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Neuroprotective effects of the amylin analogue pramlintide on Alzheimer's disease pathogenesis and cognition

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ABSTRACT

Amylin is a metabolic peptide hormone that is co-secreted with insulin from beta cells in the pancreas and activates many of the downstream targets of insulin. To investigate the relationship between this hormone and Alzheimer's disease (AD), we measured plasma human amylin levels in 206 subjects with AD, 64 subjects with mild cognitive impairment, and 111 subjects with no cognitive impairment and found significantly lower amylin levels among subjects with AD and mild cognitive impairment compared with the cognitively intact subjects. To investigate mechanisms underlying amylin's effects in the brain, we administered chronic infusions of the amylin analog pramlintide in the senescence-accelerated prone mouse, a mouse model of sporadic AD. Pramlintide administration improved performance in the novel object recognition task, a validated test of memory and cognition. The pramlintide-treated mice had increased expression of the synaptic marker synapsin I and the kinase cyclin-dependent kinase-5 in the hippocampus, as well as decreased oxidative stress and inflammatory markers in the hippocampus. A dose-dependent increase in cyclin-dependent kinase-5 and activation of extracellular-signal-regulated-kinases 1/2 by pramlintide treatment in vitro was also present indicating functionality of the amylin receptor in neurons. Together these results suggest that amylin analogs have neuroprotective properties and might be of therapeutic benefit in AD.

1. Introduction

A growing body of evidence demonstrates an association between metabolic abnormalities and Alzheimer's disease (AD). Epidemiologic studies have demonstrated that patients with type 2 diabetes are at significantly increased risk of developing AD (Arvanitakis et al., 2004; Xu et al., 2004). This association is further supported by serum studies that demonstrate altered levels of a number of metabolic hormones in patients with AD, including insulin, (Craft et al., 1998; Meneilly and Hill, 1993) cortisol, (Lupien et al., 1994), and leptin (Lieb et al., 2009). Moreover, neuropathologic studies demonstrate altered insulin signaling in AD brains consistent with insulin resistance (De la Monte and Wands, 2008; Rivera et al., 2005; Steen et al., 2005). A more central role of insulin signaling in AD is suggested by recent clinical studies that demonstrate an improvement in cognitive function in patients with AD following chronic administration of insulin (Craft et al., 2012). Two other metabolic hormones that activate insulin-related signaling pathways, leptin, and glucagon-like peptide 1 have also been shown to improve memory in vivo (Greco et al., 2010; McClean et al., 2011; Tezapsidis et al., 2009). Therefore, restoring insulin signaling in the brain with insulin or related metabolic hormones might provide a therapeutic benefit to patients with AD.

Amylin is a peptide hormone that is co-secreted with insulin from beta cells in the pancreas (Mitsukawa et al., 1990). Known effects of amylin include inhibiting glucagon (Gedulin et al., 1997), delaying gastric emptying (Young et al., 1995), and inducing satiety

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(Morley and Flood, 1991). Interestingly, human amylin has aggregative properties and is toxic in vitro due to its tendency to form amyloid fibrils (May et al., 1993). Indeed, amylin oligomers and plaques were recently reported in temporal lobe gray matter and vasculature in diabetic and nondiabetic patients with AD at autopsy, and these were independent of amyloid-β plaques (Jackson et al., 2013). However, rat and mouse amylin, which differ from the human form by 6 substitutions, and the synthetic analog pramlintide (Symlin), are nontoxic forms of amylin that do not aggregate (May et al., 1993; Nonoyama et al., 2008) and activate many of the metabolic pathways in the central nervous system (CNS) that are thought to be beneficial for memory, weight loss, mood, and other central functions. To this end, the synthetic amylin analog pramlintide causes clinical weight loss and is an approved adjunct to insulin to improve glycemic control in diabetic patients (Hollander et al., 2003; Smith et al., 2008).

The blood–brain barrier is permeable to amylin (Banks et al., 1995) and brain uptake of amylin in mice is almost 3 times greater than brain uptake of insulin (Banks and Kastin, 1998). Amylin receptors are distributed widely throughout the CNS, with the highest density of amylin binding in the area postrema, nucleus of the solitary tract, parabrachial nucleus, amygdala, hypothalamus, nucleus accumbens, and dorsal raphe (Sexton et al., 1994). Amylin also has anxiolytic and antidepressant-like (Laugero et al., 2010; Roth et al., 2009) effects and analgesic properties, (Huang et al., 2010) although the mechanism underlying these effects is not fully understood. In peripheral tissue and in the CNS, amylin interacts with the insulin signaling cascade and activates several downstream targets of insulin, including signal transducer and activator of transcription 3, AMP-activated protein kinase, and Akt, (Moon et al., 2011) cascades involved in cellular metabolism and survival (Dudek et al., 1997; Hirano et al., 2000; Mihaylova and Shaw, 2011). In addition to the insulin-signaling pathway, amylin is also a known modulator of the ERK/MAP Kinase pathway (Moon et al., 2011; Potes et al., 2012), a cascade that is thought to underlie amylin's effects on satiety (Potes et al., 2012) and that has been implicated in synaptic plasticity and memory consolidation in the hippocampus (English and Swede, 1997; Schafe et al., 2000).

