Interleukin-1β Enhances GABA$_\text{A}$ Receptor Cell-surface Expression by a Phosphatidylinositol 3-Kinase/Akt Pathway

**RELEVANCE TO SEPSIS-ASSOCIATED ENCEPHALOPATHY**

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Sepsis-associated encephalopathy (SAE) is a frequent but poorly understood neurological complication in sepsis that negatively influences survival. Here we present clinical and experimental evidence that this brain dysfunction may be related to altered neurotransmission produced by inflammatory mediators. Compared with septic patients, SAE patients had higher interleukin-1β (IL-1β) plasma levels; interestingly, these levels decreased once the encephalopathy was resolved. A putative IL-1β effect on type A γ-aminobutyric acid receptors (GABA$_\text{A}$Rs), which mediate fast synaptic transmission in most cerebral inhibitory synapses in mammals, was investigated in cultured hippocampal neurons and in *Xenopus* oocytes expressing native or foreign rat brain GABA$_\text{A}$Rs, respectively. Confocal images in both cell types revealed that IL-1β increases recruitment of GABA$_\text{A}$Rs to the cell surface. Moreover, brief applications of IL-1β to voltage-clamped oocytes yielded a delayed potentiation of the GABA-elicited chloride currents (I$_\text{GABA}$); this effect was suppressed by IL-1ra, the natural IL-1 receptor (IL-1RI) antagonist. Western blot analysis combined with I$_\text{GABA}$ recording and confocal images of GABA$_\text{A}$Rs in oocytes showed that IL-1β stimulates the IL-1RI-dependent phosphatidylinositol 3-kinase activation and the consequent facilitation of phospho-Akt-mediated insertion of GABA$_\text{A}$Rs into the cell surface. The interruption of this signaling pathway by specific phosphatidylinositol 3-kinase or Akt inhibitors suppresses the cytokine-mediated effects on GABA$_\text{A}$, whereas activation of the conditionally active form of Akt1 (myr-Akt1.ER*) with 4-hydroxytamoxifen reproduces the effects. These findings point to a previously unrecognized signaling pathway that connects IL-1β with increased “GABAergic tone.” We propose that through this mechanism IL-1β might alter synaptic strength at central GABAergic synapses and so contribute to the cognitive dysfunction observed in SAE.

Acute impairment of mental function is often the first manifestation of sepsis. This condition is best defined as “sepsis-associated encephalopathy” (SAE) in order to stress the absence of direct infection of the central nervous system. The syndrome is characterized by a marked decrease in cerebral activity resulting in confusion, somnolence, and disorientation and decreased consciousness (1). As in other kinds of metabolic encephalopathy, diagnosis of this condition is dependent on the exclusion of all other possible causes of brain dysfunction (2). The exact incidence of SAE is unknown. Sprung et al. (3) reported that 23% of a large series of 1333 septic patients enrolled in the Veterans Affairs Systemic Sepsis Study developed encephalopathy. The same authors found a significantly higher mortality (49%) in the SAE group compared with the group of patients with normal mental status (26%). A direct relation between the severity of cerebral dysfunction and increased mortality in septic patients was also reported in other studies (2, 4).

The pathophysiology of SAE remains poorly understood and is probably multifactorial. Damage to endothelial cells and breakdown of the blood-brain barrier mediated by cytokines and reactive oxygen species occur at an early stage of sepsis and can alter cerebral blood flow (5). Perimicrovessel edema, disruption of astrocyte end-feet, and neuronal injury are observed in septic encephalopathic pigs with fecal peritonitis (6), but limited data are available from human studies. An imbalance between branched chain and aromatic amino acids and reduced plasma and cerebrospinal fluid concentrations of ascorbate, probably due to excessive oxidative stress, has been reported in some patients with SAE (7, 8).

We have demonstrated previously a significant association between the intensity of the inflammatory response and outcome in patients with sepsis (9–11). Because of that observation, we hypothesized that one or more of the inflammatory mediators could play a key role in the origin of this type of encephalopathy. Our hypothesis is based on three previous observations as follows: (i) inflammatory stimuli increase the levels of mediators and their receptors in rodent brain and raise the level of circulating mediators that cross into the central nervous system (12–14); (ii) 30–50% of melanoma and renal carcinoma patients receiving high subcutaneous IL-2 doses as a coadjuvant treatment for their cancer developed confusion and delirium (15); and (iii) the effects of intracerebral injection of IL-1β in rodents mimic the electroencephalographic changes and soporic effects of SAE (16–17).

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4 The abbreviations used are: SAE, sepsis-associated encephalopathy; a.u., arbitrary units; CSF, cerebrospinal fluid; 4-HT, 4-hydroxytamoxifen; ER, estrogen receptor; GABA, γ-aminobutyric acid; GABA$_\text{A}$, GABA type A receptor; IL-1α, IL-1 receptor antagonist; IL-1β, IL-1 receptor type-1; myr-Akt1, conditionally active form of Akt1; PB, phosphate buffer; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol-3-kinase; SAPS, simplified acute physiology scoring; TNFR-I, TNF receptor type-I and II; PKC, protein kinase C; LPS, lipopolysaccharide.
Cerebral synaptic activity is decreased in SAE and because type A γ-aminobutyric acid receptors (GABA_{A}Rs) regulate fast synaptic transmission in most cerebral inhibitory synapses in mammals, we suspected that this receptor would be a good target for inflammatory mediators. In fact, increased GABAergic neurotransmission has been reported in patients with hepatic encephalopathy (18).

Therefore, the first phase of this study analyzed the plasma levels of different cytokines in septic patients with and without encephalopathy. In light of the strong association that we observed between the IL-1β plasma levels and neurological affectation, we designed a second experimental phase using cultured rat hippocampal neurons and Xenopus oocytes injected with rat brain mRNA to study the following: 1) how cytokine affects GABA_{A}Rs in these two very different cell types, and 2) the mechanism behind this putative interaction. Different experimental approaches are used in this study, including immunocytochemistry, confocal microscopy, molecular biology, and pharmacological, electrophysiological, and biochemical techniques.

