Regional difference in inflammatory response to LPS-injection in the brain: role of microglia cell density.

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Abstract

To elucidate whether density of cells could contribute to the extent of microglial activation, we performed *in vitro* assays using three different densities of N13 microglia stimulated with LPS. Our results showed that induction of pro-inflammatory factors as TNF- α and iNOS was directly related to cell density, meanwhile the induction of the anti-inflammatory IL-10 was inversely related to cell density. Accordingly, *in vivo* assays showed that after LPS-injection, iNOS expression was more intense in substantia nigra, a brain area showing specific susceptibility to neurodegeneration after microglia activation, whereas IL-10 expression was more sustained in striatum, an area resistant to damage. These results support that microglia density is pivotal to control the balance between pro- and anti-inflammatory factors release.

Key words: neurodegeneration, inflammation, microglia density, substantia nigra, striatum.

1. Introduction

Although microglial activation is the brain's major defense against immune challenge, it has been widely described that activated microglia may contribute to neurodegeneration through the release of proinflammatory and/or cytotoxic factors as interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), nitric oxide (NO) and reactive oxygen intermediates among others (Chao et al., 1992; Dickson et al., 1993; Lee et al., 1993; Brosnan et al., 1994; Matsuo et al., 1995; Espey et al., 1997; Minghetti and Levi, 1998). However, all these factors are necessary for normal function of microglia. So to preserve brain integrity it is important to keep activation of microglia under strict control (see review, Hanisch and Kettenmann, 2007). In this sense, IL-10 has emerged as an important anti-inflammatory modulator of glial activation, functioning to maintain a balance between pro- and anti-inflammatory cytokines levels in the CNS (Sawada et al., 1999). In fact, IL-10 has been reported to inhibit release of inflammatory mediators by microglia (Lodge et al., 1996; Hu et al., 1999; Ledebor et al., 2000; Molina-Holgado et al., 2001a; Sawada et al., 1999; Kremlev and Palmer, 2005) exerting a neuroprotective role in several models of CNS injury (Bachis et al., 2001; Dietrich et al., 1999; Molina-Holgado et al., 2001b; Knoblack and Faden, 1998).

Microglial production of cytotoxic factors after exposure to the proinflammatory lipopolysaccharide (LPS) has been well documented (Boje and Arora, 1992; Chao et al., 1992; Dawson et al., 1994; Kong et al., 1996; Liu et al., 2000). However, except for the substantia nigra (SN), acute administration of LPS in different brain regions does not induce an evident neuronal damage (Herrera et al., 2000; Kim et al., 2000; Liu et al., 2000; Lu et al., 2000; Ji et al., 2008; Espinosa-Oliva et al., 2011). Thus, challenge with LPS in SN has been widely used by us and others as an *in vivo* model to selectively induce neurodegeneration in this area (see Dutta et al., 2008, for extensive review).

Trying to clarify the mechanism underlining region-specific differential susceptibility to LPS, Kim et al. (2000) proposed that it may fall on differences in the number of microglia cells

within specific brain regions. They found that LPS induced neurodegeneration in SN, a region with high density of microglia cells (Lawson et al., 1990), but not in cortex or hippocampus. However, other features of the SN as a higher blood brain barrier permeability, excessive neutrophil infiltration, and lower astrocyte density cannot be excluded (Ji et al., 2008).

Based on this, we propose that in the mesencephalic scenery with a high density of microglia, LPS triggers an inflammatory response leading to over-activation of microglia and the overproduction of inflammatory mediators, which may give rise to an imbalance between pro-inflammatory and anti-inflammatory cytokines and molecules that regulate inflammatory response. In order to elucidate whether density of microglia cells could contribute to the extent of microglial activation, we performed *in vitro* assays using three different densities of N13 microglia stimulated with LPS and analyze, by real-time PCR, ELISA or immunoblots, the level of pro-inflammatory (TNF- α , iNOS) and anti-inflammatory (IL-10) factors. Finally, we tested *in vivo* the same inflammatory markers expressed in SN and striatum following LPS administration.

