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Defective Tumor Necrosis Factor-α-dependent Control of Astrocyte Glutamate Release in a Transgenic Mouse Model of Alzheimer Disease*

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The cytokine tumor necrosis factor-α (TNFα) induces Ca^{2+}-dependent glutamate release from astrocytes via the downstream action of prostaglandin (PG) E_2. By this process, astrocytes may participate in intercellular communication and neuromodulation. Acute inflammation *in vitro*, induced by adding reactive microglia to astrocyte cultures, enhances TNFα production and amplifies glutamate release, switching the pathway into a neurodamaging cascade (Bezzi, P., Domercq, M., Brambilla, L., Galli, R., Schols, D., De Clercq, E., Vescovi, A., Bagetta, G., Kollias, G., Meldolesi, J., and Volterra, A. (2001) *Nat. Neurosci.* 4, 702–710). Because glial inflammation is a component of Alzheimer disease (AD) and TNFα is overexpressed in AD brains, we investigated possible alterations of the cytokine-dependent pathway in PDAPP mice, a transgenic model of AD. Glutamate release was measured in acute hippocampal and cerebellar slices from mice at early (4-month-old) and late (12-month-old) disease stages in comparison with age-matched controls. Surprisingly, TNFα-evoked glutamate release, normal in 4-month-old PDAPP mice, was dramatically reduced in the hippocampus of 12-month-old animals. This defect correlated with the presence of numerous β-amyloid deposits and hypertrophic astrocytes. In contrast, release was normal in cerebellum, a region devoid of β-amyloid deposition and astrocytosis. The Ca^{2+}-dependent process by which TNFα evokes glutamate release in acute slices is distinct from synaptic release and displays properties identical to those observed in cultured astrocytes, notably PG dependence. However, prostaglandin E_2 induced normal glutamate release responses in 12-month-old PDAPP mice, suggesting that the pathology-associated defect involves the TNFα-dependent control of secretion rather than the secretory process itself. Reduced expression of DENN/MADD, a mediator of TNFα-PG coupling, might account for the defect. Alteration of this neuromodulatory astrocytic pathway is described here for the first time in relation to Alzheimer disease.

Studies over the last 15 years provide new insight in the neuron-astrocyte interrelations, revealing that astrocytes do not merely support neuronal functions but also actively control them (for review, see Refs. 2–6). Rapid bidirectional communication between neurons and astrocytes was first revealed in the hippocampus, where transmitters released from nerve terminals stimulate surface receptors and trigger intracellular calcium ($[Ca^{2+}]_i$) elevation in neighboring astrocytes (7–11). In turn, an increased astrocyte $[Ca^{2+}]_i$ may trigger feedback or feed-forward signaling to neurons via active release of gliotransmitters, notably the excitatory amino acid glutamate (1, 12, 13). Hippocampal astrocytes contain synaptic-like microvesicles apt at releasing glutamate via Ca^{2+}-dependent exocytosis (14–16). This regulated process may serve physiological functions, including control of neuronal excitability and synaptic transmission (17–23). However, in pathological conditions the same mechanism may become de-regulated and produce deleterious effects on neurons (1). Among the different stimuli able to trigger glutamate release from astrocytes is the cytokine tumor necrosis factor α (TNFα)[3] (1). This molecule induces glutamate release from astrocyte cultures via the stimulation of p55 TNF receptor type 1 (TNFR-1) and the activation of a complex Ca^{2+}-dependent process involving the action of prostaglandin E_2 (PGE_2) (1). We have demonstrated that, if reactive microglia is co-cultured with astrocytes, to mimic an acute glial inflammation, the TNFα levels increase dramatically. This causes a strong amplification of the TNFα-dependent glutamate release from astrocytes to an extent that can induce slow apoptotic neuronal cell death (1).

Starting from these observations, the aim of the present work was to evaluate whether alterations of astrocytic glutamate release and astrocyte-neuron coordination can take place in the context of a specific brain pathology, Alzheimer disease (AD). Indeed, a well documented chronic inflammatory glial cell reaction around β-amyloid (Aβ) plaques (24–26) as well as elevated levels of TNFα were described in the brain of AD subjects (27). We utilized transgenic mice ubiquitously overexpressing the familial AD-linked human V717F mutation of the amyloid precursor protein (APP) gene (PDAPP mice (28)). These animals show age-related deficits in spatial learning and memory retention (29, 30) and reproduce several neuropathological features of human AD, notably deposition of Aβ plaques and associated reactive gliosis (28, 31–37). Therefore, they provide a good experimental model to study changes occurring in astrocytes during the development of the disease. The

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[3] The abbreviations used are: TNFα, tumor necrosis factor-α; PGE_2, prostaglandin E_2; AD, Alzheimer disease; $[Ca^{2+}]_i$, intracellular calcium; Aβ, β-amyloid; APP, amyloid precursor protein; WT, non-transgenic littermates; GFAP, glial fibrillary acidic protein; BAPTA/AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester); COX, cyclooxygenase; Baf A1, bafilomycin A1; TeNT, tetanus neurotoxin; TNFR-1, TNF receptor-1; DENN/MADD, differentially expressed in normal versus neoplastic/mitogen-activated protein kinase activating death domain.

properties of Ca^{2+}-dependent glutamate release evoked by stimulation of the TNFα-dependent pathway were evaluated in brain slices from PDAPP mice at early and late stages of the disease and compared with those of age-matched non-transgenic littermates. We report a region-specific alteration of TNFα-dependent glutamate release in the hippocampus of aged PDAPP mice correlated to the presence of Aβ deposits and reactive astrocytosis. At variance with the observations in the acute inflammation paradigm *in vitro*, in the PDAPP mice the cytokine-dependent process was found to be dramatically impaired.

EXPERIMENTAL PROCEDURES

Animals—Homozygous transgenic mice carrying the APP^{V717F} familial AD mutation under the control of the platelet-derived growth factor-β promoter (PDAPP, 4-month-old animals, $n = 14$; 12-month-old animals, $n = 17$) (28) and age-matched non-transgenic littermates (WT) (4-month-old animals, $n = 17$; 12-month-old animals, $n = 19$) were provided by Eli Lilly (Indianapolis, IN). These mice are derived from a hybrid background representing combinations of C57BL/6J x DBA/2J x Swiss-Webster strains.

Materials—Percoll was purchased from Amersham Biosciences. TNFα was from R&D Systems. The anti-pan β-amyloid antibody was purchased from BIOSOURCE. The antibody anti-glial fibrillary acidic protein (GFAP) was from DAKO. The anti-differentially expressed in normal *versus* neoplastic (DENN)/mitogen-activated protein kinase activating death domain (MADD) polyclonal antibody was kindly provided by Dr. Ulrich Blank (INSERM U699, Paris, France). All the other chemicals were from Sigma-Aldrich.

Acute Brain Slice—Acute brain slices were prepared as previously described (1, 13). Briefly, animals were killed, their brains were removed, and the two hemispheres were separately processed for preparing acute slices or for immunohistochemistry. The hemi-brain used for preparing the slices was immersed in ice-cold artificial cerebrospinal fluid of the composition 120 mM NaCl, 3.1 mM KCl, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 4 mM glucose, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM sodium pyruvate, 0.5 mM *myo*-inositol, 0.1 mM ascorbic acid, pH 7.4, bubbled with a 95% O_2, 5% CO_2 gas mixture. Transverse thin slices (200 μm) from brain or cerebellum were prepared using a Vibratome (Campden Instruments). Hippocampal slices were carefully isolated from the coronal brain section by gently removing the extraneous cortical tissue. Slices were subsequently placed in a chamber thermostated at 37 °C and containing artificial cerebrospinal fluid continuously bubbled with 95% O_2, 5% CO_2 for at least 1 h before processing.

