Angiotensin II AT$_2$ Receptor Oligomers Mediate G-protein Dysfunction in an Animal Model of Alzheimer Disease*

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Said AbdAlla$^1$, Heinz Loether$^1$, Ahmed el Missiry$^1$, Andreas Langer$^1$, Pavel Sergeev$^6$, Yasser el Faramawy$^1$, and Ursula Quitterer$^1$††

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

Progressive neurodegeneration and decline of cognitive functions are major hallmarks of Alzheimer disease (AD). Neurodegeneration in AD correlates with dysfunction of diverse signal transduction mechanisms, such as the G-protein-stimulated phosphoinositide hydrolysis mediated by Go$_{q/11}$. We report here that impaired Go$_{q/11}$-stimulated signaling in brains of AD patients and mice correlated with the appearance of cross-linked oligomeric angiotensin II AT$_2$ receptors sequestering Go$_{q/11}$. Amyloid β (Aβ) was causal to AT$_2$ oligomerization, because cerebral microinjection of Aβ triggered AT$_2$ oligomerization in the hippocampus of mice in a dose-dependent manner. Aβ induced AT$_2$ oligomerization by a two-step process of oxidative and transglutaminase-dependent cross-linking. The induction of AT$_2$ oligomers in a transgenic mouse model with AD-like symptoms was associated with Go$_{q/11}$ dysfunction and enhanced neurodegeneration. Vice versa, stereotactic inhibition of AT$_2$ oligomers by RNA interference prevented the impairment of Go$_{q/11}$ and delayed Tau phosphorylation. Thus, Aβ induces the formation of cross-linked AT$_2$ oligomers that contribute to the dysfunction of Go$_{q/11}$ in an animal model of Alzheimer disease.

Alzheimer disease (AD) is a protein aggregation disease that is characterized by profound neuropathological changes in the brain, including neurodegeneration, neurofibrillary tangles, and the accumulation of fibrillar β-amyloid (Aβ) in extracellular senile plaques. Although some neuropathological features of AD, such as tangles and plaques, are also detected in brains of elderly people without major symptoms of dementia (1), neurodegeneration and neuronal loss of AD patients are associated with the major AD symptoms of memory impairment and dementia (2, 3). Neurodegeneration in AD is accompanied by dysfunction of diverse signal transduction mechanisms, such as the G-protein-stimulated phosphoinositide hydrolysis mediated by Go$_{q/11}$ (4–8). Go$_{q/11}$-stimulated signal transduction pathways are important for neuronal communication, synaptic plasticity, and neuronal survival (9, 10). Therefore, it is likely that the Go$_{q/11}$ signaling defect of AD patients plays a role in the disease process leading to neurodegeneration and dementia. In agreement with this notion, G-protein dysfunction is directly associated with disease severity of AD patients (8).

Further insight into the pathological role of the G-protein dysfunction of AD patients is lacking, because the underlying mechanism is barely understood. Several observations point to a specific defect at the level of Go$_{q/11}$: (i) protein levels of Go$_{q/11}$ are not changed (7); (ii) downstream receptor/G-protein-independent phosphoinositide hydrolysis is intact (7); and (iii) receptor-mediated activation of other G-proteins, such as the Go$_{i/o}$ proteins, is not affected (5). In view of a putative role in the pathogenesis, we investigated the mechanism accounting for defective Go$_{q/11}$ activation in AD.

Go$_{q/11}$ activation is under control of specific receptors. Although most receptors interacting with Go$_{q/11}$ stimulate guanine nucleotide exchange and thereby trigger Go$_{q/11}$-mediated signaling, the angiotensin II AT$_2$ receptor is inhibitory and capable of disrupting receptor-stimulated Go$_{q/11}$ activation under specific conditions (11). Moreover, several studies link the AT$_2$ receptor to diverse neuronal functions related to behavior (12), apoptosis (13), and memory (14). Focusing on the inhibitory AT$_2$ receptor as a potential candidate accounting for defective Go$_{q/11}$ activation in AD, we identified cross-linked oligomeric AT$_2$ receptors that form by a two-step cross-linking process triggered by aggregated Aβ.

EXPERIMENTAL PROCEDURES

Functional Assays—Basal and stimulated binding of $^{[35]}$S-GTPγS (specific activity 1250 Ci/mmol; final concentration 0.5 mM) to Go$_{q/11}$ was determined in the presence of 0.1 μM GDP in a volume of 200 μl in triplicates with membranes (25 μg of protein/point) prepared from human prefrontal cortex specimens and mouse hippocampal tissue as described (15), followed by immunofinity enrichment of Go$_{q/11}$. The method measures specifically the activation of Go$_{q/11}$, because the applied antibodies cross-react specifically with Go$_{q/11}$ as determined in immunoblot. The assay of $^{[3}H$]phosphatidylsinositol hydrolysis was carried out in triplicates with membranes (100 μg of protein/point) of human prefrontal cortex specimens, as
detailed previously (6). Transglutaminase activity was determined by a \(^{3}H\)putrescine incorporation assay (15). Immunoaffinity enrichment of Gaq/11 followed by immunoblot detection of co-enriched AT\(_2\) was performed by a method that was described (16).

Activities of \(\alpha\)-secretase and \(\beta\)-secretase present in hippocampal tissue of 13-month-old nonstressed and stressed APP\(^{Sw}\) mice were determined using commercially available secretase kits (R&D Systems). Soluble and aggregated A\(\beta\) peptide levels (A\(\beta\)-(1–40) and A\(\beta\)-(1–42)) were determined in supernatant (SDS-soluble) and the formic acid extract of the pellet (SDS-insoluble) of hippocampal tissue preparations from 13-month-old nonstressed and stressed APP\(^{Sw}\) mice using a sandwich enzyme-linked immunosorbent assay (Invitrogen).