2. Materials and methods

2.1. Cell Culture and LPS-stimulation

The N13 microglial line was a kind gift of Dr. David Pozo-Pérez (Dpto. Bioquímica Médica y Biología Molecular. Facultad de Medicina, US). After stimulation with LPS, N13 microglia produces a repertoire of cytokines similar to primary microglia (Righi et al., 1989). Cells were grown in RPMI 1640 (PAA, Linz, Austria) supplemented with 2 mM glutamine (PAA, Linz, Austria) and 5% (v/v) fetal bovine serum (PAA, Linz, Austria), 100 U/ml penicillin and 100 μ g/ml streptomycin (PAA, Linz, Austria) at 37°C and 5% CO₂.

Cells were grown to confluence in tissue flasks and then plated on 6-wells plastic plates (Nunc, Thermo Fisher Scientific, USA) in culture media, at different cellular densities. Densities of cells were: 0.25x 10⁶ cells/well (low), 0.5x10⁶ cells/well (medium) and 10⁶ cells/well (high).

After adhering, cells were treated with LPS in culture media (0.01 µg/ml) and finally collected at different times after stimulation (15 min, 30 min, 1, 3, 6, 9 and 24 hours), to isolate RNA and/or proteins. For each cell density and at any time point studied, cells treated only with vehicle but no LPS were used as control.

2.2. Animals and surgery

Adult male Wistar rats (200–250 g; n= 70) purchased from the University of Seville (Centro de Producción y Experimentación Animal (Espartinas, Sevilla), Universidad de Sevilla, Spain) were used. The rats were anesthetized with 400 mg kg⁻¹ chloral hydrate and positioned in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA) to conform with the brain atlas of Paxinos and Watson (1986). LPS (from *Escherichia coli*, serotype 026:B6; Sigma, USA) was dissolved (2 µg/µl) in a solution of 1% Monostral Blue (Sigma; 1% in phosphate-buffered saline, PBS) in PBS and 2.0 µl injected into left and right striatum. The injection needle was lowered through a drill hole 0.6 mm posterior, ±1.5 mm lateral and 8.3 mm ventral to the bregma. The injections were delivered over a period of 2 min and after each the needle was left in situ for an additional 5 min to avoid reflux along the injection track. Animals were decapitated or perfused at different times post-injection (3, 6, 14 and 24 h; and 3 and 7 days) and brains quickly removed. A second group of animals were injected with LPS (2 µg/injection) into the SN, according to the coordinates: 5.5 mm posterior, ±1.5 mm lateral and 8.3 mm ventral to the bregma. In a further set of experiments, rats were co-injected into SN with 2 µg of LPS along with SB203580 (0.25 and 2.5 nmol, Calbiochem, UK) or S-methylisothioureia (3 nmol, Sigma). In these experiments animals were killed 7 days post-injection.

In all experiments, additional animals were injected with saline into the striatum or SN. Experiments were carried out in accordance with the Guidelines of the European Union Council (86/609/EU), following the Spanish regulations (BOE 67/8509–12, 1988) for the use of laboratory animals and approved by the Scientific Committee of the *Universidad de Sevilla*. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.3. RNA extraction and reverse transcription

For PCR analysis, young and aged rats were killed by decapitation and both striatum or SN were dissected, frozen in liquid N₂ and stored at –80° C until use. Total RNA was extracted using the Tripure™ Isolation Reagent (Roche, Germany), according to the instructions of the manufacturer. This procedure allows the isolation of total RNA, DNA and protein fractions from a single sample. After isolation, the integrity of the RNA samples was assessed by agarose gel electrophoresis. The yield of total RNA was determined by measuring the absorbance (260/280 nm) of ethanol precipitated aliquots of the samples.

