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The amyloid precursor protein modulates α_{2A}-adrenergic receptor endocytosis and signaling through disrupting arrestin 3 recruitment

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ABSTRACT: The amyloid precursor protein (APP) has long been appreciated for its role in Alzheimer's disease (AD) pathology. However, less is known about the physiologic function of APP outside of AD. Particularly, whether and how APP may regulate functions of cell surface receptors, including GPCRs, remains largely unclear. In this study, we identified a novel direct interaction between APP and the α_{2A}-adrenergic receptor (α_{2A}AR) that occurs at the intracellular domains of both proteins. The APP interaction with α_{2A}AR is promoted by agonist stimulation and competes with arrestin 3 binding to the receptor. Consequently, the presence of APP attenuates α_{2A}AR internalization and desensitization, which are arrestin-dependent processes. Furthermore, in neuroblastoma neuro-2A cells and primary superior cervical ganglion neurons, where APP is highly expressed, the lack of APP leads to a dramatic increase in plasma membrane recruitment of endogenous arrestin 3 following α_{2A}AR activation. Concomitantly, agonist-induced internalization of α_{2A}AR is significantly enhanced in these neuronal cells. Our study provided the first evidence that APP fine tunes GPCR signaling and trafficking. Given the important role of α_{2A}AR in controlling norepinephrine release and response, this novel regulation of α_{2A}AR by APP may have an impact on modulation of noradrenergic activity and sympathetic tone.—Zhang, F., Gannon, M., Chen, Y., Zhou, L., Jiao, K., Wang, Q. The amyloid precursor protein modulates α_{2A}-adrenergic receptor endocytosis and signaling through disrupting arrestin 3 recruitment. FASEB J. 31, 4434–4446 (2017). www.fasebj.org

KEY WORDS: internalization · desensitization · interactions · cell surface receptor

As the source of amyloid-β peptides, the amyloid precursor protein (APP) has been well appreciated for its function in AD. Outside of the disease, APP plays an important role in neuronal development, regulating neurite outgrowth, and synaptogenesis (1, 2). APP-deficient mice show several neurologic phenotypes that could be related to synaptogenesis, including decreased grip strength (3), alterations in spine density, and diminished performance on spatial memory tasks (4, 5). In mammals, the APP family also includes 2 APP-like proteins, APP-like protein (APLP)-1 and -2, which share partially overlapping biologic activities with APP (6). Despite significant progress being made in understanding the physiologic significance of APP after several decades of research on the protein, the mechanisms behind its functions are still unclear, and our understanding of the diverse activities of APP remains incomplete.

APP has been shown to interact with several transmembrane and cytosolic proteins (7, 8). Although more efforts have been made to understand how such interactions regulate APP trafficking and processing, less is known about the role of APP in regulating the functions of its partners. A previous finding on regulation of the NGF/TrkA signaling (9) by APP suggests that interacting with other cell surface receptors and regulating their downstream signaling may represent an important means for APP to elicit its physiologic functions. Despite GPCRs being the largest family of cell surface receptors, whether and how APP may regulate GPCR functions has not been investigated.

The α_{2A}-adrenergic receptor (α_{2A}AR) is a prototypical GPCR, and mediates a variety of critical physiologic/pharmacological responses, which include lowering blood pressure, evoking sedation, reducing pain perception, and decreasing epileptogenesis and anxiety (10–12).

ABBREVIATIONS: 3iloop, third intracellular loop; α_{2A}AR, α_{2A}-adrenergic receptor; Ab, antibody; AD, Alzheimer's disease; APLP1, APP-like protein 1; APLP2, APP-like protein 2; APP, amyloid precursor protein; APP-C, C terminus of APP; co-IP, coimmunoprecipitation; DIV, days *in vitro*; GFP, green fluorescent protein; GST, glutathione *S*-transferase; HA, hemagglutinin; IP, immunoprecipitation; KO, knockout; N2a, neuro-2A; NE, norepinephrine; siRNA, small interfering RNA; SCG, superior cervical ganglion; t-ERK, total ERK

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Positioned at both the presynaptic noradrenergic terminal and the postsynaptic compartments of targeting neurons, the α_{2A}AR controls both noradrenergic input to the cerebrum and the resulting response in this brain region (13). In addition, α_{2A}AR serves as the primary autoreceptor in sympathetic neurons, controlling norepinephrine (NE) release and sympathetic tone (14, 15). Like many GPCRs, the non-G-protein–interacting partners of α_{2A}AR modulate multiple aspects of the receptor function both *in vitro* and *in vivo* (16–20). Among these partners, a universal GPCR regulator, arrestin 3, binds to α_{2A}AR after receptor activation and mediates agonist-induced endocytosis and desensitization of α_{2A}AR (17, 18, 21). As a result, arrestin 3 determines the response sensitivity of α_{2A}AR in multiple pharmacological settings *in vivo* (18, 20).

In this study, we discovered a novel direct interaction between APP and the α_{2A}AR through the intracellular portions of each protein. We hypothesized that APP binding to α_{2A}AR has functional consequences on receptor trafficking and signaling. Using both gain- and loss-of-function approaches, we demonstrated that the presence of APP antagonizes arrestin-dependent endocytosis and desensitization of α_{2A}AR. Consistent with these observations, we discovered that the interaction of APP with α_{2A}AR competes with the interaction of arrestin and α_{2A}AR. Furthermore, we extended our studies to primary superior cervical ganglion (SCG) neurons, where the α_{2A}AR is the major autoreceptor and demonstrated the APP antagonism of arrestin function in this native setting.

MATERIALS AND METHODS

Antibodies and chemicals

Antibodies (Abs) for GAPDH and APP (22C11) were purchased from EMD Millipore (Billerica, MA, USA); APP rabbit mAb (Y188) from Abcam (Cambridge, United Kingdom); HA.11 Ab for detecting HA-tagged α_{2A}AR from Covance (Princeton, NJ, USA); Abs for phospho-ERK1/2 (Thr202/Tyr204), ERK, and the green fluorescent protein (GFP) mAb from Cell Signaling Technology (Danvers, MA, USA); Flag M2 Ab from Sigma-Aldrich (St. Louis, MO, USA); secondary Abs used for immunostaining (Alexa Fluor 488- and 594-conjugated) from Thermo Fisher Scientific (Waltham, MA, USA); secondary Abs used for Western blot with the Li-Cor Odyssey Imaging System (IRDye 680 and 800; Li-Cor Biosciences, Lincoln, NE, USA); Lipofectamine 2000 from Thermo Fisher Scientific; NE, clonidine, guanfacine, UK14304, yohimbine, propranolol, and prazosin from Sigma-Aldrich; and [^{35}S]Methionine from GE Healthcare (Little Chalfont, United Kingdom).

Cell culture

Neuro-2A (N2a) cells were cultured in 1:1 DMEM/Opti-MEM mix (Thermo Fisher Scientific) supplemented with 5% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. HEK293 cells were cultured in DMEM with 10% fetal bovine serum plus 100 U/ml penicillin, and 100 μg/ml streptomycin (Thermo Fisher Scientific).

N2a-HA-α_{2A}AR-APP-CRISPR cells are a stable N2a cell line expressing HA-α_{2A}AR with APP knocked out by the CRISPR/Cas9 system. This cell line was generated according to a published protocol (22). Two target genomic DNA sequence primers were designed and annealed (forward: 5′-CACCACTGCAGATCACAAACGTGG-3′ and reverse: 5′-AAACCCACGTTTGTGATCTGCAGT-3′). Using the *Bbs*I restriction enzyme, the primers were introduced to the pSpCas9(BB)-2A-GFP plasmid. An N2a-HA-α_{2A}AR stable cell line (23) was transfected with the resulting plasmid using Lipofectamine 2000 and selected for GFP expression by fluorescence-activated cell sorting. Single clones were allowed to grow and screened for loss of expression of APP. N2a-HA-α_{2A}AR cells transfected with the empty pSpCas9(BB)-2A-GFP vector were established as a control cell line. APP knockout (KO) in the N2a-HA-α_{2A}AR-APP-CRISPR line was confirmed using both Western blot and immunostaining, and continued expression of APP in the N2a-HA-α_{2A}AR-control-CRISPR was confirmed in the same way.

For small interfering RNA (siRNA) treatment, siRNAs targeting mouse APP were purchased from Integrated DNA Technologies (Coralville, IA, USA) (TriFecta Kit DsiRNA Duplex), Duplex sequences: 5′-ACUAGUGCAUGAAUAGAUUCUCUCC-3′ and 3′-UUUGAUCACGUACUUAUCUAAGAGAGG-5′; 5′-GGAGAUUCAAGAUGAAGUCGAUGAG-3′ and 3′-CUCCUCUAAGUUCUACUUCAGCUACUC-5′ and were transfected into cells using Lipofectamine 2000. A negative control siRNA, also from Integrated DNA Technologies (NC1 Control Duplex) was transfected into cells to use as the control.

Animals and drug treatment

Mice were housed in the Association for Assessment and Accreditation of Laboratory Animal Care–accredited Animal Resources Program facility at the University of Alabama at Birmingham, in accordance with procedures of the Animal Welfare Act and the 1989 amendments to the Act, and all studies followed protocols approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

The generation of α_{2A}AR-knock-in mice has been described in Lu *et al*. (24). This line has been backcrossed for more than 10 generations to a pure C57BL/6 background. The mice (6 mo old) were treated though intraperitoneal injection with saline, clonidine, guanfacine, or UK14304 (1 mg/kg) 1 h before euthanizing for the experiments. Postnatal d 5–7 pups were used to culture SCG neurons.

Primary culture of SCG neurons

SCG neurons were cultured from HA-α_{2A}AR-knock-in (24) mouse pups at postnatal d 5–7, as described elsewhere (24–26), with slight modifications. SCGs were dissected and placed into HBSS (Thermo Fisher Scientific) with 25 mM glucose and 20 mM HEPES (pH 7.3). The neurons were enzymatically digested with 3 mg/ml collagenase (Sigma-Aldrich) and 1 mg/ml trypsin (Sigma-Aldrich). Using trituration with a fire-polished siliconized Pasteur pipette, we dissociated the neurons by trituration. A preplating step was then performed to reduce non-neuronal cell types in the final culture. The neurons were then plated onto coverslips treated with poly-D-lysine (Sigma-Aldrich) and laminin (Thermo Fisher Scientific). Growth medium was L-15 base medium (Thermo Fisher Scientific) supplemented with 10% Nu-serum (Corning, Corning, NY, USA), 30% glucose, 2% GlutaMax (Thermo Fisher Scientific), 1% insulin/transferrin/selenium supplement (Thermo Fisher Scientific), 25 ng/ml nerve growth factor (Thermo Fisher Scientific), and 24 nM $NaHCO_3$. Medium was changed on days *in vitro* (DIV) 1, 4, and 6. On DIV 1 and 4, 10 μM 5-fluoro-2′-deoxyuridine (Sigma-Aldrich) was added to control nonneuronal cell growth, and on DIV

4 and 6, 1 μM yohimbine (α_{2A}AR antagonist) was added to preserve cell surface α_{2A}ARs. All experiments were performed on DIV 8.

