

Interleukin-1 β Released by gp120 Drives Neural Death through Tyrosine Phosphorylation and Trafficking of NMDA Receptors*

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Barbara Viviani^{1,2}, Fabrizio Gardoni¹, Stefano Bartesaghi, Emanuela Corsini, Alessandra Facchi, Corrado L. Galli, Monica Di Luca, and Marina Marinovich

From the Laboratory of Toxicology and Center of Excellence on Neurodegenerative Diseases, Department of Pharmacological Sciences, University of Milan, 20133 Milan, Italy

Interleukin-1 β is a proinflammatory cytokine implicated under pathological conditions involving NMDA receptor activation, including the AIDS dementia complex (HAD). No information is available on the molecular mechanisms recruited by native interleukin-1 β produced under this type of condition. Using a sandwich co-culture of primary hippocampal neurons and glia, we investigated whether native interleukin-1 β released by HIV-gp120-activated glia (i) affects NMDAR functions and (ii) the relevance on neuronal spine density and survival, two specific traits of HAD. Increased phosphorylation of NR2B Tyr-1472 was observed after 24 h of exposure of neurons to 600 pM gp120. This effect occurred only when neurons were treated in the presence of glial cells and was abolished by the interleukin-1 receptor antagonist (IL-1ra). Gp120-induced phosphorylation of NR2B resulted in a sustained elevation of intracellular Ca²⁺ in neurons and in a significant increase of NR2B binding to PSD95. Increased intracellular Ca²⁺ was prevented by 10 μ M ifenprodil, that selectively inhibits receptors containing the NR2B, by interleukin-1ra and by Ca-pYEEIE, a Src family SH2 inhibitor peptide. These last two inhibitors, prevented also NR2B binding to PSD95. Finally, gp120 reduced by 35% of the total PSD95 positive spine density after 48 h of treatment and induced by 30% of the neuronal death. Again, both of these effects were blocked by Ca-pYEEIE. Altogether, our data show that gp120 releasing interleukin-1 β from glia increases tyrosine phosphorylation of NMDAR. Thus, tyrosine phosphorylation may contribute to the sensitization of the receptor increasing its function and synaptic localization. Both of these effects are relevant for neurodegeneration.

Interleukin-1 β (IL-1 β)³ has been proposed as a novel neuromodulator involved in the communication between glia and

neurons (1–4), opening up new perspective in the current view of brain behavior. This cytokine, locally produced by glial cells as a consequence of CNS diseases and/or in response to neuronal activities (4), exerts a profound impact on neuronal functionality through a specific receptor. So far, IL-1 β has been implicated in the exacerbation of neuronal damage caused by excitotoxic, ischemic, traumatic brain injury (5, 6), and viral infection (AIDS dementia complex: HAD) (7–9), in seizures (10) as well as in physiological events such as long-term potentiation (11, 12), sleep (13), memory consolidation (14, 15), and pain (16). Although IL-1 β may offer exciting potential in the discovery of novel targets to modulate brain behavior, the biochemical pathways specifically recruited in neurons by this cytokine have not been explored in detail.

Recombinant IL-1 β potentiates calcium response and neuronal death achieved through NMDA receptor activation in primary hippocampal neurons by increasing phosphorylation of NR2B tyrosine 1472 (17). Tyrosine phosphorylation of NR2 subunits has been involved in NMDAR channel gating, protection of NR2 subunits against degradation by calpain, assembly with signaling proteins and NMDAR trafficking (18–20), and it is therefore relevant in the dynamic modulation of NMDARs. The ability of IL-1 β to specifically recruit NR2 tyrosine phosphorylation in neurons (17, 18) could provide a plausible biochemical explanation of the effects exerted *in vivo* by this cytokine and supports IL-1 β as a neuromodulator possibly involved in those physiological and pathological aspects relying on NMDAR.

IL-1 β enhancement of NMDA functions may represent an important part of the complex cascade of events in AIDS dementia complex (HAD). It is well established that overstimulation of NMDAR represents a key event in HAD (21, 22), the most common cause of dementia worldwide among people aged 40 or less (23). Behavioral symptoms characteristic of HAD such as cognitive decline, personality change, and motor deficits (24, 25) appear to be consequent to decreased synaptic density, spine loss, dendritic simplification, and neuronal loss (26, 27) all of which are thought to result from release of toxic factors from activated macrophages and glial cells (21, 28). At the moment, no information is available on the possible recruit-

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¹ These two authors contributed equally to this work.

² To whom correspondence should be addressed: Dept. of Pharmacological Sciences, Via Balzaretti 9, 20133 Milan, Italy. Tel.: 39-0-250318356; Fax: 39-0-2503183260; E-mail: Barbara.Viviani@unimi.it.

³ The abbreviations used are: IL-1 β , interleukin-1 β ; HIV, human immunodeficiency virus; ANOVA, analysis of variance; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide; IL-1ra, interleukin-1 receptor antag-

onist; PSD, postsynaptic density; TIF, Triton-insoluble fractions; NMDA, N-methyl-D-aspartate.

ment of NR2 subunits, Tyr phosphorylation in HAD, and the involvement of glial released IL-1 β .

To test this hypothesis we exposed primary hippocampal neurons and glial cells to HIV-envelope glycoprotein gp120, considered one of the pathological agents of HAD (29). We then examined NR2B Tyr-1472 phosphorylation in neurons, part of the pathway leading to this process and the relevance on intracellular Ca²⁺ homeostasis. Finally, we evaluated the functional consequences of two hallmarks of HAD: dendritic spine density and neuronal survival.

EXPERIMENTAL PROCEDURES

Sandwich Co-cultures of Hippocampal Neurons and Glial Cells—Primary cultures of glial cells were prepared from 1- to 2-day-old newborn rats (Sprague-Dawley). All animal care procedures were in accordance with the local Animal Care Committee, and no weight loss or death was observed after receipt of rats in our animal facility. Pregnant rats were housed over wood chip bedding, acclimatized to a 12-h light-dark cycle and allowed food and water *ad libitum*. All efforts were made to minimize the suffering of animals.

Cerebral hemispheres were freed from the meninges and mechanically disrupted. Cells were dispersed in a solution of trypsin 2.5% and DNase 1%, filtered through a 100- μ m nylon mesh and plated (140,000 cells per 35-mm dish) in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum, 0.6% glucose, streptomycin (0.1 mg/ml), and penicillin (100 international units/ml). Glial cultures were fed twice a week and grown at 37 °C in a humidified incubator with 5% CO₂.

Neuronal cultures were established from the hippocampus of 18-day rat fetuses. Briefly, brains were removed and freed from meninges, and the hippocampus was isolated. Cells were then dispersed by incubation for 5 min at 37 °C in a 2.5% trypsin solution followed by trituration. The cell suspension was diluted in Neurobasal media supplemented with 1% B-27 (Invitrogen) and plated onto polyornithine-coated coverslips at a density of 80,000 cells per coverslip. Three days before treatment with gp120, coverslips were transferred to dishes containing a glial monolayer in neuron maintenance medium. Coverslips were inverted so that the hippocampal neurons faced the glia monolayer; both cell preparations were immersed in the same culture medium. Paraffin dots adhering to the coverslips supported them above the glia, creating a narrow gap that prevented the two cell types from contacting each other but allowed the diffusion of soluble substances. These culture conditions allowed us to grow differentiated neuronal cultures with >96% homogeneity, as assessed by immunocytochemistry of microtubule-associated protein 2 and glial fibrillary acidic protein (Molecular Probes). Hippocampal neurons were used after a culturing period of 14 days.

Gp120 and Treatments—Recombinant HIV-gp120 protein type IIIB (Bartels, Carlsbad, CA) was used in all the experiments. Neurons were exposed to drugs in their culture medium (Neurobasal media supplemented with 1% B-27). Treatment with the glycoprotein was mainly performed in the presence of glia. To this purpose, primary hippocampal neurons were transferred on a glial monolayer for 3 days before exposure to

gp120. Glass coverslips with primary hippocampal neurons were detached from the glial monolayer at the end of the exposure and assessed for NMDAR Tyr phosphorylation and trafficking, Ca²⁺ homeostasis, cell fractionation, confocal microscopy, and cell death.