**Antibodies for Immunoblotting and Immunohistochemistry**—The following antibodies were used for immunoblotting, affinity purification, and immunohistochemistry (11, 15, 16): affinity-purified rabbit/rat polyclonal anti-AT\(_2\) antibodies (raised against an antigen encompassing amino acids 320–349 of the human AT\(_2\) receptor); affinity-purified rabbit polyclonal anti-AT\(_2\) antibodies (raised against an antigen encompassing amino acids 16–35 of the human or mouse AT\(_2\) receptor); affinity-purified rabbit/rat polyclonal anti-M\(_1\) antibodies (raised against an antigen encompassing amino acids 231–350 of the human M\(_1\) 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-Gaq/11 antibodies (raised against the C terminus of Gaq/11). Immunoblotting and immunohistochemistry were routinely used to determine and confirm cross-reactivity of the antibodies with the respective proteins (11, 15, 16).

**Protein Detection in Immunoblot**—Insoluble, formic acid (70%)-extractable A\(\beta\) in the prefrontal cortex specimens indicative of A\(\beta\) plaque load was assessed by immunoblot with A\(\beta\)-specific rat polyclonal antibodies after serial extraction in the presence of protease inhibitors by high salt (150 mM Tris, pH 7.6, 750 mM NaCl, 2 mM EDTA) and detergents (1% Triton X-100) in high salt buffer followed by radioimmune precipitation buffer and 2% SDS in 150 mM Tris, pH 7.6.

Membranes were prepared by sucrose density gradient centrifugation at 4 \(^{\circ}\)C, followed by partial enrichment (16). Briefly, membranes (500 \(\mu\)g of protein/1.5 ml containing \(\sim\)300–400 fmol/mg AT\(_3\)) were solubilized by 20 mM CHAPS in 50 mM Tris, pH 7.4, supplemented with 1 mM EDTA and protease inhibitor mixture (Sigma). Particulate material was removed by centrifugation at 20,000 \(\times\) g for 15 min at 4 \(^{\circ}\)C. The solubilized proteins were precipitated and delipidated by acetone/methanol (12:2; final concentration 83%) for 90 min at 4 \(^{\circ}\)C. The precipitate was collected by centrifugation, followed by three washing steps with 0.2 ml of cold acetone. The pellet was dissolved in SDS-sample buffer containing 2% SDS, 5% \(\beta\)-mercaptoethanol and 6 M urea for 90 min at room temperature and stored for further use at –70 \(^{\circ}\)C. 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 (15). Affinity-purified antibodies or F(ab\(_2\)) fragments of the respective antibodies preabsorbed to human and mouse proteins, respectively, were used for detection of AT\(_2\), AT\(_1\) receptors, and Gaq/11. Applied antibodies were characterized in previous studies (11, 15–17). 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).

**Receptor Quantification**—The number of AT\(_2\) receptors was determined with membranes (50 \(\mu\)g of protein/point) suspended in phosphate-buffered saline containing 1% bovine serum albumin and 1 mM EDTA (including protease inhibitor mixture) by incubation for 4 h at 4 \(^{\circ}\)C with 50 nm \(^{125}\)I-labeled F(ab\(_2\)) fragments of affinity-purified AT\(_2\)-specific antibodies (\(\sim\)1 \(\mu\)Ci) in a total volume of 100 \(\mu\)l in the presence or absence of a 100-fold excess of the unlabeled antibodies to determine nonspecific binding. Receptor-bound antibodies were separated from free antibodies by rapid filtration over glass fiber filters (GF/B; Whatman), followed by washing with ice-cold incubation buffer. Bound radioactivity was measured in a \(\gamma\)-counter. The binding assay was standardized with affinity-purified recombinant AT\(_2\) receptors expressed in Spodoptera frugiperda (SF9) cells infected with a recombinant baculovirus encoding AT\(_2\).

**Patients**—Prefrontal cortex specimens were obtained at autopsy from 10 demented individuals with clinical diagnosis of (probable) AD according to NINCDS-ADRDA criteria (male/female ratio 4:6, average age 73 \pm 2 years, range 63–81 years, postmortem interval 3–10 h) and from 10 nondemented patients (male/female ratio 5:5, average age 71 \pm 2 years, range 60–78 years, postmortem interval 4–10 h) used as a control group. Informed consent was obtained from all patients and control individuals (or subjects’ relatives), and the study was approved by the Ethical Committee, Faculty of Medicine, Ain Shams University.

**Transgenic Animals**—Transgenic mice (Tg2576; Taconic) 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 (18). Nontransgenic mice served as a control group for immunoblotting studies.

**Lentiviral Vector Production**—Vector plasmids were constructed for the production of third generation lentiviruses expressing HA-transglutaminase-2 under control of the cytomegalovirus promoter (Invitrogen). For RNA interference, lentiviral constructs with RNA polymerase II promoter-driven expression of micro-RNAs targeting AT\(_2\) (nucleotides 296–316) or \(\beta\)-galactosidase (nucleotides 1298–1318) were used. The generation of viral particles pseudotyped with the vesicular stomatitis virus G glycoprotein was performed with a four-plasmid system by transient transfection of 293T cells. Forty-eight hours later, the supernatants were collected. Titers of the lentiviral stocks were determined by transduction efficiency of HT1080 cells. High titer stocks were obtained by ultracentrifugation. For stereotactic injection in mice, high titer lentiviral stocks were used (\(>1 \times 10^7\) transduction units/\(\mu\)l).

