THE PRESENCE OF THE APP\textsuperscript{SWE} MUTATION IN MICE DOES NOT INCREASE THE VULNERABILITY OF CHOLINERGIC BASAL FOREBRAIN NEURONS TO NEUROINFLAMMATION

G. L. WENK,* K. MCGANN-GRAMLING AND B. HAUSS-WERZYNIAK

Division of Neural Systems, Memory and Aging, University of Arizona, 350 Life Sciences North Building, Tucson, AZ 85724, USA

Abstract—Neuroinflammation, and elevated levels of inflammatory proteins, such as tumor necrosis factor-α, and the deposition of β-amyloid may interact to contribute to the pathogenesis of Alzheimer’s disease. We reproduced a component of the neuroinflammatory state within the basal forebrain cholinergic system, a region that is vulnerable to degeneration in Alzheimer’s disease, of transgenic Tg2576 mice that express the Swedish double mutation of the human amyloid precursor protein (APPswe). We have previously shown that basal forebrain cholinergic neurons are selectively vulnerable to the consequences of neuroinflammation. In the current study, tumor necrosis factor-α was infused into the basal forebrain region of APPswe and nontransgenic control mice for 20 days with the expectation that the presence of the transgene would enhance the loss of cholinergic neurons. Chronic infusion of tumor necrosis factor-α significantly decreased cortical choline acetyltransferase activity, reduced the number of cholinergic cells and increased the number of activated astrocytes and microglia within the basal forebrain. The presence of the APPswe gene did not enhance the vulnerability of forebrain cholinergic neurons to the chronic neuroinflammation. Furthermore, combined treatment of these mice with memantine demonstrated that the neurotoxic effects of tumor necrosis factor-α upon cholinergic cells did not require the activation of the N\text-superscript-methyl-D-aspartate receptors. In contrast, we have previously shown that memantine was able to provide neuroprotection to cholinergic forebrain neurons from the consequences of exposure to the inflamмogen lipopolysaccharide.

These results provide insight into the mechanism by which neuroinflammation may selectively target specific neural systems during the progression of Alzheimer’s disease.

Key words: neuroinflammation, transgenic mice, glia, basal forebrain, acetylcholine.

Among the most important challenges in research on Alzheimer’s disease (AD) today are to define why specific neural systems and selected brain regions are affected with the progression of the symptoms of dementia and to determine what mechanisms will slow the progression of these processes. AD is characterized by specific neuro-pathological changes, including widespread chronic inflammation, a profound degeneration of cholinergic neurons within the basal forebrain and the presence of β-amyloid deposits (Whitehouse et al., 1981; Selkoe, 1998; Akiyama et al., 2000). The decline in the number of cholinergic cells within the basal forebrain may contribute to aspects of the cognitive impairments associated with AD. Neuroinflammation may play an important role in the pathogenesis of AD (Aisen and Davis, 1994; Akiyama et al., 2000) and the degeneration of the basal forebrain cholinergic system (Wenk et al., 2000). For example, head trauma in humans is a significant risk factor for AD (Rasmussen et al., 1995) and is associated with increased levels of inflammatory proteins (Griffin et al., 1998) and a decline in the number of basal forebrain cholinergic neurons (Murdoch et al., 1998). The brains of AD patients have elevated levels of inflammatory proteins, such as tumor necrosis factor-α (TNF-α; McGeer and McGeer, 1998; Griffin et al., 1989) that may contribute to the neurodegenerative process (Blasko et al., 1997; Gahtan and Overmier, 1999) as well as to the pattern of degeneration (Cagnin et al., 2001). For example, those brain regions that demonstrated the greatest degree of microglial activation early in the disease progression ultimately showed the highest rate of atrophy and appearance of pathology (Cagnin et al., 2001).

