Dominant Negative AT\textsubscript{2} Receptor Oligomers Induce G-protein Arrest and Symptoms of Neurodegeneration* 

Said AbdAlla\textsuperscript{4}, Heinz Lothert\textsuperscript{1}, Ahmed el Missiry\textsuperscript{3}, Pavel Sergeev\textsuperscript{5}, Andreas Langer\textsuperscript{6}, Yasser el Faramawy\textsuperscript{2}, and Ursula Quitterer\textsuperscript{4,7} 

From the \textsuperscript{4}Heinrich-Pette-Institute, Martinistrasse 52, D-20251 Hamburg, Germany, the \textsuperscript{5}Medical Research Center, Ain Shams University Hospital, Cairo, Egypt, and the \textsuperscript{6}Department of Molecular Pharmacology, Swiss Federal Institute of Technology and University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

Neurodegeneration in Alzheimer’s disease (AD) correlates with dysfunction of signaling mediated by Go\textsubscript{q/11}. Nondissociable angiotensin II AT\textsubscript{2} receptor oligomers are linked to the impaired Go\textsubscript{q/11}-stimulated signaling of AD patients and transgenic mice with AD-like symptoms. To further analyze the role of AT\textsubscript{2} receptor oligomers, we induced the formation of AT\textsubscript{2} oligomers in an \textit{in vitro} cell system. Similarly as in vivo, sequential oxidative and transglutaminase-dependent cross-linking steps triggered the formation of AT\textsubscript{2} oligomers \textit{in vitro}. Elevated reactive oxygen species mediated oxidative cross-linking of AT\textsubscript{2} monomers to dimers involving tyrosine residues located at putative interreceptor contact sites of the cytoplasmic loop connecting transmembrane helices III/IV. Cross-linked AT\textsubscript{2} dimers were subsequently a substrate of activated transglutaminase-2, which targeted the carboxyl terminus of AT\textsubscript{2} dimers, as assessed by truncated and chimeric AT\textsubscript{2} receptors, respectively. AT\textsubscript{2} oligomers acted as dominant negative receptors \textit{in vitro} by mediating Go\textsubscript{q/11} protein sequestration and Go\textsubscript{q/11} protein arrest. The formation of AT\textsubscript{2} oligomers and G-protein dysfunction could be suppressed \textit{in vitro} and \textit{in vivo} by an AT\textsubscript{2} receptor mutant. Inhibition of AT\textsubscript{2} oligomerization upon stereotactic expression of the AT\textsubscript{2} receptor mutant revealed that Go\textsubscript{q/11} sequestering AT\textsubscript{2} oligomers enhanced the development of neurodegenerative symptoms in the hippocampus of transgenic mice with AD-like pathology. Thus, AT\textsubscript{2} oligomers inducing Go\textsubscript{q/11} arrest are causally involved in inducing symptoms of neurodegeneration.

Dysfunction of Go\textsubscript{q/11} is a characteristic feature of AD\textsuperscript{2} patients and transgenic mice with AD-like symptoms (see the accompanying article (26) and Refs. 1–5). The Go\textsubscript{q/11} protein defect is a well established hallmark of clinical AD (5). However, the pathophysiological role of the G-protein defect is barely understood. A direct relationship between Go\textsubscript{q/11} dysfunction and the pathogenesis of AD leading to neurodegeneration and dementia is suggested by the important role of Go\textsubscript{q/11} proteins in neuronal survival and memory (6, 7). In addition, many of the cognition-enhancing effects of acetylcholine are mediated by the Go\textsubscript{q/11}-coupled muscarinic receptors (8). The M\textsubscript{1} receptor is a major target of Go\textsubscript{q/11}, dysfunction of AD patients and mice, and impaired Go\textsubscript{q/11} coupling of M\textsubscript{1} receptors correlates with disease severity (5).

To better understand the pathophysiological function of the G-protein defect in AD, we analyzed the mechanism accounting for the G-protein defect in AD. In the first article (26), we identified cross-linked AT\textsubscript{2} receptor oligomers in the brains of AD patients and transgenic mice with AD-like symptoms. Coenrichment studies showed that AT\textsubscript{2} receptor oligomers resembled dominant negative receptors by sequestering Go\textsubscript{q/11} in the absence of agonist. The cross-linked AT\textsubscript{2} receptors were directly linked to the progression of AD, because (aggregated) amyloid \textit{β} (A\textit{β}) triggered AT\textsubscript{2} oligomerization \textit{in vitro} in a dose-dependent manner by a two-step process of oxidative and transglutaminase-dependent cross-linking. Down-regulation of AT\textsubscript{2} receptors by RNA interference revealed that AT\textsubscript{2} receptor oligomers (oligomers) contributed to Go\textsubscript{q/11} dysfunction of transgenic mice with AD-like symptoms. Specifically, AT\textsubscript{2} receptor oligomers impaired signaling of Go\textsubscript{q/11}-coupled muscarinic M\textsubscript{1} receptors in the hippocampus.