**Immunotherapy with A\(\beta\)-(1–40) Peptide Immunization**—Immunotherapy of APP\(^{Sw}\) mice with A\(\beta\)-(1–40) peptide immunization was performed essentially as described (19),...
starting at 12 months of age. Prior to immunization, \( \alpha \beta-(1–40) \) (2 mg/ml in phosphate-buffered saline) was incubated for 24 h at 37 °C. Immunization was performed with \( \alpha \beta-(1–40) \) (100 \( \mu \)g/injection) or phosphate-buffered saline (control) mixed 1:1 with complete Freund’s adjuvant. Booster injections in incomplete Freund’s adjuvant followed 2 weeks after the first injection and monthly thereafter up to 18 months. Blood samples were collected prior to immunization and 3 weeks after the initial immunization. Titters of anti-\( \alpha \beta \) antibodies were determined by enzyme-linked immunosorbent assay with serum samples from \( \alpha \beta \)- and control- immunized APP\(^{sw} \) mice. \( \alpha \beta \)-immunized mice, which did not exhibit high titters of anti-\( \alpha \beta \) antibodies, were not included in the study.

At 18 months of age, APP\(^{sw} \) transgenic mice were anesthetized and perfused intracardially with 0.1 \( m \) sodium phosphate buffer, pH 7.2, followed by 4% (w/v) paraformaldehyde in the same buffer. Brains were removed, fixed >48 h in 10% neutral buffered formalin, and paraffin-embedded by standard methods. Immunoblotting and functional studies were performed with hippocampal membrane tissue isolated from nonfixed brain samples.

**Immunohistochemistry, Radioimmunohistochemistry, and Immunofluorescence**—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 (15). Immunohistochemical staining of transglutaminase-2, \( \alpha T2 \) receptor, and \( M1 \) receptor were performed with F(ab)\(^2 \) fragments of preabsorbed affinity-purified polyclonal antibodies (11, 15). Sections were stained for \( \alpha \beta \) plaques with monoclonal antibodies cross-reacting with residues 1–12 of the \( \alpha \beta \) peptide (clone BAM-10; Sigma). Phosphorylated Tau was visualized with AT8 antibodies (Pierce). 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 DFC350FX camera. Plaque burden was analyzed at 18 months of age by computerized quantitative image analysis of immunolabeled hippocampal areas.

Assessment of the site and area of lentivirus infection was performed with paraffin sections obtained from brains of 17-month-old APP\(^{sw} \) mice 5 weeks after injection into the CA3 area of 2 \( \mu l \) of a lentivirus preparation (\(~1 \times 10^7\) transduction units/\( \mu l \)) encoding HA-transglutaminase-2. Lentivirus-driven expression of HA-transglutaminase-2 was visualized by radioimmunohistochemistry applying anti-HA antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), followed by 125I-labeled secondary antibodies (specific activity ~3000 Ci/mmol) and autoradiography.

Immunofluorescence staining of phosphorylated Tau, \( \alpha T2 \), and \( M1 \) receptors was performed with cryosections (8 \( \mu m \)) of postfixed and frozen brains obtained from 13- or 15-month-old APP\(^{sw} \) mice subjected to stress for 4 weeks (short term) or 12 weeks (long term), respectively. For co-localization of \( \alpha T2 \) and \( M1 \), affinity-purified rat anti-\( \alpha T2 \) antibodies and rabbit anti-M1 antibodies were applied (dilution 1:4000), followed by second-
the dark phase, once a week; noise in the room for 3 h, three times a week; flashing light for 30 min, three times a week. Stressed mice with a significant decrease in sucrose preference (≤50% of sucrose consumption compared with nonstressed controls) were included in the study. Stressed mice also exhibited a significant increase in plasma corticosterone levels (11.4 ± 0.6 μg/dl of stressed APP<sup>Sw</sup> mice and 5.2 ± 0.5 μg/dl of nonstressed APP<sup>Sw</sup> mice; n = 12 mice/group). After 28 days of stress, 4 h before the beginning of the dark phase, brains from stressed and nonstressed APP<sup>Sw</sup> mice were removed and processed for immunoblotting, functional studies, and immunohistochemistry as described above. Twelve animals of each subgroup were used for behavioral studies.

Behavioral Studies—We used the standard water maze task (hidden platform) to test for spatial memory (24). 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. All behavioral studies were performed essentially as described (25).

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

Impaired G<sub>A</sub><sub>q/11</sub>-stimulated Signaling on Prefrontal Cortex Specimens of AD Patients—Prefrontal cortex specimens of AD patients with significant levels of “insoluble” amyloid β (Aβ) characteristic of Aβ plaque load (Fig. 1A) were applied to analyze the G-protein signaling defect. Specimens of AD patients were characterized by impaired G<sub>A</sub><sub>q/11</sub>-stimulated signaling, as revealed by significantly reduced phosphoinositide hydrolysis under basal conditions or upon stimulation with the muscarinic receptor agonist carbachol and the G-protein activator GTPγS or AlF<sub>4</sub><sup>-</sup> (Fig. 1B). By contrast, direct stimulation of phospholipases C by calcium was not different between AD cases and nondemented controls (Fig. 1B). Post-mortem time (≤10 h) did not significantly affect the phosphoinositide signal (data not shown). These findings with prefrontal cortex specimens from AD patients confirm previous data of defective G<sub>A</sub><sub>q/11</sub>-stimulated phosphoinositide signaling in AD (4–8).

