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Inflammatory processes induce β-amyloid precursor protein changes in mouse brain

(neurodegeneration/cerebellum/staggerer mice/interleukin 1/interleukin 6)

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ABSTRACT **In Alzheimer disease, a combination of genetic predisposition and environmental factors may contribute to changes in β-amyloid precursor protein (APP) expression, β-amyloid peptide deposition, and neuronal loss. Factors such as head injury or acute infection that trigger inflammatory processes may play a crucial role in development of the disease. In the present *in vivo* study, we showed that, in mouse brain, peripheral stimulation with lipopolysaccharide (LPS) induced a transient increase in the inflammatory cytokine mRNAs (interleukin 1β and interleukin 6), followed by changes in expression of APP isoforms in the cerebellum but not in the cerebral cortex. These changes consisted of a decrease in the APP-695 and an increase in the Kunitz protease inhibitor-bearing isoforms (KPI-APP). In the cerebellum of the staggerer mouse mutant, where a severe loss of Purkinje and granule cells occurs, basal mRNA levels of these interleukins were elevated and an increase in the KPI-APP/APP-695 ratio compared to wild-type mice was observed. These abnormalities were further accentuated by LPS stimulation. This study shows that acute and chronic inflammatory processes play an important role in changes in APP expression possibly associated with neurodegeneration.**

Elevated amounts of two inflammatory cytokines, interleukin 1β (IL-1β) and interleukin 6 (IL-6), have been described in neurodegenerative diseases such as Alzheimer disease (AD) and Down syndrome (1, 2). Recent epidemiological studies have shown that antiinflammatory drugs may be an antirisk factor for AD (3). This suggests that, besides genetic factors (4), inflammatory processes (head injury, acute infections) may be involved in the development of AD (5–8). It is generally admitted that a 4-kDa peptide, the amyloid βA4, which is derived from the β-amyloid precursor protein (APP) (9, 10), plays a crucial role in the pathogenesis of AD (11, 12). In addition to the initially cloned 695-residue isoform of the APP (APP-695), major alternative transcripts (APP-751 and APP-770) are present that contain an exon with strong homology to the Kunitz family of serine protease inhibitors, named KPI-APP (13–15). The corresponding transmembrane proteins can be cleaved within the βA4 region by the α-secretase leading to soluble secreted derivatives of APP (16). An increase in the KPI-APP/APP-695 ratio could be involved in a shift toward a different proteolytic process leading to βA4 deposition and neuronal death (17, 18). Cell culture studies suggest that IL-1β is responsible for altered APP gene regulation in endothelial cells, astrocytes, and neurons (19–21). These results are supported by the finding that the APP gene has the same transcriptional factor, AP-1, found in the promoter region of most acute-phase proteins, which can be induced by IL-1β and IL-6 (19, 22). However, it has not yet been demonstrated whether cytokines and APP expression are linked *in vivo*.

We studied APP, IL-1β, and IL-6 expression in mouse brain following an acute inflammation, induced by systemic lipopolysaccharide (LPS) injection (23). As a possible model for chronic inflammation associated with neuronal degeneration, we also studied APP, IL-1β, and IL-6 expression levels in the neurological mouse mutant staggerer. Homozygous staggerer (*sg/sg*) animals show a severe Purkinje cell deficiency in the cerebellum, whereas the cerebral cortex is not affected. Due to the absence of 60–80% of their Purkinje cell targets, almost two-thirds of the inferior olivary neurons and all the cerebellar granule neurons die during the first postnatal month (24–27). Besides the pronounced astrogliosis described in the cerebellum (2, 28, 29), there is an abnormal increased production of inflammatory cytokines in the immune system (30–32). These data suggest that neurodegeneration is associated with a chronic inflammatory process in the staggerer mutant.

MATERIALS AND METHODS

Animals. Experiments were carried out with 30-day-old C57BL mice. Staggerer (*sg/sg*) mutant mice (PN30; postnatal day 30) were obtained by intercrossing heterozygous (+/*sg*) animals and were identified by clinical symptoms and macroscopic examination of the brain. The staggerer mutation was bred on a C57BL/6J background.

LPS Stimulation. In each experiment, repeated three times, five C57BL mice and five staggerer mice were injected intraperitoneally with LPS (0.5 μg per g of body weight; carrier, physiological saline) or carrier alone. Eight hours or 24 h later, the animals were sacrificed and brain regions were pooled and analyzed.