We have infused the proinflamмagen lipopolysaccharide (LPS) into the basal forebrain of rats to reproduce aspects of the chronic neuroinflammation seen in the AD brain. This inflammation leads to a selective degeneration of cholinergic neurons that could be attenuated by chronic treatment with either anti-inflammatory drugs or glutamate receptor antagonists (Wenk and Willard, 1998; Willard et al., 1999; Wenk et al., 2000). We hypothesize that TNF-α is the primary agent responsible for the selective cell death of cholinergic neurons following infusion of LPS (Wenk and Hauss-Werzywniak, 2003; Wenk et al., 2003). A recent study found that chronic infusion of TNF-α alone into the basal forebrain of young mice increased the number of activated microglia and astrocytes and decreased the number of cholinergic neurons (Wenk et al., 2003). Although chronic inflammatory processes may contribute to the overall pathogenesis of AD, the specific mechanism underlying the degeneration of basal forebrain cholinergic cells in the AD brain is unknown. A potential role for neuroinflammation, and the specificity of its effects upon cholinergic neurons, was suggested by a study that iso-
lated antibodies from the sera of AD patients that selectively recognized and destroyed basal forebrain cholinergic cells when injected into a rat brain (Foley et al., 1988).

β-Amyloid deposits can act as an inflammatory stimulus (Matsuoka et al., 2001) to activate glia to release cytokines and initiate a cascade of processes that are toxic to nearby cells (Goossens et al., 1995; Mrak et al., 1995). Recent hypotheses suggest that soluble β-amyloid fibrils may play a critical role in the long term induction of a chronic inflammatory condition that exposes the brain to low levels of cytokines for many decades (Lambert et al., 1998) possibly leading to a progressive decline in cholinergic function (Buttini et al., 2002). Transgenic mice that overexpress the human gene for β-amyloid develop plaques that are associated with activated microglia and astrocytes and other biomarkers of inflammation (Benzing et al., 1999; Matsuoka et al., 2001), as well as the degeneration of cholinergic fibers (Boncristiano et al., 2002; Lüth et al., 2003); however, the number of cholinergic neurons in the basal forebrain is unaltered (Jaffar et al., 2001; Boncristiano et al., 2002).

Given this evidence, the current study investigated the interaction of the effects of inflammation in the presence of elevated expression of the amyloid precursor protein (APP) gene within a brain region known to be vulnerable to degeneration in AD, i.e., the basal forebrain cholinergic system. We predicted that the toxic action of TNF-α would be synergistic with the presence of the transgene for APP. Finally, we determined whether the neurotoxic mechanism of TNF-α upon basal forebrain cholinergic neurons depended upon the activation of N-methyl-D-aspartate (NMDA)-sensitive glutamate receptors; an open-channel low-affinity, uncompetitive NMDA receptor channel antagonist, 1-amino-3,5-dimethyladamantane hydrochloride (memantine), was administered to mice that were infused with TNF-α. Memantine has been used previously to demonstrate the role of the NMDA receptor in the neurotoxicity due to inflammation induced by an infusion of LPS (Willard et al., 2000).

**EXPERIMENTAL PROCEDURES**

**Subjects**

A total of 30 mice were involved in this study. Fifteen young (approximately 3 months old, 30 g) male, transgenic mice (tg: B6SJ-TgN(APP_6925)/2576; Taconic Transgenics, Germantown, NY, USA) and 15 young (age-matched) male non-transgenic (wt: B6SJLF1; Taconic) mice were housed singly in a colony room with a 12-h light/dark cycle with lights off at 10:00 a.m. and food and water provided ad libitum. Three months old tg2576 mice do not demonstrate amyloid deposition (Benzing et al., 1999) but do show a three-fold elevation in β-amyloid_{1–40} (Lehman et al., 2003).