1 μ g/ml human recombinant IL-1 α (R&D Systems, Minneapolis, MN), 10 μ M ifenprodil hemitartrate (Tocris, Bristol, UK), 10 μ M TAT-2B (Primm, Milano, Italy) were incubated with gp120 and kept in the incubation medium until the end of the treatment. In contrast, 10 μ g/ml caffeic acid-pYEEIE (Ca-pYEEIE; Tocris) was delivered only in primary hippocampal neurons, prior to gp120 treatment, by means of the lipid base transfection reagent Chariot (Active Motif, Rixensart, Belgium) according to supplier instructions. Briefly, caffeic acid-pYEEIE was incubated with Chariot dilution at room temperature for 30 min to allow the formation of the Chariot-molecule complex. Neuronal cells without glia were then overlaid with the Chariot-macromolecule complex solution diluted in Neurobasal and incubated at 37 °C for 1 h. At the end, complete growth medium was added, and incubation continued for another hour. Ca-pYEEIE loaded neurons were then washed to remove extracellular Ca-pYEEIE and transferred to the glial monolayer for gp120 exposure. Control neuronal cultures were run in parallel under each experimental condition and incubated for the appropriate times in the presence or absence of glia.

IL-1 β Assay—IL-1 β release was measured by means of an interleukin-1L rat ELISA system (Quantikine, R&D Systems, Abingdon, UK).

Determination of the Cytosolic-free Ca²⁺ Concentration [Ca²⁺]_i—At the end of exposure to gp120, neurons on glass coverslips were detached from glial cells and loaded with 10 μ M Fura 2-AM (Sigma) for 1 h at 37 °C in the treatment culture medium supplemented with 1% bovine serum albumin, 0.1% pluronic (Sigma). [Ca²⁺]_i measurement was performed in neurons only in Hepes buffer, pH 7.4, as previously described (9). The Fura 2 fluorescence ratio signal was measured in a PerkinElmer Life Sciences 50 B double wavelength fluorometer and calibrated in terms of [Ca²⁺]_i as described by Grynkiewicz *et al.* (30).

Immunofluorescence Labeling, Image Acquisition, and Quantification—Hippocampal neurons were fixed in 100% methanol at -20 °C for 15 min. Primary and secondary antibodies were applied in GDB buffer (30 mM phosphate buffer (pH 7.4) containing 0.2% gelatin, 0.5% Triton X-100, and 0.8 M NaCl). Fluorescence images were acquired using Bio-Rad Radiance 2100 confocal microscope. Confocal images were obtained using a Nikon 60 \times objective with sequential acquisition setting at 1024 \times 1024 pixel resolution. Each image consisted of a z-stack of pictures taken at a depth interval of 0.1 μ m and then projected into one image. Both image acquisition and quantification of the fluorescence signal were performed by operators blind to the experimental condition. PSD-95 staining puncta were defined by thresholding images at three times the background measured within the dendritic shaft. The ratio of average immunofluorescence intensity between the spine head and the dendritic shaft next to it was measured on manually selected spine head and dendritic

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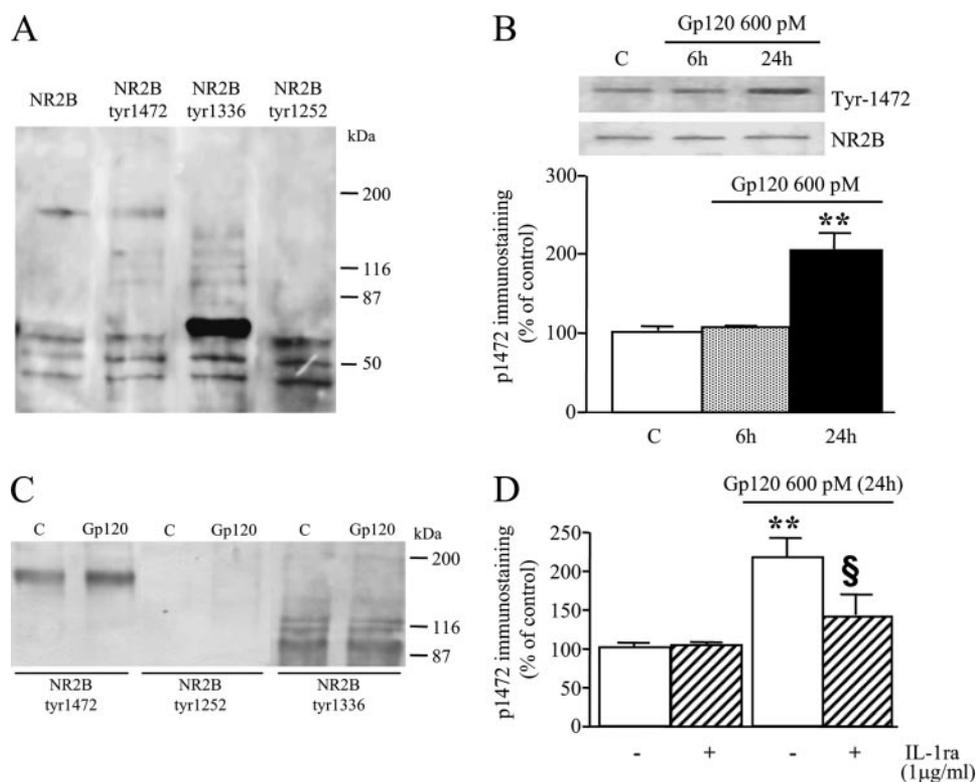


FIGURE 1. Gp120 effect on Tyr-1472 phosphorylation of NR2B subunits. *A*, Western blotting analysis for NR2B, NR2B-Tyr1472, NR2B-Tyr-1336 and NR2B-Tyr-1252 performed from cultured hippocampal neurons homogenate. *B*, representative Western blotting and quantification of NR2B-Tyr-1472 phosphorylation after exposure for 6 and 24 h of hippocampal neurons to gp120 in the presence of glia. At the end of treatment, neurons were detached from glia and lysed for Western blotting. Values are mean \pm S.E. of three independent experiments (**, $p < 0.01$ gp120 versus C; ANOVA followed by Tukey's test). *C*, Western blotting analysis for NR2B-Tyr-1472, NR2B-Tyr-1336 and NR2B-Tyr-1252 from control and 24-h gp120-treated hippocampal neurons. *D*, quantification of NR2B-Tyr-1472 phosphorylation after exposure of hippocampal neurons in the presence of glia \pm IL-1ra to gp120 for 24 h. Values are mean \pm S.E. of three independent experiments (**, $p < 0.01$ gp120 versus C, §, $p < 0.05$ gp120 + IL-1ra versus gp120; ANOVA followed by Tukey's test).

shaft areas. Neurons selected for analysis had a similar local environment; synapses on soma were not analyzed. For statistical analysis of spine number, a dendritic shaft of 100 μ m starting from the cell soma from 12 neurons for each experimental condition was analyzed. Colocalization analysis was performed using Bio-Rad Laserpix software.

Subcellular Fractionation—Triton-insoluble fractions (TIF) were isolated from neurons harvested at 14 DIV as previously described (31). PSDs from rat hippocampus were purified as previously described (32).

Immunoprecipitation Experiments—Hippocampal neurons were harvested in ice-cold solubilization buffer containing 50 mM HEPES, pH 7.4, 0.5 M NaCl, 0.5% Triton X-100, 0.1% deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, in the presence of a complete set of proteases inhibitors (CompleteTM). Each solubilized sample was incubated overnight with NR2B polyclonal antibody and then for 2 h with protein A-Sepharose beads. Following this incubation, the supernatants were removed, and the beads were washed five times with solubilization buffer. After the final wash, the beads were resuspended in sample buffer for SDS-PAGE and briefly centrifuged; the supernatants were loaded on 7% SDS-PAGE gels. Western blot analysis was performed by computer-assisted imaging (Quantity-One^R System; Bio-Rad).

assay (33). MTT tetrazolium salt was dissolved in serum-free medium to a final concentration of 0.75 mg/ml and added to the cells before the end of the experiment for 3 h at 37 $^{\circ}$ C. The medium was then removed and the formazan was extracted with 1N HCl:isopropyl alcohol (1:24). Absorbance at 560 nm was read on a Multiscan reader.

Statistical Analysis—Statistical significance of differences was determined by one-way or two-way analysis of variance (ANOVA) followed by a multiple comparison test (Tukey's test). A significance level of 95% ($p < 0.05$) was accepted.

RESULTS

gp120 Effect on Tyr-1472 Phosphorylation of NR2B Subunits: Relevance of Glial Cells and Role of IL-1 β —In the last few years, several potential Src phosphosites have been described within the NR2B C-terminal tail (34, 35). Not only Tyr-1472 but also Tyr-1252 and Tyr-1336 have been demonstrated as Src family phosphorylation sites at least in transfected cells. To check for endogenous NR2B tyrosine phosphorylation in cultured hippocampal neurons co-cultured with glia, Western blotting analysis was performed from DIV14 neuronal lysates.

