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Imipramine and fluoxetine inhibit LPS-induced activation and affect morphology of microglial cells in the rat glial culture

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ABSTRACT

Background: Recent evidence has suggested that antidepressants evoke neuroprotective and immunomodulatory effects in the brain, partly at least, by inhibiting glia activation. This study has been conducted on the lipopolysaccharide (LPS)-stimulated primary rat mixed glial cell culture in order to better recognize the influence of imipramine (a tricyclic antidepressant) and fluoxetine (a selective serotonin reuptake inhibitor) on the important balance between pro- and anti-inflammatory cytokines produced by the glial cells. Moreover, microscopic observations were made to describe the morphological alterations in the studied cell cultures exposed to the drugs.

Methods: The effect of both antidepressants on TNF-α, IL-1β and IL-10 levels was determined by ELISA. The mRNA levels of mentioned cytokines were evaluated by qRT-PCR assay. Moreover, drug influence on the LPS-stimulated level of NF-κB p65 subunit in nuclear fraction was determined by the colorimetric transcription factor assay.

Results: After LPS-stimulation both drugs decreased concentration of TNF-α and IL-1β in culture medium and expression of TNF-α and IL-1β mRNAs in cellular extracts. They also diminished the LPS-induced nuclear translocation of NF-κB p65 subunit. In contrast, imipramine and fluoxetine induced a few-fold weaker suppressing effect on the levels of IL-10. Parallelly to the inhibition of the LPS-induced inflammatory response, the antidepressants prevented the morphological alterations of cells elicited by LPS. Moreover, in unstimulated cultures imipramine but not fluoxetine caused transformation of microglia cells into cells with neuron-like morphology.

Conclusions: Imipramine and fluoxetine, by modulating glia activation, may exert anti-inflammatory effects in the CNS. It also seems that microglia cells are important target particularly for imipramine.

Introduction

Glial cells (astrocytes, microglia and oligodendrocytes) constitute about 90% of all cells of the central nervous system (CNS). They provide essential physical support for neurons and affect their function. Though microglia cells represent only about 10% of the total glial population, they are the major immune cells in the CNS which possess phagocytic activity and survey the parenchyma. Several lines of evidence indicate the involvement of microglia in the regulation of synaptogenesis and neural plasticity [21]. Microglia cells, due to their neuroprotective potential, promote neuronal survival in brain injury [10]. However, it is also certainly known that microglia becomes activated in response to inflammation, stress, infection, and neuronal damage in various CNS pathologies. In opposite to quiescent form, 'classically' activated microglia is a principal source of toxic substances, such as: nitric oxide, reactive oxygen species, eicosanoides and pro-inflammatory cytokines, like interleukin (IL)-1β, tumor necrosis factor-α (TNF-α), IL-6. Neuroinflammation, driven mainly by an activated microglia, is believed to be a crucial mechanism leading to CNS injury seen in various psychiatric and neurodegenerative diseases [6,20,23]. In the brain, overexpressed pro-inflammatory cytokines, such as IL-1β and TNF-α not only contribute to the neighboring neuronal damage but also play an essential role in microglia activation and recruit immune cells into the CNS [11]. Recently, it has been revealed that not only

Abbreviations: ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; IL, interleukin; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor-kappa B; PBS, phosphate buffered saline; qRT PCR, quantitative reverse transcription-polymerase chain reaction; TNF-α, tumor necrosis factor-α.

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'classically' activated microglia able to produce and release proinflammatory cytotoxic mediators but also 'alternatively' activated microglia exhibiting some anti-inflammatory properties important for repair processes appears in the same regions of the diseased brain during chronic neuroinflammation [4]. For example, IL-10 also produced mainly by microglia is a well-characterized anti-inflammatory cytokine which elicits immunosuppressive and neuroprotective effects [33]. Therefore, an effective pharmacological conversion of the 'classically' activated microglia to the other forms – alternative activation state or resting form – may be of therapeutic utility in the CNS diseases.

Microglial cells are in close contact with other cells in the brain, mainly astrocytes which constitute the majority of glial cells. Astrocytes are intimately involved in maintaining the extracellular milieu. They also modulate synaptic transmission, play a role as neural stem cells in adult neurogenic zones, secrete growth factor and release cytokines but they perform the latter functions less intensely than microglia cells [36].

Though antidepressants have been used in therapy for more than 50 years, their mechanism of action remains a mystery. Recently, new pharmacological effects induced by these drugs were discovered. It has been shown that despite different mechanism of action on the monoaminergic systems, some antidepressants elicit anti-inflammatory [13] and neuro/cytoprotective [25] effects which are at least partly related to their influence on cytokine production. Until now only a few in vitro studies have directly demonstrated that some antidepressants are able to resilience glia activation what seems to be of significance for their therapeutic efficacy (for review see [22]). Moreover, recent studies suggest a putative role for glia in antidepressant-stimulated neurogenesis [3,27].

Imipramine and fluoxetine are well-known antidepressants. Imipramine, the oldest tricyclic antidepressant is used as a drug of second choice in therapy of endogenous depression and illness-associated depression and is recommended for use in neuropathic pain treatment. This drug is also frequently applied in pharmacological studies as a reference antidepressant drug [37]. Fluoxetine is a representative of the selective serotonin reuptake inhibitors which belong to the first-choice drugs in therapy of depression. It is also used in the treatment of obsessive-compulsive disorders, bipolar affective disorders and neurodegenerative diseases [37]. A very few in vitro studies concern the effects of both mentioned drugs on production of only pro-inflammatory cytokines by glial cells [12,14,18,24,26]. In general, these studies provide evidence that imipramine and fluoxetine suppress production or expression of such cytokines as TNF-α, IL-1β, IL-6.

In order to better recognize the influence of imipramine and fluoxetine on the important balance between pro- and anti-inflammatory cytokines produced by the glial cells we investigated their effect on release and expression of TNF-α, IL-1β and IL-10 by lipopolysaccharide (LPS)-stimulated primary rat mixed glial cell cultures. Subsequently, we attempted to explain the cellular mechanism of the observed effects. We also conducted the microscopic observation of the effect of both antidepressants on morphology of microglia cells in unstimulated and LPS-stimulated glial cell culture.

