCNA was performed on the list of 975 AIG, each of which had a significant expression difference ($p < 0.05$) in the APP-amylin versus APP-saline treated mice. Four modules were identified and color-coded. We determined the hub genes for turquoise and yellow modules, i.e., the two modules that were shown to have significant influence on mouse AD symptomatic traits (see below). Briefly, we uploaded the 300 connections with the highest TOs within the module onto the VisANT program [24, 25] and screened out less important genes with connection values within the module (k_{in}) < 0.38 and 0.30 for the turquoise and yellow modules, respectively. Hub genes were then determined to have at least five connections with other genes in the module network.

Statistical analysis

Statistical analyses were performed using R as indicated above.

RESULTS

Amylin treatment attenuates neuroinflammation in the AD brain

We conducted i.p. treatment on two mouse lines on daily basis for 10 weeks: 1) wild type mice treated with saline (WT-saline) and AD mice treated 2) with saline (APP-saline) or 3) with amylin (APP-amylin). We were specifically interested in the amylin effect on Cd68 in the context of the AD pathology since Cd68 is a proinflammatory indicator for activated microglia in the brain but its pathological function and regulation is unclear in AD. Immunohistochemistry staining of Cd68 showed that the AD mouse model (APP-saline) had significantly increased Cd68 expression in microglia cells in the cortex compared to the WT-saline mice, indicating that the AD pathology caused neuroinflammation in the brain (Fig. 1A). Interestingly, amylin treatment reduced the Cd68 expression towards the WT level of Cd68. We verified the amylin treatment decreased the expression of Cd68 both by using RT-PCR (Fig. 1B) and microarray (Fig. 1C).

The amylin-mediated change in Cd68 expression in neuroinflammation was further investigated by using a mouse microglia line, BV-2 cells. We activated BV-2 cells by adding LPS in the presence of different concentrations of amylin for 3 h. As expected, LPS-mediated activation significantly increased the expression of Cd68 (Fig. 2A). The addition of one to 100 μ M amylin attenuated the LPS-induced expression of Cd68 (Fig. 2A). We further incubated BV-2 cells in 100 μ M amylin for different time durations, and the results showed that the optimum treatment effect was observed 3 h after cells were dosed with amylin (Fig. 2B). The addition of amylin also decreased the expression of Iba-1 protein (Fig. 2C), another indicator of microglia cell activation. These results suggest that amylin treatment attenuated the LPS-induced proinflammatory state of the microglia in a dose- and time-dependent manner.

We further studied if amylin effect on Cd68 is mediated by the amylin receptor. The amylin receptor is a heterodimer, consisting of a calcitonin receptor and one of three receptor activity modifying proteins (RAMP) [26]. There are two types of amylin receptors, AMY1 or AMY3, based on whether RAMP1 or 3 is in the complex. We found that BV-2 cells expressed Ramp3, but not Ramp1 (Supplementary Figure 1). We used siRNAs to specifically knock down the expression of RAMPs, thus disrupting the function of their respective amylin receptors AMYs. As shown in Fig. 2D, the siRNA targeting of Ramp3 in BV-2 cells nearly abolished Ramp3 expression, whereas siCK (i.e., a negative control siRNA with random sequences) had negligible effects on the expression of Ramp3. Knocking down Ramp3, but not Ramp1, expression abolished the amylin-mediated inhibition of Cd68 in BV-2 cells (Fig. 2E). Together, these results revealed that amylin treatment reduced Cd68 expression through amylin/Ramp3 receptor in microglia BV-2 cells. These data also provide a molecular mechanism for our observations showing amelioration of the abnormal Cd68 mRNA expression in the brain of AD mouse by amylin treatment (Fig. 1).

Amylin treatment influences the abnormal gene expression pattern linked with Cd68 expression in the AD brain

We then hypothesized that amylin treatment could ameliorate the abnormal pattern of gene expressions linked with Cd68 and with the pathology in the AD mouse brain. Amylin treatment significantly reduced the amyloid pathology in the cortex of APP-amylin mice (Fig. 3A) and also decreased the levels of endogenous phosphorylated tau protein (p-tau) (Fig. 3C). We used microarray assay to show that amylin treatment influenced the expression of a total of 975 genes ($p < 0.05$ as the cut-off criteria as the gene expressions were compared between APP-amylin versus APP-saline mice), as the gene expression level in the WT treatment group was used as a reference. These 975 genes were termed AIGs.