Because activation of hippocampal Go\textsubscript{q/11}-stimulated signaling retards the development of AD-like symptoms in transgenic mice (9), we thought that AT\textsubscript{2} receptor oligomers were directly involved in symptoms of neurodegeneration. To address this question, we used an AT\textsubscript{2} receptor mutant to suppress AT\textsubscript{2} oligomerization \textit{in vitro} and \textit{in vivo}. We show here that inhibition of AT\textsubscript{2} oligomerization by stereotactic expression of a mutated AT\textsubscript{2} receptor retarded the development of neurodegenerative symptoms in the hippocampus of transgenic mice with AD-like pathology. These observations provide strong evidence for an involvement of AT\textsubscript{2} oligomers in mediating symptoms of neurodegeneration.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Protein Expression, and Functional Assays—** Cultivation of human embryonic kidney (HEK) cells and transfection with plasmids encoding AT\textsubscript{2}\textsubscript{α} AT\textsubscript{2}\textsubscript{ΔCTerm}, M\textsubscript{1}, Go\textsubscript{q/11}Q209L, or transglutaminase-2 under control of the cytomegalovirus promoter was performed as described (10, 11). Reactive oxygen...
species (ROS) was induced by expression of NOX-3. Changes in total inositol phosphates and measurement of [Ca\(^{2+}\)] were determined with intact cells (11, 12). Basal and stimulated binding of \[^{35}S\]GTP\(_{\gamma}\)S (specific activity 1250 Ci/mmol; final concentration 0.5 nM) to \(\alpha_q\) was determined in the presence of 0.1 mM GDP in a volume of 200 \(\mu\)L in triplicates with membranes (25 \(\mu\)g of protein/point) prepared from cells and mouse hippocampal tissue followed by immunoaffinity enrichment of \(\alpha_q\). The method measures specifically the activation of \(\alpha_q\), because the applied antibodies cross-react specifically with \(\alpha_q\) as determined in immunoblot. Transglutaminase activity was determined by a \[^3H\]putrescine incorporation assay (11). Immunoaffinity enrichment of \(\alpha_q\) followed by immunoblot detection of co-enriched \(\alpha_q\) was performed by a method that was described (12).

Quantification of the carbachol-stimulated M1-\(\alpha_q\) interaction in the presence of increasing levels of oligomeric \(\alpha_q\) (0–300 fmol/mg protein) was performed after immunoaffinity enrichment of M1 receptors from M1 receptor-expressing HEK cells (−90 fmol/mg protein) co-expressing oligomeric \(\alpha_q\), as indicated. Briefly, HEK cell membranes (50 \(\mu\)g/point) in GTP-free buffer supplemented with 2 mM MgCl\(_2\) were incubated in the absence or presence of 1 mM carbachol for 60 min at 12 °C followed by 20 min at 24 °C and the addition of 1 mM DSP. Membranes were washed three times with 20 mM Tris (pH 7.5) supplemented with 1 mM EDTA and protease inhibitors, collected by centrifugation, and solubilized in 200 \(\mu\)L of buffer (1% deoxycholate, 0.1% Nonidet P-40, 0.1% SDS in 20 mM Tris, pH 7.4, supplemented with protease inhibitors). The solubilisate was incubated for 2 h at 4 °C with an immunoaffinity matrix of M1-specific antibodies coupled to Affi-Gel 10. After washing, \(\alpha_q\) co-enriched with the M1 receptor was quantified by the addition of \(^{125}\)I-labeled F(ab\(_2\)) fragments of immunoselected anti-\(\alpha_q\) antibodies (1 \(\times\) 10\(^{-8}\) M, specific activity ~0.02 Ci/mg) to the affinity matrix. After 2 h of incubation at 4 °C, the affinity matrix was washed three times with ice cold buffer, and radioactivity was determined in a \(\gamma\)-counter. To determine the carbachol-stimulated M1-\(\alpha_q\) interaction, bound radioactivity in the absence of carbachol (<10%) was subtracted. In a parallel approach, \(\alpha_q\) co-enriched with the M1 receptor was quantified in immunoblot with \(\alpha_q\)-specific antibodies after elution from the affinity matrix and SDS-PAGE under reducing conditions. Activities of \(\alpha\)-secretase present in hippocampal tissue of 13-month-old stressed APP\(^{Sw}\) mice were determined using a commercially available kit (R&D Systems).

Antibodies for Immunoblotting and Immunohistochemistry—The following antibodies were used for immunoblotting and immunohistochemistry (10–12): affinity-purified rabbit/rat polyclonal anti-\(\alpha_q\) antibodies (raised against an antigen encompassing amino acids 320–349 of the human \(\alpha_q\) receptor); affinity-purified rabbit polyclonal anti-\(\alpha_q\) antibodies (raised against an antigen encompassing amino acids 326–349 of the human \(\alpha_q\) receptor); affinity-purified rabbit polyclonal anti-\(\alpha_q\) antibodies (raised against an antigen encompassing amino acids 231–350 of the human \(\alpha_q\) receptor); affinity-purified rabbit polyclonal anti-transglutaminase antibodies (raised against an antigen encompassing amino acids 1–20 of mouse transglutaminase-2); affinity-purified rabbit polyclonal anti-\(\alpha_q\) antibodies (raised against the COOH terminus of \(\alpha_q\)). Immunoblotting and immunohistochemistry were routinely used to determine and confirm cross-reactivity of the antibodies with the respective proteins (10–12).

Protein Detection in Immunoblot—Membranes were prepared by sucrose density gradient centrifugation at 4 °C followed by partial enrichment (see accompanying manuscript (26)). For immunoblotting analysis, protein samples were separated by SDS-PAGE under reducing conditions and supplemented with urea followed by transfer to polyvinylidene difluoride membranes, as described (11). Affinity-purified antibodies or F(ab\(_2\)) fragments of the respective antibodies preabsorbed to human and mouse proteins, respectively, were used for detection of \(\alpha_q\), \(\alpha_1\) receptors, and \(\alpha_q\). Antibodies were characterized in previous studies (accompanying manuscript (26) and Refs. 10–13). Bound antibody was visualized by preabsorbed F(ab\(_2\)) fragments of enzyme-coupled secondary antibodies or by enzyme-coupled Protein A followed by enhanced chemiluminescence detection (ECL plus).