Materials and methods

Cell cultures

Study was performed on the primary mixed glial culture which through keeping microglia-astrocytes integrity and cross-talking is considered to be a valuable experimental model because it better imitates the natural brain environment than isolated microglia cultures [35].

Primary mixed glial cultures were prepared from the cerebral hemispheres of one-day old Wistar rat pups and cells were cultured according to the method described previously [1]. Briefly, brains were excised aseptically from the skull, separated from the blood vessels and membranes and mechanically disrupted by trituration in ice-cold Dulbecco's Modified Eagle's Medium (Gibco-BRL, USA) supplemented with 20% of heat inactivated fetal bovine serum (FBS) (Gibco-BRL) containing 100 IU/ml penicillin, 100 μg/ml streptomycin and 25 μg/ml fungizone (Gibco-BRL). The suspension was filtered through sterile cell strainers (Becton-Dickinson, San Jose, CA, USA) with pores sizes: 70 μm and 10 μm. Cell count of the cell suspension was adjusted to 1.4×10^6 cells/ml and a volume of 0.1 ml was poured into each well of 96-well tissue culture plates (Becton Dickinson). Cultures were incubated at 37 °C in 95% air and 5% CO_2. The medium was replenished on day 1 after plating and every 3rd day thereafter with medium supplemented with 10% of heat inactivated FBS and abovementioned antibiotics. The experiments were conducted on 13-day cultures that contained the mature microglia being the most reactive to LPS and immature astrocytes which had not expressed typical phenotype yet, because they became mature on about the 21st day of culture. On day 13 the cultures were stained with *Ricinus communis* agglutinin-1(Vector, Burlingame, CA, USA), a lectin that binds to the surface of glycoproteins on microglia, the antibodies against glial fibrillary acidic protein (GFAP) (Sigma–Aldrich, St Louis, MO, USA), a marker of astrocytes and the antibodies against mitogen activated protein-2 (MAP-2) (Promega, USA), a marker of neurons. Approximately 60–65% of cells reacted with agglutinin-1 and 30–35% of cells were GFAP positive. No neurons were detected.

This study was approved by the Local Ethical Committee for the Animal Experiments of the Medical University of Silesia.

Evaluation of the effect of imipramine or fluoxetine on TNF-α, IL-1β and IL-10 concentration in the culture medium

On day 13, the culture medium was replaced with medium containing 10% of FBS, antibiotics and additionally LPS (*Escherichia coli* serotype 0111: B4; Sigma–Aldrich) or LPS + antidepressant: imipramine hydrochloride (Sigma–Aldrich, St Louis, MO, USA) or fluoxetine hydrochloride (Pliva, Kraków, Poland). Both drugs were dissolved in sterile water and then diluted in culture medium. LPS, a bacterial endotoxin, is frequently applied in experimental studies as a potent unspecific immunostimulator which enhances production of inflammatory mediators including pro-inflammatory cytokines, chemokines, nitric oxide and reactive oxygen species [32]. LPS was applied at a concentration of 1 or 2 μg/ml for TNF-α or IL-1β and IL-10 stimulation, respectively. LPS concentration and time of incubation were determined experimentally to achieve the strongest stimulation of cytokine release as described previously [1]. TNF-α release was 34-fold increased after 6-h incubation with LPS at a concentration of 1 μg/ml. A release of IL-1β and IL-10 was 15-fold and 2-fold increased, respectively after 48-h incubation with LPS at a concentration of 2 μg/ml. Imipramine was used in a concentration from 10^{-8} to 100 μM but fluoxetine in a concentration from 10^{-8} to 10 μM because in the higher concentration this drug induced cytotoxic effect as it was evidenced before in cell viability assays. After incubation with LPS or LPS + antidepressant, the culture medium was harvested, centrifuged ($2000 \times g$, 5 min) and assayed for TNF-α or IL-1β and IL-10 respectively.

Cytokine concentrations were measured with rat TNF-α, IL-1β and IL-10 enzyme-linked immunosorbent (ELISA) kits (R&D Systems, Minneapolis, MN, USA). The assays were carried out according to the manufacturer's protocol. Absorbance of each well was measured at the wavelength of 450 nm using a microplate reader Multiskan RC (Labsystems, Helsinki, Finland). The intra-assay

precision coefficient variations for TNF-α, IL-1β and IL-10 were 7.4%, 8.7% and 5.5%, respectively. The sensitivity of the assays for TNF-α and IL-1β was 5 pg/ml and for IL-10 was 10 pg/ml.

Cell viability

Three methods such as 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) conversion, lactate dehydrogenase (LDH) release, and trypan blue exclusion method were used to determine whether imipramine or fluoxetine at concentrations from 10^{-8} to 100 μM are toxic to the mixed glial cell cultures. Viability of cells was evaluated after 48-h exposure to each drug or LPS at a dose of 2 μg/ml. The effect of imipramine or fluoxetine on cell viability was evaluated in two independent experiments, each with four to six determinations.

In the MTT assay, the ability of the cells to convert MTT (Sigma–Aldrich) indicates mitochondrial activity and in consequence cell viability [28]. MTT (0.25 mg/ml) was added to the medium for 3 h (37 °C) before the end of the experiment. After being washed with phosphate buffered saline (PBS), cells were lysed in 100 μl of dimethyl sulfoxide (Sigma–Aldrich) which enabled the release of the blue reaction product – formazan. Absorbance at the wavelength of 570 nm was read on a microplate reader Multiskan RC (Labsystems).

LDH is rapidly released into the culture medium from necrotic cells. In this method maximum LDH release is compared with spontaneous LDH release evaluated in the culture medium and cell lysates. Absorbance at the wavelength of 490 nm was read on a microplate reader Multiskan RC (Labsystems).

In trypan blue method, cells failing to exclude 4% trypan blue solution after 5 min were considered as nonviable. The quantity of alive cells was counted using a Burker's chamber under light microscope.