**EXPERIMENTAL PROCEDURES**

**Patients**—Over a 1-year period, a total of 75 consecutive patients admitted to our medical ward in a European university hospital with a diagnosis of sepsis were screened daily for the onset of acute mental impairment. Our institutional review board approved the study, and informed consent was obtained from the patient and/or his/her guardian (family member). Sepsis was defined according to standardized international criteria (19), and SAE was defined as acute alteration of mental status (i.e., inattention, disorganized thinking, and altered level of consciousness) secondary to sepsis. Patients with SAE were included in the study if neurological symptoms had begun within 12 h of admission, and they did not meet the following exclusion criteria: 1) alcohol- or having received medications known to cause mental confusion at admission to hospital; 2) a history consistent with significant memory impairment before hospitalization, i.e., dementia, psychosis, or neurological disease; 3) metabolic encephalopathy because of renal or liver failure, complete respiratory failure (oxygen saturation of <90 mm Hg), or electrolytic disturbance; and/or 4) immunosuppression because of poorly controlled diabetes or treatment with oral corticosteroids or cytotoxic drugs within the previous month. The diagnosis of SAE was established by ruling out other possible causes of encephalopathy through standard procedures, including blood examinations as well as cranial computed tomography scanning and electroencephalographic recordings (20). Twenty-one patients with SAE were finally entered in the study. Encephalopathy was scored daily by two classic neuropsychological tests: the Glasgow Coma Scale (21) and the Reaction Level Scale (22) until the neuropsychological tests were included as controls; both groups of patients have similar ages, duration of fever before the collection of blood samples, and severity of disease. The Folstein Mini–Mental State Examination (23) was performed to exclude dementia in the control group (score ≥24) and to estimate base-line cognitive performance in patients after SAE had resolved. Cerebrospinal fluid (CSF) was analyzed in patients whose encephalopathy persisted beyond the first 48 h after study enrollment to exclude a direct infection of the brain. The simplified acute physiology scoring (SAPS) II system (24) was used to assess illness severity for each patient at the time of study entry and at 48 h after entering the study.

A venous blood sample of 10 ml was obtained within 24 h after severe alterations in mental status had been noted (day 1), repeated 48 h later (day 3), and again on the day the encephalopathy resolved completely. Resolution was confirmed by normal performance in all neuropsychological tests. CSF samples (1 ml) were obtained from the 10 patients who remained encephalopathic 48 h after entering the study. A cell count of <5/ml and a protein level of <0.45 g/liter were considered normal. TNF-α, IL-1β, and IL-6 levels were determined by an enzyme-linked immunoassay (Medgenix Diagnostic, Fleurus, Belgium) as described elsewhere (10, 11). Concentrations of soluble TNF receptor type I and II (TNFR I + II) and IL-1 receptor antagonist (IL-1ra) were measured using a quantitative sandwich enzyme immunoassay (Quantikine; R & D Systems, Minneapolis, MN).

**Primary Neuronal Culture**—Primary cultures of hippocampal neurons (for immunocytochemistry experiments) or mixed cortical plus hippocampal neurons (for immunoprecipitation experiments) were prepared from the hippocampi or from the cerebral cortices of Sprague-Dawley rat fetuses at embryonic day 18. Brains were removed and freed from the meninges, and the tissues were then dissected under a binocular microscope. Neurons were mechanically dispersed and plated on poly-l-lysine and laminin–treated 12-mm glass coverslips (at a density of 30,000/cm²) in B-27-supplemented Neurobasal media. The neurons were kept in an incubator at 5% CO₂ without medium change for 14 days; experimental treatments were begun after this period.

**Immunocytochemistry and Confocal Imaging of Rat Hippocampal Neurons**—After treatment with the cytokine, neurons were either fixed with 3% paraformaldehyde in phosphate buffer (PB) for 10 min at room temperature and permeabilized for 5 min in 0.25% Triton X-100 (in PBS, 10% normal goat serum) or fixed without permeabilization. Non-specific immunolabeling sites were blocked by incubation with 10% normal goat serum in PBS. The overnight incubation with either the mouse monoclonal anti-GABA_{A}R β2/β3 subunit antibody (bd-17, 20 µg/ml) or the rabbit polyclonal antibody raised against the type I receptor for IL-1β, IL-1RI (1:200), at 4 °C, was followed by incubation with Oregon Green 488 goat anti-mouse IgG (1:200) or the AlexaFluor 546 goat anti-rabbit IgG (1:300), respectively. Neurons treated as above but not incubated with the primary antibodies were used as negative controls. The stained neurons were imaged using a Leica TCS SP2 spectral confocal laser scanning microscope. All images were captured with the Leica confocal software as reported previously (25), using the same adjustments of laser intensity and photomultiplier sensitivity and then processed with Adobe Photoshop 6.0 software using identical contrast and brightness values for both control and IL-1β-treated neurons. Fluorescence intensity, expressed as arbitrary units (a.u.), was measured in a region of interest of 10 µm² on the cell body membrane or within the soma. In other cases, fluorescence intensity was measured along the x axis of the entire cell body.

For quantification of the number of GABA_{A}R clusters in proximal neurites, hippocampal neurons were plated on coverslips and maintained in culture in the same conditions as above. After IL-1β treatment for 1 h, “live neurons” were incubated for 1 h at 37 °C with the primary anti-GABA_{A}R β-chain antibody, at the same dilution as above. After rinsing, neurons were fixed with 3% paraformaldehyde in PB (5 min) followed by incubation with the secondary antibody for 45 min at room temperature. Coverslips were washed twice with PBS and mounted in glycerol/buffer (1:1). Images were collected with a DXM1200F digital camera incorporated to a personal computer, using the ×40 objective of a Nikon TE2000-S microscope. Image files were processed using Scion Image software (Scion Corp., Frederick, MD). The number of clusters was determined along dendritic segments of an area of 10 µm², starting >5 µm from the soma; a single mean value was obtained for each neuron after analyzing three to four different fields per cell. At least five
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different neurons from each culture of three independent cultures were used in this analysis.