Western Blot Analysis. For β-APP protein detection and quantification, different brain regions from C57BL and staggerer mice were homogenized in a glass homogenizer with 5 vol of 50 mM Tris·HCl buffer (pH 7.4) containing 0.5 M NaCl, 10 mM $MgCl_2$, 2 mM EDTA, 2 mM EGTA, 1 μg of pepstatin per ml, and 2 mM phenylmethylsulfonyl fluoride. The homogenate was subjected to ultracentrifugation at 100,000 × *g* for 30 min at 4°C. The resulting supernatant, termed the soluble fraction, was mixed with 1/8th vol of 10× sample buffer (33) and boiled for 10 min. The remaining pellet was directly adjusted to sample buffer without 2-mercaptoethanol (ME) and bromophenol blue (BPB) but containing 8 M urea, boiled for 10 min, and finally dialyzed against the same buffer for 4 h at room temperature. Prior to electrophoresis, ME and BPB

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Abbreviations: AD, Alzheimer disease; APP, β-amyloid precursor protein; LPS, lipopolysaccharide; IL, interleukin; KPI, Kunitz protease inhibitor; RT, reverse transcriptase; PN30, postnatal day 30; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

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were added to the samples. Protein concentrations were determined in triplicate according to the method of Lowry *et al.* (34) modified for the presence of SDS (35). Fifty micrograms of supernatant protein or 50 μg of the membrane fractions per lane was separated by SDS/7% polyacrylamide gel electrophoresis (33). Proteins were transferred to nitrocellulose membrane according to the method of Towbin *et al.* (36), except that 0.1 g of SDS per liter and 100 ml of methanol per liter were included in the transfer buffer. The membrane was stained with Ponceau red to visualize proteins and then destained; nonspecific binding sites were blocked using 50 g of skim milk powder per liter in phosphate-buffered saline (PBS) for 30 min. Membranes were incubated first with a rabbit anti-KPI (0.1 μg/ml) (37), or anti-APP (0.8 μg/ml; Boehringer Mannheim) antibody and then with biotinylated second antibody (Boehringer Mannheim) and horseradish peroxidase-conjugated streptavidin (Boehringer Mannheim). Immunoreactive bands were compared densitometrically (A_{595}). Measurements were performed in the linear range, which was determined by using dilution curves of brain extracts.

Reverse Transcriptase (RT)-PCR Analysis. Total RNA was isolated with RNA^B (Bioprobe, Paris) from different brain regions of C57BL mice and staggerer mutant mice. RNA concentration and quality were checked by spectrometry and electrophoresis on 1% agarose/formamide gels. RNA was reverse transcribed by the Riboclone cDNA synthesis system with oligo(dT)$_{15}$ primers and avian myeloblastosis virus RT (Promega). PCR amplification was carried out in the presence of [α-^{32}P]dATP (Amersham), control glyceraldehyde-3-phosphate dehydrogenase (G3PDH) primers, mouse IL-1β and IL-6 amplimer sets, and competitor DNA for IL-1β and IL-6 (Clontech) (38). PCR primers to detect the different APP isoforms (APP-770, APP-751, and APP-695) were used as described elsewhere (39). The PCR mixture contained in a final vol of 50 μl, 10 mM Tris·HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 nM each dNTP, 0.4 μM each primer, 1 μg of cDNA, and 2.0 units of *Taq* DNA polymerase (Bioprobe, Paris). Amplification was carried out on a Perkin–Elmer DNA thermal cycler. The PCR were carried out in a linear amplification range and control reactions were performed in the absence of RT to rule out genomic DNA contamination. The PCR products were analyzed on 1.5% agarose gels. The ethidium bromide-stained bands, containing the ^{32}P-labeled PCR products, were cut out of the agarose gel and measured in a β-counter. The resulting APP isoform, IL-1β, and IL-6 mRNA data were normalized by using the corresponding competitor DNA and G3PDH values.

RESULTS

The secreted derivatives and membrane-bound isoforms of APP, in cerebral cortex and cerebellum of normal and staggerer animals, were characterized by Western blot analysis. Expression of APP, IL-1β, and IL-6 mRNAs were analyzed by quantitative RT-PCR.

Normal Mice. In PN30 normal mice, APP-695 was the predominant secreted derivative in brain-soluble proteins, more abundant in the cerebral cortex than in the cerebellum (Fig. 1*A*), whereas KPI-APP isoforms were predominant in membrane-bound proteins (Fig. 1 *C* and *D*). Densitometric analysis revealed a total (membrane-bound and secreted proteins) KPI-APP/APP-695 ratio of ≈1 in both brain regions (Fig. 2*A*). At the mRNA level, APP-695 mRNA was always the predominant form (Fig. 3*A*). Basal IL-1β mRNA levels in cerebral cortex and cerebellum were very low, in the range of 1×10^{-21} mol per μg of total RNA; IL-6 mRNA could not be detected (Fig. 3 *B* and *C*).

