JBC RESEARCH ARTICLE

Selective coactivation of α7- and α4β2-nicotinic acetylcholine receptors reverses beta-amyloid–induced synaptic dysfunction

Received for publication, November 4, 2020, and in revised form, February 2, 2021 Published, Papers in Press, February 9, 2021,
https://doi.org/10.1016/j.jbc.2021.100402

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Edited by Paul Fraser

Beta-amyloid (Aβ) has been recognized as an early trigger in the pathogenesis of Alzheimer's disease (AD) leading to synaptic and cognitive impairments. Aβ can alter neuronal signaling through interactions with nicotinic acetylcholine receptors (nAChRs), contributing to synaptic dysfunction in AD. The three major nAChR subtypes in the hippocampus are composed of α7-, α4β2-, and α3β4-nAChRs. Aβ selectively affects α7- and α4β2-nAChRs, but not α3β4-nAChRs in hippocampal neurons, resulting in neuronal hyperexcitation. However, how nAChR subtype selectivity for Aβ affects synaptic function in AD is not completely understood. Here, we showed that Aβ associated with α7- and α4β2-nAChRs but not α3β4-nAChRs. Computational modeling suggested that two amino acids in α7-nAChRs, arginine 208 and glutamate 211, were important for the interaction between Aβ and α7-containing nAChRs. These residues are conserved only in the α7 and α4 subunits. We therefore mutated these amino acids in α7-containing nAChRs to mimic the α3 subunit and found that mutant α7-containing receptors were unable to interact with Aβ. In addition, mutant α3-containing nAChRs mimicking the α7 subunit interact with Aβ. This provides direct molecular evidence for how Aβ selectively interacted with α7- and α4β2-nAChRs, but not α3β4-nAChRs. Selective coactivation of α7- and α4β2-nAChRs also sufficiently reversed Aβ-induced AMPA receptor dysfunction, including Aβ-induced reduction of AMPA receptor phosphorylation and surface expression in hippocampal neurons. Moreover, costimulation of α7- and α4β2-nAChRs reversed the Aβ-induced disruption of long-term potentiation. These findings support a novel mechanism for Aβ's impact on synaptic function in AD, namely, the differential regulation of nAChR subtypes.

Alzheimer's disease (AD) is the predominant cause of dementia in the elderly, which is characterized by two histopathological hallmarks, beta-amyloid peptide (Aβ)-containing senile plaques and hyperphosphorylated tau-based neurofibrillary tangles (1). One of the early cognitive symptoms of AD is hippocampus-dependent memory impairments (2). Although neurodegeneration in AD is associated with multiple cellular abnormalities including tauopathies, mitochondrial dysfunction and oxidative stress, neuroinflammation, and gliosis (3, 4), many studies have provided evidence that oligomeric Aβ triggers synaptic dysfunction and loss of hippocampus-dependent memory in AD (4–6). In particular, accumulation of Aβ in the prodromic stage of AD is strongly associated with Aβ's contribution to the synaptic dysfunction (6, 7). Although deficits in many neurotransmitter systems, including γ-aminobutyric acid (GABA) and serotonin, are associated with the progression of AD, the early symptoms appear to correlate strongly with dysfunction of cholinergic and glutamatergic synapses (6). However, the precise mechanisms of Aβ-induced deficits in these synapses remain to be determined.

The cholinergic system has been postulated to be a primary target in AD (8). A loss of cholinergic function is strongly associated with the onset of memory deficits in AD (9). Specifically, the loss of basal forebrain cholinergic neurons and altered nicotinic acetylcholine receptor (nAChR) expression in multiple regions of the brain, including in the hippocampus, are prominent pathological hallmarks in AD (10–12). In contrast, the expression of most muscarinic acetylcholine receptor subtypes is relatively unaltered in AD (13, 14). The nAChR-mediated cholinergic modulation of hippocampal synaptic plasticity, such as long-term potentiation (LTP) and long-term depression, plays a critical role in learning and memory (15). Of importance, cholinergic synapses in the hippocampus are impaired by Aβ in the early stage of AD (16). Indeed, Aβ can alter neuronal signaling through interactions with nAChRs, ultimately contributing to synaptic dysfunction in AD (reviewed in (17)). There are diverse lines of evidence that molecular interactions between Aβ and nAChRs affect receptor function in the early stages of AD (18–20). Nonetheless, contradictory results have been reported describing the effects of Aβ on nAChR physiology. For example, Aβ has been reported to bind to these receptors and produce functional receptor activation or inhibitory effects, depending on Aβ concentration, type of preparation (*i.e.*, monomers, soluble oligomers, or fibrils), and incubation times (21–23). Therefore,

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there is a need to determine how Aβ specifically affects nAChRs and contributes to AD pathogenesis.

Although nearly 30 subtypes of neuronal nAChRs have been reported, the three major nAChR subtypes in the hippocampus are composed of α7, α4β2, and α3β4 subunits (24–26). Most of the current US Food and Drug Administration-approved drugs for AD (acetylcholinesterase inhibitors) inhibit the general breakdown of acetylcholine, which potentially stimulates all types of nAChRs. Thus, it is not surprising that these receptor modulators are only moderately effective (12, 27, 28). In addition, the observation that Aβ accumulates in brain regions enriched for α4β2- and α7-nAChRs may provide an important clue for the selective vulnerability of the hippocampus to Aβ toxicity given the high-affinity interaction between Aβ and these nAChRs (29–32).

Our previous work using Ca^{2+} imaging in cultured hippocampal neurons has shown that Aβ selectively inhibits α7- and α4β2-nAChRs together, but not α3β4-nAChRs (32), indicating that distinct nAChR subtypes are differentially affected in AD. As nAChRs are more prominently expressed in inhibitory interneurons than excitatory cells in the hippocampus (33, 34), nAChR-mediated cholinergic activity in the hippocampus may be biased toward altering the excitability of inhibitory interneurons. In line with this idea, our previous work demonstrates that Aβ induces neuronal hyperexcitation, an important characteristic in AD linked to network hyperexcitability and consequential dysfunction in brain rhythms (5), in cultured hippocampal excitatory neurons by predominantly reducing neuronal activity in inhibitory neurons *via* selective inhibition of α7- and α4β2-nAChRs, but not α3β4-nAChRs (32). Consistent with these findings, considerable evidence suggests that Aβ exerts subtype-specific inhibition of α7- and/or α4β2-nAChR function without affecting α3β4-nAChRs (21–23, 32, 35–39). The expression of α7 and α4 subtypes is also more significantly reduced in the cortex and hippocampus of patients with AD compared with α3-type receptors (40, 41). This suggests that Aβ-induced disruption of selective nAChR function may induce synaptic and neuronal dysfunction in the hippocampus, leading to cognitive decline in AD. Therefore, strategies that selectively regulate nAChRs in the hippocampus can reverse the pathological Aβ effects on AD pathology, which may improve cognitive function. However, how nAChR subtype selectivity of Aβ affects synaptic function in AD is not completely understood.

Previous work using structure–function analysis has shown that the hydrophilic N-terminal domain of Aβ affects α7- and α4β2-nAChR function, elevating presynaptic Ca^{2+} levels in a model reconstituted rodent neuroblastoma cell line and isolated mouse nerve terminals (42). Furthermore, the activity of the Aβ N terminus largely comes from a sequence surrounding a putative histidine-based metal binding site, YEVHHQ (42). More importantly, this hexapeptide Aβ core sequence (Aβcore) is found to dock into the ligand-binding site of nAChRs and reverse Aβ-induced neuronal apoptotic death, synaptic plasticity, and fear memory deficits (43). In addition, mutations of tyrosine to serine and the two histidine residues to alanines in Aβcore (SEVAAQ) substantially reduce its neuroprotective effects, identifying these residues as critical to the neuroprotective actions of the Aβcore (43). These findings are consistent with earlier evidence showing that a different core Aβ fragment, $Aβ_{12–28}$, that contains the critical residues of the Aβcore is sufficient to prevent Aβ from binding to α7-nAChRs and reverse Aβ-induced inhibition of α7-nAChRs (44). Finally, a recent study shows that the formation of the Aβ–α4β2-nAChRs complex is based on the interaction of a part of Aβcore sequence (EVHH) with α4β2-containing receptors, and blocking this interaction prevents Aβ42-induced inhibition of α4β2-nAChRs (45). This thus suggests that interrupting the association of Aβ with nAChRs may be neuroprotective against Aβ-induced neuronal dysfunction in AD, although the differential impact of the Aβcore on the three major nAChRs in the hippocampus remains to be explored.