**Surgical procedures**

Each mouse was anesthetized with isoﬂurane gas and placed in a stereotactic instrument equipped with an appropriate nose cone attachment to deliver a constant supply of isoﬂurane and oxygen during the surgical procedure. The mouse rested upon a heating pad to maintain its body temperature. This research was conducted under the supervision, and with the approval, of the University of Arizona Institutional Animal Care and Use Committee. All efforts were taken in order to minimize the number of animals used and their level of discomfort. The scalp was shaved, incised and retracted and a hole was drilled in an appropriate location in the skull with a dental drill. An Alzet (Palo Alto, CA, USA) osmotic minipump (model 1002; to deliver 0.25 μl/h for approximately 20 days, as estimated by information provided by Alzet) containing TNF-α (20 ng/μl; Sigma, St. Louis, MO, USA) was implanted into the dorsal abdomen and attached via Tygon tubing (0.060 inch O.D.) to a chronic indwelling cannula (Model 3280PM, osmotic pump connect, 28 gauge; Plastics One, Inc., Roanoke, VA, USA) that had been positioned stereotaxically so that the cannula tip extended into the left basal forebrain at the following coordinates: AP: +1.2 mm from Bregma; ML: +1.2 mm from midline suture; and DV: −5.2 mm from the top of the skull. Our previous study demonstrated that this dose of TNF-α can produce a significant decline in the number of cholinergic neurons within this brain region (Wenk et al., 2003). Control mice were infused with the artificial cerebrospinal fluid (CSF) vehicle: (in mM) 140 NaCl; 3.0 KCl; 2.5 CaCl_2; 1.0 MgCl_2; 1.2 Na_2HPO_4; pH 7.4. The cannula was secured into place by dental cement that was anchored around a small screw inserted into the skull. The scalp was then closed around the top of the low profile pump connect. A volume overload to the brain was minimal using this procedure because the 0.25 μl/h administered contributes only about 0.2% of the total CSF volume produced by the mouse each hour and is only 0.12% of the mouse’s total CSF volume. A second Alzet osmotic minipump (model 1002) was implanted on the opposite side of the mouse’s lower back and administered either saline or memantine. These pumps allow a steady state level of the drug to be achieved in the plasma and brain. The following six groups of five mice/group, 12 weeks old at the beginning of the experiment, were prepared: CSF-infused into wt (wt CSF); CSF-infused into tg (tg CSF); TNF-α-infused into wt (wt TNF); TNF-α-infused into tg (tg TNF); TNF-α-infused into wt, memantine (10 mg/kg/day; wt TNF+Mem); TNF-α-infused into tg, memantine (10 mg/kg/day; tg TNF+Mem). The dose of memantine was determined according to our previous experience with this drug in neuroprotection studies involving the basal forebrain cholinergic system (Wenk et al., 1996, 1997; Willard et al., 2000).

**Biochemistry**

Twenty days after surgery the mice were deeply and quickly anesthetized using isoﬂurane gas and killed by decapitation. Tissue samples were taken from right and left frontal motor neocortex and then assayed separately for choline acetyltransferase (ChAT) activity by the formation of [1-14C]acetylcholine from [1-14C]acetyl-coenzyme-A and choline (Fonnum, 1969). The ChAT enzyme is specific for cholinergic cells; its decline is used as a standard measure of cholinergic cellular dysfunction in the basal forebrain. The frontal motor sensorimotor cortex was chosen for examination of ChAT activity because preliminary studies have found that the cholinergic cells at the coordinates of the injections send afferents to this region of the cortex (Wenk et al., 1984). The protein concentration was determined in each sample (Lowry et al., 1951). All assays were performed in triplicate.

The results were analyzed by analysis of variance (ANOVA) followed by pair-wise comparisons between groups with adjustment for multiple comparisons by Bonferroni’s correction (SigmaStat software; Jandel Scientific, San Rafael, CA, USA).