No signal for both NR2B Tyr-1336 and NR2B Tyr-1252 was found at the expected 170-kDa molecular mass (Fig. 1A). On the other hand, a nice immunosignal for both total NR2B and NR2B Tyr-1472 was present in the same samples. These data

Antibodies—Polyclonal IL-1R antibody was purchased from Santa Cruz Biotechnology; α -CaMKII monoclonal antibody was purchased from Chemicon International, Inc., (Temecula, CA); monoclonal PSD-95 antibody was purchased from Affinity BioReagents Inc. (Golden, CO); monoclonal PY20 antibody and polyclonal pTyr-1472-NR2B antibody were purchased from Calbiochem (Nottingham, UK), polyclonal anti- β -tubulin was purchased from Covance (Berkeley, CA); monoclonal anti-NR2A was purchased from Zymed Laboratories Inc.; monoclonal anti-synaptophysin was purchased from Sigma-Aldrich; polyclonal anti-NR2A, polyclonal anti-NR2B, and AlexaFluor 488, 555, and 568 secondary antibodies were purchased from Molecular Probes (Eugene, Oregon).

Viability Assay—Neuronal cell death was monitored over 6, 24, 48, and 72 h. In a set of experiments neurons were loaded with caffeic acid-pYEEIE or IL-1ra, and exposed to 600 pM gp120 for 72 h. Cell viability was measured by the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

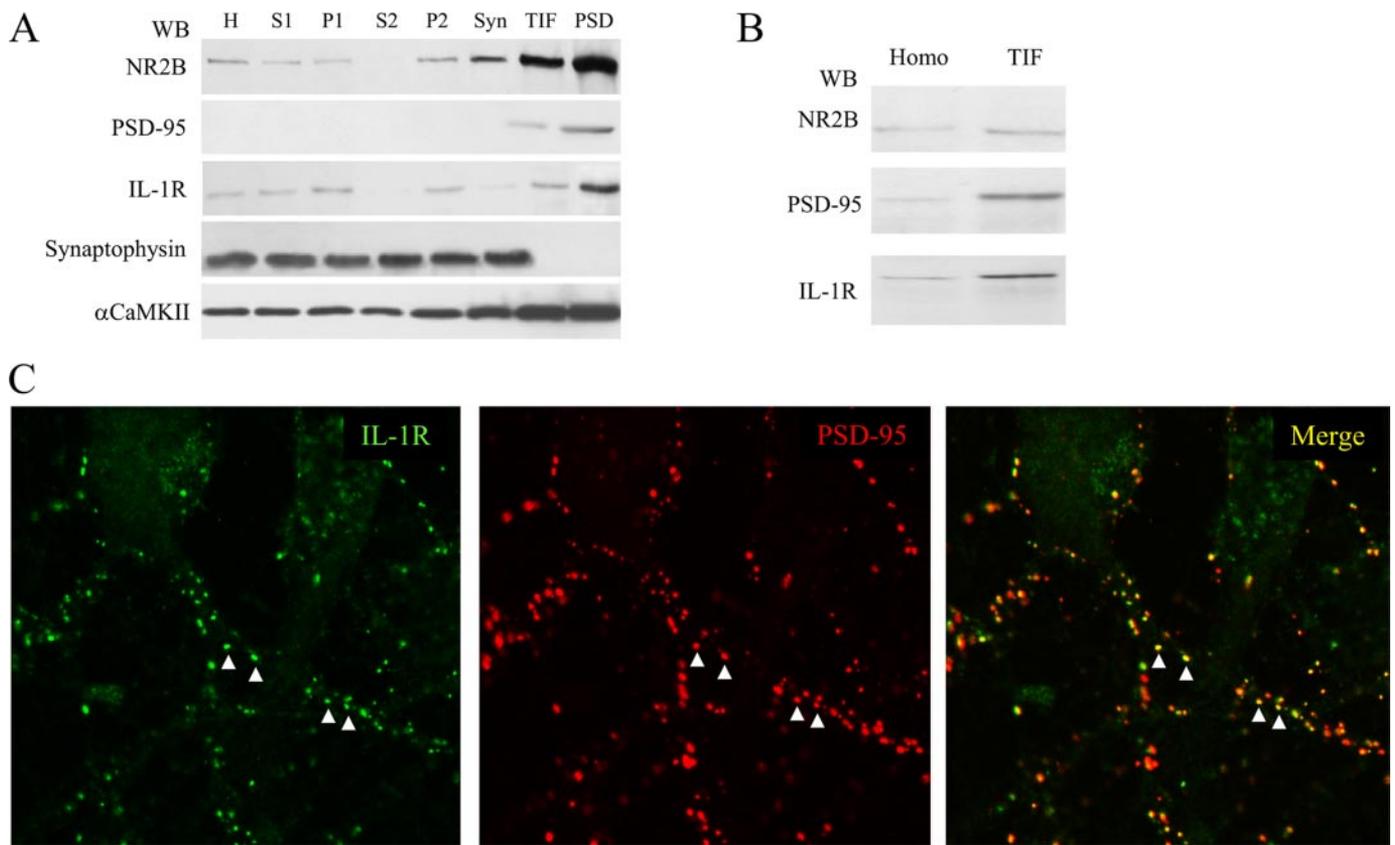


FIGURE 2. **Characterization of IL-1R, NR2B, and PSD-95 colocalization in neurons.** *A*, isolated biochemical fractions from hippocampal tissues were separated by SDS-PAGE, and the blots were probed with antibodies against NR2B, PSD-95, IL-1R, synaptophysin, and α CaMKII. *H*, homogenate; *S1*, low speed supernatant; *P1*, nuclei-associated membranes; *S2*, cytosol; *P2*, crude membrane fraction; *Syn*, synaptosomes. *B*, representative Western blot analysis of NR2B, PSD-95 and IL-1R from homogenate (Homo, left lane) and TIF (right lane) obtained from *DIV14* hippocampal cultures. The same amount of protein was loaded in each lane. *C*, *DIV14* hippocampal neurons were immunolabeled for IL-1R (left panels) or PSD-95 (middle panels). Merge data are shown on the right. Areas of overlap in merge panels appear yellow.

suggest that Tyr-1252 and Tyr-1336 could represent Src family phosphosites only *in vitro* in transfected cells, but not under our experimental conditions in neurons.

Hippocampal neurons co-cultured with glia were treated with 600 pM gp120 for 6 and 24 h, and NR2B subunit Tyr phosphorylation was evaluated. Neurons were then separated by glia and lysed, and phosphorylation at NR2B Tyr-1472 was assessed with a specific antibody. A significant increase of NR2B Tyr-1472 phosphorylation was evident 24 h after gp120 treatment (Fig. 1B, **, $p < 0.01$ gp120 versus control), whereas 6 h of treatment was ineffective (Fig. 1B). This effect was specifically limited to phosphorylation processes because NR2B protein level was not affected at the above time points (Fig. 1B). No phosphorylation of NR2B Tyr-1336 and NR2B Tyr-1252 was observed after 24 h of gp120 treatment (Fig. 1C), suggesting that these two phosphosites were not involved in the effects induced by gp120 treatment.

The effect of gp120 on NR2B Tyr-1472 phosphorylation in neurons required the presence of glia, because it was undetectable when primary hippocampal neurons were exposed alone to 600 pM gp120 for 24 h (p1472 immunostaining % of control: controls 100 ± 31.6 , gp120: 88 ± 33.0 $n = 3$).

Exposure of primary glial cells to 600 pM gp120 induces a significant release of IL-1 β within 24 h (IL1 β , pg/ml: controls: 62.4 ± 3.19 , gp120: 139 ± 18 ($n = 6$); *, $p < 0.05$ gp versus

control). The glial necessity for gp120 to trigger Tyr-1472 phosphorylation of the NR2B subunit together with the promotion of IL-1 β release suggest the possible involvement of this cytokine in the observed effect. Hippocampal neurons co-cultured with glia were then incubated with 600 pM gp120 in the presence or absence of 1 μ g/ml IL-1 receptor antagonist (IL-1ra). IL-1ra *per se* did not affect basal p1472 immunostaining but prevented gp120-induced increase of Tyr-1472 phosphorylation (Fig. 1D; **, $p < 0.01$ versus control, §, $p < 0.05$ versus gp120), supporting our hypothesis.