Immunofluorescence staining

To examine colocalization between α_{2A}AR and APP on the plasma membrane, live cells were first incubated with a hemagglutinin (HA; rat anti-HA.11) and APP (mouse 22C11) Ab to label cell surface HA-α_{2A}AR and APP, respectively. Cells were then treated with vehicle or clonidine (1 μM) for 5 min. After stimulation, the cells were fixed and then incubated with Alexa Fluor 488–conjugated anti-mouse and Alexa Fluor 594–conjugated anti-rat secondary Abs. Images were obtained using an LSM 710 confocal microscope (Zeiss, Oberkochen, Germany), with a ×63 oil magnification. Colocalization was estimated with Pearson's correlation coefficient in ImageJ software (27).

For the arrestin recruitment staining, N2a cells or SCG neurons were treated with NE (10 μM) in the presence of prazosin (1 μM) and propranolol (1 μM) for various times. Cell were then fixed, permeabilized, and incubated with rabbit arrestin 3 Ab (kindly provided by the J. Benovic laboratory at Thomas Jefferson University, Philadelphia PA, USA) and mouse APP Ab (22C11) followed by Alexa Fluor 488-conjugated anti-rabbit and Alexa Fluor 594-conjugated anti-mouse secondary Ab. Images were obtained with a U-TBI90 confocal microscope (Olympus Corporation, Shinjuku, Tokyo, Japan) at ×63 oil magnification. The percentage of recruitment was calculated with ImageJ to determine both surface and internal fluorescence activity, and the relative amount of fluorescence activity on the membrane compared to the total fluorescence activity was calculated.

α_{2A}AR internalization assays

Immunofluorescent staining

To assess α_{2A}AR internalization, a published prelabeling method was used (24, 25, 28). In brief, the HA-tagged α_{2A}ARs on the surface of live cells were labeled with a HA Ab (HA.11) before treatment. After labeling of the receptor, the cells were stimulated with 10 μM NE (plus 1 μM prazosin and 1 μM propranolol to selectively activate the α_{2A}AR) for various time points. After fixation and permeabilization, the cells were incubated with a rabbit APP Ab (Y188) followed by anti-mouse Alexa Fluor 488- and anti-rabbit Alexa Fluor 594 conjugated secondary Abs. Images were obtained with an LSM 710 confocal microscope (Zeiss), with ×63 oil magnification. Quantification of internalization was performed with MetaMorph software (Molecular Devices, Sunnyvale, CA, USA), to calculate both surface and internal fluorescence activity, and a ratio of internal fluorescence over total fluorescence was determined to give an arbitrary unit of internalization.

Intact cell surface ELISA

Internalization of α_{2A}AR was examined by intact cell surface ELISA, according to our procedure (21, 28). In brief, HEK293 cells coexpressing HA-α_{2A}AR, with or without APP, were plated onto 96-well culture plates at 1×10^4 cells/well. Cells were stimulated acutely with NE for the indicated time, followed by fixation. Samples were then subjected to blocking, primary Ab (HA.11), and secondary Ab (horseradish peroxidase–conjugated anti-mouse) and then incubated with *o*-phenylenediamine substrate (Thermo Fisher Scientific). Cell surface receptor density was determined by measuring absorbance at 490 nm.

In vitro glutathione *S*-transferase pull-down assay

cDNA encoding the C terminus of APP (APP-C; aa 639–695) was cloned into the pGEX4T vector. Preparation of glutathione *S*-transferase (GST) and GST-fused APP-C was performed as previously described (16, 29). [^{35}S]-labeled, *in vitro* translated, α_{2A}AR third intracellular loop (3iloop) was prepared with the TNT rabbit reticulocyte lysate kit (Promega, Madison, WI, USA) (16). Pull-down assays with 2 μg of GST or GST-fused APP-C in each sample were performed according to procedures described elsewhere (16, 29). In each assay, the same SDS-PAGE gel for autoradiography was stained with Coomassie Blue to confirm that a comparable amount of GST or GST-fusion protein was loaded in each lane.

Coimmunoprecipitation to detect APP-α_{2A}AR interaction

Coimmunoprecipitation (co-IP) assays were performed according to published procedures (28, 29). In brief, after stimulation, HEK or N2a cells were lysed on ice in immunoprecipitation (IP) buffer [10 mM Tris (pH = 7.4), 10% glycerol, 5 mM EGTA, 5 mM EDTA, 0.3% NP-40 and protease inhibitors], after stimulation with the appropriate ligands or vehicle. After 20 min of centrifugation at 14,800 rpm at 4°C, the supernatant was subjected to IP. For anti-HA IP, cell lysates were incubated with rat Anti-HA Affinity Matrix (Roche, Basel, Switzerland) overnight at 4°C. For anti-APP IP, cell lysates were incubated with anti-APP-C Ab (1:100 dilution; Sigma-Aldrich), followed by incubation with protein A/G beads. HA-α_{2A}AR and the full-length APP in the IP complex or total lysates were detected by Western blot with mouse HA.11 and rabbit anti-APP (Y188; Abcam) Ab, respectively. Western blots were quantified with the Odyssey Imaging System (Li-Cor) according to the manufacturer's instruction.

To examine α_{2A}AR interaction with APPΔC, the *Hin*dIII–*Apa*I fragment coding aa 1–649 of APP was amplified by PCR, using (forward) 5′-CCCAAGCTTATGCTGCCCGGTTTGGCACTGCTCCTG-3′ and (reverse) 5′-CCAGGGCCCCTACTTGTCGTCGTCGTCCTTGTAATCTGCTGCCTTCAGCATCACCAAGGTGATG-3′, and cloned into the pCMV-tag2 vector (Agilent Technologies, La Jolla, CA, USA) to generate the FLAG-APPΔC construct.

Quantification of ERK1/2 activation

To test ERK1/2 activation, both HEK293 and N2a-HA-α_{2A}AR-APP-CRISPR or control cells with the empty vector were used. HEK293 cells were transfected with either the empty vector or APP. Cells were stimulated by 10 μM NE (plus 1 μM prazosin and 1 μM propranolol) for various time points. Cell lysates were collected and run in a Western blot, using Abs for p-ERK and t-ERK. Western blots were quantified using the Odyssey Imaging System (Li-Cor) according to the manufacturer's instructions. Activation of ERK1/2 was calculated by dividing p-ERK1/2 by t-ERK1/2. To calculate the relative change of the ERK1/2 activation, the percentage of activation remaining at 10 min was determined by dividing the amount of activated ERK at 10 min by the amount of activated ERK at the peak activation, or 5 min.

Statistical analyses

Statistical analyses were performed with Prism software (GraphPad, La Jolla, CA, USA). All values are presented as means ± SEM. To determine difference between 2 groups with

1 variable, an unpaired Student's t test was performed, with $P < 0.05$ considered significant. Intact cell surface ELISA was analyzed by 2-way ANOVA followed by Sidak's multiple comparisons test.

RESULTS

APP interacts with the α_{2A}AR in an α_{2A}AR agonist-regulated manner

Both APP and α_{2A}AR are transmembrane proteins. We used an intact-cell Ab-prelabeling method to specifically detect α_{2A}AR and APP localized on the plasma membrane. A significant amount of APP (endogenously expressed) and HA-tagged α_{2A}AR (exogenously transfected) were colocalized in the same compartment on the cell surface of N2a cells (**Fig. 1*A***). This colocalization was further enhanced with stimulation of the receptor with an α_{2A}AR agonist, clonidine, as revealed by a significant increase in Pearson's correlation coefficient in clonidine-treated cells compared to vehicle-treated cells (Fig. 1*B*). Although colocalization of α_{2A}AR with another cell surface protein, epidermal growth factor receptor, was detected (Fig. 1*C*), clonidine stimulation did not change the degree of the colocalization between these two proteins (Fig. 1*D*).

Since APP and α_{2A}AR appear to be located in the same compartment on the plasma membrane, we next determined whether these two proteins actually interact with each other by co-IP assays. In HEK293 cells coexpressing APP and HA-α_{2A}AR, but not in cells expressing APP alone, APP was coimmunoisolated with HA-α_{2A}AR by an anti-HA Ab (**Fig. 2*A*** and Supplemental Fig. 1*A*). Furthermore, when α_{2A}AR was stimulated with an agonist, NE (in the presence of propranolol and prazosin to selectively activate α_{2A}AR) or clonidine, the level of APP coimmunoprecipitated with HA-α_{2A}AR was markedly increased (Fig. 2*B*). The quantification shows that 5 min of NE stimulation led to a nearly 5-fold increase in the level of

