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Neurotrophic and Neurotoxic Effects of A β 42 and Its Oligomers on Neuronal Survival: Revealed by Their Opposite Influence on the Potency of Extracellular BDNF

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Abstract: Brain-derived neurotrophic factor (BDNF) is critical for neuronal survival. Amyloid- β monomers (A β 42M) and oligomers (A β 42O) have trophic and toxic effects on neuronal survival, respectively. Branched oligosaccharides (BOs) and catechins (CAs) can specifically bind to A β 42M/A β 42O, influencing both effects. However, whether and how A β 42M/A β 42O influences BDNF remains unknown. This study investigated the interaction between A β 42M/A β 42O and BDNF, the effects of A β 42M and A β 42O on BDNF binding to the TrkB/p75 receptor and their impact on BDNF-supported cell survival, and the roles of BOs and CAs in these processes. BDNF exhibited stronger binding affinity for A β 42M and A β 42O than BOs/CAs. A β 42M increased neuronal viability by synergistically enhancing BDNF binding to TrkB and p75, whereas A β 42O decreased neuronal viability by inactivating/consuming BDNF, thereby reducing its binding to these receptors. BDNF-A β 42O binding appeared to mutually neutralize/counteract each other's biological effects; therefore, increasing BDNF levels might reduce A β 42O's neurotoxicity. By competitively targeting A β 42M/A β 42O rather than BDNF or its receptors, BOs and CAs enhanced these effects. These findings suggest that A β 42M's neurotrophicity was directly linked to its synergistic enhancement of BDNF activity, whereas A β 42O's neurotoxicity was primarily due to its inactivation or consumption of BDNF. This study provided valuable insights for developing BOs/CAs-based neuroprotective therapeutics or nanomaterials against AD.

Keywords: amyloid- β protein (A β 42); brain-derived neurotrophic factor (BDNF); catechins (CAs); branched oligosaccharides (BOs); tyrosine kinase receptor B (TrkB); p75; Alzheimer's disease (AD)



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1. Introduction

Alzheimer's disease (AD) is a complex neurodegenerative disorder characterized by the loss of hippocampal neurons and the presence of various types of amyloid- β protein 1–42 (A β 42) aggregates and amyloid plaques, primarily in the hippocampus. Since the C-terminal fragment of the A β 42 molecule is hydrophobic [1,2], A β 42 monomers (A β 42M) tend to self-assemble into aggregates in the absence of molecular chaperone-like substances, gradually forming physiologically harmful oligomers (A β 42O), protofibrils (A β 42P), fibrils (A β 42F), and plaques, even though they function like neurotrophins for neuronal survival and development [3,4]. These A β 42 aggregates, particularly A β 42O, are the primary cause of neuronal loss in AD [5], disrupting synapses and causing neuronal death both in vitro

and in vivo. Therefore, the main medications should prevent A β 42 from aggregating and depositing or stop A β 42 aggregates from causing injury and reducing cell viability [6].

A β 42M has beneficial effects on various properties of neuronal cells, such as supporting cell survival [4,7], motility, and adhesion [8,9]. In contrast, A β 42 aggregates adversely affect multiple aspects of neuronal function and directly disrupt a range of membrane receptor-mediated pathways [10]. Although the physiological functions of these A β 42 species are primarily manifested in their effects (whether positive or negative) on neuronal cell viability or survival, the key mechanisms underlying these effects remain poorly understood, significantly hindering the development of effective prevention and treatment strategies for AD.

Neurotrophins (NTs), called growth factors, are essential for synaptic plasticity and mediate neuron survival and development in the peripheral and central nervous systems (CNS). Brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), NT3, and NT4/5 are the four main NTs in the brain. In addition to preserving neuronal viability and function, NTs—particularly BDNF—contribute to the repair of injured cells in adults [11–13]. Thus, injured but viable CNS neurons can be repaired with strong support from neurotrophic factors. The impairment and death of neurons occur when effective BDNF levels are insufficient or when its binding to TrkB (tyrosine kinase receptor B) and p75 receptors is blocked, which is a major cause of the onset and progression of neurodegenerative diseases [13]. Therefore, NTs—particularly BDNF—may be a key or primary target for AD prevention and treatment [14].

Clarifying whether A β 42 or its aggregates specifically interact with BDNF and how these interactions influence the cytological effects of BDNF or its correlation with neuronal survival is crucial, as both A β 42 and BDNF significantly impact neuronal viability. We recently found that branched oligosaccharides (BOs), particularly isomaltotriose (IG3) and biantennary N-linked core pentasaccharide (BCP), enhance neuronal survival by functioning as molecular chaperone-like substances for A β 42M or antagonistic-binding partners for A β 42O [15]. Additionally, catechins (CAs), especially catechin (CA) and epigallocatechin gallate (EGCG), have been reported to bind to A β 42 and attenuate the cytotoxicity associated with A β 42 aggregates [16–18]. CAs are a class of tea polyphenols that cross the BBB [19], and their chemical properties, molecular conformations, and binding properties to A β 42 or its aggregates are very similar to those of BOs [16,20]. To develop new strategies for AD treatment, we investigated in this study the interaction of A β 42 and its aggregates with BDNF, examined the effects of these A β 42 species on the cytological potency of extracellular BDNF, and explored the roles of BOs and CAs in these processes.

2. Results

2.1. Examination of the Binding Specificity of BDNF to Four A β 42 Species

BDNF, a basic protein with an isoelectric point of 9–10, and A β 42, a weakly acidic small-molecule protein (pI \approx 5.3), exhibit inherent chemical interaction potential under physiological conditions. Different A β 42 species (A β 42M, A β 42O, A β 42P, and A β 42F) exhibit distinct neurotoxic/neuroprotective effects on neuronal cell viability, and different BOs (e.g., IG3 and BCP) or CAs (e.g., CA and EGCG) have distinct binding specificities and affinities for various A β 42 species [15–17]. Therefore, the binding rate of BDNF to each A β 42 species was determined using indirect ELISA in the presence or absence of these modulators (IG3, BCP, CA, and EGCG), and the results were expressed as the binding specificity of BDNF for each A β 42 species. HSA served as the negative control for the A β 42 species.

Under the conditions as shown in Figure 1, after 24 h of co-incubation, the binding rate of BDNF to HSA was less than 3% but it was greater than 10% for both A β 42M

and A β 42O, indicating binding specificity between BDNF and A β 42M/A β 42O. However, BDNF showed minimal binding capabilities to larger A β 42 aggregates, as indicated by a binding rate of less than 5% for both A β 42P and A β 42F. Furthermore, Figure 1 illustrates that the binding rate of BDNF to A β 42O (*n* in Figure 1) was slightly higher than to A β 42M (*e* in Figure 1), although this difference was not statistically significant within 24 h. The results for the A β 42M group likely reflect the combined effects of A β 42M and the newly formed A β 42O generated by A β 42M aggregation during the 24 h incubation period. This implies that BDNF had a higher binding specificity for A β 42O than A β 42M.

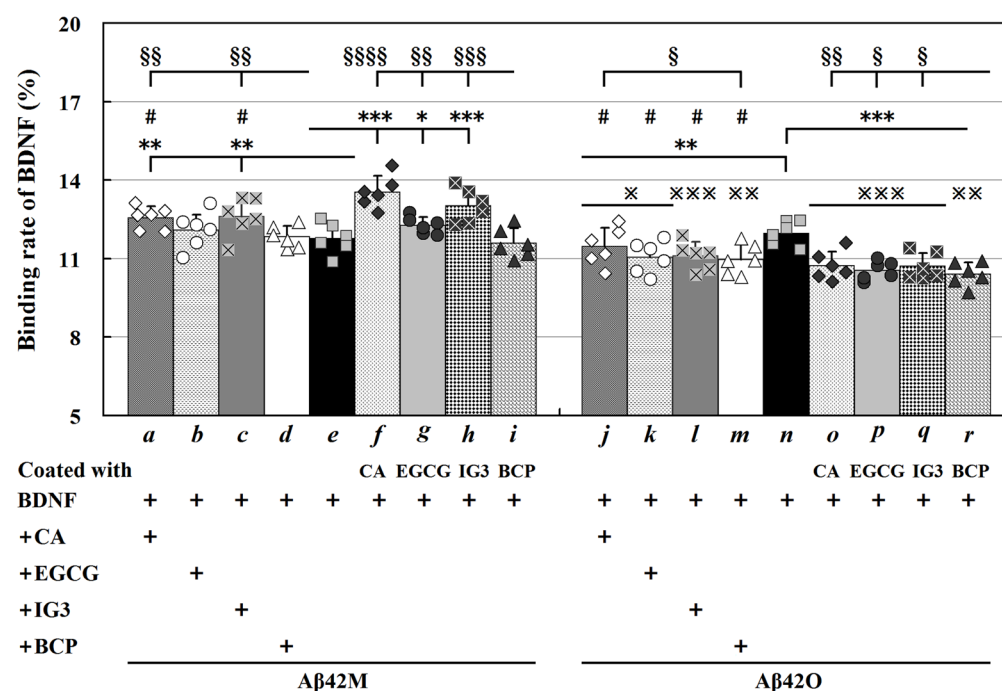


Figure 1. Binding rate of BDNF for A β 42M and A β 42O by indirect ELISA. The letters *a* through *r* indicate the corresponding groups. Binding rates of BDNF (final concentration: 20 pg/mL) to A β 42M and A β 42O (final concentration: 2.0 μ M) with and without CA/EGCG/IG3/BCP (final concentration: 50 μ M for CA/EGCG, 2.0 μ M for IG3/BCP). The average amount of BDNF in the supernatants of the blank control groups was regarded as 100%. Each assay was performed in triplicate and repeated across six different batches of A β 42. Values are presented as the mean \pm SEM. Symbols *, #, \$, and * indicate significant differences between groups with and without CA/EGCG/IG3/BCP, between groups with and without CA/EGCG/IG3/BCP in the coated A β 42M/A β 42O, between corresponding groups with CA/EGCG/IG3 and with BCP, and between corresponding A β 42M and A β 42O groups, respectively. */#/\$/* $p < 0.05$, **/\$\$/** $p < 0.01$, ***/\$\$\$/** $p < 0.001$, \$\$\$\$ $p < 0.0001$.

The results in Figure 1 show that the binding specificity of BDNF for A β 42M and A β 42O was affected differently by the four polyhydroxy compounds (IG3, BCP, CA, and EGCG). Overall, IG3, CA, and EGCG enhanced the binding rate of BDNF to A β 42M (*a*, *c*, and *f–h*, Figure 1) (* $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$); however, all of these compounds, especially BCP and EGCG, decreased the binding rate of BDNF to A β 42O (*j–m* and *o–r*, Figure 1) (** $p < 0.01$, or *** $p < 0.001$). These differing effects appear to result from the distinct binding properties of the compounds to A β 42M because IG3 exhibits relatively high binding affinity for A β 42M, whereas BCP shows relatively high binding affinity for A β 42O [15].

Furthermore, when A β 42M/A β 42O was pre-incubated (co-coated) with these compounds for 12 h at 4 $^{\circ}$ C (*f–i* and *o–r*, Figure 1), the effect of these compounds on the binding rate (increase/decrease) of BDNF to A β 42M/A β 42O became more noticeable.