Similarly, samples from *in vitro* assays were extracted with Tripure. For that, after removing supernatant, whole cells were collected by adding 0.5 ml/well of Tripure.

Reverse transcription (RT) was performed using random hexamers primers, 3 µg of total RNA as template and the High-Capacity cDNA Archive Kit (Applied Biosystems) following the manufacturer's recommendations as previously described (Gavilan et al. 2009).

2.4. Real-time PCR

After RT, the cDNA was diluted in sterile water and used as template for the amplification by the polymerase chain reaction. Optimization and amplification of each specific gene product was performed using the ABI Prism 7000 sequence detector (Applied Biosystems, Madrid, Spain) and Sybr green™, as previously described (Gavilán et al., 2009). Primers were designed using the ProbeFinder software™ (Roche Applied Science) and are listed in Table 1. All of them flanked an intronic sequence to ensure the absence of genomic contamination. The cDNA levels of the different samples were determined using β-actin as housekeeper. The amplification of the housekeeper was done in parallel with the gene to be analysed. Thus, the results were normalized using the β-actin expression. Threshold cycle (Ct) values were calculated using the software supplied by Applied Biosystems.

Table 1. Sequences of the primer pairs used for the real-time PCR experiments

Subunit	Forward	Reverse
TNF-α	TCATTCCTGCTCGTGGCGGG	CGGCTGACGGTGTGGGTGAG
IL-10	CAAGGCAGTGGAGCAGGTGAAG	ACAAACGAGGTTTTCCAAGGAGTTG
iNOS	TCCCAAGTACGAGTGGTTCCA	GGCAGCGCATACCACTTCA
GAD65	TCTTTTCTCCTGGTGGTGCC	CCCCAAGCAGCATCCACAT
β-Actin	CGGAACCGCTCATTGCC	ACCCCACTGTGCCCATCTA

2.5. Immunoblot

Protein pellets obtained using the TripureTM isolation reagent, were resuspended in 4% SDS and 8 M urea in 40 mM Tris–HCl. The total recovery and integrity of these fractions were determined by Lowry et al. (1951) and SDS–polyacrylamide gel electrophoresis. Immunoblots blots were done as described elsewhere (Ruano et al., 2006). Briefly, proteins from striatum, SN or cell culture samples were loaded on a 12% polyacrylamide gel for electrophoresis (SDS-PAGE, Bio-Rad, USA). Then, proteins were transferred to a nitrocellulose membrane (Hybond-C Extra, Amersham, Sweden). After block, membranes were incubated overnight at 4 °C, with the rabbit polyclonal antibody anti-iNOS (BD Bioscience, USA, 1/1000) or the mouse monoclonal antibody against β -actin (Sigma-Aldrich) at a dilution of 1 / 10000, or the monoclonal antibody against TNF- α (Santa Cruz Biotechnology, inc. USA) at a dilution of 1 / 1000. Then, membranes were incubated with the appropriate secondary antibody (Dako, Denmark) (anti-rabbit or anti-mouse horseradish–peroxidase-conjugated, 1/6000). The blots were developed using the ECL-plus detection method (Amersham).

2.6. Enzyme-linked immunoabsorbent assay (ELISA)

To assay IL-10 by quantitative ELISA, LPS stimulation was performed in serum-free cultures. Aliquots of supernatants were collected at different times after LPS stimulation (6, 9 and 24 hours) and assays were developed according to manufacturers kit (mouse IL-10 Platinum ELISA; eBioscience[®]). Data are expressed as the mean of cytokine concentration (pg) per numbers of cells in the well, from three different experiments.

2.7. Statistical analysis

Data were expressed individually, as mean $\pm$ SD., or as percentage with respect to control. Data comparison were done between LPS-treated cells and control cells at each time point and for each cell density studied, as well as between LPS-injected and saline-injected animals, or between groups. Data comparison was analyzed by one-way ANOVA followed by Bonferroni post hoc multiple comparisons test. The significance was set at 95% of confidence.