Given amylin's multiple metabolic targets in the insulin signaling pathway of relevance to neuroplasticity and its newly recognized accumulation in the AD brain, we investigated how levels of circulating amylin might relate to AD diagnosis and sought to better understand the mechanisms by which this emerging relationship might occur. We first studied the association between plasma human amylin levels and AD in a large cohort of subjects with AD, mild cognitive impairment or normal cognition. To determine the direct effects of amylin on cognition and AD pathogenesis, we studied the effects of the amylin analog pramlintide on memory in the senescence-accelerated prone (SAMP8) mouse, a rodent model of accelerated senescence that is an useful model of sporadic AD (Morley et al., 2012a, 2012b; Pallas et al., 2008). We investigated signaling changes in vitro to verify receptor functionality and in vivo to determine potential mechanisms of relevance to cognitive improvement.

2. Methods

2.1. Human amylin plasma analysis

All subjects were community-based volunteers who were individually recruited to participate in plasma donation and cognitive evaluation at the University of Pennsylvania's Alzheimer's Disease Center. Verbal informed consent was obtained from all study participants at the time of enrollment. Subjects were eligible to participate if they were aged above 50 years and in generally good health. Subjects underwent cognitive and neurologic examinations by experts in the evaluation of neurodegenerative dementias according to National Alzheimer's Coordinating Center's protocols. This included neuropsychological testing of multiple cognitive domains, physical and neurologic examinations, and history obtained from the subject and a research partner. Of the 450 participants who were initially enrolled in our study, 49 participants had cognitive impairment primarily due to a condition other than AD or MCI and were excluded from analysis (e.g., psychiatric, frontotemporal dementia, Lewy body dementias, medication-induced). The remaining subjects were classified into principal neuropathologic diagnosis groups according to established criteria such as: normal cognition, MCI, or AD (McKhann et al., 1984; Petersen, 2007). Of the 64 subjects classified as MCI, 43 were amnestic MCI, 5 were single domain nonamnestic MCI, and 12 were MCI not otherwise specified. The study protocol was approved by the Institutional Review Board at the University of Pennsylvania.

Blood samples were obtained during initial clinical evaluation or at a scheduled time for biofluid donation for research purposes. All samples were collected during the daytime without prior overnight fasting. Plasma was collected in 10 mL BD Vacutainer K2EDTA (Franklin Lakes, NJ) and centrifuged at 4 °C into plasma and cellular components. Plasma was subsequently stored at −80 °C in 1 mL polypropylene vial aliquots until the time of analysis.

A commercial enzyme-linked immunosorbent assay (ELISA) kit (Millipore [Billerica, MA] Human Amylin ELISA) was used to determine the concentration of amylin following standard ELISA kit procedures. The ELISA capture antibody recognizes amylin and deamidated amylin (1–20 fragment), but not reduced amylin. A 4-parameter logistic equation was used for the dose-response curve of this assay. The lower sensitivity limit of the assay was 1 pM and the interassay coefficient of variation ranged from 3.7% to 6.9%.

2.2. Animals and drug treatment

The senescence-accelerated prone mouse was selected as a model of age-related dementia because it displays multiple features of AD-like neurodegeneration including severe deficits of learning and memory, increased oxidative stress, cortical atrophy, amyloid-β accumulation (Del Valle et al., 2010), and tau phosphorylation (Butterfield and Poon, 2005; Kawamata et al., 1997; Pallas et al., 2008; Takeda et al., 1994). SAMP8 mice were obtained from an established colony at Case Western Reserve University. All animals were group housed, provided ad libitum access to a standard diet and water, and were exposed to a light-dark (12:12) cycle. The mice were housed under pathogen-free conditions at a temperature of 21 ± 1.5 °C. All animals were treated in accordance with the protocols by The Institutional Animal Care and Use Committee of Case Western Reserve University.

Twenty SAMP8 mice equally distributed by sex were treated with either pramlintide (n = 10) or saline (n = 10) infusions beginning at 6 months of age for a total of 5 weeks. A subcutaneous ALZET osmotic minipump (Model 2002, Durect Corp., Cupertino, CA, USA) was surgically implanted into all mice as described previously (Fewlass et al., 2004). The osmotic mini-pump infused 0.24 mg/kg per day pramlintide acetate (0.6 mg/mL pramlintide, Amylin Pharmaceuticals, Inc, San Diego, CA) or saline at a rate of 0.5 μL/hr. Pumps were replaced with new refilled pumps every 2 weeks throughout the study duration. All animals were weighed weekly throughout the course of the experiment.

2.3. Object recognition task

Behavioral testing with the object recognition task occurred during the last week of the 5-week treatment period when the mice were 7 months old. The object recognition task was performed as

described previously (Bevins and Besheer, 2006). The testing set-up involved 4 adjacent open-field boxes measuring 20 × 20 × 17 × 4 in (San Diego Instruments, San Diego, CA) illuminated by indirect dim lighting. On the day before testing, mice were individually placed in 1 of the boxes for 15 minutes for habituation. The following day during the training session, the mice were again placed into 1 of the boxes but this time with 2 plastic sample objects approximately 12 in apart. The mice were allowed to explore the environment for 10 minutes, during which their movements were recorded with a tracking system. The box in which animals were placed was counterbalanced by treatment to avoid location bias. After each trial, the objects and open-field were cleaned with 70% ethanol to eliminate any olfactory cues. The retention test was conducted 3 hours later. The mice were once again placed in the open-field, but this time a novel object of similar size and complexity replaced 1 of the objects that was present during the training session. The mice were allowed to explore the environment for 5 minutes, after which they were returned to their cages.