Synaptosomal Preparation—Synaptosomes from mouse hippocampus were prepared using the Percoll gradient method (38). Briefly, 15–20 hippocampi/experiment were removed, placed in ice-cold sucrose buffer (0.32 M sucrose, 5 mM Hepes, pH 7.4), and homogenized in a Teflon glass homogenizer (12 times at 2800 rpm). The homogenate was centrifuged at 1000 × *g* for 5 min, and the resulting pellet was resuspended and spun again at 15,700 × *g* for 10 min. Supernatants were pooled, layered onto discontinuous sucrose-Percoll gradients (2.5 ml of 23% Percoll, 3 ml of 10%, and 2.5 ml of 3%) and centrifuged at 38,600 × *g*. The synaptosomal fraction was recovered from the 23%, 10% Percoll interface. Synaptosomes were washed twice in 30 volumes of ice-cold Krebs buffer (135 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 12.5 mM Hepes, 10 mM glucose, pH 7.4) and stored in suspension at 4 °C before use (≤6 h).

Cell Cultures—Astrocyte-pure cultures (>99% of GFAP-positive cells) and microglia-pure cultures (>99% cells positive for the *Griffonia Simplicifolia* isolectin B4) were obtained as described (1). Briefly, confluent monolayers of cultured astrocytes from the cerebral cortex of newborn animals were depleted of microglial cells by mechanical shaking and treatment with the microglial toxin L-leucine methyl ester (7.5 mM, 12 h). Free-floating microglia were collected from shaken astrocyte flasks, purified by pre-plating on plastic dishes for 1 h, seeded on 35-mm dishes (300,000 cells/dish), and used in experiments within 12–24 h. Cultures enriched in mature, galactocerebroside-positive oligodendrocytes were obtained by differentiation of the bipotential CG-4 glial precursor cell line as described by Louis and co-workers (39). Finally, mixed neuron-glia cultures were obtained from embryonic day 16 mouse brains. Hippocampi were dissociated by treatment with trypsin (0.25%) plus DNase (0.05%) in Hanks' balanced salt solution (HBSS, 15 min, 37 °C). The reaction was stopped with 10% fetal bovine serum followed by gentle mechanical pipetting. Cells were then plated at a density of 3×10^5 onto astrocyte monolayers. Cultures were then grown in Dulbecco's modified Eagle's medium supplemented with 10% horse serum and N2 (Invitrogen) and treated on day 3 with cytosine arabinoside (5 μM; 24 h) to prevent glial proliferation. In part of the experiments we depleted the neuronal component of the mixed neuron-glia cultures (40). Briefly, at 7 days *in vitro*, cultures were exposed to *N*-methyl-D-aspartate (300 μM) for 1 h in a Mg^{2+}-free medium and utilized 24 h later, after all neurons had died, and debris was removed by several pipette washes.

Enzymatic Assay of Endogenous Glutamate Release—Efflux of endogenous glutamate from synaptosomal suspensions, cell cultures, or tissue slices in response to various stimuli was monitored on-line by a specific enzymatic assay, as previously described (1, 13). Briefly, each type of preparation was lodged in a 1 × 1-cm cuvette (1.3-ml volume: Hellma Italia s.r.l., Italy) inside a LS55 computerized spectrofluorometer (PerkinElmer Life Sciences) at 37 °C under continuous stirring in a buffer containing 120 mM NaCl, 3.1 mM KCl, 1.25 mM NaH_2PO_4, 25 mM Na-HEPES, 1 mM $MgCl_2$, 4 mM glucose, 2 mM $CaCl_2$ at pH 7.4 with glutamate dehydrogenase (15.5 units/ml) and 1 mM $NADP^+$. Synaptosomal pellets were resuspended in Krebs buffer and, shortly before the assay, preincubated for 30 min at 37 °C with bovine serum albumin (16 μM) to bind any released free fatty acids. Glutamate released from the preparations was immediately oxidized by glutamate dehydrogenase to α-ketoglutarate with formation of NADPH and fluorescence emission at 430 nm (excitation light 335 nm). This procedure allows reliable detection of the glutamate released without important subtraction by re-uptake even in thin slices (41). Release was quantified referring to standard curves constructed with exogenous glutamate, and values were normalized relative to the total protein content of each sample. Agonists inducing glutamate release were added directly in the cuvette. Agents acting intracellularly, such as 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA/AM), cyclooxygenase (COX) inhibitors, tetanus neurotoxin (TeNT), and bafilomycin A1 (Baf A1), required a pretreatment that was usually performed by incubating brain slices in artificial cerebrospinal fluid at 37 °C under constant bubbling with 95% O_2, 5% CO_2. BAPTA/AM and COX inhibitors were preincubated for 30 min, whereas TeNT required 40 min. The effect of Baf A1 was assessed using two different experimental protocols; hippocampal slices were (*a*) exposed to the drug for 2 or 6 h in the dark and stimulated with agonists inducing glutamate release at the end of the incubation period or (*b*) exposed to the drug for 2 h and stimulated 2 times with an agonist (either TNFα or PGE_2) at the start and at the end of the incubation period.

Immunohistochemistry—Brain hemispheres used for histological analyses were fixed in Carnoy solution (ethanol, chloroform, glacial acetic acid, 6:3:1) and embedded in paraplast. 5-μm-thick serial sections from paraplast-embedded blocks were stained with hematoxylin-eosin, cresyl violet for Nissl substance, and thioflavin S for amyloid. For immu-

nohistochemistry, sections were incubated with a polyclonal antibody to a synthetic peptide homologous to residues 1–40 of human amyloid-β peptide (anti-pan β-amyloid, 1:800, overnight) or a polyclonal antibody against GFAP (1:800, overnight). Before Aβ-immunostaining, the sections were pretreated with guanidine thiocyanate (3 M, 25 °C, 30 min). Immunoreactivity for GFAP was enhanced by pretreatment with 4% formaldehyde solution. Immunoreactions were revealed by Envision system (DAKO, Denmark) for rabbit immunoglobulins using 3–3′-diaminobenzidine as chromogen.

The relative area of GFAP immunoreactive structures was measured on the 5-μm-thick sections immunostained with anti-GFAP antibody using a Nikon Eclipse E800 microscope equipped with a color video camera (Nikon DMX 1200) and a computer-based image analysis system (Lucia Measurement, Version 4.60, Laboratory Imaging, Czech Republic). After establishing a density threshold, the software calculated the percentage of area occupied by the reaction product by dividing the area of immunopositivity by the total area. Using a 20× objective, we measured 6–8 fields in the hippocampus of 4- and 12-month-old PDAPP and control mice.