3. Results

3.1. Microglial cell density determines the LPS-induction of pro- and anti-inflammatory factors

We first investigated whether microglial cell density could influence the pro-/anti-inflammatory balance produced following LPS stimulation. For that we evaluated the expression (mRNA and protein) of the proinflammatory factors TNF- α and iNOS, as well as the anti-inflammatory cytokine IL-10, in murine N13 microglia stimulated with LPS, at three different cell densities: 0.25, 0.5 and 1×10^6 cells/well. Both, cell density and LPS concentration were previously established in pilot experiments (data not shown). As shown in Fig. 1A, TNF- α mRNA was significantly up-regulated from 30 min to 3 h following LPS treatment. Importantly, level of induction was directly related to cell density. Significant differences were observed between cell densities at 30 min, 1h and 3h following LPS stimulation (Bonferroni $P < 0.05$). Expression of TNF- α was also evaluated in protein samples. Accordingly to mRNA expression, protein values showed a positive correlation with cell density that was evident at 6 hours postinjection (Fig. 2A). It is worth to note that immunoblots of supernatant samples revealed a band of TNF- α at 17 kDa corresponding to the secreted TNF- α , meanwhile in samples of proteins isolated from cells, TNF- α antibody revealed a band at 26 kDa corresponding to

membrane-bound TNF- α . Released TNF- α was only evident at the highest density (1.0×10^6 cells/well) and at the later time studied (24 hours; lower panel in Fig. 2A).

LPS stimulation also up-regulated the mRNA expression of iNOS in N13 microglia (Fig. 1B). The induction was observed later than for TNF- α , but it was also directly related to cell density, reaching the highest expression in cultures with high N13 density. Also, immunoblots revealed that expression of iNOS was highly dependent on the cell density, showing a positive correlation with microglia density at the three times studied (Fig. 2B). Although at 6 hours poststimulation the increase was not observed at low density culture, it was already evident in cultures with medium and high densities of cells. Significant differences were observed between cell densities at 3h, 6h and 9h following LPS stimulation for mRNA and at 6h, 9h, and 24 h poststimulation for protein values (Bonferroni $P < 0.05$).

The mRNA expression of the anti-inflammatory cytokine IL-10 was also upregulated after LPS stimulation. Interestingly, the level of induction was inversely related to cell density. A significant increase (Fig. 1C; Bonferroni $P < 0.05$) in IL-10 mRNA was observed from 1h to 6h in cultures with low or medium density. However, in cell cultures with high cellular density LPS stimulation produced a significant down-regulation of IL-10 mRNA expression.

Release of IL-10 was assayed in supernatants by ELISA. Data revealed that protein values strongly correlated with the mRNA results. So, at 24 hours after stimulation with LPS, IL-10 reached maximum values that were inversely correlated to cell density. Significant difference between groups were observed at 9 h and 24 h after LPS treatment (Fig. 2C; Bonferroni $P < 0.05$).

These *in vitro* data show evidence supporting that microglial cell density influences the balance between anti- and pro-inflammatory cytokines. In this sense brain areas with high density of microglial cells could be more prone to inflammation.

3.2. TNF- α , iNOS and IL-10 are differentially up-regulated in SN and striatum after LPS injection

We next searched for in vivo regional differences between induction of pro- and anti-inflammatory factors following LPS injection, similarly as observed in cell cultures. As shown in Fig.3A real-time PCR analysis demonstrated that LPS induced a robust increase in the mRNA expression of TNF- α at the earliest time points studied. TNF- α mRNA was significantly increased from 3 to 6 hours in both, SN and striatum, peaking at 3 h in striatum and later in SN (57 and 27 folds in striatum and 81 and 61 folds in SN upon saline-injected level, respectively; Bonferroni $P<0.05$). The mRNA expression of iNOS was also up-regulated by LPS injection (Fig 3B). A significant increase was first observed in SN (from 3 to 24 hours: Bonferroni $P<0.05$) and later in striatum (from 6 to 24 hours: Bonferroni $P<0.05$). Importantly, the magnitude of iNOS up-regulation was higher than that of TNF- α , and contrary to TNF- α , significant increases were observed in SN with respect to striatum (Bonferroni $P<0.05$).