Oligomeric AT<sub>2</sub> Receptor Receptors in AD Patients—We hypothesized that the neuronal angiotensin II AT<sub>2</sub> receptor could be involved in the G<sub>A</sub><sub>q/11</sub> signaling defect of AD patients, because the AT<sub>2</sub> receptor is one of the few inhibitory receptors capable of disrupting receptor-stimulated G<sub>A</sub><sub>q/11</sub> activation under specific conditions (11). Moreover, several studies link the AT<sub>2</sub> receptor to cerebral functions related to behavior, neuronal apoptosis, and memory (12–14). To determine the AT<sub>2</sub> receptor protein in immunoblot, we applied AT<sub>2</sub>-specific antibodies (11). The AT<sub>2</sub>-specific antibodies cross-reacted with the AT<sub>2</sub> receptor of ~65 ± 5 kDa of receptor-transfected human embryonic kidney (HEK) cells, whereas AT<sub>2</sub> was absent in the mock control (Fig. 1C, left). AT<sub>2</sub> receptors expressed in HEK cells appeared predominantly as a monomer of ~65 kDa, even when receptor expression levels were >5 pmol/mg protein (Fig. 1C, left). In contrast to the monomeric AT<sub>2</sub> receptor of HEK cells, the AT<sub>2</sub> protein on AD specimens showed significant levels of a high molecular mass AT<sub>2</sub> receptor of ~250 kDa under urea-supplemented reducing conditions of SDS-PAGE (Fig. 1C, left versus right). In addition to the high molecular mass AT<sub>2</sub> receptor, the monomeric AT<sub>2</sub> receptor of ~65 ± 5 kDa and minute amounts of an SDS-stable dimeric form of ~130 kDa were detected (Fig. 1C, right). The high molecular mass AT<sub>2</sub> receptor was completely absent on specimens of nondemented control individuals (Fig. 1C, right). Preabsorption of the antibodies with the immunizing antigen abolished the specific interaction confirming antibody specificity (Fig. 1C, right). As a control and in agreement with previous results (7), protein levels of membrane-bound G<sub>A<sub>q/11</sub></sub> were similar between the two groups (Fig. 1C, bottom). Post-mortem time (≤10 h) and gender did not significantly affect AT<sub>2</sub> and G<sub>A<sub>q/11</sub></sub> levels (data not shown).

Together, these findings demonstrate a high molecular mass oligomeric AT<sub>2</sub> receptor protein in AD patients that is absent in nondemented individuals.

In contrast to the AT<sub>2</sub> receptor, the AT<sub>1</sub> receptor on prefrontal cortex specimens of AD patients appeared as a pure monomer when detected in immunoblot with AT<sub>1</sub>-specific antibodies (Fig. 1D). Thus, AT<sub>2</sub> receptors are specifically altered in AD, whereas the closely related AT<sub>1</sub> receptor is not affected.

Sequestration of G<sub>A<sub>q/11</sub></sub> by AT<sub>2</sub> Receptor Oligomers in AD Patients—The oligomeric AT<sub>2</sub> receptor on prefrontal cortex specimens of AD patients bound specifically to G<sub>A<sub>q/11</sub></sub> in the absence of agonist as determined by co-enrichment, whereas AT<sub>2</sub> receptors of control individuals did not interact significantly with G<sub>A<sub>q/11</sub></sub> (Fig. 1E). The interaction of oligomeric AT<sub>2</sub> with G<sub>A<sub>q/11</sub></sub> in AD was paralleled by G<sub>A<sub>q/11</sub></sub> dysfunction as detected by significantly reduced G<sub>A<sub>q/11</sub></sub> activation either under basal conditions or upon stimulation with carbachol (Fig. 1F). These findings suggest that the AT<sub>2</sub> oligomer of AD patients could act as a dominant negative receptor that inhibits G-proteins by sequestration (26).

Aβ Triggers AT<sub>2</sub> Oligomerization in Vivo—Since the previous experiments suggested an involvement of AT<sub>2</sub> oligomers in the G-protein dysfunction of AD patients, we investigated the possibility of a causal relationship between AT<sub>2</sub> oligomers and aggregated Aβ as a prominent feature of AD. Cerebral microinjection of (aggregated) Aβ sufficient to induce symptoms of cognitive impairment and neuronal degeneration (21, 22) into the hippocampus of nontransgenic mice triggered the sequential dimerization/oligomerization of AT<sub>2</sub> receptors in a dose-dependent manner (Fig. 2A). This experiment provides strong evidence for a direct involvement of Aβ in inducing AT<sub>2</sub> oligomers in vivo.

Formation of AT<sub>2</sub> Oligomers in an Animal Model of AD—To further analyze the formation of AT<sub>2</sub> oligomers in vivo, we chose APP<sup>Sw</sup> transgenic mice as an animal model of AD (18). These mice develop Aβ plaques with increasing age (18); however, major symptoms of clinical AD (i.e. neurodegeneration and memory impairment) are not profound in APP<sup>Sw</sup> transgenic mice (25, 27). To enhance the Aβ-dependent neurode-
Generative process as a typical feature of AD, stress was used as an environmental factor that is known to promote the progression of AD in patients and mice (28–30). Stress enhanced the formation of aggregated SDS-insoluble Aβ and SDS-soluble Aβ in APPsw transgenic mice (Fig. 2, B and C). Stress also marginally promoted the amyloidogenic processing of Aβ by decreasing the activity of α-secretase and increasing the activity of β-secretase (Fig. 2, D and E). The stress-induced increase in