We next performed supervised WGCNA to depict functional networks among the 975 AIGs. As shown in Fig. 3B, we detected four modules from the WGCNA, i.e., blue, brown, turquoise, and yellow, each containing 264, 189, 367, and 155 genes, respectively (Supplementary Table 1). Then, for each module, we summarized the variations in gene expressions into a single value, i.e., the module (ME; Supplementary Table 2), to roughly represent the major variation in the module so that the correlation between a module ME and a sample AD phenotypical trait (e.g., the A β burden or p-tau) or amylin treatment applied to that sample could be determined. As a group of genes, turquoise module had a negative coefficient ($r = -0.93$, $p = 0.0002$) and yellow module had a positive coefficient ($r = +0.97$, $p = 0.000003$), but the other two modules did not show a statistically significant correlation, with A β burden in the brain (Fig. 3D). Consistent with amyloid pathology, while the turquoise module tended to show a negative correlation with endogenous p-tau ($r = -0.63$, $p = 0.07$), the yellow module showed a positive correlation with the levels of endogenous p-tau ($r = +0.76$, $p = 0.02$) (Fig. 3D). These results suggested that the turquoise and yellow modules contained genes involved in AD pathogenesis and were influenced by amylin treatment.

Interestingly the turquoise module included the Cd68 gene expression. Figure 4A and B show the expression heat map for each of the 367 genes in the turquoise module and the summarized ME values across the treatments, respectively. Each mouse in the WT-saline treatment had a positive ME value, while those in the APP-saline treatment possessed negative ME values (Fig. 4B). All mice in the APP-amylin group had ME values between those for the WT-saline and APP-saline mice. These data suggest that amylin treatment brought the expression level of this gene cluster as a functional group, from AD toward the levels observed in the WT mice. We performed a GO enrichment analysis on the gene clusters in the turquoise modules (Supplementary Table 3) and found that consistent with Cd68 function, cellular defense reaction that includes proinflammation was among the 10 GO categories with the best enrichment p values. We further identified 15 hub genes for this module and found that amylin treatment influenced the expression levels of all hub gene expression levels from those observed in the AD mouse model ($p < 0.05$) toward to the ones in WT mice (Supplementary Table 5, Fig. 5A, B). While the expressions of seven of 15 hub genes including Cd68 were significantly higher (Fig. 5A), the expression of the other eight genes were lower (Fig. 5B) in the APP-saline treatment group when compared with the WT-saline treatment group ($p < 0.05$).

Amylin treatment affects the AD gene expressions in another gene module related to metabolism

Since amylin is a gut-brain axis hormone regulating metabolism, we also hypothesized that amylin treatment would influence a group of genes related to brain metabolism. Functional annotation via gene ontology analysis revealed that the yellow module was defined as a gene cluster for mitochondria because the representative GO terms were related to cell energy production and respiration (Supplementary Table 3). Figure 6A shows the expression heat map for each of the 155 genes in the yellow module. While the gene expressions were significantly different between WT-saline and APP-saline, the gene expression patterns between WT-saline and APP-amylin were indistinguishable, indicating that amylin treatment corrected the gene expressions in the yellow module caused by the AD pathology in the brain. Additionally, while each mouse in the WT-saline treatment had a negative ME value, each animal in the APP-saline group possessed a positive ME value, and amylin treatment reversed these abnormal gene expression patterns to ME values similar to WT-saline group (Fig. 6B).

As delineated in Fig. 6C, we identified eight hub genes for the yellow module. While five of the eight hub genes including *Atp13a5* were significantly increased (Fig. 5C) in the APP-saline treatment group when compared with the WT-saline treatment group, the remaining three including *Atp5b*, *Atpaf1*, and *Ppp2ca* were significantly reduced (Fig. 5D) ($p < 0.05$). Again, amylin treatment influenced the expression levels of these hub gene expression levels from those observed in the AD mouse model ($p < 0.05$) toward to the ones in WT mice.