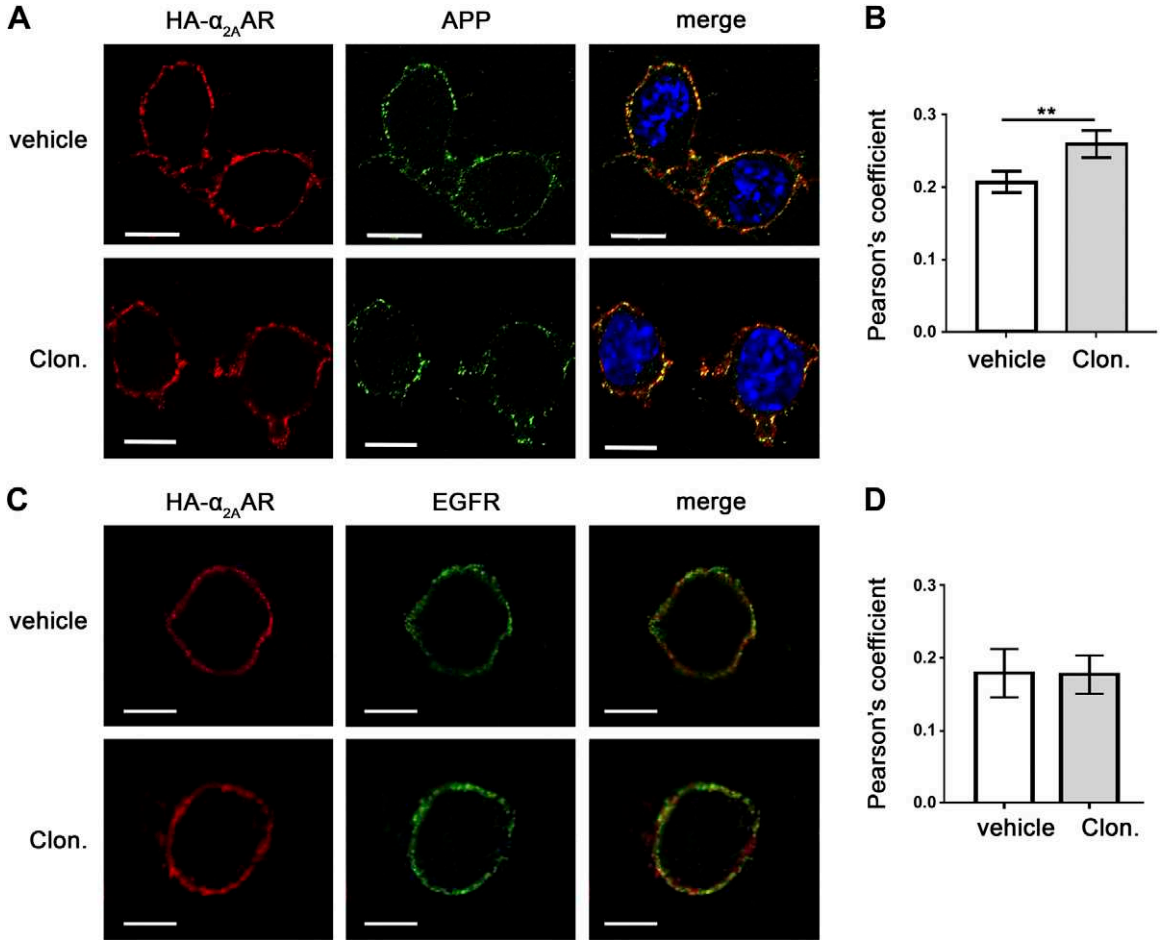

Figure 1. Agonist stimulation enhances APP and α_{2A}AR colocalization on the plasma membrane. *A*, *B*) N2a cells stably expressing HA-α_{2A}AR were first labeled with primary Abs against HA (rat) and APP (22C11, mouse) and then treated with vehicle or 1 μM clonidine for 5 min. After treatment, cells were fixed and stained with fluorescence-conjugated secondary Abs. *A*) Representative images showing localization of HA-α_{2A}AR and endogenous APP in cells. *B*) Quantification of colocalization between APP and α_{2A}AR, using Pearson's coefficient. Data are means ± SEM (n = 11–23 cells per condition). $^{**}P < 0.01$ (Student's t test). *C*, *D*) N2a cells stably expressing the HA-α_{2A}AR were first labeled with primary Abs against HA and then stimulated. Cells were fixed, permeabilized, and incubated with rabbit anti–epidermal growth factor receptor Ab, followed by fluorescence-conjugated secondary Abs. *C*) Representative images showing localization of HA-α_{2A}AR and endogenous APP in cells. *D*) Quantification of colocalization between APP and α_{2A}AR, using Pearson's coefficient. Data are means ± SEM (n = 12–14 per condition). Scale bars, 10 μm.

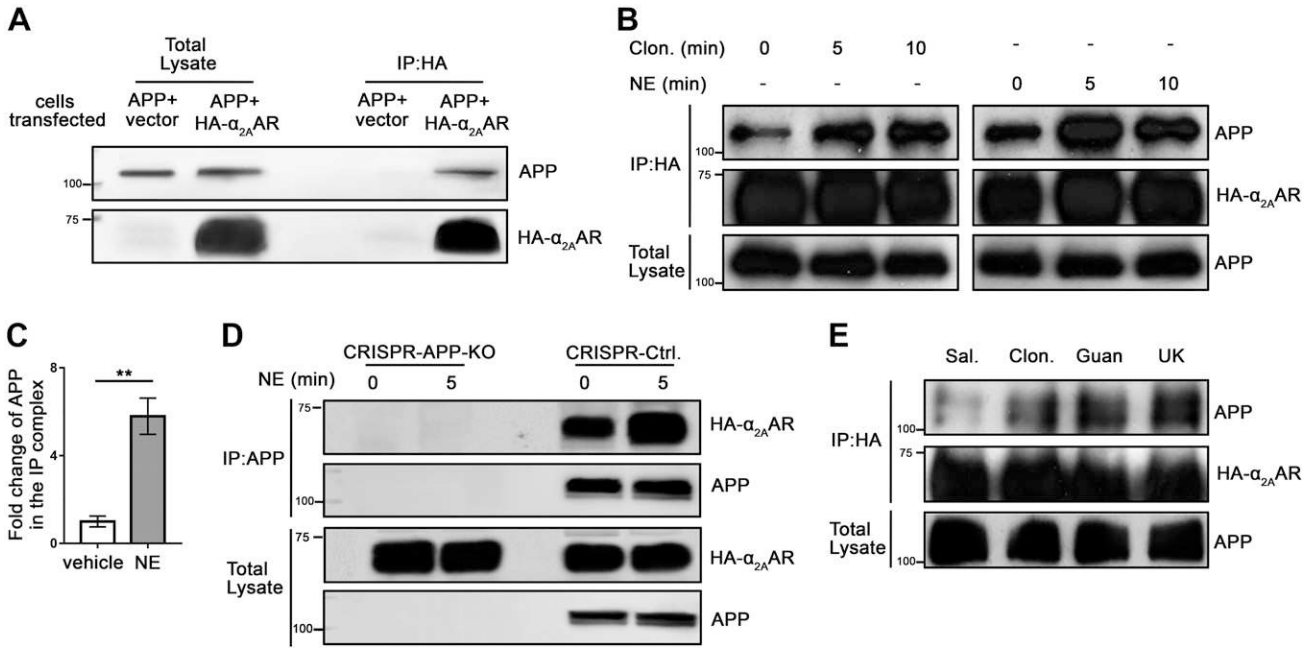

Figure 2. α_{2A}AR agonists promote complex formation between APP and α_{2A}AR in cells and in the mouse brain. *A–C*) HEK293 cells coexpressing APP with or without HA-α_{2A}AR were treated with 10 μM NE (plus 1 μM prazosin and 1 μM propranolol), clonidine (1 μM), or vehicle. Cell lysates were then subjected to co-IP assays with anti-HA affinity matrix. *A*, *B*) Representative Western blots. *C*) Quantification of the amount of APP coimmunoprecipitated with HA-α_{2A}AR in cells treated with NE for 5 min. Data (means ± SEM) are expressed as fold change compared with vehicle control (defined as 1.0) ($n = 8$ in each group). $^{**}P < 0.01$, NE *vs.* control (Student's *t* test). *D*) Control or APP-KO N2a cells expressing HA-α_{2A}AR were stimulated with vehicle or 10 μM NE (plus 1 μM prazosin and 1 μM propranolol) for 5 min. Cell lysates were subjected to co-IP assays using an anti-APP Ab. Representative Western blots are shown. *E*) Adult HA-α_{2A}AR-knock-in mice were injected (i.p. with saline or 1 mg/kg of clonidine, guanfacine, or UK14304). At 1 h after injection, cortices were dissected and homogenized. Cortical lysates were subjected to IP assays using anti-HA matrix. Representative Western blots are shown.

APP in the IP complex over the control (Fig. 2*C*). We then tested the interaction between APP (endogenously expressed) and HA-α_{2A}AR (exogenously transfected) in N2a cells by co-IP with an APP Ab. To control the specificity of the IP assays, we generated an N2a cell line with the *App* gene inactivated (APP-KO) using the CRISPR/Cas9 technique (Fig. 2*D*). In control N2a cells (generated in parallel with the empty CRISPR vector), but not in APP-KO cells, HA-α_{2A}AR was coimmunoisolated with APP by an anti-APP Ab, and the amount of α_{2A}AR in the immunoprecipitation complex was further increased by NE stimulation (Supplemental Fig. 1*B*). Together, these results indicate that α_{2A}AR and APP form a complex in cells and activation of α_{2A}AR enhances the formation of this complex.

We further examined the interaction between endogenous APP and α_{2A}AR *in vivo* in the brain by exploiting an HA-α_{2A}AR-knock-in mouse line that we had generated (24). Mice were injected with saline or one of 3 different α_2 agonists: clonidine, guanfacine, and UK14304 (1 mg/kg, i.p.). One hour after injection, the cortices were collected, lysed, and subjected to co-IP assays using an HA Ab. Stimulation with any one of the 3 agonists markedly increased the amount of APP in the IP complex with α_{2A}AR (Fig. 2*E*). These data clearly demonstrate endogenous interaction between APP and α_2AR in the mouse brain, which is promoted by α_2AR agonist treatment.

APP and α_{2A}AR directly interact through their intracellular domains

We have reported that the 3iloop of α_{2A}AR can interact with multiple regulatory proteins (16–18). We therefore sought to test whether this region is also involved in the interaction with APP. We performed co-IP assays with HEK293 cells coexpressing APP and HA-tagged mutant α_{2A}AR with the 3iloop deleted (HA-α_{2A}AR-Δ3iloop) (30). The removal of the 3iloop significantly decreased the level of APP coimmunoprecipitated with α_{2A}AR (**Fig. 3*A*, *B***), indicating that this portion of the receptor is essential for its interaction with APP. This result also suggests that the interaction between APP and α_{2A}AR likely occurs intracellularly. In this case, the intracellular domain of APP would be necessary for its interaction with α_{2A}AR. To test this idea, we examined the interaction between HA-α_{2A}AR and Flag-tagged mutant APP, in which the C-terminal intracellular domain was deleted. Although we readily detected the full-length APP in the IP complex with α_{2A}AR, we failed to detect the APPΔC-α_{2A}AR interaction in parallel experiments using the same experimental conditions (Fig. 3*C*, *D*), suggesting the requirement of APP-C for interaction with α_{2A}AR. Furthermore, when coexpressed with HA-α_{2A}AR, the C99 fragment of APP, which contains the transmembrane and intracellular domains of APP, also formed a complex with the receptor (Fig. 3*E*). Complex

