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Interleukin-1 β affects calcium signaling and “in vitro” cell migration of astrocyte progenitors

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Abstract

Spontaneous calcium activity of neural progenitors is largely dependent on a paracrine signaling mechanism involving release of ATP and activation of purinergic receptors. Although it is well documented that, in mature astrocytes, cytokines modulate the expression levels of certain purinergic receptors, nothing is known about their impact during early stages of development. Here we provide evidence that conditioned medium from activated microglia as well as interleukin-1 β , but not tumor necrosis factor- α , decrease the frequency of calcium oscillations and reduce the rate of “in vitro” migration of astrocyte progenitors. Such alterations were due to changes in activity of two purinergic P2 receptors, and not to the amount of released ATP. These results indicate that Interleukin-1 β plays important role during early stages of CNS development, modulating calcium signaling and cell migration.

Keywords

cytokine; microglia; neurospheres; purinergic receptors; IL-1 β ; TNF α

1. Introduction

Studies on repair mechanisms have indicated that the inflammatory microenvironment is an important component involved in the engagement of endogenous and/or exogenous neural stem/progenitor cells. These include observations that neural progenitor cells (NPC) increase their proliferation and migrate toward pathologic sites derived from diverse etiologies in *in vivo* and *in vitro* experiments (Picard-Riera et al., 2002; Ben-Hur et al., 2003b; Kokaia and Lindvall, 2003; Imitola et al., 2004; Kelly et al., 2004; Pluchino et al., 2005). These observations have led some authors (Imitola et al., 2004) to propose that the inflammatory cues guide the behavior of potentially reparative stem cells. However, brain injury can also lead to the establishment of a “non-permissive” molecular *milieu* that could favor apoptotic cell death and would prevent migration, proliferation and differentiation of NPC (Fok-Seang et al., 1998; Fawcett and Asher 1999; Monje et al., 2003). Consequently, the ultimate effect of the neuroinflammatory *milieu* on the development and fate of neural progenitors is complex and likely to depend on a balance between proliferation and apoptotic-inducing factors, and differentiation-inducing factors. The inflammatory response in the central nervous system

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(CNS) involves the activation of microglia, which releases inflammatory mediators, such as acute phase proteins, eicosanoids, complement, chemokines, free radicals, and pro-inflammatory cytokines (Allan and Rothwell, 2001; Monje et al., 2003).

Several lines of evidence indicated that the migration of neuronal and glial progenitor cells is dependent on intracellular calcium elevation induced by the release of neurotransmitters and modulators (Komuro and Rakic, 1998; Komuro and Kumada, 2005; Kumada and Komuro, 2004; Scemes et al., 2003; Agresti et al., 2005a,b; Striedinger et al., 2007). At least for glial progenitor cell migration, stimulation of purinergic P2Y₁ receptor by extracellular ATP have been proposed as a key component mediating their rate of migration (Scemes et al., 2003; Agresti et al., 2005a,b; Striedinger et al., 2007).

Because purinergic receptor expression and activity can be modulated by pro-inflammatory cytokines (John et al., 1999; Narcisse et al., 2005; Scemes, 2008), we evaluated the effects of conditioned medium of activated microglia (CM), as well as those induced by interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF α) on astrocyte progenitor cell migration and calcium signaling. Our results indicate that, similarly to the effects of conditioned medium of activated microglia, the pro-inflammatory cytokine IL-1 β , but not TNF α reduced the rate of *in vitro* progenitor cell migration. Moreover, the reduced cell migration induced by IL-1 β was paralleled by decreased frequency of spontaneous calcium oscillations due to reduced activity but not expression level of the P2Y₁ receptor.

2. Materials and Methods

Neurospheres and neural progenitor cultures

Neurospheres were prepared as previously described (Striedinger et al., 2007). Briefly, neural progenitor cells were obtained by aspiration of forebrain tissues of 14 day-old (E14) wild-type C57Bl/6 mouse embryos (time-pregnant C57Bl/6 females obtained from Charles River) and mechanically dissociated into single cells in ice-cold Hanks balanced solution (HBSS Ca²⁺- and Mg²⁺-free). The Albert Einstein College of Medicine (AECOM) Animal Care and Use Committee approved all experimental procedures performed. Viable cells were transferred to tissue culture dishes containing Dulbecco Modified Eagle Medium Nutrient Mixture F12 (DMEM-F12, Gibco, Invitrogen, Carlsbad, CA) supplemented with 5% B27 (Gibco), 1% antibiotics and 20ng/ml human recombinant epidermal growth factor (EGF; Sigma, St. Louis, MO), and allowed to grow into floating neurospheres. Culture medium was changed twice a week, and neurospheres were mechanically dissociated into smaller neurospheres once a week. Neurosphere cultures were maintained for no longer than 2 months. For *in vitro* cell differentiation, floating neurospheres were plated on glass bottom microwells (MaTek Co, Ashland, MA) coated with poly-D-lysine- (10 μ g/ml; Sigma) and fibronectin- (10 μ g/ml; Sigma) and bathed in DMEM-F12 in the absence of EGF. After adhesion of neurospheres to the substrate, cells migrating out of the neurospheres differentiate into glia and neuronal progenitors.

Conditioned medium from activated microglia

Purified cultures of murine microglia were kindly provided by Dr. Kostantin Dobrenis (Department of Neuroscience, Albert Einstein College of Medicine), and prepared as previously described (Dobrenis et al., 2005) from neocortex of 2-day postnatal C57Bl/6 mice. Purified microglia cultures were maintained in Macrophage-SPM Medium (Gibco) supplemented with 10ng/ml recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF; R&D, Minneapolis, MN), 5% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin. Conditioned media (CM) from GM-CSF activated microglia were obtained by

replacing the Macrophage medium with the neurospheres DMEM-F12 medium, which was then collected 24 hrs after medium substitution, filtered and stored at -20°C until further use.