Fluorescence-activated Cell Sorting Analysis—Cell surface M1 receptors of transfected HEK cells were determined by fluorescence-activated cell sorting analysis using affinity-purified M1-specific antibodies raised against an epitope corresponding to the receptor’s amino-terminal region according to standard methods (11).

Transgenic Animals—Transgenic mice used in this study express human APP695 with the double mutation (K670N/ M671L; APP\(^{Sw}\)) that was identified in a Swedish family with early onset Alzheimer disease (14).

Lentiviral Vector Production—Vector plasmids were constructed for the production of third generation lentiviruses expressing wild-type \(\alpha_q\) and \(\alpha_{AC\alpha}^{CTe}\) under control of the cytomegalovirus promoter. The generation of viral particles pseudotyped with the vesicular stomatitis virus G glycoprotein was described in the accompanying article (26). For stereotactic injection in mice, high titer lentiviral stocks were used (>1 \(\times\) 10\(^7\) transduction units/\(\mu\)l).

Immunohistochemistry—For immunohistochemistry, paraffin-embedded sections (8 \(\mu\)m, taken at 50-\(\mu\)m intervals for analyses, 10–15 sections/set) were deparaffinized, followed by antigen retrieval (11). Immunohistochemical staining of \(\alpha_q\) receptor was performed with F(ab\(_2\)) fragments of preabsorbed affinity-purified polyclonal antibodies (10, 11). Immunohistochemistry with antibodies to MAP2 (microtubule-associated protein 2) was used as a marker of neuronal cell bodies and dendrites (anti-MAP2 antibodies; Sigma). DNA strand breaks were determined in situ, applying TUNEL technology (Roche Applied Science). All sections were imaged with a Leica DMi6000 microscope equipped with a DFC420 camera.

Cerebral Injection of Lentiviruses—To inhibit the formation of cross-linked \(\alpha_q\) oligomers, lentivirus preparations encoding \(\alpha_{AC\alpha}^{CTe}\) were injected bilaterally into the CA1 area of 12-month-old APP\(^{Sw}\) mice similarly as described in the accompanying article (26) (anteroposterior −1.6 to −2.3; lateral 1; dorsoventral −1.8). The control group received an injection of
a control lentivirus encoding β-galactosidase or wild-type AT2 receptor. After 1 week of recovery, mice were subjected to the stress paradigm for 4 weeks. Five weeks after the lentiviral injection, behavioral analysis, biochemical analysis, and immunohistochemistry were performed.

Protein expression levels of lentivirus-driven expression were routinely monitored in immunoblot with anti-AT2 receptor antibodies (~1.8–2.3-fold hippocampal overexpression compared with mice injected with a control lentivirus encoding β-galactosidase).

**Induction of Stress**—The generation of Aβ was enhanced in 12-month-old APPsw mice by 4 weeks of stress as described in the accompanying article (26). Stressed mice with a significant decrease in sucrose preference (~50% of sucrose consumption compared with nonstressed controls) were included in the study. After 28 days of stress, 4 h before the beginning of the dark phase, brains from stressed and nonstressed APPsw mice were removed and processed for immunoblotting, functional studies, and immunohistochemistry as described above.

**Behavioral Studies**—We used the standard water maze task (hidden platform) to test for spatial memory (15). Testing involved four trials per day over 10 days starting after the stress period. On the day following the 10 days of acquisition testing, memory retention was determined in a single 60-s probe trial for which the submerged platform was removed (16).

Animal experiments were reviewed and approved by the committees on animal research at the Universities of Hamburg and Cairo and were conducted in accordance with National Institutes of Health guidelines.

**Statistics**—Unless otherwise stated, data are expressed as mean ± S.E. To determine significance between two groups, we made comparisons using the unpaired two-tailed Student’s t test. p values of <0.05 were considered significant.

**RESULTS**

**Oxidative and Transglutaminase-mediated Cross-linking of AT2 Receptors in Vitro**—In the accompanying article (26) we have shown that (aggregated) Aβ induces the formation of AT2 receptor oligomers in vivo in a dose-dependent manner. The formation of AT2 oligomers in vivo is due to a two-step process of oxidative and transglutaminase-mediated cross-linking. To analyze whether ROS and transglutaminase were indeed sufficient to support the formation of AT2 oligomers, we reconstituted the cross-linking in a nonneuronal in vitro system. For detection of AT2 receptors in immunoblot, we applied AT2-specific antibodies cross-reacting specifically with the AT2 receptor (A) or mutated AT2 receptor (B). ROS was induced by expression of NOX-3. As indicated, pretreatment with the antioxidant difluoromethylene (40 μM) was performed. The arrowheads mark monomorphic (M), dimeric (D), and oligomeric (O) AT2 receptor. C and D, immunoblot of AT2 receptors (IB: anti-AT2) on membranes of HEK cells expressing wild-type AT2 receptor (AT2; C) or AT2 receptor (AT2; D). ROS was induced in HEK cells by co-transfection of NOX-3 in cells expressing activated transglutaminase-2 (NOX-3/Tg). As indicated, cells were treated with the transglutaminase inhibitor monodansyl cadaverine (Tg-inhib, 200 μM). E, the AT2 receptor is not significantly cross-linked by ROS/transglutaminase, as revealed by immunoblots of AT2 receptors (IB: anti-AT2) on membranes of HEK cells expressing wild-type AT2 receptor. As indicated, cells were treated with the transglutaminase inhibitor monodansyl cadaverine (200 μM) or the antioxidant diterfluoromethylene (40 μM). F, chimeric AT2/AT1 receptor is not a target of transglutaminase-2. ROS was induced in HEK cells by co-transfection of NOX-3 in cells expressing activated transglutaminase-2. As indicated, cells were treated with the transglutaminase inhibitor monodansyl cadaverine.