However, the binding rate of BDNF to A β 42M/A β 42O remained unchanged regardless of the pre-incubation of the four compounds with BDNF. This implies that the compounds targeted A β 42M/A β 42O rather than BDNF, thereby altering the binding specificity of BDNF for A β 42M/A β 42O. Consequently, compared to the matching in-site systems co-incorporated with A β 42M/A β 42O and BDNF (*a–d* and *j–m*, Figure 1), these compounds had a more profound effect on A β 42M/A β 42O in their pre-incubated (co-coated) systems with A β 42M/A β 42O (*f–i* and *o–r*, Figure 1) (# $p < 0.05$). This suggests that the simultaneous binding of IG3/BCP/CA/EGCG and BDNF to an A β 42 molecule or an A β 42O unit was nearly impossible.

The results in Figure 1 indicate that IG3, CA, and EGCG increased the interaction between BDNF and A β 42M most likely by preserving the native (or active) conformation of A β 42 molecules, while they decreased the interaction between BDNF and an A β 42O unit most likely by disrupting the integrated conformation of the A β 42O unit according to previous findings [15]. Consequently, as Figure 1 illustrates, the binding rate of BDNF to A β 42M was higher than that to A β 42O when IG3, BCP, CA, or EGCG were present (* $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$). As BCP has a relatively low binding efficiency compared to A β 42M [15], it had a minimal effect on BDNF binding to A β 42M over 24 h. Indeed, BCP significantly increased the binding rate of BDNF to A β 42M when the experiment duration was prolonged to 48 h. Thus, the results in Figure 1 suggest that additional extracellular substances, such as BOs or CAs, that interact with A β 42M/A β 42O may influence the binding specificity between extracellular BDNF and A β 42M/A β 42O.

2.2. Binding Affinity of BDNF for A β 42M and A β 42O

The specific binding between BDNF and A β 42M/A β 42O depends not only on specific, particularly polar interactions, but also on their conformation and the spatial arrangement or orientation of the interacting groups. These factors influence the strength and extent of the interaction force between them or their binding affinity. To assess the binding affinity of BDNF for A β 42M and A β 42O, the equilibrium dissociation constants (K_D) of BDNF with A β 42M and A β 42O in the presence and absence of CA, EGCG, IG3, and BCP, respectively, were determined using indirect competitive ELISA and were then used to inversely express the binding affinity of BDNF for A β 42M and A β 42O.

The amount of BDNF in the well that was not bound to the immobilized A β 42M/A β 42O was measured using the anti-BDNF antibody after the establishment of equilibrium relationships of BDNF between immobilized and free A β 42M/A β 42O were established. The results are shown in the supplementary materials (Figure S1).

The values on these curves corresponding to the 50% maximum absorbance were used to calculate the K_D values for each group. As shown in Table 1, the K_D value of BDNF for A β 42M (6.67×10^{-7} M) was marginally higher than that for A β 42O (2.88×10^{-7} M), indicating that the binding affinity between BDNF and A β 42O was marginally higher than that between BDNF and A β 42M, and vice versa. Combined with the results in Figure 1, which show that the binding specificity of BDNF for A β 42O was higher than that for A β 42M (*n* and *e*, Figure 1), it could be concluded that BDNF and A β 42O interacted or bound more strongly and stably than BDNF and A β 42M. In contrast, BDNF dissociates from A β 42M more quickly and easily than from A β 42O.

Furthermore, the K_D values of BDNF for A β 42M and A β 42O changed in opposite directions when CA, EGCG, IG3, or BCP was present; specifically, the K_D values decreased for A β 42M and increased for A β 42O (Table 1). The lowest K_D values were observed in the A β 42M with IG3 group (3.89×10^{-7} M), whereas the highest was in the A β 42O with BCP group (2.34×10^{-5} M), respectively. According to these findings, the polyhydroxy compounds increased the binding affinity of BDNF for A β 42M (particularly with IG3) while

reducing the binding affinity of BDNF for A β 42O (particularly with BCP). This implies that these compounds enhanced the interaction between BDNF and A β 42M while blocking or reducing the interaction between BDNF and A β 42O. These results can be explained as follows: for A β 42M, these compounds preserved or maintained the conformation of A β 42M, which increased the binding affinity between A β 42M and BDNF in addition to improving the binding efficiency between them; for A β 42O, they blocked or disrupted the integrated conformation of an A β 42O unit, which decreased the binding affinity between A β 42O and BDNF as well as the binding efficiency between them. The BDNF-A β 42M complex should have a significantly smaller interface area than the BDNF-A β 42O complex because A β 42 is a small-molecule protein. This could make the BDNF-A β 42M complex easier to dissociate than the BDNF-A β 42O complex.

Table 1. K_D values (M) for BDNF.

A β 42 Species	In the Presence of ^a CA (or EGCG), ^b IG3, or ^c BCP	In the Absence of CA/EGCG/IG3/BCP
A β 42M	^a $4.91 \pm 0.57 \times 10^{-7}$ ^b $3.89 \pm 0.43 \times 10^{-7}$ ^c $6.17 \pm 0.13 \times 10^{-7}$	$6.67 \pm 0.26 \times 10^{-7}$
A β 42O	^a $3.36 \pm 0.27 \times 10^{-6}$ ^b $2.19 \pm 0.12 \times 10^{-6}$ ^c $2.34 \pm 0.28 \times 10^{-5}$	$2.88 \pm 0.33 \times 10^{-7}$

A β 42M, Amyloid- β monomers; A β 42O, oligomers; CA, catechins; EGCG, epigallocatechin gallate; IG3, isomaltotriose; BCP, biantennary N-linked core pentasaccharide; M, molar concentration; ^a, CA or EGCG group; ^b, IG3; ^c, BCP group.

The binding affinity between BDNF and A β 42M/A β 42O was significantly higher than that of BOs and A β 42M/A β 42O as the K_D values of BOs for A β 42M/A β 42O were 10- to 1000-fold higher than those of BDNF for A β 42M/A β 42O [15], and was higher than that between A β 42O and human anti-A β 42O single-chain antibodies, whose K_D values of the antibodies were roughly 10-fold higher than those of BDNF for A β 42O [21–24].

2.3. Differential Effects of A β 42M and A β 42O on BDNF-Supported Neural Cell Survival

It is well established that the viability or survival of neuronal cells in vitro and in vivo is directly correlated with the neurotoxicity of A β 42O and the neurotrophicity of A β 42M. We have shown that BOs (such as IG3 and BCP) have strong binding capabilities for both A β 42M and A β 42O and either protect or enhance neuronal cell viability in the presence of A β 42M and A β 42O [15]. However, the results in Table 1 indicate that the binding affinity of BDNF for A β 42M and A β 42O is higher than that of BOs and that CA/EGCG/IG3/BCP also modulate the binding affinity of BDNF as well.

As physiological BDNF levels in the CNS and in SH-SY5Y and HT22 cultures are extremely low (picogram levels) [25–27], even slight fluctuations in BDNF levels may critically influence neuronal cell survival. To determine whether A β 42M and A β 42O influence the supportive efficacies of BDNF on neuronal cell survival and whether CA/EGCG/IG3/BCP preserves or potentiates the cytological functions of BDNF via A β 42M and A β 42O, we systematically examined the combinatorial effects of these substances or anti-BDNF antibody (Ab) with A β 42M and A β 42O on the viability of SH-SY5Y and HT22 cells under distinct intervention sequences.

When compared to their respective controls (100%), CA/EGCG/IG3/BCP alone did not affect the viability of SH-SY5Y or HT22 cells. However, Ab and BDNF alone significantly decreased (down to ~91%) and increased (up to ~110%) the viability, respectively (① and ②, Figure 2A,B). Meanwhile, the viability under the co-treatment with Ab/BDNF and

CA/IG3 (⑥, Figure 2A), other was identical to that with Ab or BDNF alone. These results imply that the viability of these neuronal cells was positively correlated with the level of free BDNF, either endogenous or exogenous BDNF, and that an insufficient amount of BDNF in the culture medium directly reduced the cell viability. Additionally, the cell viability in the group co-treated with Ab and BDNF (⑤, Figure 2A) was identical to that of the control group, indicating that Ab and exogenous BDNF had equivalent effectiveness under these experimental settings.

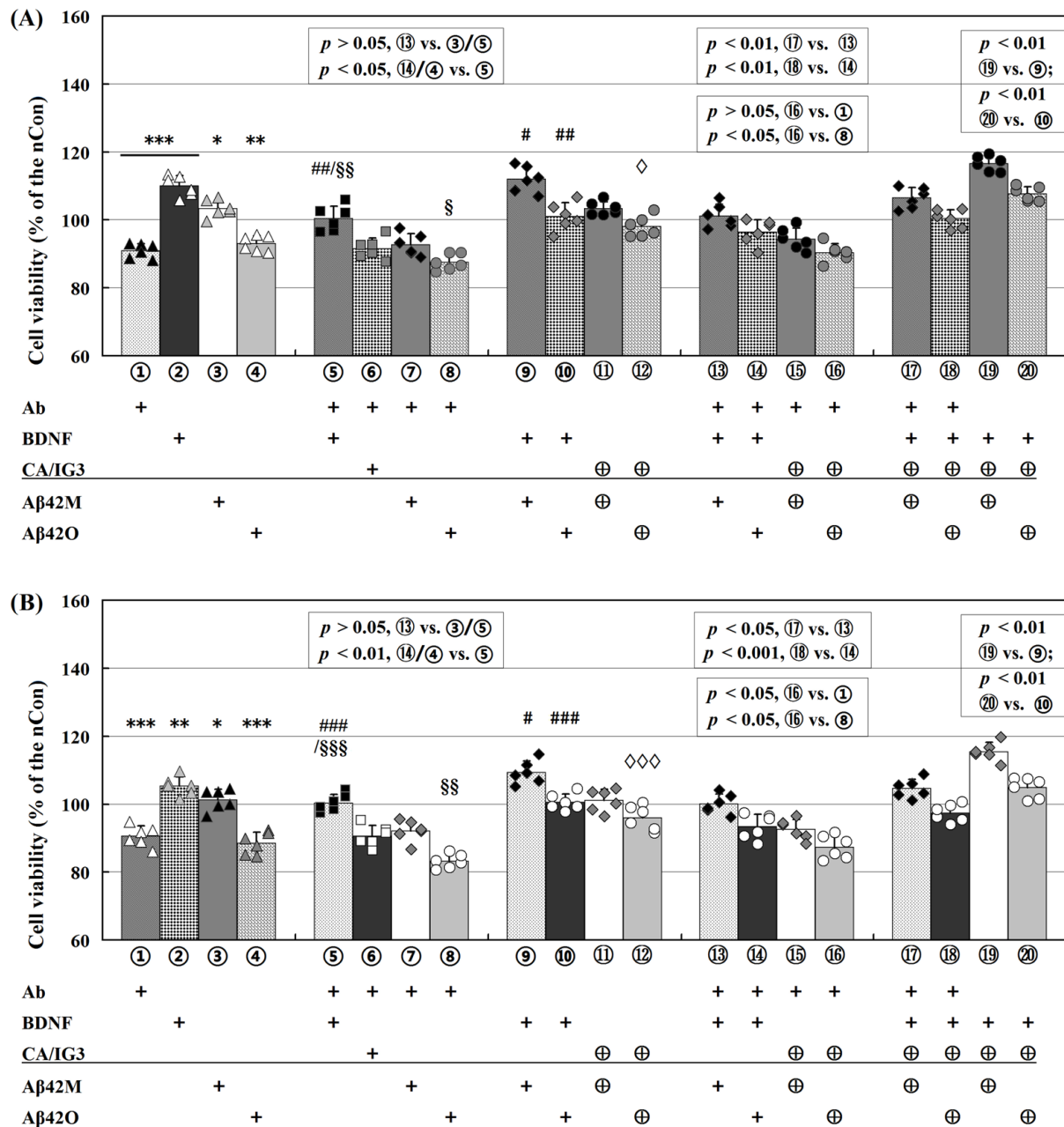


Figure 2. Differential effects of Aβ42M and Aβ42O on BDNF-supported neural cell survival. Viability of SH-SY5Y (A) and HT22 (B) cells in the presence of anti-BDNF antibody (Ab), BDNF, and Aβ42M/Aβ42O with and without CA and IG3 for 2 h at 37 °C quantified by MTT assay. The viability of the blank control group was defined as 100%. Symbol ⊕ indicates addition after being pre-incubated for 0.5 h at 4 °C. Symbols *, #, §, and ◇ indicate significant differences between the experimental and control groups, between BDNF alone and other groups, between Ab alone and other groups, and between Aβ42O alone and other groups, respectively. */#/§/◇ $p < 0.05$, **/##/§§ $p < 0.01$, and ***/###/§§§/◇◇◇ $p < 0.001$.

