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β-Amyloid Peptide Binding Protein Does Not Couple to G Protein in a Heterologous *Xenopus* Expression System

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Alzheimer's disease is a neurodegenerative disorder related to the formation of protein aggregates. β-Amyloid protein (Aβ), generated by enzymatic cleavage of amyloid precursor protein (APP), can cause such aggregation, and these aggregates may cause neuronal cell death by inducing apoptosis. However, Aβ-induced intracellular signaling pathways involved in the neuronal death are not well understood. Recently it was shown that Aβ aggregates induce neuronal cell death via β-amyloid peptide-binding protein (BBP), a receptor for Aβ in BBP-transfected cells, which is known to be sensitive to pertussis toxin, a $G\alpha_{i/o}$ family inhibitor. However, the actual coupling of BBP to the pertussis-sensitive G protein was not demonstrated. In this study, we performed electrophysiological recordings using the two-electrode voltage-clamp technique to test whether human or *Drosophila* BBPs, singly or in combination with APP, are coupled to a specific type of G protein. Our results suggest that BBP is not directly coupled to $G\alpha_{i/o}$, $G\alpha_s$, or $G\alpha_q$ proteins and that BBP may need a component other than APP to exert its toxic effect in concert with Aβ.

Key words: Alzheimer's disease; β-amyloid peptide binding protein; amyloid precursor protein; G protein

Alzheimer's disease is a neurodegenerative disorder related to the formation of two main protein aggregates, neuritic plaques and neurofibrillary tangles (Maccioni et al., 2001). β-Amyloid peptide (Aβ), generated by enzymatic cleavage of amyloid precursor protein (APP), is the major component of the neuritic plaque (Suh and Checler, 2002). Mutations in genes encoding amyloid precursor protein (APP), presenillin 1, and presenillin 2 cause abnormal processing of APP to produce Aβ, which aggregates and causes neuronal cell death. There are many hypotheses on neurotoxic mechanisms of Aβ, such as oxidative stress (Suh and Checler, 2002), calpain-activated cdk5 pathways (Lee et al., 2000), and caspase-dependent pathways (Nakagawa et al., 2000). However, the precise mechanisms of Aβ toxicity are not well known.

Kajkowski and coworkers have recently shown that a novel β-amyloid peptide-binding protein (BBP) is an essential component for Aβ-induced apoptosis and that Aβ toxicity via BBP is eliminated by pertussis toxin, a $G\alpha_{i/o}$ family inhibitor. Consequently, these authors proposed that the binding of Aβ to BBP, mediated by G protein, causes apoptosis in cultured cells (Kajkowski et al., 2001). However, they did not show whether BBP is directly coupled to pertussis-sensitive G protein.

In this regard, we performed electrophysiological recordings using the two-electrode voltage-clamp technique to test whether human or *Drosophila* BBPs, singly or in combination with APP, are coupled to a specific type of G protein in the heterologous *Xenopus* expression system. Our data indicate that BBP is not directly coupled to $G\alpha_{i/o}$, $G\alpha_s$, or $G\alpha_q$ proteins in this heterologous system. Furthermore, our results suggest that BBP may need a component(s) other than APP to exert its toxic effect in concert with Aβ.

MATERIALS AND METHODS

Preparation of Aβ Peptides

$A\beta_{1-42}$ and $A\beta_{1-40}$ were purchased from U.S. peptide (Fullerton, CA). As previously described (Kim and Suh, 1996),

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peptides were initially dissolved in distilled water and further diluted in phosphate-buffered saline (pH 7.4) immediately before use. Peptides were aged by incubation at 37°C for 7 days, which caused Aβ aggregation to mimic monomeric, dimeric, and trimeric components from neuritic and vascular amyloids of Alzheimer's disease brain (Roher et al., 1996).

Cell Culture and Treatment With the Purified Aβ

Rat pheochromocytoma PC12 cells were plated on polyethyleneimine-coated 96-well dishes in Dulbecco's modified Eagle's medium (DMEM) media supplemented with 10% fetal bovine serum, 10 μg/ml penicillin/streptomycin, and 0.025 g/ml amphotericin B (Gibco BRL, Grand Island, NY) at 37°C in 5% CO_2. Differentiation of PC12 cells, a model system for postmitotic neurons, was induced in the presence of low-serum nerve growth factor (NGF) media [DMEM containing 50 ng/ml NGF (Calbiochem, Darmstadt, Germany), 0.3% serum, and 10 μg/ml penicillin/streptomycin]. Two days later, culture media were exchanged with fresh low-serum NGF media, and then 10 μM or 50 μM concentrations of the purified Aβ were applied to the cultures for 48 hr.