**FIGURE 1.** AT$_2$ receptor oligomers and G-protein dysfunction in AD patients. A, immunoblot of “insoluble” Aβ on prefrontal cortex specimens (Cortex) of five AD patients (AD) indicative of plaque load, whereas “insoluble” Aβ was barely detectable in samples of five nondemented control individuals (Controls). Positions of Aβ-(1–40) and Aβ-(1–42) are marked by arrowheads. As a control, preabsorption of the antibodies by the immunizing antigen (+a) abolished the specific staining. B, significantly reduced phosphatidylinositol hydrolysis (PI hydrolysis) on prefrontal cortex membranes of 10 AD patients compared with 10 nondemented controls (Con) under basal conditions and upon stimulation with carbachol (Cch; 1 mM) or the G-protein activator GTPγS (3 μM) or AlF$_4$ (10 μM). PI hydrolysis after direct PLC stimulation by Ca$^{2+}$ (10 μM) was not significantly different. Data represent mean ± S.E., n = 10 patients/group (*, p < 0.03; **, p < 0.003). C, left, immunoblot detection of AT$_2$ receptors with affinity-purified Fab$_2$ fragments of AT$_2$-specific antibodies preabsorbed to human proteins (IB; anti-AT2) on membranes of HEK cells (HEK) overexpressing high levels (~5 pmol/mg) of AT$_2$ (lane 1) compared with mock-transfected cells (lane 2). Right, immunoblot detection of partially enriched AT$_2$ receptors on prefrontal cortex membranes of five AD patients compared with five nondemented control individuals. Arrowheads, oligomeric (O), dimeric (D), and monomeric (M) AT$_2$. Preabsorption of the antibodies by the immunizing antigen abolished the specific staining. The lower panel shows a control immunoblot detecting Gα$_{q/11}$. D, left, immunoblot of AT$_2$, receptors detected with affinity-purified Fab$_2$, fragments of AT$_2$-specific antibodies (anti-AT2) on membranes of HEK cells (HEK) expressing nontransfected cells (lane 2). Right, immunoblot detection of partially enriched AT$_2$ receptors on prefrontal cortex membranes of five AD patients compared with five nondemented control individuals. Preabsorption of the antibodies by the immunizing antigen abolished the specific staining. E, immunoblot of AT$_1$, receptors co-enriched with affinity-purified Gα$_{q/11}$ (AP; IB: anti-q/11; IB: anti-AT2) from prefrontal cortex membranes of five AD patients and five nondemented control individuals. The lower panel shows equal enrichment of Gα$_{q/11}$ (AP, IB: anti-q/11). F, decreased Gα$_{q/11}$ activation on prefrontal cortex membranes of 10 AD patients compared with 10 control individuals as determined by [35S]GTPγS binding under basal conditions and upon carbachol stimulation, followed by immunoaffinity enrichment of Gα$_{q/11}$. Data represent mean ± S.E., n = 10. **, p < 0.001.
aggregated Aβ species was accompanied by the induction of AT2 oligomers (Fig. 2F). In contrast, the AT2 receptors of nonstressed APPSw transgenic mice with significantly lower levels of aggregated Aβ appeared as a monomer and dimer (Fig. 2F). These findings are complementary to the sequential induction of AT2 dimers/oligomers by microinjected Aβ-(1–40) in nontransgenic mice (cf. Fig. 2A).

Progressive Neurodegeneration in Stressed APPSw Mice—In view of the increased Aβ generation and the induction of AT2 oligomers by stress, we asked whether the characteristic AD-related neurodegenerative process was also enhanced in APPSw mice by stress. Four weeks of stress led to increased symptoms of dendritic degeneration in the hippocampal CA1 area of 13-month-old APPSw transgenic mice as revealed by immunohistochemistry with MAP2-specific antibodies (Fig. 3A). Stress also induced DNA-strand breaks, as determined by a TUNEL assay (Fig. 3B). Tau phosphorylation as a marker of degenerating neurons (31) was also significantly higher in the hippocampal CA1–CA3 area of stressed APPSw mice, as determined by immunohistochemistry with AT8 antibodies (Fig. 3C). Quantification with 125I-labeled AT8 antibodies confirmed the increase in phosphorylated Tau levels in the CA1 area of stressed APPSw mice (Table 1). Concomitantly to neurodegenerative symptoms, APPSw transgenic mice with stress-enhanced Aβ aggregation showed significant impairment of a typical CA1-mediated learning task, as measured by the water maze acquisition (Fig. 3D) and memory retention test (Fig. 3E).

Analogously to AD patients (32), Tau phosphorylation preceded neuronal loss in the CA1 area of APPSw mice (Fig. 3F). Quantification with neuron-specific NeuN antibodies showed a decrease of neuronal cell bodies by ~22% in the CA1 area of stressed APPSw mice compared with nonstressed mice (Table 1). Intraneuronal accumulation of phosphorylated Tau was evident in the remaining CA1 neurons (Fig. 3G). In agreement with a potential role of AT2 oligomers in Tau-positive degenerating neurons, immunofluorescence revealed prominent AT2 receptor membrane localization in CA-1 neurons that were

**FIGURE 2.** Aβ-induced AT2 oligomers in mice. A, partially enriched AT2 receptors on hippocampal membranes of nontransgenic mice 7 days after cerebral microinjection of the indicated amounts of Aβ-(1–40) (2 μl) into the hippocampus as determined by immunoblot with F(ab)2 fragments of affinity-purified AT2-specific antibodies (IB: anti-AT2 preabsorbed to mouse proteins. B and C, 4 weeks of stress (Stressed) led to increased levels of SDS-insoluble (B) and SDS-soluble (C) Aβ (Aβ-(1–40) and Aβ-(1–42)) in the hippocampus of 13-month-old APPSw mice compared with nonstressed APPSw mice (Non-stressed). Data represent mean ± S.E., n = 4. **, p < 0.001; *, p < 0.05. D and E, stress decreased the activity of hippocampal α-secretase in APPSw mice (D) and led to increased activity of hippocampal β-secretase (E). Data are given as percentage of control (i.e., the α-secretase or β-secretase activity of nonstressed mice, respectively (i.e. 100%)) and represent mean ± S.E., n = 4, **, p < 0.01. F, formation of AT2 oligomers in the hippocampus of stressed APPSw mice as determined in immunoblot of partially enriched AT2 receptors from hippocampal membranes of stressed APPSw transgenic mice compared with nonstressed APPSw transgenic mice applying F(ab)2 fragments of affinity-purified AT2-specific antibodies preabsorbed to mouse proteins.
AT₂ Receptor Oligomers

AT₂-positive (Fig. 3H). Thus, stress induced AT₂ oligomerization and triggered a pathological sequence of neurodegenerative events in APPsw mice that resembled the AD pathology of patients.

Stress Induces G-protein Sequestration by AT₂ Receptor Oligomers and Gα₁q/11 Dysfunction in APPsw Mice—Similarly to AD patients (cf. Fig. 1E), AT₂ oligomers of stressed APPsw mice sequestered Gα₁q/11, whereas AT₂ receptor monomers and dimers did not interact with Gα₁q/11 (Fig. 4, A and B).