Evaluation of the effect of imipramine or fluoxetine on the levels of TNF-α, IL-1β and IL-10 mRNAs

The dissociated cells from cerebral hemispheres of one-old day rats were plated in 35-mm Petri dishes (1.4×10^6 cells per dish)(Becton Dickinson) and cultured as described for primary mixed glial cultures. On day 13, the culture medium was replaced with medium containing additionally imipramine or fluoxetine, LPS alone or LPS + antidepressant. The cultures were incubated for 4 h with medium containing LPS in a dose of 1 μg/ml for stimulation of TNF-α or LPS in a dose of 2 μg/ml for 24 h to induce IL-1β and IL-10 mRNAs. Antidepressants were used at a concentration of 10 μM. mRNA copies were determined by qRT-PCR assay in cellular total RNA extracts.

Total RNA was extracted from harvested cells using TRIzol (Invitrogen) according to the manufacturer's protocol. After the 4-h (for TNF-α mRNA) or 24-h (for IL-1β and IL-10 mRNAs) incubation, the cells were washed up with PBS and lysed by addition 0.75 ml of TRIzol. Then samples were incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. After extraction with chloroform (Sigma–Aldrich) (1:5) by vigorously shaking for 15 s and incubation for 10 min at room temperature, the samples were centrifuged (12,000 × *g*, 15 min, 4 °C). The aqueous phase was transferred to a clean tube and the RNA was precipitated with isopropyl alcohol (Sigma–Aldrich) (1:1) at room temperature for 10 min. After centrifugation (12,000 × *g*, 10 min, 4 °C) the supernatants were removed and the RNA pellets were washed twice with 75% ethanol and dried. The RNA extracts were qualitatively evaluated by electrophoresis in 1% agarose gel and quantitatively using the BioPhotometer (Eppendorf).

Primers and probes for amplification of TNF-α (amplimer length: 108 bp), IL-1β (amplimer length: 149 bp), IL-10 (amplimer length: 101 bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (amplimer length: 87 bp) mRNA were applied (TaqMan Gene Expression Assays; Applied Biosystems, Foster City, CA, USA). Total RNA was reverse-transcribed for single strand cDNA using MultiScribe polymerase and cDNA copies were amplified using the TaqMan One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems). The reaction mixture (25 μl) was composed of 1.25 μl of sequence-specific primers and probes (20× Target Primers and Probes; Applied Biosystems), 12.5 μl of 2× RT-PCR Master Mix, 0.625 μl of 40× MultiScribe and RNA-se Inhibitor, 2.5 μl (10 ng) of total RNA and 8.125 μl RNA-se free water. qRT-PCR was performed using the ABI PRISM7700 Sequence Detection System (Perkin Elmer, San Antonio, TX, USA) (RT: 48 °C – 30 min; PCR: 95 °C – 10 min; 40 cycles: 95 °C – 15 s, 60 °C – 1 min). The quantified transcripts of the gene encoding rat GAPDH as an endogenous RNA control were carried out. The copy number of each sample is inferred from the CT and by using the standard curve performed during the same experiment. The number of TNF-α, IL-1β and IL-10 mRNA copies was calculated based on the calibration curve for the β-actin standards (TaqMan DNA Template Reagents; Applied Biosystems) performed during the same experiment using TaqMan Universal PCR Master Mix (Applied Biosystems).

Determination of NF-κB p65 subunit translocation

The nuclear level of p65 subunit that correlates positively with the activation of NF-κB p65 pathway was determined using the colorimetric Transcription Factor Assay (Millipore, Temecula, CA, USA). Glial cell cultures were incubated with imipramine or fluoxetine in a concentration of 10 μM, LPS alone in a dose of 2 μg/ml or LPS + antidepressant for 1 h. Then nuclear extracts were prepared according to the manufacturer's protocol. Protein concentration was estimated with Bradford's method [2]. Absorbance of the samples at the wavelength at 450 nm was measured using a microplate reader Multiskan RC (Labsystems).

Estimation of the influence of imipramine or fluoxetine on morphology of microglial cells in mixed glial cell culture

On day 13, the culture medium was replaced with medium containing additionally imipramine or fluoxetine in a concentration of 10 μM, LPS alone in a dose of 2 μg/ml, or LPS + antidepressant. After 24-h incubation, the medium was removed and culture plates were fixed using a frozen mixture of methanol and ethanol. Before observation under an inverted fluorescence confocal microscope Olympus X70 or fluorescence microscope Nikon TS-100F the cultures were stained with *R. communis* agglutinin-1, marker for microglial cells. In cultures treated with imipramine alone, microglia cells with the altered morphology were counted on three culture plates, using 200× objective.

Statistical analysis

Statistical analysis of data was performed using one-way analysis of variance (ANOVA) followed by the post hoc Bonferroni test. Differences were considered significant for $p < 0.05$. Statistical analysis was performed using GraphPad Prism 4.01 software (GraphPad Software, San Diego, CA, USA).

Results

Effect of imipramine or fluoxetine on LPS-induced concentration of TNF-α and IL-1β in the culture medium

According to the results of three cell viability tests (results of the MTT conversion test are shown as representative data in Fig. 1), imipramine was not cytotoxic to the glia cells at concentrations