To quantify the number of astrocytes present in the hippocampus, sections were immunostained with anti-GFAP antibody and visualized with a Cy3-conjugated secondary antibody (1:400, Jackson Immunoresearch Laboratories). Nuclei were subsequently labeled with Hoechst 33342 staining (10 μg/ml, 15 min). Immunopositive cells were counted in 6 different fields per each hippocampus using a 40× objective mounted on Axioskop 40 fluorescence microscope.

Reverse Transcription-PCR Analysis—Total RNA was extracted from astrocyte-pure cultures using RNeasy Mini kit according to manufacturer's guidelines (Qiagen, Italy). 1 μg of extracted RNA was reverse-transcribed using RETROscript according to the manufacturer's instructions (Ambion Europe Ltd). 1 μl of the resulting cDNA was amplified using DENN/MADD (sense, 5′-CAGCCCATCCATTATCGAGTC; antisense, 5′-CTCCACTTCACTGGCATCTCCC) and β-actin (sense, 5′-GTGGGCCGCTCTAGGCACAA; antisense, 5′-CTCTTTGATGTCACGCACGATTTC) primers.

Western Blot Analysis—Samples were electrophoresed through 7.5% SDS-polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (Amersham Biosciences). DENN/MADD was detected by incubation with 1 μg/ml polyclonal anti-DENN/MADD antibody followed by horseradish peroxidase-conjugated swine anti-rabbit IgG (DAKO, 5000-fold dilution in TBST (10 mM Tris·HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) containing 1% dry milk) at room temperature for 1 h. The immune complex was visualized by enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Pierce). Membranes were stripped and incubated with an anti-β-actin monoclonal antibody (Sigma-Aldrich). Appropriate film exposures were acquired with Versadoc 3000 (Bio-Rad) and quantified with Quantity-One 4.4.1 software.

Statistical Analyses—Student's *t* test was used for comparisons between two groups; one-way analysis of variance followed by Scheffe's *F*-test was used for *post hoc* comparisons of multiple groups. Analyses were performed with Statview Version 1.2.

RESULTS

TNFα-dependent Glutamate Release in Hippocampal Slices—Previous work in our laboratory established that stimulation of TNFR-1 with TNFα evokes glutamate release from astrocyte-pure cultures via a Ca^{2+}-dependent process that requires the production of PGE_2 (1). Here we show that the cytokine evokes an analogous release from acute hippocampal slices (Fig. 1*A*) and that such a glutamate release has a non-

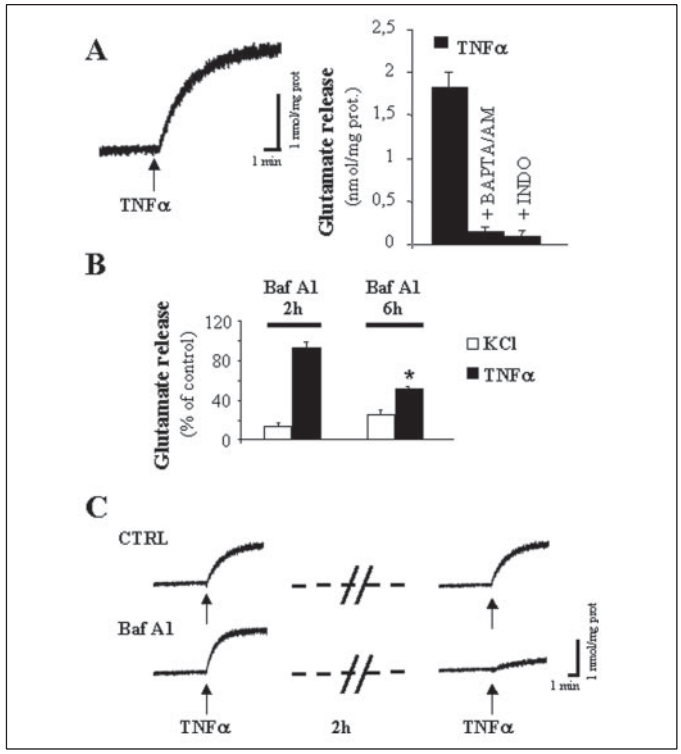

FIGURE 1. **TNFα-dependent glutamate release in hippocampal slices: pharmacological characterization.** *A*, *left*, representative fluorescence trace of the rapid glutamate release response induced by application of TNFα (30 ng/ml). *Right*, TNFα-induced glutamate release is inhibited in the presence of either BAPTA/AM (50 μM, 30 min) or indomethacin (INDO 5 μM, 30 min). Data are expressed in nmol/mg of tissue protein (mean ± S.E.; $n = 3$–10 for each condition). *B*, effect of Baf A1 (1 μM) on the glutamate release responses induced by KCl (20 mM, *white histograms*) or TNFα (30 ng/ml, *black histograms*). Hippocampal slices were pre-exposed to Baf A1 for either 2 h (*left*) or 6 h (*right*). Data are expressed as percentage (mean ± S.E.) of the glutamate release responses induced by KCl (2.11 ± 0.11 nmol/mg of protein) or TNFα (1.42 ± 0.03 nmol/mg of protein) in untreated slices; $n = 3$ for each experimental condition. The *asterisk* highlights significant decrease of TNFα-evoked glutamate release in slices incubated for 6 h in the presence of Baf A1 with respect to slices similarly incubated in control conditions ($p < 0.001$, Student's *t* test). *C*, Baf A1 abolishes TNFα-induced glutamate release in pre-stimulated slices. Representative glutamate release responses induced in hippocampal slices by two sequential stimulations with TNFα (*arrows*) separated by 2 h of incubation in the absence (*CTRL*) or presence of Baf A1.

neuronal, most likely astrocytic origin. First, we directly demonstrate that the soluble cytokine does not evoke glutamate release from nerve terminals. In these experiments we used synaptosomal suspensions prepared from mouse hippocampus. As expected, both $[Ca^{2+}]_i$ elevation with the Ca^{2+} ionophore, ionomycin (2 μM), and membrane depolarization with high K^+ (20 mM) elicited a large glutamate release response from the synaptosomes (Fig. 2, *A* and *B*). In contrast, TNFα (30 ng/ml; $n = 6$) did not induce detectable glutamate release (Fig. 2*A*). Second, we confirm that TNFα does not elicit detectable glutamate release from neurons by studies in hippocampal cultures. Thus, administration of the cytokine (30 ng/ml) to mixed hippocampal cultures induced a rapid glutamate release response (1.22 ± 0.18 nmol/mg of protein, $n = 3$), whose extent was identical to the response obtained from the same cultures after selective depletion of the neuronal component (see "Experimental Procedures"; 1.20 ± 0.08 nmol/mg of protein, $n = 3$). Third, by comparing the glutamate release responses to TNFα in purified cultures of different glial cell types, astrocytes, microglia, or oligodendrocytes (see "Experimental procedures"), we find that the cytokine (30 ng/ml) evokes glutamate release exclusively from astrocytes (1.32 ± 0.2 in nmol/mg of protein from astrocytes; 0.04 ± 0.02 in nmol/mg of protein from microglia; 0.02 ± 0.02 in nmol/mg of protein from oligodendrocytes; $n = 4$–6 for each cell culture type). Finally, we show that