We next analyzed whether the induction of AT₂ oligomers led to Gα₁q/11 impairment. To assess Gα₁q/11 dysfunction, we chose the G(α)ₑq/11-coupled muscarinic M₁ receptor, because impaired G-protein coupling of M₁ correlates with disease severity in AD (8). The muscarinic agonist carbachol stimulated the activation of Gα₁q/11 proteins in the hippocampus of APPsw mice (Fig. 4C, columns 1 and 2), and this signal was predominantly mediated by the M₁ receptor as evidenced by inhibition with the M₁-selective antagonist MT-7 (Fig. 4C, columns 3 and 4). The activation of Gα₁q/11 under basal conditions and after M₁ stimulation was significantly reduced in stressed APPsw mice with Gα₁q/11-sequestering AT₂ oligomers compared with nonstressed APPsw mice (Fig. 4D).

Thus, APPsw mice with stress-enhanced neurodegeneration displayed G-protein-sequestering AT₂ oligomers and Gα₁q/11 dysfunction.

To assess the direct relationship between Gα₁q/11-sequestering AT₂ oligomers and impaired M₁ dysfunction in APPsw mice, we determined the localization of M₁ and AT₂. Immunohistochemistry revealed hippocampal localization of the M₁ receptors in the CA1–CA4 area and the dentate gyrus of APPsw mice with stress-enhanced neurodegeneration (Fig. 4E, left). In contrast, the AT₂ receptor was only prominent in those regions of the hippocampus that also displayed Tau phosphorylation (i.e. CA1–CA3) (Fig. 4E, right; cf. Fig. 3C). Pre-
absorption of the antibodies confirmed antibody specificity (Fig. 4E, lower panels).

**Hippocampal Co-localization of AT2 and Gq/11-coupled M1 Receptors**—We next investigated the co-localization of AT2 and M1. Immunofluorescence revealed extensive co-localization of AT2 and M1 receptors in CA1 neurons of the hippocampus of stressed APPsw mice (Fig. 5A). Notably, all neurons that expressed AT2 were also positive for M1 (Fig. 5A). As a control, preabsorption of the antibodies with the immunizing antigen abolished the specific staining (Fig. 5B).

The expression levels of hippocampal AT2 and M1 receptors were comparable (63 ± 4 fmol/mg protein and 91 ± 7 fmol/mg protein, respectively; n = 4 mice/group; data not shown), and stress did not alter the total number of M1 receptors (91 ± 7 and 89 ± 5 fmol/mg protein of stressed and nonstressed APPsw mice, respectively; n = 4 mice/group; data not shown). Thus, AT2 and M1 receptors are co-localized in the hippocampal CA1 area of APPsw mice with impaired M1-stimulated Gq/11 activation.

**Down-regulation of AT2 Oligomers by RNA Interference Delayed the Development of Gq/11 Dysfunction and Tau Phosphorylation**—To explore whether Gq/11-sequestering AT2 receptors were directly involved in the impairment of Gq/11, we specifically down-regulated the expression of hippocampal AT2 in stressed APPsw mice by RNA interference. Injection of a lentivirus into the CA1 area targeting the APPSw mice was associated with a reduced hippocampal expression of AT2 in stressed APPsw mice compared with nonstressed mice (Fig. 6A). Concomitantly, the amount of AT2 receptor oligomers was substantially reduced (Fig. 6C). Upon down-regulation of AT2, the development of Gq/11 dysfunction was prevented (Fig. 6D). Complementally to previous findings (33), intact M1 receptor signaling upon down-regulation of AT2 was accompanied by significantly lower levels of phosphorylated Tau as a marker of degenerating neurons compared with control mice with impaired M1 receptor signaling (Fig. 6E).

**Down-regulation of AT2 receptor(s) (oligomers) also prevented the stress-induced decrease in α-secretase activity** (Fig. 6F) and delayed the generation of SDS-soluble and -insoluble Aβ (Fig. 6G and H). The latter effects of AT2 oligomers seem to be directly linked to Gq/11 dysfunction, because intact Gq/11-stimulated signaling is known to mediate activation of α-secretase (34, 35). Together, these findings provide strong evidence
for a causal role of AT<sub>2</sub> receptor oligomers in the AD-related G<sub>q/11</sub> dysfunction <em>in vivo</em>.

**Aβ Induces Oxidative and Transglutaminase-dependent Cross-linking of AT<sub>2</sub> Receptors in Vivo**—In view of the pathophysiological importance of AT<sub>2</sub> oligomers, we determined the mechanism of AT<sub>2</sub> oligomerization. A two-step process seemed to underlie the formation of AT<sub>2</sub> oligomers, because cerebral microinjection of Aβ induced the sequential dimerization-oligomerization of AT<sub>2</sub> receptors in a dose-dependent manner (cf. Fig. 2A). Accordingly, nontransgenic mice devoid of aggregated Aβ showed predominantly monomeric AT<sub>2</sub> receptors, whereas APP<sup>Sw</sup> mice with moderate levels of (aggregated) Aβ displayed only monomers and cross-linked AT<sub>2</sub> receptor dimers (Fig. 7, A, lanes 1–4 versus 5–8; cf. Fig. 2, A and F). The initial cross-linking of AT<sub>2</sub> receptors leading to dimers was due to reactive oxygen species (ROS) generated as a consequence of aggregated Aβ (36), because reduction of Aβ plaques or ROS by immunization with an Aβ peptide or by antioxidant treatment with diferuloylmethane, respectively, led to significantly decreased levels of nondissociable (cross-linked) AT<sub>2</sub> receptor dimers (Fig. 7, A (lanes 9–12 and lanes 13 and 14) and B).