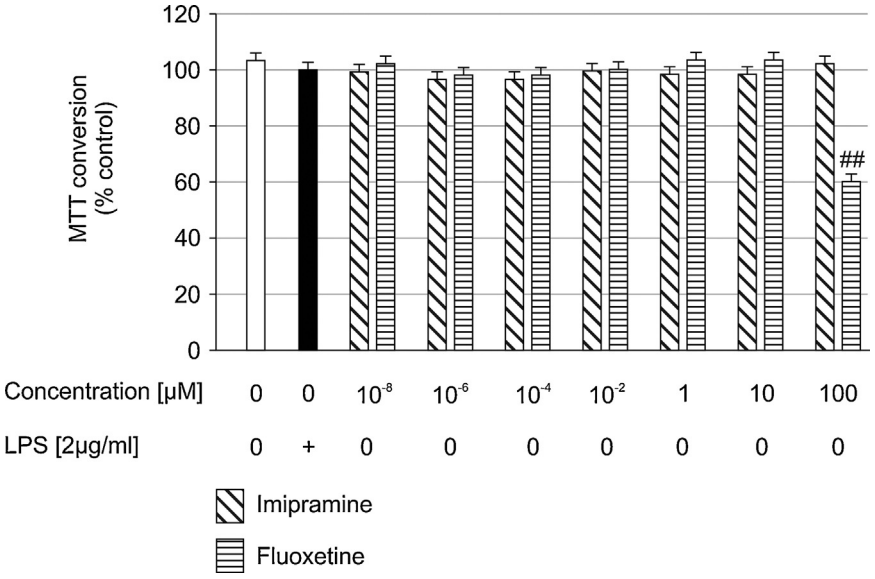

Fig. 1. Cell viability in the primary mixed glial cell cultures exposed to LPS (2 μg/ml) or imipramine or fluoxetine (from 10^{-8} to 100 μM) for 48 h. Cell survival was determined by the colorimetric measurement of the reduction product of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). Each bar represents the mean ± SEM of two independent experiments ($n = 12$ for each group). $^{\#\#}p < 0.01$ significantly different from the medium-treated cultures.

from 10^{-8} to 100 μM and fluoxetine at concentrations from 10^{-8} to 10 μM. The antidepressants remarkably decreased the LPS-stimulated levels of both pro-inflammatory cytokines in the culture medium. The maximal suppression of TNF-α by 60–70% *vs* LPS alone was noted when imipramine was present in the medium in the concentration range from 1 to 100 μM (Fig. 2A) while the strongest decrease in IL-1β level by 60–80% *vs* LPS alone was elicited at imipramine concentration from 10^{-2} to 100 μM (Fig. 2B). The maximal suppression of TNF-α by 40–55% *vs* LPS alone was observed when fluoxetine was used at a concentration from 10^{-2} to 10 μM (Fig. 2A). The maximal decrease in IL-1β concentration by 40–60% *vs* LPS alone was produced by fluoxetine at a concentration from 10^{-2} to 10 μM (Fig. 2B).

Effect of imipramine or fluoxetine on LPS-stimulated concentration of IL-10 in the culture medium

Imipramine at a concentration from 10^{-8} to 100 μM and fluoxetine at a concentration from 10^{-8} to 10^{-2} μM reduced LPS-stimulated IL-10 level in the culture medium by 15–20% *vs* LPS alone (Fig. 2C). At a higher concentration, from 1 to 10 μM fluoxetine had no influence on IL-10 level (Fig. 2C).

Effect of imipramine or fluoxetine on TNF-α, IL-1β and IL-10 mRNAs in mixed glial cell cultures

In comparison to the LPS alone, imipramine and fluoxetine at a concentration of 10 μM decreased the LPS-stimulated level of pro-inflammatory cytokine mRNAs. Imipramine reduced TNF-α mRNA by 35% (Fig. 3A) and IL-1β mRNA by 50% *vs* LPS alone (Fig. 3B). Fluoxetine reduced TNF-α mRNA by 60% (Fig. 3A) and IL-1β mRNA by 62% *vs* LPS effect alone (Fig. 3B). Only imipramine slightly attenuated IL – 10 mRNA level by 14% *vs* LPS alone (Fig. 3C). Both antidepressants did not alter the constitutive levels of mRNA for the studied cytokines (Fig. 3A–C).

Inhibitory effect of imipramine or fluoxetine on NF-κB p65 subunit translocation into the cellular nuclei fraction of glial cells

Imipramine and fluoxetine at a concentration of 10 μM diminished the LPS-induced nuclear translocation of NF-κB p65 subunit by 40% and 15% *vs* LPS alone, respectively. The studied antidepressants did not alter the constitutive level of p65 subunit in nuclear fraction (Fig. 4).

Effect of imipramine or fluoxetine on morphology of microglia cells in mixed glial cell cultures

In the LPS-unstimulated mixed glial cell cultures, microglia cells stained with agglutinin-1 were stratified and tight-packed. On top surface round-shaped active cells were situated and on the bottom, less visible branched-ramified resting forms were present (Fig. 5A). Imipramine at a concentration of 10 μM added to the medium of the unstimulated culture induced transformation 40–45% of microglia cells into cells with a neuron-like morphology. Their stoma with longer, thinner and more branched processes were smaller than in controls (Fig. 5B). Fluoxetine at the same concentration altered only arrangement of cells. As in the unstimulated mixed glia culture, microglia cells positively stained with agglutinin-1 looked like round-shaped active cells or resting forms but they were atypically arranged in the chain-like forms (Fig. 5C). In stimulated cultures, cells were round and enlarged, without any processes and arranged in a "grape-chain" structure (Fig. 5D). Morphology of the cultures exposed to LPS + antidepressant was altered. Cultures exposed to LPS + antidepressant looked like inactivated cultures with cells not conglomerated in "grape structure". The tight connections of cells were extended (Fig. 5E and F)

Discussion

The present study shows for the first time that imipramine and fluoxetine induce similar effects on cytokines in the LPS-stimulated rat primary mixed glial cell cultures but have different influences on morphology of unstimulated microglia cells. Both drugs shifted the balance between pro-inflammatory cytokines TNF-α, IL-1β and anti-inflammatory cytokine IL-10 toward the latter. They remarkably and dose-dependently inhibited LPS-induced production of TNF-α and IL-1β, and only slightly diminished the LPS-stimulated level of IL-10.

