BMP9 ameliorates amyloidosis and the cholinergic defect in a mouse model of Alzheimer's disease

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Bone morphogenetic protein 9 (BMP9) promotes the acquisition of the cholinergic phenotype in basal forebrain cholinergic neurons (BFCN) during development and protects these neurons from cholinergic dedifferentiation following axotomy when administered in vivo. A decline in BFCN function occurs in patients with Alzheimer's disease (AD) and contributes to the AD-associated memory deficits. We infused BMP9 intracerebroventricularly for 7 d in transgenic AD model mice expressing green fluorescent protein specifically in cholinergic neurons (APP.PS1/CHGFP) and in wild-type littermate controls (WT/CHGFP). We used 5-mo-old mice, an age when the AD transgenics display early amyloid deposition and few cholinergic defects, and 10-mo-old mice, by which time these mice exhibit established disease. BMP9 infusion reduced the number of Aβ42-positive amyloid plaques in the hippocampus and cerebral cortex of 5- and 10-mo-old APP.PS1/CHGFP mice and reversed the reductions in choline acetyltransferase protein levels in the hippocampus of 10-mo-old APP.PS1/CHGFP mice. The treatment increased cholinergic fiber density in the hippocampus of both WT/CHGFP and APP.PS1/CHGFP mice at both ages. BMP9 infusion also increased hippocampal levels of neurotrophin 3, insulin-like growth factor 1, and nerve growth factor and of the nerve growth factor receptors, tyrosine kinase receptor A and p75/NGFR, irrespective of the genotype of the mice. These data show that BMP9 administration is effective in reducing the Aβ42 amyloid plaque burden, reversing cholinergic neuron abnormalities, and generating a neurotrophic milieu for BFCN in a mouse model of AD and provide evidence that the BMP9-signaling pathway may constitute a therapeutic target for AD.

acetylcholine | APPswe PS1dE9 mice | growth/differentiation factor 2 | juvenile protective factors

Basal forebrain cholinergic neurons (BFCN) project to the hippocampus and cerebral cortex where the release of their neurotransmitter, acetylcholine (ACh), is central for the processes of learning, memory, and attention throughout life (1). The acquisition of the cholinergic phenotype by these neurons during development is induced by bone morphogenetic protein 9 (BMP9), also known as growth/differentiation factor 2 (GDF2), which is expressed in the fetal basal forebrain (2). Application of BMP9 to basal forebrain cell cultures and into the cerebral ventricles of developing mouse embryos (2) and adult mice (3) significantly increases ACh production. Moreover, the idea that the BMP9-signaling pathway may be a master regulator of the BFCN phenotype is supported by evidence showing that BMP9 is sufficient to induce BFCN-like features in human embryonic stem cell-derived neural progenitor cells (4) and that, in rodent basal forebrain cell cultures, BMP9 induces the BFCN transcriptome (5). Recent studies indicate that intracerebroventricularly (i.c.v.)-infused BMP9 fully prevents the cholinergic dedifferentiation (i.e., loss of cholinergic markers) of axotomized BFCN in mice (3). Taken together, these data provide a compelling motivation to determine the efficacy of BMP9 as a treatment of diseases characterized by BFCN dysfunction and/or degeneration.

A prominent example of such an illness, for which effective therapies are critically needed, is Alzheimer's disease (AD). A decline in BFCN function and diminished cholinergic marker expression occurs in brains of patients with AD (6, 7) and is similarly observed in AD animal models (6, 8–13). This dysfunction and/or degeneration of BFCN contribute to the memory deficits that are a hallmark of AD (6, 7, 14). Some of the current treatments for AD are designed to increase the intrasynaptic levels of ACh by inhibiting the enzyme acetylcholinesterase that breaks down the neurotransmitter (15). This approach, however, is only mildly effective, presumably because the pathophysiologic process that underlies AD is progressive, leading to inevitable loss of synapses (16, 17). Therefore, the use of trophic factors [e.g., nerve growth factor (NGF)] (18) that support BFCN survival and maintain the differentiated state of these neurons represents a possible strategy for the treatment of AD.