We thought that the Aβ-induced oxidative dimerization could assemble AT<sub>2</sub> receptors for cross-linking by a transglutaminase, because transglutaminases are capable of cross-linking preassembled G-protein-coupled receptors (15), and high transglutaminase activity is a characteristic feature of clinical AD with neurodegeneration (37, 38). In agreement with this concept, the formation of AT<sub>2</sub> oligomers was paralleled by the activation/induction of transglutaminase in APP<sup>Sw</sup> mice upon stress-enhanced Aβ aggregation (Fig. 7C, left) and in nontransgenic mice upon cerebral microinjection of more than 10 pg of (aggregated) Aβ (Fig. 7C, right). Vice versa, expression of transglutaminase in nonstressed APP<sup>Sw</sup> transgenic mice (lacking AT<sub>2</sub> oligomers and elevated transglutaminase activity) by stereotactic injection of a lentivirus encoding HA-transglutaminase-2 into the CA3 area of the hippocampus (Fig. 7, D and E) was not only followed by DNA strand breaks as a marker of neuronal degeneration but also induced G<sub>q/11</sub>-sequestering AT<sub>2</sub> oligomers and G<sub>q/11</sub> dysfunction (Fig. 7, F–H). Thus, transglutaminase accounts for the formation of G<sub>q/11</sub>-sequestering AT<sub>2</sub> oligomers <em>in vivo</em> in APP<sup>Sw</sup> mice.

Altogether, the experiments are compatible with a two-step process leading to AT<sub>2</sub> oligomers <em>in vivo</em>. In a first step, AT<sub>2</sub> receptors are oxidized by ROS. In a second step, the assembled AT<sub>2</sub> receptors (dimers) are targeted by activated transglutaminase, resulting in nondissociable tetramers (oligomers). Both steps are related to the pathogenesis of AD by the sequential induction of ROS and transglutaminase activity with increasing doses of (aggregated) Aβ.

**DISCUSSION**

The current study identified and characterized AT<sub>2</sub> receptor oligomers in brains of AD patients and mice. AT<sub>2</sub> receptor oligomers assemble in brain by a two-step process of oxidative and transglutaminase-dependent cross-linking that is triggered consecutively by (aggregated) Aβ in a dose-dependent manner. An initial oxidative cross-linking step accounts for the formation of AT<sub>2</sub> receptor dimers from monomers. The oxidation of AT<sub>2</sub> is linked to the pathogenesis of AD, because oxidation is due to high levels of ROS generated as a consequence of (aggregated) Aβ (i.e., reduction of Aβ plaque burden and antioxidant treatment of APP<sup>Sw</sup> transgenic mice reduced the amount of oxidized AT<sub>2</sub> receptors). By assembling AT<sub>2</sub> receptors, oxidative cross-linking generates the target for the second transglutaminase-mediated cross-linking step leading finally to AT<sub>2</sub> tetramers/oligomers. Relying on activated transglutaminase, the second cross-linking step is also linked to aggregated Aβ and the pathogenesis of AD, because transglutaminase activity is elevated in the brains of AD patients (37, 38), and cerebral microinjection of (aggregated) Aβ or stress-enhanced generation of (aggregated) Aβ in APP<sup>Sw</sup> transgenic mice was accompanied by the activation/induction of transglutaminase.

Aβ-induced AT<sub>2</sub> oligomers seem to contribute to the well known G<sub>q/11</sub> dysfunction in AD: (i) impaired activation of G<sub>q/11</sub> in prefrontal cortex specimens of AD patients and hip-
AT2 Receptor Oligomers

FIGURE 6. Down-regulation of AT2 oligomers by RNA interference delayed the development of Goq11 dysfunction and Tau phosphorylation. A, AT2 protein expression (anti-AT2) was determined by immunohistochemistry in the CA1 area of stressed APPsw mice injected with a control lentivirus targeting β-galactosidase (left; Control RNAi) or with a lentivirus targeting AT2 by RNA interference (right; AT2-RNAi; original magnification, ×600). B, M1 receptor protein expression was determined by immunohistochemistry (anti-M1) in the CA1 area of stressed APPsw mice injected with a control lentivirus (left; control RNAi) or with a lentivirus targeting AT2 by RNA interference (right; AT2 RNAi; original magnification, ×600). C, immunoblot detection of AT2 receptors with anti-AT2 antibodies (IB: anti-AT2) in the hippocampus of stressed APPsw mice (n = 4 mice/group) injected with a control lentivirus targeting β-galactosidase (Control-RNAi) or with a lentivirus targeting AT2 by RNA interference (AT2-RNAi). The control immunoblot (bottom) shows similar protein levels of Goq11, of the two groups (IB: anti-Goq11). D, basal and carbachol (Cch)-stimulated Goq11 activation in the hippocampus of stressed APPsw mice injected with a control lentivirus or with a lentivirus targeting AT2 by RNA interference. Data represent mean ± S.E., n = 4 mice/group. *, p < 0.05; **, p < 0.001. E, Tau phosphorylation (top) was determined by immunoblot (IB: AT8) in the hippocampus of stressed APPsw mice (n = 4 mice/group) injected with a control lentivirus or with a lentivirus targeting AT2 by RNA interference. A control immunoblot (IB: anti-Tau; bottom) shows equal protein levels of (dephosphorylated) Tau of the two groups. F, down-regulation of AT2 expression by RNA interference prevented the decrease in the activity of α-secretase of stressed APPsw mice (AT2-RNAi versus Control-RNAi). Data are given as percentage of control (i.e. the α-secretase activity of nonstressed mice (Non-str.) injected with a control lentivirus (i.e. 100%)) and represent mean ± S.E., n = 4. *, p < 0.01. G and H, levels of SDS-soluble (G) and SDS-insoluble Aβ (H) in the hippocampus of stressed APPsw mice injected with a control lentivirus targeting β-galactosidase or with a lentivirus targeting AT2 by RNA interference. Data represent mean ± S.E., n = 4. *, p < 0.05; **, p < 0.002.