In contrast to other studies [12,14,18,24,26] that investigated effect of a narrow range of antidepressant concentrations (maximal from 10^{-2} to 100 μM) on glial cytokine production

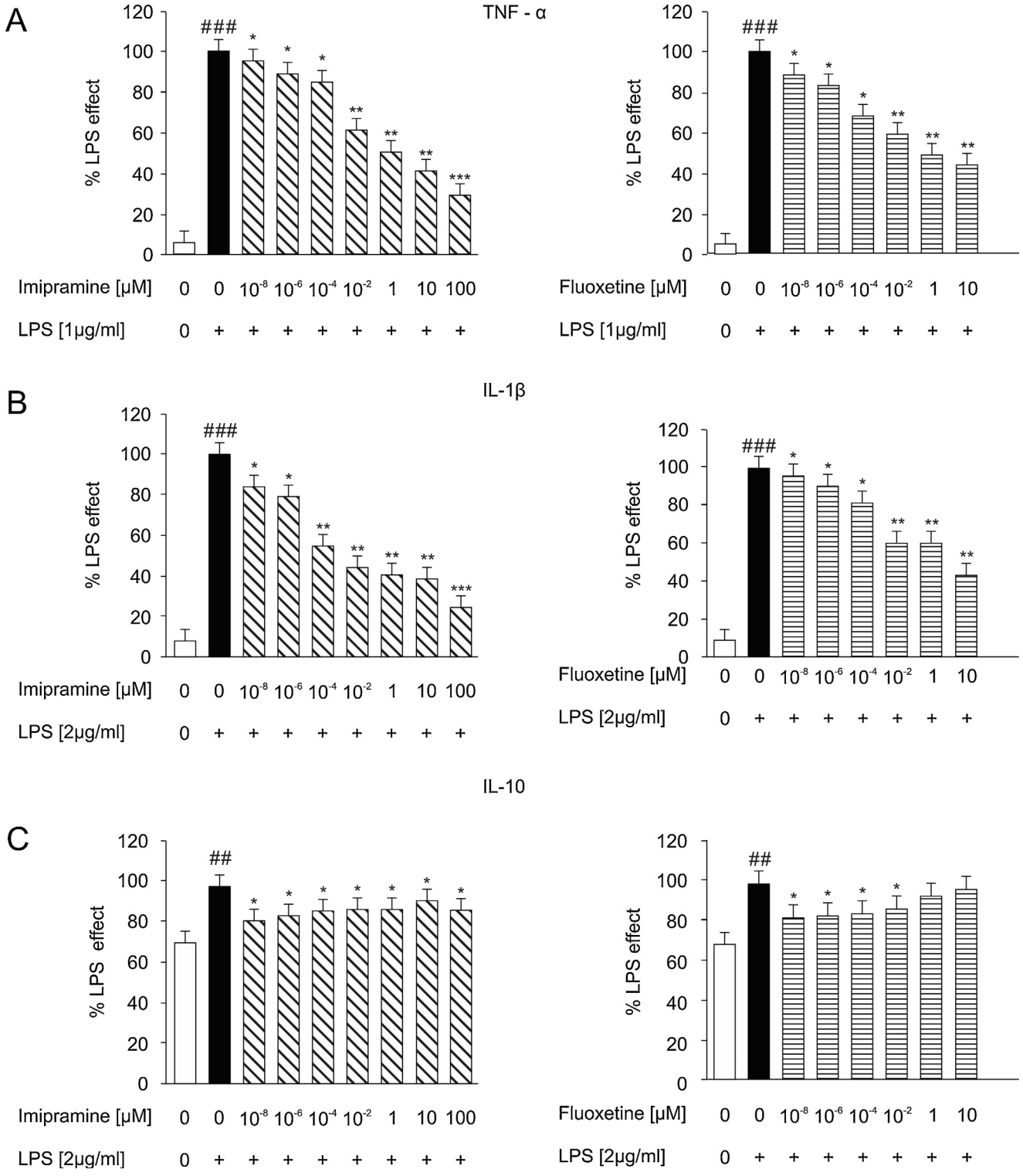

Fig. 2. The effect of imipramine or fluoxetine on LPS-stimulated TNF-α (A), IL-1β (B), IL-10 (C) release in the primary mixed glial cell culture. Cells were exposed to imipramine (from 10^{-8} to 100 μM) or fluoxetine (from 10^{-8} to 10 μM). LPS (1 μg/ml) was used for 6 h to induce TNF-α release. LPS (2 μg/ml) was applied for 48 h to induce IL-β and IL-10 release. Cytokine release was determined using an ELISA assay. Each bar represents the mean ± SEM from three independent experiments (n = 9–12 for LPS + imipramine or fluoxetine; n = 12 for medium or medium + LPS). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ significantly different from the vehicle (medium + LPS)-treated cultures; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ significantly different from the medium-treated cultures.

we studied the drug effects on the LPS-stimulated cytokine levels in culture medium at a broad drug concentration range, namely from 10^{-8} to 100 μM (imipramine) or to 10 μM (fluoxetine). We found that both drugs suppressed LPS-induced cytokine production even at a very low concentration what suggests that this effect may be long-lasting in the CNS. However, in order to verify this suggestion further studies conducted in an in vivo model or on adult glial cell cultures would be needed.

In the subsequent experiments aimed to partly explain the mechanism of the observed effects, both antidepressants were applied at a concentration of 10 μM. On the basis of pharmacokinetic studies, it may be concluded that the effects induced