In the current study we explored the potential of BMP9 to serve as a therapeutic agent for AD using the APPswe/PS1deltaE9 (APP.PS1) transgenic mouse model of AD. These mice were engineered to express murine amyloid precursor protein (APP) with the human β-amyloid (Aβ) amino acid sequence harboring mutations that cause a familial form of AD [the Swedish mutation APP(K595N/M596L): APPswe] together with a mutated form of presenilin 1 (PS1 with exon 9 deleted: PS1dE9) (19). Although no model of AD fully recapitulates the human disease (20), APP.PS1 mice are well suited for our investigations because they exhibit (*i*) cholinergic defects (8–12), (*ii*) high production of Aβ peptides in brain and accumulation of amyloid plaques (21), and (*iii*) cognitive impairments (12, 22–26). We elected to study animals at 5 and 10 mo of age. At 5 mo, the APP.PS1 mice begin to exhibit amyloid accumulation (27) and have normal choline

Significance

Bone morphogenetic protein 9 (BMP9) is a trophic factor for basal forebrain cholinergic neurons (BFCN) and constitutes a candidate therapeutic molecule for diseases characterized by BFCN dysfunction. A prominent example of such an illness, for which effective therapies are critically needed, is Alzheimer's disease (AD). A decline in BFCN function and diminished cholinergic marker expression occurs in brains of patients with AD and is observed in AD animal models. We report that BMP9 administration is effective in reducing the amyloid plaque burden, reversing cholinergic neuron abnormalities, and generating a neurotrophic milieu for cholinergic neurons in a mouse model of AD, thus providing evidence that the BMP9-signaling pathway may constitute a novel therapeutic target in AD.

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acetyltransferase (CHAT) activity, although they show some impairment of the BFCN projections (8). This time point represents early stages of pathogenesis. The 10-mo-old APP.PS1 mice exhibit high amyloid deposition (27) and cholinergic impairment characterized by reduced CHAT activity and dystrophic cholinergic neurites in the terminal fields within the hippocampus and cerebral cortex (8). To facilitate studies of the cholinergic phenotype in this model, we crossed these mice with a transgenic strain that expresses enhanced green fluorescent protein specifically in cholinergic cells (28, 29). Thus, our studies were performed either on wild-type control mice that did not express the AD-related transgenes, designated WT/CHGFP, or on their AD model littermates, designated APP.PS1/CHGFP.

Intracerebroventricular infusion of human recombinant BMP9 in these mice for a brief period of 7 d fully reversed AD-like cholinergic pathology and induced the expression of procholinergic growth factors including NGF and its receptors (p75/NGFR and TRKA), neurotrophin 3 (NT3), and insulin-like growth factor 1 (IGF1). Unexpectedly, BMP9 infusion also reduced Aβ42 amyloid plaque number in the hippocampus and cerebral cortex of APP.PS1/CHGFP mice. Taken together, these data indicate that BMP9 exhibits efficacy as a therapeutic agent for AD.

Results

BMP9 Infusion Reduces the Number of Amyloid Plaques in the Hippocampus and Cerebral Cortex. We determined the amyloid burden in APP.PS1/CHGFP mice using immunohistochemical staining. Aβ42-positive plaques were present in the cortex and hippocampus of these animals. Consistent with previous studies in APP.PS1 mice, the plaque number increased with age (30), and females were more vulnerable to the amyloidosis than the males (30) (Figs. 1 and 2). We determined plaque number in the anterior (bregma approximately −1.8 mm) and posterior (bregma approximately −3 mm) brain sections, focusing on the hippocampus of each and on the somatosensory and primary visual cortex, respectively (Figs. 1 and 2). Remarkably, a 7-d infusion of BMP9 caused highly significant reductions (~45% overall) in the number of Aβ42-positive plaques in all of those brain areas in both 5-mo-old (Fig. 1) and 10-mo-old (Fig. 2) APP.PS1/CHGFP mice. The effect of sex was statistically significant as well, and no treatment by sex interaction was found (Figs. 1 and 2). We also measured the hippocampal levels of the solubilized Aβ40 and Aβ42 peptides using ELISA. The levels of the peptides rose with the age of APP.PS1/CHGFP mice and were higher in females than in males (Fig. S1). We found no significant effects of BMP9 infusion on Aβ40 and Aβ42 levels by this method (Fig. S1).

In contrast to the sexual dimorphism in brain amyloid deposition, we found no differences between the sexes in other measures and thus the subsequently reported data are not stratified by sex.

BMP9 Infusion Increases CHAT Expression and Cholinergic Fiber Density in the Hippocampus. Consistent with published data (8), CHAT levels were reduced by 20% in 10-mo-old APP.PS1/CHGFP mice compared with the WT/CHGFP animals (Fig. 3*A*). BMP9 increased hippocampal CHAT protein levels at both ages in the WT/CHGFP and APP.PS1/CHGFP mice (Fig. 3*A*). Notably, infusion of BMP9 increased hippocampal CHAT levels in APP.PS1/CHGFP mice by 54%, i.e., more than fully counteracting the CHAT deficit observed in the APP.PS1/CHGFP mice (Fig. 3*A*). Moreover, confocal microscopy of fluorescent GFP-expressing cholinergic fibers within the CA1 region revealed that BMP9 increased their density in both the WT/CHGFP and APP.PS1/CHGFP mice (Fig. 3 *B* and *C* and Fig. 4). Consistent with studies in other AD models, the cholinergic fibers from APP.PS1/CHGFP mice (visualized by GFP fluorescence) displayed multiple dystrophic features (8) and were absent in the areas occupied by amyloid plaques (31) (Fig. 4). Qualitatively, we observed fewer dystrophic neurites in BMP9-treated mice than in the controls (Fig. 4).