We also assayed iNOS expression by Western blot. As shown in Fig. 3C, iNOS protein was induced in both areas from 14 to 24 hours after LPS-injection. It should be noted that at 24 hours postinjection the induction of protein was clearly more intense in SN than in CD, a data that correlates with the iNOS mRNA.

Finally, we also analyzed the expression of the cytokine IL-10, an anti-inflammatory modulator of glial response that keeps a balance between pro- and anti-inflammatory factors in the CNS (Sawada et al., 1999). As shown in Fig. 3D, IL-10 mRNA expression was significantly up-regulated following LPS-injection in SN from 3 to 6 hours post-injection (Bonferroni $P<0.05$). However, in striatum the increase lasted until 24 hours (Bonferroni $P<0.05$).

In summary, data revealed that LPS injection induces the mRNA up-regulation of both, pro- and anti-inflammatory factors in SN and striatum. However, iNOS expression was more persistent in SN whereas IL-10 expression was more sustained in striatum.

3.3. LPS injection down-regulates GAD65 mRNA expression in SN but not in striatum

Based on previous data, we next analyzed whether LPS injection could affect differentially the expression of GAD65, a neuronal marker common in SN and striatum. Saline injection did not modify basal level of GAD65 mRNA either in striatum or SN (data not shown). However, LPS-injection differentially affected GAD65 mRNA levels in striatum and SN. As shown in Figure 4A, LPS injection in SN decreased GAD65 mRNA expression as early as 14 hours ($53.12 \pm 10.56\%$ of saline value; Bonferroni; $P < 0.05$) and still lasted at 7 days ($22.39 \pm 2\%$ of saline value; Bonferroni; $P < 0.05$). However, in striatum (Figure 4B) LPS injection did not modify GAD65 mRNA expression, or even significantly increased it ($233.84 \pm 28.58\%$ upon saline value for 7 days, Bonferroni; $P < 0.05$).

S-methylisothiourea and SB203580 attenuated GAD65 mRNA decreases in SN.

We have previously reported that both drugs, S-methylisothiourea and SB203580, block the LPS-induction of iNOS, caspase-11 and proinflammatory cytokines, and partially attenuated dopaminergic damage after LPS injection in SN (Ruano et al., 2006). So, we evaluate the role of iNOS and p38 MAPK in the LPS-induced effect on the GAD65 mRNA in SN. As shown in figure 4C, seven days after LPS-injection co-administration of 3 nmol of S-methylisothiourea partially reversed the decrease induced by LPS in GAD65 mRNA expression (16% of recovery respect to LPS injected animals; Bonferroni; $P < 0.05$; Fig. 1C). Similarly, co-injection of 2.5 nmol of SB203580 with LPS also exerted an attenuating effect (30% of recovery; $P < 0.05$; Fig 4C).

The intranigral injection of S-methylisothiourea or SB203580 had no effect with respect to saline injection on GAD65 mRNA levels (data not shown).

These results corroborate previous studies about region-specific differential susceptibility to LPS and again reveal SN as an area highly susceptible to neurotoxicity exerted by inflammatory response.

4. Discussion

The present study demonstrates that, in vitro, inflammatory response is highly influenced by microglial cell density. High density cultures determined a pronounced proinflammatory (TNF- α , NO) response whereas low density cultures displayed a higher anti-inflammatory response (IL-10). These data support the hypothesis that differences in susceptibility to inflammatory neurodegenerative effects could be related to differences in the microglial cell density within specific brain regions.