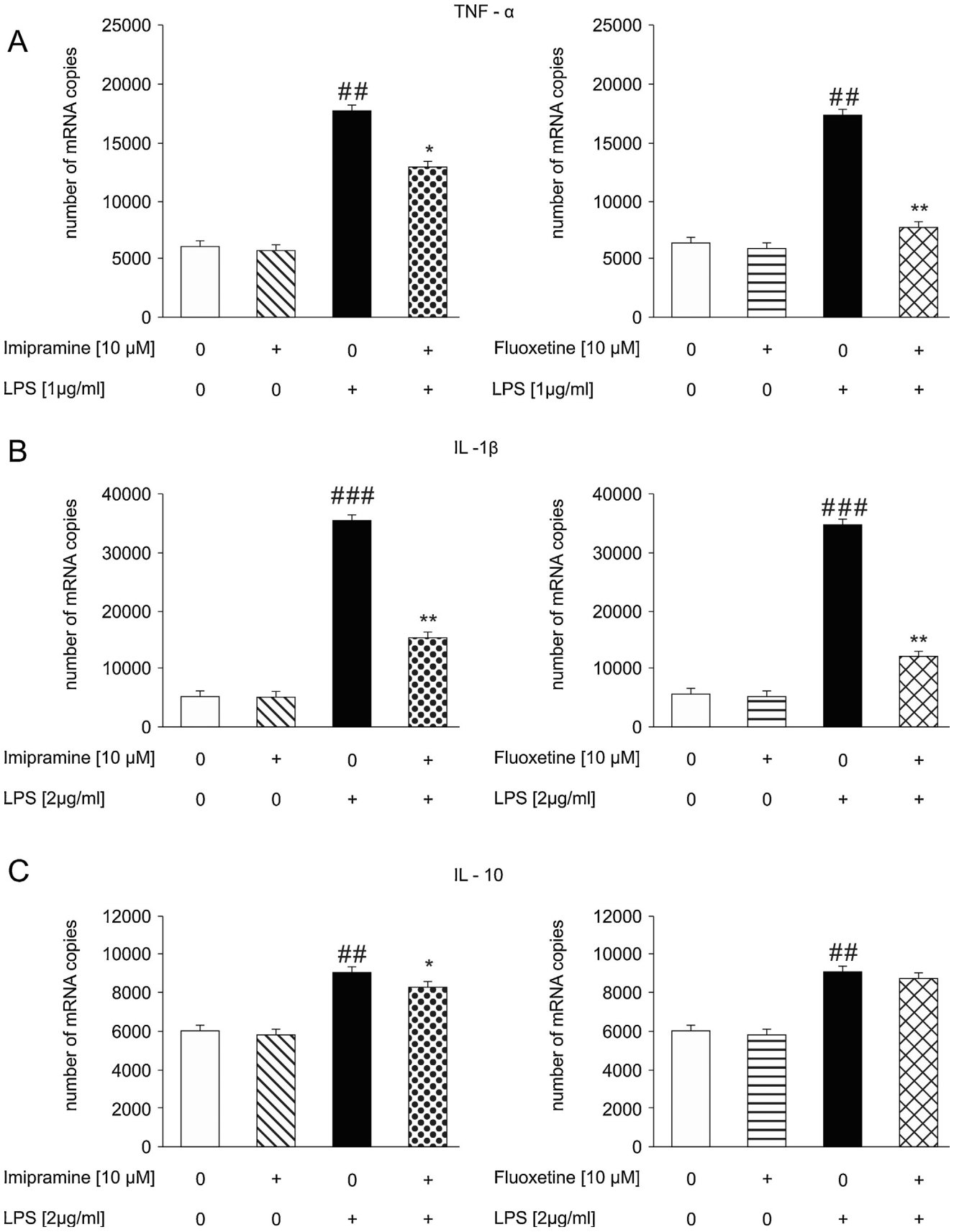

Fig. 3. The influence of imipramine or fluoxetine on TNF-α mRNA copies (A), IL-1β mRNA copies (B), IL-10 mRNA copies (C) in the primary mixed glial cell cultures. The cultures were incubated with medium containing LPS (1 μg/ml) for 4 h to induce TNF-α mRNA or LPS (2 μg/ml) for 24 h to induce IL-1β and IL-10 mRNAs. Imipramine or fluoxetine (10 μM) were used alone or with LPS. mRNA copies were detected by quantitative RT-PCR. Each bar represents the mean ± SEM of two independent experiments ($n = 6$ for each group). $^{*}p < 0.05$, $^{**}p < 0.01$ significantly different from the vehicle (medium + LPS)-treated cultures; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ significantly different from the medium-treated cultures.

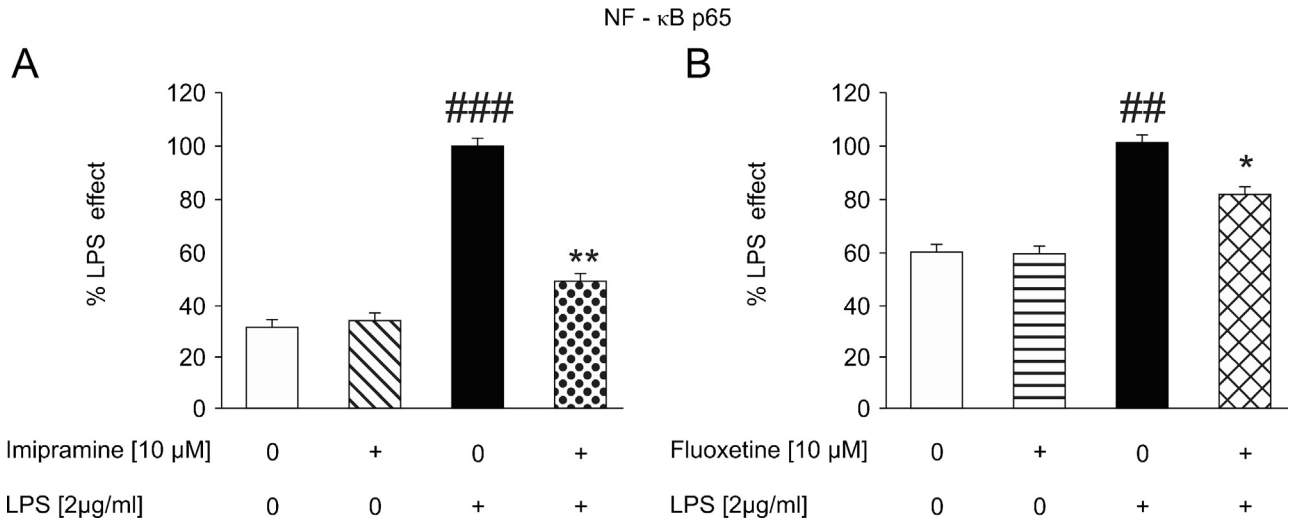

Fig. 4. The effect of imipramine or fluoxetine on the level of NF-κB p65 subunit in nuclear fraction of cells in the primary mixed glial cell cultures stimulated by LPS. The cultures were incubated with medium containing LPS (2 μg/ml) and/or imipramine or fluoxetine (10 μM) for 1 h. NF-κB p65 subunit level was determined by colorimetric transcription factor assay. Each bar represents the mean ± SEM from two independent experiments (n = 9–12 for each group). $^{*}p < 0.05$, $^{**}p < 0.01$ significantly different from the vehicle (medium + LPS)-treated cultures; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ significantly different from the medium-treated cultures.

by the studied drugs at this concentration are of great significance because such level may be achieved in the brain during treatment. Plasma level of imipramine used in therapeutic doses was estimated at 0.16–0.54 μM [37]. Since experimental studies indicate that the level of imipramine in brain tissue is 32 times higher than in plasma [5] it may be assumed that during therapy imipramine reaches the levels of about 5–17 μM in the brain. In turn, in the brain of healthy volunteers receiving fluoxetine in therapeutic dose for 5 weeks, its concentration was determined at about 20 μM by using magnetic resonance spectroscopy [15].