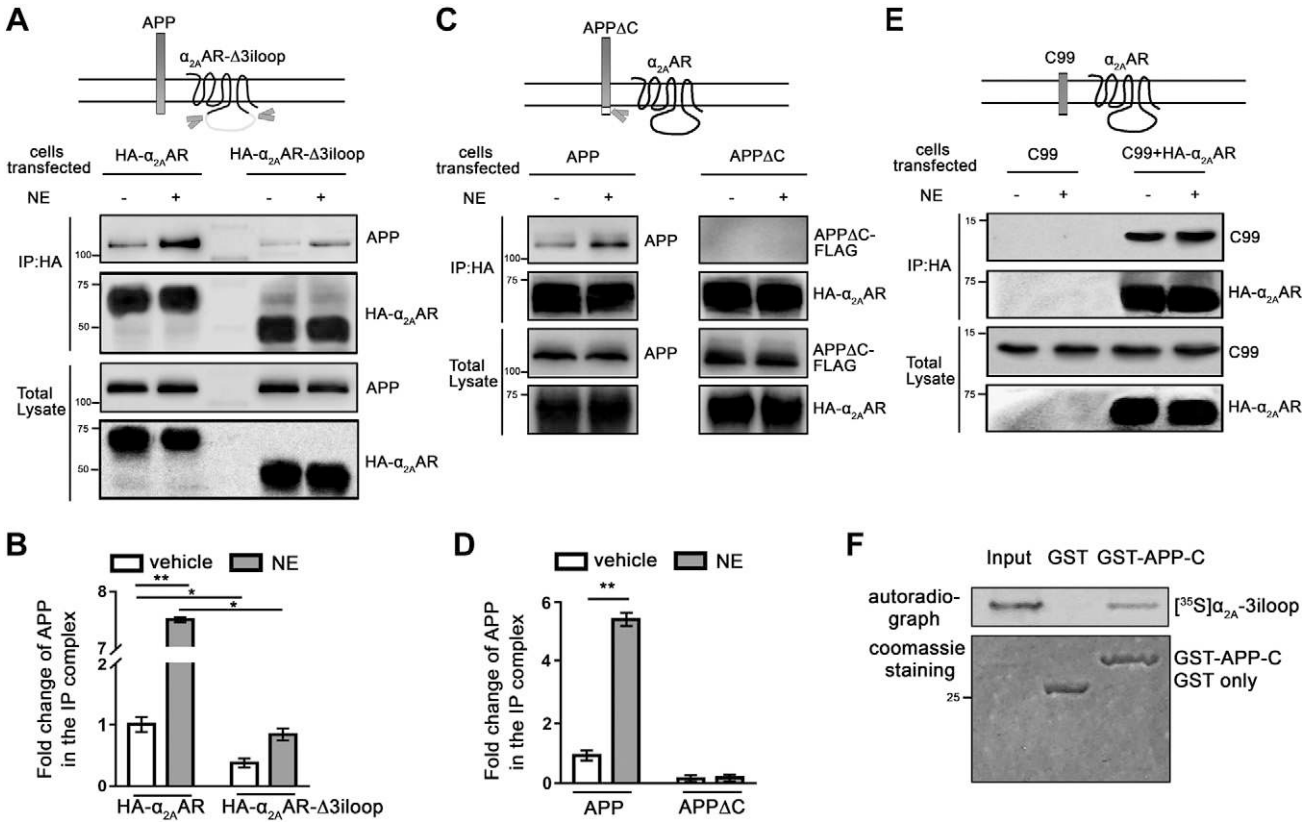

Figure 3. APP and α_{2A}AR interact through their intracellular domains. *A*, *B*) Deletion of the α_{2A}AR 3iloop significantly reduced its ability to interact with APP. HEK293 cells coexpressing APP with HA-α_{2A}AR or HA-α_{2A}AR-Δ3iloop were treated with 10 μM NE (plus 1 μM prazosin and 1 μM propranolol) or vehicle for 5 min. Cell lysates were subjected to IP assays with an HA Ab. *A*) Representative Western blots. *B*) Quantification of the amount of APP in the IP complex. Data (means ± SEM) are expressed as fold change *vs.* vehicle control (defined as 1.0) ($n = 3$ in each group). $^*P < 0.05$; $^{**}P < 0.01$ (Student's *t* test). *C*, *D*) Removal of the APP-C abolished its interaction with the α_{2A}AR. HEK293 cells coexpressing HA-α_{2A}AR with APP or APPΔC were treated with 10 μM NE (plus 1 μM prazosin and 1 μM propranolol) or vehicle for 5 min. Cell lysates were subjected to IP assays. *C*) Representative Western blots. *D*) Quantification of the amount of APP and APPΔC in the IP complex. Data (means ± SEM) are expressed as fold change *vs.* vehicle control (defined as 1.0) ($n = 3$ in each group). $^{**}P < 0.01$ (Student's *t* test). *E*) The C99 fragment of APP formed a complex with HA-α_{2A}AR. HEK293 cells coexpressing C99, with or without HA-α_{2A}AR were stimulated with vehicle or 10 μM NE (plus 1 μM prazosin and 1 μM propranolol), and cell lysates were subjected to IP assays. Representative Western blots are shown. *F*) *In vitro* GST pull-down assay showing direct interaction between the α_{2A}AR 3iloop and APP C-terminal domain. Purified GST or GST-fused APP-C was incubated with [^{35}S]-labeled, *in vitro* translated α_{2A}AR-3iloop. Input probe represents one-tenth of the total input in each reaction.

formation between α_{2A}AR and C99 seemed not to be affected by agonist stimulation of the receptor.

We next examined whether the α_{2A}AR 3iloop and C-terminal intracellular domain of APP directly interact with each other by *in vitro* GST pull-down assays. [^{35}S]-labeled, *in vitro* translated α_{2A}AR-3iloop was successfully pulled down with purified GST-fused APP-C but not with GST alone (Fig. 3*F*). Taken together, these results reveal a direct interaction between APP and α_{2A}AR that requires the intracellular domains of these 2 proteins.

APP reduces agonist-dependent endocytosis of α_{2A}AR

Having discovered this novel α_{2A}AR–APP interacting pair, we next sought to determine the impact of this interaction on receptor trafficking and signaling. We first examined agonist-induced endocytosis of the receptor using a receptor prelabeling method that we have described in several publications (24, 25, 28). We used 2 different types of cell lines, HEK293 and N2a, for these studies, taking advantage of the different expression levels of endogenous APP in these cell lines. In HEK293 cells, the endogenous APP level is very low and undetectable by Western blot (**Fig. 4*A***). In these cells, a significant portion of α_{2A}AR internalized after treatment with NE (in the presence of prazosin and propranolol), as detected using 2 independent methods, intact cell surface ELISA (Fig. 4*B*) and Ab-prelabeling immunofluorescence (Fig. 4*C*). The expression of exogenous APP in HEK293 cells significantly reduced the amount of α_{2A}AR that was internalized after NE treatment for both 10 and 30 min when compared to that in vehicle-treated control cells in both intact cell surface ELISA (Fig. 4*B*) and Ab-prelabeling immunofluorescence assays (Fig. 4*C*, *D*).

Contrary to HEK293 cells, N2a cells have a relatively high level of endogenous APP (Figs. 2*D* and **5*A***). We used siRNAs against APP to knock down its expression in these cells (Fig. 5*A*) and examined α_{2A}AR endocytosis in

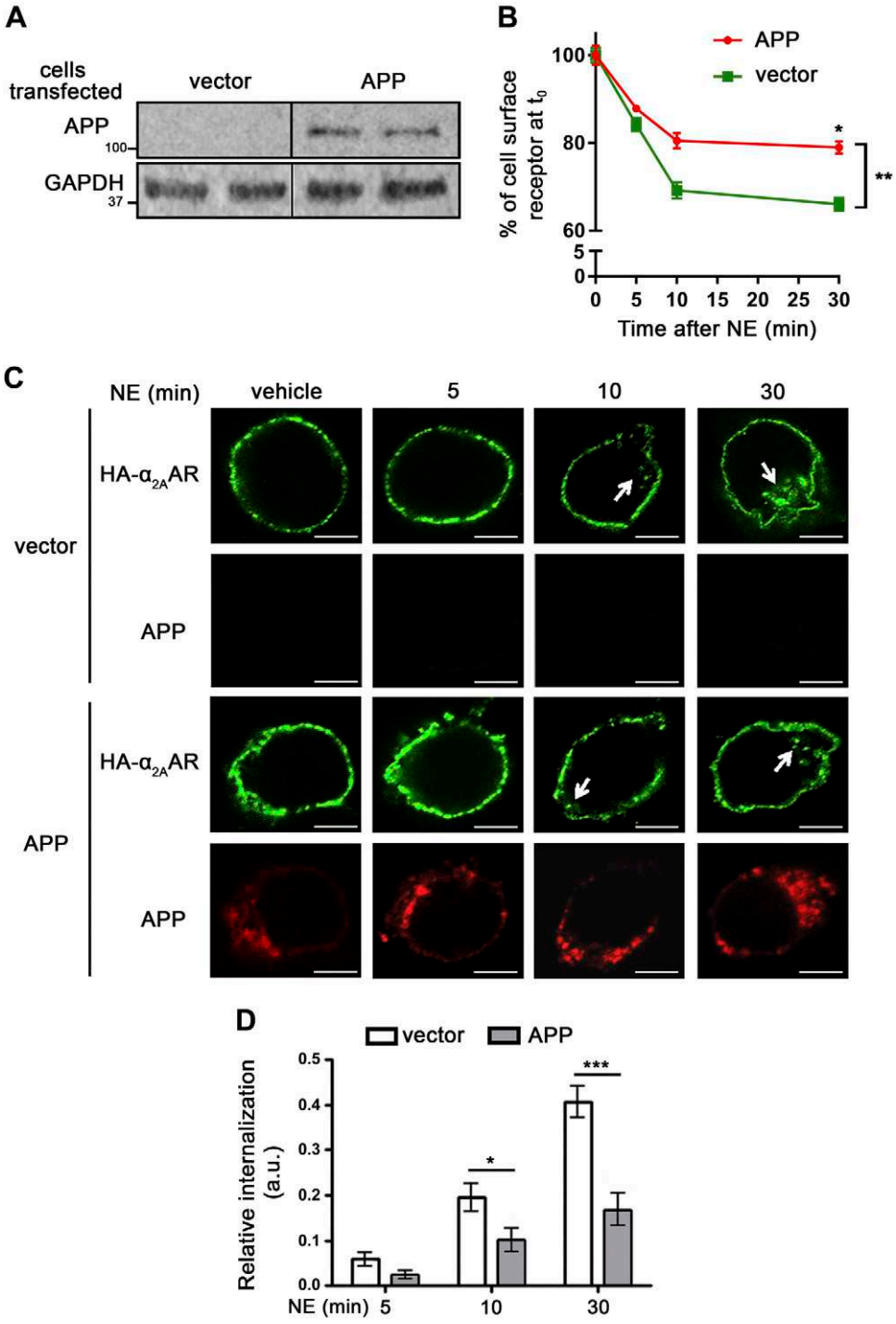

Figure 4. Overexpression of APP in HEK293 cells reduces α_{2A}AR internalization. HEK293 cells were cotransfected with cDNA vectors encoding HA-α_{2A}AR, together with APP or empty vector. *A*) Western blot image showing expression of exogenous APP in HEK293 cells, which have a very low level of endogenous APP. *B*) Internalization of cell surface HA-α_{2A}AR was assessed by intact cell surface ELISA. Internalization is indicated by the decrease in the percentage of surface receptor (with $t = 0$ set as 100%). Data are means ± SEM ($n = 4$ per condition). $^*P < 0.05$ by Sidak's multiple comparisons test; $^{**}P < 0.01$ by 2-way ANOVA. *C*, *D*) Internalization of HA-α_{2A}AR was examined by an Ab-prelabeling method. *C*) Representative images showing internalization of cell surface HA-α_{2A}AR after 10 μM NE treatment (plus 1 μM prazosin and 1 μM propranolol). Arrows: perinuclear punctate staining, which is indicative of endocytosed receptors. Scale bars, 5 μm. *D*) Quantitation of α_{2A}AR endocytosis. Data are means ± SEM (n = 17–23 cells per condition). $^*P < 0.05$, $^{***}P < 0.001$ (Student's t test).

response to the agonist clonidine. In N2a cells transfected with siRNA1, α_{2A}AR internalization in response to 30 min of clonidine stimulation was significantly increased compared with that in cells treated with scrambled siRNA (Fig. 5*B*, *C*). Similar results were obtained with siRNA2 (data not shown). We further examined α_{2A}AR internalization in APP-KO N2a cells, in which NE-induced α_{2A}AR internalization was significantly enhanced compared to that in control cells that were generated in parallel with the empty CRISPR vector (Fig. 5*D*, *E*). Taken together, our experiments using both gain- and loss-of-function approaches strongly suggest that APP reduces agonist-dependent endocytosis of α_{2A}AR and stabilizes the receptor on the cell surface.