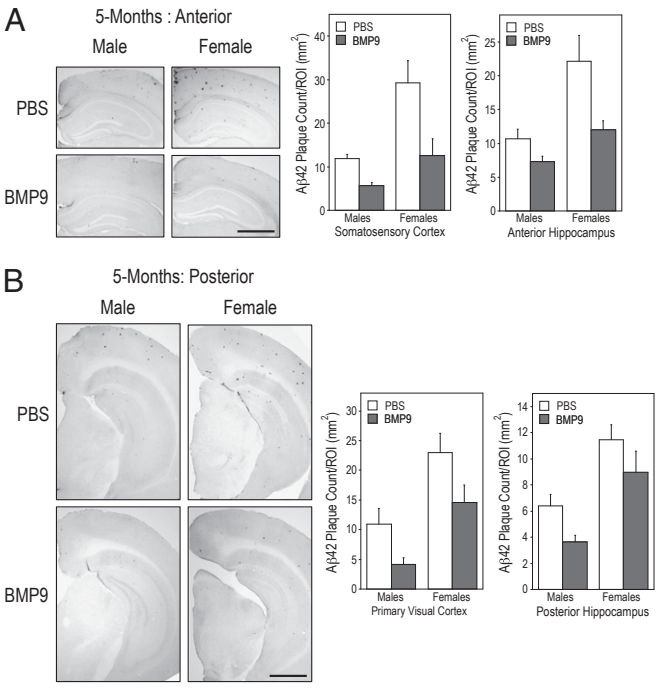

Fig. 1. BMP9 reduces the number of Aβ42 plaques in the hippocampus and cortex of 5-mo-old APP.PS1/CHGFP mice. Immunohistochemical staining of Aβ42 was performed on anterior (*A*) and posterior (*B*) brain sections from 5-mo-old male and female APP.PS1/CHGFP mice as described in *Materials and Methods*. Representative images from each sex and treatment group are shown for the anterior and posterior sections. (Scale bar, 1 mm). The number of Aβ42 plaques was counted in the anterior and posterior hippocampus and in somatosensory cortex (anterior) and primary visual cortex (posterior). Means ± SEM per treatment group and sex are presented for each of the brain subregions. The data were analyzed by two-way ANOVA and revealed significant effects of treatment ($P = 0.005$ in the anterior hippocampus, $P = 0.023$ in the posterior hippocampus, $P = 0.001$ in the somatosensory cortex, and $P = 0.009$ in the primary visual cortex) and sex ($P = 0.001$, $P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively), and no significant effect of treatment by sex interaction. ROI, region of interest.

BMP9 Infusion Generates a Trophic Environment for Basal Forebrain Cholinergic Neurons. Because almost all BFCN express the signaling TRKA NGF receptor (32, 33) and the NGF-binding neurotrophin receptor, p75/NGFR (34–36), we analyzed these proteins in the hippocampi of our experimental subjects using immunoblotting. The levels of TRKA and p75/NGFR proteins were similar in the WT/CHGFP and APP.PS1/CHGFP mice. BMP9 infusion increased those levels in a statistically significant fashion as determined by ANOVA at 5 mo of age, whereas in 10-mo-old mice BMP9 infusion was ineffective (Fig. 5).

Moreover, BMP9 infusion increased the levels of NGF protein in both 5- and 10-mo-old WT/CHGFP and APP.PS1/CHGFP mice by ~15–20% (Fig. 6*A*). Note that 5-mo-old APP.PS1/CHGFP mice were characterized by a small (15%) reduction of NGF levels in the hippocampus and that BMP9 infusion fully restored these levels to control values. In addition, BMP9 increased the hippocampal levels of NT3 in both the WT/CHGFP and APP.PS1/CHGFP mice (Fig. 6*B*). This action of BMP9 was particularly striking in 5-mo-old APP.PS1/CHGFP mice (close to a twofold increase). Furthermore, BMP9 increased hippocampal IGF1 expression by ~24% in 5-mo-old APP.PS1/CHGFP mice, but exhibited no efficacy in 10-mo-old animals (Fig. 6*C*).