Characterization of IL-1R, NR2B, and PSD-95 Colocalization—The ability of IL-1ra to prevent Tyr-1472 phosphorylation induced by gp120, suggests the recruitment of IL-1 receptor (IL-1R) in the observed effect. We first examined the relative abundance of IL-1R in rat hippocampal subcellular compartments by a biochemical fractionation method, as described previously (32). PSD was purified from rat hippocampus, and the expression of IL-1R, NR2B, as well as, pre- and postsynaptic marker proteins in several subcellular compartments was investigated by Western blot analysis. As shown in Fig. 2A, IL-1R was present with a similar distribution in the total homogenate (H) and in the crude membrane fraction (P2), at a low level in the synaptosomal membrane (Syn) and in the low speed supernatant (S1) fractions. IL-1R was enriched in the PSD fraction and in the Triton-insoluble PSD-enriched frac-

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tion (TIF). In the same samples, we examined the subcellular distribution of NR2B, α CaMKII, synaptophysin (a presynaptic marker), and PSD-95 (a postsynaptic marker). As expected, synaptophysin was present in all subcellular compartments analyzed, but not in the PSD or in the TIF-purified fractions, whereas PSD-95 and α CaMKII showed a similar distribution pattern with enrichment in synaptosomes and PSD fractions (Fig. 2A). The partition pattern of NR2B subunit closely resembled IL-1R being similarly distributed in the different subcellular fractions. A modified fractionation method was then used to analyze the presence of endogenous IL-1R at synaptic sites also in primary hippocampal cultures; in particular, a Triton-insoluble postsynaptic fraction (TIF) was prepared and the presence of IL-1R, NR2B, and PSD-95 evaluated by Western blot analysis. As shown in Fig. 2B, IL-1R has a similar enrichment in the TIF fraction compared with both NR2B and PSD-95, confirming the results obtained in rat hippocampal tissue (Fig. 2A).

We then examined by confocal labeling the distribution pattern of IL-1R in cultured hippocampal neurons (Fig. 2C); PSD-95 was used as a marker of postsynaptic structures. IL-1R displayed a very low labeling in the somatic cytoplasm; immunoreactivity was present in dendrites where a punctate staining was present. A nice colocalization pattern with PSD-95 was observed (arrowheads, Fig. 2C), confirming the presence of endogenous IL-1R at the postsynaptic side of cultured neurons.

Effect of gp120-induced Phosphorylation of NR2B Subunits at Tyr-1472 on Intracellular Ca^{2+} ($[Ca^{2+}]_i$) Homeostasis—Tyr phosphorylation is a well recognized pathway adopted from neurons to upregulate NMDA receptor function (for review see Ref. 18) and has been involved in pathological conditions such as ischemic neuronal death (36, 37). Neuronal injury consequent to gp120 is also predominantly mediated by overactivation of NMDAR-coupled ion channels that allows excessive Ca^{2+} influx and a general imbalance of its homeostasis (21, 22). We thus evaluated whether gp120-induced NR2B Tyr phosphorylation, through the release of IL-1 β , could be implicated in the altered $[Ca^{2+}]_i$ homeostasis in neurons. Primary hippocampal neurons were treated with 600 pM gp120 in the presence or absence of glia, separated at the end of treatment and monitored for $[Ca^{2+}]_i$. The exposure of primary hippocampal neurons to 600 pM gp120 in the presence of glia significantly increased neuronal $[Ca^{2+}]_i$ (Fig. 3A) after 24 h. This effect was caused predominantly by Ca^{2+} influx through the NR2B-NMDA receptor channels because the addition of 10 μ M ifenprodil, a channel blocker that binds only to the NR2B subunit of NMDA receptors, inhibited the rise of gp120-induced neuronal $[Ca^{2+}]_i$ (Fig. 3A).

To investigate whether NR2B Tyr phosphorylation was implicated in gp120 modulation of the NMDAR-dependent Ca^{2+} response, we examined the rise of gp120-induced neuronal $[Ca^{2+}]_i$ in the presence of Ca-pYEEIE, a peptide inhibitor for Src family SH2 domain (38). Thus, primary hippocampal neurons were loaded in the absence of glia with 10 μ g/ml Ca-pYEEIE, washed to remove extracellular Ca-pYEEIE, and then exposed for 24 h in the presence of glia to 600 pM gp120. This experimental approach allowed us to act on neurons without interfering with the Src family signal transduction in glia. The classical tyrosine kinases inhibitors PP1 and PP2 could not be

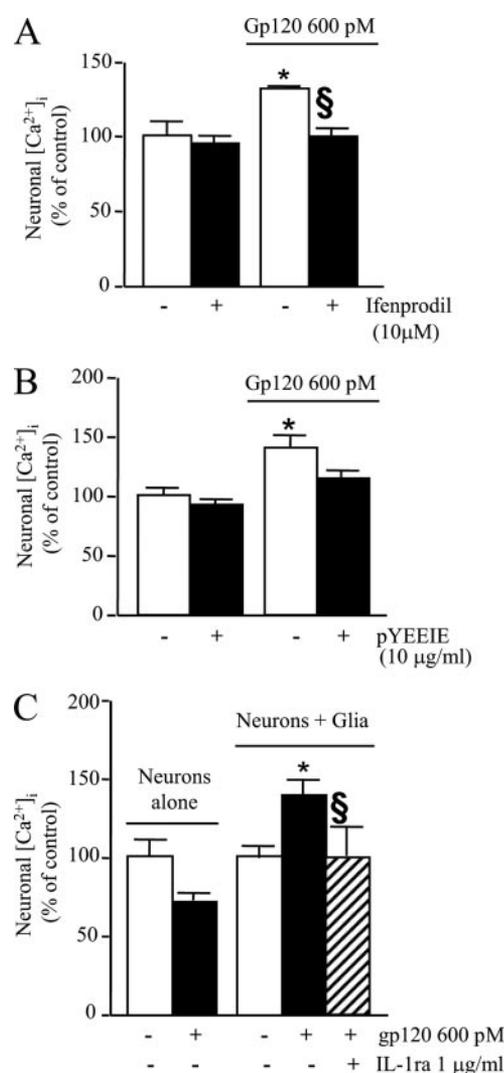


FIGURE 3. Involvement of NR2B subunit Tyr phosphorylation, and IL-1 β in gp120-induced $[Ca^{2+}]_i$ increase in primary cultures of hippocampal neurons. A, hippocampal neurons were exposed to gp120 for 24 h in the presence of glia with (black columns) or without (white columns) 10 μ M ifenprodil. At the end of treatment, hippocampal neurons were detached from the glial monolayer and assayed for $[Ca^{2+}]_i$. Values are means \pm S.E. of nine independent samples and represent the $[Ca^{2+}]_i$ levels in neurons at the end of treatment (*, $p < 0.05$ gp120 versus C, \S , $p < 0.05$ gp120 + ifenprodil versus gp120; ANOVA followed by Tukey's test). B, hippocampal neurons were loaded with Ca-pYEEIE, washed to remove extracellular Ca-pYEEIE and then exposed to gp120 for 24 h in the presence of glia. Values are means \pm S.E. of fifteen independent samples and represent the $[Ca^{2+}]_i$ levels in neurons at the end of treatment (*, $p < 0.05$ gp120 versus C; ANOVA followed by Tukey's test). C, hippocampal neurons were exposed for 24 h in the presence (right) or absence (left) of glia to gp120. IL-1ra was added in part of the co-cultures (striped column). At the end of treatment, co-cultured neurons were detached from glia and assessed for $[Ca^{2+}]_i$. IL-1ra did not affect $[Ca^{2+}]_i$ levels per se ($[Ca^{2+}]_i$, % of control: 112 ± 9.64). Values are means \pm S.E. of nine independent samples and represent the $[Ca^{2+}]_i$ levels in neurons at the end of treatment (*, $p < 0.05$ gp120 versus C, \S , $p < 0.05$ gp120 + IL-1ra versus gp120; ANOVA followed by Tukey's test).

used for this purpose because of the reversibility of their action upon removal (39, 40). As shown in Fig. 3B, in Ca-pYEEIE-pretreated neurons, $[Ca^{2+}]_i$ levels were not altered by gp120 treatment. As expected, treatment with Ca-pYEEIE also prevented Tyr-1472 phosphorylation (data not shown).