Video recordings of the retention session were scored by an investigator blinded to the treatment groups. Object exploration, defined as the duration of time in which the head of the mouse faced less than half cm from the object, was measured during the training and retention sessions. The frequency and time spent rearing and grooming were also recorded to determine general exploratory behavior. Mice that spent less than 5 seconds total exploring the objects and mice that exhibited stereotypic behavior such as spinning were discarded from the analysis. The recognition index was calculated as the percentage of time spent exploring the novel object versus the total time spent exploring the objects.

2.4. Protein extraction and western blotting

The same SAMP8 mice that were tested in the object recognition task were sacrificed by lethal overdose of Avertin (10 g tribromoethanol, 10 mL tert amyl alcohol) (Acros Organics, Geel, Belgium) (500 mg/kg) 5 weeks after the initiation of the pramlintide and saline treatments. The brains were harvested and the hippocampus was dissected and homogenized in protease and phosphatase inhibitor-supplemented 1× RIPA lysis/extraction buffer (Pierce, Rockford, IL). Total protein was quantified using the BCA Protein Assay Kit (Pierce). Protien (10–20 μg) was run in a 10% sodium dodecyl sulfate- polyacrylamide gel electrophoresis SDS-PAGE gel and the protein was transferred onto Polyvinylidene fluoride membranes (Millipore). After blocking for 1 hour in 5% milk, the membranes were incubated overnight at 4 °C in the primary antibody at a 1:1000 dilution, followed by 1-hour incubation with horseradish peroxide (HRP)-conjugated IgG at a 1:10,000 dilution. Primary antibodies used were cyclin-dependent kinase-5 (CDK5) (Millipore), P35/P25 (Millipore), Synapsin I (Santa Cruz, Dallas, TX), heme-oxygenase-1 (HO-1, [Gift from Drs Smith/Zhu]), pERK1/2, and total ERK1/2 (Cell Signaling). The blots were developed using Immobilon Western Chemiluminescent HRP Substrate (Millipore) and imaged using FluorChem M (ProteinSimple, Santa Clara, CA). Quantifications were performed using ImageJ 1.44 software.

2.5. Cellular stress immunohistochemistry

To further explore pramlintide's anti-inflammatory properties, the effects of pramlintide on the cellular stress proteins 4-Hydroxynonenal (HNE) and cyclooxygenase 2 (COX-2) were evaluated. Twelve mice were used, 6 of which received chronic infusions of pramlintide, and 6 of which received saline infusions for 2 weeks. At the end of the 2 weeks, the mice were deeply anesthetized and transcranially perfused using 4% paraformaldehyde. The brains were extracted and postfixed in 4% paraformaldehyde for 24 hours, then 30% sucrose for 3 days. All brains were quick-frozen, cut into 40 μm sagittal sections, and stored as floating sections.

Three hippocampal sections were selected from each mouse (n = 6 per group) for staining. Staining and quantification were conducted according to established protocols as previously described (Greco et al., 2010). Briefly, floating sections were incubated in 1% H_2O_2 for 1 hour to block endogenous peroxidase activity, then blocked in 3% normal goat serum (NGS) + 0.5% Triton for 1 hour, incubated with the primary antibodies HNE and COX-2 (Gifts from Dr Smith/Zhu) at a 1:200 dilution overnight, then incubated with the secondary antibody Biotinylated Anti-Rat IgG for 1 hour. The ABC reagent and chromogen diaminobenzidine Vector Kit (Vector Labs, Burlingame, CA) were used to complete the staining. A single highly skilled operator (HH) blinded to treatment group captured 10× images of 6 fields in the dentate gyrus and CA1 regions using a light microscope equipped with the QCapture Pro Imaging System (QImaging, British Columbia, Canada). The captured fields were preselected using gross landmarks to prevent repeated analysis of the same field. Percentage of staining of these images was quantified using Metamorph software, version 6.3 (Molecular Devices, Sunnyvale, CA).

2.6. Primary rat cortical neuron culture

Primary cortical neuron cultures were grown from Sprague/Dawley rat brains at embryonic day 18 as previously described (Lee et al., 2009). Neuron cultures were grown in 6 well plates coated with Poly-D-Lysine/Laminin (BD, Franklin Lakes, NJ) in neurobasal medium supplemented with 2% B27 (Invitrogen, Carlsbad, CA) per 0.5 mM glutamine. Cultures were maintained at 37 °C in a humidified, 5% CO_2 atmosphere for 6 days. On day 7 cultures were treated in parallel with pramlintide (Sigma) for 1 or 3 hours with concentrations (nM) of 0, 10, 100, 300, 500, and 1000. All treatment groups were run in duplicate. After treatment, cells were lyzed, collected, and frozen for immunoblotting as described previously. Experiments were repeated twice.