Toxicity Assay

The toxicity of the purified Aβ was determined using the lactate dehydrogenase (LDH) assay. After incubation of NGF-treated PC12 neuronal cells with 10 μM or 50 μM concentrations of the purified $A\beta_{1-42}$, LDH activity in the medium was measured by a Cytotox 96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI) according to the manufacturer's instructions. The LDH release obtained by vehicle treatment was taken as 0%. The results were expressed as percentages of maximum LDH release obtained by the complete cell lysis following 10% Triton X-100 treatment.

cRNA Preparation and Expression and Voltage-Clamp Electrophysiology in *Xenopus* Oocytes

cDNAs encoding full-length human BBP (hBBP) and *Drosophila* BBP (dBBP) were subcloned as Acc65I and XhoI fragments into the *Xenopus* oocyte expression vector pSDR-ER modified from pSP64T (Krieg and Melton, 1984; Lee et al., 1999). The oocyte expression DNA construct of full-length human APP was generated by polymerase chain reaction (PCR)-based subcloning into the NotI and SpeI sites of pSDR-ER. For the hBBP-EGFP fusion construct, cDNA sequences encoding both proteins were PCR amplified and were then fused in frame via the common linker sequence, 5′-GGGGATATCGGG-3′. The fused DNA flanked by a 5′ EcoRI and a 3′ XhoI site was inserted into the EcoRI and XhoI sites of pSDR-ER. After plasmid linearization by XbaI, the cRNAs of human and *Drosophila* BBPs, hBBP-EGFP, and APP were prepared by in vitro transcription, using the cRNA Megascript kit (Ambion). The cRNAs of the G protein-gated inwardly rectifying K^+ channel (GIRK1; Dascal et al., 1993) and the octopamine/tyramine receptor (Arakawa et al., 1990; Robb et al., 1994) were prepared as described previously (Lee et al., 1999). *Xenopus* oocyte preparation and cRNA microinjection were carried out as described previously (Lee et al., 1999). Five nanograms of each GIRK channel and octopamine/tyramine receptor cRNA were coinjected with 5 ng BBP cRNA into a *Xenopus* oocyte. Ten nanograms of hBBP-EGFP cRNA were injected into oocytes. EGFP fluorescence was examined by confocal microscopy 3 days after injection. Fluorescence intensity was determined by LaserPix software (Image-Pro Plus 4.0; Bio-Rad, Hercules, CA).

The two-electrode voltage-clamp experiment was performed with a Geneclamp 500 amplifier (Axon Instruments, Burlingame, CA) as described previously (Lee et al., 1999). The holding potential was set at −70 mV. In case of GIRK current recording, the bath solution was exchanged to high-K^+ (HK; 96 mM KCl, 2 mM NaCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5) solution from ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5). Data were collected on a chart recorder (Pharmacia LKB REC1) and stored on a video cassette recorder tape using Digidata (Instrutech) for later analysis.

Data Analysis

Electrophysiological recording data and cellular toxicity data were analyzed statistically by two-tailed unpaired Student's *t*-test. Data were considered significantly different at $P < .05$.

RESULTS

Determination of Aβ Toxicity in NGF-Treated PC12

We evaluated the toxic activity of Aβ in both its aggregated and its nonaggregated forms, by using PC12 cells differentiated into neurons in the presence of NGF. After treatment with 10 μM or 50 μM $A\beta_{1-42}$ for 48 hr, we performed the LDH assay to measure the toxic activity of $A\beta_{1-42}$. The treatment with 50 μM aggregated or nonaggregated $A\beta_{1-42}$ induced cell death in 53.3% ± 7.1% (n = 18) and 34.2% ± 5.5% (n = 19) of the total cells, respectively. A Student's *t*-test analysis shows that these are significantly different (unpaired, $P < .05$). On the other hand, 10 μM $A\beta_{1-42}$ showed reduced cell toxicity (death rate: aggregated, 10.0% ± 1.9%, n = 19; nonaggregated, −1.6% ± 2.0%, n = 18; unpaired Student's *t*-test, $P < .001$). For voltage-clamp experiments (see below), we mainly used 10 μM aggregated or nonaggregated $A\beta_{1-42}$, the same low concentration that was previously used in a study of G protein coupling (Kajkowski et al., 2001).