As previously observed for NR2B-induced phosphorylation, gp120 ability to increase $[Ca^{2+}]_i$ requires the presence of glia,

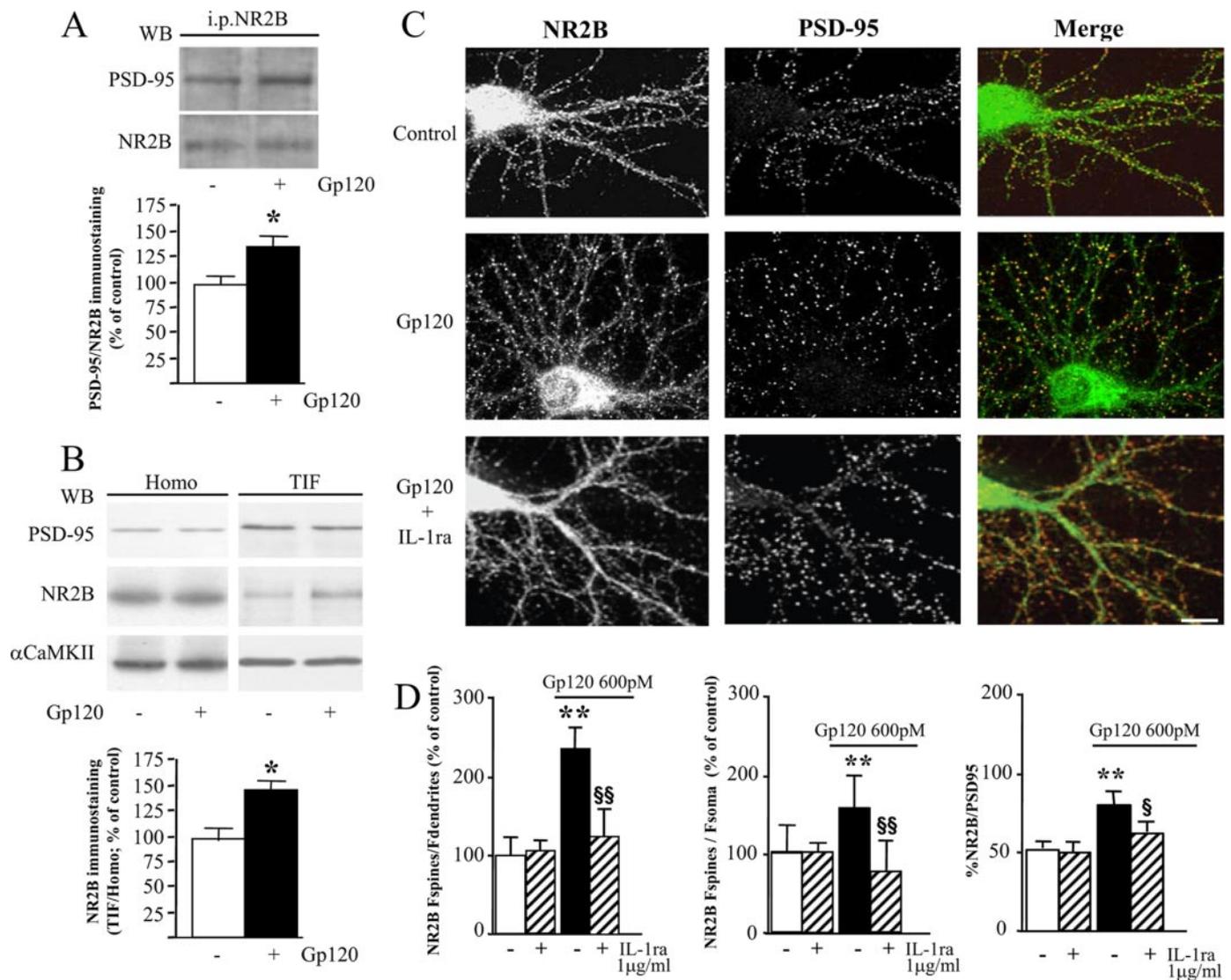


FIGURE 4. gp120 effect on NR2B subcellular localization. *A*, control and gp120-treated neuronal lysates were immunoprecipitated with NR2B polyclonal antibody. Western blot analysis was performed in the immunoprecipitated material with NR2B (lower panel) and PSD-95 (upper panel) antibodies. Histogram shows the quantification of Western blotting for PSD-95 performed in the co-immunoprecipitated material. Data are expressed as PSD-95/NR2B ratio staining in the immunocomplex (*, $p < 0.05$). *B*, Western blotting analysis performed in the homogenate (Homo) and in the TIF fraction obtained from control and gp120-treated hippocampal cultures. The same amount of protein was loaded in each lane. gp120 treatment leads to a higher NR2B localization in the TIF fraction (*, $p < 0.01$) leaving unaffected α CaMKII and PSD-95 immunostaining in the TIF fraction. Values are means \pm S.E. of eight independent samples and represent the NR2B TIF/Homo ratio staining expressed as percentage of control neurons. *C*, hippocampal neurons were either left untreated (control) or treated for 24 h with gp120 in the presence of glia + IL-1ra, fixed, and immunolabeled for NR2B (left panels) or PSD-95 (middle panels). Scale bar, 10 μ m. Merge data are shown on the right. Areas of overlap in merge panels appear yellow. 12 neurons for each experimental condition were analyzed. *D*, quantification of the confocal experiments. gp120 administration results in a redistribution of NR2B into spine-like clusters; IL-1ra treatment interferes with gp120-mediated NR2B trafficking toward spines. The ratio of spines to cell soma or dendrites fluorescence was computed and averaged (**, $p < 0.01$, gp120 versus control; §§, $p < 0.01$, gp120 + IL1ra versus gp120). Gp120 treatment also causes a significant increase in the percentage of NR2B clusters of the total number of PSD-95-positive spines compared with control values (**, $p < 0.01$, gp120 versus control; §, $p < 0.01$, gp120 + IL1ra versus gp120).

because 24 h of treatment with 600 pM gp120 did not affect basal $[Ca^{2+}]_i$ in neurons exposed alone (Fig. 3C). Again, gp120-induced $[Ca^{2+}]_i$ increase in neurons was prevented by 1 μ g/ml IL-1ra (Fig. 3C), confirming the role of IL-1 β and IL-1R as possible mediators of gp120 action on the NMDA receptor.

Effect of gp120 on NR2B Subcellular Localization: Role of IL-1 β —It has been shown that the stability of NMDA receptors at the synaptic membranes is regulated by both tyrosine phosphorylation and interaction with PSD-95 (41). In particular, association of NMDA receptor NR2B subunits with PSD-95 and, as a direct consequence, inhibition of NR2B subunit endocytosis has been suggested to be modulated by phosphorylation

of NR2B Tyr-1472 (42–44). Based on these considerations, we first measured by co-immunoprecipitation assay the effect of 24 h of gp120 treatment on NR2B/PSD-95 interaction. Because of the relevant role exerted by glial cells, neurons were exposed to gp120 in co-culture with glia under all the experimental conditions (even if not stated) and separated at the end of the treatment to measure the parameters of interest. As shown in Fig. 4A, gp120 induced a significant increase of PSD-95 binding to NR2B (+31.1% \pm 6.4%, $p < 0.05$). To confirm this result and to examine the role of gp120 in promoting the synaptic localization of NR2B, TIF was obtained from the control and 24-h gp120-treated neurons and protein levels were measured in the