Here, we investigated the Aβ interaction with nAChRs in Aβ-induced Ca^{2+} hyperexcitation in cultured hippocampal neurons, assessing the impact of the neuroprotective, nontoxic N-terminal Aβcore to reverse the hyperexcitation. In addition, we assessed the selective interaction of Aβ with specific nAChRs and identified the amino acids, arginine and glutamate, within the loop C of the α7 and α4 subunits to be critical for these interactions. Moreover, we examined the impact of selective coactivation of α7- and α4β2-nAChRs on Aβ-induced synaptic dysfunction, including regulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamate receptors (AMPARs). The findings have implications for regulation of nAChRs as therapeutic targets in the hippocampus for neuroprotection in AD.

Results

Interaction between Aβ and nAChRs in Aβ-induced Ca^{2+} hyperexcitation and reversal by the neuroprotective Aβcore

Altering the interaction of Aβ with α7- and α4β2-nAChRs may be neuroprotective against Aβ-induced neuronal dysfunction in AD. We thus examined whether the interaction of $Aβ_{1–42}$ (Aβ42) with nAChRs is important for neuronal hyperactivity by using the Aβcore peptide (42, 43). As neuronal Ca^{2+} indicates neuronal activity (46), we measured Ca^{2+} activity in cultured 12 to 14 days *in vitro* (DIV) mouse hippocampal pyramidal neurons transfected with GCaMP6f (a genetically encoded Ca^{2+} indicator) as described previously (32, 47). We treated neurons with soluble Aβ42 oligomers (oAβ42) and determined Ca^{2+} activity in hippocampal neurons immediately after treatment. We found active spontaneous Ca^{2+} transients in the control condition (250 nM scrambled Aβ42; sAβ42) (Fig. 1). Consistent with the previous findings (32), the total Ca^{2+} activity in 250 nM oAβ42-treated cells was significantly higher than in sAβ42-treated controls (sAβ42, 1.00 ± 0.65 $\Delta F/F_{min}$ and oAβ42, 1.48 ± 0.95 $\Delta F/F_{min}$, p = 0.0009), confirming that soluble 250 nM Aβ42 oligomers were sufficient to increase neuronal Ca^{2+} activity (Fig. 1).

Of note, when 1 μM of Aβcore (YEVHHQ) was added in conjunction with 250 nM oAβ42, Aβcore treatment was able to reverse oAβ42-induced Ca^{2+} hyperexcitation (oAβ42+Aβcore, 0.98 ± 0.93 $\Delta F/F_{min}$, p = 0.04) (Fig. 1). However, the Aβcore had no effect on GCaMP6f activity in sAβ42-treated

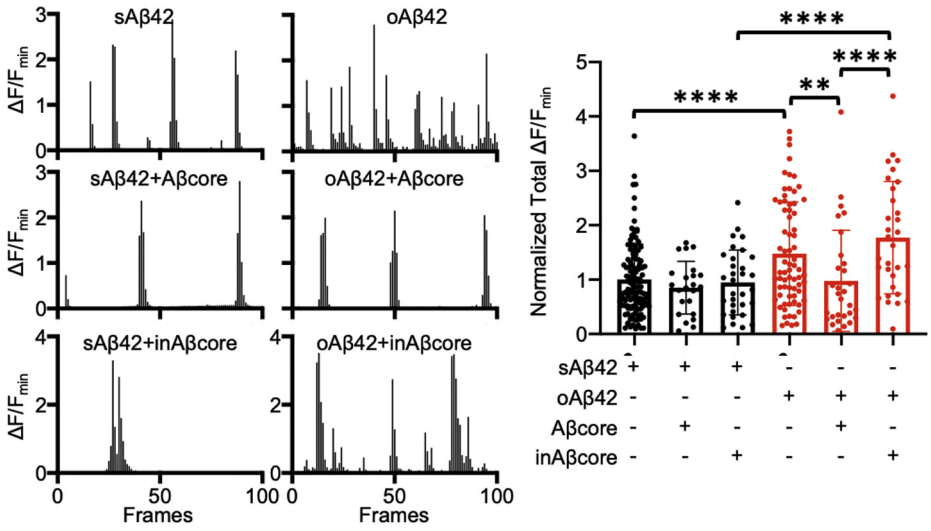

Figure 1. Interaction between Aβ42 and nAChRs is important for Aβ42-induced Ca^{2+} hyperexcitation. Representative traces of GCaMP6f fluorescence intensity in hippocampal neurons in each condition and a summary graph of the normalized average of total Ca^{2+} activity in neurons treated with either 250 nM sAβ42 (*black*) or 250 nM oAβ42 (*red*) in the absence or presence of 1 μM Aβcore or inactive 1 μM Aβcore (inAβcore) (n = number of neurons [sAβ42, n = 127; sAβ42+Aβcore, n = 23; sAβ42+inAβcore, n = 32; oAβ42, n = 71; oAβ42+Aβcore, n = 30; and oAβ42+inAβcore, n = 31], $^{**}p < 0.01$ and $^{****}p < 0.0001$ and, one-way ANOVA, Fisher's least significant difference test).

control neurons (sAβ42+Aβcore, 0.85 ± 0.48 $\Delta F/F_{min}$) (Fig. 1), which is consistent with the previous finding that Aβcore treatment had no prolonged effect on Ca^{2+} levels in differentiated mouse neuroblastoma cells (43). Next, we added 1 μM inactive Aβcore (SEVAAQ) in oAβ42-treated neurons and found that it was unable to reverse oAβ42 effects on Ca^{2+} activity (oAβ42+inactive Aβcore, 1.77 ± 1.03 $\Delta F/F_{min}$) (Fig. 1). Finally, inactive Aβcore treatment had no effect on neuronal activity in sAβ42-treated control neurons (sAβ42+inactive Aβcore, 0.95 ± 0.60 $\Delta F/F_{min}$) (Fig. 1). We thus demonstrated that cotreatment with the Aβcore peptide following application of oAβ42 significantly attenuated oAβ42-induced Ca^{2+} hyperactivity, possibly owing to the inhibition of the interaction between Aβ42 and nAChRs.

Aβ selectively interacts with α7- and α4β2-nAChRs but not α3β4-nAChRs

Many studies support that Aβ can physically interact with α7-, α4- and β2-containing nAChRs in various model systems (17, 31, 48, 49), whereas Aβ is unable to affect α3- and β4-containing receptor function when heterologously expressed in *Xenopus* oocytes (23). Nonetheless, the exact nature of the selective Aβ interaction with nAChRs is not fully defined. To directly measure interactions of Aβ with nAChR subunits, we carried out a series of coimmunoprecipitation (co-IP) analyses in transfected human embryo kidney (HEK293) cells, as described (50). Lysates from cells overexpressing human α7-nAChR-GFP receptors were incubated with 2 μM Aβ42 for 18 h and immunoprecipitated with an anti-GFP antibody. We found that the antibody pulled down α7-nAChR-GFP receptors along with Aβ42 (Fig. 2*A*), consistent with previous evidence for an interaction between Aβ42 and α7-nAChR in a neuronal cell line (51). Although the α7-nAChR subunit mainly assembles into homopentamers *in vivo*, α3 or α4 subunits require β subunits, coassembling to form heteropentamers (31, 52). Without β subunits, α3- or α4-nAChR subunits are unable to form functional surface receptors. We thus expressed mouse α4-nAChR-CFP receptors with mouse β2-mCherry, and co-IP experiments showed that Aβ42 associated with α4β2-nAChRs (Fig. 2*B*). Similar analysis using cells overexpressing human α3-nAChR-GFP receptors with human β4 subunits yielded no Aβ42 as a coimmunoprecipitate (Fig. 2*C*). As a separate control, we showed that the anti-actin antibody failed to pull down Aβ42 or α7-nAChRs (Fig. S1). These data suggest that Aβ42 can associate with the α4β2- and α7-containing receptors but is unable to interact with α3β4-nAChRs.

Given that coincubation with the Aβcore peptide was sufficient to reverse the Aβ42 effects on neuronal activity (Fig. 1), we carried out co-IP experiments with the Aβcore peptide to determine whether it could displace the interaction between Aβ42 and the receptors. Lysates from cells overexpressing human α7-nAChR-GFP receptors were incubated with 2 μM Aβ42 and 5 μM active Aβcore peptide for 18 h and immunoprecipitated with an anti-GFP antibody. We found that the antibody pulled down α7-nAChR-GFP receptors but not Aβ42 (Fig. S2*A*). We next used the inactive Aβcore peptide and found that it had no effect on the association between Aβ42 and α7-nAChRs (Fig. S2*B*). This suggests that the Aβcore peptide can inhibit the interaction between Aβ42 and α7-nAChRs, which may underlie, at least in part, the neuroprotective effects of the Aβcore peptide, in addition to its direct action on Aβ-regulated receptors (43).