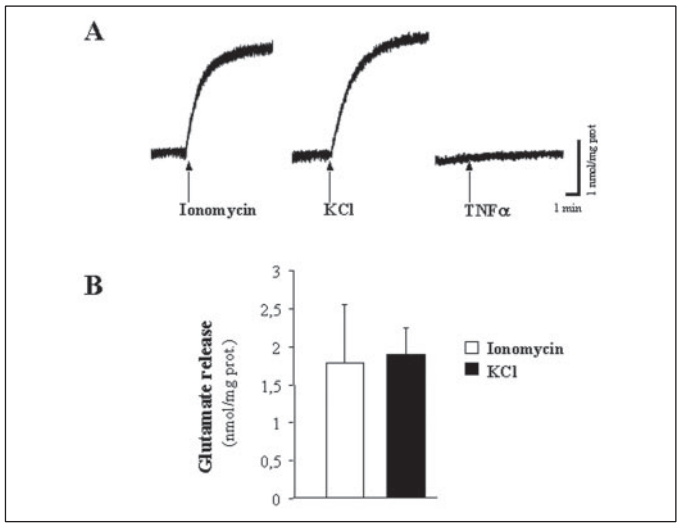

FIGURE 2. **TNFα does not induce glutamate release from nerve terminals.** *A*, representative fluorescence traces of glutamate release from hippocampal synaptosomes in response to ionomycin (2 μM), KCl (20 mM), or TNFα (30 ng/ml). *B*, the histogram indicates the glutamate release induced by ionomycin (2 μM, $n = 3$) and KCl (20 mM, $n = 6$) in hippocampal synaptosomes. The amount of glutamate released is expressed in nmol/mg of tissue protein.

the pharmacological properties of the release processes evoked by TNFα in cultured astrocytes (1) and in hippocampal slices are identical. In this respect the release *in situ* was drastically inhibited by preincubating the slices with the intracellular Ca^{2+} chelator BAPTA/AM (50 μM, 30 min), confirming the expected Ca^{2+} dependence of the process (Fig. 1*A*). Furthermore, in cultured astrocytes, TNFα induces the formation of PGE_2, which is essential for the glutamate-releasing effect of the cytokine (1). Therefore, we next tested whether TNFα-evoked glutamate release in hippocampal slices was sensitive to inhibitors of the PG-forming enzymes, the cyclooxygenases. In slices pre-exposed for 30 min to 5 μM indomethacin, glutamate release in response to TNFα was nearly abolished (Fig. 1*A*). In addition, two other chemically unrelated COX blockers, aspirin (10 μM) and ibuprofen (70 μM), potently inhibited TNFα-dependent glutamate release (−84 ± 6%, $n = 3$ and −86 ± 7%, $n = 3$, respectively).

Another property of the glutamate release evoked by TNFα from cultured astrocytes is sensitivity to long exposures with blockers of neuronal exocytotic release such as tetanus neurotoxin and bafilomycin A1 (1). In the present experiments we focused on the effects of Baf A1 (Fig. 1, *B* and *C*), which prevents uptake of transmitter into synaptic vesicles by blocking vesicular H^+-ATPase (42, 43). Given that hippocampal astrocytes contain microvesicles expressing proton-dependent vesicular glutamate transporters (VGLUTs (14)) and Baf A1 inhibits VGLUT-dependent glutamate uptake (44), the drug is expected to inhibit TNFα-dependent glutamate release if this requires that the transmitter is taken up into exocytic vesicles. A 2-h exposure of the slices to 1 μM Baf A1 almost abolished neuronal glutamate release in response to high K^+ (−85.5 ± 3%; Fig. 1*B*, see the legend for absolute values), confirming the efficacy of the drug in depleting the transmitter content of recycling synaptic vesicles (45). Baf A1 partly inhibited also TNFα-evoked release; however, with a slower time course: after a 2-h exposure the release was only weakly affected, and after 6 h it was inhibited by 49 ± 3% (Fig. 1*B*). Because there may be concerns about the specificity of an inhibitory effect that develops after several hours, we set up a different experimental protocol. Basing on the hypothesis that Baf A1 acts on the refilling of glutamatergic vesicles, we thought that if we induced glutamate release during the incubation with Baf A1 the drug effectiveness would increase, because of the speeding up of the recycling and refilling process (45). Therefore, hippocampal slices were stimulated 2 times with 30 ng/ml TNFα, waiting 2 h between the two stimulations. In control experiments without Baf A1, the first and second glutamate release responses to TNFα were identical, indicating that the slices responded reliably over time to challenges with the cytokine (Fig. 1*C*; first response, 1.49 ± 0.05; second response, 1.36 ± 0.04 nmol/mg of protein.; $n = 4$). In contrast, when slices were challenged in the presence of Baf A1, the second response to TNFα was nearly abolished (−93.2 ± 7%; $n = 6$; Fig. 1*C*); the first one, induced at the beginning of the exposure to Baf A1, was normal (1.47 ± 0.1 nmol/mg of protein; $n = 6$). If the inhibitory action of Baf A1 depends on rapid recycling and refilling of exocytic vesicles, then stimulating TNFα-dependent glutamate release before starting the exposure to Baf A1 should modify the efficacy of the drug even if the inter-challenge interval (2 h) remains constant. Indeed, in experiments when slices were challenged with TNFα 10 min before the start of Baf A1 incubation, the second response to TNFα was significantly less inhibited (−49 ± 8%, $n = 5$). These data indicate that the inhibitory action of Baf A1 takes place rapidly after an initial release of glutamate has occurred. We, therefore, propose that Baf A1 prevents the rapid refilling of vesicles and the subsequent exocytosis of glutamate induced by a second challenge with TNFα.

Amyloid-β Peptide Deposition and Astrocytosis in PDAPP Transgenic Mice—The cytokine TNFα is overexpressed in the brain of AD patients (27). Furthermore, Aβ peptides cause TNFα release from microglia in culture (46, 47).

On the basis of these observations, we next investigated whether the TNFα-dependent glutamate release *in situ* was affected during disease progression in PDAPP mice (28). Functional and histological experiments were performed in parallel using the two brain hemispheres of each animal independently, one for immunohistochemistry and the other for glutamate release assays.

For histopathological analyses, brain sections from 4- and 12-month-old homozygous PDAPP or non-transgenic littermates were immunolabeled with a polyclonal antibody against human Aβ (anti-pan β; Fig. 3) to assess the characteristics of age-dependent deposition of Aβ in our PDAPP mice. The patterns observed were significantly reproducible within groups of animals. No Aβ immunostaining was detected in WT mice at both ages (Fig. 3, *A* and *C*), and only rare Aβ deposits were present in the cingulate cortex, frontal cortex, and in the hippocampus of 4-month-old PDAPP mice (Fig. 3*B*). In contrast, 12-month-old PDAPP mice showed numerous Aβ deposits of different size in the above brain structures, with a diffuse deposition of Aβ in the outer molecular layer of the dentate gyrus (Fig. 3*D*). Most Aβ-immunoreactive deposits showed no fluorescent staining after thioflavin S treatment. Quantitative analysis of Aβ burden in the hippocampus of our PDAPP mice gave values within the previously reported range (data not shown (34, 48)). Also in agreement with previous reports (33, 35), Aβ immunoreactivity was below detection levels in the cerebellum of WT and PDAPP animals at all ages examined (Fig. 3, *E* and *F*).