**Histology**

After removal of the cortical samples, a slab of brain 3 mm thick was taken that included the basal forebrain region and immersed in 4% paraformaldehyde. After fixation, free-floating, serial coronal sections (40 μm) were taken by vibratome, washed in phosphate-buffered saline (PBS) to include the entire extent of the basal...
forebrain of both hemispheres. Activated astroglia were visualized by examination for glial fibrillary acidic protein (GFAP). Activated astrocytes rapidly upregulate their expression of GFAP in response to inflammatory stimuli (Kettenmann and Ransom, 1995). Activated astrocytes were visualized using a polyclonal antibody against GFAP (1:5000 dilution; Sigma). Activated microglia rapidly upregulate their expression of tissue antigens in response to inflammatory stimuli (Finsen et al., 1993). Activated microglia were visualized using CR3 (CD-11; antibody Mac-1) rat anti-mouse monoclonal IgG (Serotec Inc., Raleigh, NC, USA). Control sections were incubated without the primary antibody to Mac-1.

Immunocytochemical analyses were also used to confirm the presence of the ChAT-containing neurons within the basal forebrain region. Sections were placed directly into the primary antisera, a polyclonal antibody against ChAT (1:100 dilution; Chemicon, Temecula, CA, USA). Following incubations of 24 h at 4 °C, sections were washed in chilled PBS (three times for 10 min each) and placed for 1 h at 4 °C in a secondary antibody prepared in 0.1% Triton X-100 in PBS and 1% blocker. Sections were then rinsed three times for 10 min each in PBS and placed in peroxidase–antiperoxidase in PBS (1:100) and 1% blocker for 1 h at 4 °C. Sections were washed (three times for 10 min each) in chilled PBS and then reacted in 0.05% diaminobenzidine (Sigma) in PBS for 6 min (20 °C). The preparations were then mounted onto chrome-alum subbed slides, air dried at room temperature (20 °C), quickly dehydrated through a graded series of alcohols and xylol, and coverslipped.

RESULTS

Biochemistry

The level of ChAT activity within the frontal cortex on the side of the brain (left side) that was infused with CSF did not differ significantly (P > 0.1) from the level of activity on the side of the brain (right side) that did not receive an infusion. Chronic infusion of TNF-α into the basal forebrain region for 20 days, significantly (by ANOVA) decreased cortical ChAT activity on the side of the brain ipsilateral to the infusion by approximately 20% (see Fig. 1), as compared with the CSF-infused mice (F(1,22) = 12.42, P < 0.001). Chronic co-treatment with memantine did not attenuate the decline in cortical ChAT activity, i.e. both wild type and transgenic mice infused with TNF-α and also treated with memantine were significantly different from CSF-infused mice (P < 0.05 for all groups). Although there was a trend for an attenuated reduction in ChAT activity in memantine-treated mice, the histological results did not suggest such a trend (see below). Overall, wild type and transgenic mice, with or without memantine treatment, were not differentially vulnerable to the effects of chronic infusion of TNF-α, i.e. the decline in cortical ChAT activity was statistically equivalent in all of the mice.

Histology

Chronic infusion of TNF-α produced a noticeable decline in the number of ChAT-immunoreactive neurons (see Fig. 2) throughout the basal forebrain region. Treatment with memantine had no neuroprotective effect upon the apparent number of ChAT-immunoreactive cells within the basal forebrain region. This immunocytochemical stain was performed in order to confirm the quantitative results of the biochemical assay for the ChAT enzyme and also provide an estimate of the number of cholinergic cells that survived near the injection site and their morphology. The very few surviving ChAT-immunoreactive cells near the border of the injection site of TNF-α were enlarged and had distended and tortuous processes, in contrast to other immunoreactive cells further from the injection site or in mice infused with only CSF.