Computer-simulated docking studies using the homology model of the human α7-nAChRs derived from the X-ray structure of the acetylcholine-binding protein (AChBP) and human Aβ show that the N terminus of Aβ is predicted to bind to the loop C of the α7 subunit, which is located within the binding interface of two α7 subunits (43, 51, 53–55). We used the CABS-dock server for flexible protein–peptide docking (56) to analyze interactions of the α7 nAChR-AChBP chimera

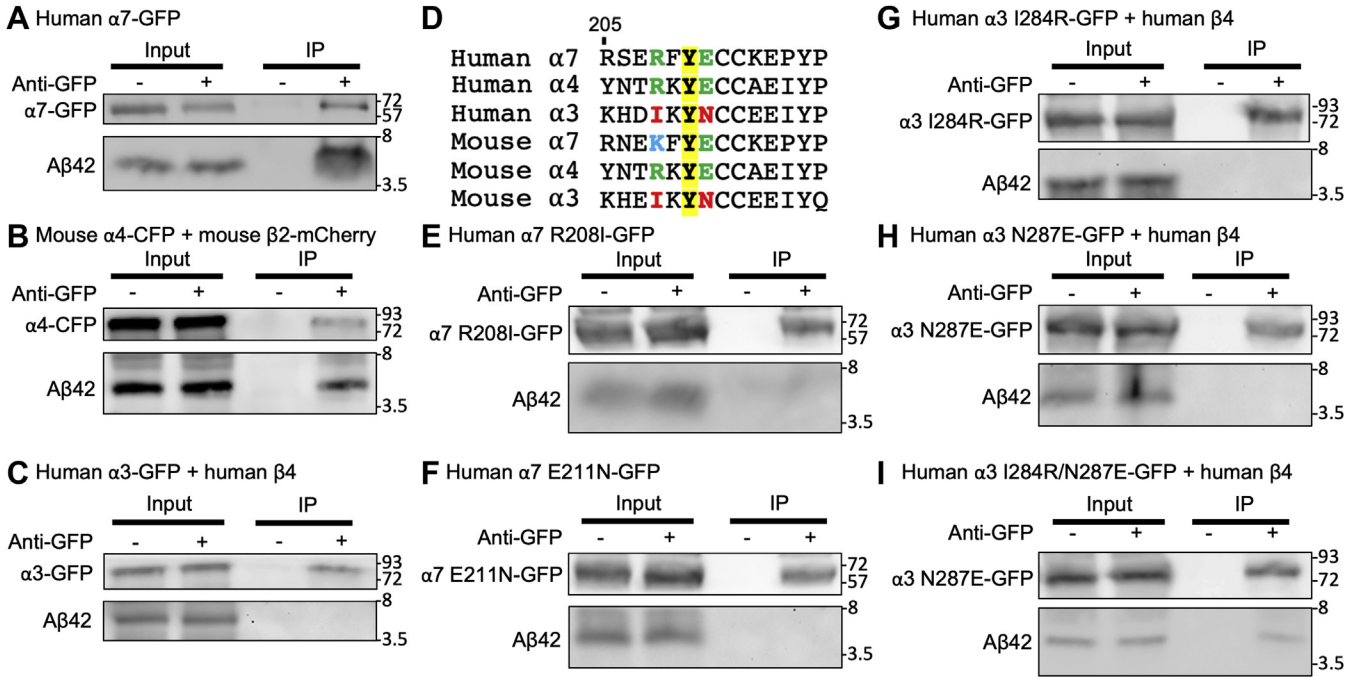

Figure 2. Aβ42 selectively interacts with α7- and α4β2-nAChRs, but not α3β4-nAChRs. *A*, Co-IP shows Aβ42 interacts with α7-nAChRs. *B*, Co-IP shows Aβ42 binds to α4β2-nAChRs. *C*, Co-IP shows Aβ42 is unable to interact with α3β4-nAChRs. *D*, sequence and numbering of human α7-nAChRs in the loop C region and its alignment with related human and mouse nAChR sequences. Y210 (*bold*) is the ligand-binding residue and conserved in all human and mouse α subunits. R208 (*blue*) and E211 (*green*) are predicted to be critical for interaction with the N terminus of Aβ, which are conserved only in both human and mouse α4 and α7 subunits except mouse α7 receptors that have positive-charged lysine (*light blue*), which is similar to positive-charged arginine. However, both mouse and human α3 receptors have uncharged residues in both positions (*red*). *E*, Co-IP shows Aβ42 is unable to interact with the α7 R208I mutant. *F*, Co-IP shows Aβ42 is unable to bind to the α7 E211N mutant. *G*, Co-IP shows Aβ42 is unable to interact with the α3 I284R mutant. *H*, Co-IP shows Aβ42 cannot interact with the α3 N287E mutant. *I*, double α3 I284R/N287E mutant is able to pull down Aβ42. Co-IP, coimmunoprecipitation.

(Protein Data Bank code: 1UW6) (55) and human N terminus of Aβ (Fig. S3). Three amino acids in the loop C of α7 nAChRs, arginine (R208), tyrosine (Y210), and glutamate (E211), were predicted to be critical for interactions of the α7 subunit with Aβ (Fig. 2*D*). Among them, Y210 is the ligand-binding residue and conserved in all human and mouse α3, α4, and α7 subunits (55) (Fig. 2*D*). Mutation studies have shown that Y210 is essential for acetylcholine binding and Aβ interactions (51, 55). Of interest, both R208 and E211, noncontact residues, are only conserved in human α4 and α7 subunits but not in the α3 subunit (Fig. 2*D*). Of importance, R208 in the human α7 subunit contains a positively charged side chain, and mouse α7 subunits contain lysine (K), a positively charged amino acid, instead of arginine (Fig. 2*D*). However, both mouse and human α3 subunits contain hydrophobic isoleucine (I) instead of positively charged arginine in the human α7 subunit or lysine in the mouse α7 subunit (Fig. 2*D*). Moreover, E211 in the human α7 subunit contains a negatively charged side chain, which is conserved in both human and mouse α4 and α7 subunits, while both mouse and human α3 subunits include uncharged asparagine (N) (Fig. 2*D*). Mutations in R208 and E211 in α7-nAChRs alter the binding affinity of the receptor to acetylcholine (55). Thus, it is possible that these two charged residues are responsible for the nAChR subtype selectivity of Aβ interactions. To test this idea, we generated mutant α7 subunits by substituting R208 for isoleucine (R208I) or E211 for asparagine (E211N) to mimic the α3 subunit (Fig. 2, *E* and *F*). Co-IP experiments were performed with lysates from HEK293 cells overexpressing human α7-nAChR-R208I-GFP or α7-nAChR-E211N-GFP receptors. We found that Aβ42 was unable to interact with either mutant α7-nAChR, a loss-of-function effect (Fig. 2, *E* and *F*). Furthermore, we made mutant α3 subunits by substituting I284 for arginine (I284R) or N287 for glutamate (N287E) to mimic the α7 subunit to test whether these mutants would show gain-of-function effects on the interaction between Aβ42 and the receptors. We carried out co-IP experiments with lysates from HEK293 cells overexpressing human α3-nAChR-I284R-GFP or α3-nAChR-N287E-GFP receptors with human β4 subunits. Both mutant receptors were unable to pull down Aβ42 (Fig. 2, *G* and *H*). We next generated a double mutant receptor that contained both I284R and N287E (Fig. 2*I*). In contrast to the single mutations, we found that the double α3 mutant was able to interact with Aβ42 when α3-nAChR-I284R/N287E-GFP and the β4 subunits were expressed in HEK293 cells (Fig. 2*I*). These data suggest that the charged arginine and glutamate residues in the loop C in the α4 and α7 subunits play important roles in the interaction between Aβ and nAChRs, providing direct molecular evidence of how Aβ selectively interacts with α7- and α4β2-nAChRs, but not α3β4-nAChRs.