The hippocampal tissue of mice with AD-like pathology correlated with the appearance of AT2 oligomers; (ii) the formation of Goq11-sequestering AT2 oligomers was causally linked to the pathogenesis of AD, because (aggregated) Aβ-induced ROS and transglutaminase were required for the formation of AT2 oligomers; and (iii) stereotactic inhibition of cross-linked AT2 oligomers by RNA interference (this work) or a mutated AT2 receptor (see accompanying article (41)) prevented the development of Goq11 dysfunction. By sequestering Goq11 proteins, AT2 oligomers of AD patients and mice seem to act as dominant negative receptors, because Goq11-sequestering AT2 oligomers induced the virtual arrest of a constitutively activated Goq11-protein in vitro (cf. accompanying article (41)).

Although the Goq11-protein defect per se is a well-established hallmark of clinical AD in patients (4–8), its pathophysiological role is barely understood. A direct relationship between Goq11 dysfunction and the pathogenesis of AD leading to neurodegeneration and dementia is suggested by the important role of Goq11 proteins in neuronal survival and memory (9, 10, 39, 40). In addition, many of the cognition-enhancing effects of acetylcholine in mice are mediated by Goq11-coupled muscarinic receptors (39). The M1 receptor is a major target of Goq11 dysfunction of AD patients, and impaired Goq11 coupling of M1 receptors correlates with disease severity (8).

The current study supports a pathophysiological role of the Goq11 signaling defect in AD, because Goq11-sequestering AT2 receptor oligomers seem to be part of the Aβ-induced neurodegenerative process during the progression of AD. (i) A threshold dose of aggregated Aβ triggers the formation of AT2 oligomers from dimers. (ii) The induced AT2 oligomers mediate G-protein sequestration and dysfunction. (iii) The resulting
AT$_2$ Receptor Oligomers

**FIGURE 7.** Aβ induces oxidative and transglutaminase-dependent cross-linking of AT$_2$ receptors in vivo. A, immunoblot of partially enriched hippocampal AT$_2$ receptors of 18-month-old nontransgenic mice (Non-transgenic, lanes 1–4), APP$_{Sw}$ transgenic mice (Non-treated, lanes 5–8) and APP$_{Sw}$ transgenic mice subjected to immunization with the Aβ peptide (Immunized, lanes 9–12) to reduce plaque burden (n = 4 mice/group). The lane marked with + a controls immunoblot specificity. Lane 14 shows hippocampal AT$_2$ of a representative 18-month-old APP$_{Sw}$ mouse treated with the antioxidant diferuloylmethane for 6 months (500 ppm in diet) compared with a nontreated control APP$_{Sw}$ transgenic mouse (lane 13). B, immunization of APP$_{Sw}$ transgenic mice with the Aβ peptide significantly decreased Aβ plaque load compared with nontreated APP$_{Sw}$ mice, as determined by immunohistochemistry with anti-Aβ antibodies (Anti-Aβ). Shown are brain sections representing the median degree of pathology as determined with 4 mice/group (original magnification, ×20). C, left, increased hippocampal transglutaminase activity of APP$_{Sw}$ transgenic mice subjected to stress (Stressed) compared with control APP$_{Sw}$ transgenic mice housed in standard conditions (Non-stressed). Data are from eight mice in each group. *, p < 0.0001. Right, cerebral injection of Aβ induced activation/induction of hippocampal transglutaminase in nontransgenic mice. Data are from 4 mice/group. *, p < 0.001. D, expression of HA-transglutaminase in the hippocampus of a representative APP$_{Sw}$ mouse 5 weeks after unilateral injection into the CA3 area of a lentivirus encoding HA-transglutaminase was determined by radioimmunohistochemistry applying anti-HA antibodies (anti-HA) followed by 125I-labeled secondary antibodies and autoradiography. E, expression of transglutaminase in the hippocampal CA3 area of nonstressed APP$_{Sw}$ mice injected with a transglutaminase-2-encoding lentivirus (Anti-Transglut.; left) or with a control lentivirus encoding β-galactosidase (Control; right) was determined by immunohistochemistry with transglutaminase-2-specific antibodies (Anti-Transglut.; original magnification, ×600). F, DNA strand breaks were determined by in situ TUNEL labeling in the CA3 area of the hippocampus of APP$_{Sw}$ mice injected with a transglutaminase-encoding lentivirus (Transglut.; left) or with a control lentivirus (right; original magnification, ×600). G, co-enrichment of oligomeric AT$_2$ receptors with affinity-purified Go$_{q/11}$-specific antibodies (AP/IB+q/11) from the hippocampus of four APP$_{Sw}$ transgenic mice injected with a transglutaminase-encoding lentivirus (Transglut.) compared with mice receiving an injection of a control lentivirus. The lower panel shows equal enrichment of Go$_{q/11}$ from both groups (AP/IB+q/11). H, reduced hippocampal activation of Go$_{q/11}$ in APP$_{Sw}$ transgenic mice injected with a transglutaminase-2-encoding lentivirus compared with mice injected with a control lentivirus. Data represent mean ± S.E., n = 4 mice/group. *, p < 0.003.

Impairment of neuroprotective Go$_{q/11}$-dependent signaling (10, 40) may enhance the neurodegenerative process, as revealed by a decreased Tau phosphorylation and Aβ aggregation upon down-regulation of AT$_2$ oligomers. And indeed, additional experiments confirmed a role of Go$_{q/11}$-inhibitory AT$_2$ receptor oligomers in neurodegeneration (i.e., specific inhibition of AT$_2$ oligomerization by an AT$_2$ mutant delayed the development of neurodegenerative symptoms in “AD mice”) (cf. accompanying article (41)). Aβ-induced AT$_2$ oligomers may thus constitute a previously unrecognized signature of ongoing neurodegeneration in AD.

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