As shown in Figure 2A (SH-SY5Y cells), the neurotrophic effect of A β 42M on the cell viability appeared to depend on the BDNF level (③, ⑦, ⑨, and ⑬, Figure 2A). The corresponding groups with and without A β 42M did not differ in cell viability while Ab was present ($p > 0.05$, ① vs. ⑦/⑤ vs. ⑬, Figure 2A). However, the effect of A β 42M became more apparent as the BDNF levels increased ($p < 0.05$, ③ vs. control/② vs. ⑨, Figure 2A), suggesting that A β 42M enhanced the BDNF efficacy. These results indicate a synergistic effect of A β 42M on BDNF, i.e., the binding of A β 42M to BDNF might enhance the cytological efficacy of BDNF in targeting and binding to cells. This suggests that the neurotrophic effects of A β 42M might be primarily related to its synergistic effects on BDNF. This could be the cause of the dependence of A β 42M's neurotrophic effects on the BDNF levels. Additionally, the synergistic effect of A β 42M on BDNF was more significant after pre-incubating with CA or IG3 ($p < 0.01$, ⑨ vs. ⑲/⑰ vs. ⑬, Figure 2A). However, there was no difference in the cell viability between the A β 42M alone group and A β 42M with CA/IG3 groups when the BDNF level was low (③ and ⑪, ⑦ and ⑮, Figure 2A). These findings were consistent with the results showing that BOs and CAs facilitated the binding between A β 42M and BDNF (Figure 1 and Table 1).

In contrast, A β 42O continued to impair the cell viability in the presence of Ab, suggesting that A β 42O-induced neurotoxicity was somewhat dependent on the BDNF levels ($p < 0.05$, ① vs. ⑧/⑤ vs. ⑭, Figure 2A). However, as the BDNF level increased, the damage caused by A β 42O to the cell viability was progressively reduced (④ and ⑩, Figure 2A), which was inevitable because A β 42O and BDNF bound together (Figure 1 and Table 1). These results imply that a substantial portion of the neurotoxicity of A β 42O was caused by a reduction in BDNF effectiveness rather than merely a mix of the individual impacts of A β 42O and BDNF on the cell viability. Furthermore, regardless of whether these impairments were related to BDNF, the binding of CA/IG3 to A β 42O (during pre-incubation) significantly decreased the impairment of cell viability caused by A β 42O ($p < 0.05$, ⑧ vs. ⑩/④ vs. ⑫, Figure 2A). Regarding the BDNF-related A β 42O neurotoxicity, the protective effect of CA/IG3 on BDNF against A β 42O appeared to be positively correlated with BDNF levels [$p < 0.05$, ⑧ vs. ⑩; $p < 0.01$, ⑮ vs. ⑭] or ($p < 0.05$, ④ vs. ⑫; $p < 0.01$, ⑩ vs. ⑲), Figure 2A].

The results shown in Figure 2B and the results mentioned above were also confirmed in HT22 cells. Overall, the results of HT22 cells were almost identical to those of SH-SY5Y cells, with some minor differences. In HT22 cells, the synergistic effect of A β 42M on BDNF was comparable to or marginally weaker than that in SH-SY5Y cells (③, ⑨, and ⑬, Figure 2B), whereas the toxic effect of A β 42O, regardless of its relation to BDNF, was marginally stronger than that in SH-SY5Y cells (④, ⑩, and ⑭, Figure 2B). These patterns were observed in how CA/IG3 influenced A β 42M's synergistic effect on BDNF (⑪, ⑰, and ⑲, Figure 2B) and A β 42O's toxic effect (⑫, ⑱, and ⑲, Figure 2B). This suggests that the effects of A β 42M or A β 42O on the cytological efficacy of BDNF, as reflected in the cell viability or survival, were identical across different neuronal cell systems.

Furthermore, EGCG and BCP were systematically substituted for CA and IG3 within the established protocol. Quantitative analyses indicated that the neuroprotective effects were comparable to those in Figure 2, with BCP demonstrating a marginally enhanced cytoprotective effect against A β 42O relative to other test compounds. Furthermore, dose-response characterization demonstrated concentration-dependent therapeutic effects across all investigated agents: CA and EGCG exhibited optimal activity within the 0–100 μ M ranges, while IG3 and BCP showed maximal efficacy at 0–2.0 μ M concentrations.

Taken together, the results in Figure 2 suggest that the beneficial effect of A β 42M and the detrimental effect of A β 42O on neuronal cell viability or survival were substantially mediated by their binding to BDNF. A significant part, if not all, of the mechanism under-

lying the neurotrophicity of A β 42M and the neurotoxicity of A β 42O appeared to involve increased A β 42M, but A β 42O decreased the supportive efficacy of BDNF on neuronal cell survival through their specific binding to BDNF.

2.4. Examination of the Sites and Properties of the Interaction Between A β 42M/A β 42O and BDNF by Molecular Docking

Our previous studies have shown that both BOs bind to the A β 42 chain(s) axially or in a forked form [15]. However, BDNF also binds to A β 42M and A β 42O with greater affinity than BOs and CAs (Figure 1 and Table 1). To elucidate the mechanistic effects of distinct molecular interactions on the cytological function of BDNF and establish their correlations, we conducted systematic molecular docking analyses between BDNF and three critical targets: A β 42M, A β 42 trimer, and its receptor TrkB. These computational findings were subsequently compared with the integrated conformation of the NT4-TrkB reference complex.

Docking models of BDNF and A β 42M (Figure 3A(ii,iii)) showed that only the bottom portion of the BDNF molecule bound to the N-terminal (1–16 residues) and middle (17–28 residues) regions of the A β 42 molecule. This suggested that the interactions between polar or hydrophilic groups on the main and side chains in their interfacial regions—with or without the formation of H-bonds—were the main forces driving and stabilizing their binding (Figure 3A(i)). Furthermore, the docking model of BDNF and A β 42M showed that the binding between A β 42M and BDNF did not appear to spatially affect the binding of BDNF to the extracellular binding domain of its receptor TrkB, nor did it produce a spatial barrier to the dimerization of BDNF for the stable binding of BDNF to TrkB, as demonstrated in Figure 3B. This implies that the combination of A β 42M and BDNF might stabilize the conformation of BDNF and enhance its potency without diminishing its cytological effects. Combined with the beneficial effects of A β 42M on cell viability (③ and ⑨, Figure 2A,B), the BDNF-A β 42M docking model suggests that A β 42M binding to BDNF facilitated or enhanced the efficiency of BDNF to target and bind to its receptors in the cellular system. Thus, the apparent depletion of BDNF by A β 42O may be primarily due to their unique binding pattern in addition to their high binding affinity. This might be the mechanism underlying the synergistic effect of A β 42M on BDNF.

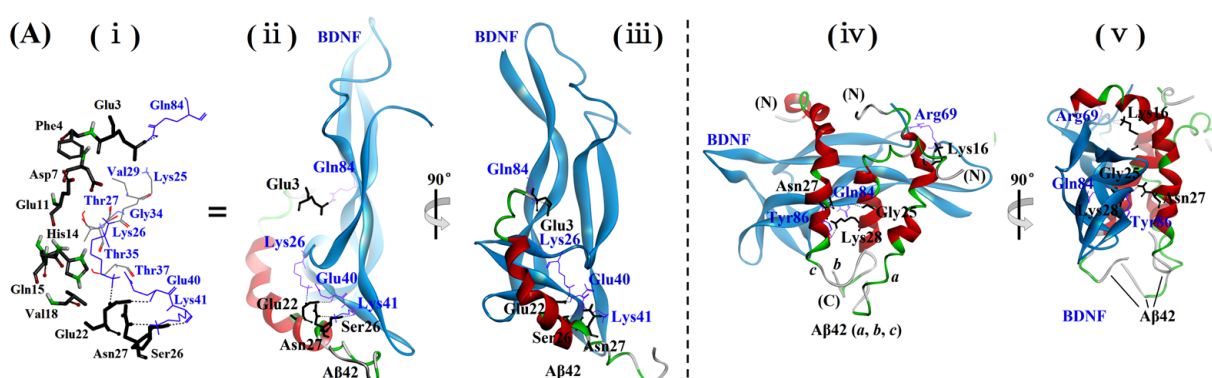


Figure 3. Cont.

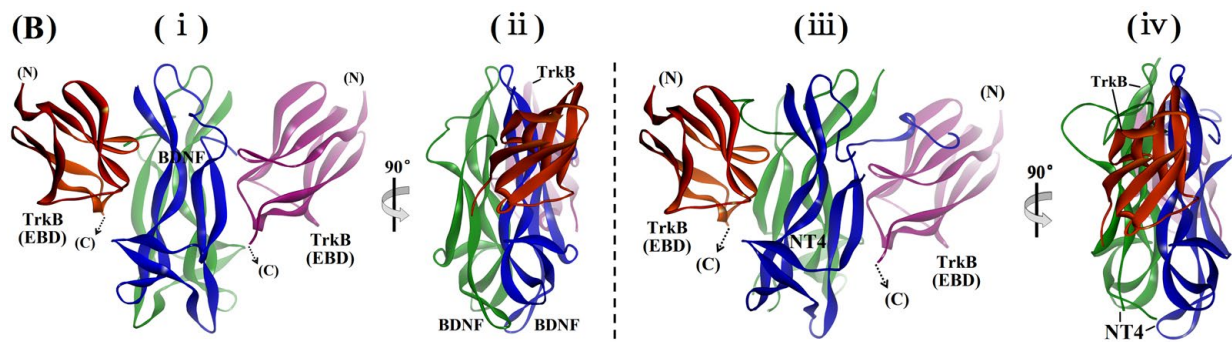


Figure 3. Molecular docking models of BDNF to Aβ42M, Aβ42 trimer, and the extracellular binding domain of TrkB. (A) Docking models of BDNF to Aβ42M chain and Aβ42 trimer (peptide chains were marked with symbols *a*, *b*, and *c*, respectively), showing the intermolecular hydrogen bonds (dashed lines) between BDNF's (lines) and the Aβ42 trimer's (stick) amino acid residues. (i) main amino acid residues involved in the interaction between BDNF and Aβ42; (ii,iii) docking model of BDNF with Aβ42; (iv,v) docking model of BDNF with Aβ42 trimer. (B) Docking models of BDNF to the extracellular binding domain (EBD) of TrkB (i,ii) and the crystal structure of NT4-TrkB complex downloaded from PDB (ID: 1HCF) (iii,iv). N or C: N or C terminus of Aβ42 chain.