In sections from the same animals, GFAP immunostaining was performed to obtain parallel information on the astrocytes. In the molecular cortical layer, paraventricular white matter and hippocampus of WT mice (both 4- and 12-month-old), GFAP-positive cells showed scanty cytoplasm and thin and short processes. GFAP immunoreactivity was slightly more pronounced in 4-month-old WT mice than in those 12 months old (Fig. 4, *A* and *C*). In 4-month-old PDAPP mice, GFAP immunoreactivity was similar to WT mice of the same age (Fig. 4*B*). In contrast, 12-month-old PDAPP mice showed increased GFAP immunoreactivity in the hippocampus and in the cerebral cortex. GFAP-

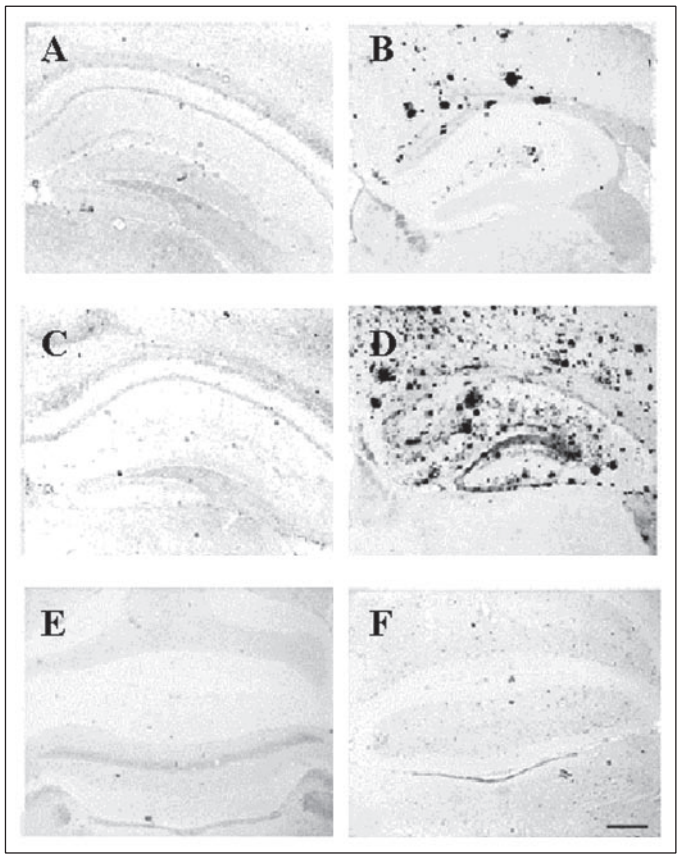

FIGURE 3. **Age- and area-dependent Aβ deposition in the brain of PDAPP mice.** Aβ immunohistochemical analysis in hippocampal sections of 4-month-old (*panel A*, WT; *panel B*, PDAPP) and 12-month-old mice (*panel C*, WT; *panel D*, PDAPP). Note the increasing Aβ deposition in the cortex and hippocampus of PDAPP mice from rare deposits in 4-month-old animals to diffuse plaques in 12-month-old mice. Cerebellar sections from 12-month-old WT (*E*) and PDAPP (*F*) animals show no Aβ plaques similar to those observed in the hippocampus. *Scale bar*, 500 μm.

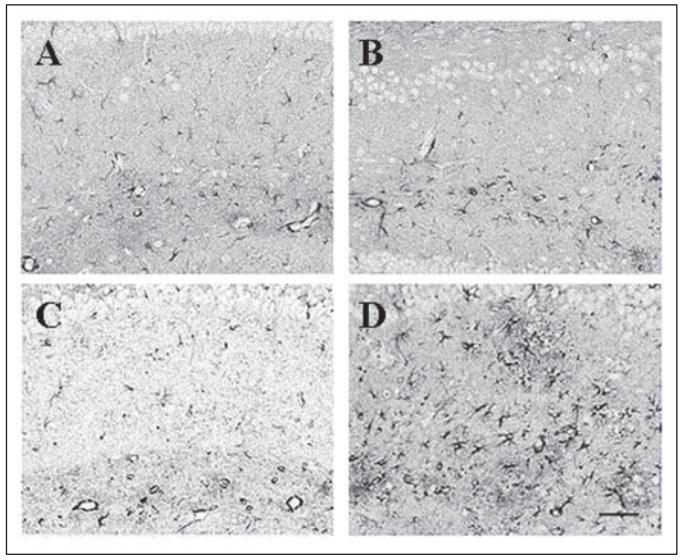

FIGURE 4. **Astrocytosis in the hippocampus of 12-month-old PDAPP mice.** GFAP immunoreactivity in the hippocampus of 4-month-old (*panel A*, WT; *panel B*, PDAPP) and 12-month-old mice (*panel C*, WT; *panel D*, PDAPP). Note the different arrangement and morphology of the GFAP-positive cells in 12-month-old WT and PDAPP mice. *Scale bar*, 20 μm.

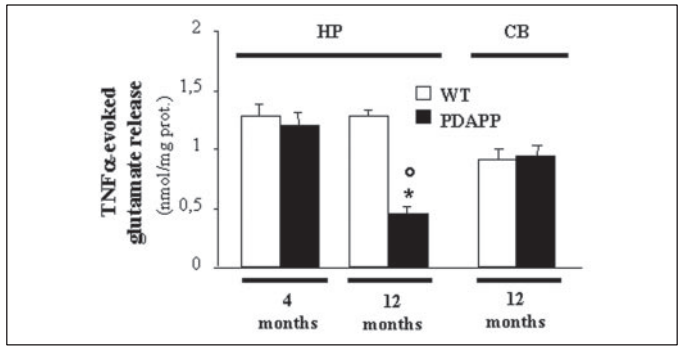

FIGURE 5. **Comparison of the glutamate release responses evoked by TNFα in wild-type and PDAPP mice of 4 and 12 months of age.** Histograms indicate the glutamate release induced by TNFα (30 ng/ml) in hippocampal slices (*HP*) of 4-month-old (*4 months*) animals and in both hippocampal and cerebellar (*CB*) slices of 12-month-old (*12 months*) animals. The amount of glutamate released is expressed in nmol/mg of tissue protein (mean ± S.E.). *White bars*, WT mice: *black bars*, PDAPP mice; $n = 36–44$ for each experimental group. *Symbols* denote a significant reduction of the TNFα-evoked glutamate release in the hippocampus of 12-month-old PDAPP mice with respect to both 4-month-old PDAPP mice and 12-month-old WT mice ($p < 0.01$, one-way analysis of variance followed by Scheffe's *F*-test for multiple comparisons). In contrast, the glutamate release responses induced by TNFα in the cerebellum of 12-month-old WT and PDAPP mice are not statistically different ($n = 9–11$).

positive cells displayed typical features of hypertrophic astrocytes, with an enlarged cytoplasm and thick processes often surrounding the Aβ deposits (Fig. 4*D*). Morphometric analysis was utilized to obtain a more quantitative estimation of the surface occupied by GFAP immunoreactivity in the hippocampus of 12-month-old animals. In WT mice the relative surface of GFAP-positive tissue ranged between 0.5 and 1.1% ($0.73 \pm 0.29\%$, $n = 4$), whereas that in PDAPP mice ranged from 3.6 to 9.8% ($7.77 \pm 2.82\%$; $n = 4$; $p < 0.03$, two-tailed Student's t test) of the total hippocampal surface. Noteworthy, such an increased surface occupation by GFAP immunoreactivity was not paralleled by a significant difference in the number of GFAP-immunopositive cells in the hippocampus of PDAPP mice (21.2 ± 0.7 cells/field; $n = 6$) compared with 12-month-old WT (22.6 ± 0.44 cells/field; $n = 6$). Moreover, the difference in GFAP expression seen in the hippocampus of PDAPP mice was not observed in other areas such as thalamus, cerebellum, and brainstem (data not shown). These data indicate that Aβ deposition in the hippocampus is accompanied by GFAP overexpression and the transformation of astrocytes into the reactive phenotype.