The *Cd68* and *Atp5b* genes influenced by amylin treatment have relevance for the human AD pathology

We used an existing human brain microarray dataset of Blalock et al. [16] to study the relevance of the hub genes targeted by amylin treatment to AD pathology in humans. The dataset from the human AD study was collected from microarrays assays performed on the CA1 region of the hippocampus obtained from postmortem subjects with relatively well-defined clinical and pathological stages. Among the 23 hub genes influenced by amylin treatment in two modules described above, 14 had overlapped human orthologues that had probe sets/genes including *Cd68* and *Atp5b* in the microarrays conducted in humans (Supplementary Table 4).

We found that more than 85% of the hub gene human orthologues were linked with either NFT values or MMSE scores ($r > 0.2000$), and each of them possessed an opposite correlation with NFT versus MMSE (Supplementary Table 4). While *Atp5b*, *Ppp2ca*, *Clip3*, and *Igf1* reached statistical significance for both NFT and MMSE ($p < 0.05$) (Supplementary Table 4), *Cd68*, *Map2k7*, and *Tnpo2* were significantly correlated with NFT, but showed the trends with MMSE. Figure 7 shows the relationships of two representative hub genes, *Cd68* and *Atp5b*, which were influenced by amylin treatment in the AD mouse model, with NFT values and MMSE scores in the human AD study [3]. Compared to the controls, while the expression level of *Cd68* was increased, the expression level of *Atp5b* was decreased in the CA1 region with the increased severity of AD (Fig. 7A, B left panels). While amylin treatment reduced the expression of *Cd68* in an AD mouse model (Fig. 1), *Cd68* expression

was positively associated with NFT (Fig. 7A, middle panel), and only tended to be negatively associated with MMSE scores (Fig. 7A, right panel) in humans. In contrast to Cd68, while amylin treatment increased the expression of Atp5b in an AD mouse model (Fig. 5D), the expression of Atp5b was negatively associated with NFT (Fig. 7B, middle panel), and positively with MMSE scores (Fig. 7B, right panel) in humans. All these suggested that amylin treatment can be beneficial for AD in humans.

DISCUSSION

This study demonstrates that chronic, peripheral amylin treatment attenuated neuroinflammation in addition to reducing typical amyloid pathology in an AD mouse model. Our data indicate that amylin treatment produced strong decreases in the expressions of Cd68 and Iba-1 (Figs. 1 and 2), both of which are accepted markers of microglia activity [27]. Cd68 is a marker for activated microglia [28, 29]. Human neuropathological studies show increased hippocampal expression of Cd68 in AD, as well as in frontotemporal dementia [30]. Although Iba-1 is present in both resting and activated microglia, and its function is unclear [31–33], its levels correlate with pTau pathology and neuronal apoptosis in the AD brain [34, 35]. A recent study demonstrates that Cd68+ and Iba-1+ microglia cells correlate with A β oligomers and reduced synaptic density in AD [36]. The ability of amylin to reduce each of these markers demonstrates the strong anti-inflammatory actions.

Our experiments also demonstrated that the amylin effect on Cd68 expression in microglia through binding the amylin receptor in parallel with negative correlations with Map2k7 expression and tau phosphorylation (Figs. 2 and 4). A recent paper demonstrated that vesicular transport, microglia, and tau phosphorylation are linked cellular activities that spread tauopathy in the AD brain [37]. Cd68 is a heavily glycosylated transmembrane protein and is mainly expressed in late endosomes and lysosomes (the human protein ATLAS 2016). Cd68 is also a member of a scavenger receptor family of proteins that not only functions to clear cellular debris, but also relates to activation of microglia [38]. Although the function of Cd68 protein in activated microglia is unclear and could be just a marker for neuroinflammation in the brain, we posit that because of its proinflammatory state, Cd68+ microglia could be harmful leading to cognitive decline [39], and attenuating its expression in microglia by amylin treatment could be beneficial for AD.