Selective coactivation of α7- and α4β2-nAChRs reverses Aβ-induced reduction of AMPAR surface expression

Aβ has been reported to affect the function of AMPARs, which are important in synaptic plasticity (57). Several studies

suggest that Aβ-induced Ca^{2+} hyperexcitation promotes AMPAR endocytosis, which ultimately decreases the surface expression of AMPA receptor subunits GluA1 and GluA2, a cellular mechanism underlying Aβ-induced depression of AMPAR-mediated synaptic transmission (32, 58–61). Given that selective coactivation of α7- and α4β2-nAChRs reverses Ca^{2+} hyperexcitation in cultured neurons (32), we examined whether selective coactivation of α7- and α4β2-nAChRs reversed the Aβ effects on surface expression of AMPARs. We measured surface expression of AMPARs by biotinylation after 1 μM soluble Aβ42 oligomers (oAβ42) were applied to cultured hippocampal neurons for 1 h. Scrambled Aβ42 (sAβ42) was treated in neurons as the control. Consistent with the previous findings (32), oAβ42 treatment reduced surface expression of AMPAR subunits GluA1 and GluA2 (Fig. 3, *A–C* and Tables 1–3). Next, subtype-specific nAChR agonists, 1 μM PNU-282987 (α7), 2 μM RJR-2403 Oxalate (α4β2), or 1 μM NS-3861 (α3β4), were incubated with oAβ42 or sAβ42 for 1 h to activate each nAChR subtype. Activation of α7- or α4β2- or α3β4-nAChRs singularly was unable to reverse the Aβ effects on GluA1 and GluA2 surface levels (Fig. 3*A* and Table 1). Stimulation of each receptor by themselves also had no effect on GluA1 and GluA2 surface expression in control neurons (Fig. 3*A* and Table 1). Of importance, when we concurrently activated α7- and α4β2-nAChRs for 1 h using 1 μM PNU-282987 and 2 μM RJR-2403 Oxalate, GluA1 and GluA2 surface levels were restored to normal levels in cells treated with oAβ42 (Fig. 3*B* and Table 2). However, stimulation of α7- and α3β4-nAChRs or α4β2- and α3β4-nAChRs was unable to reverse the Aβ effects (Fig. 3*B* and Table 2). In addition, costimulation of two nAChR subtypes had no effect on GluA1 and GluA2 surface levels in sAβ42-treated neurons (Fig. 3*B* and Table 2). Next, we activated all three types of nAChRs together by treating neurons with the three agonists for 1 h and found no neuroprotective effect on Aβ-induced reduction of AMPAR surface levels (Fig. 3*C* and Table 3). Stimulation of α7-, α3β4- and α4β2-nAChRs together was unable to alter surface GluA1 and GluA2 levels in sAβ42-treated control cells (Fig. 3*C* and Table 3). Of interest, 1 μM carbachol, a cholinergic agonist, was also unable to reverse the Aβ effects on AMPAR surface expression (Fig. 3*C* and Table 3). Furthermore, carbachol was sufficient to reduce surface GluA1 but not GluA2 expression in control cells, suggesting that global stimulation of acetylcholine receptors may exacerbate the Aβ effects in neurons (Fig. 3*C* and Table 3). This suggests that selective coactivation of α7- and α4β2-nAChRs is required to abolish the Aβ effects on AMPAR surface expression.

Coactivation of α7- and α4β2-nAChRs reverses Aβ-induced impaired AMPAR phosphorylation and synaptic plasticity

Several studies suggest that Aβ-induced Ca^{2+} hyperexcitation elevates the activity of Ca^{2+}-dependent phosphatase, calcineurin, which, in turn, will promote AMPAR endocytosis *via* dephosphorylation of AMPAR subunit GluA1 at serine

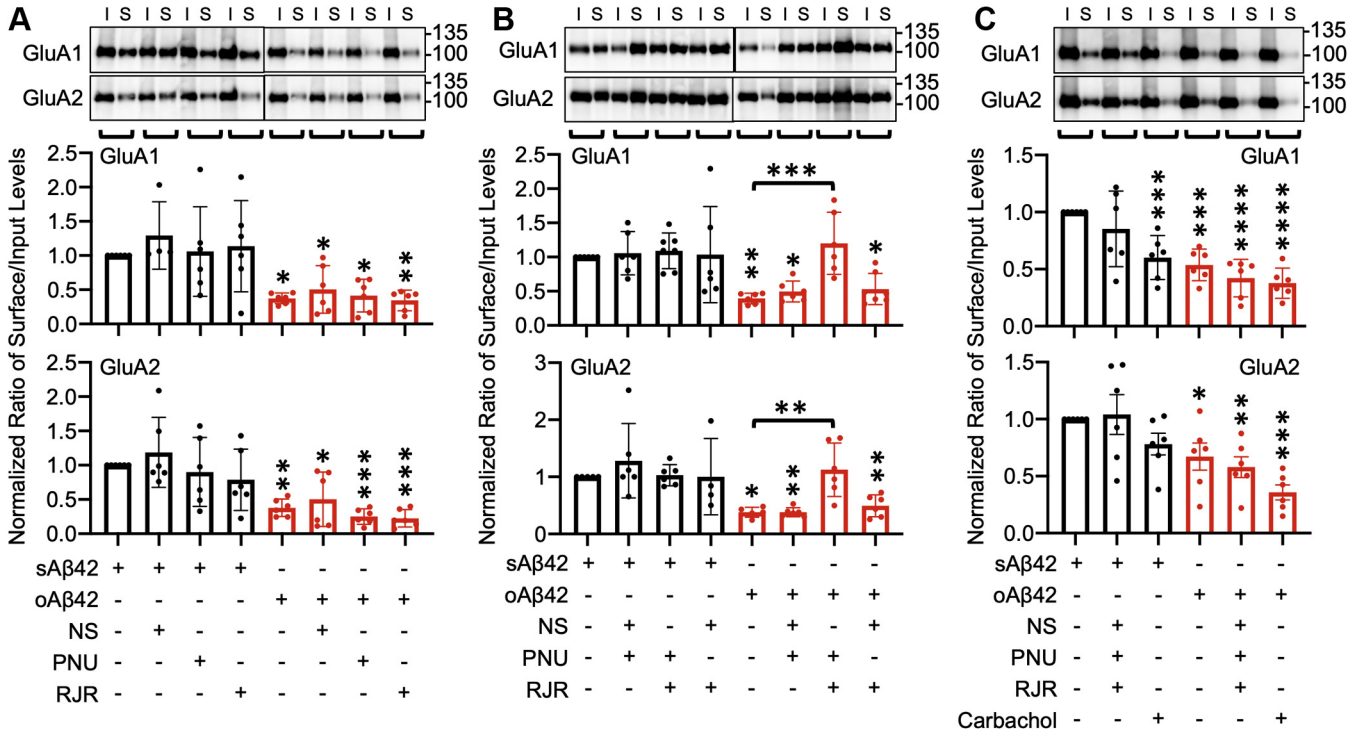

Figure 3. Selective coactivation of α7- and α4β2-nAChRs reverses Aβ-induced reduction of AMPAR surface expression. Representative immunoblots of input (I) and surface (S) levels and quantitative analysis in (*A*) single activation of each nAChRs (n = 6 immunoblots from three independent cultures duplicated, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, one-way ANOVA, Fisher's least significant difference [LSD] test). *B*, double activation of each nAChRs (n = 6 immunoblots from three independent cultures duplicated, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, one-way ANOVA, Fisher's LSD test). *C*, triple activation and cholinergic stimulation (n = 6 immunoblots from three independent cultures duplicated, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$, one-way ANOVA, Fisher's LSD test).

Table 1
Effects of single agonist application on Aβ-induced reduction of AMPA receptor surface expression

	Normalized ratio of surface/input levels
GluA1	
sAβ42	1.00
sAβ42+NS-3861	1.29 ± 0.49
sAβ42+PNU-282987	1.06 ± 0.65
sAβ42+RJR-2403 Oxalate	1.14 ± 0.67
oAβ42	0.37 ± 0.08
oAβ42+NS-3861	0.51 ± 0.35
oAβ42+PNU-282987	0.41 ± 0.24
oAβ42+RJR-2403 Oxalate	0.34 ± 0.15
GluA2	
sAβ42	1.00
sAβ42+NS-3861	1.19 ± 0.51
sAβ42+PNU-282987	0.90 ± 0.50
sAβ42+RJR-2403 Oxalate	0.79 ± 0.48
oAβ42	0.38 ± 0.13
oAβ42+NS-3861	0.50 ± 0.40
oAβ42+PNU-282987	0.25 ± 0.11
oAβ42+RJR-2403 Oxalate	0.23 ± 0.13