BBP Does Not Couple to the Heterotrimeric G Protein in *Xenopus* Oocytes

To examine whether BBP is directly coupled to a G protein, we coexpressed BBP with G protein-coupled channels in the *Xenopus* oocyte. We first examined whether BBP was properly targeted to the plasma membrane of the *Xenopus* oocyte. As shown in Figure 1, BBP fused to enhanced green fluorescent protein (EGFP) was largely targeted to the membrane.

We then investigated whether hBBP is coupled to $G\alpha_{i/o}$ protein. For this purpose, G protein-activated inward-rectifying K^+ (GIRK1) channel coupled to $G_{i/o}$ (Dascal et al., 1993; Lee et al., 1999) was coexpressed with hBBP. As a control, the cRNA of the octopamine/

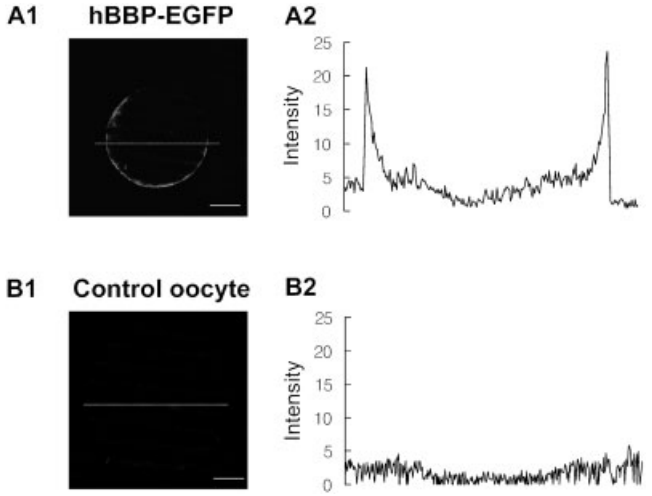

Fig. 1. BBP is targeted to the plasma membrane in *Xenopus* oocytes. **A:** cRNA of hBBP conjugated to EGFP was injected into the *Xenopus* oocyte. This confocal microscopic image shows EGFP-fused hBBP expressed on the plasma membrane of a *Xenopus* oocyte. **B:** No fluorescence was detected in the control oocyte. The dashed lines in the confocal fluorescence images (A1,B1) indicate the paths along which the fluorescence intensities of the corresponding images were plotted in the corresponding A2,B2. EGFP-fused hBBP is localized primarily on the plasma membrane, whereas no fluorescence was observed in non-injected control oocytes. Cross sections of fluorescence intensity in confocal images were analyzed using LaserPix (Bio-Rad). F.I., fluorescence intensity in arbitrary units. Scale bars = 200 μm.