Immunoprecipitation Assay—Cortical cultures, containing a mixed population of cortical plus hippocampal neurons, were prepared and maintained in culture for 14 days as described previously. After this period, neurons were homogenized in RIPA buffer (10 mM Na2HPO4, pH 7.2, 150 mM NaCl, 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.2 mM, 1,10-phenanthroline, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 mM benzamidine). The neuronal lysates (300 μg of protein) were then immunoprecipitated using the monoclonal anti-GABAAR β2/β3-chain antibody (2 μg) for 4 h at 4 °C, followed by the addition of 20 μl of a mixture of γ-bind protein G-Sepharose (Amersham Biosciences) and γ-bind protein A-agarose (Sigma) for 16 h at 4 °C. After three washings in lysis buffer, the immunoprecipitates were boiled in Laemmli sample buffer containing β-mercaptoethanol and resolved in a 12% SDS-PAGE. Proteins were transferred to Immobilon membranes (Millipore, Billerica, MA) and immunoblotted with the phospho-Ser detection antibody set (Biomol, Plymouth Meeting, PA). The efficiency of receptor immunoprecipitation was determined by reprobing the same membrane with the anti-γ2/γ3-chain antibody.

Preparation of mRNA—Techniques for poly(A)＋RNA isolation from tissues as well as in vitro mRNA synthesis have been described elsewhere (25–27). Briefly, poly(A)＋RNA was purified from mixed cortex plus hippocampus regions of brain from 3-week-old Sprague-Dawley rats using the FastTrack mRNA 2.0 kit from Invitrogen. The plasmid vector containing the entire coding region of the myristoylated conditionally active form of Akt1 (pcDNA3.1-myr-Akt1.ER*) was linearized with NotI and transcribed with T7 polymerase using the mCAP RNA cap-active form of Akt1 (pcDNA3.1-myr-Akt1.ER*) was linearized with NotI and transcribed with T7 polymerase using the mCAP RNA cap-active form of Akt1.ER* was constructed by fusing the c-Src myristoylation targeting sequence to a constitutively active form of Akt1 lacking the pleckstrin homology domain. Conditional activity was conferred in a manner similar to that described for other protein kinases by fusing the myr-Akt1 to a modified form of the hormone binding domain of the mouse estrogen receptor that binds 4-hydroxystyroxifen (4-HT) but is refractory to estrogen. This fusion protein is expressed in an inactive form and becomes activated in the presence of 4-HT as reported in a previous paper (28). As a result, myr-Akt1.ER* is rapidly activated in response to 4-HT and elicits effects that have been attributed to endogenous cellular Akt.

Electrophysiology in Oocytes—Techniques for mRNA injection and electrophysiological recordings of foreign receptors expressed in Xenopus oocytes are described in detail elsewhere (25–27, 29, 30). Mature female Xenopus laevis frogs obtained from a commercial supplier (Xenopus Express, Haute-Loire, France) were anesthetized with tricaine mesa (Express, Haute-Loire, France) and were perfused through the left ventricle with saline, 4% paraformaldehyde. The animal experiments were conducted in accordance with European Community Council Directives 86/609/EEC and 2003/65/EC guidelines and sacrificed 14–20 h post-injection following deep sodium pentobarbital anesthesia. The rats were perfused through the left ventricle with saline, 4% paraformaldehyde in 0.1 M PB, and a graded series of cold sucrose solutions in PB. The brains were blocked in the coronal stereotaxic plane, cryoprotected by immersion in 30% sucrose until they sank, and frozen sectioned at 40 μm. Adjacent series of sections were collected and processed for GABAAR immunocytochemistry, acetylcholinesterase histochemistry, and cresyl violet staining. GABAAR immunohistochemistry was performed in two series of the control and two series of the septic rats. In order to facilitate comparison of results between the control and septic rats, tissues from both were processed simultaneously using identical solutions and incubation times. Immunocytochemistry was performed following a protocol described previously for dopamine transporter (32). In this study, the primary antibody was the monoclonal anti-GABAAR β-chain antibody, diluted 1:100–1:200. The sections from control and septic brains were analyzed and photographed with a Zeiss Axiophot microscope, using identical contrast and brightness values. Negative control sections that omitted primary antibody were run in parallel and showed no immunostaining.

Chemicals—Unless otherwise indicated, all products not specified were purchased from Sigma. The anti-GABAAR and anti-IL-1RI anti-
bodies were purchased from Chemicon (Temecula, CA) and from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The secondary antibodies Oregon Green 488 goat anti-mouse IgG and AlexaFluor 546 goat anti-rabbit IgG were purchased from Molecular Probes (Eugene, OR). The anti-Akt polyclonal antibody was obtained from BD Biosciences, and the anti-phospho-Akt-S473 was from Cell Signaling Technology, Inc. (Beverly, MA). We obtained Neurobasal media from Invitrogen, and paraformaldehyde was from Merck. The human recombinant IL-1ra were purchased from PeproTech (London, UK); LY294002 was from Cayman Chemical. The Akt inhibitors (SH-5, specific inhibitor of the Akt1 isoform; API-2, an inhibitor of the three Akt isoforms) and the PKC inhibitor (Go6850) were purchased from Calbiochem.

Statistical Analysis—For comparison of gender distribution, source of infection, and causative microorganisms, severity of illness, and biochemical parameters were observed between the two patient groups (Table 1). Septic encephalopathy was considered severe in four cases, nonsevere in 13, and mild in 4. The encephalopathy reversed in all patients, and the average time to symptom resolution was 5 days, with a range of 2–10 days. Patients with SAE had significantly higher plasma levels of IL-1β and lower levels of IL-1ra at base line than did patients without encephalopathy (Table 2). No significant difference was found for plasma IL-6, TNF-α, or soluble TNFR(I + II) levels between the two groups. Interestingly, only the IL-1β concentration remained significantly high, whereas the concentration of its endogenous antagonist, IL-1ra, was lower at day 3 in those SAE patients whose mental changes had persisted as compared with patients whose mental disturbances had cleared up. Plasma cytokine concentrations had returned to normal values in all patients the day before discharge (Table 2). A significant correlation between the base-line (r = 0.620) and 48-h (r = 0.530) plasma IL-6 levels and the severity of disease, as measured by the SAPS II score, was found in patients with SAE. Base-line plasma IL-6 also correlated (r = 0.577) with the SAPS II value in non-SAE patients. In contrast, plasma concentrations of the other cytokines were not associ-
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ate with disease severity in either of the two patient groups. Cytokine concentrations in CSF were measured in the 10 patients who still had encephalopathy on day 3 (Table 3). Compared with normal CSF cytokine values, the IL-1β concentration was significantly elevated in all patients, and IL-1ra was detectable in seven, IL-6 was detectable in five, and TNF-α was undetectable in any patient. Concerning the severity of cerebral symptoms, the scores from all of the neurological tests were assessed against plasma or CSF concentrations of the different cytokines. There was a significant correlation between IL-1β plasma concentrations and the Glasgow Coma Scale score at study entry ($r = 0.350$, $p = 0.05$) and on day 3 in patients who remained encephalopathic ($r = 0.340$, $p = 0.05$). Neither IL-6 nor TNF-α plasma levels correlate with the severity of the neurological symptoms as measured by any of the neuropsychological tests on both admission and day 3.