845, a residue that plays a crucial role in AMPAR surface expression during synaptic plasticity (32, 58, 59, 62). In fact, previous studies reveal that Aβ reduces AMPAR GluA1 phosphorylation at serine 845 (pGluA1), which is strongly associated with disrupted LTP in AD (32, 58, 61). Consistently, hippocampal LTP can be blocked by either direct exogenous Aβ application at high levels or abnormally high levels of Aβ produced in AD transgenic mouse models (58, 59, 63–66). This may contribute to AD-associated synaptic dysfunction and memory deficits (6). Given that coactivation of α7- and α4β2-nAChRs was sufficient to restore normal AMPAR surface levels (Fig. 3*B*) and neuronal Ca^{2+} activity in Aβ-treated cultured neurons (32), we examined whether costimulation of these receptors reversed the effects of a high concentration of Aβ on AMPAR phosphorylation and LTP. First, we treated cultured hippocampal neurons with 1 μM oAβ42 or sAβ42 for

Table 2
Effects of double agonist application on Aβ-induced reduction of AMPA receptor surface expression

	Normalized ratio of surface/input levels
GluA1	
sAβ42	1.00
sAβ42+NS-3861+PNU-282987	1.06 ± 0.32
sAβ42+PNU-282987+RJR-2403 Oxalate	1.09 ± 0.26
sAβ42+RJR-2403 Oxalate+NS-3861	1.04 ± 0.70
oAβ42	0.39 ± 0.08
oAβ42+NS-3861+PNU-282987	0.50 ± 0.15
oAβ42+PNU-282987+RJR-2403 Oxalate	1.21 ± 0.45
oAβ42+RJR-2403 Oxalate+NS-3861	0.53 ± 0.23
GluA2	
sAβ42	1.00
sAβ42+NS-3861+PNU-282987	1.28 ± 0.65
sAβ42+PNU-282987+RJR-2403 Oxalate	1.02 ± 0.18
sAβ42+RJR-2403 Oxalate+NS-3861	1.00 ± 0.67
oAβ42	0.38 ± 0.09
oAβ42+NS-3861+PNU-282987	0.38 ± 0.08
oAβ42+PNU-282987+RJR-2403 Oxalate	1.12 ± 0.47
oAβ42+RJR-2403 Oxalate+NS-3861	0.49 ± 0.19

Table 3
Effects of triple agonist application and cholinergic agonist treatment on Aβ-induced reduction of AMPA receptor surface expression

	Normalized ratio of surface/input levels
GluA1	
sAβ42	1.00
sAβ42+NS-3861+PNU-282987+RJR-2403 Oxalate	0.85 ± 0.33
sAβ42+Carbachol	0.6 ± 0.19
oAβ42	0.54 ± 0.14
oAβ42+NS-3861+PNU-282987+RJR-2403 Oxalate	0.42 ± 0.17
oAβ42+Carbachol	0.38 ± 0.13
GluA2	
sAβ42	1.00
sAβ42+NS-3861+PNU-282987+RJR-2403 Oxalate	1.04 ± 0.43
sAβ42+Carbachol	0.78 ± 0.23
oAβ42	0.67 ± 0.29
oAβ42+NS-3861+PNU-282987+RJR-2403 Oxalate	0.58 ± 0.22
oAβ42+Carbachol	0.36 ± 0.16

1 h and measured basal pGluA1 levels (Fig. 4*A*). As shown before (32), oAβ42 treatment decreased pGluA1 compared with the sAβ42-treated control (sAβ42, 1.00 and oAβ42, 0.48 ± 0.25, $p = 0.0424$) (Fig. 4, *A* and *B*). Of significance, Aβ-induced reduction of pGluA1 was reversed by costimulation of α7- and α4β2-nAChRs when we treated neurons with 1 μM PNU-282987 and 2 μM RJR-2403 Oxalate for 1 h (oAβ42+agonists, 1.27 ± 0.91, $p = 0.003$) (Fig. 4, *A* and *B*). However, coactivation of these receptors in control cells had no effect on

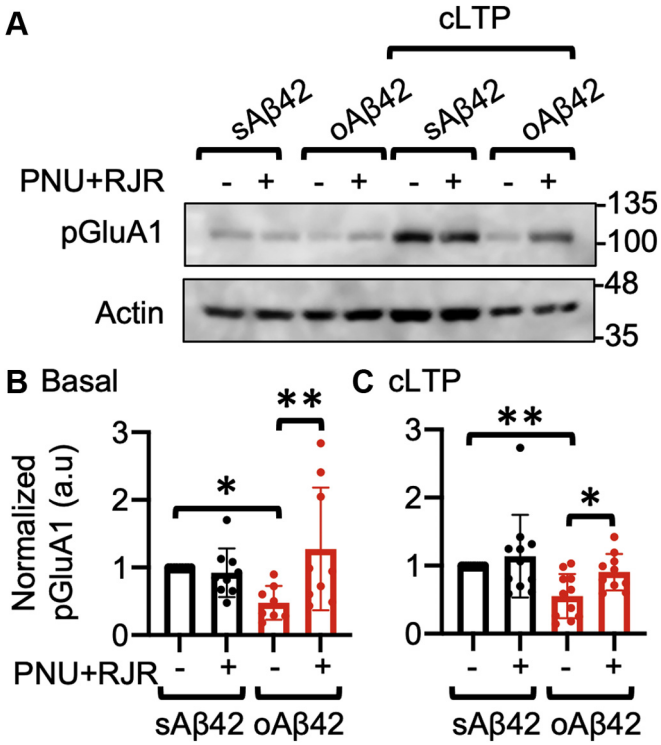

Figure 4. Coactivation of α7- and α4β2-nAChRs reverses Aβ-induced impaired AMPA receptor phosphorylation and synaptic plasticity. *A*, representative immunoblots of pGluA1 levels in each condition. *B*, quantitative analysis of pGluA1 levels under the basal condition in each condition (n = 9 immunoblots from four independent cultures, $*p < 0.05$ and $**p < 0.01$, one-way ANOVA, Fisher's least significant difference test). *C*, quantitative analysis of pGluA1 levels following cLTP induction in each condition (n = 11 immunoblots from five independent cultures, $*p < 0.05$ and $**p < 0.01$, one-way ANOVA, Fisher's least significant difference test).

pGluA1 levels (sAβ42+agonists, 0.92 ± 0.36) (Fig. 4, *A* and *B*). We next treated neurons with a glycine-based medium, well established to induce a form of chemical LTP (cLTP), as shown previously (62, 67). We treated neurons with 1 μM oAβ42 or sAβ42 for 1 h, induced cLTP, and measured pGluA1 levels (Fig. 4*A*). As shown previously (62, 67), following cLTP induction, pGluA1 levels were significantly elevated in control neurons, an indication of LTP expression (Fig. 4*A*). However, pGluA1 levels were significantly lower in oAβ42-treated neurons compared with sAβ42-treated control cells after cLTP induction (sAβ42, 1.00 and oAβ42, 0.55 ± 0.33, $p = 0.0075$) (Fig. 4, *A* and *C*), an indication of impaired synaptic plasticity. Of importance, normal pGluA1 levels were restored when we activated both α7- and α4β2-nAChRs and induced cLTP in oAβ42-treated neurons (oAβ42+agonists, 0.91 ± 0.27, $p = 0.0359$) (Fig. 4, *A* and *C*). However, coactivation of these receptors in control cells had no effect on pGluA1 levels following cLTP induction (sAβ42+agonists, 1.14 ± 0.61) (Fig. 4, *A* and *C*). Thus, coactivation of α7- and α4β2-nAChRs was sufficient to reverse the Aβ effects on AMPAR phosphorylation and cLTP.

Discussion

Current therapeutic approaches to AD suffer from lack of specificity and poor efficacy. Preclinical approaches based on altering Aβ have failed in clinical trials. Consequently, novel approaches are being explored, including targeting receptors regulated by Aβ. Nicotinic receptors have emerged as potential targets for reversing cognitive deficits in AD (68), owing to their noted potent regulation by Aβ, but there remains a need to determine the roles of specific nAChR subtypes in AD. In this study, we demonstrate that the interaction between Aβ and nAChRs plays an important role in Aβ-induced alteration of synaptic and neuronal activity. We provided further evidence for Aβ's selective interaction with α7- and α4β2-nAChRs but not α3β4-receptors, and selective stimulation of α7- and α4-containing nAChRs was shown to be neuroprotective against the Aβ effects on synaptic function. Of note, we identified two key amino acids, arginine and glutamate, present in the loop C of the α7 and α4 subunits, but not the α3 subunit, that are important for interaction with Aβ, providing a molecular mechanism for Aβ's selective inhibition of α7- and α4β2-nAChRs.