ELISA Assay

The level of interleukin-1 β (IL-1 β) present on the microglia conditioned media (CM) was measured using the quantitative sandwich enzyme immunoassay technique (Quantikine, R&D Systems) as described by the manufacture. Briefly, 50 μl of samples (CM and microglia cell lysates) and 50 μl of a buffer solution were added to a 96 well plate pre-coated with a polyclonal antibody specific for the mouse IL-1 β and incubated for 2 hr at room temperature (RT). After several washes, 100 μl of a polyclonal HRP-conjugated antibody against mouse IL-1 β were added to each well. Following 2hr incubation and several washes, 100 μl chromogen-hydrogen peroxide solution was added to each well and reaction incubated for 30 min before the addition of a hydrochloric acid (stop) solution. Optical densities were obtained using a microplate reader (Dynex Revelation 4.21) and the concentration (pg/ml) of IL-1 β calculated based on a standard curve. All samples were run in triplicates.

Cytokine treatment

Neurospheres (100–200 μm diameters) were treated in suspension for 24 hrs with CM, IL-1 β (20ng/mL; Cedarlane) or tumor necrosis factor- α (TNF α : 10ng/mL; Chemicon) and thereafter plated on coated dishes, in the continuous presence of these cytokines, for migration and calcium assays (see below). Parallel experiments were performed on untreated neurospheres. In some experiments the neutralizing IL-1 receptor antibody (100ng/mL; Antigenix Inc.) was used.

Migration assay

To evaluate the distances of emigration of progenitor cells from the neurospheres, the outgrowth index (OI) was calculated as the ratio between the distance of the nucleus of the foremost cells to the center of the sphere and the radius of the sphere, as previously described (Scemes et al., 2003; Striedinger et al., 2007). *In vitro* cell migration was induced by withdrawal of EGF from the culture medium of untreated and treated neurospheres plated on poly-D-lysine/fibronectin coated glass bottom microwells. Twenty-four hrs CM-, IL-1 β -, or TNF α -treated floating neurospheres were then seeded on coated dishes and the OI measured daily during three days in culture in the continuous presence of these inflammatory mediators. In some experiments, neurospheres were treated with the P2X $_7$ R agonist (100 μM BzATP) as described above for the cytokines.

Spontaneous calcium oscillations

Spontaneous intracellular Ca $^{2+}$ transients were measured in adherent, Cd44S immun-identified astrocyte progenitors, as previously described (Striedinger et al., 2007). Briefly, adherent cultures of progenitors were incubated in serum-free DMEM-F12 containing monoclonal anti-Cd44S (1:200) antibodies for 30 min at 37 $^{\circ}\text{C}$. After several washes with DMEM-F12, the cultures were incubated with Alexa fluor 594-tagged secondary antibodies, diluted 1:500 and concomitantly loaded with the Ca $^{2+}$ indicator Fluo-3-AM (5 μM ; Molecular Probes-Invitrogen) at 37 $^{\circ}\text{C}$ for 40 min. Cells were then washed with Dulbecco's phosphate buffered saline (DPBS, pH 7.4; Cellgro, Herndon, VA) and imaged on an epifluorescence microscope (Eclipse TE2000-S; Nikon, Japan), equipped with FITC and Texas red filter sets. Images were acquired with a CCD camera (Orca-ER; Hamamatsu, Japan). Fluo-3 fluorescence intensity emitted at one excitation wavelength (488 nm) was continuously acquired at a rate of 1.0 Hz for 5 min period using combined systems of filters and shutter (Lambda DG-4 Diaphot, Sutter Instruments Co., Burlingame, CA) driven by a computer through Metafluor software (Universal Imaging Systems, Downingtown, PA). Fluo-3 fluorescence intensity

obtained from regions of interest placed on Cd44S positive cells were normalized to initial values and expressed as relative changes in fluorescence intensity (F/F0).

Ca²⁺ mobilization induced by P2 receptor agonists

P2 receptor agonists with affinity for the metabotropic receptor P2Y₁R, 2-MeSATP (100nM; 2-methylthioadenosine 5'-triphosphate; Sigma-Aldrich, St Louis, MO) and for the ionotropic receptor P2X₇R, BzATP (100μM; 3'-O-4-benzoylbenzoyl-ATP; Sigma) were employed to evaluate the effects of IL-1β on P2Y₁ and P2X₇R-mediated Ca²⁺ mobilization of 3 day-old astrocyte Cd44S positive progenitors. For that, cells were loaded with the ratiometric Ca²⁺ indicator, Fura-2-AM (2.5 μM; Molecular Probes-Invitrogen) for 40 min. Fura-2 ratio values were translated into intracellular Ca²⁺ concentrations according to an *in vitro* calibration curve of the form: $[Ca^{2+}]_i = K_D \{ (R - R_{min}) / (R_{max} - R) \} (F_{380_{min}} / F_{380_{max}})$, where $[Ca^{2+}]_i$ is the calculated intracellular calcium concentration, K_D is the dissociation constant of free-Ca²⁺ for Fura-2 ($K_D = 224$ nM; Grynkiewicz et al., 1985), R is the ratio intensity, R_{min} is the ratio of the intensity obtained at zero calcium, R_{max} is the ratio of the intensity at saturated calcium, $F_{380_{min}}$ is the fluorescence intensity measured with zero calcium at 380nm, and $F_{380_{max}}$ is the fluorescence intensity measured with saturated calcium at 380nm. At least three independent cultures of neurospheres were used to measure the amplitudes of Ca²⁺ transients induced by the P2R agonists.