**Table 3**

| Cytokine     | Encephalopathic patients | Normal values |
|--------------|--------------------------|---------------|
| TNF-α        | 3.5 ± 1.0 (0.5–4.5)       | <0.5          |
| IL-1β        | 250 ± 270 (<50–780)       | <50–270       |
| IL-1ra       | 250 ± 270 (<50–780)       | <50–270       |
| IL-6         | 15 ± 20 (0–35)            | <0.5          |

**Figure 1.** Surface expression of GABA<sub>AR</sub> containing β2/β3 subunits in hippocampal neurons is selectively increased by IL-1β. A, representative confocal images showing the basal distribution of IL-1RI in a primary culture of neurons fixed and permeabilized for immunostaining as described under "Experimental Procedures." B, negative control in which the primary anti-IL-1RI antibody was omitted; it shows no fluorescent signal. C–J, distribution of GABA<sub>AR</sub>Rs in a primary culture of neurons under basal conditions or after treatment with IL-1β (3 nM, 1 h). Neurons were either fixed and permeabilized before immunostaining or fixed without permeabilization. After this, neurons were first incubated with the primary antibody raised against an extracellular epitope of β2/β3 subunits of the GABA<sub>AR</sub>, followed by a secondary antibody conjugated with Oregon Green as described in detail under "Experimental Procedures." C and D, confocal images of GABA<sub>AR</sub> staining in the intracellular compartment and at the cell membrane surface in permeabilized neurons under control or after IL-1β stimulation. Quantification of the ratio of GABA<sub>AR</sub> staining fluorescence intensity on the membrane to that in the cytoplasm in both groups of neurons is shown in E. Each bar represents the mean ± S.E. of different neurons (n = 8–10 neurons from three different cultures). F and G, scanned fluorescence intensity corresponding to GABA<sub>AR</sub> along the x axis of two permeabilized neurons incubated or not with IL-1β as above. Note that the cytokine increases the fluorescent signal at the membrane, and it reduces the intracellular fluorescence. H and I, confocal images showing GABA<sub>AR</sub> distribution in nonpermeabilized neurons, under control or IL-1β-stimulated conditions. Note that only surface GABA<sub>AR</sub>Rs were labeled because the incubation with the primary antibody, which recognizes an extracellular epitope of the receptor β3/β6 subunits, was performed before cell permeabilization. J, histograms of fluorescence intensity (a.u.) at the somata membrane and dendrites from nonpermeabilized neurons, under control conditions or after IL-1β stimulation. *, $p \leq 0.05$; ***, $p \leq 0.001$ upon comparing control versus stimulated neurons.
the anti-GABA<sub>α</sub>R β2/β3 antibody show the expression of plasma membrane-associated receptors; however, large numbers of the receptors were also found to be located intracellularly throughout the cell soma (Fig. 1C). Thus, the possibility exists that IL-1β, by acting directly on GABA<sub>α</sub>R and/or on its specific IL-1RI receptor, could be regulating GABAergic activity.

To examine the above hypothesis, we first investigated the potential effect of IL-1β on expression and distribution of native GABA<sub>α</sub>Rs in situ. Incubation of the neurons with IL-1β (3 nM, 1 h) increased the ratio of GABA<sub>α</sub>R labeling on the cell membrane surface to the labeling in the cytoplasmic compartment (Fig. 1, C–E). Moreover, scanning the fluorescence intensity along the x axis in IL-1β-treated neurons provided evidence for translocation to the neuronal membrane by intracellularly located receptors (Fig. 1, F and G). Confocal images of neurons immunostained with the anti-GABA<sub>α</sub>R antibody under nonpermeabilized conditions demonstrated that IL-1β increased the receptor labeling on the plasma membrane surface, and this increase in surface labeling was prominent on the proximal dendrite (Fig. 1, H–J), sites where GABAergic synapses have been shown to be predominantly localized.

By using a standard epifluorescence microscope to visualize hippocampal neurons stained with the primary anti-GABA<sub>α</sub>R antibody following the "live cell" immunolabeling protocol described under "Experimental Procedures," it is possible to identify individual GABA<sub>α</sub>R clusters on the cell membrane surface and neurites under basal conditions (Fig. 2A). The treatment with IL-1β (3 nM, 1 h) significantly increased the number of clusters in both proximal neurites (Fig. 2B, bottom) and somatic membrane surface (Fig. 2B, inset). Fig. 2C shows the cytokine-mediated effect on the number of clusters in proximal neurites of different neurons in three different cultures assayed as above. The high density of clusters on the membrane surface of cytokine-treated neurons made their recount difficult.