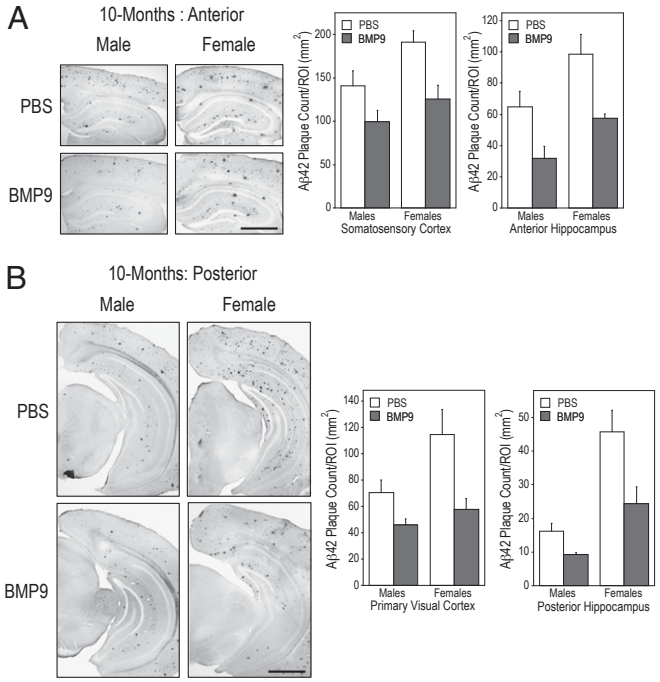

Fig. 2. BMP9 reduces the number of Aβ42 plaques in the hippocampus and cortex of 10-mo-old APP.PS1/CHGFP mice. Immunohistochemical staining of Aβ42 was performed in 10-mo-old male and female APP.PS1/CHGFP mice, and the data are presented as described in Fig. 1. Two-way ANOVA revealed significant effects of treatment ($P = 0.002$ in the anterior hippocampus, $P = 0.004$ in the posterior hippocampus, $P = 0.003$ in the somatosensory cortex, and $P = 0.004$ in the primary visual cortex) and sex ($P = 0.007$, $P < 0.001$, $P = 0.022$, and $P = 0.034$, respectively) and no significant effect of treatment by sex interaction. ROI, region of interest.

BMP9 Infusion Does Not Affect Hippocampal Gliosis. APP.PS1 mice are reportedly characterized by hippocampal gliosis that increases with age, as determined using GFAP immunostaining and quantitative PCR assays (37, 38). Indeed, we found increased GFAP expression in 10-mo-old (but not in 5-mo-old) APP.PS1/CHGFP mice using immunofluorescence (Fig. S2*A*) in hippocampal sections costained with thioflavin T to visualize the amyloid plaques and by GFAP immunoblotting (Fig. S2*B*). BMP9 infusion had no effect on hippocampal GFAP levels (Fig. S2*B*).

Discussion

These data show that administration of BMP9 to the brain in AD model APP.PS1/CHGFP mice ameliorates two central pathophysiological features of AD: reductions of CHAT levels and accumulation of amyloid plaques in the hippocampus and cerebral cortex. The actions of BMP9 on hippocampal CHAT levels are consistent with prior investigations showing the induction of the cholinergic phenotype by this protein in multiple in vitro systems (2, 4, 5, 29) as well as in in vivo models (2, 3), such as axotomized septohippocampal cholinergic neurons (3). BMP9 was highly effective at increasing CHAT levels in the hippocampus of both WT/CHGFP and APP.PS1/CHGFP mice at 5 mo of age. However, these mice had not yet developed a cholinergic deficit. It is only later, at 10 mo of age, when the APP.PS1/CHGFP mice show reduced CHAT levels, that BMP9 reverses the CHAT defect. Moreover, the APP.PS1/CHGFP mice were characterized by an increase in the density of cholinergic nerve fibers in response to BMP9 infusion (three- to fourfold greater density than that in the controls), whereas the wild-type animals had a robust, but smaller (1.5- to 1.9-fold), response to BMP9. Thus, it is apparent that BFCN in the APP.PS1/CHGFP mice are poised to respond to BMP9 by both increasing their CHAT levels and elaborating their terminal projections in the hippocampus. These observations point to unexplored trophic actions of BMP9 on cholinergic neuronal morphology in vivo and provide evidence that the BFCN vulnerability to the AD pathophysiologic process, modeled by the APP.PS1/CHGFP mice, is accompanied by BFCN hypersensitivity to BMP9.