Alteration of TNFα-dependent Glutamate Release in PDAPP Mice—The TNFα-dependent glutamate release process was studied in acute hippocampal slices from 4- and 12-month-old PDAPP mice and age-matched WT mice (Fig. 5, *HP*). Similar responses were evoked by TNFα in 4-month-old WT and PDAPP mice. However, 12-month-old PDAPP mice showed a major impairment of the TNFα-induced process compared with both non-transgenic littermates of the same age and 4-month-old PDAPP mice ($-64 \pm 5\%$ and $-62 \pm 6\%$, respectively).

To assess whether the defect of glutamate release in aged PDAPP mice is related to the region-specific deposition of Aβ or to ubiquitous overexpression of the mutant amyloid precursor protein, we checked whether the defect was present in a different brain region. We selected the cerebellum because, in 12-month-old PDAPP mice this area is devoid of Aβ deposits and reactive astrocytes (Fig. 3, *E* and *F*). Similar to the effect induced in the hippocampus, the cytokine evoked a rapid glutamate release response from acute cerebellar slices of 4-month-old WT animals (1.12 ± 0.11 nmol/mg of protein, $n = 4$). Also the pharmacological properties of this response were similar to those observed in the hippocampus (Fig. 1*A*; Ref. 1), notably the response was strongly inhibited in the presence of BAPTA/AM (50 μM, 30 min) (data not shown). Based on this evidence, we compared TNFα-evoked glutamate

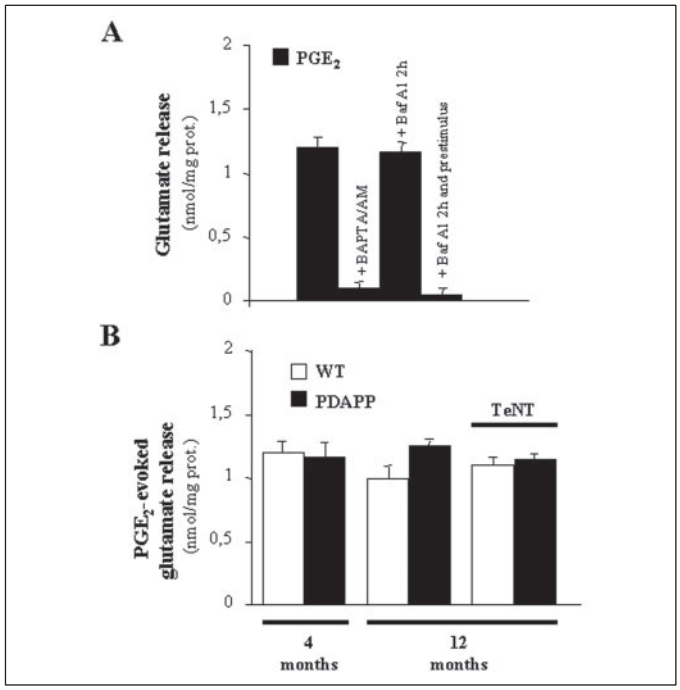

FIGURE 6. **PGE_2-evoked glutamate release in hippocampal slices: pharmacological analogy with the process stimulated by TNFα and lack of defect in the 12-month-old PDAPP mice.** *A*, PGE_2 (50 μM) induces glutamate release from mouse hippocampal slices that is inhibited in the presence of BAPTA/AM (50 μM, 30 min). Exposure to Baf A1 (1 μM, 2 h) inhibits PGE_2-evoked glutamate release in slices stimulated with the PGE_2 (50 μM) at the beginning of the incubation (Baf A1, 2 h and prestimulus) but not in unstimulated slices (Baf A1, 2 h). Prestimulation has no effect in control slices (not shown). Data are expressed in nmol/mg of tissue protein (mean ± S.E.); $n = 10–15$ for each experimental condition. *B*, PGE_2-evoked glutamate release from the hippocampus of wild-type and PDAPP mice. The histogram represents glutamate release responses to PGE_2 (in nmol/mg of protein; mean ± S.E.) from hippocampal slices of 4-month-old (*4 months*) or 12-month-old (*12 months*) animals. Acute slices from 12-month-old mice were either untreated or treated with TeNT (100 μg/ml, 40 min.). *White bars*, WT mice; *black bars*, PDAPP mice; $n = 13–25$ for each experimental group.

release in cerebellar slices from 12-month-old WT and PDAPP mice. Unlike in the hippocampus, no significant differences were observed between the two genotypes (Fig. 5, *CB*). Lack of difference was not due to reduced release in 12-month-old WT animals, as the response in those animals was comparable with that observed in 4-month-old mice.

Altered Signal Transduction Control of Glutamate Release in PDAPP Mice—In cultured astrocytes, PGE_2 participates to the mechanism that couples TNFR-1 activation to Ca^{2+}-dependent glutamate release (1). The fact that TNFα-evoked glutamate release in hippocampal slices is blocked by COX inhibitors (Fig. 1*A*) suggests that the process *in situ* involves analogous molecular events. Thus, in the next set of experiments we investigated whether the PGE_2-dependent signaling was involved in the defect observed in 12-month-old PDAPP mice.

First of all, we confirmed that PGE_2 reproduces the effects induced by TNFα. Thus, hippocampal slices, but not synaptosomes, responded with rapid and large glutamate release (1.20 ± 0.09 nmol/mg of protein; $n = 6$) when stimulated with 50 μM PGE_2. Such response was strongly inhibited in the presence of BAPTA/AM (50 μM, 30 min, Fig. 6*A*; see also Ref. 13). Moreover, the response to the PG was abolished in slices exposed to Baf A1 (1 μM, 2 h) provided that the pre-stimulation paradigm described for the experiments with TNFα was utilized (Fig. 6*A*). Therefore, the same pharmacological properties associate Ca^{2+}-dependent glutamate release induced by PGE_2 to the one evoked by TNFα. A striking difference was, however, observed when we analyzed PGE_2-induced glutamate release responses in the hippocampus of 12-month-old animals, as no defect could be evidenced in PDAPP mice compared with age-matched WT mice. Moreover, the responses induced by PGE_2 in both genotypes were of similar amplitude whatever the age examined (Fig. 6*B*). We explored the possibility that the lack of defect in the release of glutamate evoked by PGE_2 was due to a contribution of Ca^{2+}-dependent exocytosis from neuronal cells. We, therefore, repeated the experiments after preincubation of the slices with tetanus neurotoxin (100 μg/ml) for 40 min, a treatment known to abolish neuronal exocytosis selectively (13). Indeed, in this situation, high K^+-evoked neuronal exocytosis was fully blocked (high K^+, 2.08 ± 0.08 nmol/mg of protein, $n = 14$; high K^+ in TeNT 40 min, undetectable, $n = 13$). However, PGE_2 induced an equal amount of glutamate release as the one evoked in the absence of TeNT in the same slices (Fig. 6*B*) (13). These data exclude a neuronal contribution to the PGE_2-evoked glutamate release, suggesting that the alteration observed in PDAPP mice involves the signal transduction process in astrocytes, notably the events controlling PG formation, rather than the coupling between PG signaling and glutamate release.