Based on the present findings, we propose the following model (Fig. 5). Given that nAChRs are more prominently expressed in inhibitory interneurons in the hippocampus, soluble Aβ42 oligomers selectively interact with α7- and α4β2-nAChRs but not α3β4-nAChRs and reduce neuronal activity in inhibitory cells, leading to a decrease in the release of GABA onto hippocampal excitatory neurons (Fig. 5*A*). This is supported by our previous work in which stimulation of $GABA_A$ receptors is sufficient to reverse Aβ42-induced Ca^{2+} hyperactivity in cultured neurons (32). Excitatory cells will thus have increased neuronal Ca^{2+} activity, consequently elevating the activity of calcineurin (32) (Fig. 5*A*) and other Ca^{2+} signaling pathways. This promotes the dephosphorylation of the AMPAR subunit, GluA1, which allows for AMPAR endocytosis, resulting in an overall decrease of AMPAR surface expression (Fig. 5*A*). This ultimately contributes to disruption of LTP (Fig. 5*A*) and may lead to cognitive decline. As Aβ42 inhibits both α7- and α4β2-nAChRs but not α3β4-nAChRs, costimulation of α7- and α4β2-nAChRs by using selective agonists can reverse the Aβ effects on synapses by restoring normal activity of both hippocampal inhibitory and excitatory cells (Fig. 5*B*). With restoration of normal Ca^{2+} activity, calcineurin activity decreases, leading to AMPAR phosphorylation and decreased AMPAR endocytosis, ultimately restoring normal LTP (Fig. 5*B*). Given that Aβ42 inhibits both α7- and α4β2-nAChRs, stimulation of each receptor by themselves has no neuroprotective effect (Fig. 3*A*).

Our co-IP experiments using mutant α7- and α3-containing receptors suggest that the charged arginine and glutamate residues in the loop C of α7- and α4β2 nAChRs are critical for the interaction of Aβ with these receptors. However, it is important to note that, as Aβ was added to the cell lysates and not the cell culture medium during co-IP, we are unable to rule out the possibility that Aβ may interact differently with surface nAChRs *in situ* on neurons. Nonetheless, a previous study identified that arginine 182 (R182) and glutamate 185 (E185), located in the loop C region of the α7-nAChR-AChBP chimera (equivalent to R208 and E211 in the native α7 subunits), do not have direct contact to agonists but impact the affinity of α7-nAChRs for ligands (55). In fact, the study revealed that R182 pairs with lysine 141 (K141), and E185 pairs with glutamate (E158) and aspartic acid 160 (D160), and these interactions provide electrostatic repulsion, which in native α7 may favor the open conformation of loop C, contributing to lower agonist affinity (55). It was further shown that mutations in any of these residues shift the concentration dependence of acetylcholine binding to the receptor to lower concentrations (higher affinity) by relief of electrostatic repulsion (55). Thus, we suggest that electrostatic repulsion generated by these residues may favor the open conformation of loop C, contributing to Aβ affinity. Therefore, a loss of one of these charged residues in the α7 subunit (R208I or E211N) disrupts the interaction of mutant α7-containing receptors with Aβ. Where α7-nAChRs are primarily present as homopentamers (31, 52), electrostatic repulsion would be established within the α7 subunits, and thus mutations of R208 or E211 disrupt this electrostatic repulsion, which may contribute to loss of binding to Aβ. In contrast to homomeric α7-nAChRs, α4- and α3-containing receptors exist as heteropentamers incorporating β subunits (69, 70). Of interest, glutamate in the loop C of the α4 subunit is likely to pair with a negatively charged residue in the β subunits, providing the electrostatic repulsion similar to α7 homopentamers (69). Thus, the β subunits may contribute to the binding affinity of the receptor to Aβ in α4β2- or α7β2-nAChRs expressed in hippocampal inhibitory neurons (71). Given that the α3 subunit contains noncharged amino acids in the loop C, a gain of charged residues in the loop C of α3-containing receptors may underlie an increased affinity of the receptors for Aβ42. In addition, other computational modeling studies show that E211 in the loop C of the α7 subunit is able to interact with Aβ42 and further suggest that

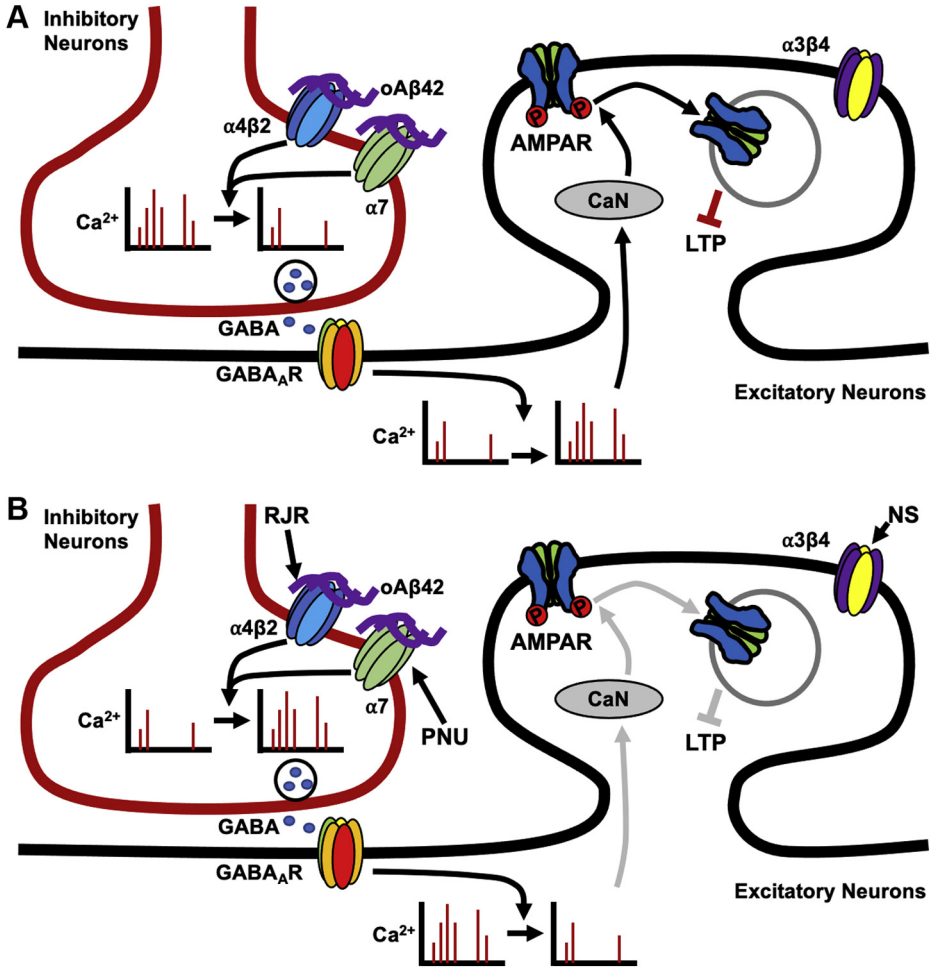

Figure 5. Schematic model. *A*, impact of Aβ oligomers. In the hippocampus, α7- and α4β2-nAChRs are prominently expressed on inhibitory interneurons; thus, selective binding of soluble Aβ42 oligomers (oAβ42) to α7- and α4β2-nAChRs but not α3β4-nAChRs, reduces neuronal activity in inhibitory cells, leading to a decrease in the release of GABA onto hippocampal excitatory neurons. Consequently, excitatory cells have increased frequency of Ca^{2+} transients, resulting in elevated calcineurin (CaN) activity. Calcineurin then dephosphorylates the AMPA receptor (AMPAR) subunit, GluA1, promoting AMPAR endocytosis and resulting in an overall decrease of AMPAR surface expression. This ultimately contributes to disruptions of long-term potentiation. *B*, reversal of Aβ-induced synaptic and neuronal dysfunction by costimulation with α7- and α4β2-nAChRs agonists. As Aβ42 inhibits both α7- and α4β2-nAChRs but not α3β4-nAChRs, costimulation of α7- and α4β2-nAChRs by selective agonists, PNU-282987 (PNU) and RJR-2403 Oxalate (RJR), can restore normal activity of both hippocampal inhibitory and excitatory cells, reversing Aβ-induced synaptic dysfunction. This restoration of normal Ca^{2+} activity prompts a decrease in calcineurin activity, leading to a decrease in AMPAR dephosphorylation and AMPAR endocytosis, ultimately restoring normal long-term potentiation. However, an agonist for α3β4-nAChRs, NS-3861 (NS), does not appear to have neuroprotective effects. Moreover, nonspecific stimulation of nAChRs by using three agonists together or carbachol is unable to reverse the Aβ effects on neuronal activity and synaptic function, emphasizing the importance of selective costimulation of nAChRs as potential therapeutic approaches.

the similar interactions apply to α4β2-AChRs (72, 73). In sum, these findings suggest that the charged residues, arginine and glutamate, in the loop C of the α7- and α4β2-nAChRs are critical for Aβ interaction and its effect on the receptors. However, as dynamic conformational changes cannot be accurately predicted from static models, we are unable to exclude other conformations that might contribute to the interaction between Aβ and the receptors.