Our data suggest the possibility that the anti-amyloidogenic action of BMP9, observed in APP.PS1/CHGFP mice, may be the direct consequence of the increased cholinergic hippocampal innervation caused by this protein. This notion is based on mechanistic studies showing that cholinergic neurotransmission directs APP processing to the nonamyloidogenic pathway by activating α-secretase, which hydrolyzes APP within the Aβ sequence (39), and on in vivo investigations showing that in AD model mice cholinergic activity slows down plaque deposition. For example, in mice that do not express the m1 ACh muscarinic receptors, hippocampal Aβ levels and amyloid plaque numbers are increased (40). Moreover, APP.PS1 mice subjected to a

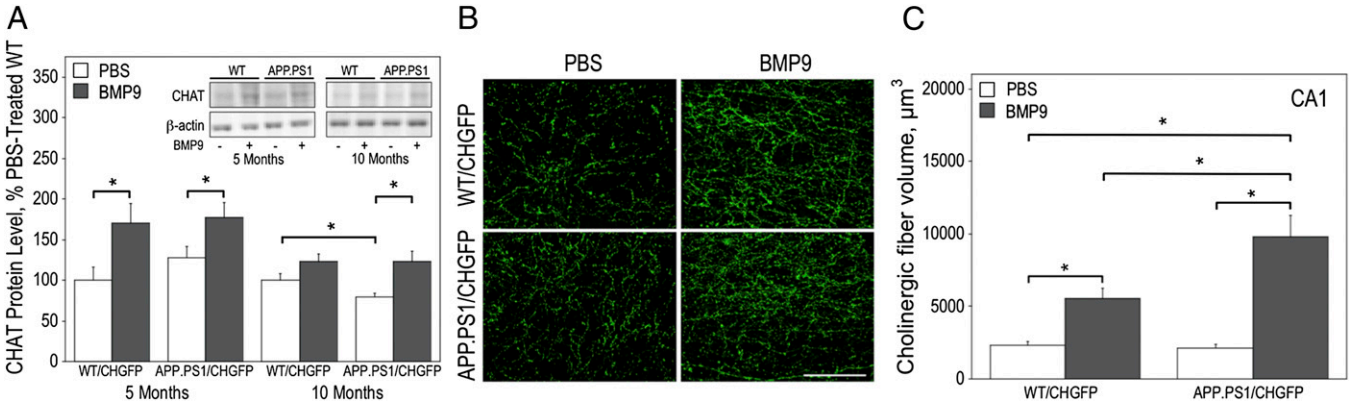

Fig. 3. BMP9 prevents the reductions of CHAT protein levels in the hippocampus of APP.PS1/CHGFP mice and increases cholinergic fiber density in both WT/CHGFP and APP.PS1/CHGFP mice. Hippocampal lysates from 5- and 10-mo-old WT/CHGFP and APP.PS1/CHGFP mice were used to determine CHAT protein levels by immunoblot (*A*). GFP in anterior hippocampal sections from 10-mo-old mice was visualized (*B*) and quantified in the stratum radiatum of the CA1 region of the hippocampus (*C*). CHAT levels were lower in APP.PS1/CHGFP mice than in WT/CHGFP mice at 10 mo ($P < 0.05$). Infusion of BMP9 significantly increased protein levels of CHAT ($P < 0.005$ in 5 mo, $P < 0.001$ in 10 mo) and cholinergic fiber volume in the CA1 region ($P < 0.005$) as determined by ANOVA. Significant differences, determined by a post hoc Tukey test, are indicated by the brackets (*$P < 0.05$). (Scale bar, 50 μm.)

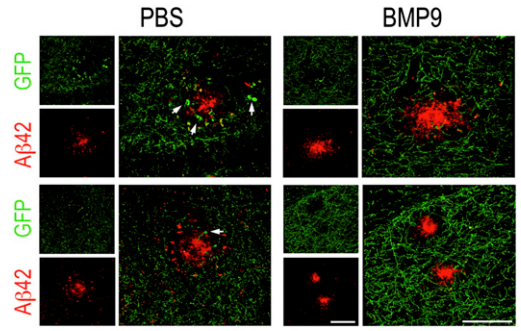

Fig. 4. BMP9 attenuates the Aβ42-mediated disruptions of the cholinergic fiber network in the hippocampus. Z-stacks (10 μm) were acquired using laser-scanning confocal microscopy to visualize Aβ42 immunofluorescence (red) and cholinergic fibers (green) in the hippocampus of 10-mo-old APP.PS1/CHGFP mice following a 7-d infusion with either PBS or BMP9. Cholinergic fibers avoid the amyloid plaques [compare single-channel (*Left*) and dual-channel (*Right*) images]. Dystrophic cholinergic neurites are evident (white arrows). In BMP9-infused mice, cholinergic fibers exhibit fewer dystrophic features, higher density, and are more robust than in the controls. (Scale bar, 50 μm.)

specific BFCN lesion produced by the immunotoxin anti–p75-saporin display a rapid (within days) acceleration of amyloid plaque deposition in the hippocampus (41, 42).