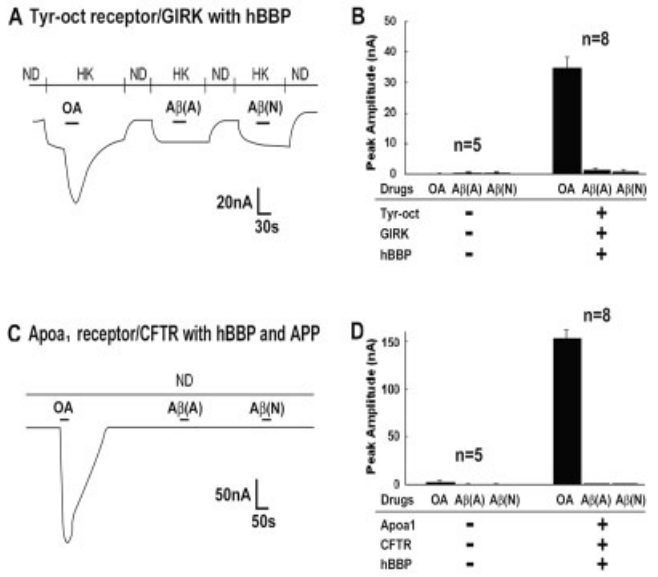

Fig. 2. BBP is not coupled to the heterotrimeric G protein in a heterologous *Xenopus* oocyte system. Treatment with aggregated or nonaggregated Aβ did not produce a significant GIRK current (A,B) or CFTR current (C,D) compared with the noninjected control oocytes. **A:** Representative recording data in oocytes expressing hBBP, Tyr-oct receptor, and GIRK. Octopamine treatment induced a GIRK current, which confirms the expression of the injected cRNAs. ND, ND96 solution; HK, high-K^+ solution; OA, octopamine; Aβ(A), aggregated $A\beta_{1-42}$; Aβ(N), nonaggregated $A\beta_{1-42}$. **B:** Group data showing that hBBP is not coupled to $G_{i/o}$ proteins. Bars represent mean ± SEM; n, number of oocytes. Three independent experiments were carried out by using oocytes prepared from six female animals. Tyr-oct, Tyr-oct receptor. **C:** Representative recording data in oocytes expressing hBBP, CFTR, and the $Apoa_1$ receptor. cRNA expression was confirmed by CFTR current induced by octopamine treatment. **D:** Group data showing that BBP is not directly coupled to G_s proteins. Bars represent mean ± SEM; n, number of oocytes. Three independent experiments were carried out using oocytes prepared from six animals.

tyramine (Tyr-oct) receptor (Arakawa et al., 1990; Robb et al., 1994) coupled to $G\alpha_{i/o}$ was coinjected with hBBP and GIRK. When oocytes expressing hBBP, Tyr-oct receptor, and GIRK were treated with 10 μM octopamine, a substantial GIRK current (35.0 ± 3.1 nA, n = 8) was detected. Neither 10 μM aggregated $A\beta_{1-42}$ (1.1 ± 0.6 nA, n = 8) nor 10 μM nonaggregated $A\beta_{1-42}$ (0.6 ± 0.6 nA, n = 8) treatment produced a significant current compared with control vehicle treatment (1.1 ± 0.3 nA, n = 5; Fig. 2A,B). Moreover, $A\beta_{1-42}$ did not produce a significant response even at the relatively high concentration of 50 μM (aggregated, $A\beta_{1-42}$, 0.0 ± 0.0 nA, n = 3; nonaggregated, $A\beta_{1-42}$, 0.0 ± 0.0 nA, n = 3). Similarly, 10 μM of neither aggregated nor nonaggregated $A\beta_{1-40}$, which is known to be less toxic than $A\beta_{1-42}$ (Suh and Checler, 2002), produced a significant current (aggregated, $A\beta_{1-40}$, 1.7 ± 1.7 nA, n = 3; nonaggregated, $A\beta_{1-40}$, 0.0 ± 0.0 nA, n = 3). Oocytes expressing only hBBP and GIRK were also treated with 10 μM aggregated or nonaggregated $A\beta_{1-42}$, but no significant difference compared with the other experimental groups was detected (data not shown). These results suggest that hBBP may not be directly coupled to $G\alpha_{i/o}$ in these oocytes. *Drosophila* BBP (dBBP) was also tested. Neither 10 μM aggregated $A\beta_{1-42}$ treatment nor 10 μM nonaggregated $A\beta_{1-42}$ treatment produced a significant response (aggregated, 0.0 ± 0.5 nA, n = 5; nonaggregated, 1.0 ± 0.4 nA, n = 7) in oocytes expressing dBBP, Tyr-oct receptor, and GIRK. In oocytes expressing only the Tyr-oct receptor and GIRK, a current of 1.0 ± 0.4 nA (n = 4) was detected on treating with 10 μM aggregated $A\beta_{1-42}$. Similarly, dBBP did not seem to be coupled to $G_{i/o}$ protein either.

To test whether hBBP is coupled to the G_s protein, hBBP was coexpressed with the *Aplysia* octopamine receptor ($Apoa_1$; Chang et al., 2000) and the cystic fibrosis transmembrane regulator (CFTR; Uezono et al., 1993). $Apoa_1$ and CFTR were previously shown to be coupled to G_s protein signaling (Chang et al., 2000). In oocytes expressing hBBP, $Apoa_1$, and CFTR, a CFTR current of 153.1 ± 12.2 nA (n = 8) was detected in response to 10 μM octopamine. However, 10 μM of neither aggregated nor nonaggregated $A\beta_{1-42}$ treatment activated the CFTR current (aggregated, 0.6 ± 0.3 nA, n = 8; nonaggregated, 0.3 ± 0.2 nA, n = 8) in oocytes expressing hBBP, $Apoa_1$, and CFTR (Fig. 2C,D), nor did 50 μM of aggregated or nonaggregated $A\beta_{1-42}$ (aggregated, $A\beta_{1-42}$, 0.0 ± 0.0 nA, n = 3; nonaggregated, $A\beta_{1-42}$, 0.0 ± 0.0 nA, n = 3).