APP enhances α_{2A}AR signaling intensity and duration

We next addressed how APP may affect α_{2A}AR signaling by measuring α_{2A}AR-mediated ERK1/2 activation, which is a G-protein-dependent process (18, 31). In HEK293 cells

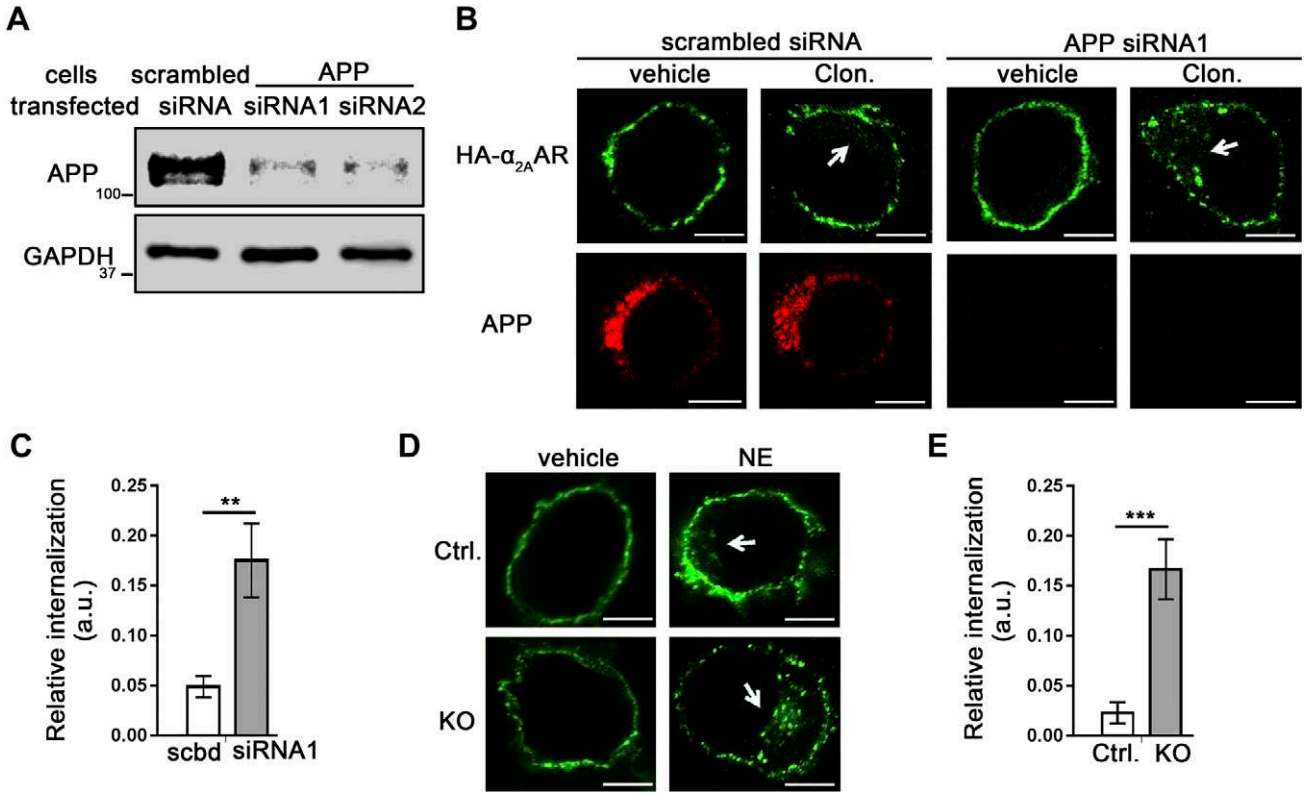

Figure 5. Loss of endogenous APP in N2a cells increases α_{2A}AR internalization. *A–C*) N2a cells stably expressing HA-α_{2A}AR were transfected with APP siRNAs or a scrambled siRNA. Internalization of surface HA-α_{2A}AR was examined by an Ab-prelabeling method. *A*) Western blot image showing expression of the knockdown efficiency of scrambled or APP siRNAs. *B*) Representative images showing internalization of cell surface HA-α_{2A}AR after 1 μM clonidine treatment for 30 min. Arrows: perinuclear punctate staining, which is indicative of endocytosed receptors. *C*) Quantitation of α_{2A}AR endocytosis. Data are means ± SEM (n = 11–13 cells per condition). *D, E*) α_{2A}AR internalization was examined in APP-KO and control N2a cells. *D*) Representative images showing internalization of cell surface HA-α_{2A}AR after 30 min 10 μM NE treatment (plus 1 μM prazosin and 1 μM propranolol). Arrows: perinuclear punctate staining, indicative of endocytosed receptors. *E*) Quantitation of α_{2A}AR endocytosis. Data are means ± SEM (n = 14–22 cells per condition). $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's t test). Scale bars, 10 μm.

expressing exogenous APP, 5 min treatment of NE (in the presence of prazosin and propranolol) was able to induce a significantly higher level of ERK1/2 activation (reflected by p-ERK/t-ERK ratio) compared to that in control cells (**Fig. 6*A, B***). Moreover, the decrease in ERK1/2 signals from the 5-min (peak) to 10-min time point (indicating signal desensitization) in APP-overexpressing cells was reduced compared with that in control cells (Fig. 6*A*, *C*). On the other hand, α_{2A}AR-mediated ERK1/2 activation after 5 min NE treatment was significantly reduced in APP-KO N2a cells compared with that in control cells transfected with the empty vector (**Fig. 7*A, B***). In addition, loss of APP led to a stronger reduction of ERK1/2 signals from the 5- to 10-min time point, compared with that in control cells transfected with the empty vector (Fig. 7*A*, *C*). Taken together, our data suggest that the presence of APP both strengthens and prolongs α_{2A}AR signaling.

APP interaction with the α_{2A}AR competes with arrestin binding to the receptor

Because APP affects endocytosis and desensitization of α_{2A}AR, which are arrestin-dependent processes (18, 21, 32), we speculate that the APP-α_{2A}AR interaction may alter the α_{2A}AR interaction with arrestin 3. We first examined arrestin 3 translocation after α_{2A}AR stimulation by NE in N2a cells. In control cells transfected with the empty vector, 10 min NE treatment (in the presence of prazosin and propranolol) induced a slight but significant translocation of the endogenous arrestin 3 from cytoplasm to the plasma membrane (**Fig. 8*A, B***). However, in APP-KO cells, almost all visible arrestin 3 was translocated to the plasma membrane after α_{2A}AR activation by NE (Fig. 8*A*, *B*). These data suggest that the presence of APP blocks arrestin 3 recruitment to the α_{2A}AR on the plasma membrane.

We next examined the α_{2A}AR-arrestin 3 interaction in HEK293 cells, with or without APP overexpression by co-IP assays. In control cells, we were able to readily coimmunoisolate GFP-tagged arrestin 3 with HA-α_{2A}AR using an HA Ab, and the amount of arrestin 3 in the IP complex was enhanced by 5 min of NE stimulation (Fig. 8*C*, *D*). However, in APP-overexpressing cells, the level of arrestin 3 immunoprecipitated with α_{2A}AR was significantly reduced compared with that in controls, both with and without NE stimulation (Fig. 8*C*, *D*). These data suggest that APP competes with arrestin 3 for binding to α_{2A}AR.

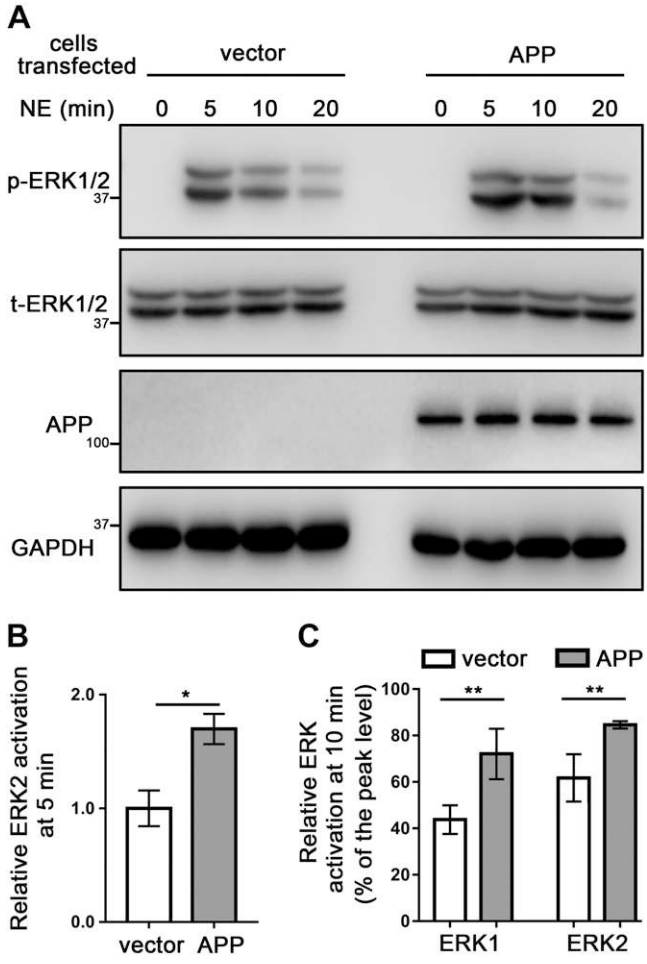

Figure 6. Overexpression of APP increases the strength and duration of α_{2A}AR-mediated ERK signaling. HEK293 cells were cotransfected with cDNA vectors encoding HA-α_{2A}AR together with APP or empty vector. *A*) Representative Western blots showing p- and t-ERK in HEK293 cells, with or without APP overexpression. *B*) Quantification of ERK2 activation with 10 μM NE (plus 1 μM prazosin and 1 μM propranolol) treatment for 5 min. Data (means ± SEM) are expressed as fold change *vs.* control cells expressing empty vector (defined as 1.0). *C*) Quantification of the relative change in ERK1/2 activation at 10 *vs.* 5 min of NE treatment. $^*P < 0.05$, $^{**}P < 0.01$ (Student's *t* test; $n = 8$ per group).