Interestingly, Cd68, an ancient marker for proinflammatory microglia, was identified as a hub gene linked with a group of other genes (the turquoise module) together influenced by amylin treatment to reduce the AD pathology (Figs. 1, 2, 4, and 5). At the late stage, AD brains had increased levels of Cd68 expression (Fig. 7A), but we are cautious regarding its correlation with NFT or MMSE due to the small sample size. We found that turquoise module was functionally categorized as vesicle/transport processes and cellular defense reaction. Hub genes in the turquoise module, such as Cd68, Map2k7, Cfh, Cd4, Igf1, and Orai2, have been implicated to be factors in AD pathogenesis [16, 40–43]. While Cd68 and Cd4 are biomarkers for proinflammatory state or elevated phagocytic activity of microglia, Map2k7 is likely to be involved in the phosphorylation of tau protein. Amylin treatment caused a general reversion in the expression of hundreds of genes related to neuroinflammation, transcription regulation, energy homeostasis, and cellular transport/

vesicle processes in the AD pathological process (Fig. 3). Malfunction of these enriched gene pathways could play critical roles in the pathogenesis of AD. A large amount of A β in the AD brain probably blocks amylin's neuropeptide function, due to their competitive binding for shared amylin receptors [44]. Thus giving exogenous amylin influences the gene expressions in an opposite direction with the AD pathological cascade in the brain and with cognitive decline.

As amylin is metabolic regulating hormone, it is logical that yellow module impacted by amylin treatment was classified in gene clustering as associated with mitochondria processes and AD pathology (Fig. 6). Mitochondria possess a number of functions, including energy metabolism, calcium homeostasis, generation of reactive oxygen species, and apoptotic signaling [33, 45, 46, 49]. Mitochondrial dysfunction and oxidative stress were reported to be early indicators of AD pathogenesis, even detectable in human blood [47]. It is striking that treatment with amylin, a gut-brain hormone-regulating metabolism, almost completely corrected the abnormal expressions of these mitochondria genes in AD (Fig. 6). Inspection of the AIG list (Supplementary Table 1) revealed that two AIGs (Atp5b and Idh3a) in our study were among the hub genes associated with the synaptic mitochondrial module described by [48]. The Atp5b gene encodes a subunit of mitochondrial ATP synthase. The decreased expression of Atp5b has been correlated with the severity of AD [16, 49]. In situ hybridization data indicated that in adult mouse brains, Atp5b is primarily present in the hippocampal region [50] and that the Atp5b protein interacts with Atpaf1 (Breast Cancer Database, <http://www.itb.cnr.it/breastcancer/php/geneReport.php?id=64756>), another hub gene in the yellow module (Fig. 6C). Ppp2ca, a yellow module hub gene, is a catalytic subunit of the holoenzyme Pp2a and binds to and dephosphorylates phosphorylated tau protein [51]. The dysfunction of Ppp2ca has been implied to contribute to neuronal tau pathology, general inflammatory processes, and amyloidogenesis. Future work is necessary to further elaborate the functional interactions among the hub genes in relation to the pathogenesis of AD.

AD is a neurodegenerative disorder that may take decades to develop clinical symptoms and thus evaluation of the drug effects for AD requires large numbers of patients and extended treatment periods in clinical trials [52]. Current cell- and animal-based disease models targeting a single component of AD pathology are poor at translating a positive AD treatment response in humans. To help bridge the gap between AD mouse models and costly clinical trials, gene expression studies focused on the entire AD pathological cascade rather than a single component of AD pathology may be of value. Together with recent novel findings on amylin's positive effect on cognition and neuropathology [3, 4], the current study provides transcriptomic evidence on proinflammation and mitochondria function to further support that amylin type peptides have a therapeutic potential for AD.

The beneficial effects of amylin might be surprising to some because that amylin deposits have been observed in the pancreas in type 2 diabetic patients [53] and that presence of amylin associated with amyloid deposits and the cerebrovascular system in AD [54]. While our study shows the benefits of amylin for AD, we have no doubt that an extremely high concentration of human amylin leads to aggregation and causes neurotoxicity. It is shown that the concentration (50 μ M) of amylin can cause neurotoxicity in rat cortical neurons after