In an attempt to outline the molecular step responsible for the signal transduction defect, we focused on DENN/MADD, a protein involved in transducing TNFα signaling downstream to activation of TNFR-1 (see Fig. 8). The reasons for focusing on DENN/MADD are 2-fold: (i) by binding TNFR-1 through the death domain, this protein activates multiple signaling pathways, including the one responsible for phosphorylation and activation of cytosolic phospholipase A_2, the enzyme deputed to arachidonic acid release and prostaglandin production (49), and (ii) reduced levels of DENN/MADD have been recently reported in the hippocampi from Alzheimer subjects (50). By using a specific non-commercial antibody and Western blot analysis we found that (*a*) DENN/MADD immunoreactivity is significantly reduced in the hippocampi (−52.81 ± 16.26%; Fig. 7*A*, *HP*) but not in the cerebella (+1,50 ± 12.77%; Fig. 7*A*, *CB*) of 12-month-old PDAPP mice ($n = 7$) compared with age-matched WT mice ($n = 7$), and (*b*) in cell cultures, DENN/MADD is present not only in neurons, as reported previously (50), but also in astrocytes at the mRNA as well as at the protein level (Fig. 7, *B* and C). These data suggest that reduced levels of DENN/MADD in astrocytes of PDAPP mice could lead to a defective TNFα-PGE_2 coupling.

DISCUSSION

An altered expression of both the cytokine TNFα and its receptor TNFR-1 has been reported in the brains from AD patients (27, 50). The present work identifies in the hippocampus of aged PDAPP mice a pathology-related alteration of the Ca^{2+}-dependent glutamate release process evoked by this cytokine.

Because TNFR-1 is expressed on neuronal as well as on glial cells (51–53), we first investigated the cellular origin of the glutamate release. Three independent lines of evidence exclude a neuronal origin: (*a*) depletion of the neuronal component of mixed hippocampal cultures did not affect the TNFα-evoked release; (*b*) TNFα did not evoke glutamate release from hippocampal synaptosomes; (*c*) the TNFα-evoked release from hippocampal slices was different in a number of properties from the Ca^{2+}-dependent process of synaptic origin induced by depolarization with high K^+. These include dependence on PG signaling and differential sensitivity to exocytosis blockers. The latter properties match those of the Ca^{2+}-dependent process evoked by the cytokine from cultured astrocytes (but not from other glial cells, microglia, or oligodendrocytes). Therefore, we conclude that the TNFα-dependent glutamate release *in situ* originates from astrocytes.

The described results with Baf A1 suggest that the release occurs via exocytosis. The issue of whether astrocytes *in situ* possess such a finely

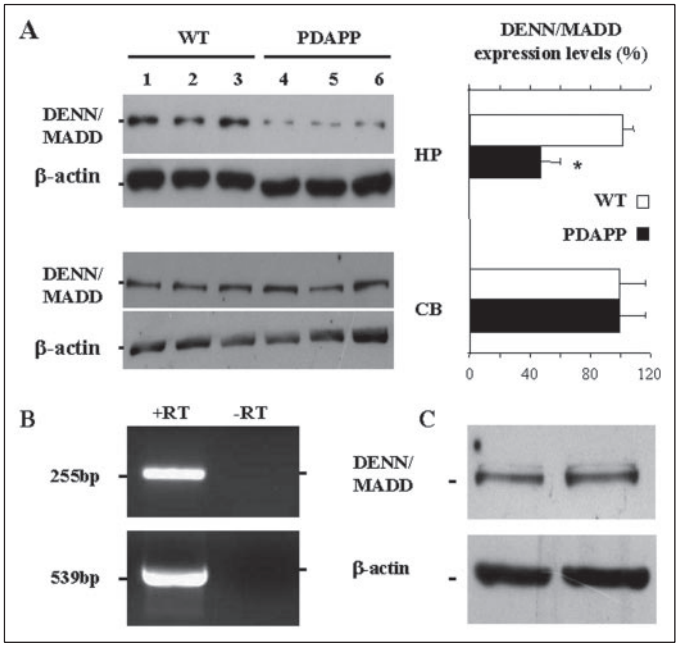

FIGURE 7. **DENN/MADD expression in astrocyte cultures and down-regulation in the hippocampus of 12-month-old PDAPP mice.** *A*, *left*, representative immunoblots of DENN/MADD in the hippocampus (*HP*) and cerebellum (*CB*) of three different WT (*lanes 1–3*) and PDAPP (*lanes 4–6*) mice at the age of 12 months. Samples (5 μg of total protein) were immunoblotted with antibodies specific for DENN/MADD or β-actin. *Right*, Western blots were quantified by densitometric analysis. Values were normalized relative to β-actin and expressed as percentage of DENN/MADD expression levels in WT mice; *white bars*, WT mice: *black bars*, PDAPP mice. The *asterisk* indicates a significant reduction of DENN/MADD in the hippocampus of PDAPP mice ($n = 7$) with respect to WT mice ($n = 7$) of the same age ($p < 0.05$, Student's t test). By contrast, the levels of expression of DENN/MADD in the cerebellum of 12-month-old WT ($n = 7$) and PDAPP ($n = 7$) mice were not statistically different. *B*, expression of DENN/MADD mRNA in cultured astrocytes; reverse transcription (*RT*)-PCR analysis was performed on total RNA from astrocyte-pure cultures in the presence (+*RT*) or absence (−*RT*) of reverse transcriptase. *C*, Western blot analysis reveals DENN/MADD expression in cultured astrocytes. 20 μg of total protein were loaded in each lane. The sample was loaded in duplicate.

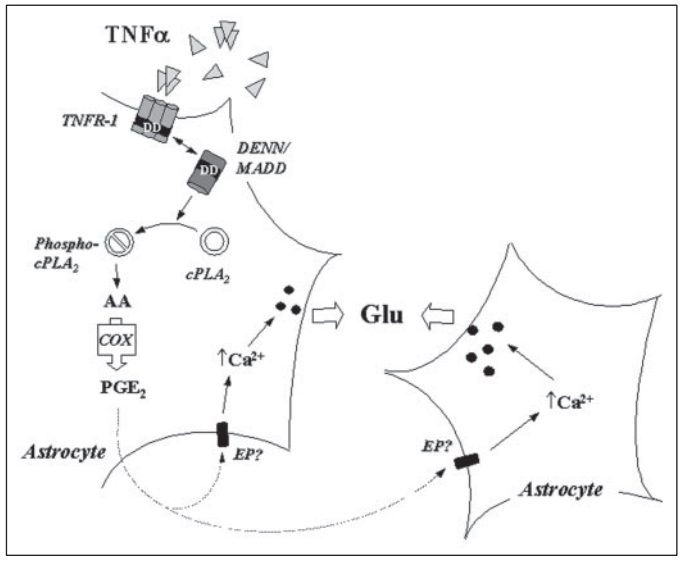

FIGURE 8. **Hypothetical scheme of the signaling events coupling TNFR-1 activation to glutamate release from astrocytes (see also Ref. 1).** *DD*, death domain; *cPLA$_2$*, cytosolic phospholipase A$_2$; *AA*, arachidonic acid; *EP*, E-type prostanoid receptor.