ROS targets tyrosine residues of proteins, leading to dityrosine formation. Tyrosine residues of AT2 are located at putative interreceptor contact sites (i.e. the cytoplasmic loop connecting transmembrane helices III/IV (18)). Those interreceptor contact sites appear crucial for ROS-mediated cross-linking of AT2, because oxidative cross-linking was strongly diminished in a mutated AT2 receptor lacking three tyrosines of connecting loop III-IV, AT2 Tyr→Ala143,148,162 (Fig. 1, B versus A). ROS-dependent cross-linking assembled AT2 for transglutaminase-mediated cross-linking, because the concomitant action of ROS and transglutaminase-2 led to AT2 receptor oligomers, whereas inhibition of transglutaminase-2 by monodansyl cadaverine prevented only the appearance of AT2 oligomers and left the oxidized AT2 dimers intact (Fig. 1C). Thus, transglutaminase targeted AT2 receptors preassembled by oxidative cross-linking.
Transglutaminase-dependent cross-linking of oxidized AT2 receptors seemed to require the receptor's carboxyl terminus, because a mutated AT2 receptor, AT2<sup>ACTer</sup>, lacking the carboxyl terminus was not significantly cross-linked by transglutaminase-2, although oxidative dimerization was evident (Fig. 1D). This finding is in agreement with a model of receptor oligomers identifying COOH-terminal residues as contact sites between dimers (18).

ROS/transglutaminase targeted specifically the AT2 receptor, because the related AT1 receptor appeared as a pure monomer under similar cross-linking conditions (Fig. 1E). The specificity of transglutaminase for oxidized AT2 receptor dimers was further analyzed by a chimeric AT2 receptor with the carboxyl terminus of AT1. The chimeric AT2 receptor, AT2<sup>ACTer</sup>/AT1<sup>COO</sup>, was not significantly cross-linked to oligomers by concomitant ROS/transglutaminase action, although AT2<sup>ACTer</sup>/AT1<sup>COO</sup> dimers were detected in the presence of the transglutaminase inhibitor (Fig. 1F). This observation further confirms that the carboxyl terminus of the AT2 receptor is required for transglutaminase-dependent cross-linking of (oxidized) AT2 receptor dimers.

Altogether, a two-step process of oxidative and transglutaminase-dependent cross-linking leads to AT2 oligomers in vitro (this work) and in vivo (accompanying article (26)).

**In Vivo**—The biochemical similarity between in vitro formed AT2 oligomers and in vivo detected AT2 oligomers was further analyzed. Similarly to AD patients and mice, AT2 oligomers of HEK cells sequestered Gα<sub>q/11</sub>, as determined by co-enrichment with Gα<sub>q/11</sub> proteins, whereas AT2 monomers/dimers did not significantly interact with Gα<sub>q/11</sub> (Fig. 2, A (lanes 2 and 3) and B).

The interaction with an activated G-protein-coupled receptor is an essential part of the G-protein activation step. In agreement with this paradigm, stimulation of muscarinic M1 receptors by carbachol strongly enhanced the interaction of M1 receptors with Gα<sub>q</sub> as determined by co-enrichment (Fig. 2C). The presence of AT2 oligomers prevented the carbachol-stimulated M1/Gα<sub>q</sub> interaction almost entirely (Fig. 2C). Thus, oligomeric AT2 is capable of suppressing the interaction of Gα<sub>q</sub> with an activated heterologous receptor, as would be expected from its Gα<sub>q/11</sub>-sequestering capacity.

Next, we quantified the carbachol-stimulated interaction of Gα<sub>q</sub> with the M1 receptor (∼900 fmol/mg) in the presence of increasing levels of AT2 oligomers. We chose the Gα<sub>q/11</sub>-coupled muscarinic M1 receptor, because impaired G-protein coupling of M1 correlates with disease severity in AD (5). Cross-linked AT2 oligomers potently inhibited the stimulated M1<sub>Gα<sub>q</sub></sub> interaction in a concentration-dependent manner with an EC<sub>50</sub> of ∼30 fmol/mg protein (Fig. 2D). As a control, comparable levels of the AT2<sup>ACTer</sup> mutant that was not cross-linked to oligomers under similar conditions (cf. Fig. 1D) had no significant effect on the carbachol-stimulated M1<sub>Gα<sub>q</sub></sub> interaction (Fig. 2E). These observations strongly indicate that physiological AT2 receptor expression levels of AD brain are suffi-
Dominant Negative \( \text{AT}_2 \) Receptors

FIGURE 3. \( \text{AT}_2 \) receptor oligomers inhibit basal and carbachol-stimulated activation of \( \text{G}\alpha_q \). A and B, activation of \( \text{G}\alpha_q \) as determined by basal (columns 1 and 2) and carbachol-stimulated (Cch; columns 3 and 4) \[^{35}\text{S}\]GTP\( \gamma \)-binding followed by immunooaffinity enrichment of \( \text{G}\alpha_q \), with membranes of M1 receptor-expressing HEK cells (100 fmol/mg) co-expression (−150 fmol/mg protein of \( \text{AT}_2 \)) or \( \text{AT}_2 \) (B). As indicated, \[^{35}\text{S}\]GTP\( \gamma \)-binding was determined with membranes of cells co-expressing NOX-3 and activated transglutaminase-2 (±NOX-3/Tg). Data represent mean ± S.E., \( n = 6 \), * \( p < 0.0001 \).