Among other factors affecting GABA<sub>α</sub>R trafficking, it has been reported that phosphorylation of different β subunits of the receptor by diverse protein kinases and extracellular signals plays an important role in the dynamic regulation of GABA<sub>α</sub>R numbers in the postsynaptic domain (reviewed in Ref. 33). To determine whether this could be the mechanism implicated in the IL-1β effect on GABA<sub>α</sub>R redistribution in neurons, we next examined the phosphorylation of native GABA<sub>α</sub>R β-chain in cultured cortical neurons with and without IL-1β treatment (3 nM, 10 min). After treatment, the native β2/β3 subunits of GABA<sub>α</sub>R were immunoprecipitated with the monoclonal anti-β2/β3 GABA<sub>α</sub>R antibody from the culture lysates. When immunoprecipitates were immunoblotted with anti-phospho-Ser antibody, it was possible to distinguish a low level of phosphorylation of β subunits under the basal conditions, and this level was markedly increased by IL-1β treatment (Fig. 2D). Interestingly, phosphorylation of the subunit by the cytokine treatment was not affected by a 15-min preincubation of the neurons with 125 nM Go66850 (not shown). This bisindolylmaleimide derivative is a selective and potent inhibitor (IC<sub>50</sub> = 20 nM) of all the subtypes of PKC (34). This result indicates that this protein kinase is not implicated in the IL-1β-induced phosphorylation of native GABA<sub>α</sub>Rs. To study whether the IL-1β-mediated GABA<sub>α</sub>R phosphorylation and trafficking are two connected processes, we used a heterologous cell system that efficiently expresses receptors and proteins from neuronal tissues.

The IL-1β Effect on Native GABA<sub>α</sub>Rs in Neurons Is Reproduced in Oocytes Expressing the Foreign Receptors Transplanted from Rat Brain—Oocytes injected with rat cortex/hippocampus mRNA efficiently express both IL-1RI and GABA<sub>α</sub>Rs on their surface, as shown in the confocal images of Fig. 3, A and B. These images were captured from the animal hemispheres of two oocytes permeabilized and immunostained with the specific antibodies as described under "Experimental Procedures." In contrast with the cultured neurons, no intracellular fluorescence signal was detected in these mRNA-injected oocytes for either receptor type under any experimental condition. Our results on GABA<sub>α</sub>R distribution in immunostained mRNA-injected oocytes agree with those obtained in a previous study performed in oocytes expressing recombinant GABA<sub>α</sub>R and reporting a strong fluorescence corresponding to the receptor on the surface at the animal pole, although it was almost absent inside the oocyte (35). The absence of any intracellular fluorescence signal in the oocyte might be due to the following: 1) the inability of the laser to penetrate far into a large 1-mm cell; 2) the quenching of the fluorescent signal by the oocyte yolk/pigment, or 3) the fact that the intracellular distribution of antibody may be so diffuse that it is below detection levels.

Moreover, our results demonstrate that the fluorescent signal for IL-1RI and GABA<sub>α</sub>Rs at the animal surface of mRNA-injected oocytes (Fig. 3, A and B) is specific because it was not detectable in either non-
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FIGURE 3. Interleukin-1β induces a rapid incorporation of GABAARs to the cell surface and potentiates GABAAR-mediated currents in oocytes expressing rat brain receptors. A and B, overall confocal (2-scan) images of the animal hemispheres of two typical intact oocytes (out of 5) injected with rat brain cortex/hippocampus mRNA and immunostained for IL-1RI or GABAAR. Images reveal that these cells successfully express both types of neuronal receptors. The fluorescent signals for both receptors seem to be restricted to the cell surface. C, representative confocal image showing that IL-1β (3 nM, 1 h) drastically increases GABAAR expression at the oocyte surface. Right, oocyte orientation on the confocal microscope stage; a and v indicate animal or vegetal hemisphere, respectively. D, original traces of current obtained in mRNA-injected oocytes, voltage-clamped at −60 mV, and stimulated with regular pulses of GABA (○). Although the control current elicited by the agonist remained stabilized throughout the experiment (G, Control), three brief and separate reintroductions of IL-1β (1 nM, 60 s) immediately before the corresponding GABA pulse produced a retarded increase in IgABA (D and G). E, it is worth noting that the cytokine modified the amplitude but not the kinetics of the current, as can be deduced upon analyzing the ratio between IgABA/Max in six oocytes treated with IL-1β as above (inset); values show the mean ± S.E. F and G, the specific antagonist of the receptor for IL-1β, IL-1ra, applied as indicated by the black bars, inhibits the IL-1β effect on IgABA completely and in a reversible manner. G shows pooled results obtained in several oocytes assayed as above in control conditions (n = 5) or treated with IL-1β in the presence (n = 4) or absence of the antagonist (n = 6); each value represents the mean ± S.E.

Consistent with the confocal images on GABAAR redistribution obtained in IL-1β-treated neurons and oocytes, the cytokine upregulates GABAAR-mediated currents in injected oocytes (see Fig. 3, D, E, and G). Thus, when voltage-clamped oocytes expressing foreign GABAARs were exposed to successive GABA pulses (100 μM, 20 s), applied at regular intervals of 3 min to prevent receptor desensitization, the reproducible GABA-elicited inward chloride currents (IgABA) that were obtained lasted more than 2 h (Fig. 3G, Control). The current elicited by GABA is mediated by the activation of the GABAAR subtype because it is completely blocked, in a reversible manner, by the specific antagonist bicuculline (10 μM), applied 1 min before and during the agonist pulse (not shown). Two or three separate and brief exposures to IL-1β (1 nM, 1 min), applied immediately before the corresponding GABA pulse, are enough to yield a significant increase of IgABA (Fig. 3, D and G) that can be prevented by the specific IL-1 receptor antagonist IL-1ra (Fig. 3, F and G). Blockade of the cytokine effect on IgABA by the antagonist is completely reversible by washout (Fig. 3F). Pooled data collected from different oocytes stimulated with IL-1β show the time course of the cytokine effect on the amplitude and on the kinetics of IgABA (Fig. 3, G and E). Because of the low decay of the current induced by GABA, an exponential function could not be suitably fitted to the current time course; thus, kinetics analysis simply estimated the amplitude of the current at the end of the pulse (Ilate) as a fraction of the peak.
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mRNA were preincubated with the inhibitor (10 μM) for 20 min and then stimulated with IL-1β (3 nM, 60 min) in the presence of LY294002. Fig. 4A illustrates representative confocal images showing the different GABAAR distribution in the animal and vegetal hemispheres of three oocytes under both basal and IL-1β-stimulated conditions. It should be noted that the cytokine significantly increases GABAAR transport to both hemispheres, although it is much more efficient in activating receptor translocation to the animal surface (Fig. 4B; pooled results of several oocytes from two donors), suggesting a clear polarization of receptor distribution. The presence of LY294002 practically suppresses the IL-1β effect on receptor transport. The same result was obtained when I_GABA was recorded in voltage-clamped oocytes stimulated with IL-1β; the presence of LY294002 completely prevents the cytokine-mediated effect on I_GABA. Furthermore, the continuous perfusion of LY294002 reduced slightly, but significantly, the currents in a reversible manner, irrespective of the presence or absence of the cytokine. Pooled results of the LY294002 effect on GABA-elicted current observed in several oocytes assayed as above are summarized in Fig. 4C.