ATP assays

The amount of ATP released in the bathing solution by untreated and cytokine-treated 3 day-adherent progenitor cells was measured for 2 min in 200μl DPBS, as previously described (Striedinger et al., 2007). The amount of ATP was measured using the luciferin/luciferase assay (Molecular Probes- Invitrogen). Fifty microliters of a buffered solution containing luciferin (50μM) and luciferase (1.25μg/ml) were placed in triplicates in a 96-well plate luminometer (Veritas, Turner) for background luminescence subtraction. Reactions were started by adding 5μl of the experimental samples and luminescence values acquired during 1 sec integration time. The amount of ATP in the samples was calculated from standard curves and normalized for the total amount of protein, using the BCA assay (Pierce, Rockford, IL).

Western Blots

Untreated and IL-1β-(20ng/ml) treated 3-day adherent progenitor cells were lysed in lyses buffer (150mM NaCl, 10mM Tris-HCl, 1% Triton-X-100, Roche complete protease inhibitor cocktail). Samples of whole cell lysates were mixed with 2XLaemli buffer and subjected to electrophoresis using a 4–20% SDS-PAGE mini-gel (BioRad). After transfer of proteins to nitrocellulose membranes (Schleicher & Schuell, Keene, NH), membranes were incubated for 1hr at RT with blocking solution (1XPBS, 0.5% Tween-Twenty, 2% non-fat dry milk) prior to exposure to primary antibodies. Membranes were blotted for 2hrs at RT with anti-P2Y₁ (1:200) and anti-P2X₇ (1:500) antibodies (Alomone labs) prepared in blocking solutions. After three washes (1XPBS, 0.5% Tween-Twenty), membranes were incubated with goat-anti-rabbit-HRP conjugated secondary antibodies (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA) for 1hr at RT. After several washes, detection of bands was performed on X-ray films (Kodak, Rochester, NY) following incubation with enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia Biotechnology, Piscataway, NJ). Membranes were re-probed with anti-GAPDH (1:2000; Fitzgerald). Quantification was performed by densitometric analysis of bands using Image-J and P2R levels normalized to that of GAPDH.

Statistical analyses

All data are expressed as mean±SE. GraphPad Prism version 4 was used for statistical analysis, consisting of one-way ANOVA analysis of variance, followed by Newman-Keuls' multiple comparison test. T-test comparison was also employed in some cases.

3. Results

Conditioned medium from activated microglia (CM) and IL1- β but not TNF α decrease the migration distances of neural progenitor cells

We have previously reported (Striedinger et al., 2007) that the great majority (> 80%) of progenitor cells derived from fibronectin adherent neurospheres are of the astrocytic lineage that express the extracellular glycoprotein Cd44S (a marker for astrocyte progenitors: Rao and Mayer-Proschel, 1997; Liu et al., 2002; Liu and Rao, 2004).

Using this neurosphere system, we evaluated the effects of microglia conditioned medium and of two cytokines on the migration of astrocyte progenitors.

When adherent neurosphere cultures were exposed for 3 days to CM, a significant decrease on the distance of migration of astrocyte progenitor cells was observed compared to untreated cultures (Fig. 1A). Under these conditions, the outgrowth index (OI) of CM-treated neurospheres was 7.66 ± 0.94 (N=29 neurospheres), while the OI of control neurospheres was 11.04 ± 0.26 (N=66 neurospheres). To further investigate which of the main inflammatory mediators contained in the conditioned media from activated microglia was mediating the reduced OI, we treated neurospheres with two pro-inflammatory cytokines, IL-1 β and TNF α that have been previously reported to be released from activated microglia (Aloisi et al., 1997; Degousee et al., 2001; Meme et al., 2006).

After 3 days of adhesion to poly-D-lysine/fibronectin substrate, IL-1 β -treated progenitors displayed a significant reduction of the outgrowth index (OI= 6.27 ± 0.20 ; N=32 neurospheres) compared to untreated cells (OI= 11.04 ± 0.26 ; N=66 neurospheres, $p<0.001$; Fig. 1A). This decreased OI was prevented by co-treating the neurospheres with the neutralizing IL-1 receptor antibody (OI= 9.86 ± 0.26 ; N=24 neurospheres). In contrast to the effects of IL-1 β , after three days of adhesion, the outgrowth index of TNF α -treated progenitors (OI= 10.58 ± 1.06 ; N=29 neurospheres; Fig. 1A) was not significantly different to that of control, untreated progenitors.

Using an ELISA assay we quantified the amount of IL-1 β present on the CM obtained from two cultures of microglia cells. As shown in the table displayed in Fig. 1B, about 30–40pg/ml IL-1 β were detected in CM, and about 900pg/ml were found in the cytosol of microglia. To evaluate whether lower concentrations of IL-1 β than those used in the experiments described above could also reduce the outgrowth index, neurospheres were treated for 3 days with 0.31 and 3.1 ng/ml IL-1 β . Even at lowest concentration used (0.31ng/ml), IL-1 β caused a similar reduction in the outgrowth index seen in cultures treated with CM (0.03ng/ml IL-1 β) (Fig. 1B). These results suggest that the levels of IL-1 β found in CM are sufficient to maximally reduce progenitor cell migration.

Given that 10ng/ml TNF α (a concentration similar - 23ng/ml - to that found in CM of mouse microglia: Meme et al., 2006) did not cause any effect on astrocyte progenitor cell migration, we did not quantify the levels of this cytokine in CM.

Spontaneous Ca²⁺ oscillations in astrocyte progenitors are attenuated by CM and IL-1 β

We and others have previously shown that the frequency of spontaneous calcium oscillations of neural progenitor cells dictates their rate of migration (Komuro and Rakic, 1998; Kumada

and Komuro, 2004; Komuro and Kumada, 2005; Scemes et al., 2003; Striedinger et al., 2007). To evaluate whether the effect of CM and IL-1 β on the outgrowth index of astrocyte progenitors was related to reduced Ca²⁺ activity, we measured the number of astrocyte progenitors displaying spontaneous Ca²⁺ transients, as well as the frequencies of these Ca²⁺ oscillations. For that, we loaded the cells with the Ca²⁺ indicator Fluo-3-AM and recorded Ca²⁺ activity from Cd44S positive cells.