In contrast, the binding between Aβ42O and BDNF was stronger and more stable than that between Aβ42M and BDNF, as demonstrated by the docking model of BDNF with Aβ42O (Figure 3A(iv,v)), which also revealed that the interaction sites and interface area between Aβ42O and BDNF were substantially greater than those between Aβ42M and BDNF. This finding explains why the binding performance of BDNF to Aβ42O was higher than that for Aβ42M (Figure 1 and Table 1). Additionally, nearly the entire flank of BDNF—specifically, the interface where it dimerizes (Figure 3B(i,ii))—was involved in the binding between Aβ42 trimer and BDNF (Figure 3A(iv,v)). This suggests that BDNF was rendered inactive, inhibiting its biological activity by Aβ42O binding, which lowered the level of effective BDNF. This is evidenced by the reduced cell viability observed in the Aβ42O groups containing either endogenous or exogenous BDNF, as shown in Figure 2A,B (③ and ⑨). Compared with the electrostatic energy (Eelec) (−428.5 kcal/mol) of the Aβ42 trimer-BDNF complex (Table S1), the slightly higher Eelec (−413.0 kcal/mol) of the Aβ42M-BDNF complex suggests that the Aβ42M-BDNF complex might have slightly lower stability than the Aβ42 trimer-BDNF complex. This was consistent with the difference in their binding affinities, as shown in Table 1.

Furthermore, the regions on the Aβ42M chain and Aβ42O unit where BDNF was bound were similar to or overlapped with those where IG3/BCP was bound to an Aβ42M chain or Aβ42O unit [15], particularly on the Aβ42M chain, as indicated by the docking models shown in Figure 3A. This suggests that competition between BDNF and BOs/CAs for binding to Aβ42M or Aβ42O is inevitable. Consequently, in the presence of both BDNF and BOs/CAs, their binding efficiency to Aβ42M or Aβ42O depended on their concentration and binding affinity to Aβ42M or Aβ42O, similar to the interaction between an enzyme's substrate and its competitive inhibitor. This was confirmed by the corresponding results for the Aβ42M and Aβ42O groups shown in Figure 2A,B (⑪–⑬ and ⑲–⑳). Combined with the results in Figure 2A,B, it could be concluded from the docking models in Figure 3A that BOs/CAs bound to Aβ42M were easily displaced by BDNF, whereas those bound to Aβ42O were not. As a result, only a fraction of BOs/CAs in the Aβ42O groups shown in Figure 2A,B might be displaced by BDNF.

BDNF and NT4 share the same receptors on neuronal cells and bind to them in a similar pattern [28]. BDNF and NT4 are structurally related proteins, sharing more than 50% identity in their amino acid sequences; therefore, their three-dimensional (3D)

structures have very similar features. The docking model of BDNF with the extracellular binding domain of TrkB shown in Figure 3B suggests that the docked conformations of the BDNF-TrkB complex (Figure 3B(i,ii)) closely matched those of the crystal structure of the NT4-TrkB complex (Figure 3B(iii,iv)). Therefore, the 3D structural model of the BDNF-TrkB complex is credible.

2.5. Effects of A β 42M and A β 42O on the Receptor-Targeting Performance of BDNF in the Presence and Absence of IG3/BCP/CA/EGCG by IF Staining

The supporting effects of BDNF on neuronal cell survival are mediated by its two receptors: the high-affinity TrkB and low-affinity p75. The activation of this coordinated BDNF/TrkB/p75 signaling axis plays a critical role in maintaining neuronal viability and regulating synaptic plasticity. Despite the characteristically low basal levels of BDNF in the CNS and the relatively sparse membrane expression of TrkB and p75 receptors under physiological conditions [25], the binding affinity between BDNF and TrkB/p75 is significantly higher than that between BDNF and A β 42M/A β 42O according to their extremely low dissociation constants of 10^{-11} mol/L and 10^{-9} mol/L for TrkB and p75, respectively [29]. Our earlier studies have demonstrated that both A β 42M and A β 42O can target cells [8], but neither colocalized with TrkB or p75 in our preliminary IF experiments. Given the effects of A β 42M and A β 42O on BDNF-related cell viability (Figure 2) and the distinct binding patterns of BDNF in the A β 42M-BDNF and A β 42O-BDNF complexes (Figure 3A), it is crucial to determine whether A β 42M and A β 42O (with or without IG3/BCP/CA/EGCG) influence the targeting and binding of BDNF to TrkB/p75 at the SH-SY5Y plasma membranes. This is essential for clarifying the mechanisms by which different A β 42 species affect neuronal cells.

The colocalization of the extracellular BDNF with the membrane TrkB/p75, which can be identified at the cellular level via IF staining, indicates their binding on the plasma membrane. Figure 4 shows representative images of SH-SY5Y cells in which BDNF and membrane TrkB/p75 were fluorescently labeled with specific anti-BDNF (green) and anti-TrkB/p75 (red) antibodies, respectively. Their spatial overlap at the same pixel was considered evidence of colocalization (yellow). Overall, in all four groups (Figure 4A–D), BDNF colocalized more with TrkB than with p75, indicating a positive correlation with BDNF affinity for both receptors. Compared to the levels of BDNF colocalization with TrkB and p75 in the BDNF group without A β 42M or A β 42O (Figure 4B), the levels of BDNF colocalization with TrkB or p75 appeared to be elevated in the A β 42M group (Figure 4C, column 1), whereas they were reduced in the A β 42O group (Figure 4D, column 1). These phenomena suggest that A β 42M and A β 42O had specific but opposite effects on the binding of BDNF to the receptors; A β 42M promoted binding, while A β 42O inhibited it. The parameters MOC and the colocalization coefficient M2 (for BDNF) quantitatively confirmed these results (group 1, 2, 9, 14, 19, and 24, Table 2). This supports our conclusion from the A β 42M and A β 42O docking models with BDNF (Figure 3A) that A β 42O inhibits BDNF's ability to bind to its receptors, whereas A β 42M does not. More specifically, by binding to BDNF in a different way (Figure 3A), A β 42M might function to synergistically provide BDNF for binding to its receptors by binding to BDNF differently than A β 42O, which instead consumed BDNF. This was consistent with the result that A β 42O had a slightly higher affinity for BDNF than A β 42M (Table 1). Clearly, the impacts of A β 42M and A β 42O on the colocalization of BDNF with its receptors were consistent with their effects on BDNF-related neuronal cell viability (③, ④, ⑨, ⑩, Figure 2A,B). Additionally, the colocalization coefficient M1 (for TrkB/p75) of the A β 42M group was marginally lower than that of the BDNF group (Table 2). This suggests that the presence of A β 42M increased the binding effectiveness of BDNF to its receptors, which in turn increased cell survival ($p < 0.05$, ③ vs. control/⑨ vs. ②, Figure 2A,B).

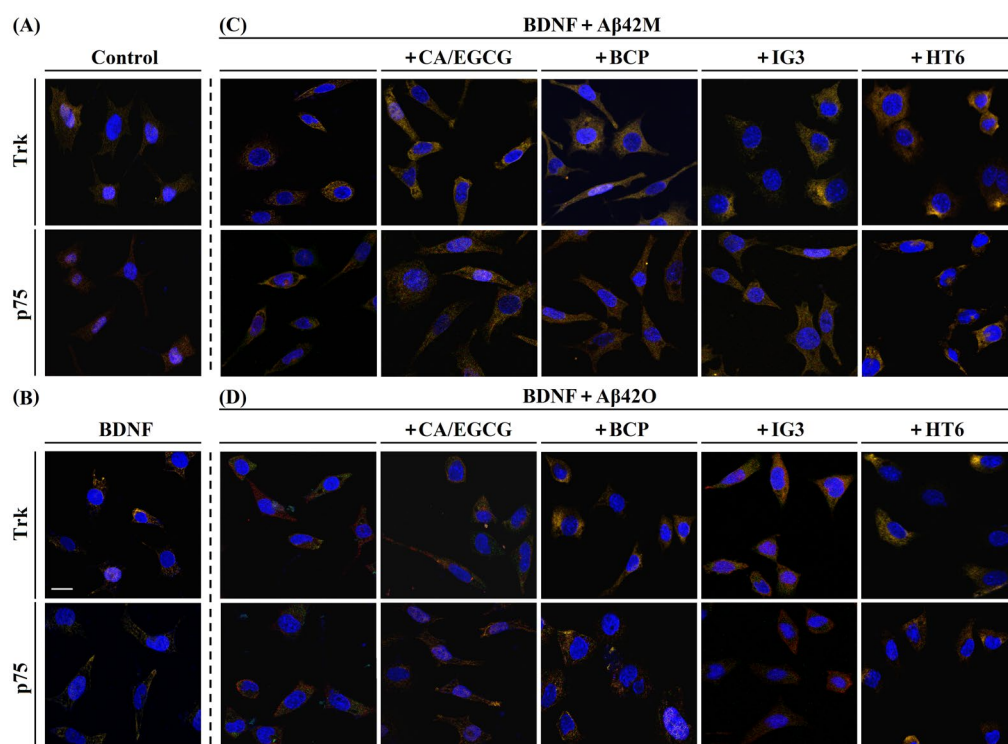


Figure 4. Representative confocal images of double-labeling immunofluorescence of BDNF (green) and TrkB/p75 (red) in SH-SY5Y cells. The merged images include BDNF (green), TrkB/p75 (red), nucleus (blue), and the BDNF-TrkB/p75 complex (yellow). (A) Double-IF images of control group. (B) Double-IF images of BDNF group. (C,D) Double-IF images of A β 42M and A β 42O groups with or without IG3/BCP/CA/EGCG/HT6, respectively. The first and second rows in (A–D) represent TrkB and p75 groups, respectively. Scale bar = 20 μ m.

Furthermore, BDNF colocalization with both receptors was significantly increased in the A β 42M or A β 42O groups when IG3/BCP/CA/EGCG/HT6 were present (Figure 4C,D), especially in the HT6 subgroups. In contrast, BDNF colocalization with TrkB or p75 remained unchanged when IG3/BCP/CA/EGCG/HT6 were present without A β 42M/A β 42O. These findings demonstrated that IG3/BCP/CA/EGCG competitively influence A β 42M or A β 42O binding to BDNF, thereby enhancing BDNF binding to its receptors. HT6, an scFv anti-A β 42O antibody, reduced A β 42O binding to BDNF by inducing the integrated conformation of the (freshly formed) A β 42O units to be disrupted [24]. As listed in Table 2, the MOC, M1, and M2 parameters values in the A β 42M/A β 42O groups with IG3/BCP/CA/EGCG/HT6 (groups 5–8, 10–13, 15–18, and 20–23) were higher than those in the A β 42M/A β 42O group without those compounds (group 9, 14, 19, and 24), and in the presence of IG3/BCP/CA/EGCG/HT6 alone, these parameter values were almost identical to those of the control group. This finding indicates that these substances increased either the proportion of BDNF bound to its receptors or the amount of BDNF-TrkB/p75 complexes on the plasma membrane of neuronal cells. This may be because these substances competitively bound to A β 42M and A β 42O, increasing the A β 42M presentation of BDNF to its receptors and/or decreasing the A β 42O consumption of BDNF. This effect could also enhance BDNF's sensitivity to its receptor and increase its binding rate. Therefore, IG3/BCP/CA/EGCG had consistent impacts on the role of A β 42M/A β 42O in BDNF binding to its receptors (Figure 4, Table 2) and on neuronal cell viability or survival (Figure 2).