During the acute inflammation induced by LPS, we did not observe any changes in APP expression at the protein and mRNA levels in the cerebral cortex (Fig. 1 *C* and *D*, Fig. 2*A*, and Fig. 3*A*). In contrast, in the cerebellum, the total KPI-APP/APP-695 ratio increased to ≈2.2 24 h after LPS injection (Fig. 2*A*). Secreted APP-695 derivative and membrane-bound APP-695 decreased (Fig. 1 *A* and *C*) and only secreted KPI-APP derivatives increased (Fig. 1 *B* and *D*), but the total amount of APP did not change (data not shown). The overproduction of secreted KPI-APP derivatives induced by acute inflammation suggests that the membrane-bound KPI-APP forms are immediately processed by the α-secretase (16). The cerebellar APP mRNAs (Figs. 3*A* and 4) showed the same tendency as the protein levels, with a significant decrease in APP-695 and an increase in APP-770. No changes were observed for the APP-751 isoform (Fig. 4). The cerebellar changes of the APP mRNAs were relatively small compared to

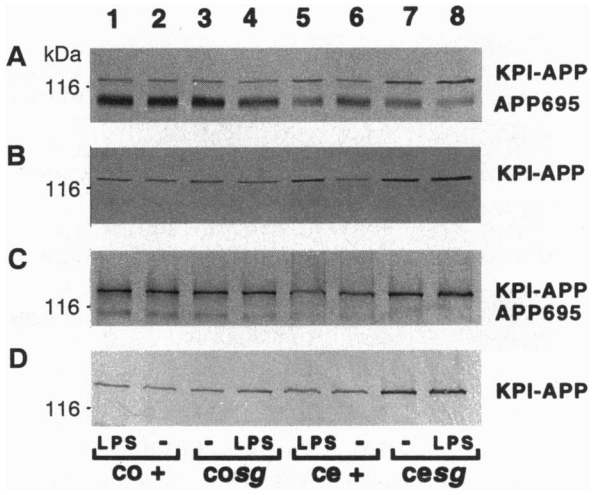

FIG. 1. Western blots of APP isoforms in the cerebellum (ce) and cerebral cortex (co) of PN30 C57BL mice (+) and PN30 staggerer mutant mice (*sg*), before (lanes 2, 3, 6, and 7) and 24 h after (lanes 1, 4, 5, and 8) LPS injection. A monoclonal antibody (anti-APP) was used to detect secreted derivatives (*A*: APP-695, 105–110 kDa; KPI-APP, 120 kDa) and membrane-bound full-length forms of APP (*C*: APP-695, 110–116 kDa; KPI-APP, 125 kDa). An affinity-purified polyclonal antibody (anti-KPI) made against synthetic peptide (37) of the KPI domain was used to detect specifically KPI-APP in secreted derivatives (*B*) and membrane-bound full-length forms (*D*) as described elsewhere (37).

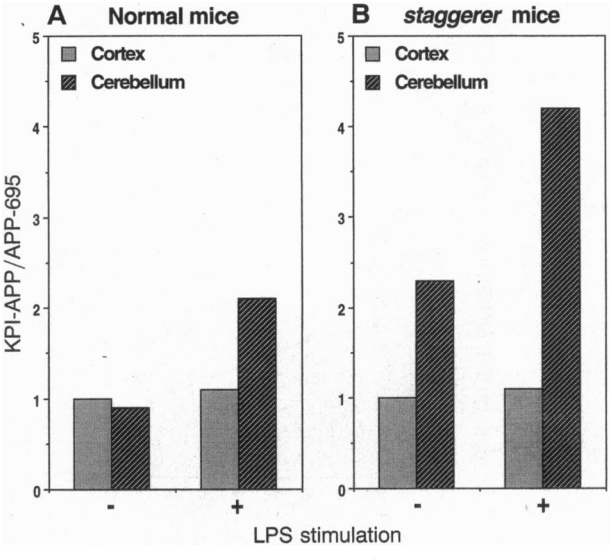

FIG. 2. Ratio of total (secreted and membrane-bound) APP isoforms, A_{595} KPI-APP/A_{595} APP-695, in different brain regions of normal mice (*A*) and staggerer mutant mice (*B*) before (−) and 24 h after (+) LPS administration.