Staining for GFAP demonstrated highly activated astrogia distributed throughout the entire forebrain of mice, both wild type and transgenic, which were infused with TNF-α. The greatest concentration of activated astrogia was near the infusion site (see Fig. 3, upper panel). Treatment with memantine did not influence the apparent degree of activation of the astrogia. Mice treated with CSF
Fig. 2. Chronic infusion of TNF-α produced a decline in the number of ChAT-immunoreactive neurons throughout the basal forebrain region. High magnification is on the left; low magnification is on the right. The photomicrographs show that very few ChAT-immunoreactive neurons survived near the site of the infusion of TNF-α (arrows). ChAT-immunoreactive cells near the border of the injection site of TNF-α were distended and had tortuous processes in contrast to other immunoreactive cells further from the injection site or those in mice infused with CSF. Memantine had no effect upon the number of immunoreactive cells of TNF-α-infused mice. Low magnification scale bar = 150 μm; high magnification scale bar = 70 μm.
had only a few GFAP-positive astroglia scattered throughout the region. A section from a TNF-α-memantine mouse is shown, which is representative of all mice that received infusions of TNF-α.

Microgliosis within the basal forebrain region was evaluated using Mac-1. The CSF infusion only very slightly increased the level of Mac-1 immunoreactivity (see Fig. 3, lower panel). The chronic infusion of TNF-α, with or without simultaneous treatment with memantine, significantly elevated the degree of Mac-1 immunoreactivity throughout the basal forebrain region.

**DISCUSSION**

In the present study, chronic infusion of TNF-α into the basal forebrain of young mice produced extensive inflammation, as evidenced by the widespread astrogliosis, and a significant decline in the number of ChAT-immunoreactive cholinergic neurons. These results are consistent with our previous findings in rats infused with either LPS or TNF-α into the basal forebrain (Wenk and Willard, 1998; Willard et al., 1999, 2000; Wenk et al., 2003) and further extend our understanding of the
mechanisms by which chronic neuroinflammation might contribute to the progressive dysfunction and degeneration of basal forebrain cholinergic neurons. The results are also consistent with the hypothesis that the presence of the human transgene for APP does not contribute to the vulnerability of basal forebrain cholinergic neurons in the presence of the cytokine TNF-α. These results are similar to those in recent reports showing that transgenic mice that overexpress the human gene for β-amyloid demonstrate biomarkers of inflammation (Benzing et al., 1999; Matsuoka et al., 2001) but do not show evidence for enhanced degeneration of cholinergic neurons in the basal forebrain, as compared with nontransgenic mice (Jaffar et al., 2001; Boncristiano et al., 2002). One potential explanation for the lack of interaction between these factors is that the mice in the current study were too young and did not, as yet, demonstrate extensive plaque deposition that is typically associated with an inflammatory response (Jantzen et al., 2002). We speculate that much older mice might demonstrate an increased vulnerability to the presence of enhanced inflammatory processes.

The neurotoxic actions of TNF-α are both time- and dose-dependent (Bemelmans et al., 1993; Viel et al., 2001); in addition, the half-life of the exogenously applied cytokine is probably less than 30 min in mice (Bemelmans et al., 1993; Pan et al., 1997). The current study maximized the influence of these factors by infusing a relatively high concentration of TNF-α over a comparatively long period of time. Chronic neuroinflammation associated with activated glia and elevated levels of extracellular inflammatory proteins, including TNF-α, are characteristic of the conditions that exist with the brains of patients with AD (Aisen and Davis, 1994; Akiyama et al., 2000). We hypothesized that the prolonged presence of TNF-α could lead to neurodegeneration within the basal forebrain region by many synergistic processes, such as the inhibition of extracellular glutamate reuptake (Fine et al., 1996) or the direct activation of the TNF-α type I receptor leading to the activation of apoptosis related systems within the neurons. However, the results of a recent double-immunofluorescence study indicated that TNF-α type I receptors are not found on cholinergic neurons in the basal forebrain region (Wenk et al., 2003). Therefore, we speculate that the neurotoxic effects of elevated levels of TNF-α were most likely indirect, possibly via its effects upon astroglia similar to that recently shown for the neurotoxicity of β-amyloid (Abramov et al., 2003). β-Amyloid may directly alter the calcium currents across astrocyte plasma membranes; this leads to the depletion of glutathione in both neurons and glia, ultimately increasing neuronal vulnerability to oxidative stress (Abramov et al., 2003).