2.7. Statistics

For the human plasma analysis, amylin levels had a skewed distribution and were therefore logarithmically transformed for all statistical analyses and are reported as log-transformed values. The associations of amylin with demographic, clinical, and potential confounder variables were examined using Pearson correlations for continuous variables and analysis of variance for categorical variables. Multinomial logistic regression analysis was used to determine the relationship between logarithmically transformed amylin level and disease category membership, with the adjustment for possible confounders including age, sex, education, apolipoprotein E4 genotype, and diabetes. Data from mouse studies, including the object recognition task, western blotting, and cellular stress immunohistochemistry, were analyzed using a Student *t* test. The cell culture experiments were analyzed using a bivariate fit model using the log of pramlintide concentrations as the independent variable. All statistical analyses were performed using JMP 9.0 for Windows (SAS Institute Inc, Cary, NC, USA). The *p*-values <0.05 were considered significant and all statistical tests were 2-sided. Results are depicted as mean ± standard error of mean.

3. Results

3.1. Circulating plasma amylin is reduced in human subjects with MCI and AD

Circulating plasma amylin levels were measured in 206 subjects with AD, 64 subjects with MCI, and 111 subjects with no cognitive

Table 1
Demographic and clinical characteristics of the participant groups

Charateristics	AD	MCI	Cognitively intact
N	206	64	111
Age	74.7 (7.7)	71 (8.6)	70.2 (10.0)
Age range	49–94	50–86	48–93
Female	123/59.7%	34/53.1%	75/67.6%
Years of education	13.9 (4.0)	13.0 (5.4)	15.6 (3.5)
APO E4+	127/59.9%	29/45.3%	31/25.4%
BMI	26.5 (5.7)	27.3 (5.3)	26.2 (3.93)
Diabetes mellitus	85/41.3%	38/59.4%	55/49.6%
Hypertension	77/37.4%	28/43.8%	57/51.4%
Hyperlipidemia	88/42.7%	37/57.8%	56/50.4%
MMSE	22.9 (4.2)	25.8 (3.8)	29.0 (1.4)

Key: AD, Alzheimer's disease; APO E4, apolipoprotein E; BMI, body mass index; MCI, mild cognitive impairment; MMSE, Mini Mental State Examination.

impairment. Demographic and clinical characteristics of the participant groups are displayed in Table 1. The average plasma amylin for all patients in our study was 0.77 (standard deviation = 0.69). We assessed possible confounders but found no statistically significant relationships between plasma amylin concentration and age ($F_{[1,379]} = 0.021$; $p = 0.88$), sex ($F_{[1,379]} = 1.45$; $p = 0.22$), or years of education ($F_{[1,379]} = 0.006$; $p = 0.93$) across the entire cohort or within any diagnostic category.

Mean plasma amylin levels in each participant group are depicted graphically in Fig. 1. In between-group comparisons, plasma amylin levels were significantly lower in the AD group ($F_{[1,315]} = 4.44$, $p = 0.036$) and the MCI group ($F_{[1,173]} = 5.88$, $p = 0.016$) as compared with the cognitively intact group. There was no significant difference in amylin levels between the MCI and AD groups ($F_{[1,268]} = 0.64$; $p = 0.43$). Using a multivariable regression analysis, we repeated these between-group comparisons with adjustment for age, sex, and years of education. In this multivariate analysis, plasma amylin levels remained significantly lower in the AD group ($F_{[4,311]} = 2.51$; $p = 0.012$) and the MCI group ($F_{[4,170]} = 2.57$, $p = 0.011$) compared with the cognitively intact group.

We repeated these multivariable regression analyses with adjustment for 2 additional possible covariates, the apolipoprotein E4 genotype and diabetes. Apolipoprotein E4 is a class of apolipoprotein that is highly associated with AD (Poirier et al., 1993). In our sample, there was a nonsignificant trend toward higher plasma amylin levels in carriers of the apolipoprotein E4 genotype ($F_{[1,376]} = 3.56$; $p = 0.06$) and among subjects with diabetes ($F_{[1,307]} = 3.00$; $p = 0.084$). After additionally adjusting for all of these possible covariates, significant differences in plasma amylin levels remained between the 3 diagnostic groups ($F_{[6,302]} = 3.69$; $p = 0.026$).

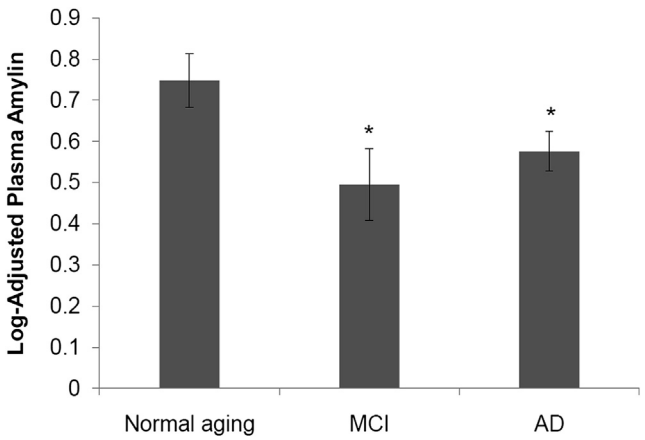

Fig. 1. Plasma amylin levels measured in human subjects categorized as cognitively intact (n = 111), mild cognitive impairment (MCI) (n = 64), or Alzheimer's disease (AD) (n = 206). Amylin levels were significantly lower in the AD group ($p = 0.036$) and the MCI group ($p = 0.016$) compared with the cognitively intact group, with no significant difference in amylin levels between the AD and MCI groups ($p = 0.43$). Multinomial logistic regression analysis was used to determine the relationship between logarithmically transformed amylin level and disease category membership. The results are depicted as mean ± standard error of mean (SEM). (* = $p < 0.05$). Abbreviations: AD, Alzheimer's disease; MCI, mild cognitive impairment; SEM, standard error of mean.