four days' exposure in another study [55], but the concentration of exogenous amylin we gave to the mice was much lower than this and the average fasting plasma amylin concentrations are in the range of 4–25 pmol/l in healthy humans [56]. Thus the potential benefits of amylin type peptides as a physiological neuropeptide and as a drug at a right concentration against the AD neurodegeneration should not be ignored. Indeed, we did not observe increased amyloids in the brain by amylin treatment (data not shown). The pharmaceutical industry optimized the protective actions of amylin by substituting prolines at positions 25, 28, and 29 of human amylin, which prevent amylin from oligomerizing or aggregating [57]. The approval of the amylin analog pramlintide by the FDA combined with its safe profile and successful use for the treatment of type 2 diabetes emphasizes the clinically beneficial actions of amylin type peptides [12, 58, 59]. The importance of diabetes as a risk factor for AD [60] highlights yet another potentially beneficial action of the amylin cascade in AD and this action was examined in the current study. Pramlintide is also found to regulate vascular and inflammatory reaction [61]. Ultimately, whether amylin type peptides can be a new and novel avenue of therapeutic for AD should only be concluded through a double blind, placebo controlled clinical trial in humans [44].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Amylin treatment reduced the Cd68 gene expression in the Alzheimer's disease mouse cortex. A) Cd68 expression in microglia cells at cortex is stained by using immunohistochemistry. Illustrated in (B) is the Cd 68 mRNA level via RT-PCR amplification by primer set specific to Cd68 gene. Gapdh was amplified as loading control. Bar graphs in (C) depicted RT-PCR quantification versus mouse array values across the samples/treatments for individual genes shown in (A). * $p < 0.05$ and ** $p < 0.01$ indicate statistical significance.

Fig. 2.

Amylin downregulates the Cd68 expression in microglia BV-2 cells. A) BV-2 cells were cultured in absence or presence of LPS; and various concentrations of amylin. Three hours post treatment, total cellular protein was extracted and analyzed by western blotting with an antibody against Cd68. Gapdh was used as the loading control. B) BV-2 cells were treated with 0 or 100 nM human amylin in the presence or absence of LPS for different time periods. Western blots using antibodies against Cd68 or β -actin (loading control) were performed on total cellular protein extracted from cells harvested at different time points. C) LPS-activated BV-2 cells were treated with 0 or 100 nM amylin. Total cellular protein was extracted from cells harvested three hours post treatment and probed by antibodies to Iba-1 and Gapdh (loading control). D) RT-PCRs using primer sets specific to Ramp3 or Gapdh (loading control) were performed on total RNAs prepared from BV-2 cells transfected with different siRNAs as indicated. E) LPS-activated BV-2 cells were treated with siRNAs specific to Ramp1 (E, left panel) or Ramp3 (E, right panel) for 48 hours. LPS, and amylin or mock treatment (1x PBS) were added 3 hours before the cells were harvested. Western blots using antibodies against Cd68 or β -actin (loading control) were performed on the total cellular protein harvested. Shown here are one representative western blot pictures of three independent experiments.

Fig. 3.

Amylin treatment on Alzheimer's disease neuropathology and WGCNA analysis of the treatment data. A) Amylin treatment reduced the amyloid pathology in the brain. Upper panel, Aβ staining of the cerebral cortex of WT-saline (upper), APP-saline (middle), and APP-amylin (bottom). Bottom panel, bar graph summarizing total area of amyloid burden in mouse cortex for different treatment groups. Amyloid burden in APP-saline mice was set to one. **stands for significance at 1% level. B) Clustering analysis and module detection on the gene expressions by amylin treatment. WGCNA was performed using the 975 AIGs, and gene modules were detected from the dendrogram using a dynamic tree-cutting algorithm. Same color clusters were combined. A total of four modules were identified, and labeled by different colors: blue, brown, turquoise, and yellow, each containing 264, 189, 367, and 155 genes, respectively. C) Amylin treatment reduced endogenous tau protein phosphorylation in mouse cortex. Upper panel, western blot using antibodies against phosphoserine 202 tau (CP-13) and β-actin (loading control) on total cellular protein extracted from mouse cortex (top panel). Bottom panel, bar graph summarizing the degree of tau protein phosphorylation across different treatment groups. The amount of phosphorylated tau protein was divided by the amount of β-actin, resulting in the phosphorylated tau percentage (p-tau, %). The average p-tau from 3 different mice per treatment was calculated. The p-tau in WT-saline was set as one. *stands for significant at 5% level while ns, not significant. D) Correlation between Module eigengenes (MEs) and Alzheimer's disease pathology. MEs, the first

principal component, across all nine samples/treatments were calculated, and their correlations with samples traits were determined. Numbers shown in each block are the correlation coefficients with a p value in the brackets. Blocks were painted with different colors, representing the degrees of significance, i.e., red, positive and green, negative.