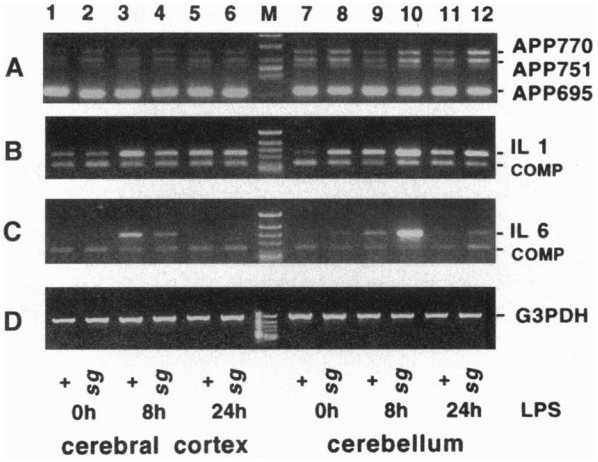

FIG. 3. RT-PCR detection of APP, IL-1β, and IL-6 mRNA levels in cerebral cortex and cerebellum of C57BL mice (+) and staggerer mutant mice (*sg*), before (lanes 1, 2, 7, and 8), 8 h after (lanes 3, 4, 9, and 10), and 24 h after (lanes 5, 6, 11, and 12) LPS stimulation. (*A*) Amplified fragments corresponding to APP-770, APP-751, and APP-695 (39). (*B*) Amplified IL-1β and IL-1β competitor cDNA (2×10^{-21} mol). (*C*) Amplified IL-6 and IL-6 competitor cDNA (2×10^{-22} mol). (*D*) Amplified G3PDH control. Lane M, molecular weight DNA markers. φX174 DNA digested by *Hin*cII.

the changes of the APP protein, suggesting that regulation of the different APP protein isoforms occurs at posttranscriptional levels.

These changes were observed 24 h after induction of the acute inflammation, whereas after 8 h neither APP mRNA (Figs. 3*A* and 4) nor protein isoforms were changed (data not shown). In contrast, mRNAs for the IL-1β and IL-6 were already increased in both brain regions 8 h after LPS injection (Fig. 3 *B* and *C*) and decreased to control levels at 24 h (Fig. 3 *B* and *C*). The time delay with which the change in ratio of APP forms appears is too short to correspond to a change in cell numbers, such as IL-1β-induced astrocyte proliferation (40). It is probably due to a cell activation, as suggested by cell culture studies, where it has been shown that IL-1 induces KPI-APP isoforms in cortical neurons, brain-derived endothelial cells, and astrocytes (19–21).

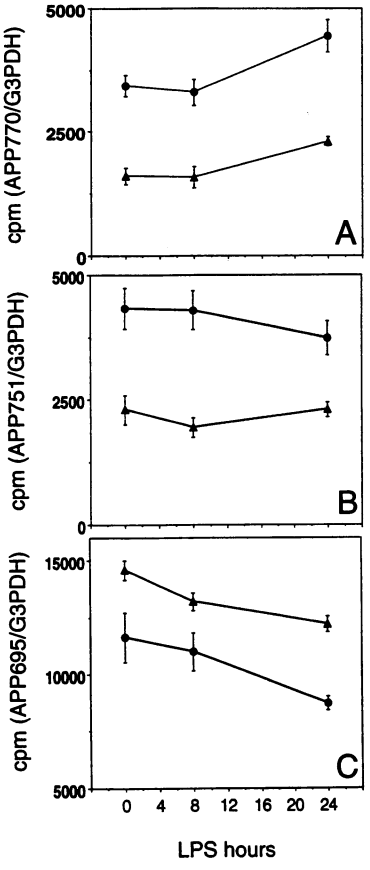

FIG. 4. Changes of the different APP mRNA isoforms [APP-770 (*A*), APP-751 (*B*), and APP-695 (*C*)]. Expression in the cerebellum of C57BL mice (▲) and staggerer mutant (●) after intraperitoneal LPS injection. Each value is the mean ± SDM of three determinations.