The decreased LPS-stimulated TNF-α and IL-1β mRNA levels in cultured cells exposed to antidepressants (10 μM) detected by qRT-PCR analysis evidence that the reduced concentration of both cytokines found in culture medium was secondary to the reduction of their synthesis at the transcriptional level. Similarly, parallel results concerning the influence of both drugs (10 μM) on IL-10 concentration and its mRNA levels were also found. Namely, in stimulated cultures imipramine suppressed but fluoxetine did not alter these parameters. Importantly, imipramine as well as fluoxetine had no influence on the unstimulated mRNA levels of any cytokine studied what suggests that these drugs should not disturb production of the above-mentioned cytokines in physiological circumstances. Furthermore, we also found that anti-inflammatory effects of both antidepressants are mediated by the inhibition of LPS-activated NF-κB pathway because they decreased the level of nuclear subunit p65 of NF-κB. It is well known that LPS enhances pro-inflammatory cytokine gene expression by degradation of IκB subunit of transcription factor NF-κB followed by nuclear translocation of subunit p65 [29]. Our results are consistent with previous evidence from other research groups that antidepressants, such as imipramine, clomipramine [18] and fluoxetine [24,26] negatively regulate NF-κB pathway in LPS-activated BV2 microglial cell line.

Up to now only a few studies dealing with the effect of imipramine or fluoxetine on pro-inflammatory cytokines produced by cells derived from the CNS have been published. It has been found that imipramine decreased the production of IL-1β, TNF-α by the LPS-activated murine BV-2 microglia cells [18] or reduced IL-6 release by IFN-γ-stimulated murine microglial 6–3 cells [14] and inhibited LPS-stimulated IL-6, IL-1β, TNF-α release by neural stem cells having originated from the hippocampal tissue [31]. In vitro studies on the effect of fluoxetine did not bring unequivocal results. Fluoxetine decreased TNF-α and IL-6 production by the LPS-activated BV-2 microglial cell line and primary microglia culture [26], blocked LPS-induced expression of TNF-α and IL-1β in primary microglial culture [24] but increased unstimulated IL-6 and TNF-α mRNA levels in the BV-2 microglial cells [12]. The results of in vivo studies indicated inhibitory effects of fluoxetine on pro-inflammatory cytokines, namely in rats with middle cerebral arterial occlusion this drug given iv. caused a marked suppression of microglia activation, expression of IL-1β and TNF-α and NF-κB activity [24]. When given *ip* fluoxetine suppressed IL-1β and TNF-α production induced in the hippocampus by *icv* injection of kainic acid [19].

Our results confirmed suppressing effect of both antidepressants on the LPS-stimulated production of pro-inflammatory cytokines IL-1β and TNF-α and, importantly, they showed a few-fold weaker effect on the LPS-stimulated production of the anti-inflammatory cytokine IL-10. These findings directly support the previous suggestion about anti-inflammatory properties of the studied drugs. In the above mentioned other studies conclusion about anti-inflammatory effect evoked by antidepressants in the CNS has been drawn indirectly on the basis of data showing that they inhibit expression of inflammatory mediators or glial activation (for review see [13,22]).

In the light of data from experimental and clinical studies, anti-inflammatory properties of antidepressants caused partially by modulation of cytokine production seem to be of significance for their therapeutic efficacy in depression, neurodegenerative diseases and neuropathic pain because nowadays neuroinflammation is believed to be involved in the pathogenesis and/or development of such disorders. Experimental data indicate that inflammatory process and neurogenesis [8,39], neuroplastic processes [9] or apoptosis [17] are closely connected and that anti-inflammatory drug activity enhances its neuroprotective effects [17]. These findings reveal an essential interplay between cytokines, neurotrophic factors and proteins involved in apoptosis.

The meta-analysis published by Dowlati et al. [7] and Howren et al. [16] brought evidence that major depression is accompanied by activation of the inflammatory response system. Three causal pathways: depression to inflammation, inflammation to depression and bidirectional relationships were suggested [16]. The authors reported the higher concentration of TNF-α and IL-6 [7] or