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Fig. 4.

Gene network analysis for turquoise module under amylin treatment in Alzheimer's disease mice. A) Heat map showing the expression of all 367 genes in the module were influenced by amylin treatment. Each row represents one gene; each column represents one sample. Colors within the heat map depict the relative gene expression level, with red indicating a high expression level whereas green represents a low level of expression. B) The weighted summary of gene expression is exhibited and shows that amylin treatment brought the expression level of this gene cluster from Alzheimer's disease toward the levels observed in the WT mice. The bar height in the bar plot describes the ME value for each sample. C) Gene networks within the module were revealed by the program VisANT [27, 28] to reveal the gene expressions and their connections influenced by amylin treatment. The hub genes are represented by enlarged pentagons in blue, with the exception of Cd68 in red, while other genes are depicted by squares in dark green. The occurrence of a connection between genes is described by lines in turquoise while those connections to Cd68 are depicted in purple.

Fig. 5. Relative expression of hub genes under amylin treatment in Alzheimer's disease mice by using mouse microarray assays. (A) and (B) for the hub genes in the turquoise module while (C) and (D) for those hub genes in the yellow module. Sample averages across treatments for hub genes that were upregulated (A, C) or downregulated (B, D) in APP-saline versus WT-saline treatments, respectively. For each of the hub genes, the sample average expression level in WT-saline was set as one. For all hub genes in A, B, C, and D, significant differences were observed in APP-saline versus WT-saline treatments and APP-saline versus APP-amylin treatments with a p value < 0.05 (Supplementary Table 4).

Fig. 6.

Gene network analysis for yellow module under amylin treatment in Alzheimer's disease mice. A) Heat map showing the expression of all 155 genes in the module were influenced by amylin treatment in Alzheimer's disease mice toward to the levels of wild type mice. Each row represents one gene; each column represents one sample. Colors within the heat map depict the relative gene expression level, with red indicating a high level of expression, whereas green represents a low level of expression. B) The weighted summary of gene expression shows that the ME values in Alzheimer's disease mice were not distinguishable from the ones in wild type mice. The bar height in the bar plot indicates the ME values for each sample. C) Gene networks within the module were revealed by the program VisANT [27, 28]. The hub genes are represented by enlarged pentagons in blue, except for *Atp5b*, which is represented by an enlarged red square, while other genes are depicted by squares in dark green. The occurrence of a connection between genes is indicated by lines in turquoise while the connections to *Atp5b* is depicted in purple.

Fig. 7.

The gene expressions influenced by amylin treatment have relevance in Alzheimer's disease in humans. Relative expression of hub gene human orthologue groups with different Alzheimer's disease severities, and correlation with NFT and MMSE in the published human Alzheimer's disease study. An existing human brain microarray dataset [3] was used. Two hub genes, Cd68 (A) and Atp5b (B), from the turquoise and yellow modules for amylin treatment, respectively, were chosen to study their possible relevance with Alzheimer's disease. For each hub gene, array intensities across subjects were obtained and averaged for different groups: C, control subjects; I, subjects with incipient Alzheimer's disease; M, subjects with moderate Alzheimer's disease; and S, subjects with severe Alzheimer's disease. The average expression of the control group was set to one, and the expression levels of other groups relative to controls are shown. T-test was used to compare the control and a disease group with different severities. * $p < 0.05$ and ** $p < 0.01$ indicate statistical significance. The associations between gene expression and neurofibrillary tangles (NFT; middle panels) or Mini-Mental State Examination (MMSE; right panels) are shown. Trend lines are indicated in each graph on the middle and right panels. Numbers inside the inset box on the middle and right graphs are the correlation coefficients and p values, respectively.