Table 2. Parameters ^a MOC, ^b M1, and ^c M2.

No.	Group	MOC	M1	M2	Receptor
1	Control	0.622 ± 0.013	0.587 ± 0.004	0.611 ± 0.001	TrkB
2	Control	0.614 ± 0.007	0.556 ± 0.006	0.200 ± 0.003	p75
3	BDNF (or with BOs/CAs)	0.778 ± 0.009	0.653 ± 0.002	0.638 ± 0.004	TrkB
4	BDNF (or with BOs/CAs)	0.654 ± 0.011	0.565 ± 0.004	0.208 ± 0.007	p75
5	AβM+BDNF+CA/EGCG	0.714 ± 0.015	0.586 ± 0.006	0.668 ± 0.004	TrkB
6	AβM+BDNF+BCP	0.823 ± 0.009	0.592 ± 0.003	0.639 ± 0.002	TrkB
7	AβM+BDNF+IG3	0.871 ± 0.012	0.597 ± 0.001	0.702 ± 0.005	TrkB
8	AβM+BDNF+HT6	0.731 ± 0.005	0.593 ± 0.004	0.670 ± 0.003	TrkB
9	AβM+BDNF	0.721 ± 0.006	0.551 ± 0.007	0.650 ± 0.005	TrkB
10	AβM+BDNF+CA/EGCG	0.875 ± 0.010	0.598 ± 0.006	0.316 ± 0.002	p75
11	AβM+BDNF+BCP	0.726 ± 0.007	0.583 ± 0.002	0.295 ± 0.003	p75
12	AβM+BDNF+IG3	0.746 ± 0.012	0.566 ± 0.005	0.309 ± 0.002	p75
13	AβM+BDNF+HT6	0.726 ± 0.012	0.534 ± 0.005	0.328 ± 0.003	p75
14	AβM+BDNF	0.710 ± 0.005	0.536 ± 0.003	0.271 ± 0.002	p75
15	AβO+BDNF+CA/EGCG	0.676 ± 0.011	0.517 ± 0.005	0.523/0.581 ± 0.004	TrkB
16	AβO+BDNF+BCP	0.619 ± 0.012	0.623 ± 0.005	0.686 ± 0.004	TrkB
17	AβO+BDNF+IG3	0.623 ± 0.011	0.557 ± 0.004	0.656 ± 0.003	TrkB
18	AβO+BDNF+HT6	0.769 ± 0.015	0.550 ± 0.005	0.673 ± 0.004	TrkB
19	AβO+BDNF	0.617 ± 0.008	0.410 ± 0.003	0.572 ± 0.001	TrkB
20	AβO+BDNF+CA/EGCG	0.661 ± 0.009	0.524 ± 0.002	0.300 ± 0.002	p75
21	AβO+BDNF+BCP	0.672 ± 0.006	0.533 ± 0.006	0.302 ± 0.003	p75
22	AβO+BDNF+IG3	0.712 ± 0.015	0.544 ± 0.004	0.327 ± 0.005	p75
23	AβO+BDNF+HT6	0.817 ± 0.011	0.561 ± 0.007	0.311 ± 0.003	p75
24	AβO+BDNF	0.615 ± 0.007	0.485 ± 0.004	0.163 ± 0.002	p75

^a MOCs (Manders' overlap coefficients) were calculated based on the merged whole images. The MOC value from 0 to 0.6 indicates absence of colocalization, and that from 0.6 to 1.0 indicates colocalization. ^b M1 and ^c M2 (Manders' colocalization coefficients) were calculated by analyzing the corresponding fluorescence signals corresponding to the merged images. M1: fraction of TrkB or p75 overlapping BDNF; M2: fraction of BDNF overlapping TrkB or p75. All data were the mean ± SEM. BOs/CAs: branched oligosaccharides/catechins.

2.6. Effects of Aβ42M and Aβ42O on BDNF Binding to TrkB/p75 in the Presence and Absence of IG3/BCP/CA/EGCG by Co-IP

To further confirm the impact of Aβ42M and Aβ42O on BDNF binding to TrkB/p75 in the presence and absence of IG3/BCP/CA/EGCG, BDNF bound to TrkB/p75 was then identified by a Co-IP assay using an anti-TrkB/p75 antibody, followed by Western blotting with an anti-BDNF antibody. Figure 5 illustrated that only the WCL, SFC, and Co-IP groups treated with the anti-BDNF antibody exhibited a BDNF signal (a and b in Figure 5), whereas the control IgG group did not (c in Figure 5) (control IgG data for all other experimental groups were not presented). Additionally, BDNF was detected in both Co-IP and SFC samples, confirming that it binds to TrkB and p75 receptors simultaneously. Immunoblot densities in Figure 5 were analyzed and BDNF levels were quantified, as presented in Table 3.

The comparison of BDNF signals in the control (a and b in Figure 5 and Table 3) and BDNF (d and e in Figure 5 and Table 3) groups indicated that the degree of BDNF co-precipitated with TrkB and p75 increased as the BDNF level in the culture medium increased, revealing a positive correlation between the amount of BDNF bound to the receptors and the amount of free BDNF in the extracellular matrix. Overall, the Aβ42M group (f and i in Figure 5 and Table 3) showed greater BDNF co-precipitation with TrkB or p75 than the control BDNF group, while the Aβ42O group showed less (l and o in Figure 5 and Table 3). These results confirmed that Aβ42M promoted BDNF binding to

both receptors, whereas A β 42O inhibited it. Apparently, the Co-IP results were consistent with those of IF (Figure 4 and Table 2).

Table 3. Mean percentage of BDNF bound to TrkB and p75 by quantitative analysis of BDNF immunoblots in Co-IP.

Group	Bait	BDNF	A β 42M	A β 42O	CA/ EGCG	IG3/ BCP	WCL	Co-IP	SFC
a	TrkB	—	—	—	—	—	100	44.07 \pm 0.79	10.87 \pm 1.09
d		+	—	—	—	—	243.90 \pm 1.31	85.03 \pm 0.58	63.22 \pm 0.64
f		+	+	—	—	—	248.71 \pm 1.51	88.33 \pm 0.96	51.63 \pm 0.74
g		+	+	—	+	—	242.51 \pm 0.89	89.27 \pm 0.65	47.72 \pm 0.88
h		+	+	—	—	+	245.97 \pm 1.37	92.01 \pm 0.84	46.39 \pm 0.73
l		+	—	+	—	—	210.06 \pm 1.08	58.12 \pm 0.74	56.13 \pm 0.61
m		+	—	+	+	—	221.36 \pm 0.95	72.18 \pm 0.87	45.60 \pm 0.72
n		+	—	+	—	+	212.38 \pm 1.35	73.44 \pm 0.63	49.61 \pm 0.76
b	P75	—	—	—	—	—	100	31.95 \pm 1.04	12.57 \pm 0.42
e		+	—	—	—	—	243.90 \pm 1.31	70.00 \pm 0.69	22.81 \pm 0.73
i		+	+	—	—	—	248.29 \pm 1.31	71.88 \pm 0.67	39.11 \pm 0.78
j		+	+	—	+	—	242.65 \pm 1.26	73.40 \pm 0.78	38.07 \pm 0.91
k		+	+	—	—	+	246.20 \pm 1.53	83.23 \pm 0.68	37.58 \pm 0.73
o		+	—	+	—	—	208.18 \pm 1.38	64.28 \pm 0.63	43.90 \pm 0.54
p		+	—	+	+	—	230.02 \pm 1.32	62.07 \pm 0.76	47.02 \pm 0.62
q		+	—	+	—	+	231.36 \pm 0.62	61.68 \pm 0.98	41.85 \pm 0.81

The grayscale intensity of the corresponding BDNF bands from the control WCL samples was considered as 100%. The symbols indicating groups listed in Table 3 correspond to those in Figure 5. WCL: whole-cell lysate samples. SFC: supernatants from Co-IP. Co-IP: Co-IP beads. Data for BDNF+A β 42M and BDNF+A β 42O+IG3 groups in Table 3 (n and i) were normalized with their multiplicative scores shown in Figure 5 (n and i). —, absent; +, present. All data were the mean \pm SEM.

Based on the binding specificity of A β 42M and A β 42O for BDNF (Figure 1) and preliminary IF results showing that they are neither colocalized with TrkB or p75, it is evident that A β 42M and A β 42O exerted opposite effects on BDNF-receptor binding due to their specific binding to BDNF rather than to the receptors. Given the differences in the binding strength between A β 42M and A β 42O to BDNF (Figure 3A) and the lower total BDNF levels in the WCL samples of the A β 42O group (l and o in Table 3) compared to the BDNF group (d and e in Table 3), it is suggested that A β 42M increases the binding rate of BDNF to its receptors by binding more reversibly to extracellular BDNF, whereas A β 42O may consume BDNF by binding less reversibly to extracellular BDNF. Here, the action of A β 42M enhanced the sensitivity or targeting of BDNF to its receptors, effectively synergizing BDNF binding and enhancing the attachment of BDNF to its receptors. The A β 42O consumption of BDNF may be explained by the stronger chemical and physical (or stacking) interactions between BDNF molecules and A β 42 chains in the A β 42O-BDNF complex (Figure 3A), which likely prevented BDNF from being (fully) released in the Co-IP experiment and prevented recognition by the anti-BDNF antibody. This indicated that the amount or efficiency of BDNF binding to its receptors depends on the level of free or effective BDNF, rather than total BDNF, in the extracellular matrix. Thus, BDNF molecules in A β 42M-BDNF complexes may be regarded as effective BDNF molecules, that is, BDNF molecules that attach to their receptors favorably, whereas those BDNF molecules in the A β 42O-BDNF complexes may be considered as ineffective or consumed.