FIGURE 4. \( \text{AT}_2 \) receptor oligomers suppress \( \text{G}\alpha_q \)-stimulated signaling. A, inositol phosphate signal of M1 receptor-expressing HEK cells co-expressing dissociable \( \text{AT}_2 \) receptors (+AT2mono) or cross-linked \( \text{AT}_2 \) receptor oligomers (+AT2d olig). Total cellular inositol phosphate levels were determined under basal conditions (−), upon stimulation with carbachol (Cch; 100 \( \mu \)M), GTP\( \gamma \)-S (100 \( \mu \)M in a nominally Ca\(^{2+}\)-free permeabilization buffer containing 10 \( \mu \)M digitonin), AIF\(_{4}^+\) (300 \( \mu \)M), or Ca\(^{2+}\) (10 \( \mu \)M in permeabilization buffer) or upon expression of the constitutively active mutant of \( \text{G}\alpha_q (Q209L) \). Data are the mean ± S.D., \( n = 6 \). *, \( p < 0.0002 \). B, concentration-response relationship of the carbachol-stimulated inositol phosphate signal of M1 receptor-expressing HEK cells co-expressing dissociable \( \text{AT}_2 \) receptors or \( \text{AT}_2 \) receptor oligomers. Inositol phosphates are expressed as percentage of maximum stimulation (i.e. the signal obtained with 100 \( \mu \)M carbachol on cells with dissociable \( \text{AT}_2 \) receptors) (12,710 ± 420 dpm). Data represent mean ± S.D., \( n = 3 \). C, carbachol-stimulated (100 \( \mu \)M) rise in the free intracellular calcium concentration (\([\text{Ca}^{2+}]_i\)) of M1 receptor-expressing HEK cells co-expressing dissociable \( \text{AT}_2 \) receptors (panels 1−3) or \( \text{AT}_2 \) oligomers (panels 4−6). As indicated, car cells were co-stimulated with 100 nm angiotensin II (Ang; panels 2 and 5). In the experiments of panels 3 and 6, cells were desensitized by prior stimulation with carbachol for 30 min at 37 °C, followed by washing steps (Cch desens.). Single experiments are representative of three independent experiments, each with similar results. D, fluorescence-activated cell sorting analysis (FACS) of cell surface M1 receptors of HEK cells co-expressing dissociable \( \text{AT}_2 \) receptors (panels 1 and 3) or \( \text{AT}_2 \) oligomers (panels 2 and 4). In the experiments of panels 3 and 4, M1 receptor redistribution (Redistrib.) was induced by stimulation with 1 \( \mu \)M carbachol for 60 min at 37 °C. Cell surface M1 receptors were determined by FACS using affinity-purified Fab\(_{2}\) fragments of M1-specific antibodies.
arrest of an activated Gαq/11 protein. The dominant negative activity of the nondissociable oligomeric AT2 receptor platform could account for the potent inhibition of Gαq/11-stimulated signaling at the cell membrane, which is dependent on the release of a limited number of activated Gαq/11 proteins triggered by stimulation of specific receptors.

Inhibition of Calcium Signaling and Desensitization by AT2 Oligomers—Impaired M1 receptor-stimulated Gαq/11-protein activation in the presence of AT2 oligomers was also reflected by a decreased rise in the intracellular free calcium concentration, [Ca2+], (Fig. 4C, panels 1 and 2 versus panels 4 and 5). The Gαq/11 inhibitory effect of the cross-linked AT2 oligomers was independent of the activation of AT2 by angiotensin II (Fig. 4C, panels 4 and 5).

Activated G-proteins trigger signal generation as well as signal desensitization. To determine whether AT2 oligomers inhibited the desensitization of the M1 receptor, we pretreated the cells with carbachol. Carbachol pretreatment led to a significant decrease of the M1-stimulated rise in [Ca2+], on cells expressing dissociable AT2 receptors indicative of M1 receptor desensitization (Fig. 4C, panel 3 versus panels 1 and 2). In contrast, the pretreatment with carbachol did not markedly decrease the M1 signal on cells expressing oligomeric AT2 receptors (Fig. 4C, panel 6 versus panels 4 and 5). Thus, AT2 oligomers inhibit signal generation and desensitization of the Gαq/11-coupled M1 receptor.

Receptor desensitization is followed by receptor redistribution. Stimulation with carbachol induced prominent M1 receptor redistribution/internalization on cells expressing dissociable AT2 receptors (Fig. 4D, panel 3 versus panel 1). In contrast, inhibition of Gαq/11 proteins by AT2 oligomers led to a significantly reduced carbachol-stimulated M1 receptor redistribution (Fig. 4D, panel 4 versus panel 2). Together, these findings show that cross-linked AT2 oligomers arrest Gαq/11-stimulated signaling, as reflected by inhibition of different signal transduction events triggered by a prototypic Gαq/11-coupled receptor (i.e. the muscarinic M1 receptor).