Loss of APP promotes arrestin recruitment to plasma membrane and enhances α_{2A}AR endocytosis in primary neurons

The above studies demonstrate regulation of α_{2A}AR by APP in heterologous cells. We next sought to determine whether such regulation exists in native SCG neurons, which have a relatively high level of expression of both APP and α_{2A}AR. siRNA was used to knock down endogenous APP. We first examined translocation of endogenous arrestin 3 after α_{2A}AR stimulation. In control neurons transfected with scrambled siRNA, 10 min NE treatment slightly enriched arrestin 3 at the plasma membrane. However, in neurons with APP knocked down, almost all detectable arrestin 3 was localized onto the plasma membrane after NE treatment (**Fig. 9*A, B***). These data suggest that APP blocks arrestin 3 recruitment to α_{2A}AR in native neurons.

Taking advantage of our HA-α_{2A}AR-knock-in mouse line (24), we examined agonist-dependent endocytosis of endogenous α_{2A}AR in SCG neurons, with or without APP knockdown. After 10 min of NE stimulation (plus prazosin and propranolol), internalization of endogenous α_{2A}AR was significantly increased in neurons treated with the APP siRNA, compared that in neurons treated with the scrambled siRNA (Fig. 9*C, D*). These data suggest that, as in the heterologous cells tested, APP stabilizes α_{2A}AR to the cell surface in native neurons.

DISCUSSION

In the present study, we identified a novel function of APP in regulating trafficking and signaling of a GPCR, namely α_{2A}AR. APP directly interacts with the 3iloop of α_{2A}AR

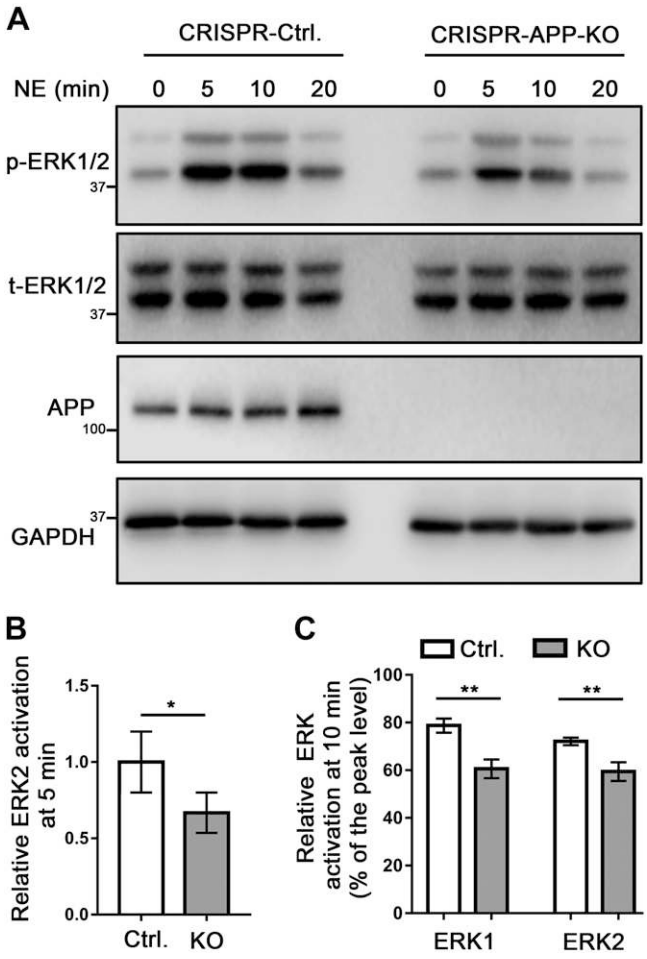

Figure 7. KO of endogenous APP in N2a cells decreases the strength and duration of α_{2A}AR-mediated ERK signaling. *A*) Representative Western blots showing p-ERK and t-ERK in control and APP-KO N2a cells. *B*) Quantification of ERK2 activation with 10 μM NE (plus 1 μM prazosin and 1 μM propranolol) treatment for 5 min. Data (means ± SEM) are expressed as fold change *vs.* that in WT control cells (defined as 1.0). *C*) Quantification of the relative change in ERK1/2 activation at 10 *vs.* 5 min of NE treatment. $n = 5$ in each group. $^*P < 0.05$, $^{**}P < 0.01$ (Student's *t* test).

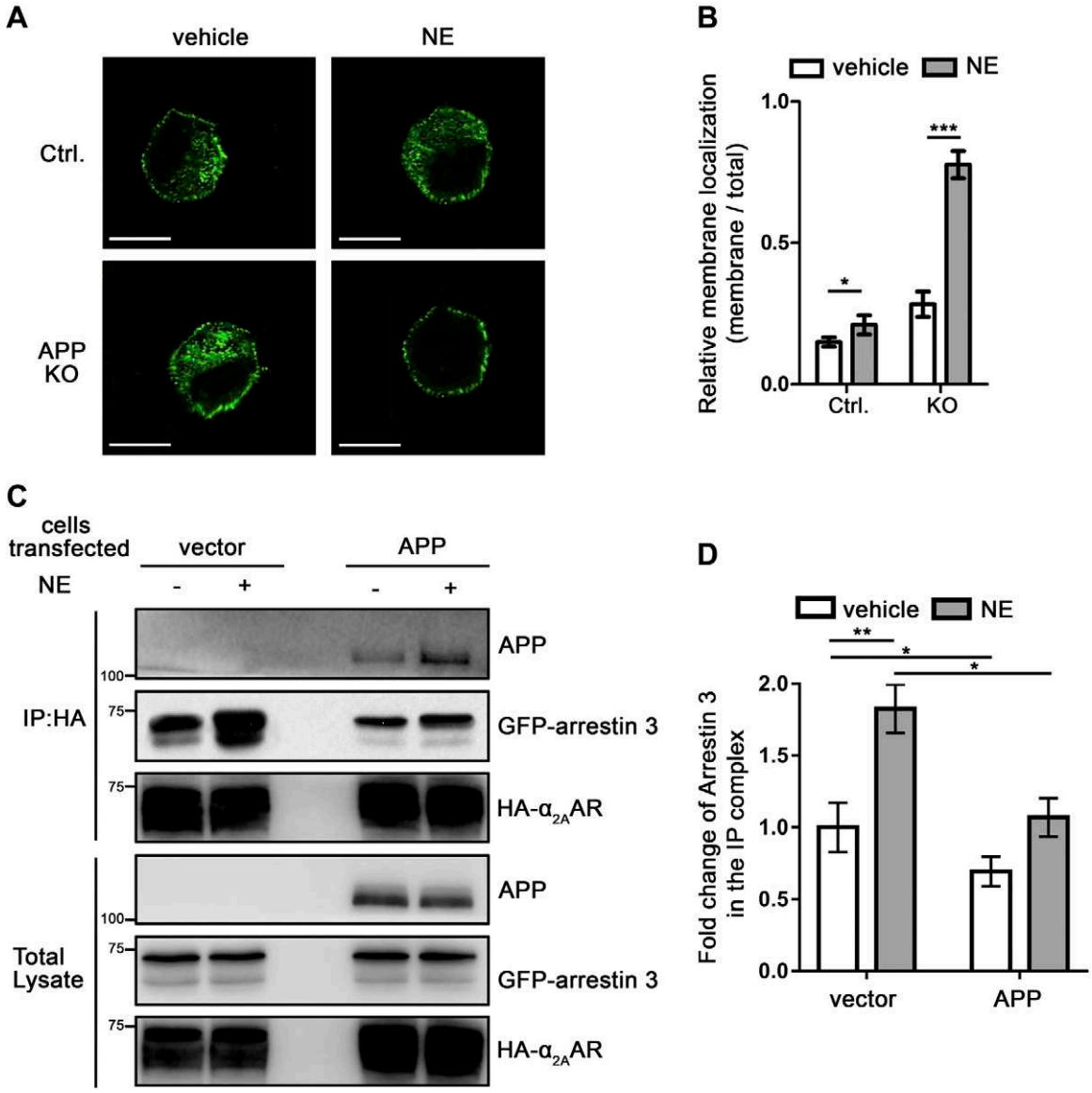

Figure 8. APP reduces the interaction between α_{2A}AR and arrestin in cells. *A*, *B*) APP-KO and control N2a cells were simulated with 10 μM NE (plus 1 μM prazosin and 1 μM propranolol) for 10 min. Endogenous arrestin 3 and APP were examined. *A*) Representative image of control and APP-KO N2a cells showing endogenous arrestin. Scale bars, 15 μm. *B*) Quantification of arrestin membrane recruitment. Data are means ± SEM. ($n = 16$ cells per condition). *C*, *D*) HEK293 cells coexpressing HA-α_{2A}AR and GFP-arrestin 3, together, with or without exogenous APP, were treated with 10 μM NE (plus 1 μM prazosin and 1 μM propranolol) for 5 min. Cell lysates were subjected to co-IP assays. *C*) Representative Western blots are shown. *D*) Quantification of the amount of arrestin 3 coimmunoprecipitated with HA-α_{2A}AR. Data (means ± SEM) are expressed as fold change *vs.* vehicle control without APP expression (defined as 1.0) ($n = 5$ in each group). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t* test).

through its C-terminal intracellular domain (Fig. 3). The APP-α_{2A}AR interaction occurs in an agonist-dependent fashion in both cultured cells and the mouse brain (Figs. 1 and 2), and competes with arrestin 3 binding to the receptor (Figs. 8 and 9). Consequently, the presence of APP blocks arrestin translocation to the plasma membrane (Figs. 8 and 9) and attenuates arrestin-mediated internalization (Figs. 4, 5, and 9) and desensitization (Figs. 6 and 7) of α_{2A}AR. Our study provided the first evidence demonstrating that APP stabilizes a GPCR on the cell surface and potentiates its signaling. Furthermore, the APP antagonism of arrestin translocation and α_{2A}AR endocytosis exists in primary SCG neurons (Fig. 9), demonstrating the physiologic relevance of APP-α_{2A}AR interaction.