Staggerer Mice. In the cerebella of PN30 *sg/sg* mice, the IL-1β mRNA level was 5-fold higher than in normal mice, and IL-6 mRNA was detectable (in the range of 2.5×10^{-22} mol per μg of total RNA; Fig. 3 *B* and *C*). These elevated expression levels are unlikely to be due to a change in the number of either cytokine-producing granule neurons (41), which disappear in the mutant (27), or microglia cells, which do not increase in number in *sg/sg* cerebellum (J.M., unpublished data). They could be explained partly by the astrogliosis existing in the staggerer cerebellum and partly by a change in the activity of cytokine-producing cells in the brain similar to the one we previously described in peripheral macrophages of this mutant (31). As seen in induced acute inflammation, these elevated levels of cytokine mRNAs were associated with changes in APP expression. The total KPI-APP/APP-695 ratio was ≈2.3 (Fig. 2*B*) compared to the ratio of ≈1 in normal mice. Secreted APP-695 derivative and membrane-bound APP-695 were decreased and both secreted KPI-APP derivatives and membrane-bound KPI-APP isoforms were increased (Fig. 1), whereas the total amount of APP protein did not change (data not shown). At the mRNA level, the APP-695 was decreased and KPI-APP forms were increased 2-fold (Figs. 3*A* and 4). In the cerebral cortex, where no degeneration occurs, basal cytokine mRNA levels (Fig. 3 *B* and *C*) and APP expression (Fig. 1) were the same as in healthy mice. These results suggest that the neuronal degeneration in staggerer is associated with a chronic inflammatory process and consequent changes in APP expression.

In the staggerer cerebellum, we wondered whether an additional acute inflammation could still change cytokine mRNA levels and APP expression. Eight hours after LPS injection, IL-1β and IL-6 mRNA levels increased (3- and 5-fold; Fig. 3 *B* and *C*) and dropped to initial levels after 24 h (Fig. 3 *B* and *C*). One day after LPS injection, total KPI-APP/APP-695 increased from ≈2.3 to ≈4.3 (Fig. 2*B*), secreted APP-695 derivative decreased, and only the secreted KPI-APP derivatives increased (Fig. 1). At the mRNA level (Figs. 3*A* and 4), we observed after LPS stimulation changes similar to the one of control mice, indicating that the staggerer cerebellum is still responsive to peripheral LPS induction.

DISCUSSION

This study shows *in vivo* that high levels of cytokines, induced by two different processes, are related to changes in APP expression. A shift toward KPI-APP isoforms is observed following an experimentally induced acute inflammation and in a genetically induced chronic inflammation associated with neuronal degeneration (Table 1). A similar shift has also been described in AD brain regions where neuronal loss is observed and thus could be involved in an aberrant proteolytic process leading to β-amyloid deposition and neuronal death (17, 18). This shift in APP isoforms could be related to a change in cellular distribution and/or activation of certain cell types during the degeneration process.

In normal animals subjected to an acute inflammation that caused only a transient increase in IL-1β and IL-6, the shift was observed in the cerebellum but not in the cerebral cortex, indicating that a still unknown regulatory mechanism is dif-

Table 1. Expression of IL-1β and IL-6 mRNA and changes in total APP isoform ratio, before and after LPS injection, in normal and staggerer mouse brains

	Normal mice		Staggerer mice	
	Cortex	Cerebellum	Cortex	Cerebellum
IL-1β mRNA				
Basal level	+	+	+	++
After 8 h of LPS	++	++	++	++++
IL-6 mRNA				
Basal level	ND	ND	ND	+
After 8 h of LPS	+	+	+	++++
KPI-APP/APP-695*				
Basal level	1	0.9	1	2.3
After 24 h of LPS	1.1	2.2	1.1	4.3

ND, not detected; +, detected; ++, induced; ++++, strongly induced.
*Total of membrane-bound and secreted APP forms.

ferent in both brain regions. In the cerebellum, the shift concerned only the secreted forms of APP, the overproduced KPI-APP being entirely cleaved by the α-secretase. In contrast, a characteristic feature of the staggerer cerebellum was the increase in membrane-bound KPI-APP. This accumulation in neurons and/or astrocytes could result from an inhibition of α-secretase either directly by the KPI domain of the APP itself or by the IL-6-induced expression of α_2-macroglobulin, known to be a potent inhibitor of the α-secretase (42). A sustained increase of cytokine levels might induce these phenomena in the staggerer cerebellum as well as in AD brains where it could be triggered by factors such as repeated infections, injury, stroke, and stress.

In our staggerer mutant model, chronic inflammation occurred in the cerebellum, and it would be relevant to study a chronic situation in the cerebral cortex to determine whether sustained elevated cytokines are associated with changes in APP expression. A description of transgenic mice overexpressing IL-6 in the brain has been recently published and could be such a model (43).

In conclusion, the present data support the hypothesis of a loop between inflammation processes and APP regulation in the mouse cerebellum. The cascade of interactions between cytokines, APP isoforms, and a mutated staggerer gene that could ultimately lead to the neurodegeneration observed has now to be elucidated.

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