Our results indicate that the fraction of astrocyte progenitors (Cd44S+) displaying spontaneous Ca²⁺ oscillations recorded under control conditions (0.54 \pm 0.04; N=4 experiments) was significantly reduced to 0.38 \pm 0.04 and to 0.38 \pm 0.02 (P<0.05; ANOVA analysis of variance and Newman Keuls' multiple comparison test) when treated with CM and with IL-1 β , respectively. TNF α , in contrast, did not alter the fraction of astrocyte progenitors displaying spontaneous Ca²⁺ oscillations (0.55 \pm 0.09; p>0.05) compared to control untreated cells. The frequency of spontaneous Ca²⁺ oscillations was also greatly reduced after treating the cells with CM and IL-1 β . As shown in Figure 2, the frequency of Ca²⁺ oscillations (10.81 \pm 0.34 mHz; N=571 cells) recorded from untreated cultures was significantly reduced in CM- (6.69 \pm 0.36 mHz; N=122 cells; p<0.001) and IL-1 β - (7.09 \pm 0.35 mHz; N=203 cells; p<0.001) treated cells; TNF α did not significantly alter the frequency of spontaneous Ca²⁺ oscillations (11.17 \pm 0.63 mHz; N=161 cells; p >0.05) compared to that obtained from control cells.

These data indicate that the reduced frequency of spontaneous Ca²⁺ oscillations, together with the reduced number of Ca²⁺ oscillating astrocyte progenitors contribute to the reduced cell migration observed by treating the cells with CM and IL-1 β .

IL-1 β does not alter the amount of ATP released from progenitor cells but altered purinergic receptor agonist mediated calcium transients

Given that we have previously shown that spontaneous Ca²⁺ oscillations of astrocyte progenitors are dependent upon ATP release and subsequent activation of purinergic P2Y₁ receptors (Scemes et al., 2003; Striedinger et al., 2007), we investigated whether IL-1 β and TNF α affected the amount of ATP released from these cells. For that, we measured the amount of ATP present on the bathing solution of 3-days adherent progenitors using the luciferase assay. As shown in Figure 3, no significant changes in the amount of released ATP were observed compared to control untreated cells.

To evaluate whether changes in spontaneous calcium activity induced by IL-1 β were related to changes in purinergic receptor activity, the amplitudes of Ca²⁺ transients induced by P2Y₁ and P2X₇ receptor agonists were measured on Cd44S positive progenitors derived from 3-days adherent neurospheres that were untreated or treated with this cytokine. As shown in Figure 4, intracellular Ca²⁺ levels mobilized by the P2Y₁ receptor agonist 2-Me-S-ATP (100nM) were significantly reduced in IL-1 β (394.71 \pm 35.87nM; N=175 cells; P<0.01) treated progenitors compared to controls (977 \pm 55.78nM; N=175 cells). In contrast to the action of P2Y₁R agonist, the P2X₇ R agonist BzATP (100 μ M) caused a significant sustained increase in intracellular Ca²⁺ levels in IL-1 β -treated cells (612 \pm 45.06nM; N=192 cells; P<0.01; Fig. 4) compared to control cells.

These data indicate that IL-1 β attenuates P2Y₁ receptor mediated intracellular calcium mobilization and increases the activity of P2X₇ receptor.

In order to evaluate whether changes in activity of the two P2Rs were related to altered protein expression levels, western blots were performed in 3-days adherent progenitors untreated and treated with 20ng/ml IL-1 β . Western blot analysis revealed that the expression levels of P2X₇ and P2Y₁ receptors were unchanged following IL-1 β treatment, compared to control, untreated progenitors (Fig. 5).

Contribution of P2R to neural progenitor cell migration

Regarding the contribution of these P2 receptors to astrocyte progenitor migration, it is likely that the P2Y₁ and not the P2X₇ receptors are involved in this process. This is because we have previously reported that a P2Y₁R antagonist, MRS2179, but not the P2X₇R antagonist KN62, reduced progenitor cell migration (Scemes et al., 2003). Further support that the P2X₇R does not contribute to this process was obtained from experiments in which adherent neurospheres were treated for three days with BzATP (100μM). Thus, after 3 days of adhesion, BzATP-treated cells featured an outgrowth index (OI) of 12.35±0.82 (N=16 neurospheres from 3 independent experiments), which was not statistically different (P>0.05; unpaired T-test) from that recorded from untreated cells (OI=13.16±1.26; N=11 neurospheres from 3 independent experiments).

Thus, our previous (Scemes et al., 2003) and current results support the hypothesis that migration of astrocyte progenitors is dependent upon the proper function of P2Y₁Rs and that the P2X₇Rs do not play a major role in this process.

4. Discussion

A relevant aspect of the response of neural precursor cells to CNS inflammation is their migration towards lesion sites (Imitola et al., 2004), a feature that is also common of transplanted precursor cells (Ben-Hur et al., 2003b). Microglia and astrocytes are the main sources of pro-inflammatory cytokines in the CNS (Aloisi, 2001; Aloisi et al., 1992; Degousee et al., 2001; Gehrmann et al., 1995; Meme et al., 2007) and some of these inflammatory mediators, such as IL-1β and TNFα, were reported to affect *in vitro* neural progenitor cell proliferation (Wang et al., 2007; Widera et al., 2006) and migration (Ben-Hur et al., 2003a).

Besides the well established roles of cell adhesion and extracellular molecules on cell migration, other signaling mechanisms have been identified; most prominent is the intracellular Ca²⁺ fluctuation resultant from ion channel and membrane receptor activation (Komuro and Rakic, 1998; Kumada and Komuro, 2004). Spontaneous intracellular Ca²⁺ oscillations and waves are prominent events during early CNS development that modulate cell proliferation and migration (Komuro and Rakic, 1998; Owens and Kriegstein, 1998; Catsicas et al., 1998; Pearson et al., 2002; Scemes et al., 2003; Webb et al., 2003; Kumada and Komuro, 2004; Weissman et al., 2004; Agresti et al., 2005a,b). It has been suggested that during these early stages of development, glial cells, by releasing transmitters, would provide the necessary inputs leading to calcium transients and thus influencing neurogenesis (Komuro and Rakic, 1998; Weissman et al., 2004).