Of interest, costimulation of α4β2-and α7-nAChRs can prevent the Aβ effects in cultured neurons, whereas coadministration of agonists for three nAChR subtypes has no effect (Fig. 3*C*). Cholinergic signaling in GABAergic inhibitory networks is generally stronger than direct actions on glutamatergic neurons in the hippocampus, as nAChRs are more densely expressed on inhibitory interneurons than on excitatory cells (33, 74–78). It has been suggested that, in the hippocampus, α7-nAChRs are located on GABAergic inhibitory interneurons and a subset of glutamatergic neurons, whereas α4β2-nAChRs are mainly located on GABAergic cell bodies and nerve terminals. By contrast, α3β4-nAChRs appear to be primarily associated with glutamatergic neurons, although their precise localization remains to be determined (74, 76, 77, 79–88) (Fig. 5). Thus, costimulation of α7- and α4β2-nAChRs predominantly activates GABAergic interneurons, in turn inhibiting excitatory neurons in the hippocampus. In contrast, activation of α3β4-nAChRs would directly stimulate glutamatergic neurons. Given that the majority of cells in hippocampal cultures are excitatory neurons (89), reduction of the activity in excitatory neurons by costimulation of α7- and α4β2-nAChRs can be offset by direct activation of α3β4-nAChRs on glutamatergic neurons. Therefore, the net activity in hippocampal excitatory neurons

treated with three agonists together would not change significantly, contributing to the unaltered AMPAR surface expression (Fig. 3*C*).

The current study focuses on the differential effects of Aβ on nAChR subtypes that lead to alterations in neuronal excitability and synaptic function. Consistent with previous findings using acetylcholinesterase inhibitors (74), we found that nonselective cholinergic activation exacerbates Aβ effects on hippocampal neurons (Fig. 3*C*), pointing to confounding effects of activating α3β4-nAChRs, which may result in unexpected side effects, including imbalance of excitation and inhibition in hippocampal circuits. Therefore, acetylcholinesterase inhibitors are not very effective in slowing AD progression due to nonselective stimulation of acetylcholine receptors (90, 91). There are also discrepancies involving the use of nicotine treatment to stimulate nAChRs to alter cognitive function. For example, nicotinic agonists have been found to improve performance in a variety of memory tasks in rodents and nonhuman primate studies (68), whereas several other studies have failed to find significant enhancement of learning and memory by nicotine treatment (92). Part of the discrepancy may lay in the antagonistic effect of prolonged nicotine exposure, owing to induction of nAChR inactivation (desensitization). The extent to which nAChR subtype-selective agonists drive receptor inactivation, and their impact on intracellular signaling in Aβ neurotoxicity, remains to be determined (93). Nonetheless, nAChR agonists have consistently been suggested as promising approaches in the treatment of AD (94). However, clinical trials thus far have been challenged by adverse effects or minimal improvement (95). In particular, stimulating only one type of nAChR by using a subtype-specific agonist was found to either enhance cognitive performance or have no beneficial effect. For instance, selective α7-nAChR agonists have been reported to improve cognition in a variety of animal models (96–98), whereas another study has found they have almost no beneficial effect on learning and memory in mice (99). An α4β2-nAChR agonist alone can improve working memory only in young rats but not in older animals (100). It is thus not yet clear whether single activation of specific nAChR subtypes provides optimal efficacy in AD (94). We have also shown that activation of either α7- or α4β2-nAChRs singularly had no effect on Aβ-induced hyperexcitation (32) or α7- and α4β2-nAChR Aβ-induced reduction of AMPAR surface expression (Fig. 3*A*). By contrast, selective coactivation of α7- and α4β2-nAChRs was sufficient to reverse Aβ-induced neuronal hyperexcitation (32) and synaptic dysfunction (Figs. 3*B* and 4). Several subtype-specific agonists have been developed for clinical trials, but coactivation of nAChRs has not been applied for clinical trials yet (94); thus, the current study may lead to an innovative and novel therapeutic strategy for AD.

Experimental procedures

Mouse hippocampal neuron culture

Mouse hippocampal neuron cultures were prepared as described (32, 47, 101–103). Hippocampi were isolated from postnatal day 0 (P0) CD-1 (Charles River) mouse brain tissues and digested with 10 U/ml papain (Worthington Biochemical Corp). For Ca^{2+} imaging, mouse hippocampal neurons were plated on poly-D-lysine-coated glass-bottom dishes (5×10^5 cells) and imaged on DIV 12 to 14. For biotinylation assays and cLTP, hippocampal neurons were plated in 6-cm dishes (3×10^6 cells) and used on DIV 14. Cells were grown in Neurobasal medium (Life Technologies) with B27 supplement (Life Technologies), 0.5 mM Glutamax (Life Technologies), and 1% penicillin/streptomycin (Life Technologies). Colorado State University's Institutional Animal Care and Use Committee reviewed and approved the animal care and protocol (978).

Reagents

Soluble Aβ42 oligomers or soluble scrambled Aβ42 oligomers were prepared as described (32, 104). One milligram of lyophilized human Aβ42 (Anaspec) or scrambled Aβ42 (Anaspec) was dissolved in 1 ml of 1,1,1,3,3,3-hexafluoro-2-propanol (Fisher Scientific) to prevent aggregation, portioned into 10-μg aliquots, air dried, and stored at −80 °C. For use in experiments, an aliquot was thawed at room temperature and then dissolved in 100% dimethyl sulfoxide, then diluted into PBS to make a 100 μM solution. The solution was incubated for 16 h at 4 °C and then diluted to a final concentration for use in experiments. The following agonists were used in this study: 1 μM PNU-120596 (Alomone Labs), 2 μM RJR-2403 Oxalate (Alomone labs), 1 μM NS-3861 (Tocris Bioscience), and 1 μM Carbamoylcholine chloride (carbachol) (Tocris Bioscience). Aβcore (YEVHHQ) and inactive Aβcore (SEVAAQ) peptides were prepared as described (43).

HEK293 cell culture and transfection

HEK293 cells were grown in Dulbecco's modified Eagle's medium with fetal bovine serum (Life Technologies) and 1% penicillin/streptomycin (Life Technologies) and transfected by jetPRIME DNA and siRNA transfection reagent (Polyplus) according to the manufacturer's protocol. Cells used for each experiment were from more than three independently prepared cultures. Two micrograms of human α7-nAChR-GFP plasmid was transfected into HEK293 cells to express the homopentameric form of α7-nAChRs. Both α3β4- and α4β2-nAChRs expressed by 1:1 α:β transfection ratio formed functional channels in HEK293 cells (105–108). Therefore, the α3 to β4 or α4 to β2 cDNA ratio in the mixture was kept equal (1:1, 1 μg each) for transfection to express α3β4- or α4β2-nAChRs in HEK293 cells.

DNA plasmids and mutagenesis

Human pcDNA3.1-CHRNA7-mGFP was a gift from Henry Lester (Addgene plasmid # 62629; http://n2t.net/addgene:62629; RRID:Addgene_62629) (109). Mouse nAChR alpha4 CFP was a gift from Henry Lester (Addgene plasmid # 15244; http://n2t.net/addgene:15244; RRID:Addgene_15244) (110). pCI-neoBeta2mcherry was a gift from Henry Lester (Addgene plasmid # 45097; http://n2t.net/addgene:45097; RRID:Addgene_45097). β4-nAChR (DPM negative control) was a gift

from Jaime Modiano (Addgene plasmid # 86651; http://n2t.net/addgene:86651; RRID:Addgene_86651) (111). Human α3-nAChR-GFP was obtained from Sino Biological (HG29719-ACG). Human α7-nAChR R208I-GFP and E211N-GFP were generated from pcDNA3.1-CHRNA7-mGFP, and human α3-nAChR I284R-GFP and N287E-GFP were generated from human α3-nAChR-GFP by PCR-based QuikChange Site-Directed Mutagenesis Kit (Agilent) according to the manufacturer's protocol. Human α3-nAChR I284R/N287E-GFP was generated by two sequential mutagenesis from human α3-nAChR-GFP. The following primers were used for mutagenesis; R208I (5′-GGA ATC CCC GGC AAG AGG AGT GAA ***ATA*** TTC TAT GAG TGC TGC-3′ and 5′-CCT TAG GGG CCG TTC TCC TCA CTT ***TAT*** AAG ATA CTC ACG ACG-3′), E211N (5′-AGG TTC TAT ***AAC*** TGC TGC AAA GAG CCC TAC CCC GAT GTC-3′ and 5′-TCC AAG ATA ***TTG*** ACG ACG TTT CTC GGG ATG GGG CTA CAG-3′), I208R (5′-CCA GGC TAC AAA CAC GAC ***CGC*** AAG TAC AAC TGC TGC-3′ and 5′-GCA GCA GTT GTA CTT ***GCG*** GTC GTG TTT GTA GCC TGG-3′) and N211E (5′-GGC TAC AAA CAC GAC ATC AAG TAC ***GAG*** TGC TGC GAG GAG-3′ and 5′-CTC CTC GCA GCA ***CTC*** GTA CTT GAT GTC GTG TTT GTA GCC-3′); bold and italic nucleotides indicate mutations introduced.