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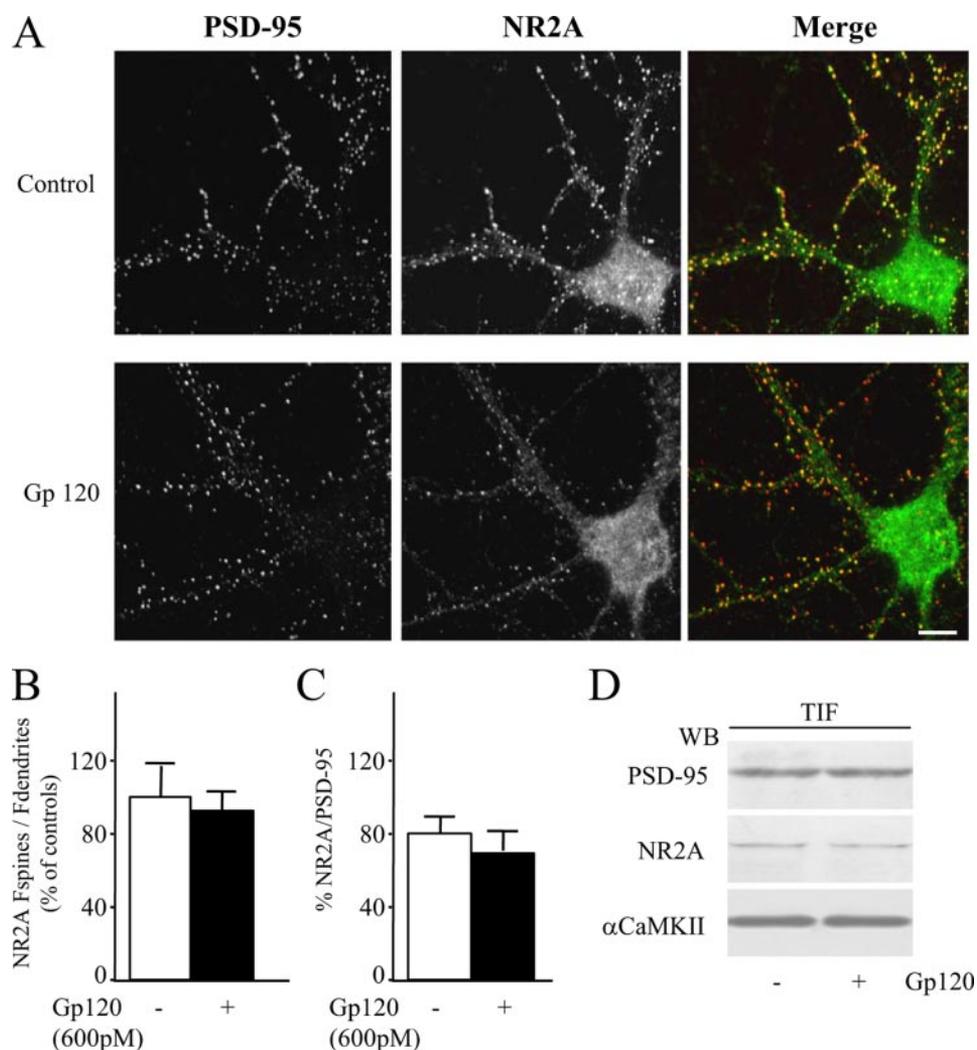


FIGURE 5. NR2A colocalization with PSD-95 after gp120 treatment. *A*, hippocampal neurons were either left untreated (*upper panels*) or treated for 24 h with gp120 (*lower panels*) in the presence of glia and immunolabeled for PSD-95 (*left panels*) or NR2A (*middle panels*). Scale bar, 10 μ m. Merge data are shown on the *right*. Areas of overlap in merge panels appear yellow. *B*, quantification of the confocal experiments. The ratio of spines to dendrite fluorescence was computed and averaged. *C*, gp120 administration does not affect the percentage of NR2A clusters on the total number of PSD-95-positive spines; 12 neurons for each experimental condition were analyzed. *D*, Western blot analysis performed in the TIF fraction obtained from control and gp120-treated hippocampal cultures. The same amount of protein was loaded in each lane. gp120 treatment does not affect NR2A, PSD-95, and α CaMKII localization in the TIF fraction.

homogenate and TIF. The same amount of proteins from homogenate and TIF was loaded on SDS-PAGE gels for Western blot analysis. As shown in Fig. 4*B*, gp120 treatment significantly increased NR2B immunostaining in TIF without affecting the total NR2B protein level in the homogenate (**, $p < 0.01$; +47.2 \pm 8.1%, gp120 *versus* control expressed as NR2B ratio TIF/homogenate). No alteration of both PSD-95 and α CaMKII immunostaining in both homogenate and TIF was observed following gp120 treatment.

To further confirm these data by confocal labeling, primary hippocampal neurons were treated for 24 h with gp120, fixed and double-labeled for NR2B and PSD-95 (Fig. 4*C*). In control neurons (Fig. 4*C*, *upper panels*), NR2B signal was of a greater intensity in the soma, but signals were clearly seen also in PSD-95 positive “spine-like” puncta as well as, in the dendritic shaft. In addition, NR2B immunofluorescence in “spine-like” structures overlapped NR1 immunosignal, further confirming

the presence of the NMDA receptor complex in the postsynaptic site (data not shown). 24-h gp120 treatment produced a more pronounced NR2B punctuated pattern associated with a higher colocalization degree with PSD-95 (Fig. 4*C*). Quantification of NR2B relative fluorescence intensity in spine *versus* dendritic shaft or *versus* soma (Fig. 4*D*) revealed that gp120-treated neurons exhibited an increased fluorescent signal in these spine-like structures (Fig. 4*D*, **, $p < 0.01$, gp120 *versus* control). In addition, quantification of NR2B punctuated staining revealed a significant increase of NR2B immunoreactivity in PSD-95-positive dendritic spines compared with control values (Fig. 4*D*; **, $p < 0.01$, gp120 *versus* Control). Both sets of data suggest a promotion of NMDA NR2B subunit redistribution to synaptic sites by gp120.

To assess whether the effects on NR2B localization were mediated by IL-1 β , gp120 treatments were performed in the presence of IL-1ra. Fig. 4*D* shows that IL-1ra prevented gp120-induced trafficking of the NMDA receptor NR2B subunit (§§, $p < 0.01$ and §, $p < 0.05$, gp120 + IL-1ra *versus* gp120); both NR2B relative fluorescence in spines *versus* dendrites or soma and NR2B immunoreactivity in PSD-95 positive dendritic spines were significantly reduced by IL-1ra. IL-1ra *per se* did not affect NR2B subcellular distribution (Fig. 4*D*) or cell survival

(% of cell survival, control: 100 \pm 7.2; 24h IL-1ra: 97.4 + 4.45).

Previous studies have shown that membrane stabilization of NMDA receptors containing the NR2B subunit is regulated differently from those containing the NR2A subunit (45). Recent results suggest that the differential regulation of NR2A- and NR2B-containing receptors at the synapse may depend upon specific interactions of NR2B with PDZ proteins and AP-2 (44). We thus analyzed the effects of gp120 on NR2A subunit, lacking the Tyr-1472 phosphosite (Fig. 5*A*). No alteration of both spine *versus* dendritic localization, (Fig. 5*B*) as well as colocalization with PSD-95 was observed (Fig. 5*C*). In addition, no alteration of NR2A immunostaining in TIF was observed following gp120 treatment (Fig. 5*D*) suggesting a specific effect of the glycoprotein on NR2B subunit.

Role of Tyr-1472 Phosphorylation and Binding to PSD-95 in NR2B Redistribution Induced by gp120—Possible molecular mechanisms responsible for gp120-induced synaptic redistribi-

bution of NR2B subunit include Tyr-1472 phosphorylation (see Fig. 1A) and NR2B binding with specific postsynaptic scaffolding proteins such as PSD-95 (see Fig. 4A). These two mechanisms can converge and lead to the same final effect; in fact, it has been demonstrated that mutation of NR2B Tyr-1472 disrupts the endocytic AP-2 binding site on NR2B and that PSD-95 inhibit NR2B-mediated endocytosis (45). In addition, recent data (44) clearly confirm that anchoring to a PDZ protein such as PSD-95, AP-2-mediated internalization, and phosphorylation of Tyr-1472 together play a key role in the regulation of NR2B-containing synaptic NMDA receptors.

Based on these observations, Ca-pYEEIE and TAT2B peptides, corresponding to the last 9 amino acids C-terminal of NR2B (fused to the TAT peptide to facilitate internalization into living cells), were used to unravel the mechanisms modulating NR2B localization in spines after 24 h of gp120 treatment. In neurons preloaded with Ca-pYEEIE and treated with gp120, both NR2B trafficking to spines and colocalization of NR2B with PSD-95 were reduced (Fig. 6, A and B, **, $p < 0.01$ versus control; §§, $p < 0.01$ versus gp-120). Similarly, TAT2B treatment which reduces NR2B binding to PSD-95 (data not shown) rescues NR2B neuronal distribution to control levels (Fig. 6, A and C, **, $p < 0.01$ versus control; §§, $p < 0.01$ versus gp-120) confirming the key role for PSD-95 to confine NR2B inside the postsynaptic compartment. Furthermore, as previously observed with Ca-pYEEIE, TAT2B also prevented gp120-induced $[Ca^{2+}]_i$ increase in neurons (neuronal $[Ca^{2+}]_i$ as % of controls: 100 ± 8.6 , $n = 6$; gp120-treated: 152 ± 12.7 , $n = 7$; TAT2B + gp120-treated: 106 ± 10.8 , $n = 6$).