The Truncated AT2ΔCTer Mutant Suppresses AT2 Oligomerization in Vivo and in Vivo—We further assessed the impact of AT2 oligomers on Gαq/11 activity and applied the truncated AT2ΔCTer mutant to interfere with AT2 oligomerization. The AT2ΔCTer receptor when co-expressed with wild-type AT2 prevented the transglutaminase-dependent cross-linking step accounting for AT2 dimer/oligomer transition, as determined in immunoblot with AT2-specific antibodies detecting specifically wild-type AT2 receptors (Fig. 5A). Upon inhibition of AT2 oligomerization, the basal and M1 receptor-stimulated Gαq/11 activation were not significantly impaired by AT2ΔCTer even in the presence of ROS and activated transglutaminase (Fig. 5B; cf. Fig. 3A). These findings confirm that AT2 oligomers prevent Gαq/11 activation.

To suppress Aβ-induced AT2 oligomerization in vivo, we expressed the AT2ΔCTer mutant (that prevented AT2 oligomerization in vitro) in the hippocampal CA1 area of stressed APPSw mice by stereotactic injection of a lentivirus. Similarly as in HEK cells, expression of AT2ΔCTer led to a marked decrease of AT2 oligomerization in the hippocampus of treated “AD mice,” as determined by immunoblotting (Fig. 6A). Thus, AT2ΔCTer interfered with transglutaminase-mediated cross-linking of AT2 receptors in vitro and in vivo.

Inhibition of AT2 Oligomerization Slows Progression of Neurodegeneration in AD Mice—Detection of the endogenous AT2 receptor and the transduced AT2ΔCTer by immunohistochemistry revealed prominent neuritic/dendritic AT2 staining in a representative treated AD mouse expressing AT2ΔCTer that was not visible in the control (i.e. a representative APPSw mouse with stress-enhanced Aβ generation and injection of a control lentivirus) (Fig. 6B). Notably, there was prominent neuritic/dendritic AT2 staining in treated AD mice expressing AT2ΔCTer that was not visible in the controls (Fig. 6B). Immunohistochemistry applying MAP2-specific antibodies confirmed this observation and revealed largely intact neurites/dendrites in the injected CA1 area of mice expressing AT2ΔCTer, whereas there was extensive neuritic/dendritic degeneration in the control mice (Fig. 6C; cf. Fig. 7A). These findings strongly suggest that the AT2ΔCTer mutant retarded the development of neurodegenerative symptoms in stressed APPSw mice (Fig. 6C).

In accordance with these observations, TUNEL-positive cells were barely detectable in the injected CA1 area of treated AD mice expressing AT2ΔCTer, whereas CA1 neurons of control mice showed prominent TUNEL labeling (Fig. 6D, right versus left panel). Decreased symptoms of neuronal degeneration correlated with significantly reduced hippocampal transglutaminase activity (Fig. 6E).

Treated AD mice expressing AT2ΔCTer also displayed a marginally better performance in the CA1-dependent learning task of spatial memory acquisition and retention compared with control AD mice with a high load of cross-linked AT2 receptor oligomers (Fig. 6, F and G). Together these findings strongly suggest that inhibition of AT2 oligomerization by expression of a protein inhibitor (i.e. the AT2ΔCTer mutant) retarded the
Inhibition of AT$_2$ oligomerization prevents G-protein dysfunction in transgenic mice with AD-like symptoms.

In agreement with a role of M$_1$ in nonamyloidogenic processing of APP (19, 20), dysfunctional M$_1$ receptor-stimulated G-protein activation of AT$_2$ receptor-expressing mice was accompanied by a marginally decreased hippocampal $\alpha$-secretase activity compared with mice with expression of AT$_2^{\Delta CTer}$ and intact M$_1$-stimulated signaling (Fig. 7C). Thus, the $\alpha$-secretase activity of AD mice was preserved upon inhibition of AT$_2$ oligomerization by expression of an AT$_2$ mutant (this work) and also upon down-regulation of AT$_2$ (oligomerization) by RNA interference (cf. accompanying article (26)) (Fig. 6). Altogether, the experiments reveal that targeting of AT$_2$ receptor oligomers by expression of a protein inhibitor is feasible and slows the development of neurodegenerative symptoms in transgenic mice with AD-like pathology.

**DISCUSSION**

G-protein dysfunction in the brains of AD patients is a characteristic hallmark of clinical AD with neurodegeneration (5). In the accompanying article (26), G-protein dysfunction was linked to AT$_2$ oligomers that form in the brains of AD patients and mice with AD-like symptoms. AT$_2$ receptor oligomers assemble in vivo by a two-step process of oxidative and transglutaminase-dependent cross-linking, which is triggered consecutively by (aggregated) A$\beta$ in a dose-dependent manner (see the accompanying article (26)). To further characterize the function of AT$_2$ oligomers, the current study reconstructed the two-step formation of AT$_2$ receptor oligomers in vitro in a transfected cell system. The in vitro experiments revealed that the initial oxidative cross-linking step accounting for the formation of AT$_2$ receptor dimers targets specifically AT$_2$ with its tyrosine residues at putative interreceptor contact sites (18), because a mutated AT$_2$ receptor lacking the tyrosines