Using an *in vitro* model of neural cell development we have previously shown that migrating neural progenitor cells display spontaneous calcium oscillations that are blunted by bath application of apyrase, an ATP degrading enzyme, and by the specific P2Y₁ receptor antagonist MRS-2179, which also reduced progenitor cell migration (Scemes et al., 2003). In this same study, we also showed that exogenous expression of P2Y₁R in P2Y₁R-deficient cells, rescued their frequency of Ca²⁺ oscillations and migration rate. Moreover, interference with ATP release machinery was also shown to attenuate the rate of *in vitro* astrocyte progenitor cell migration and calcium oscillations (Striedinger et al., 2007). These studies, therefore, strongly indicate that spontaneous Ca²⁺ oscillations mediated by the release of ATP acting on P2Y₁ receptors influence the migration of astrocyte progenitor cells.

We here show that conditioned medium derived from activated microglia as well as IL-1β, but not TNFα, reduced the migration distances of astrocyte progenitor cell compared to controls, untreated cells. Evidence is also provided indicating that the reduced *in vitro* cell migration induced by IL-1β is accompany by a decrease in the frequency of spontaneous Ca²⁺ oscillations.

It is likely that the changes in spontaneous Ca^{2+} oscillations observed in the present study are related to the altered P2Y_1 receptor activity rather than changes in its expression levels or to the ATP release machinery. Evidence favoring such possibility is based on the observations that, in IL-1 β treated progenitors, a $\text{P2Y}_1\text{R}$ agonist induced Ca^{2+} transients of smaller amplitudes than those recorded from untreated cells, and on measurements showing that neither the expression levels of P2Y_1 receptors nor the amount of ATP released from progenitor cells were affected by this pro-inflammatory cytokine.

In contrast to the effect of IL-1 β on P2Y_1 receptor activity, we found that this cytokine enhanced Ca^{2+} mobilization induced by a $\text{P2X}_7\text{R}$ agonist. Because activation of P2X_7 receptors, differently from P2Y_1 receptors, leads to prolonged/sustained calcium elevation, it is likely that the reduced frequency of spontaneous Ca^{2+} oscillations recorded from IL-1 β -treated progenitors is a reflection of the combined changes in activity of these two P2 receptors. However, despite the increased activity of $\text{P2X}_7\text{R}$, these receptors are unlikely to be involved in the migration of astrocyte progenitors. This is based on our current and previous studies showing that neither the $\text{P2X}_7\text{R}$ agonist BzATP (this study) nor its antagonist KN-62 (Scemes et al., 2003) had an impact on progenitor cell migration. Although the P2X_7 receptors have been proposed to be a component of the inflammatory response in astrocytes (Narcisse et al., 2005), and a contributor to pathogenic Ca^{2+} entry (Yeung et al., 2006), and cell death (Zhang et al., 2005; Wang et al., 2004), further studies are needed to disclose the impact of these ionotropic receptors on neural progenitor cell (patho) physiology.

It is well established that activation of G-coupled surface membrane receptors is the initial step leading to cell polarization and migration triggered by a chemo-attractant. The downstream signal transduction pathway mediating cell motility and migration involves members of the Rho family of small GTPases including Rho, Rac and Cdc42, which are involved in cytoskeletal remodeling (Bokoch, 2005; Barber and Welch, 2006). Although nothing is known about the role of P2R on the triggering of small GTPases during astrocyte progenitor cell migration, at least in platelet, it has been shown that stimulation of $\text{P2Y}_1\text{R}$ leads to rapid activation of Rac through a G_q and Ca^{2+} -dependent pathway (Soulet et al., 2005). Given that Rac is primarily involved in lamellipodia protrusion at the leading edge (Bokoch, 2005; Barber and Welch, 2006), reduced activity of $\text{P2Y}_1\text{R}$ is expected to attenuate cell migration. Clearly, further studies are necessary to evaluate such hypothesis and to elucidate whether the deactivating effect of IL-1 β on Rho GTPase seen in mature astrocytes (John et al., 2004) is also effective in reducing astrocyte progenitor cell migration, independently of $\text{P2Y}_1\text{R}$. Nevertheless, because the effects of IL-1 β on the activity of P2 receptors reported in the present study are very similar to what has been previously reported for mature astrocytes, i.e., decreased response to agonists of P2Y_1 receptors (Scemes, 2008) and increased response of P2X_7 receptors (Narcisse et al., 2005), it is likely that astrocyte progenitors acquire in early stages of CNS development, the ability to fully respond to IL-1 β .

In summary, the present study indicates that IL-1 β is a potent negative modulator of calcium signaling and cell migration in astrocyte progenitors, two important processes for proper CNS development and repair mechanisms during pathological conditions involving neuroinflammation.