Our studies show that a relatively brief (7-d) infusion of BMP9 was sufficient to reduce the number of Aβ42 plaques in the hippocampus and cerebral cortex. This time course is consistent with the current understanding of plaque dynamics in AD model mice. It has been demonstrated that new cortical plaque formation is exceptionally rapid, occurring within 1 d in APP.PS1 mice (43). Thus, it is conceivable that BMP9 acting via cholinergic induction or other, yet-to-be-determined mechanisms could inhibit the generation of new plaques. Similarly, plaque burden may be reduced within days in APP.PS1 mice by the administration of certain drugs, e.g., a PPARγ agonist (44), indicating that, if BMP9 acted by accelerating plaque clearance, this could be observed within the time period of BMP9 administration. However, in contrast to the reduction of Aβ42 plaque density in the hippocampus of BMP9-infused mice, we found no effect of BMP9 on total Aβ40 and Aβ42 levels measured by ELISA in hippocampal extracts. We note that others have also reported relatively more robust effects of various treatments on plaque burden compared with Aβ peptide levels in other mouse models of AD (45, 46). One possibility that could explain our observations might be that BMP9 slows down the aggregation and deposition of the Aβ peptides into plaques or, alternatively, that BMP9 promotes plaque clearance (e.g., by glial cells), transferring the peptide from the plaque to an intracellular compartment where it may be ultimately degraded. In this fashion, higher amounts of Aβ peptides would be present in nonplaque form in brains of BMP9-treated mice compared with the vehicle-infused animals. Our ELISA method—which includes amyloid solubilization in guanidine hydrochloride—presumably detects all Aβ42 (i.e., that present in plaques as well as that in diffuse extracellular amyloid and the intracellular peptide).

BMP9 signaling is mediated by its principal type I receptor, ALK1/ACVRL1 (47, 48), which can interact with several type II receptors. Interestingly, we previously found that *Alk1* is specifically expressed in BFCN and not in other neuronal cells in the basal forebrain (29), and therefore exogenous BMP9 would be expected to target primarily BFCN in this brain region and have a favorable cellular specificity. BFCN also express *Bmpr2*, *Acvr2a*, and *Acvr2b* type II BMP receptors (29), which could transduce the BMP9 signal via ALK1. Recent studies indicate that ACVR2B binds BMP9 with highest affinity (48) and thus is the most likely type II receptor to mediate this interaction. Stimulation of these receptors in BFCN leads to the phosphorylation of SMAD1/5/8 proteins (29), i.e., activation of the canonical BMP-signaling pathway, and consequent changes in transcription of multiple genes, including stimulation of the expression of *Chat* (2) and several transcription factors (5). Indeed, we found that some actions of BMP9 in BFCN, e.g., stimulation of *Ngf* gene transcription, require the synthesis of an intermediate protein factor (possibly a transcriptional regulator) (29). ALK1 is also expressed on vascular endothelial cells (47), and BMP9 is known to remodel the vasculature (49) by activating this receptor. Given that Aβ clearance is mediated, in part, by the cerebral blood vessels (50), it will be interesting to determine if some of the anti-amyloidogenic effects of BMP9 observed in this study are related to its actions on the vascular endothelium.

BMP9 infusion increased the hippocampal levels of NGF in the WT/CHGFP and APP.PS1/CHGFP mice at 5 and 10 mo of age and of its receptors, p75/NGFR and TRKA (at 5 mo only). These data confirm our previous studies showing that BMP9 not only directly induces the cholinergic phenotype, but also may generate a trophic environment for cholinergic neurons by promoting the synthesis of NGF and its receptors (3, 5, 29). The BMP9-induced increase in NGF levels is of particular interest because NGF is a prototypic trophic factor for septal cholinergic neurons (51) whose utility as a therapeutic agent for AD has been actively explored (18). The increased expression of p75/NGFR in BMP9-infused mice is also noteworthy, as a previous study documented an *Ngfr* gene dosage dependence of amyloidosis with wild-type $Ngfr^{+/+}$ mice having relatively low amyloid burden and knockout $Ngfr^{-/-}$ mice having a high amyloid burden (52). Thus, the increased levels of p75/NGFR in BMP9-treated mice, although seen only at a young age in our study, could potentially contribute to the anti-amyloidogenic action of BMP9.

The BMP9-induced increase in hippocampal NT3 levels observed in both the WT/CHGFP and APP.PS1/CHGFP mice is of interest because NT3 promotes the extension of cholinergic axons toward their hippocampal and cortical targets and facilitates