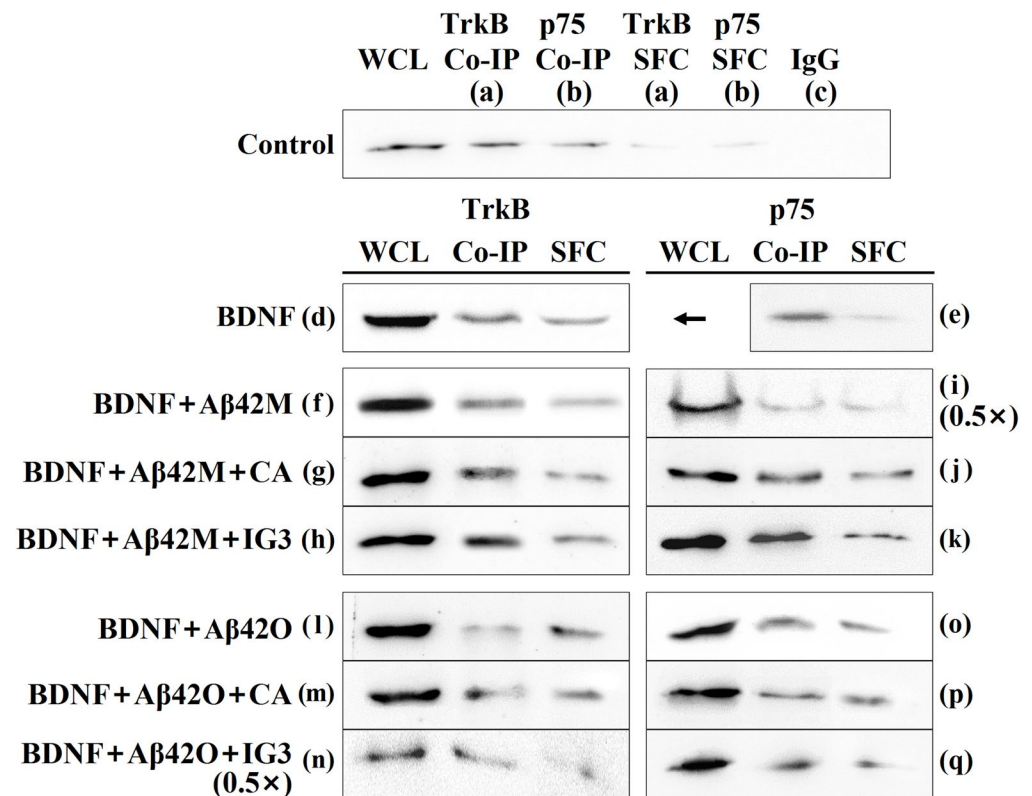


Figure 5. Detection of BDNF bound to TrkB and p75 by Western blot analysis after Co-IP. Co-IP was conducted using anti-TrkB and anti-p75 antibodies, with rabbit IgG serving as a negative control. BDNF bound to TrkB and p75 was detected using an anti-BDNF antibody. The symbols indicating groups in Figure 5 correspond to those listed in Table 3. The letters a through q indicate the corresponding groups. WCL: whole-cell lysate samples; SFC: supernatants from Co-IP; Co-IP: Co-IP beads. SFC and Co-IP samples were prepared from equal amounts of WCL within the same group, while the WCL samples were obtained from lysates of initial cells equivalent to those of the control groups. The symbol 0.5 \times indicates that half of the sample size was used. An arrow in subgroup e indicates that the WCL sample in the p75 group is the same as that in the TrkB group.

Furthermore, both the A β 42M (g, h and j, k in Figure 5 and Table 3) and A β 42O (m–n and p, q in Figure 5 and Table 3) groups showed increased BDNF co-precipitation with TrkB/p75 in the presence of CA and IG3, whereas the results in our preliminary experiments showed that CA/IG3 alone did not have any effect on either the TrkB/p75 levels or co-precipitation of BDNF with TrkB/p75. In addition, EGCG and BCP showed effects corresponding to those of CA and IG3, respectively, on the co-precipitation of BDNF with TrkB/p75.

This should be attributed to the dynamics of A β 42M/A β 42O binding to CA/EGCG/IG3/BCP and BDNF or the competition between CA/EGCG/IG3/BCP and BDNF for their binding to A β 42M or A β 42O. Consequently, CA/EGCG/IG3/BCP increased the effectiveness of A β 42M in presenting BDNF to its receptors while reducing the A β 42O consumption of BDNF. It was clear that all binding was related to the concentration of the chemicals involved in these processes.

3. Materials and Methods

3.1. A β 42 Species and Other Proteins

Human A β 42 protein was purchased from Dalian Meilun Biological Co., Ltd. (MB10425, Dalian, China), and its purity was higher than 96%. Four A β 42 species (A β 42M, A β 42O, A β 42P, and A β 42F) were prepared as described previously [3] and confirmed by electron microscopy and fluorescence spectroscopy based on the binding of thioflavin T

(ThT) as described previously [21,30]. For ThT assay, the fluorescence intensities of A β 42O, A β 42P, and A β 42F (each 5.0 μ M) were typically about 230 ± 20 , 350 ± 26 , and 500 ± 30 , respectively, corresponding to their states observed in electron microscopy as previously reported [21]. HT6 was obtained as described previously [24]. Human-brain-derived neurotrophic factor (BDNF), anti-BDNF antibody, and anti-TrkB antibody were purchased from Shanghai Abcam Trading Co., Ltd. (ab222178/ab108319/ab134155, Shanghai, China). BF488-conjugated anti-BDNF antibody (green) and Goat anti-mouse/rabbit IgG H&L/HRP were purchased from Beijing Biosynthesis Biotechnology Co., Ltd. (bs-4989R-BF488/bs-0296G-HRP/bs-0295G-HRP, Beijing, China). Anti-p75 antibody and Rabbit IgG were purchased from Shanghai Beyotime Biotechnology Co., Ltd. (AF1033/A7016, Shanghai, China). AF594-conjugated anti-TrkB antibody (red) and AF647-conjugated anti-p75 antibody (red) were purchased from Shanghai Santa Cruz Biotechnology Co., Ltd. (sc-7268 AF594/sc-271708 AF647, Shanghai, China). Protein A&G agarose beads was purchased from Shanghai Roche Co., Ltd. (11719408001, Shanghai, China). Prestained protein marker was purchased from Wuhan Servicebio Technology Co., Ltd. (G2058, Wuhan, China). Human serum albumin (HSA) was purchased from Beijing Solarbio Science and Technology Co., Ltd. (A8230, Beijing, China).

3.2. Chemicals

CA, EGCG, and IG3 were purchased from Shanghai Yuanye Biochemical Technology Co., Ltd. (B21722/B20106/B27498, Shanghai, China). BCP was purchased from Shanghai SCR-Biotech Co., Ltd. (SCGC-101239, Shanghai, China). These oligosaccharides are in D-type configuration. Unless otherwise noted, all solutions were prepared using double distilled water (ddH₂O). Their stock solutions were prepared to a concentration of 1.0 mM in ddH₂O. DAPI solution was purchased from Beijing Biosynthesis Biotechnology Co., Ltd. (C02-04003, Beijing, China). Native lysis buffer was purchased from Beijing Solarbio Science and Technology Co., Ltd. (R0030, Beijing, China). BeyoECL Star Kit was purchased from Shanghai Beyotime Biotechnology Co., Ltd. (P0018AS, Shanghai, China). All other chemicals were local products of analytical grade.

3.3. Cell Culture

In this study, human neuroblastoma cell line SH-SY5Y and mouse primary hippocampal neuronal cell line HT22 (100158/358041, BeNa Culture Collection, Beijing, China) were used as model cell lines for neural cells. Unless otherwise stated, the cells were cultured in Dulbecco's modified eagle medium (DMEM) (11965092, GIBCO, Shanghai, China) with 10% fetal bovine serum (FBS, 16140071, GIBCO, Shanghai, China) and 100 U/mL penicillin–100 μ g/mL streptomycin (15140148, GIBCO, Shanghai, China) in a humidified atmosphere of 5% CO₂ at 37 °C (standard conditions) for a specified period of time. All experiments were performed between the 3rd and 6th passages.

3.4. Determination of the Binding Specificity of BDNF to Different A β 42 Species by Indirect Enzyme-Linked Immunosorbent Assay (ELISA)

3.4.1. Construction of a Standard BDNF Curve

Seven samples of BDNF aqueous solutions (0 to 80 pg/mL) were prepared in ELISA coating buffer (C1050, Beijing Solarbio Technology Co., Ltd., Beijing, China) and coated onto 96-well plates (100 μ L per well) at 4 °C for 12 h. After aspirating the liquid, the protein content in the aspirated coating solution was determined to ensure that BDNF was completely coated on the plate. All coated BDNF was then assayed by conventional indirect ELISA using anti-BDNF antibody and goat anti-mouse IgG H&L/HRP antibody at 37 °C for 1.5 h. Finally, the absorbance of each well was measured at 450 nm on a microplate reader. The measurement was performed in triplicate. A standard curve of

BDNF concentration (pg/mL) was plotted using the averaged data, yielding an equation of $y = 0.0002x + 0.0013$ with a correlation coefficient of 0.9687.

3.4.2. Determination of the Binding Rate of BDNF to Different A β 42 Species

Four A β 42 species (A β 42M, A β 42O, A β 42P, and A β 42F) and HSA (each at a final concentration of 2.0 μ M) or their respective mixtures with CA/EGCG/IG3/BCP (final concentration: 50 μ M for CA/EGCG, 2.0 μ M for IG3/BCP) were coated onto 96-well plates and incubated at 4 °C for 12 h. PBS (10 mM, pH 7.4) was used as a blank control. The coating solution was discarded after confirming the complete coating of each protein on the plate, and the coated wells were blocked with blocking solution [5% nonfat dry milk in TBS buffer (10 mM Tris-HCl, pH 7.5, 75 mM NaCl)] (150 μ L per well) for 1 h at room temperature (21 °C). Then, 100 μ L of BDNF solution (final concentration 20 pg/mL) with or without CA, EGCG, IG3, or BCP (final concentration: 50 μ M for CA/EGCG, 2.0 μ M for IG3/BCP) was added to each coated well, and the plate was incubated at 37 °C for 24 h. The supernatant was aspirated, and the amount of BDNF bound in each well and the amount of BDNF in the supernatant from blank control wells were detected using anti-BDNF antibody by indirect ELISA as described above. Measurements for every experiment were performed in triplicate and repeated across six independent A β 42 batches.

The binding specificity of BDNF for different A β 42 species was expressed as its binding rate, determined from the standard curves for BDNF concentrations. The binding rate of BDNF for different A β 42 species or HSA was calculated by taking the difference between the mean data of the A β 42/HSA-coated wells and blank control wells (uncoated with A β 42), dividing by the amount of BDNF in 100 μ L of BDNF solution above and multiplying the result by 100%.

3.5. Measurement of the Equilibrium Dissociation Constant (K_D) of BDNF to A β 42M/A β 42O by Indirect Competitive ELISA

The equilibrium dissociation constant (K_D) of BDNF to A β 42M/A β 42O represented the binding affinity between BDNF and A β 42M/A β 42O, which was measured by indirect competitive ELISA. A β 42M/A β 42O at A β 42 concentration of 10^{-12} to 10^{-5} M were mixed with BDNF (final concentration 20.0 pg/mL) or with BDNF&CA/EGCG/IG3/BCP (final concentration: 50 μ M for CA/EGCG, 2.0 μ M for IG3/BCP), respectively, and incubated at 37 °C for 1.0 h. Then, 1.0 mL of each mixture was transferred into one well of a 96-well plate that was pre-coated by A β 42M/A β 42O (final concentration: 0.2 μ M, 100 μ L per well) with or without CA/EGCG/IG3/BCP (final concentration: 50 μ M for CA/EGCG, 2.0 μ M for IG3/BCP) at 4 °C for 12 h, and incubated for additional 24 h at 37 °C, and then blocked with blocking solution [5% nonfat dry milk in TBS buffer (10 mM Tris-HCl, pH 7.5, 75 mM NaCl)] (150 μ L per well) for 1 h at room temperature (21 °C). Next, 0.2 mL of supernatant was taken from each well, and the amount of bound BDNF was probed using anti-BDNF antibody at 37 °C for 1.0 h, followed by HRP-conjugated goat anti-mouse IgG H&L/HRP using tetramethylbenzidine (TMB). Fifteen minutes later, the absorbance of each well was measured at 450 nm. PBS (10 mM, pH 7.4) was used as a blank control for the A β 42 samples. All experiments were performed in triplicate and repeated three times with different batches of A β 42. The K_D value of BDNF was equal to the initial concentration (M) of free A β 42 at which half of the BDNF was bound to the coated A β 42M or A β 42O.