Effect of gp120 on PSD-95 Positive Clusters and Neuronal Death—Reduction of spine density and neuronal survival represents two hallmarks of HAD related to cognitive decline, personality change, and motor deficits (26, 27). Under our experimental conditions, no effect on PSD-95 positive clusters was observed after 24 h of gp120 (PSD-95 clusters/50 μ m, controls: 27 ± 3.2 ; gp120 600 pM: 25 ± 5 , $n = 12$). On the other hand, 48 h of gp120 treatment produced a significant reduction of total PSD-95 clusters (Fig. 7, A and B; from 24.9 ± 4.56 to 16.5 ± 3.28 protrusions per 50 μ m dendrite length; *, $p < 0.05$ versus control) suggesting the occurrence of spine loss at this time point. Differently, dendrites appear generally undamaged, as evidenced by co-staining with β -tubulin used in all experiments to check for normal dendritic branching following gp120 treatment (Fig. 7A). Loss of PSD-95 clusters was counteracted in neurons loaded with Ca-pYEEIE or with IL-1ra (Fig. 7, A/B, §, $p < 0.05$ versus gp120). To further strengthen this data and quantify the effective neuronal damage, we monitored gp120-induced cell death in hippocampal neurons. No significant neuronal death was found after 6 and 24 h of gp120 treatment (respectively, 99.6 ± 2.49 and $91.6 \pm 5.12\%$ of cell survival). On the other hand, 48 and 72-h gp120 treatment resulted, respectively, in about 20% ($p < 0.01$ versus C) and 30% (**, $p < 0.01$ versus C) of neuronal death (Fig. 7C). Both Ca-pYEEIE 10 μ g/ml and IL-1ra 1 μ g/ml prevented gp120-induced neuronal death (72 h, Fig. 7C; §§, $p < 0.01$ versus gp120).

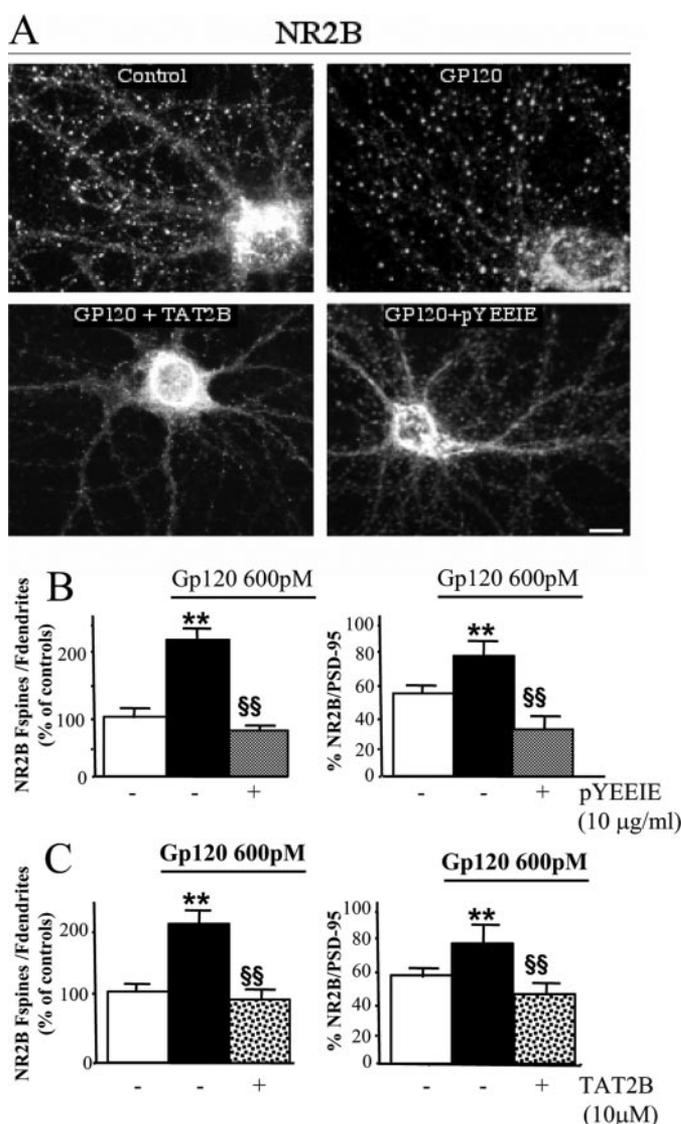


FIGURE 6. Effect of Ca-pYEEIE peptide and TAT-2B on gp120-induced NR2B trafficking to spines. A, immunofluorescence of NR2B subunit of the NMDA receptor in hippocampal neurons exposed to gp120 in the presence of glia. Hippocampal neurons were loaded with Ca-pYEEIE, washed to remove extracellular Ca-pYEEIE and exposed for 24 h to gp120 in the presence of glia or exposed to gp120 + TAT2B. Scale bar, 10 μ m. B and C, quantification of the confocal experiments. The ratio of spines to dendrite fluorescence (left panels) was computed and averaged (**, $p < 0.01$, versus control; §§, $p < 0.01$, versus gp120). Both Ca-pYEEIE and TAT2B treatments caused a significant reduction in the percentage of NR2B clusters of the total number of PSD-95-positive spines (right histograms) compared with gp120 values (**, $p < 0.01$, versus control; §§, $p < 0.01$, versus gp120).

DISCUSSION

The key finding of this study is that the HIV-1 glycoprotein gp120 increases tyrosine phosphorylation of NMDA receptor NR2B subunit through the release of IL-1 β produced by the activated glia. The activation of this pathway leads to a sustained elevation of $[Ca^{2+}]_i$ in neurons and to the stabilization of the NMDA receptor NR2B subunit at the synaptic sites, where a significant increase of NR2B colocalization and binding to PSD-95 was detected after 24 h of gp120 treatment. These effects are relevant for gp120-induced neuronal death.

Our results support previous evidence suggesting that a

Native IL-1 β Triggers NR2B Phosphorylation

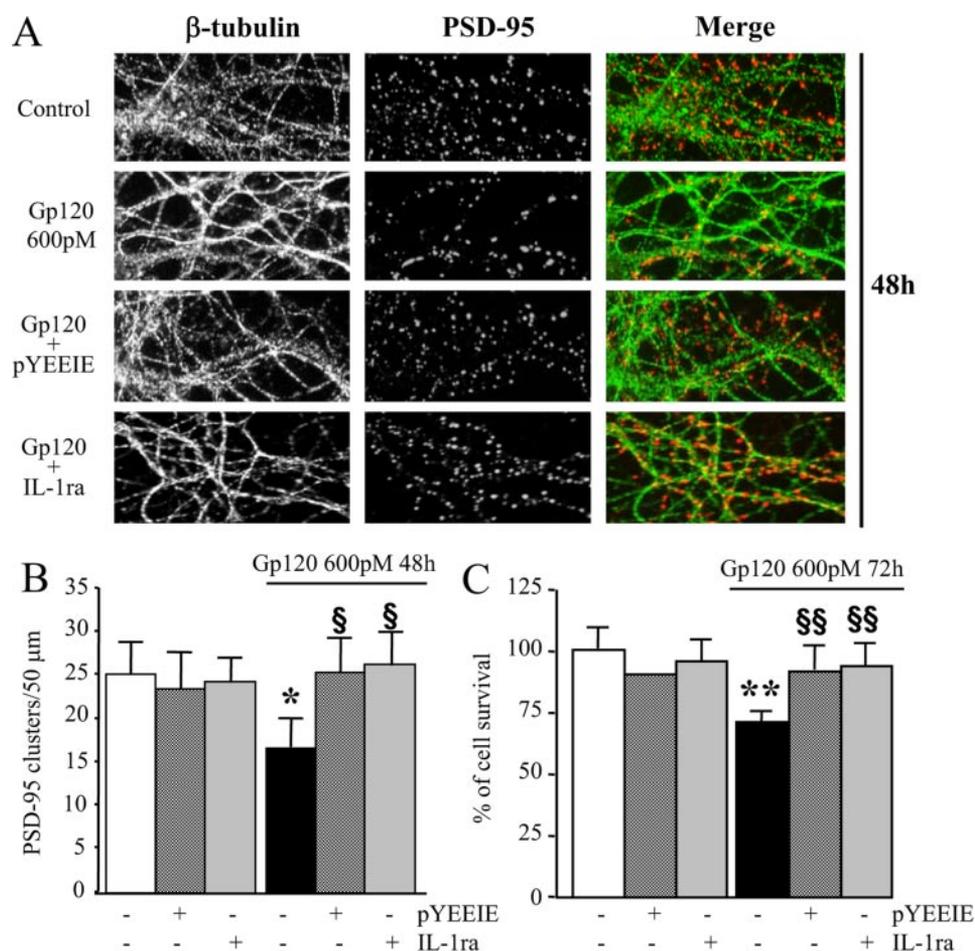


FIGURE 7. Loss of PSD-95 positive clusters and neuronal death after gp120 treatment: modulation by Ca-pYEEIE and IL-1ra. *A*, hippocampal neurons were either left untreated (*control*) or treated for 48 h with gp120 in the presence of glia, fixed, and immunolabeled for β -tubulin (*left panels*) or PSD-95 (*middle panels*). Merge data are shown on the *right*. Areas of overlap in merge panels appear yellow. *B*, number of PSD-95-positive clusters per 50 μm of dendrite length in neurons, loaded (*gray columns*) or not with Ca-pYEEIE or with IL-1ra, after 48 h of treatment with gp120 in the presence of glia (*, $p < 0.05$ versus control; §, $p < 0.05$ versus gp120). *C*, hippocampal neurons were loaded with Ca-pYEEIE or with IL-1ra and then exposed to gp120 in the presence of glia for 72 h. MTT test was performed to assay cell viability. gp120-induced cell death was prevented by both Ca-pYEEIE and IL-1ra. Values are means \pm S.E. of two independent experiments in triplicate, **, $p < 0.01$ versus control; §§, $p < 0.01$ versus gp120.