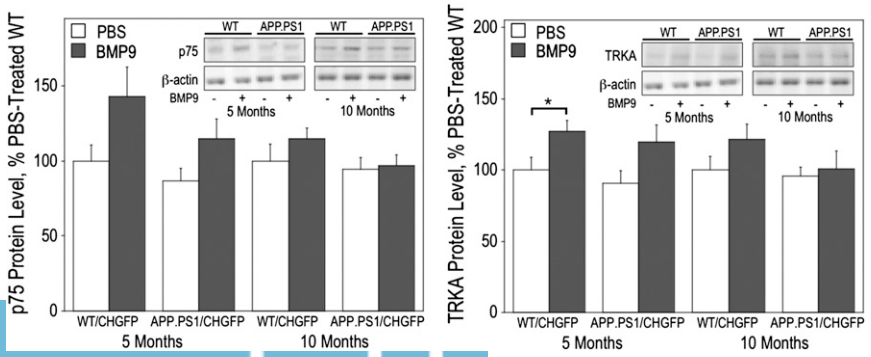

Fig. 5. BMP9 increases the expression of NGF receptors p75 and TRKA in WT/CHGFP and APP.PS1/CHGFP mice. Using immunoblot, p75 (*Left*) and TRKA protein levels (*Right*) were measured in hippocampal lysates from 5- and 10-mo-old mice. The levels of β-actin were used for normalization. At 5 mo of age only, BMP9 infusion had a significant effect on both p75 ($P < 0.05$) and TRKA expression ($P < 0.01$) in the hippocampus as determined by two-way ANOVA for treatment and genotype. (*$P < 0.05$) by a post hoc Tukey test.

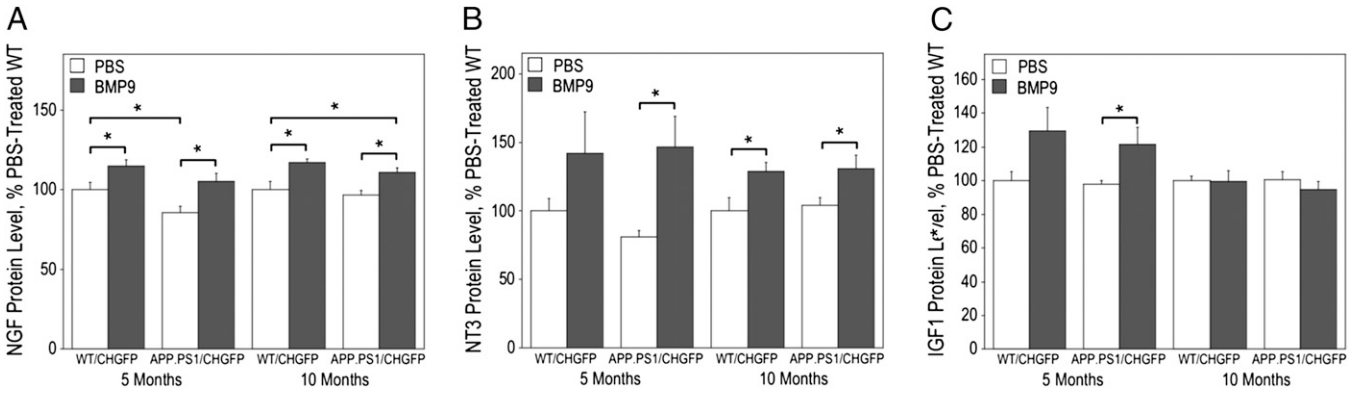

Fig. 6. BMP9 promotes the establishment of a cholinotrophic milieu in the hippocampus by increasing the levels of NGF, NT3, and IGF1 in WT/CHGFP and APP.PS1/CHGFP mice. Hippocampal lysates from 5- and 10-mo-old mice were used to assay NGF (*A*), NT3 (*B*), and IGF1 (*C*) levels by ELISA. There was a significant effect of BMP9 infusion on NGF levels at 5 and 10 mo as determined by two-way ANOVA ($P < 0.005$ and $P < 0.001$, respectively). NGF levels were reduced in the hippocampus of PBS-infused 5-mo-old APP.PS1/CHGFP mice compared with PBS-infused WT/CHGFP mice, but not in BMP9-infused mice or with either treatment at 10 mo of age. BMP9 infusion significantly increased NT3 expression at both ages ($P < 0.01$ and $P < 0.005$, respectively), but there were no significant effects of genotype as determined by two-way ANOVA. There was also a significant effect of BMP9 infusion on IGF1 levels but only in 5-mo-old mice ($P < 0.005$). Significant differences, determined by a post hoc Tukey test, are indicated by the brackets (*$P < 0.05$).

cholinergic synapse formation on these neurons (53). Thus, induction of NT3 by BMP9 may ameliorate the formation of the swollen, dystrophic cholinergic neurites seen in the APP.PS1/CHGFP mice. Furthermore, NT3 is neuroprotective for cortical neurons cultured in the presence of Aβ (54) and attenuates Aβ-mediated apoptosis of these cells (54).

Previous studies showed that levels of IGF1 are reduced in plasma of male (but not female) AD patients (55) and that APP.PS1 mice crossed with a strain engineered to have reduced circulating IGF1 levels have a high brain amyloid burden (56). In contrast, administration of IGF1 reduces brain amyloidosis in AD model mice (57). There is also evidence that IGF1 protects neurons against Aβ-induced toxicity in culture (58). Thus, the increased levels of IGF1 by BMP9 seen in 5-mo-old APP.PS1/CHGFP mice may potentially help to slow down the AD-like pathophysiologic process in these animals.