3.2. Chronic pramlintide treatment causes weight loss in SAMP8 mice

To investigate the effects of pramlintide on memory, SAMP8 mice were continually infused with saline or pramlintide for a total of 5 weeks. Consistent with pramlintide's known anorexic effect (Morley and Flood, 1991), the pramlintide-treated mice experienced a change in body weight of −5.8% ± 1.3% over the course of the treatment period as compared with a weight change of −0.1% ± 1.8% for the saline-treated mice. This difference in weight loss between the 2 groups was statistically significant ($t = 2.54$, $p = 0.019$) (data not shown).

3.3. Chronic pramlintide treatment improves object recognition memory in SAMP8 mice

During the last week of treatment, all mice were tested in the object recognition task to assess pramlintide's effects on memory. The object recognition task takes advantage of the natural tendency of mice to explore a novel object more than a familiar object to quantify recognition memory (Bevins and Besheer, 2006). The pramlintide-treated SAMP8 mice spent a greater proportion of time exploring the novel objects as compared with the familiar objects (recognition index of 0.67 ± 0.02), whereas the saline-treated mice did not differ in time spent with the novel and familiar objects (recognition index of 0.50 ± 0.07). This difference was statistically significant (Fig. 2, $t = 2.40$, $p = 0.029$). There were no differences in the total time spent exploring the objects ($t = 0.30$, $p = 0.77$) or nonspecific exploratory behaviors such as time spent rearing ($t = 1.14$, $p = 0.27$) or grooming ($t = 0.39$, $p = 0.70$) between the pramlintide and saline-treated groups.

3.4. Chronic pramlintide treatment reduces markers of inflammation in the hippocampus

We used western blotting to quantify levels of HO-1, a well-known stress-related enzyme that is a sensitive marker of

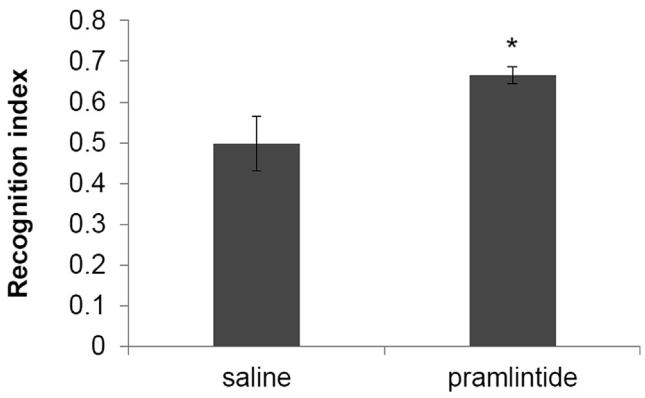

Fig. 2. Chronic treatment of SAMP8 mice with pramlintide significantly increased recognition memory in the object recognition task compared with saline-treated controls ($p = 0.029$). Recognition memory is reflected by the recognition index, which is the ratio of time spent exploring the novel object relative to the total time exploring the objects. The results are depicted as mean ± standard error mean (SEM). (Student t test, * = $p < 0.05$; n = 9 per group). Abbreviation: SEM, standard error of mean.

oxidative stress (Poon et al., 2004). Pramlintide-treated SAMP8 mice had significantly decreased expression of the protein HO-1 in the hippocampus compared with saline-treated mice, a difference that was statistically significant (Fig. 3A, $t = 2.31$, $p = 0.035$). To further explore the effects of pramlintide on oxidative stress and inflammation, we used immunostaining in the hippocampus to quantify levels of COX-2 and HNE, 2 markers of inflammation and cellular stress (Esterbauer et al., 1991; Ho et al., 1998). We demonstrate significant decreases in COX-2 expression ($t = 2.37$, $p = 0.042$) (Fig. 4A nonsignificant trend toward decreased HNE expression ($t = 1.90$, $p = 0.090$) (Fig. 4B) in the hippocampus of mice treated with pramlintide for 2 weeks compared with saline-treated mice.

3.5. Pramlintide increases synaptic protein expression in the hippocampus

Hippocampal tissues from SAMP8 mice treated with pramlintide for 5 weeks were examined by western blotting to determine its effects on synaptic integrity. Pramlintide was found to significantly increase hippocampal expression of synapsin I ($t = 3.38$, $p = 0.004$) (Fig. 3B), a protein located in neuronal synaptic vesicles that can be used as a marker of synaptic density (Moore and Bernstein, 1989). To investigate a potential mechanism underlying pramlintide's effects on synaptic density, we explored the possibility that pramlintide might alter CDK5, a protein that is strongly implicated in both neuron (Lagace et al., 2008) and synapse (Samuels et al., 2007) growth and is modulated by many growth factors (He et al., 2009). In hippocampal whole-tissue homogenates of SAMP8 mice treated with pramlintide for 5 weeks, expression of the protein CDK5 was significantly increased relative to the saline-treated group (Fig. 3C, $t = 5.01$, $p = 0.002$). There were no differences in the amount of the CDK5 activator p35 ($t = 0.64$, $p = 0.53$), its cleavage product p25 ($t = 0.22$, $p = 0.83$), or the ratio of p25/p35 ($t = 0.85$, $p = 0.41$).