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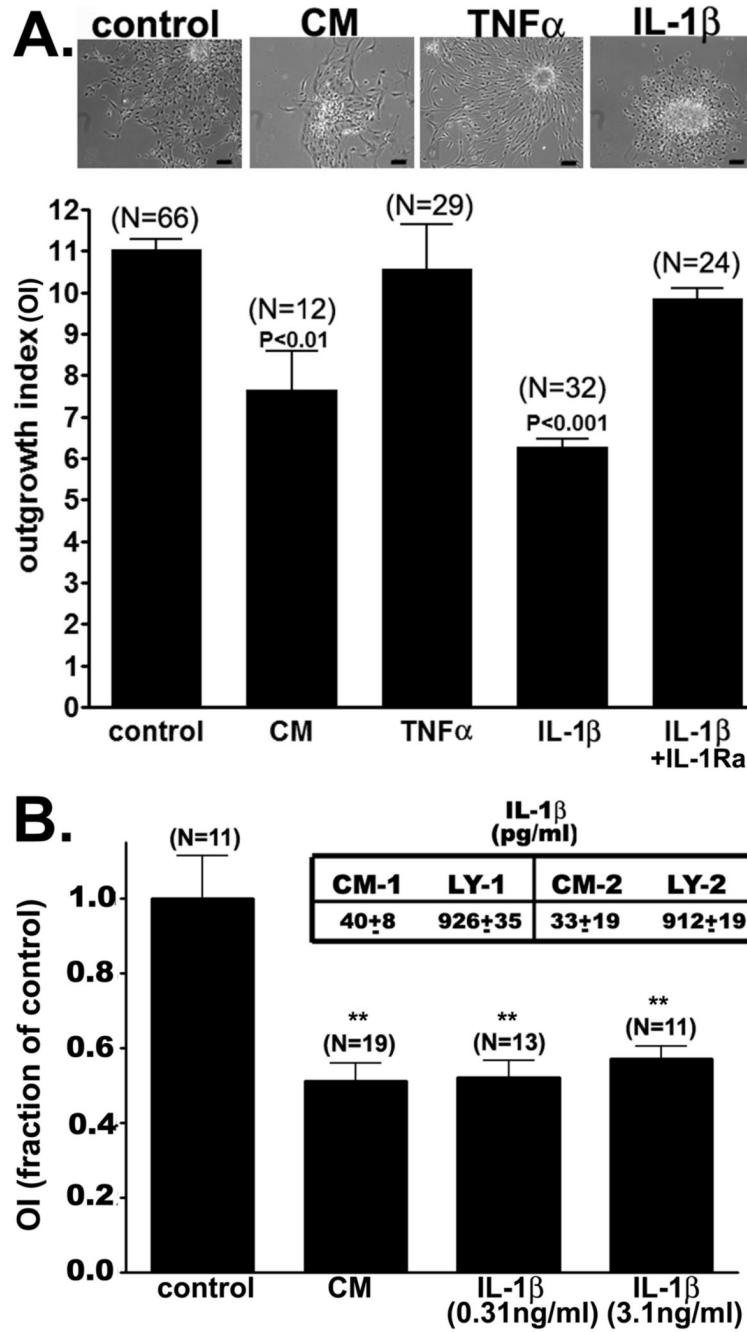


Figure 1. Effect of pro-inflammatory cytokines on the “in vitro” migration of neural progenitor cells

(A) Bar histograms showing the mean \pm SE values obtained for the outgrowth index from 3-days adherent neurospheres that were untreated (control) and treated with conditioned media from activated microglia (CM), IL-1 β (20ng/ml), TNF α (10ng/ml) and with the neutralizing IL-1 receptor antibody (100ng/ml). Note that only CM and IL-1 β caused a significant decrease in the outgrowth index compared to untreated cells (ANOVA followed by Newman-Keuls’ multiple comparison test; N represents the number of neurospheres used from at least three independent). (Top) Representative phase-contrast images obtained from 3-days adherent neurosphere cultures untreated (control) and treated with CM, IL-1 β , and TNF α . Bars: 40 μ m.

(B) Bar histograms of the mean \pm SE values obtained for the relative changes in OI from 3-day adherent neurospheres untreated (control) and treated with CM containing 0.03ng/ml IL-1 β and with IL-1 β (0.31ng/ml and 3.1ng/ml). Inset shows the mean \pm SE values (in pg/ml) of IL-1 β concentration measured in the conditioned media (CM) and in the lysate (LY) fractions obtained from two different cultures of microglia cells. (** P<0.001, ANOVA followed by Newman-Keuls' multiple comparison test; N represents the number of neurospheres used).