regulated process has been the subject of debate (54). However, there is now evidence that hippocampal astrocytes contain synaptic-like microvesicles expressing vesicular glutamate transporters (14–16). Baf A1 is known to inhibit vesicular transporter-mediated uptake of transmitter into synaptic vesicles by blocking vesicular H^+-ATPase and abolishing the proton gradient that drives the uptake process, eventually resulting in the release of transmitter-depleted vesicles (45). We confirmed the capacity of Baf A1 to inhibit synaptic glutamate release by showing that the drug nearly abolishes the response to high K^+ stimulation. However, Baf A1 also inhibited TNFα-dependent release under specific conditions. Although high K^+-evoked release was abolished within 2 h, in line with the reported time course of inhibition of miniature excitatory postsynaptic currents (45), TNFα-dependent release was inhibited more slowly, by 50% after 6 h. If glutamate release was pre-stimulated with TNFα at the same time as exposure to Baf A1, the drug, however, inhibited the release completely within 2 h. Interestingly, anticipating the pre-stimulation 10 min before the start of the incubation with Baf A1 significantly reduced the inhibitory effect of the drug. These observations indicate that the action of Baf A1 is catalyzed by glutamate release and takes place soon after it, strongly suggesting that Baf A1 acts by blocking the refilling of recycled vesicles. In conclusion, these data imply that Ca^{2+}-dependent glutamate release from hippocampal slices in response to TNFα, and similarly to PGE$_2$, takes place via exocytosis of vesicular glutamate transporter-expressing vesicles (14). As a consequence, the data with Baf A1 also suggest that spontaneous exocytosis of astrocytic vesicles occurs less frequently than spontaneous exocytic events at the synapses.

A dramatic reduction of the glutamate release response to TNFα stimulation is observed in the hippocampus of 12-month-old PDAPP mice, *i.e.* when these animals display typical age-dependent histological lesions and cognitive deficits reminiscent of AD (28, 29). In contrast, normal glutamate release responses, fully comparable with those of age-matched wild-type animals, are observed in 4-month-old PDAPP animals, *i.e.* when hippocampi are almost devoid of Aβ deposits and reactive astrocytes.

Normal responses are observed also in the cerebellum of 12-month-old PDAPP mice, an area spared of Aβ plaque burden (33, 35) and reactive astrocytes. Therefore, the impairment of glutamate release is directly correlated with the pathology affecting the hippocampus. Hippocampal astrocytes of 12-month-old PDAPP mice display a morphologically reactive phenotype, enhanced GFAP expression, and often make direct contact with Aβ plaques. It is, therefore, tempting to speculate that the identified alteration is associated with the phenotypic switch to the reactive state. Very little is known about the consequences that such a switch has on the rapid signaling properties of astrocytes. Interestingly, a recent study reports that, upon assuming a reactive phenotype in response to an acute traumatic lesion, astrocytes *in situ* lose their capacity to undergo calcium oscillations (55). This is an important observation because calcium oscillations are known to trigger glutamate release events (11, 18, 22, 56).

In aged PDAPP mice, a reduced glutamate release is observed in response to TNFα stimulation but not to PGE$_2$ stimulation. PGE$_2$ must, however, act via the same secretory process activated by TNFα in astrocytes. Thus, (*a*) abolishing neuronal exocytosis does not affect PGE$_2$-evoked glutamate release and (*b*) the PGE$_2$-evoked process has pharmacological properties identical to the TNFα-evoked process. Because the latter is abolished by COX inhibitors, PGE$_2$ must act at a step downstream of TNFR-1 activation. These observations seem to exclude a defect of the secretory process itself and point to an impairment of the stimulus secretion coupling mechanism between TNFα signaling and PGE$_2$ production.

Expression of both TNFα and TNFR-1 is enhanced in the brains from AD subjects (27, 50). One of the molecular events accompanying such

up-regulation is the down-regulation of a downstream signaling mediator, the DENN/MADD protein (50). This event may have crucial consequences for the TNFα-PGE_2 coupling because DENN/MADD is known to stimulate the arachidonic acid cascade by inducing activation of cytosolic phospholipase A_2 (Fig. 8) (49). We confirmed that in the hippocampus of 12-month-old PDAPP animals, as in the brains of AD subjects (50), the levels of DENN/MADD are reduced. Moreover, at variance with the observations of Del Villar and Miller (50), who indicated that DENN/MADD is expressed only in neurons, we found clear evidence that the protein as well as its mRNA is expressed also in astrocytes at least in culture. This finding reinforces a possible link between the AD-dependent defect of this protein and the defect of the astrocytic cascade here identified. The reason why Del Villar and Miller (50) could not observe DENN/MADD immunoreactivity in astrocytes may depend on the use of a different antibody that detected only the more pronounced neuronal expression.

Although more work is necessary to directly confirm the role of DENN/MADD in the alteration of TNFα-induced glutamate release, the present data indicate that the signal transduction mechanism that links TNFα to glutamate release is highly sensitive to pathological changes of the astrocytic environment. We have previously reported an opposite effect, *i.e.* enhanced glutamate release *in vitro* in a model of acute inflammation where reactive microglia was added to astrocyte cultures (1). Noteworthy, such an effect was observed within hours after plating microglia onto astrocytes, *i.e.* on a time-scale and in a pathological context completely different from the one in PDAPP mice, where a chronic inflammatory glial reaction accompanies the slow progression of the AD-like pathology for months. Further studies will clarify the temporal relations between alteration of glutamate release and glial inflammation in the PDAPP mice.

Recent work highlights the potential pathogenetic relevance of astrocyte alterations in AD, notably in relation to their specific interactions with Aβ. Chemically attracted to Aβ deposits, astrocytes specifically internalize and degrade amyloid-β peptides (57). This digestive function, apparently requiring apolipoprotein E (58), could be lost or perturbed in AD, with important consequences for the progression of the disease. Additional astrocyte alterations can be important for the evolution of the pathology. In particular, these cells are now known to exert a critical control on synapses, *i.e.* on their formation, maintenance, and strength, by active release of soluble factors (59–63). Notably, astrocyte-released TNFα controls the strength of excitatory synapses in the hippocampus (62, 63). Moreover, astrocytes dynamically regulate neurotransmission by rapid release of modulatory "gliotransmitters" (for review, see Refs. 2–6). Specifically, by the Ca^{2+}-dependent glutamate release process triggered by TNFα, they modulate neuronal excitability, synchronicity, and synaptic transmission in hippocampal circuits (11, 17–23),[4] particularly vulnerable in AD (64). Because of the importance of astrocyte signaling in the control of synaptic functions and of the catastrophic consequences of synaptic failure on cognitive function in AD (for review, see Refs. 65), the impairment of TNFα-dependent glutamate release reported herein may, therefore, represent a new and relevant pathological mechanism in AD.

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Defective Tumor Necrosis Factor-α-dependent Control of Astrocyte Glutamate Release in a Transgenic Mouse Model of Alzheimer Disease

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