relevant pathway to neuronal injury in HAD is indirect through release of potentially neurotoxic factors from activated glia (46–48). Thus, Tyr phosphorylation of NMDA receptor and neuronal Ca^{2+} increase were not evident in neurons exposed to gp120 in absence of glia. In these experimental conditions we did not detect any cell death following gp120 exposure.⁴ The need of glia for gp120 to affect neurons, discloses the existence of complex cell-cell interactions possibly responsible for the delayed onset of dynamic processes, such as phosphorylation, in neurons.

IL-1 β is one of the many mediators released from glia by gp120 involved in its pathogenic effect. Levels of IL-1 β are elevated in the cerebrospinal fluid of AIDS patients and at post-mortem brain examination (8, 49), as well as in several rat brain areas following intracerebroventricular infusion of gp120 (7, 50–54). Pharmacological manipulations, the use of antagonist for type I IL-1 β receptor (IL-1ra) and of specific antibodies to

neutralize the action of IL-1 β , greatly contributed to the understanding of some of the effects exerted by this cytokine in AIDS dementia complex. *In vivo*, IL-1ra and α -melanocyte-stimulating hormone (an endogenous hormone and transmitter that inhibits IL-1 β action, Ref. 55) blocked gp120-induced memory impairment (52). *In vitro*, protection from cell death was conferred by inhibiting IL-1 β action through a specific inhibitor of interleukin-1-converting enzyme, IL-1ra (7) and an antibody against IL-1 β that also prevented gp120-induced $[\text{Ca}^{2+}]_i$ increase in neurons (9). Although a relevant role for IL-1 β in sustaining gp120 neuronal damage emerges, no information is available about the molecular mechanisms recruited by this cytokine in this contest.

Under our experimental conditions, concentrations of gp120 that stimulate glial release of IL-1 β induced Tyr-1472 phosphorylation of NR2B and trigger a sustained elevation of $[\text{Ca}^{2+}]_i$ in neurons. In accordance with data showing that recombinant IL-1 β , through IL-1R, induces NMDA receptor phosphorylation by Src kinases and a consequent facilitation of NMDA-induced $[\text{Ca}^{2+}]_i$ increase (17), we observed that both IL-1ra, the antagonist of IL-1R type I, and Ca-pYEEIE, an inhibitor for Src family SH2 domains (35), prevent gp120 effects.

As previously mentioned, besides cytokines, gp-120 activated glia release other mediators, which may impair the re-uptake of glutamate by astrocytes and induce astrocytic glutamate release (56–58). Furthermore, glutamate is also spontaneously released by primary hippocampal neurons in culture. Thus, gp120-released IL-1 β may prime the NMDA receptors promoting the phosphorylation at Tyr-1472 and contributing to the potentiation of the glutamate signal through NMDAR. Overactivation of NMDA receptor-coupled ion channels and the resulting influx of Ca^{2+} appear to be central to HIV-related neuronal damage (22).

Electrophysiological recordings from neurons show that NMDA currents are governed by a balance between tyrosine phosphorylation and dephosphorylation (59). Whether phosphorylation causes the increase in NMDA receptor gating still remains unclear (18). On the other hand, tyrosine phosphorylation of NR2 subunits might also prevent the removal of signaling molecules from the NMDAR complex by protecting the subunits against degradation from the calcium-activated pro-

⁴ B. Viviani, unpublished observation.

tease, calpain (41). Furthermore, studies on recombinant NMDA receptors indicate that their association with the clathrin-mediated endocytosis machinery, a complex of proteins involved in the removal of receptors from the cell surface, is regulated by Src-mediated tyrosine phosphorylation of NMDAR subunits (60, 61). The NR2B Tyr-1472 consensus domain is part of the internalization signal motif, a binding domain for the adaptor protein complex AP2, which associates with endocytic clathrin-coated vesicles. In addition, Src family tyrosine kinases have been shown to interact with NMDA receptors by binding to the scaffolding protein PSD-95 (62). This interaction is strictly correlated to tyrosine phosphorylation of the NMDA receptors subunits (41, 43, 63). Finally, recent results show that stabilization of NR2B-containing receptors at the synapse is dynamically regulated by binding to a PDZ protein such as PSD-95 and internalization through an interaction with AP-2 (44). With this view, NR2B-PDZ protein interaction may keep Tyr-1472 phosphorylated, and consequently unable to interact with AP-2 (44). The final result of this event is an increased localization of NR2B within the postsynaptic compartment, probably through prevention of endocytosis of NR2B-containing synaptic receptors (43). Our results suggest that all these mechanisms may be recruited by gp120, through IL-1 β release. We observed a sustained elevation of Ca²⁺ in neurons exposed to gp120. Such an increase occurs through the NR2B subunit: ifenprodil, a blocker that selectively binds to this subunit, inhibited this effect. In addition, gp120 increases both the binding of NR2B subunit with PSD-95 and its enrichment in spine.

Tyrosine phosphorylation and PSD-95 binding appear to be important in the occurrence of gp120-induced NR2B localization and Ca²⁺ homeostasis unbalance because both Ca-pYEEIE and TAT2B, peptides analogous to the C-terminal domains of NR2B, prevent these effects. Therefore, gp120 by phosphorylating the NR2B tyrosine 1472 could prevent the assembly of the clathrin-mediated endocytosis machinery at the NMDA receptors and thereby prevent the internalization of NMDA receptors. Alternatively, tyrosine phosphorylation of NR2 subunits could be involved in the trafficking of NMDA receptors to the cell surface (42). Again, IL-1ra inhibits Tyr-1472 phosphorylation, NR2B enrichment of spine like structures and neuronal cell death supporting the role of this cytokine as mediator of gp120 action and its involvement in the cascade of events induced by Tyr phosphorylation.

Altered tyrosine phosphorylation of NMDA receptor NR2 subunits has been observed in pathological conditions such as transient ischemia (36, 37, 64), epilepsy (65), and in amyloid- β peptide-treated neurons (66) suggesting its involvement in neuronal damage progression.

Our data together with the observation that tyrosine phosphorylation of the NMDA receptor have been also implicated in exacerbation of neuronal cell loss induced by the pathogenic HIV-1 protein TAT (67), suggest a possible involvement of this pathway in HAD as well. Accordingly, we show that Ca-pYEEIE prevents neuronal death. It would have been interesting to evaluate whether stabilization at the membrane of NR2B-containing receptor selectively induced neuronal demise. In fact, NR2A- and NR2B-containing receptors have distinct biophys-

ical properties, including sensitivity to glutamate and deactivation time (68, 69), and interact differently with postsynaptic signal transduction complexes (70). As a result, different receptor subtypes may serve distinct physiological or pathological functions in neurons. Unfortunately, because of their toxicity, which was evident at 48 h (data not shown), we could not evaluate whether ifenprodil and TAT-2B modulated spines simplification and neuronal death. Nevertheless, the observation that gp120 enhances the presence of the NR2B subunit at the postsynaptic membrane, but not of the NR2A subunit, supports this possibility under our experimental conditions.

In conclusion, our results provide a molecular basis to link endogenous IL-1 β produced in a pathological condition and deregulation of Ca²⁺ neuronal homeostasis because of an over-activation of the NMDA response. Overall, the chance to attenuate neuronal damage blocking NMDARs has been attempted over the years but with scarce results, perhaps because of the fundamental implication of these receptors in the physiology of neurons. Thus, determining how IL-1 β , released in a pathological situation, affects neurons would provide new insight to the general comprehension of acute and chronic pathological conditions associated with specific inflammatory mediators and would offer significant promise in the identification of possible targets for a selective pharmacological intervention on NMDARs.

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