3.6. Determination of the Effect of A β 42M/A β 42O with and Without BOs and CAs on the Cytological Efficacy of BDNF in Terms of Cell Survival by MTT Assay

SH-SY5Y and HT22 cells were plated into 96-well plates at a density of approximately 2.0×10^4 cells per well in 100 μ L of DMEM and incubated under standard conditions (37 °C, 5% CO₂) for 4–6 h prior to conducting experiments. After replacing the culture medium

with a fresh medium as outlined in Table 4 and culturing the target cells accordingly, cell viability for each group was determined using the conventional MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method. For anti-BDNF antibody-treated groups shown in Table 4, 0.5 h after the addition of the anti-BDNF antibody (final concentrations: 50 pg/mL) to the cells, BDNF (final concentration: 20.0 pg/mL), A β 42M/A β 42O (final concentrations 20 nM), CA/EGCG (final concentrations 0–100 μ M), IG3/BCP (final concentrations: 0–2.0 μ M), or the corresponding mixtures, which were premixed for 0.5 h at 4 °C, were added. The cells were then incubated under standard conditions (37 °C, 5% CO₂) for 2 h. Untreated cells were used as the blank control (Ctrl0). The cultures were then washed with PBS (10 mM, pH 7.4) and cell viability in each well was assessed using conventional MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, as previously described [22]. All experiments were performed in triplicate and repeated across six independent cell batches.

Table 4. Experimental groups for MTT assay.

Group	Incubated Duration (h)	Anti-BDNF Antibody	BDNF	A β 42M/A β 42O	CA/EGCG/IG3/BCP
Ctrl0	2	—	—	—	—
①		+	—	—	—
②	2	—	+	—	—
③/④		—	—	+	—
⑤		+	+	—	—
⑥	0.5 + 1.5	+	—	—	+
⑦/⑧		+	—	+	—
⑨/⑩	2	—	+	+	—
⑪/⑫		—	—	+ premixture	
⑬/⑭		+	+	+	—
⑮/⑯	0.5 + 1.5	+	—	+ premixture	
⑰/⑱		+	+	+ premixture	
⑲/⑳		—	+	+ premixture	

Note: —, absent; +, present.

3.7. Molecular Docking

The crystal structures of A β 42 monomer (PDB ID: 1Z0Q), BDNF (PDB ID: 1B8M), and TrkB-neurotrophin-4 (NT4) (PDB ID: 1HCF) were downloaded from Protein Data Bank (<http://www.rcsb.org>) for molecular docking and comparison in this study. The 3D structure of A β 42 trimer was built from crystal structures of the A β 42 monomer using the program AutoDock 4.2.6 (<https://autodock.scripps.edu/download-autodock4/>) and compared to the model of A β trimer (PDB: 5HOX), and then, the A β 42 trimer's 3D structure was optimized through energy minimization and molecular dynamics simulation, as described previously [8]. The molecular dockings of BDNF to A β 42 monomer/trimer and to TrkB were performed as described previously [3]. The integrity of all docking models was visually examined using Discovery Studio (DS) Visualizer 3.1 and, finally, all docking images were obtained.

3.8. Immunofluorescence (IF) Microscopy

Glass slides were placed in a 48-well plate and cells were seeded at a density of approximately 2.0×10^4 per well in 200 μ L of DMEM, then incubated under standard conditions (37 °C, 5% CO₂) for 4–6 h. The culture medium was aspirated and fresh DMEM containing BDNF (final concentration: 1.0 nM) or its mixtures with A β 42M/A β 42O (final concentration: 0.2 μ M) and/or CA/EGCG/IG3/BCP (final concentration: 100 μ M for CA/EGCG and 2.0 μ M for IG3/BCP) was added to each well. Cells were further cultured

under identical atmospheric conditions for 48 h. Finally, the colocalization of BDNF with its receptor TrkB or p75 was evaluated using double-labeling immunofluorescence (IF) staining and corresponding quantitative analysis, as described previously [8]. BDNF was probed with a BF488-conjugated anti-BDNF antibody (green), while receptors TrkB and p75 were probed with AF594-conjugated anti-TrkB antibody (red) and AF647-conjugated anti-p75 antibody (red), respectively. The cellular nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole) for 10 min. Fluorescence imaging was performed using a laser scanning confocal microscope (Zeiss LSM710, Oberkochen, Germany). Each experiment was performed in at least triplicate and repeated across six independent cell batches.

To quantitatively assess the colocalization of BDNF with the membrane receptors TrkB/p75, dual-channel confocal stacks were acquired and analyzed using Manders' colocalization methodology. Specifically, Manders' overlap coefficient (MOC) and colocalization coefficients (M1 and M2) were calculated as described previously [8]. The MOC metric (scale 0–1.0) of the merged image quantifies the spatial overlap between BDNF (green) and TrkB/p75 (red), while Manders' colocalization coefficients (M1 and M2) represent the proportion of colocalized pixels for each color relative to its total pixels. All data were obtained from three biological replicates ($n = 3$).

3.9. Co-Immunoprecipitation (Co-IP) Assay

The extent of BDNF binding to its receptors in the presence or absence of A β 42M/A β 42O with or without BOs/CAs was quantitatively analyzed using a conventional Co-IP assay with protein A&G beads and anti-BDNF, anti-TrkB, and anti-p75 antibodies, following the manufacturers' instructions and our prior optimization. Briefly, cells were plated at a density of approximately 5.0×10^5 per well in 1.5 mL of DMEM and incubated under standard conditions (37 °C, 5% CO₂) for 4–6 h. The culture medium was then aspirated and fresh DMEM containing BDNF (final concentration: 1.0 nM) or its mixture with A β 42M/A β 42O (final concentration: 0.2 μ M) and/or CA/EGCG/IG3/BCP (final concentration: 100 μ M for CA/EGCG and 2.0 μ M for IG3/BCP) was added to each well. Cells not treated with the above agents were used as the control cells. Following a 48 h incubation under standard conditions (37 °C, 5% CO₂), the cell culture plates were transferred to a cold room (4 °C). The culture medium was aspirated and the cells were washed twice with 1.0 mL of ice-cold PBS (10 mM, pH 7.4). Then, 150 μ L of ice-cold lysis buffer containing protease and phosphatase inhibitors was added to each well. The cells were gently pipetted several times to ensure full contact with the lysis buffer, followed by a 10 min incubation on ice (to ensure proper lysis and solubilization of membrane proteins and lipids while maintaining structural integrity). Each cell lysate was transferred to an ice-cold 1.5 mL tube and centrifuged at 13,680 g for 10 min (4 °C). Finally, the cell lysis supernatants were collected and used as whole-cell lysate (WCL) samples for subsequent Co-IP assays.

For all Co-IP assays described in Table 5, the immunoprecipitation procedure was performed as follows: 1.0–2.0 μ L of anti-TrkB/p75 antibody (equivalent to 1.0 μ g of antibody) or a control IgG (1.0 μ L) was incubated with 100 μ L WCL in an ice-cold 1.5 mL tube with continuous rotation at 4 °C for 12 h. Subsequently, Protein-A&G beads obtained from 50 μ L of protein-A&G slurry and pre-washed twice with ice-cold lysis buffer were added to the antigen–antibody complexes and incubated with rotation at 4 °C for an additional 12 h. Following immunoprecipitation, the samples were briefly centrifuged (10 \times g, 1 min, 4 °C) and the supernatants and pelleted beads were collected separately. Finally, the original WCL, the supernatants from Co-IP (SFC), and Co-IP samples (all from equal amounts of cell lysates) were resolved on either 8% or 10% SDS-PAGE gels. The receptor-bound BDNF in these samples was then detected and visualized by conventional Western blotting using anti-BDNF antibody. All immunoreactive bands were developed using HRP-conjugated

secondary antibodies and visualized by enhanced chemiluminescence (ECL) detection. Experimental samples were normalized to the corresponding control samples (WCL, SFC, and Co-IP) obtained from control cells.

Table 5. Experimental groups for Co-IP assay.

Group	Bait	Prey	BDNF	A β 42M	A β 42O	CA/EGCG/IG3/BCP
a	TrkB		—	—	—	—
d			+	—	—	—
f			+	+	—	—
g/h			+	+	—	+
l			+	—	+	—
m/n			+	—	+	+
		BDNF				
b	p75		—	—	—	—
e			+	—	—	—
i			+	+	—	—
j/k			+	+	—	+
o			+	—	+	—
p/q			+	—	+	+

Note: —, absent; +, present.

The grayscale intensity was quantitatively analyzed using ImageJ software (Version: 1.8.0) to quantify BDNF immunoblot bands. The percentage of BDNF abundance in each WCL, SFC, and Co-IP sample was calculated by dividing the mean intensity of the target band (A) by that of the corresponding control band (A₀) (from the control group), then multiplying by 100% [(A/A₀) × 100]. All experiments were performed in triplicate and repeated across three distinct cell batches.

3.10. Statistical Analysis

All data in this study were obtained from at least three different batch experiments, each with three parallel samples, and represent the mean ± standard error of the mean (SEM). The cells in each experimental group were measured in random order by two independent investigators. During image capture and analysis, the investigators were blinded to A β 42 species and experimental group. Normality test and homogeneity of variance test were performed using the SPSS statistical analysis software (SPSS 28.0). For the data-met normality of distribution and homogeneity of variance, between-group comparisons were performed using Student's *t*-test analysis, and *p* values smaller than 0.05 were considered to be statistically significant.

4. Discussion

Although numerous in vitro and in vivo studies have shown that the massive loss of cognitive neurons in AD is closely related to A β 42 aggregates, the role of the extracellular A β 42 aggregates, particularly A β 42O, for NTs, especially BDNF, remains unknown. BDNF is the major neurotrophin in the CNS and is required for neuronal survival and growth, synaptic plasticity, and neurite outgrowth [31,32]. By binding to and activating its specific receptors, TrkB and p75, BDNF exerts powerful biological effects that maintain normal neuronal function and promote the repair of injured neuronal cells [13,33]. Numerous in vitro and in vivo studies have demonstrated that the activation or upregulation of the BDNF signaling pathway promotes neurogenesis and synaptogenesis, prevents cell death during degenerative processes, and improves learning and cognitive abilities in AD mice [34,35]. In contrast, insufficient or reduced BDNF leads to synaptic dysfunction or loss, which accelerates neuronal death and apoptosis [36,37] and has been implicated

in the pathology of several neurodegenerative diseases as well as in their physiological symptoms [38]. AD is a neuropathy caused by the lack of neurotrophic factors [39–41]. Although the neurotrophic effects of A β 42M and the neurotoxic effects of A β 42 aggregates on neuronal cell survival are well-documented, the precise relationship between A β 42M (or its aggregates) and BDNF remains poorly understood.

BOs and CAs have been shown to increase the beneficial effects of A β 42M and decrease the deleterious effects of A β 42O both in vitro and in vivo [15–18]. This study examined the interactions between A β 42M/A β 42 aggregates (especially A β 42O) and BDNF and determined the effects of A β 42M and A β 42O on the BDNF receptor-binding activity and effectiveness in supporting cell survival and the roles of BOs and CAs in these processes. It was found that the affinity of BDNF for both A β 42M and A β 42O was higher than that of BOs for A β 42M and A β 42O [15], but both were lower than that of BDNF for its receptors [29], as indicated by the dissociation constants in Table 1. Additionally, the affinity of BDNF for A β 42O was marginally higher than that for A β 42M (Table 1). BDNF is a basic protein with an isoelectric point (pI) of approximately 9.6 and consists of 119 amino acid residues, including 12 acidic (Asp + Glu) and 22 basic (Arg + Lys) residues. As a result, numerous polar groups or motifs are present on the surface of the BDNF molecule. Under physiological conditions, BDNF readily binds to the amphiphilic N-terminal fragment of amphipathic A β 42 molecules (pI \approx 5.4) [1]. However, the hydrophilic N-terminal fragment of the A β 42 peptide chain is rarely exposed on the outer surface of the larger A β 42 aggregate particles [1]. Thus, BDNF binds easily to A β 42M and smaller A β 42O particles, but hardly binds to larger A β 42P and A β 42F units (Figure 1). Furthermore, the large size of the A β 42P/A β 42F units reduces the probability of close spatial proximity to BDNF, resulting in suboptimal binding kinetics with a binding rate of <5% and low affinity. Since the accessible surface area between BDNF and A β 42O was larger than that between BDNF and A β 42M (Figure 3), the binding affinity between BDNF and A β 42O was slightly higher than that between BDNF and A β 42M (Table 1), and the stability of the BDNF-A β 42O complex was greater than that of the BDNF-A β 42M complex (Table S1). These differential binding properties may mechanistically explain the paradoxical functional outcomes of A β 42M versus A β 42O in modulating BDNF-supported neuronal survival.