3.6. Pramlintide directly increases CDK5 expression in primary cortical neuronal cultures

To determine whether pramlintide has direct effects on neurons, CDK5 expression in primary cortical neuron cultures was measured using western blot after incubation with pramlintide. Pramlintide dose-dependently increased expression of the protein CDK5 after 1-hour ($F = 5.27$; $p = 0.045$) and 3-hour incubations ($F = 7.0$; $p = 0.025$) (Fig. 5A). As a positive control, we also measured pramlintide's effects on pERK, a known downstream target of pramlintide (Potes et al., 2012). As expected, pramlintide increased expression of pERK at 3 hours of incubation ($F = 10.50$; $p = 0.009$) (Fig. 5B).

4. Discussion

Our data demonstrate, for the first time, that there is a significant association between low plasma amylin levels and MCI or AD in a sample of older adults. This association is surprising because amylin levels are positively associated with a number of classic risk

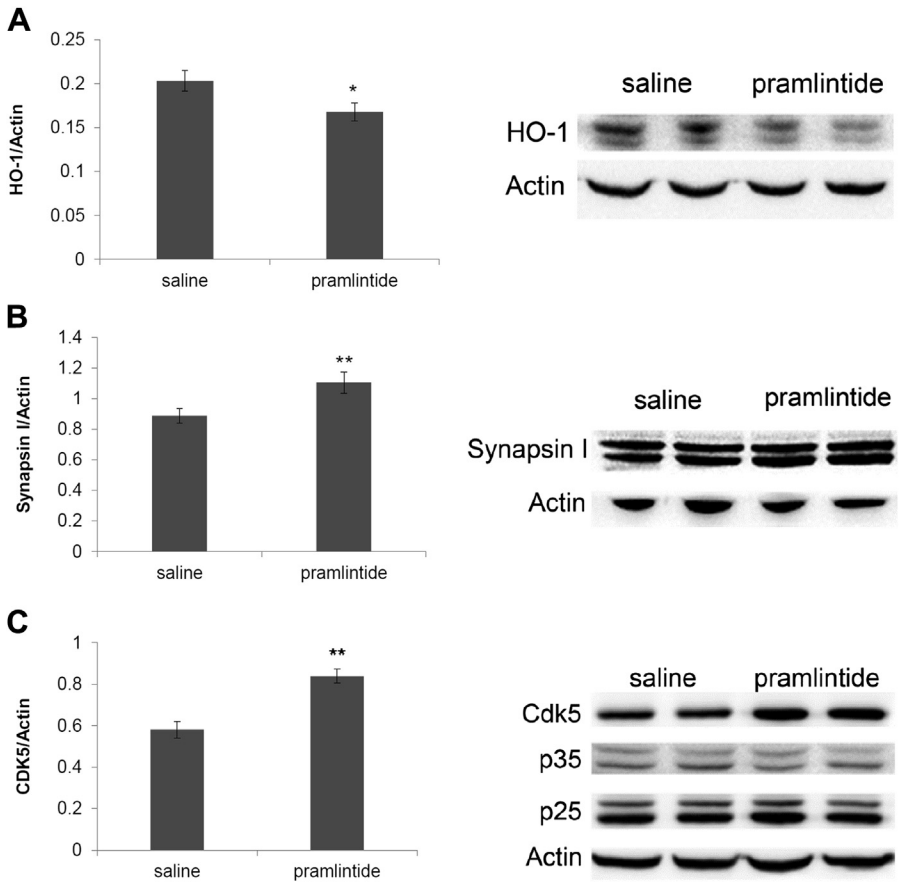

Fig. 3. Western blots of hippocampal tissue from SAMP8 mice chronically treated with pramlintide demonstrated (A) decreased hippocampal expression of the cellular stress marker heme-oxygenase-1 (HO-1) ($p = 0.035$), (B) decreased synapsin I expression ($p = 0.004$), and (C) increased expression of cyclin-dependent kinase-5 (CDK5) ($p = 0.002$). Pramlintide treatment did not have a significant effect on the expression of p35, p25, or the p25/35 ratio ($p > 0.05$). Results are depicted as mean ± standard error of mean (SEM). (Student t test, * $p < 0.05$, ** $p < 0.01$; n = 7–10 per group). Abbreviations: HO-1, heme-oxygenase-1; SEM, standard error of mean.