Coimmunoprecipitation

Co-IP experiments were performed using a Co-IP kit (Pierce) following the manufacturer's protocol with samples from three independently prepared cultures as carried out previously (50). Two micromolar soluble Aβ42 was added to total cell lysates (200 μl), and 20 μl of cell lysates with Aβ42 was collected as the input. A volume of 180 μl of cell lysates incubated with Aβ42 was pulled down with anti-GFP antibody. As a negative control, anti-actin was used. Immunoprecipitated samples were applied to immunoblots. To determine the effects of the Aβcore peptide, lysates were incubated with 2 μM Aβ42 and 5 μM active or inactive Aβcore peptide for 18 h and immunoprecipitated with an anti-GFP antibody.

Computational modeling

Computational peptide docking of the N terminus of Aβ into the α7-nAChR-AChBP (the acetylcholine-binding protein) chimera (55) was performed using the CABS-dock server for flexible protein–peptide docking (56). For the α7-nAChR-AChBP chimera, the X-ray crystallographic structure encompassing two adjacent α subunits containing the ligand-binding domain, equivalent in all five sites in the pentameric receptor, was used in a flexible protein–peptide docking.

GCaMP Ca^{2+} imaging

GCaMP Ca^{2+} imaging was carried out by the previously reported method (32, 47). DIV 4 neurons were transfected with pGP-CMV-GCaMP6f (a gift from Douglas Kim, Addgene plasmid # 40755; http://n2t.net/addgene:40755; RRID:Addgene_40755) (112) for imaging hippocampal pyramidal cells or pAAV-mDlx-GCaMP6f-Fishell-2 (a gift from Gordon Fishell, Addgene plasmid # 83899; http://n2t.net/addgene:83899; RRID:Addgene_83899) (113) for imaging interneurons by using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. Neurons were imaged DIV 12 to 14. The transfection efficiency was around 2%, and no obvious cellular toxicity has been observed. Neurons were grown in Neurobasal Medium without phenol red (Life Technologies) and with B27 supplement (Life Technologies), 0.5 mM Glutamax (Life Technologies), and 1% penicillin/streptomycin (Life Technologies) for 8 to 10 days after transfection and during the imaging. Glass-bottom dishes were mounted on a temperature-controlled stage on an Olympus IX73 microscope and maintained at 37 °C and 5% CO_2 using a Tokai-Hit heating stage and digital temperature and humidity controller. For GCaMP6f, the images were captured with a 10-ms exposure time and a total of 100 images were obtained with a 500-ms interval. F_{min} was determined as the minimum fluorescence value during the imaging. Total Ca^{2+} activity was obtained by 100 values of $\Delta F/F_{min} = (F_t - F_{min})/F_{min}$ in each image, and values of $\Delta F/F_{min} < 0.1$ were rejected due to bleaching. Ten to 20 neurons were used for imaging in each individual experiment, and one individual neuron was assayed in an image.

Surface biotinylation

Surface biotinylation was performed according to the previous studies (32, 47, 101–103). Cells were washed with ice-cold PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ and incubated with 1 mg/ml Sulfo-NHS-SS-biotin (Thermo Scientific) for 15 min on ice. Following biotin incubation, neurons were washed with 20 mM glycine to remove the excess of biotin, and cells were lysed in 300 μl radioimmunoprecipitation assay buffer for 1 h. Ten percent of the total protein was separated as an input sample, and protein lysates were incubated overnight with streptavidin-coated beads (Thermo Scientific) at 4 °C under constant rocking. The beads containing surface biotinylated proteins were separated by centrifugation. Biotinylated proteins were eluted from streptavidin beads with SDS loading buffer. Surface protein fractions and their corresponding total protein samples were analyzed by immunoblots.

Chemical LTP

cLTP was performed by a modification of the previously described method (62, 67). Cells were washed with Mg^{2+}-free buffer (150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 33 mM glucose, 10 mM Hepes [pH 7.4], 20 μM bicuculline, 1 μM strychnine), treated with 200 μM glycine in Mg^{2+}-free buffer for 5 min at 37 °C, and returned to Mg^{2+} buffer (Mg^{2+}-free buffer and 2 mM $MgCl_2$) for 20 min at 37 °C. Cells were then lysed with 100 μl radioimmunoprecipitation assay buffer, and cell lysates was used for immunoblots.

Immunoblots

Protein samples for biotinylation and cLTP were loaded on 10% glycine-SDS-PAGE gel. Co-IP samples were loaded on

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16% Tricine-SDS-PAGE gel as described (114). SDS-PAGE gels were transferred to nitrocellulose membranes. The membranes were blocked (5% powdered milk) for 1 h at room temperature, followed by overnight incubation with the primary antibodies at 4 °C. The primary antibodies consisted of anti-GluA1 (Millipore, 1:2000), anti-GluA2 (Abcam, 1:2000), anti-phosphorylated GluA1 S845 (Millipore, 1:1000), anti-GFP (Torrey Pines, 1:2000), anti-Aβ (6E10, Covance, 1:2000), and anti-actin (Abcam, 1:2000) antibodies. Membranes were subsequently incubated by secondary antibodies for 1 h at room temperature and developed with Enhanced Chemiluminescence (Thermo Fisher Scientific). Protein bands were quantified using ImageJ (https://imagej.nih.gov/ij/). Immunoblots were at least duplicated for quantitative analysis.

Statistics

Statistical comparisons were analyzed with the GraphPad Prism 9 software. Unpaired two-tailed Student *t* tests were used in single comparisons. For multiple comparisons, one-way ANOVA followed by Fisher's least significant difference (LSD) test was used to determine statistical significance. Results are represented as mean ± standard deviation (SD), and $p < 0.05$ was considered the minimum for statistical difference.

Data availability

All data are contained within the article.

Supporting information—This article contains supporting information.

Acknowledgments—We thank members of the Kim laboratory for their generous support. In particular, we thank Rosaline Danzman, Luis Gomez Wulschner, Thomas McMillan, and Caleb Wipf for initiating the project. We also thank Drs Michael Tamkun, Jozsef Vigh, Frederic Hoerndli, and Susan Tsunoda for helpful discussion and providing reagents. We specially thank Dr SeungBeom Hong at King Abdullah University of Science and Technology for his help in structural analysis.

Author contributions—S. K. and R. A. N., conceptualization; J. P. R., S. A. S., and S. K., investigation; M. F. S. and R. A. N., Resources; S. K. and R. A. N., methodology; J. P. R., S. A. S., and S. K., Writing - original draft; S. K. and R. A. N., Writing - review & editing; J. P. R., S. A. S., R. A. N., and S. K., funding acquisition; S. K., supervision.

Funding and additional information—This work is supported by the University of Hawai'i Foundation Preclinical Alzheimer's Research Fund (R. A. N.), the pilot program from the Colorado Clinical and Translational Sciences Institute (S. K.), College Research Council Shared Research Program from Colorado State University (S. K.), the Boettcher Foundation's Webb-Waring Biomedical Research Program (S. K.), The Barry Goldwater Scholarship and Excellence in Education Foundation (J. P. R.), Astronaut Scholarship Foundation (J. P. R.), and Student Experiential Learning Grant from Colorado State University (J. P. R. and S. A. S.).

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: Aβ, beta-amyloid; AChBP, acetylcholine-binding protein; AD, Alzheimer's disease; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamate receptor; cLTP, chemical long-term potentiation; co-IP, coimmunoprecipitation; DIV, days *in vitro*; GABA, γ-aminobutyric acid; LTP, long-term potentiation; nAChR, nicotinic acetylcholine receptor.

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