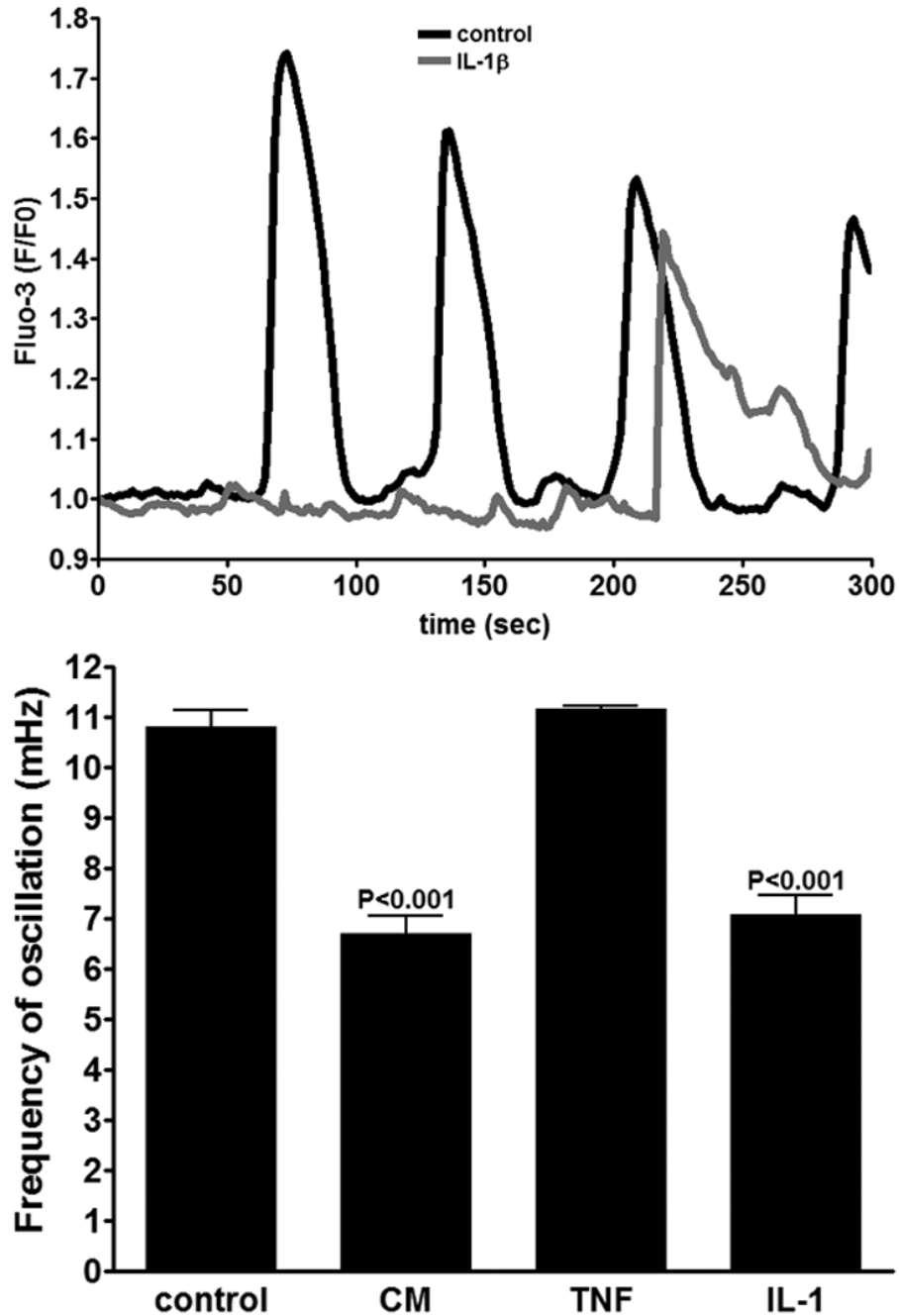


Figure 2. Frequency of spontaneous Ca^{2+} oscillations in astrocyte progenitors exposed to cytokines (A) Example of intracellular Ca^{2+} oscillations recorded from Fluo-3-AM loaded progenitor cells that were untreated and treated with IL-1 β . Each trace corresponds to a single cell. (B) Bar histograms showing the mean \pm SE values of the frequency (mHz) of spontaneous intracellular Ca^{2+} oscillations recorded from 3 days-adherent astrocyte progenitors that were untreated (control) and treated with conditioned media from activated microglia (CM), IL-1 β (20ng/ml), and TNF α (10ng/ml). Note the marked reduction in frequency of spontaneous Ca^{2+} activity in CM and IL-1 β -treated progenitors compared to control (ANOVA followed by Newman-Keuls' multiple comparison test).

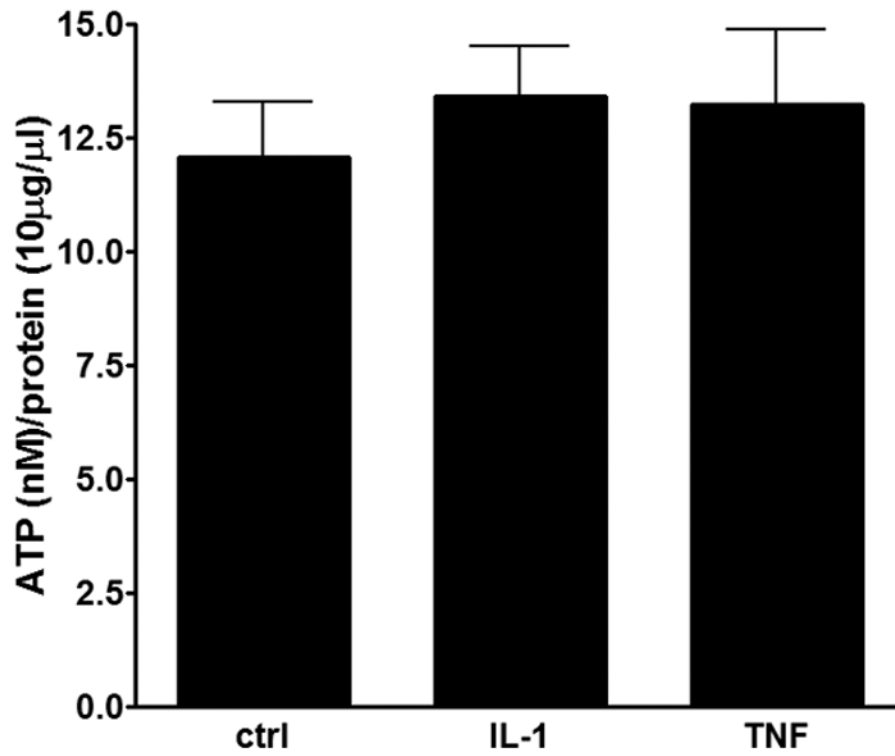


Figure 3. Effects of cytokines on the amount of ATP release
Bar histograms showing the mean \pm SE values obtained for the amount of ATP released from 3-days adherent progenitors that were untreated and treated with IL-1 β and TNF α .