IL-1β Induces Akt Phosphorylation through the PI3K Signaling Pathway in Brain mRNA-injected Oocytes—Probably the best characterized effector of PI3K is the Ser/Thr protein kinaseAkt (36, 37). To determine whether IL-1β induces PI3K-mediated Akt activation in oocytes, we assayed the phosphorylation of Akt in these cells in the absence or presence of LY294002. Three days after injection, six groups of oocytes (10 oocytes/group) were incubated with IL-1β (3 nM) for different times ranging from 0 to 60 min. Activation of Akt was monitored by the specific anti-phospho-Akt antibody that only recognizes the active, Thr-308-phosphorylated form of Akt. As shown in Fig. 5A, IL-1β induced a time-dependent activation of Akt that was maximal after 30 min. Preincubation with LY294002 (10 μM, added 20 min before and during the 30 min of oocyte incubation with 3 nM of IL-1β) drastically reduced cytokine-elicted Akt activation (Fig. 5B). It should be noted that nontreated oocytes possess a detectable basal Akt activity that was also reduced by LY294002 (Fig. 5B). These results indicate that IL-1β activates the PI3K/Akt pathway in brain mRNA-injected oocytes and

current (I_peak) in the absence (control) or presence of IL-1β. The inset of Fig. 3E shows that the ratio between peak and late I_GABA measured in several control oocytes remains unaltered in IL-1β-treated oocytes. Two interesting conclusions arise from the present electrophysiological data as follows: 1) the onset of the IL-1β action develops slowly, requiring several minutes after the cytokine application for a full effect (ranging from 40 to 50 min); 2) the cytokine increases the amplitude of I_GABA without affecting its kinetics.

Inhibition of PI3K Prevents IL-1β Effects on GABAARs—To investigate the role of PI3K in regulating the IL-1β effect on GABAARs, our next experiments focused on GABAAR expression in oocytes incubated with the PI3K inhibitor LY294002. Oocytes injected with rat brain

FIGURE 4. Effects of IL-1β on GABAAR function in oocytes involve a PI3K-mediated mechanism. A, confocal images of three intact brain mRNA-injected oocytes immunostained for GABAARs. Arrows a and v indicate the animal and vegetal hemispheres, respectively. A polarized distribution of the receptors seems evident on the animal hemisphere of the oocyte under both basal and IL-1β-stimulated conditions (3 nM, 60 min), even though the cytokine significantly increases GABAAR expression at the oocyte surface of both hemispheres. The effect of the cytokine on GABAAR incorporation to the oocyte surface is completely blocked by the PI3K inhibitory drug, LY294002 (10 μM), added beginning 20 min before and during cytokine incubation. Right, oocyte orientation on the confocal microscope stage. B, fluorescence intensity values, at the animal and vegetal surfaces of control and IL-1β-stimulated oocytes, are given in a.u. Each bar shows the mean ± S.E. of 5–6 oocytes. *, p < 0.05; ***, p < 0.001 upon comparing membrane fluorescence as indicated. C, blockade of PI3K by LY294002 reduces the IL-1β-mediated effect on GABAAR currents. Experimental procedures similar to those described in Fig. 3G for control or IL-1β-treated voltage-clamped oocytes were employed here, but this time we also assayed the effect of LY294002 alone or combined with IL-1β. The PI3K inhibitor (10 μM) was perfused 20 min before and during the cytokine pulse. Each bar shows mean ± S.E. of the normalized I_GABA obtained in the four groups of oocytes (n = 4–5) treated as indicated; in all cases, values of peak I_GABA were determined at the time at which IL-1β reached its maximum effect on the current. ***, p < 0.001; ****, p < 0.001 upon comparing different treatments of oocytes with the control group. Note that the LY294002 slightly, but significantly, reduced the current, irrespective of the presence or absence of IL-1β.
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Figure 6. Different manipulations of the Akt activity in oocytes alter GABAAR function. A and B, oocytes injected with cerebral mRNA. A, detailed confocal image of immunostained GABAARs at the animal cell surface in a control and two IL-1β-stimulated oocytes (3 μM, 1 h) in the absence or presence of the Akt inhibitor API-2 (5 μM added from 30 min before and during IL-1β incubation). B, original traces of GABA-elicited current obtained in four voltage-clamped oocytes stimulated as described in Fig. 3; panels from left to right show the potentiation of IgABA by IL-1β and the suppression of this effect by Akt but not by PKC inhibition. The Akt inhibitors (SH-5 and API-2, 5 μM) or the PKC inhibitor (Go6850, 125 nM) were perfused 30 min before and during the period of application of IL-1β. Each confocal image in A and each panel in B represents a typical oocyte from a set of 3 to 5. C, confocal image of three oocytes doubly injected with cerebral and myr-Akt1.ER* mRNAs; the images show, at a high magnification, the fluorescent signal corresponding to basal GABAAR expression at the animal surface (control); the middle and right images correspond to two oocytes incubated with 4-HT (10 μM, 1 h). Histogram shows pooled results of the 4-HT effect on GABAAR recruitment at the cell surface in 14 oocytes from two donors manipulated as described above. ***, p ≤ 0.001.