Our observations that exogenous BMP9 affects amyloid turnover raise the possibility that this process may also be regulated by endogenous BMP signaling and that AD pathophysiology may include defects of the BMP-signaling system. BMP9 mRNA is expressed in multiple brain regions of adult mice [Gene Expression Omnibus (GEO) accession GDS592] and humans (GEO accession GDS596) (59); however, the exact cell types producing BMP9 in brain remain to be determined. Choroid plexus also expresses multiple BMPs (including BMP9) (60) and is a source of these proteins in the cerebrospinal fluid (61). Although there are no data on the levels of BMP9 in AD brain, there is a single report indicating that the levels of a related protein, BMP6 (but not BMP2 and BMP7), are increased in the hippocampus of AD patients and in a mouse AD model (62). Similarly, the levels of BMP4 mRNA (63) and the number of BMP4-positive cells (64) are reportedly increased in the hippocampus of APP.PS1 mice.

Because BMP9 is not likely to cross the blood–brain barrier, its delivery to the brain of AD patients presents a challenge characteristic of protein-based therapeutic agents. However, the biological activity of BMP9 apparently resides within a 23-amino-acid-long peptide that can be used as a BMP9 agonist (65), suggesting that it may be feasible to engineer peptides or small molecules that possess BMP9 activity and are easier to deliver to the brain than full-length BMP9. In summary, our study provides a proof of concept for the use of BMP9, and possibly its analogs, to treat or prevent the cholinergic defect and amyloidosis in AD.

Materials and Methods

Detailed experimental methods can be found in *SI Materials and Methods*. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Boston University School of Medicine. Homozygous CHGFP [B6.Cg-Tg(RP23-268L19-EGFP)2Mik/J] (28, 29) females were crossed to hemizygous APP.PS1 [B6C3-Tg(APPswe,PSEN1dE9)85Dbo/Mmjax] (8, 21) males to generate WT/CHGFP and APP.PS1/CHGFP experimental subjects. The following numbers of subjects were used: 5-mo-old WT/CHGFP—male vehicle, $n = 3$; male BMP9, $n = 3$; female vehicle, $n = 4$; female BMP9, $n = 4$; 5-mo-old APP.PS1/CHGFP—male vehicle, $n = 5$; male BMP9, $n = 5$; female vehicle, $n = 4$; female BMP9, $n = 4$; 10-mo-old WT/CHGFP—male vehicle, $n = 4$; male BMP9, $n = 4$; female vehicle, $n = 4$; female BMP9, $n = 5$; 10-mo-old APP.PS1/CHGFP—male vehicle, $n = 5$; male BMP9, $n = 5$; female vehicle, $n = 5$; female BMP9, $n = 4$. We performed intraventricular infusion of vehicle or 4 ng/h of human recombinant BMP9 (Wyeth) for 7 d in male and female mice 5 or 10 mo of age using Alzet osmotic pumps (model 1002; pumping rate: 0.25 μL/h). Immunohistochemical staining of Aβ42 was performed with rabbit anti-Aβ42 (1:2,500; Invitrogen). Images of the hippocampus, somatosensory cortex (trunk region), and primary visual cortex were analyzed with ImageJ software (National Institutes of Health) to determine the number of Aβ42-positive plaques. The data are expressed as the number of plaques per square millimeter in a region of interest. Confocal microscopy and fluorescence imaging of Aβ42 or GFAP was performed using either recombinant affinity-purified primary rabbit anti-Aβ42 monoclonal antibody (1:2,500; Life Technologies) or rabbit anti-GFAP polyclonal antibody (1:250; Invitrogen). In addition, GFAP-immunostained sections were incubated in 50 μM thioflavin T to visualize aggregated amyloid plaques. To obtain estimates of cholinergic fiber density visualized by GFP fluorescence using confocal microscopy in each region of interest, total volumes occupied by the fibers were calculated. To estimate the relative amounts of proteins of interest by immunoblotting, rabbit anti-p75NTR (1:3,000; Advanced Targeting Systems), mouse anti-GFAP (1:1,000; Cell Signaling Technology), rabbit anti-TRKA (1:1,000; Millipore), or mouse anti–β-actin (1:5,000; Sigma) antibodies were used. NGF and NT3 were assayed using the Emax immunoassay system (Promega), and IGF1 was assayed using the Quantikine sandwich ELISA kit (R&D Systems). Data, presented as means ± SEM, were analyzed by *t* test or two-way ANOVA, as appropriate. Post hoc analyses were performed with Tukey's test.

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