It has now been well established that non-G-protein-interacting partners play a crucial role in fine tuning GPCR signaling and functions (17, 33–35). The nonvisual arrestins 2 and 3, have been appreciated as universal regulators of GPCRs (36, 37). Agonist-induced arrestin translocation from cytosol to the plasma membrane represents a hallmark of GPCR activation and desensitization of G-protein-dependent signaling (38–40). Like many GPCRs, agonist stimulation of α_{2A}AR leads to receptor phosphorylation by GRK2/3 and subsequent binding of arrestin 3 (17). In WT N2a cells and SCG neurons, α_{2A}AR activation was

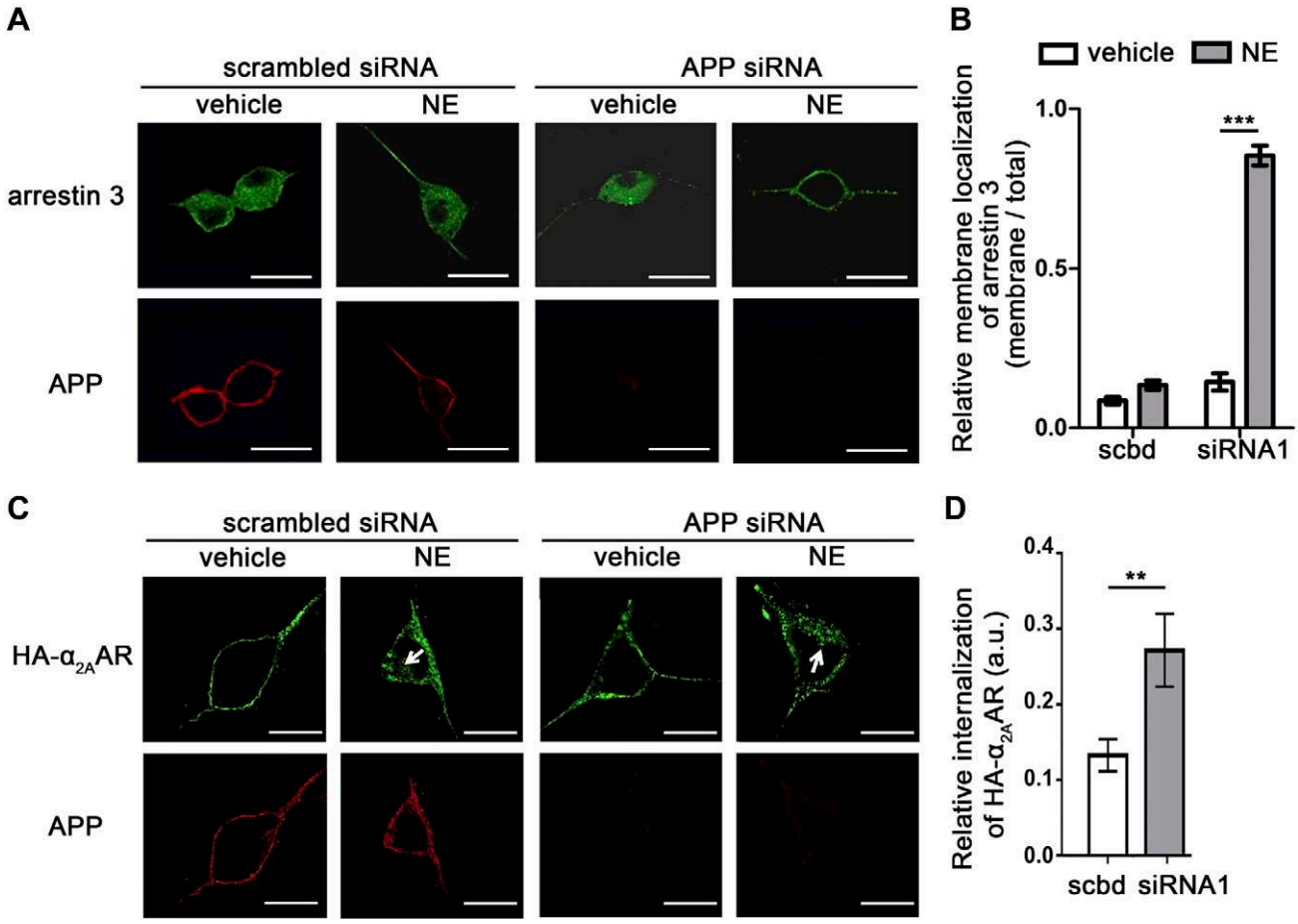

Figure 9. Loss of APP promotes α_{2A}AR-induced arrestin recruitment to the membrane and α_{2A}AR internalization in primary SCG neurons. Primary SCG neurons derived from HA-α_{2A}AR-knock-in mice were transfected with APP siRNA or scrambled siRNA and treated with 10 μM NE (plus 1 μM prazosin and 1 μM propranolol) for 10 min. *A*) Representative images showing endogenous arrestin 3 and APP. Scale bars, 25 μm. *B*) Quantification of arrestin membrane recruitment. Data are means ± SEM. $n = 12$ cell per condition. *C*) Representative images showing internalization of cell surface HA-α_{2A}AR after 10 μM NE (plus 1 μM prazosin and 1 μM propranolol) treatment for 10 min. Arrows: perinuclear punctate staining, which is indicative of endocytosed receptors. Scale bars, 15 μm. *D*) Quantitation of α_{2A}AR endocytosis. Data are means ± SEM ($n = 18$ cells per condition). $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t* test).

able to induce recruitment of only a small portion of arrestin 3 to the plasma membrane (Figs. 8 and 9). However, when APP expression is disrupted, by CRISPR KO or siRNA knockdown, almost all arrestin 3 was translocated to plasma membrane after α_{2A}AR stimulation (Figs. 8 and 9). Concomitantly, agonist-induced α_{2A}AR endocytosis was significantly increased (Figs. 4, 5 and 9). Our experiments thus revealed a novel role of APP in antagonizing arrestin-mediated regulation of α_{2A}AR in neuronal cells. We have reported that a dendritic spine-enriched scaffolding protein, spinophilin, also competes with arrestin 3 for interaction with α_{2A}AR (16, 18). Spinophilin is mainly localized in the postsynaptic compartment (41, 42), whereas APP has been shown to localize in both dendrites and axons (43, 44) and can regulate localization and activity of presynaptic membrane proteins such as the high-affinity choline transporter (45). It is plausible that spinophilin and APP play a dominant role in different neuronal compartments in impeding arrestin-mediated regulation of α_{2A}AR.

Regulation of other cell surface receptor-initiated signaling may represent an important mechanism underlying APP-elicited physiologic functions. APP has been shown to interact with the NGF receptor, TrkA (46), and this interaction is critical for TrkA activation in response to NGF (9). To the best of our knowledge, how APP could regulate GPCRs has not been investigated. APP has been reported to interact with 2 other GPCRs, the orphan GPCR, GPR3, and the prostaglandin E receptor 2 subtype EP2 (PTGER2), in HEK cells (47). However, whether APP interactions with these receptors are direct or indirect and what the functional consequences of such interactions are on receptor trafficking or signaling remain to be addressed. In our current study, we showed by multiple lines of evidence that a direct APP-α_{2A}AR interaction occurs intracellularly in an agonist-promoted manner (Figs. 1–3). Different from the case of GPR3, in which the complex formation between APP and GPR3 can be enhanced by the presence of arrestin 3 (47), α_{2A}AR interactions with APP and arrestin 3 seem to be mutually exclusive (Figs. 8 and 9). Such a result is not surprising, given that both APP

(Fig. 3) and arrestin 3 (16) directly bind to the 3iloop of α_{2A}AR. Because arrestin-dependent mechanisms are essential for α_{2A}AR internalization and desensitization (17), by competing for arrestin 3 binding to the receptor (Figs. 8 and 9), APP stabilizes α_{2A}AR on the cell surface (Figs. 4, 5, and 9) and potentiates its signaling (Figs. 6 and 7).

We have demonstrated that stimulation of α_{2A}AR promotes amyloidogenic processing of APP through activation of $G_{i/o}$ proteins (23). The direct interaction between APP and α_{2A}AR would bring APP to the proximity of α_{2A}AR downstream signaling to modulate its processing route. Meanwhile, such an interaction potentiates α_{2A}AR signaling, as suggested by the current study, which may further facilitate amyloidogenic processing of APP. The signaling molecules that mediate α_{2A}AR-promoted APP processing and potential effects of APP-α_{2A}AR interaction on activation of these signaling molecules are currently under investigation.

As the primary autoreceptor of the noradrenergic neurons, α_{2A}AR plays a key role in controlling the brain noradrenergic activity and sympathetic outflow (48). In addition, α_{2A}AR activation in sympathetic neurons inhibits high-frequency stimulus-induced NE release and reduces sympathetic tone (48, 49). Pharmacological stimulation of α_{2A}AR lowers blood pressure and represents an effective strategy for long-term control of hypertension (50, 51). Through enhancing α_{2A}AR surface retention and signaling, APP could influence noradrenergic activity and sympathetic tone. Indeed, sympathetic alterations have been indicated in APP-deficient mice (52). Future studies are necessary to understand the mechanistic contribution of impaired α_{2A}AR signaling in this process. Nonetheless, our current study using multiple complementary approaches and native neurons provides new and valuable insights into the physiologic functions of APP, and may have implications for understanding sympathetic regulation. FJ

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AUTHOR CONTRIBUTIONS

K. Jiao and Q. Wang designed research; F. Zhang, M. Gannon, and Y. Chen performed experiments; F. Zhang, M. Gannon, Y. Chen, K. Jiao, and Q. Wang analyzed data; L. Zhou provided analytic tools; and M. Gannon, F. Zhang, and Q. Wang wrote the manuscript.