Since very little BDNF is produced in the CNS and cell culture systems [25–27], even minor changes in the extracellular BDNF levels can impact cell survival. The neurotrophic effect of A β 42M may primarily result from its ability to synergistically enhance the efficacy of BDNF because this study showed that A β 42M increased neuronal cell survival via BDNF (Figure 2) and promoted BDNF binding to its receptors (Tables 2 and 3). These findings suggest that the physiological significance of the specific and reversible BDNF-A β 42M binding may stem from the A β 42M-mediated surface recruitment of BDNF and/or the synergistic potentiation of BDNF-receptor engagement. In contrast, A β 42O exhibits neurotoxic properties through high-affinity, less reversible BDNF-A β 42O binding, which competitively blocks BDNF-receptor interactions (Tables 2 and 3). Such pathological sequestration depletes bioactive BDNF in the extracellular matrix (Figure 2), thereby reducing BDNF-supported neuronal viability. Thus, BDNF binding by A β 42O is equivalent to A β 42O-induced BDNF inactivation or consumption, which may represent the primary (though not exclusive) mechanism of A β 42O's neurotoxicity. Therefore, the quantity of functional extracellular BDNF, rather than the total quantity of BDNF present, determines the cytological efficacy of BDNF.

Notably, neurotoxic A β 42O was also consumed through the formation of BDNF-A β 42O complexes; that is, A β 42O and BDNF neutralized or counteracted each other's effects on neuronal cell viability when A β 42O and BDNF specifically bound together. The level of either A β 42O or BDNF appeared to influence the extent to which the other was con-

sumed or inactivated. Consequently, BDNF would be progressively consumed as A β 42O levels in the brain gradually increase, leading to a gradual decline in neuronal cell survival. As others have reported [26,42,43], upregulating BDNF expression or secretion could potentially mitigate the neurotoxicity of extracellular A β 42O. In summary, a significant portion of the damage and death of neurons induced by A β 42O or other A β 42 aggregates may result from the depletion or inactivation of BDNF by these aggregates, whereas a significant portion of the improved neuronal survival caused by A β 42M may be due to the enhanced potency of BDNF by A β 42M.

This study also found that by boosting the beneficial synergistic effect of A β 42M on BDNF binding to its receptors and inhibiting A β 42O from consuming extracellular BDNF (Tables 2 and 3), BOs and CAs were able to increase the amount or proportion of BDNF bound to its receptors in the presence of A β 42M or A β 42O. This should be the result of their competitive displacement of BDNF for binding to the A β 42M and A β 42O, despite the lower-affinity BOs and A β 42M/A β 42O compared to BDNF (Table 1) [15]. The binding of BOs and CAs to A β 42M/A β 42O promoted the synergistic effect of A β 42M on BDNF, stabilized the bioactive conformation of A β 42M, and inactivated A β 42O. These findings demonstrate that reduced neuronal viability was likely attributable to insufficient effective BDNF caused by A β 42O. Therefore, the previously reported neuroprotective effects of BOs and CAs may be attributed to their indirect beneficial effect on BDNF activity, altering the interaction between A β 42M/A β 42O and the BDNF.

In addition to being present in the extracellular matrix, BOs are more likely to be located on the outer surface of the cytoplasmic membrane as a component of membrane glycolipids, glycoproteins, or receptors [15]. Therefore, BOs were likely to interact with A β 42M or A β 42O close to or on the outer surface of the cytoplasmic membrane. This implies that the synergistic effect of A β 42M on BDNF binding to its receptor and the consumption of BDNF by A β 42O might both occur in a membrane-proximal manner. Accordingly, A β 42M may elevate the localized concentration of bioactive BDNF within the pericellular microenvironment, whereas A β 42O depletes it. Moreover, BOs and CAs, through their interaction with both A β 42M and A β 42O, simultaneously enhance the A β 42M-mediated potentiation of BDNF-receptor engagement and attenuate A β 42O-mediated BDNF consumption, thus exerting neuroprotective effects. Therefore, although BOs/CAs improve or enhance the potency of BDNF in both A β 42M and A β 42O systems, the underlying mechanisms may differ. Figure 6 summarizes the findings of this study.

A β 42 aggregates, especially A β 42O, are considered the neurotoxic and pathogenic form in AD. Given the lack of successful therapeutic approaches for AD, targeting the elimination of A β 42O neurotoxicity at an early stage holds the potential for the development of new, effective AD treatments. However, the findings of this study also imply that while A β 42 aggregates are the characteristic pathological features of AD in the brain, making these aggregates, plaques, and downstream dysfunctions the main focus, we should also pay attention to their initial beginning, i.e., the A β 42M. The findings of this study indicate that an abnormal decrease in endogenous BOs-like molecular chaperones would inevitably increase the likelihood of A β 42M aggregation and a consequent BDNF deficiency. Therefore, further research should be completed on using nanotechnology to create nanomaterials for exogenous BOs or CAs to improve their bioabsorption or bioavailability.

This study has some limitations. First, this study focused solely on BDNF, not other neurotrophins. Although BDNF is the main neurotrophin in the CNS, other neurotrophins, such as NGF, NT3, and NT4/5, also exist. They possess similar but not identical conformational and functional properties to those of BDNF. Therefore, A β 42 or its aggregates may interact with them in similar yet distinct ways. The scope for future studies can be expanded to include investigating the effects of other neurotrophins, such as NGF and NT3, on

neuronal survival in AD. Second, this study investigated the role of CA/EGCG/IG3/BCP-represented BOs and CAs in affecting BDNF's cytological functions through A β 42M and A β 42O. These findings offer encouragement for preventing and potentially treating AD. However, the wide variety of polyhydroxy or polyphenolic chemicals in plants implies a diversity of physiological functions. The role of other polyhydroxy or polyphenolic chemicals requires further exploration.

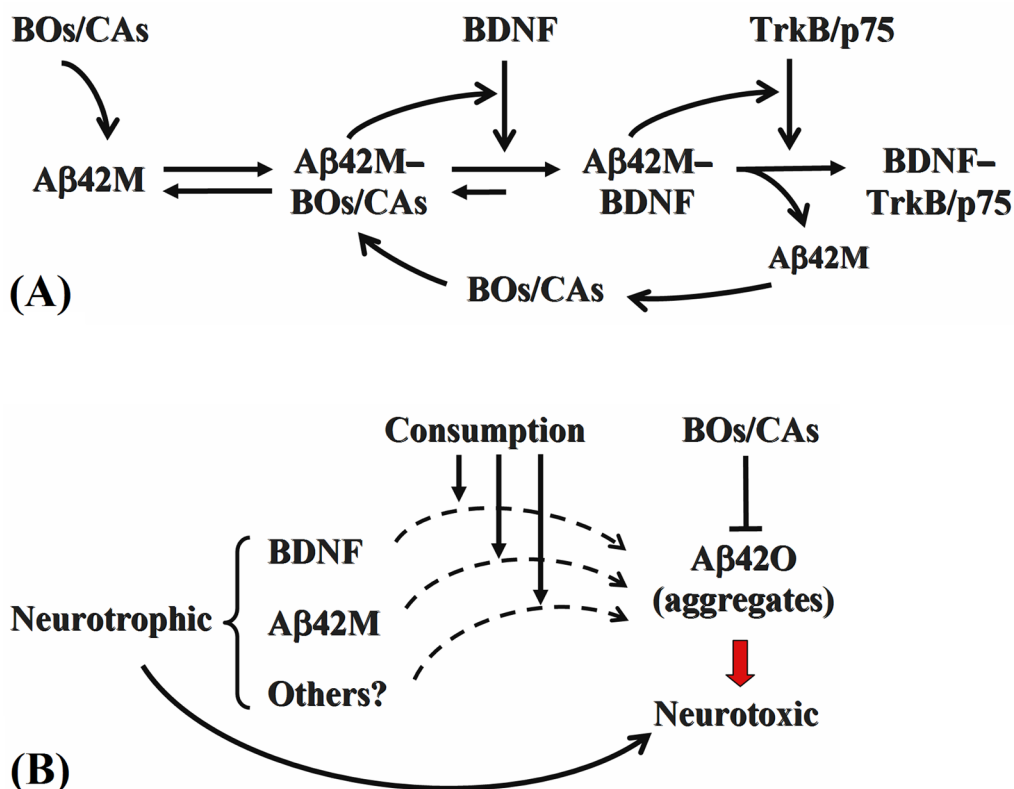


Figure 6. Schematic illustration of the different effects of A β 42M (A) and A β 42O (B) on BDNF and the modulatory roles of BOs and CAs therein.

In conclusion, this study found that (1) compared to BOs and CAs, BDNF had a higher binding specificity and affinity for A β 42M and A β 42O; (2) the neurotrophic effect of A β 42M was closely linked to A β 42M's ability to preserve the amount of effective BDNF in the extracellular matrix, close to the cytoplasmic membrane, and/or to enhancing the potency of the BDNF to bind to TrkB and p75 receptors, whereas the neurotoxicity of A β 42O was directly related to A β 42O's ability to inactivate or consume BDNF in the extracellular matrix, close to the cytoplasmic membrane, and/or reducing the efficiency of BDNF to bind to TrkB and p75 receptors; (3) BDNF and A β 42O counteracted each other's effects on neuronal cell viability when they bound together; and (4) BOs and CAs, mainly CA, EGCG, IG3, and BCP, enhanced or increased the synergistic effect of A β 42M on BDNF and inhibited or decreased the predatory or consuming effect of A β 42O on BDNF. These findings provide important insights for developing BOs- and plant polyphenol-based neuroprotective medications and their potential clinical applications in AD treatment. Clinically, these findings support novel neuroprotective strategies targeting BDNF modulation. Policy implications include prioritizing BDNF-based interventions. Future research should explore broader neurotrophin interactions. Further research on the effects of A β 42M and A β 42O on BDNF and other neurotrophins will define the critical step in the pathogenesis of AD.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms26104501/s1>.

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Abbreviations

A β 42	amyloid- β protein 1–42
AD	Alzheimer disease
CAs	catechins
EGCG	epigallocatechin gallate
BOs	branched oligosaccharides
IG3	isomaltotriose
BCP	biannennary N-linked core pentasaccharide
BDNF	brain-derived neurotrophic factor
NT	neurotrophin
NGF	nerve growth factor
TrkB	tyrosine kinase receptor B
ELISA	enzyme-linked immunosorbent assay
BBB	blood–brain barrier
DMEM	Dulbecco’s modified eagle’s medium
FBS	fetal bovine serum
HSA	human serum albumin

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