---

**FIGURE 6. Inhibition of AT$_2$ oligomerization slows the progression of neurodegeneration in AD mice.**

A, immunoblot of hippocampal AT$_2$ receptors of four stressed APP$^{\text{Sw}}$ mice upon stereotactic injection of a lentivirus encoding AT$_2^{\Delta CTer}$ (Treated) revealed decreased AT$_2$ receptor oligomerization compared with mice injected with a control lentivirus (Control). Applied antibodies cross-react with a COOH-terminal epitope of AT$_2$ that is lacking in AT$_2^{\Delta CTer}$ (IB: anti-AT$_2^{\Delta CTer}$), B, immunohistochemistry applying antibodies cross-reacting with an NH$_2$-terminal epitope of AT$_2$ (anti-AT$_2^{\Delta NTer}$) to visualize AT$_2$, and AT$_2^{\Delta CTer}$ revealed AT$_2$ receptor expression in CA1 neuronal cell bodies and largely intact dendrites of stressed APP$^{\text{Sw}}$ transgenic mice (Control) compared with mice receiving injection of a control lentivirus (Control) encoding $\beta$-galactosidase (original magnification, $\times$300). C, inhibition of AT$_2$ oligomerization by AT$_2^{\Delta CTer}$ retarded the development of symptoms of dendritic degeneration in the injected CA1 area of stressed APP$^{\text{Sw}}$ transgenic mice (Control) compared with mice injected with a control lentivirus (Control), as determined by immunohistochemistry with anti-MAP2 antibodies (original magnification, $\times$300). D, DNA strand breaks were barely detectable in the injected CA1 area of stressed APP$^{\text{Sw}}$ mice upon inhibition of AT$_2$ oligomerization by AT$_2^{\Delta CTer}$ compared with stressed APP$^{\text{Sw}}$ mice injected with a control lentivirus, as determined by in situ TUNEL labeling (original magnification, $\times$300). E, inhibition of AT$_2$ oligomerization in the CA1 area of stressed APP$^{\text{Sw}}$ transgenic mice by AT$_2^{\Delta CTer}$ (Treated) led to a significantly decreased hippocampal transglutaminase activity compared with stressed APP$^{\text{Sw}}$ transgenic mice injected with a control lentivirus (Control). Data represent mean ± S.E., n = 8. *p < 0.01, F and G, inhibition of AT$_2$ oligomerization by AT$_2^{\Delta CTer}$ led to a significantly better performance of stressed APP$^{\text{Sw}}$ transgenic mice of the water maze acquisition (F) and retention (G) test compared with mice injected with a control lentivirus. Data represent mean ± S.E., n = 12 mice per subgroup. F: *, p < 0.05; **, p < 0.008. G, *, p < 0.003; analysis of variance with Dunn’s multiple comparison test.
FIGURE 7. *Inhibition of AT$_2$ oligomerization prevents G-protein dysfunction in AD mice.* A. Immunohistochemistry with antibodies cross-reacting with an NH$_2$-terminal epitope of AT$_2$ (anti-AT$_2$ NTer) revealed dendritic pathology of CA1 neurons of stressed APP$^{sw}$ mice receiving stereotactic injection of a lentivirus encoding wild-type AT$_2$ receptor (+AT$_2$) compared with largely intact dendrites of mice expressing AT$_2$Cter (+AT$^{2}$Cter; magnification, ×300). B, strongly impaired basal and M$_1$ receptor-stimulated (Cch) activation of G$_{q/11}$ in the hippocampus of stressed APP$^{sw}$ mice injected with a lentivirus encoding AT$_2$ compared with the activation of G$_{q/11}$ in mice expressing AT$_2$Cter. Data are from four mice/group ± S.E., *p < 0.02; **p < 0.001. C, reduced activity of hippocampal α-secretase of stressed APP$^{sw}$ mice injected with a lentivirus encoding AT$_2$ compared with the unimpaired α-secretase activity of mice expressing AT$_2$Cter. Data are given as a percentage of control (i.e. the α-secretase activity of nonstressed APP$^{sw}$ mice receiving injection of a control lentivirus (i.e. 100%)) and represent mean ± S.E., n = 4, *p < 0.01.

...of the cytoplasmic loop connecting transmembrane helices III/IV was not cross-linked by ROS. Oxidative cross-linking assembles AT$_2$ receptors and thereby generates the target for the second transglutaminase-mediated cross-linking step, leading finally to AT$_2$ tetramers/oligomers. Experimental evidence suggests that transglutaminase-dependent cross-linking of oxidized AT$_2$ receptor dimers involves the carboxyl terminus of AT$_2$, which is a putative contact site between receptor dimers according to the rhodopsin model (18). Both cross-linking steps are a direct consequence of aggregated Aβ and the pathogenesis of AD; oxidative cross-linking is due to high levels of ROS triggered by aggregated Aβ (21). The second transglutaminase-mediated cross-linking step is also linked to Aβ and the pathogenesis of AD, because cerebral microinjection of Aβ or enhanced formation of (aggregated) Aβ triggered the activation induction of transglutaminase in mice (see the accompanying article (26)). In agreement with a role of Aβ in inducing transglutaminase is the observation that transglutaminase activity is elevated in the brains of AD patients (22, 23).

The Aβ-induced nondissociable oligomeric AT$_2$ receptor assembly seems to constitute a cellular platform that is kinetically favored to interact with activated G$_{q/11}$ proteins. The large interface of nondissociable AT$_2$ receptor oligomers could impede the rapid dissociation of activated G$_{q/11}$ proteins upon binding. As a consequence, the activated G$_{q/11}$ proteins are sequestered and arrested, leading to decreased receptor-stimulated signaling. Mediating G$_{q/11}$ arrest, the cross-linked AT$_2$ oligomers may thus contribute to the well-known G$_{q/11}$ dysfunction in AD.