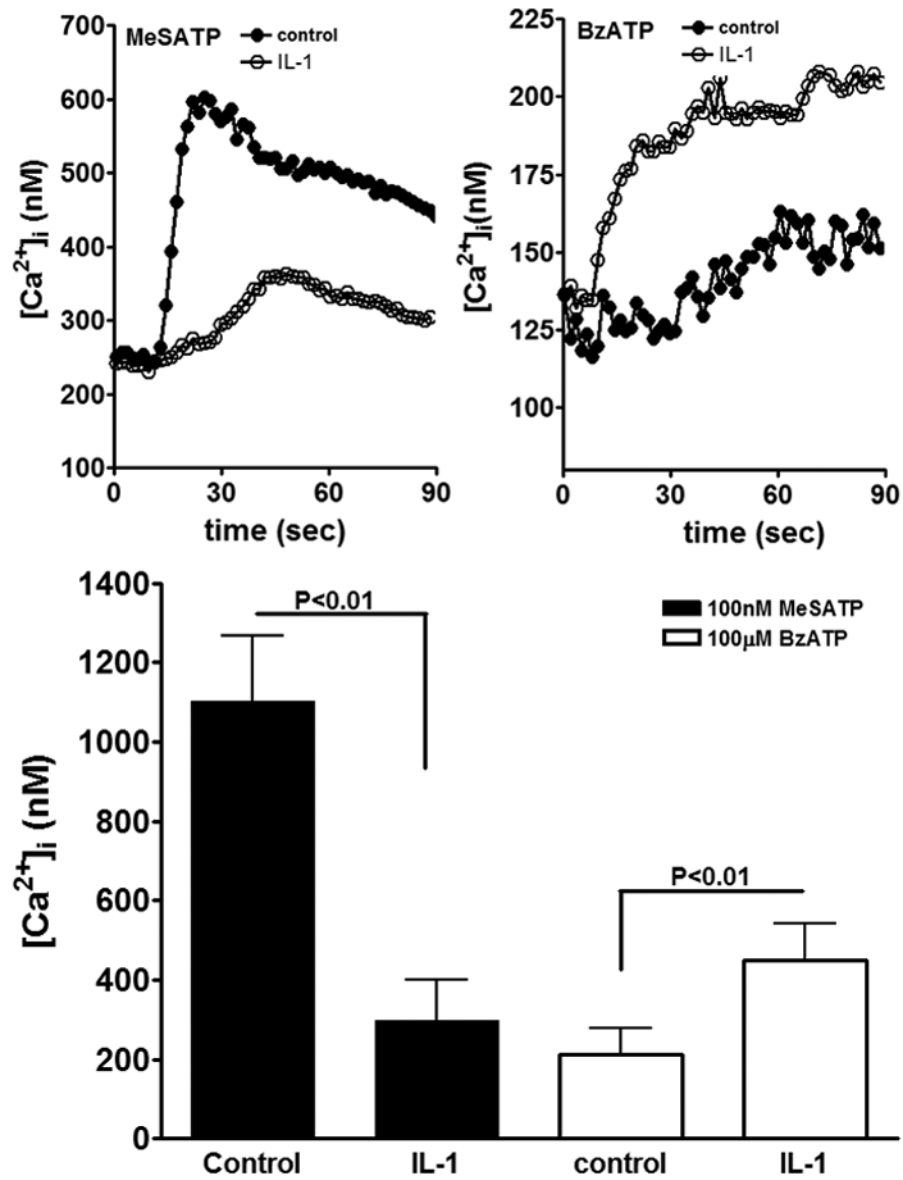


Figure 4. Changes in purinergic P2 receptor activity induced by IL-1 β

Time course of intracellular calcium changes induced by the two P2 receptor agonists showing the reduced response to the P2Y₁R agonist (2-MeSATP) (A) and the increased response to the P2X₇R agonist (B) in IL-1 β -treated progenitors compared to controls (Student T test). (C) Bar histograms showing the mean \pm SE values obtained for the amplitudes of intracellular Ca^{2+} transients induced by 100nM MeSATP and 100 μ M BzATP in untreated (control) and IL-1 β treated progenitors.

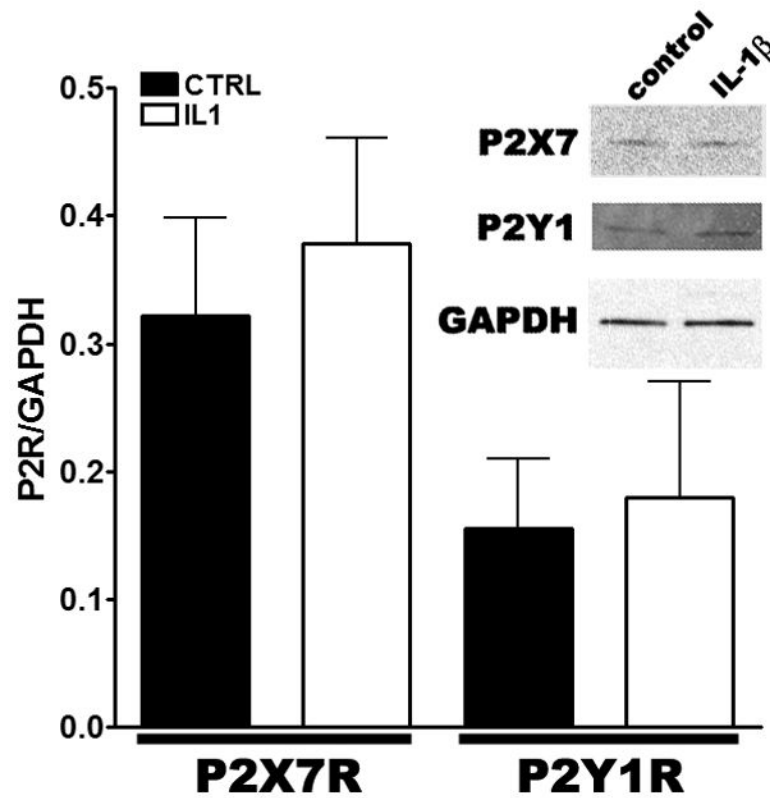


Figure 5. Expression levels of P2 receptors

Bar histograms showing the expression levels of P2 (P2X₇ and P2Y₁) receptors normalized to that of GAPDH obtained from untreated (black bars) and IL-1β treated progenitors (white bars). IL-1β did not significantly change P2 receptors expression levels ($P > 0.05$; T-test; $N = 2-5$). *Inset*. Representative Western blot showing the expression of the two P2 receptors in untreated (control) and IL-1β treated progenitors.