Elevated extracellular levels of TNF-α may also indirectly diminish available trophic support for forebrain cholinergic neurons by reducing the production of NGF (Aloe et al., 1999), an important trophic factor for forebrain cholinergic neurons (Montero and Hefti, 1988; Woolf et al., 1989). The level of ChAT activity and endogenous levels of NGF were significantly reduced in the medial septal area of transgenic mice that overexpressed TNF-α (Aloe et al., 1999). Reduced ChAT activity in this basal forebrain region would lead to the reduced production of acetylcholine (Cooper, 1994) and a reduced amount of acetylcholine available for neurotransmission in the cortex and hippocampus; the consequence would be impaired attentional abilities and impaired learning and memory (Wenk et al., 1994; Wenk, 1997).

TNF-α may also indirectly influence cholinergic neuronal function via its toxic effects upon mitochondrial function inside cholinergic neurons. TNF-α can bind to a 60-kd protein on the inner mitochondrial membrane and cause specific morphological and cytotoxic changes (Ledgerwood et al., 1998), including the generation of superoxide radicals, increased calcium ion permeability, inhibition of electron transport, swelling, and loss of cristae (Schulze-Osthoff et al., 1992; Richter, 1993). The synthesis of acetylcholine by the cytoplasmic ChAT enzyme requires the production and transport of a crucial metabolic precursor from inside mitochondria (Cooper, 1994; Prado et al., 2002); the cholinergic neuron is unique in its dependence upon normal mitochondrial function for the synthesis of its neurotransmitter (Siegel et al., 1999). Therefore, impaired mitochondrial function would likely impair the synthesis of acetylcholine (Cooper, 1994; Prado et al., 2002) and also lead to the dysfunction of cholinergic neurons within the basal forebrain.

This complex variety of potential neurotoxic mechanisms associated with elevated levels of TNF-α may explain why the NMDA channel antagonist memantine was not able to provide neuroprotection for cholinergic neurons and suggests that the neurotoxic actions of TNF-α in the current model of chronic neuroinflammation do not require the activation of the NMDA receptors. In contrast, we have previously shown that memantine was able to provide neuroprotection to cholinergic forebrain neurons from the consequences of exposure to the inflammanlg LPS (Wilkard et al., 2000). The discrepancy in these findings may be due to the timing of the exposure of the brain to elevated TNF-α levels. LPS produces a brief elevation in extracellular TNF-α that only lasts a few hours (Bencsics et al., 1997; Kowalski et al., 2003). In contrast, in the current study, the elevation in extracellular TNF-α levels lasted considerably longer. Taken together, the results of our current and previous studies are consistent with the hypothesis that the neurotoxic effects of TNF-α upon forebrain cholinergic neurons are indirect and may involve many different mechanisms (Wenk et al., 2003). For example, TNF-α limits the toxicity of calcium influx via NMDA receptors via a mechanism that requires activation of nicotinic receptors (Carlson et al., 1998).

In the brains of patients with AD, neurons are vulnerable to degeneration due to a variety of processes, including those that develop as a consequence of neuroinflammation and those that rely upon the activation of NMDA channels. Indeed, there might be some overlap in the influence of these processes. Our current results indicate that basal forebrain cholinergic neurons are vulnerable to the consequences of chronic neuroinflammation, particu-
larly the elevated expression of TNF-α, a condition that is prominent in the brain of AD patients (Akiyama et al., 2000; Yang et al., 2002).

Acknowledgements—This work was supported by the NIH, AG10546, and the Alzheimer’s Association, IIRG-01-2654 and by an unrestricted gift from Merz Pharmaceuticals, Frankfurt/Main, Germany.

REFERENCES


Tumor necrosis factor-α, a neuromodulator in the CNS. Neurosci Biobehav Rev 21:603–613.


(Accepted 18 January 2004)