Our results suggest that the G$_{q/11}$-protein defect in brain of AD patients also has a pathophysiological role. (i) The G-protein defect mediated by AT$_2$ oligomers is causally linked to the pathogenesis of AD, because it relies on aggregated Aβ-induced ROS and transglutaminase. (ii) The induction of AT$_2$ oligomers by aggregated Aβ was accompanied by hippocampal neurodegeneration, Tau phosphorylation, and neuronal loss (see the accompanying article (26)). (iii) Stereotactic inhibition of cross-linked AT$_2$ oligomers by expression of a mutated AT$_2$ receptor retarded hippocampal G$_{q/11}$ dysfunction and symptoms of hippocampal neurodegeneration.

Neurodegeneration and compromised neurite integrity may be a direct consequence of the AT$_2$ oligomer-mediated signaling protection (hippocampal) neurons from G$_{12/13}$-induced neurite damage (24, 25). Since G$_{12/13}$-dependent signaling also links microtubule and microfilament disorganization with Tau phosphorylation (25), the AT$_2$-mediated G$_{q/11}$ impairment is likely to induce symptoms of neurodegeneration and increased phospho-Tau levels via an unknown pathway.

Altogether, G$_{q/11}$-inhibitory AT$_2$ receptor oligomers are formed by two sequential cross-linking steps triggered by (aggregated) Aβ. Dominant-negative AT$_2$ oligomers may reflect the process of ongoing Aβ-induced neurodegeneration and also actively contribute to neuron damage.

REFERENCES

1. Ferrari-DiLeo, G., and Flynn, D. D. (1993) *Life Sci.* 53, PL439–PL444
2. Fowler, C. J., Cowburn, R. F., Garland, A., Winblad, B., and O’Neill, C. (1998) Mol. Cell. Biochem. 190, 287–292
3. Greenwood, A. F., Powers, R. E., and Jope, R. S. (1995) *Neuroscience* 69, 125–138
4. Jope, R. S., Song, L., Li, X., and Powers, R. (1994) *Neurobiol. Aging* 15, 221–226
5. Tsang, S. W., Lai, M. K., Kirvell, S., Francis, P. T., Essiri, M. M., Hope, T., Chen, C. P., and Wong, P. T. (2006) *Neurobiol. Aging* 27, 1216–1223
6. Miura, M., Watanabe, M., Offermanns, S., Simon, M. I., and Kano, M. (2002) *J. Neurosci.* 22, 8379–8390
7. Wettschureck, N., van der Stelt, M., Tsukobawa, K., Krestel, H., Moers, A., Petrosino, S., Schütz, G., Di Marzo, V., and Offermanns, S. (2006) *Mol. Cell. Biol.* 26, 5888–5894
8. Wess, J., Eglen, R. M., and Gautam D. (2007) *Nat. Rev. Drug Discov.* 6, 721–733
9. Caccamo, A., Oddo, S., Billings, L. M., Green, K. N., Martinez-Coria, H., Fisher, A., and LaFerla, F. M. (2006) *Neuron* 49, 671–682
10. AbdAlla, S., Lother, H., Abdel-tawab, A. M., and Quitterer, U. (2001) *J. Biol. Chem.* 276, 39721–39726
11. AbdAlla, S., Lother, H., Langer, A., el Faramawy, Y., and Quitterer, U. (2004) *Cell* 119, 343–354
12. AbdAlla, S., Lother, H., and Quitterer, U. (2000) *Nature* 407, 94–98
13. AbdAlla, S., Lother, H., el Massiery, A., and Quitterer, U. (2001) *Neurosci. Lett.* 303, 1003–1009
14. Hsiao, K., Chapman, P., Nilsen, S., Eckman, C., Harigaya, Y., Younkin, S., Yang, F., and Cole, G. (1996) *Science* 274, 99–102
15. Morris, R. G. M., Garrud, P., Rawlins, J. N. P., and O’Keefe, J. (1982) *Nature* 297, 681–683
16. King, D. L., and Arendash, G. W. (2001) *J. Alzheimers Dis.* 5, 167–181
17. Fotiadis, D., Liang Y., Filipek, S., Saperstein, D. A., Engel, A., and Palkovits, K. (2004) *FEBS Lett.* 564, 281–288
Dominant Negative AT₂ Receptors

19. Buxbaum, J. D., Oishi, M., Chen, H. I., Pinkas-Kramarski, R., Jaffe, E. A., Gandy, S. E., and Greengard, P. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10075–10078
20. Haring, R., Fisher, A., Marciano, D., Pittel, Z., Kloog, Y., Zuckerman, A., Eshhar, N., and Heldman, E. (1998) J. Neurochem. 71, 2094–2103
21. Butterfield, D. A., and Boyd-Kimball, D. (2004) Brain Pathol. 14, 426–432
22. Johnson, G. V., Cox, T. M., Lockhart, J. P., Zinnerman, M. D., Miller, M. L., and Powers, R. E. (1997) Brain Res. 751, 323–329

23. Bonelli, R. M., Aschoff, A., Niederwieser, G., Heuberger, C., and Jirikowski, G. (2002) Neurobiol. Dis. 11, 106–110
24. Nürnberg, A., Bräuer, A. U., Wettchureck, N., and Offermanns, S. (2008) J. Biol. Chem. 283, 35526–35531
25. Sayas, C. L., Moreno-Flores, M. T., Avila, J., and Wandosell, F. (1999) J. Biol. Chem. 274, 37046–37052
26. AbdAlla, S., Lother, H., el Missiry, A., Langer, A., Sergeev, P., el Faramawy, Y., and Quitterer, U. (2009) J. Biol. Chem. 284, 6554–6565