Also, 10 μM $A\beta_{1-40}$ treatment failed to induce a CFTR current (aggregated, $A\beta_{1-40}$, 0.0 ± 0.0 nA, n = 3; nonaggregated, $A\beta_{1-40}$, 0.0 ± 0.0 nA, n = 3). These results show that hBBP is not directly coupled to the G_s protein.

APP Does Not Form a Functional Receptor for Aβ With BBP

Although BBP itself is not coupled to G protein directly, it may form a functional heteromultimeric receptor with other unknown components. To examine whether APP could form a functional receptor for Aβ with BBP, we coexpressed APP with BBP, octopamine/tyramine receptor, and GIRK in *Xenopus* oocytes. No response was detected after 10 μM aggregated or nonaggregated $A\beta_{1-42}$ treatment (aggregated, $A\beta_{1-42}$, −0.3 ± 0.9 nA, n = 4; nonaggregated, $A\beta_{1-42}$, 1.0 ± 0.9 nA, n = 4; 10 μM octopamine, 75.0 ± 24.0 nA, n = 4; Fig. 3A,B). APP was also coexpressed with BBP, $Apoa_1$, and CFTR, but no response was detected after 10 μM aggregated or nonaggregated $A\beta_{1-42}$ treatment (aggregated, 0.2 ± 0.2 nA, n = 5; nonaggregated, 0.8 ± 0.6 nA, n = 5; Fig. 3C,D). Thus, APP is probably not a component of the functional BBP receptor complex.

The *Xenopus* oocyte has an endogenous Cl^- channel that is transiently activated by IP_3, a product of phospholipase C (PLC; Lubbert et al., 1987). Therefore, we can determine whether a receptor is coupled to the G_q protein by looking at IP_3-activated transient Cl^- current in the oocyte. Neither aggregated nor nonaggregated Aβ treatment produced a transient Cl^- current in oocytes expressing either hBBP only (0.0 ± 0.0 nA, n = 4) or hBBP and APP (0.0 ± 0.0 nA, n = 5). In addition, we could not observe a transient Cl^- current with Aβ treatment in the oocytes expressing CFTR and GIRK (Figs. 2, 3). Therefore, hBBP or APP is not directly coupled to G_q.