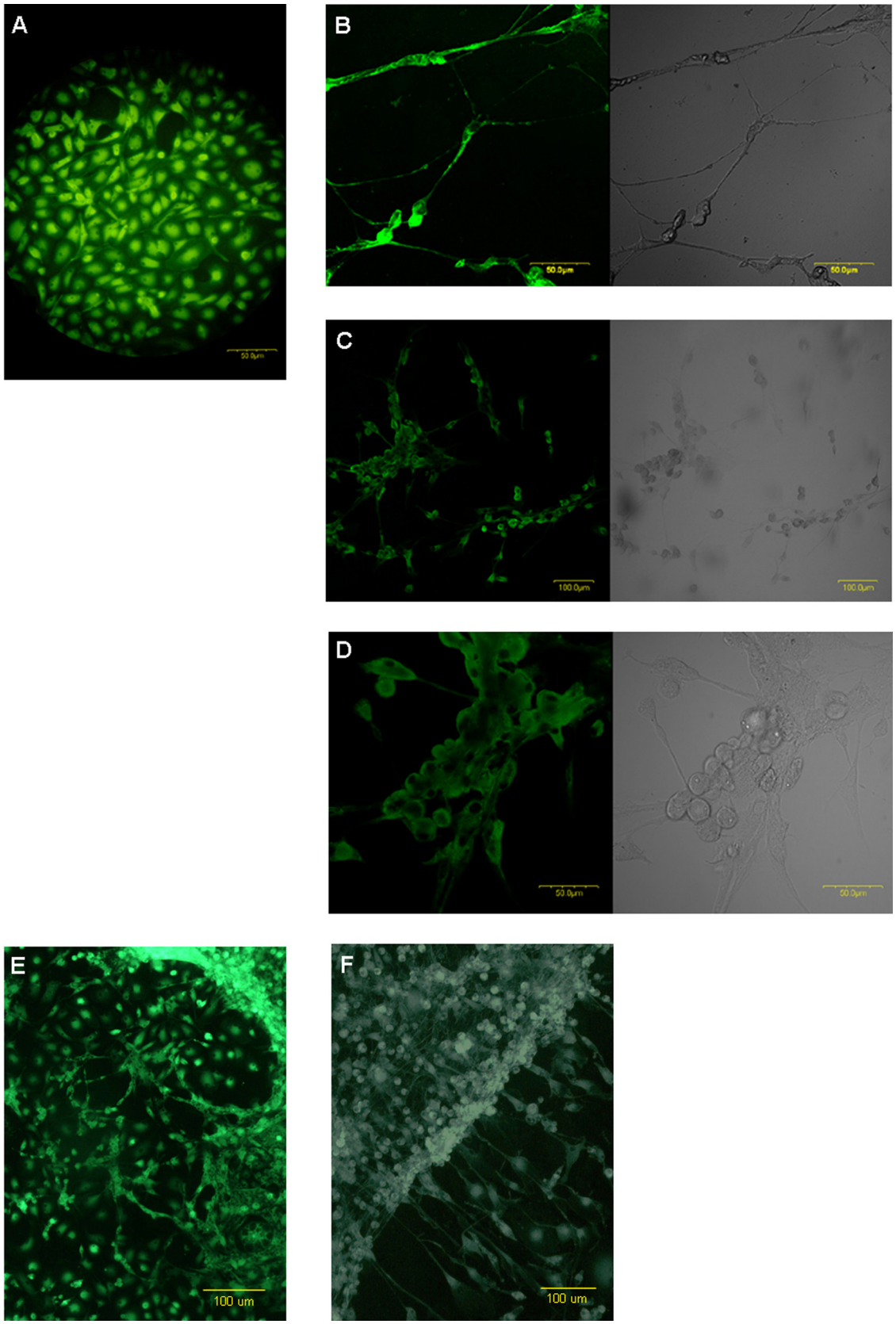

Fig. 5. The representative phase contrast image of mixed glial cell culture. Microglia cells were stained with a specific marker *Riccinus communis* agglutinin-1 (green fluorescence). In unstimulated cultures cells are tight-packed. There are activated amoeboid cells and ramified forms with pseudopodia (A). In unstimulated culture imipramine when given alone (10 μM) for 24 h altered morphology 40–45% of microglial cells which became thinner and had longer and more branched processes, like neuronal cells (B). Fluoxetine (10 μM) given for 24 h changed only arrangement of ramified resting forms of microglia cells present in unstimulated cultures. They were clustered in the star or chain structures (C). LPS (2 μg/ml) given for 24 h caused domination of ameboid cells without processes arranged in "grape-shaped" structure (D). LPS + imipramine (E) or fluoxetine (F) provoked similar separation of cells which were not conglomerated in "grape-shaped" structure as in unstimulated cultures.

IL-1 and IL-6 [16] in depressed patients compared with control subjects. Inflammation and microglial activation is postulated to contribute to pathogenesis and progression of neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease, Parkinson's disease, HIV dementia, amyotrophic lateral sclerosis. Chronic glia activation leads to neurodegeneration caused by neurotoxic, apoptotic and oxidative mechanisms [6]. For instance, it has been found that treatment of human microglia cells with β-amyloid resulted in increased expression of mRNA inflammatory molecules including pro-inflammatory cytokines and chemokines [30]. In neuropathic pain, an increased level of TNF-α in the brain via transforming function of α2-adrenergic receptors causes an inhibition of descending noradrenergic pathways activity that contributes to the pain development [34]. It has been reported that suppression of neuropathic pain is partially mediated by inhibiting of pro-inflammatory cytokines production and NF-κB activation in CNS [38].

We demonstrated for the first time that both studied drugs added to culture medium at a concentration of 10 μM influenced also morphology of microglia cells and cell connections in unstimulated and LPS-stimulated mixed glia cell culture enriched in microglia cells. Imipramine induced easily detectable transformation of some of LPS-unstimulated microglia cells into the cells with neuron-like morphology. Fluoxetine did not cause so intense alterations as imipramine but it induced atypical arrangement of cells into chain-like forms. As described in the section "Results", parallelly to the diminished inflammatory response to LPS, both drugs added to culture medium prevented also the morphological alterations of microglia cells induced by LPS. Until now, similar observation has been reported only from one in vitro study evaluating the influence of antidepressants (imipramine, fluoxetine and venlafaxine) on unstimulated primary human astrocyte culture. Cabras et al. [3] observed morphological transformation of astrocytes into cells with neuronal appearance, characterized by time-dependent increase in some neuronal markers what provided evidence that astrocytes may play a role in antidepressant-induced neurogenesis. At present, further investigations of the phenotypic changes induced by antidepressants in microglia are under way in our lab in order to support the observation, reported here for the first time, with convincing biochemical evidence. However, in our opinion the observed morphological alterations could not be without significance for the mechanism of action of the studied drugs. They indicate that microglia are the target for antidepressant drugs, especially for imipramine

Our findings support directly the previous suggestion that imipramine and fluoxetine have common anti-inflammatory properties in the CNS because as we shown they are able to silence overactivated glia via decreasing the biosynthesis of pro-inflammatory cytokines, such as TNF-α and IL-1β without a remarkable effect on anti-inflammatory cytokine IL-10. The reported microscopic observations confirm these biochemical results. It is thought that control of microglia activation which limits neuroinflammatory process may be beneficial in the treatment of CNS diseases. A better recognition of the anti-inflammatory properties of various antidepressants may help to optimize therapy or prophylaxis of neurodegenerative and psychiatric diseases associated with glia over-activation.

Conflict of interest

No conflict of interest.

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