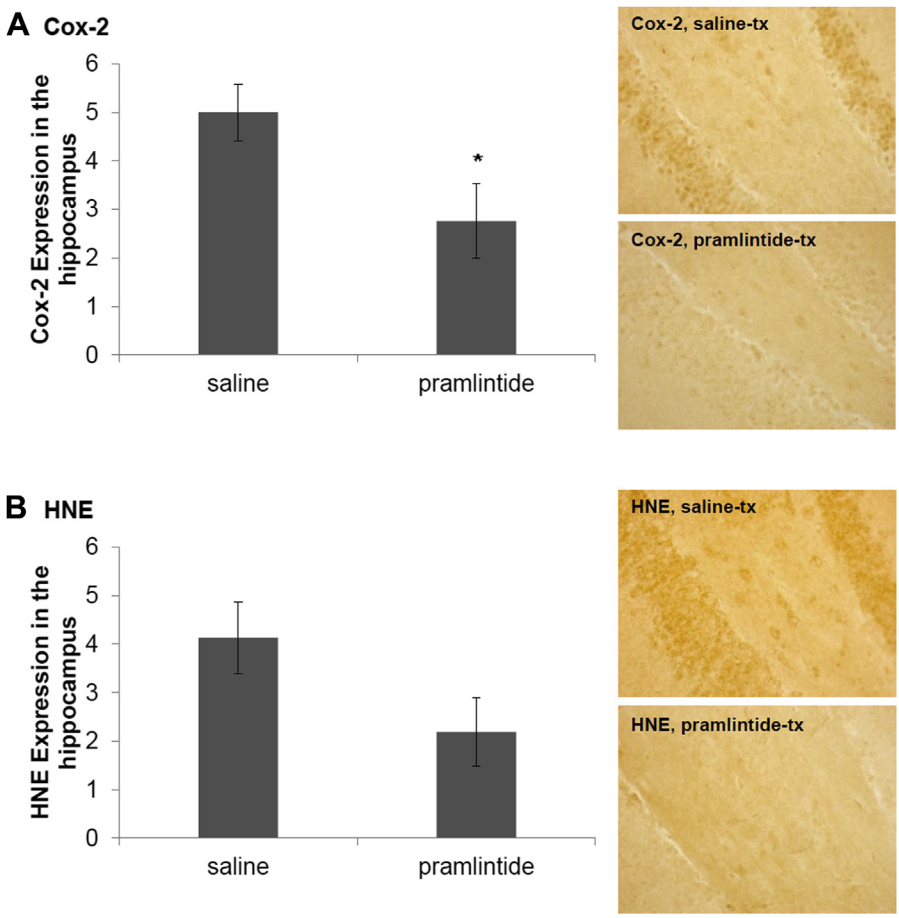

Fig. 4. (A) Chronic pramlintide treatment decreased expression of the inflammatory marker cyclooxygenase 2 (COX-2) in the hippocampus as detected by immunohistochemistry ($p = 0.042$). (B) There is a trend toward decreased expression of the oxidative stress marker 4-hydroxynonenal (HNE) in the hippocampus of pramlintide-treated mice ($p = 0.090$). The results are depicted as mean ± standard error of mean (SEM). (Student *t* test, * $p < 0.05$; n = 5–6 per group). Abbreviations: COX-2, cyclooxygenase 2; HNE, 4-hydroxynonenal; SEM, standard error of mean.

factors for AD including obesity, insulin resistance, and diabetes in middle aged adults (Hou et al., 2011; Reinehr et al., 2007). In our sample, amylin was significantly associated with another classic risk factor of AD, the apolipoprotein E4 genotype. After adjusting for diabetes and apolipoprotein E4, the significance of the association between low plasma amylin and AD remained. Therefore, the relationship between low amylin and AD appears to be independent of other risk factors for AD.

Overall, the human plasma analysis was carried out in a robust and well-characterized study population using a comprehensive

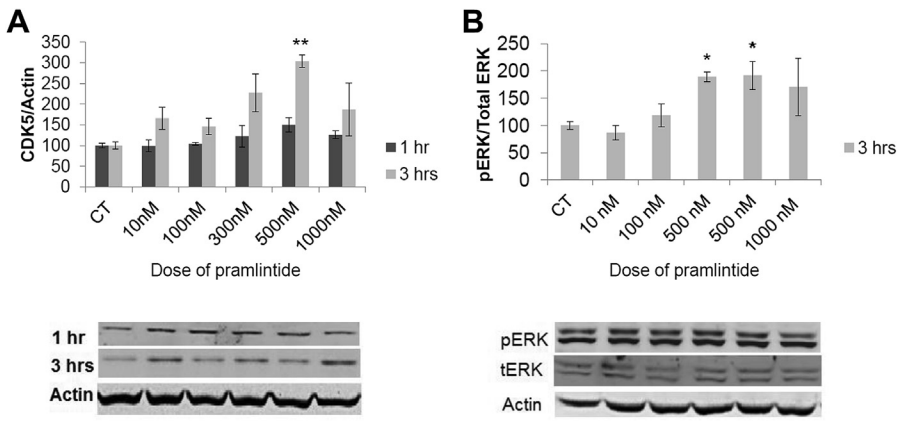

Fig. 5. Cortical neuronal cells were incubated with pramlintide at varying doses (0, 10 nM, 100 nM, 300 nM, 500 nM, 1000 nM) for 1 and 3 hours. Western blotting technique was used to measure the effects of pramlintide on cyclin-dependent kinase-5 (CDK5) and pERK protein expression. pERK is a known downstream target of pramlintide and therefore was used as a positive control. (A) pramlintide dose-dependently increased CDK5 protein expression at 1 ($p = 0.045$) and 3 hours ($p = 0.025$) of incubation and (B) pramlintide dose-dependently increased pERK expression at 3 hours of incubation ($p = 0.009$). All treatments were performed in duplicate and the experiment was repeated twice. The results are depicted as mean ± standard error of mean (SEM). (Bivariate fit model, * $p < 0.05$, ** $p < 0.01$). Abbreviations: CDK5, cyclin-dependent kinase-5; SEM, standard error of mean.

assessment of covariates. Nevertheless, future studies should carry out such analyses in samples from fasted subjects, with detailed histories of weight change, diet and physical activity, and amylin levels should be determined at the same time of the day. With this in mind, the fact that we detected a significant association with these added sources of variability suggests that a fully controlled trial may detect differences and associations of a larger magnitude.