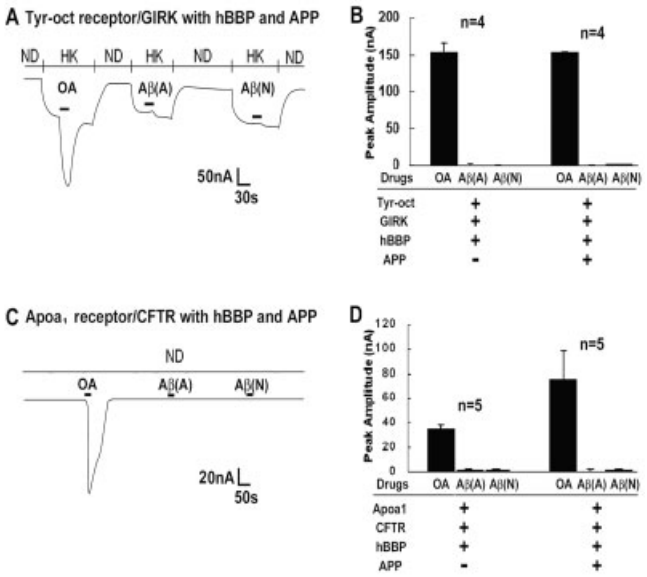

Fig. 3. APP does not form a functional receptor for Aβ with BBP. APP was expressed with hBBP to examine whether it could form a heteromultimeric G protein-coupled receptor for Aβ. Aggregated or nonaggregated Aβ treatment did not induce G protein-mediated GIRK current (A,B) or CFTR current (C,D) compared with the noninjected control oocytes. This suggests that APP does not form a functional receptor for Aβ with hBBP. **A:** Representative recording data in oocytes expressing hBBP, Tyr-oct receptor, GIRK, and APP. Octopamine treatment induced a GIRK current. However, aggregated or nonaggregated Aβ did not induce a GIRK current. ND, ND96 solution; HK, high-K^+ solution; OA, octopamine; Aβ(A), aggregated Aβ; Aβ(N), nonaggregated Aβ. **B:** Group data showing that APP and hBBP did not induce GIRK current via $G_{i/o}$ protein signaling. Bars represent mean ± SEM; n, number of oocytes. Tyr-oct, Tyr-oct receptor. Two independent experiments were carried out using oocytes prepared from four animals. **C:** Representative recording data in oocytes expressing hBBP, $Apoa_1$ receptor, CFTR, and APP. Octopamine treatment induced a CFTR current, whereas aggregated or nonaggregated Aβ treatment did not. **D:** Group data showing that APP and hBBP did not induce CFTR current via G_s protein signaling. Bars represent mean ± SEM; n, number of oocytes. Two independent experiments were carried out using oocytes prepared from two animals.

DISCUSSION

BBP has been shown to modulate Aβ toxicity by coupling to $G_{i/o}$ protein (Kajkowski et al., 2001), but it has not yet been demonstrated whether BBP is directly coupled to $G_{i/o}$. Our electrophysiological study shows that BBP is not directly coupled to $G_{i/o}$, G_s, or G_q protein in *Xenopus* oocytes. However, we cannot exclude the possibility that BBP forms a heteromultimer with other component(s) to be a functional receptor. The reasoning behind this suggestion is that common G protein-coupled receptors (GPCRs) have seven transmembrane domains; however, BBP has only two transmembrane domains, which are homologous to the third and fourths domain of the seven transmembrane domains of GPCR (Kajkowski et al., 2001). Thus, to be a functional GPCR, BBP may form a homomultimer or a heteromultimer. Our results show that BBP itself cannot form a functional receptor and that even its homomultimer may not be a functional receptor. Kajkowski and colleagues (2001) expressed BBP in SH-SY5Y cells, a human neuroblastoma cell, which might contain the other components required by BBP to form a functional GPCR. Thus, it is possible that BBP forms a heteromultimer with other membrane proteins to enable it to function as a GPCR.

APP is a possible counterpart of BBP in a functional receptor. Moreover, several mutations of the APP gene have been correlated with Alzheimer's disease; furthermore, these mutations caused apoptotic cell death when expressed in several cell lines (Yamatsuji et al., 1996a,b). In addition, apoptosis induced by APP mutant is blocked by pertussis toxin treatment (Giambarella et al., 1997; Hashimoto et al., 2000). This suggests that the APP mutant causes apoptosis mediated via G protein signaling, which is similar to the behavior of BBP. Thus, it is highly possible that BBP binds to APP to form a functional heteromultimeric receptor for Aβ, which induces neuronal apoptosis through G protein-mediated signaling. However, our data showed that oocytes coexpressing BBP and APP did not

generate any G protein-mediated response to Aβ treatment. Even though APP did not form a functional receptor complex with BBP, there are other possible receptor candidates. The membrane proteins alpha7 nicotinic acetylcholine receptor (Wang et al., 2000), p75 (NTR; Dechant and Barde, 2002), and the C-terminal domain of apolipoprotein E (Pillot et al., 1999) have previously been shown to bind with Aβ and to be involved in Alzheimer's disease. Direct evidence of the interaction between such species and Aβ in Alzheimer's disease could facilitate elucidation of the mechanism of Aβ-induced neurodegeneration.

The results of this study suggest that BBP is not directly coupled to the G protein in *Xenopus* oocytes. This does not necessarily imply that BBP and G protein coupling is absent in cells other than *Xenopus* oocytes, which may not contain a specific isotype of G protein that can interact with BBP. However, it is also possible that BBP is one component of the Aβ receptor and that other components or subunits are required to constitute a functional Aβ receptor. Therefore, we cannot rule out the possibility that the other component(s) of this hypothetic heteromultimer were missing in the *Xenopus* oocyte system.

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