REFERENCES

1. Nicolas, M., and Hassan, B. A. (2014) Amyloid precursor protein and neural development. *Development* **141**, 2543–2548
2. Van der Kant, R., and Goldstein, L. S. (2015) Cellular functions of the amyloid precursor protein from development to dementia. *Dev. Cell* **32**, 502–515; Correction **33**, 240
3. Zheng, H., Jiang, M., Trumbauer, M. E., Sirinathsinghji, D. J., Hopkins, R., Smith, D. W., Heavens, R. P., Dawson, G. R., Boyce, S., Conner, M. W., Stevens, K. A., Slunt, H. H., Sisoda, S. S., Chen, H. Y., and Van der Ploeg, L. H. (1995) beta-Amyloid precursor protein-deficient mice show reactive gliosis and decreased locomotor activity. *Cell* **81**, 525–531
4. Steinbach, J. P., Müller, U., Leist, M., Li, Z. W., Nicotera, P., and Aguzzi, A. (1998) Hypersensitivity to seizures in beta-amyloid precursor protein deficient mice. *Cell Death Differ.* **5**, 858–866
5. Dawson, G. R., Seabrook, G. R., Zheng, H., Smith, D. W., Graham, S., O'Dowd, G., Bowery, B. J., Boyce, S., Trumbauer, M. E., Chen, H. Y., Van der Ploeg, L. H., and Sirinathsinghji, D. J. (1999) Age-related cognitive deficits, impaired long-term potentiation and reduction in synaptic marker density in mice lacking the beta-amyloid precursor protein. *Neuroscience* **90**, 1–13
6. Klevanski, M., Saar, M., Baumkötter, F., Weyer, S. W., Kins, S., and Müller, U. C. (2014) Differential role of APP and APLPs for neuromuscular synaptic morphology and function. *Mol. Cell. Neurosci.* **61**, 201–210
7. Russo, C., Venezia, V., Repetto, E., Nizzari, M., Violani, E., Carlo, P., and Schettini, G. (2005) The amyloid precursor protein and its network of interacting proteins: physiological and pathological implications. *Brain Res. Brain Res. Rev.* **48**, 257–264
8. Deyts, C., Thinakaran, G., and Parent, A. T. (2016) APP receptor? to be or not to be. *Trends Pharmacol. Sci.* **37**, 390–411
9. Matrone, C., Barbagallo, A. P., La Rosa, L. R., Florenzano, F., Ciotti, M. T., Mercanti, D., Chao, M. V., Calissano, P., and D'Adamio, L. (2011) APP is phosphorylated by TrkA and regulates NGF/TrkA signaling. *J. Neurosci.* **31**, 11756–11761
10. Hein, L. (2006) Adrenoceptors and signal transduction in neurons. *Cell Tissue Res.* **326**, 541–551
11. Gyires, K., Zádori, Z. S., Török, T., and Mátyus, P. (2009) α(2)-Adrenoceptor subtypes-mediated physiological, pharmacological actions. *Neurochem. Int.* **55**, 447–453
12. Massé, F., Hascoët, M., Dailly, E., and Bourin, M. (2006) Effect of noradrenergic system on the anxiolytic-like effect of DOI (5-HT2A/2C agonists) in the four-plate test. *Psychopharmacology (Berl.)* **183**, 471–481
13. Gilsbach, R., and Hein, L. (2012) Are the pharmacology and physiology of α_2 adrenoceptors determined by α_2-heteroreceptors and autoreceptors respectively? *Br. J. Pharmacol.* **165**, 90–102
14. Schlicker, E., and Göthert, M. (1998) Interactions between the presynaptic alpha2-autoreceptor and presynaptic inhibitory heteroreceptors on noradrenergic neurones. *Brain Res. Bull.* **47**, 129–132
15. Starke, K., Göthert, M., and Kilbinger, H. (1989) Modulation of neurotransmitter release by presynaptic autoreceptors. *Physiol. Rev.* **69**, 864–989
16. Wang, Q., and Limbird, L. E. (2002) Regulated interactions of the alpha 2A adrenergic receptor with spinophilin, 14-3-3zeta, and arrestin 3. *J. Biol. Chem.* **277**, 50589–50596
17. Wang, Q., and Limbird, L. E. (2007) Regulation of alpha2AR trafficking and signaling by interacting proteins. *Biochem. Pharmacol.* **73**, 1135–1145
18. Wang, Q., Zhao, J., Brady, A. E., Feng, J., Allen, P. B., Lefkowitz, R. J., Greengard, P., and Limbird, L. E. (2004) Spinophilin blocks arrestin actions in vitro and in vivo at G protein-coupled receptors. *Science* **304**, 1940–1944
19. Lu, R., Chen, Y., Cottingham, C., Peng, N., Jiao, K., Limbird, L. E., Wyss, J. M., and Wang, Q. (2010) Enhanced hypotensive, bradycardic, and hypnotic responses to alpha2-adrenergic agonists in spinophilin-null mice are accompanied by increased G protein coupling to the alpha2A-adrenergic receptor. *Mol. Pharmacol.* **78**, 279–286
20. Cottingham, C., Li, X., and Wang, Q. (2012) Noradrenergic antidepressant responses to desipramine in vivo are reciprocally regulated by arrestin3 and spinophilin. *Neuropharmacology* **62**, 2354–2362
21. Cottingham, C., Chen, Y., Jiao, K., and Wang, Q. (2011) The antidepressant desipramine is an arrestin-biased ligand at the α(2A)-adrenergic receptor driving receptor down-regulation in vitro and in vivo. *J. Biol. Chem.* **286**, 36063–36075
22. Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., and Zhang, F. (2013) Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* **8**, 2281–2308
23. Chen, Y., Peng, Y., Che, P., Gannon, M., Liu, Y., Li, L., Bu, G., van Groen, T., Jiao, K., and Wang, Q. (2014) α(2A) adrenergic receptor promotes amyloidogenesis through disrupting APP-SorLA interaction. *Proc. Natl. Acad. Sci. USA* **111**, 17296–17301

24. Lu, R., Li, Y., Zhang, Y., Chen, Y., Shields, A. D., Winder, D. G., Angelotti, T., Jiao, K., Limbird, L. E., Zhou, Y., and Wang, Q. (2009) Epitope-tagged receptor knock-in mice reveal that differential desensitization of alpha2-adrenergic responses is because of ligand-selective internalization. *J. Biol. Chem.* **284**, 13233–13243
25. Cottingham, C., Lu, R., Jiao, K., and Wang, Q. (2013) Cross-talk from β-adrenergic receptors modulates α2A-adrenergic receptor endocytosis in sympathetic neurons via protein kinase A and spinophilin. *J. Biol. Chem.* **288**, 29193–29205
26. Brum, P. C., Hurt, C. M., Shcherbakova, O. G., Kobilka, B., and Angelotti, T. (2006) Differential targeting and function of alpha2A and alpha2C adrenergic receptor subtypes in cultured sympathetic neurons. *Neuropharmacology* **51**, 397–413
27. Zinchuk, V., Zinchuk, O., and Okada, T. (2007) Quantitative colocalization analysis of multicolor confocal immunofluorescence microscopy images: pushing pixels to explore biological phenomena. *Acta Histochem. Cytochem.* **40**, 101–111
28. Xu, J., Chen, Y., Lu, R., Cottingham, C., Jiao, K., and Wang, Q. (2008) Protein kinase A phosphorylation of spinophilin modulates its interaction with the alpha 2A-adrenergic receptor (AR) and alters temporal properties of alpha 2AAR internalization. *J. Biol. Chem.* **283**, 14516–14523
29. Chen, Y., Liu, Y., Cottingham, C., McMahon, L., Jiao, K., Greengard, P., and Wang, Q. (2012) Neurabin scaffolding of adenosine receptor and RGS4 regulates anti-seizure effect of endogenous adenosine. *J. Neurosci.* **32**, 2683–2695
30. Edwards, S. W., and Limbird, L. E. (1999) Role for the third intracellular loop in cell surface stabilization of the alpha2A-adrenergic receptor. *J. Biol. Chem.* **274**, 16331–16336
31. Wang, Q., Lu, R., Zhao, J., and Limbird, L. E. (2006) Arrestin serves as a molecular switch, linking endogenous alpha2-adrenergic receptor to SRC-dependent, but not SRC-independent, ERK activation. *J. Biol. Chem.* **281**, 25948–25955
32. DeGraff, J. L., Gagnon, A. W., Benovic, J. L., and Orsini, M. J. (1999) Role of arrestins in endocytosis and signaling of alpha2-adrenergic receptor subtypes. *J. Biol. Chem.* **274**, 11253–11259
33. Bockaert, J., Perroy, J., Bécamel, C., Marin, P., and Fagni, L. (2010) GPCR interacting proteins (GIPs) in the nervous system: roles in physiology and pathologies. *Annu. Rev. Pharmacol. Toxicol.* **50**, 89–109
34. Bockaert, J., Fagni, L., Dumuis, A., and Marin, P. (2004) GPCR interacting proteins (GIP). *Pharmacol. Ther.* **103**, 203–221
35. Ritter, S. L., and Hall, R. A. (2009) Fine-tuning of GPCR activity by receptor-interacting proteins. *Nat. Rev. Mol. Cell Biol.* **10**, 819–830
36. Lohse, M. J., and Hoffmann, C. (2014) Arrestin interactions with G protein-coupled receptors. *Handb. Exp. Pharmacol.* **219**, 15–56
37. Lefkowitz, R. J. (2013) Arrestins come of age: a personal historical perspective. *Prog. Mol. Biol. Transl. Sci.* **118**, 3–18
38. Barak, L. S., Ferguson, S. S., Zhang, J., and Caron, M. G. (1997) A beta-arrestin/green fluorescent protein biosensor for detecting G protein-coupled receptor activation. *J. Biol. Chem.* **272**, 27497–27500
39. Ma, L., and Pei, G. (2007) Beta-arrestin signaling and regulation of transcription. *J. Cell Sci.* **120**, 213–218
40. Claing, A., Laporte, S. A., Caron, M. G., and Lefkowitz, R. J. (2002) Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and beta-arrestin proteins. *Prog. Neurobiol.* **66**, 61–79
41. Muly, E. C., Smith, Y., Allen, P., and Greengard, P. (2004) Subcellular distribution of spinophilin immunolabeling in primate prefrontal cortex: localization to and within dendritic spines. *J. Comp. Neurol.* **469**, 185–197
42. Feng, J., Yan, Z., Ferreira, A., Tomizawa, K., Liauw, J. A., Zhuo, M., Allen, P. B., Ouimet, C. C., and Greengard, P. (2000) Spinophilin regulates the formation and function of dendritic spines. *Proc. Natl. Acad. Sci. USA* **97**, 9287–9292
43. Allinquant, B., Moya, K. L., Bouillot, C., and Prochiantz, A. (1994) Amyloid precursor protein in cortical neurons: coexistence of two pools differentially distributed in axons and dendrites and association with cytoskeleton. *J. Neurosci.* **14**, 6842–6854
44. Schubert, W., Prior, R., Weidemann, A., Dircksen, H., Multhaup, G., Masters, C. L., and Beyreuther, K. (1991) Localization of Alzheimer beta A4 amyloid precursor protein at central and peripheral synaptic sites. *Brain Res.* **563**, 184–194
45. Wang, B., Yang, L., Wang, Z., and Zheng, H. (2007) Amyloid precursor protein mediates presynaptic localization and activity of the high-affinity choline transporter. *Proc. Natl. Acad. Sci. USA* **104**, 14140–14145
46. Zhang, Q., Descamps, O., Hart, M. J., Poksay, K. S., Spilman, P., Kane, D. J., Gorostiza, O., John, V., and Bredesen, D. E. (2014) Paradoxical effect of TrkA inhibition in Alzheimer's disease models. *J. Alzheimers Dis.* **40**, 605–617
47. Nelson, C. D., and Sheng, M. (2013) Gpr3 stimulates Aβ production via interactions with APP and β-arrestin2. *PLoS One* **8**, e74680
48. Hein, L., Altman, J. D., and Kobilka, B. K. (1999) Two functionally distinct alpha2-adrenergic receptors regulate sympathetic neurotransmission. *Nature* **402**, 181–184
49. Trendelenburg, A. U., Nörenberg, W., Hein, L., Meyer, A., and Starke, K. (2001) Alpha2-adrenoceptor-mediated inhibition of cultured sympathetic neurons: changes in alpha2A/D-adrenoceptor-deficient mice. *Naunyn Schmiedebergs Arch. Pharmacol.* **363**, 110–119
50. MacMillan, L. B., Hein, L., Smith, M. S., Piascik, M. T., and Limbird, L. E. (1996) Central hypotensive effects of the alpha2a-adrenergic receptor subtype. *Science* **273**, 801–803
51. Giovannitti, J. A., Jr., Thoms, S. M., and Crawford, J. J. (2015) Alpha-2 adrenergic receptor agonists: a review of current clinical applications. *Anesth. Prog.* **62**, 31–39
52. Duce, J. A., Ayton, S., Miller, A. A., Tsatsanis, A., Lam, L. Q., Leone, L., Corbin, J. E., Butzkueven, H., Kilpatrick, T. J., Rogers, J. T., Barnham, K. J., Finkelstein, D. I., and Bush, A. I. (2013) Amine oxidase activity of β-amyloid precursor protein modulates systemic and local catecholamine levels. *Mol. Psychiatry* **18**, 245–254

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