FIGURE 6. Different manipulations of the Akt activity in oocytes alter GABAAR function. A and B, oocytes injected with cerebral mRNA. A, detailed confocal image of immunostained GABAARs at the animal cell surface in a control and two IL-1β-stimulated oocytes (3 μM, 1 h) in the absence or presence of the Akt inhibitor API-2 (5 μM added from 30 min before and during IL-1β incubation). B, original traces of GABA-elicited current obtained in four voltage-clamped oocytes stimulated as described in Fig. 3; panels from left to right show the potentiation of IgABA by IL-1β and the suppression of this effect by Akt but not by PKC inhibition. The Akt inhibitors (SH-5 and API-2, 5 μM) or the PKC inhibitor (Go6850, 125 nM) were perfused 30 min before and during the period of application of IL-1β. Each confocal image in A and each panel in B represents a typical oocyte from a set of 3 to 5. C, confocal image of three oocytes doubly injected with cerebral and myr-Akt1.ER* mRNAs; the images show, at a high magnification, the fluorescent signal corresponding to basal GABAAR expression at the animal surface (control); the middle and right images correspond to two oocytes incubated with 4-HT (10 μM, 1 h). Histogram shows pooled results of the 4-HT effect on GABAAR recruitment at the cell surface in 14 oocytes from two donors manipulated as described above. ***, p ≤ 0.001.

raise the possibility that the phosphorylated Akt might be responsible for GABAAR redistribution to the cell membrane surface.

To prove that the above hypothesis was true, we next examined whether interfering with Akt activity could modify the effects of IL-1β on GABAAR function. Two different strategies were followed to either reduce or mimic the IL-1β-elicited Akt activation in oocytes as follows: 1) the down-regulation of Akt activity by specific and recently commercialized inhibitors of this kinase (38, 39); 2) the expression of a conditionally active form of Akt (myr-Akt1.ER*) in oocytes. Preincubation of the oocytes with the Akt inhibitors (SH-5 or API-2, 5 μM) for 30 min and during the exposure to IL-1β (3 μM, 1 h) completely blocked the cytokine-induced effect on GABAAR insertion to the cell surface and on IgABA (Fig. 6, A and B). In contrast, the PKC inhibitor (Go6850, 125 nM) did not modify the effect of IL-1β on GABAAR function (Fig. 6B). These results suggest that the PI3K effector Akt is required for the regulation of GABAAR function by the cytokine. To determine further whether active Akt, by itself, could mimic the IL-1β effect on GABAAR trafficking, we applied a second strategy based on the double injection of oocytes with rat brain mRNA and the messenger for the conditionally active form of Akt. The incubation of these oocytes with 4-HT (10 μM, 60 min) reproduced the effect of IL-1β on GABAAR recruitment to the cell membrane surface (Fig. 6C).

Increase of GABAAR β2/β3 Subunit Immunoreactivity in Hippocampal Neurons of Septic Rats—Finally, to determine whether GABAergic neurotransmission is modified during sepsis in vivo, we next evaluated GABAAR β2/β3 subunit immunoreactivity in different brain regions, including the hippocampi of control and LPS-induced septic rats. The hippocampus was chosen because individual profiles of neuronal somata and their dendrites could be easily detected. In virtually all brain regions except the hippocampus, GABAAR immunoreactivity revealed a dense neuropil meshwork where individual neuronal profiles could not be distinguished. Fig. 7 shows that neuronal staining (most likely interneurons) was stronger in the hippocampus of septic rats (A′ and B′) than in control rats (A and B).

DISCUSSION

This study reveals for the first time the existence of a clear association between high circulating levels of IL-1β and the development of encephalopathy in septic patients. We also provide direct experimental evidence for IL-1β-mediated enhancement of plasma membrane insertion of GABAARs in situ, in both cultured neurons and oocytes expressing the recombinant receptor. Moreover, our results in oocytes show that Akt activation by IL-1β plays an important role in GABAAR redistribution. Finally, we demonstrate an increase of GABAAR β2/β3 immunoreactivity in vivo in the hippocampus of septic rats.

The manifestations of SAE resemble those referred to as “sickness behavior” in rodents consisting of lethargy-hypomotility and increased sleep and can be seen in mice inoculated intranasally with influenza virus or by injection of LPS or IL-1β into the peritoneum or the lateral ventricle of the brain (17, 40). The implication of IL-1β in this syndrome is clear because the intraventricular injection of IL-1ra, which blocks brain IL-1RI by binding with high affinity to the receptor but is devoid of any agonist activity (41, 42), has been shown to attenuate the sickness behavior induced by LPS (43). Interestingly, our findings in septic patients reflect the same picture in humans. Thus, the group of patients that developed encephalopathy had significantly higher IL-1β and lower IL-1ra plasmatic levels than the group that did not (Table 2). Moreover, the subgroup of patients whose encephalopathy resolved completely by the 3rd day showed a significant reduction in the circulating levels of IL-1β compared with their initial values as well as with the values
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observed in patients whose neurological symptoms persisted. In fact, IL-1β was the only cytokine whose concentration was elevated above basal levels in the CSF of all patients who remained encephalopathic after the 3rd day.

The above findings raised the possibility that elevated IL-1β production in septic patients may be an important factor in the development of encephalopathy. Because the encephalopathy had eventually completely reversed in all septic patients that were included in our study and their IL-1β levels had returned to the normal range (Table 2), we sought a reversible effect of the cytokine on the brain. In fact, accumulating evidence has demonstrated that, in addition to its key role in the immune system at the periphery, this cytokine has pleiotropic functions and effects in the brain, including host defense responses to neuroinflammation, fever, neuroendocrine activation, somnogenesis, behavioral depression, neuronal cell death, and modulation of synaptic transmission by its action on different classic neurotransmitter systems (44–47). Among the above referred actions, a very interesting and controversial point that could explain some of the neurological symptoms observed in SAE was the IL-1β effect on inhibitory neurotransmission. However, both decreases (48, 49) and increases (44, 50–53) in hippocampal GABAergic transmission have been reported with IL-1β. This contradiction has been attributed to the cytokine concentration, as well as to the type of cell or GABAAR subtype being investigated. Even within a well defined anatomical region, such as the hippocampus, in situ hybridization and immunoprecipitation experiments have shown the presence of multiple receptor subunits (reviewed in Ref. 54). Thus, the mechanism involved in the IL-1β/GABAAR interaction still remains to be elucidated.