To investigate the possibility that amylin is directly involved in memory, we studied the effects of chronic administration of pramlintide, a nonaggregating analog of the hormone amylin, on cognitive function in the SAMP8 mouse. The SAMP8 mouse is a senescence-accelerated mouse model of sporadic AD that displays multiple features known to occur early in the pathogenesis of AD including cortical atrophy, increased oxidative stress, amyloid-β alterations, tau phosphorylation, and severe deficits of learning and memory (Akiyama et al., 2000; Butterfield and Poon, 2005; Scheff et al., 2006). The SAMP8 mouse is increasingly recognized as a valuable model of sporadic AD because it does not rely on poorly validated mutations present only in the familial onset forms of the disease (Morley et al., 2012a, 2012b). Our data demonstrate that chronic pramlintide administration improved recognition learning and memory in the novel object recognition test in the absence of changes in total exploration of the objects or other exploratory behaviors such as grooming and rearing, which suggests that these improvements are cognition-associated rather than due to changes in general activity. The novel object recognition test measures the ability to evaluate a previously encountered item as familiar or novel and depends on the integrity of the medial temporal lobe (Hammond et al., 2004), a region affected early and severely in AD (Jack et al., 1998). Therefore, these findings suggest that pramlintide treatment may be therapeutically beneficial in MCI and/or sporadic AD.

We also demonstrate that chronic pramlintide administration ameliorates important pathologic features of AD, including synapse loss and oxidative stress. Considerable evidence indicates a role for inflammation and synaptic loss in the pathogenesis of AD (Akiyama et al., 2000; Scheff et al., 2006) and both pathologic processes are present in hippocampal tissue of SAMP8 mice (Butterfield and Poon, 2005). Our data show that in the SAMP8 mouse, pramlintide treatment significantly reduces the expression of HO-1. HO-1 is a cellular stress protein that is activated during high oxidative stress and inflammatory states, and is known to be increased in the hippocampus and cerebral cortex of AD brains (Schipper et al., 1995) and age-dependently increased in the SAMP8 mouse (Cuesta et al., 2010; Li et al., 2009). In further support of pramlintide's anti-inflammatory properties, pramlintide decreased levels of the lipid peroxidation adduct HNE, a protein that is known to be an early and abundant cellular stress marker in the AD brain (Sayre et al., 1997), as well as COX-2, a classic marker of inflammation that is increased in aging and AD brains (Ho et al, 1999). Although previous in vitro studies have demonstrated that pramlintide treatment may reduce markers of oxidative stress and inflammation in peripheral tissues (Ceriello et al., 2005), to our knowledge this is the first time that pramlintide has been shown to reduce these markers in vivo and within the CNS.

Pramlintide was also found to increase the expression of hippocampal synapsin I, a protein located in neuronal synaptic vesicles that is involved in synapse formation, neurotransmitter release, and learning and memory (Corradi et al., 2008). The increase in synapsin I expression in pramlintide-treated SAMP8 mice suggests that pramlintide either has a protective effect on synapses or induces synaptogenesis. Whether pramlintide's effects on synapsin I are secondary to the observed anti-inflammatory and antioxidant effects of this drug or are driven directly through receptor-specific signaling should be a focus of future research.

One possible mechanism underlying pramlintide's described effects may be the robust increase in CDK5 observed both in the hippocampus of treated SAMP8 mice and in primary cortical neurons, where the effect on CDK5 expression was observed to be dose-dependent. CDK5 is a proline-directed serine and/or threonine kinase involved in dendritic growth and neural migration during neocortical development (Dhavan and Tsai, 2001) that also plays an intimate role in synaptic plasticity, learning and memory in the adult brain. In particular, CDK5 is implicated in synaptic vesicle cycling, neurotransmitter synthesis, axonal transport, neuronal survival and cell death (Cheung et al., 2008), and hippocampal neurogenesis (Jessberger et al., 2008). More recently CDK5 has been shown to play an important role in synaptogenesis (Samuels et al., 2007), in part through its downstream effects on synapsin I and other synaptic proteins. Although CDK5 is thought to have neuroprotective effects, dysregulation of CDK5 through accumulation of its cleavage product p25 may promote tau and neurofilament hyperphosphorylation (Ahlijanian et al., 2000; Patrick et al., 1999). In the present study, we observed no significant effect of chronic pramlintide administration on p25 or p35 expression, or the ratio of p35/p25, suggesting that pramlintide may selectively increase CDK5 expression without promoting dysregulation of this kinase. Future studies are needed to clarify pramlintide's effect on tau phosphorylation and to determine whether the effects of amylin receptor activation on CDK5 drive the observed changes in markers of synaptic plasticity such as synapsin I.

In conclusion, here we show that plasma levels of the native hormone amylin are reduced in human subjects with MCI or AD. We also demonstrate for the first time that chronic administration of the approved diabetes medication pramlintide, a nonaggregating analog of the hormone amylin, improves cognitive deficits in a senescence mouse model, decreases inflammation and oxidative stress, increases synaptic protein expression in the hippocampus, and has direct effects on hippocampal CDK5 expression. If our results are confirmed in other models of AD and independent human plasma samples, the hormone amylin and its analogs might provide for a novel pathway for the study and treatment of AD.

Disclosure statement

The authors have no actual or potential conflicts of interest.

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