Our data from rat hippocampal neurons provide evidence supporting the view that the cytokine might increase central GABAergic activity (Figs. 1 and 2). Interestingly, the cytokine effect on GABAAR redistribution in neurons was also reproduced in a completely different cell type, the frog oocytes expressing recombinant GABAARs (Fig. 3, B and C). The similar response to IL-1β, no matter the cell type or the origin of the GABAAR protein, most likely reflects a generalized cross-talk mechanism connecting the cytokine with the neurotransmitter receptor.

To explore such a mechanism we used brain mRNA-injected oocytes that express most of the neurotransmitter receptors and proteins of cerebral tissue, including GABAARs and IL-1RI (Fig. 3, A–C). In fact, the use of Xenopus oocytes to characterize recombinant mammalian GABAARs dates back to the 1980s (55, 56). Since then, numerous studies have used this expression system to characterize particular GABAAR subunit compositions in functional and pharmacological terms (57–60). Moreover, biochemical regulation of recombinant GABAAR expression has often been studied in oocytes (61, 62), and it has been demonstrated that these receptors, either expressed in human embryonic kidney cells or in Xenopus oocytes, are similarly modulated by PKC (63).

Our results in oocytes show that IL-1β-elicited enhancement of GABAAR expression at the cell surface ran parallel to the potentiation of IGABA (Fig. 3, D–G); this finding unequivocally demonstrates that the new receptors that are recruited to the oocyte surface by the cytokine are fully functional. Moreover, the slow onset of the IL-1β effect on IGABA rules out a direct IL-1β action on GABAAR, supporting the alternative notion that a cascade of intracellular signals connects IL-1β with receptor mobilization. The next question was to ascertain whether IL-1β initiates this signaling pathway by interacting with its specific receptor IL-1RI, which, according to the present results, is expressed natively in the neurons (Fig. 1A) and can be efficiently transplanted in brain mRNA-injected oocytes (Fig. 3A). Our findings reveal that this was the case because the endogenous IL-1RI antagonist, IL-1ra, completely, but in a reversible manner, prevented the IL-1β effect on IGABA (Fig. 3, F and G).

The polarized distribution of brain GABAARs expressed in mRNA-injected oocytes, both in basal conditions or after the cell treatment with IL-1β, is interesting (Fig. 4). This finding agrees with previous electrophysiological data showing a polarization of currents through the foreign GABAAR and ion channels expressed in this cellular model (64). Although still undefined, the mechanism behind this polarized distribution of exogenous proteins in the oocyte seems to be associated to microtubule networks because depolymerization results in a random distribution of otherwise selectively targeted voltage-dependent sodium channels across the oocyte membrane.

The existence of an inducible up-regulation of the GABAAR function triggered by the IL-1β/IL-1RI interaction raises questions on the nature of the signaling pathway connecting the cytokine with the GABAAR. There are studies reporting that insulin recruits GABAARs to postsynaptic domains of hippocampal neurons as the result of Akt-mediated phosphorylation of the receptor (65) and that, through Akt activation, IL-1β has an in vivo neuroprotective effect in retinal ganglion cells (66). Thus, the possibility exists that Akt activation might be involved in the IL-1β effects on GABAAR redistribution. Our results in oocytes provide direct evidence that this is the case because the inhibition of the Akt upstream signaling molecule PI3K attenuates IL-1β-mediated Akt phosphorylation in oocytes (Fig. 5B) and, consequently, blocked the IL-1β effect on GABAAR redistribution and IGABA (Fig. 4). Moreover, the incubation of oocytes with Akt inhibitors prevents all of the above-described effects of IL-1β (Fig. 6, A and B). An opposite finding to the one obtained with the Akt inhibitors was obtained in oocytes coinjected with the brain and myr-Akt1.ER* mRNAs and then stimulated with...
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4-HT; this stimulus mimicked the effect of IL-1β on GABAAR expression at the oocyte surface (Fig. 6C).

Accordingly, our findings indicate that activated Akt is “necessary” and “sufficient” to mediate the IL-1β-induced potentiation of GABAAR function in oocytes and, most probably, in neurons. Because recent studies have demonstrated that upon activation of Akt by insulin, this kinase phosphorylates β2 GABAAR subunit at Ser-410 and this phosphorylation results in enhancement of the number of GABAARs on the plasma membrane surface in HEK293 cells and in neurons (65), then the possibility exists that a similar mechanism may also play a critical role in the IL-1β-mediated GABAAR redistribution observed in oocytes and in neurons. In fact, we have found that IL-1β induces phosphorylation of native GABAAR β2/β3 subunits in neurons (Fig. 2D). However, whether Akt is the main kinase implicated in the receptor phosphorylation by the cytokine and, if so, which GABAAR subunit is the substrate for the kinase or whether receptor phosphorylation by IL-1β results in increased plasma membrane insertion of the receptor remains to be determined in future studies. In this sense, we are planning plasmid-transfection experiments with GABAAR subunits, kindly provided by Dr. Y. T. Wang, using a cell line that natively expresses IL-1RI.

In summary, our clinical and experimental data suggest a sequence of events by which certain patients suffering sepsis might produce elevated IL-1β together with a reduced release of its endogenous antagonist IL-1ra. This “imbalance” would activate the entire signaling cascade that increases Akt-mediated GABAergic activity, and if this occurs it would markedly inhibit brain synaptic activity. This mechanism could underlie the enhanced GABAergic activity observed in the hippocampus of septic rats (present results; see Fig. 7) or both the increased GABAergic transmission in rat hippocampus evoked by chronic LPS exposure (67) and the long lasting depression of EPSPs in the CA1 region elicited by IL-1α (53). However, at this time, we cannot rule out the possibility that the cytokine might also reduce postsynaptic (AMPA)ergic (where AMPA is α-methyl-D-aspartate-dependent excitatory synaptic transmission, as has been reported by other studies (52, 68–70). We propose that by one or both mechanisms, IL-1β might alter synaptic strength at central synapses and so contribute to the cognitive dysfunction observed in SAE and other infectious and inflammatory disorders that course with elevated IL-1β levels.

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