Our results show that the induction of proinflammatory mediators (TNF- α and iNOS) displayed a positive correlation with microglial density, meanwhile the expression of the anti-inflammatory cytokines IL-10 was related to lower cell densities. High density of cells promote cell to cell contact increasing in turn the activation of the cells. Indeed, accumulating evidence suggests that not only soluble TNF- α , but also its precursor form, transmembrane TNF- α , is involved in the inflammatory response exerting its biological function in a cell-to-cell contact fashion (for review see, Horiouchi et al., 2010).

Intensity of the inflammatory response to LPS-challenge and the consequent neurotoxicity is highly region dependent (Herrera et al., 2000; Kim et al., 2000; Liu et al., 2000; Lu et al., 2000; Ji et al., 2008; Espinosa-Oliva et al., 2011). In this sense, SN is the brain region with the highest density of microglia cells (Lawson et al., 1990). Moreover, the blood brain barrier (BBB) in SN has been described to be more permissive to cell infiltration than in other areas (Ross et al., 1995; Tomas-Camardiel et al., 2004; Vizuite et al., 2000), allowing an intense recruitment of mononuclear cells through the blood-vessels after LPS injection (Herrera et al., 2004). After infiltration, monocytes differentiate into resident microglia (Djukic et al., 2006; Simard and Rivest, 2004).

Kim et al. (2000) found that LPS induced neurodegeneration in SN but not in cortex or hippocampus, whereas the capacity of microglia to produce cytokines in response to LPS was equal, regardless of whether microglial cells were derived from hippocampus, cortex or

mesencephalon, thus supporting that difference in the number of microglia cells within specific brain regions contribute to specific susceptibility. Our in vivo results demonstrate that upregulation of proinflammatory factors, particularly iNOS, were more intense in SN than striatum (see also Ruano et al., 2006). By contrast, in striatum the expression of the anti-inflammatory IL-10 was more sustained. In this sense, in vivo data match satisfactorily well with in vitro experiments. Thus, we could speculate that in the mesencephalic scenery with a higher density of microglia and a more permeable BBB, a proinflammatory agents (like LPS) could trigger an inflammatory response leading to an over-activation of microglia with overproduction of inflammatory mediators that could produce an imbalance of pro-/anti-inflammatory factors. Importantly, we have previously demonstrate that LPS-induced neuroinflammation produced a decrease of TH (mRNA and protein) in SN (Ruano et al., 2006), but not of GAD 65 mRNA in rat hippocampus (Gavilan et al., 2007). Here we show that GAD 65 mRNA was also decreased in SN following LPS injection but not in rat striatum. Similarly, thrombin injection into the SN induced microglial activation along with a significant loss of TH-immunopositive cells in SN compacta and of GAD-immunopositive cells (GABAergic neurons) in the SN reticulata (Choi et al., 2003). All these data are supporting SN as one of the most reactive brain region to neuroinflammation (Kim et al 2000). Among the factors involved in the higher susceptibility of SN to LPS, both higher density of microglial cells and BBB permeability could be considered.

Co-injection of LPS with the iNOS inhibitor S-methylisothiourea, or with the p38 MAP kinase inhibitor SB203580, partially reversed the LPS-dependent loss of GAD 65 mRNA. Importantly, similar treatment has also shown to rescue dopaminergic neurons after LPS injection in SN, at the same time that attenuated microglial activation and decreased pro-inflammatory cytokines, iNOS and caspase-11 expression (Ruano et al., 2006). Thus, our data support that iNOS seems to play a prominent role in mediating the potential death or loss of

functionality of GABAergic and dopaminergic neurons in SN. However, we cannot discard that other factors could be involved.

Our findings also show regional differences in LPS-induced mRNA up-regulation of the anti-inflammatory cytokine IL-10. IL-10 is an immunosuppressive cytokine produced by microglia upon stimulation with LPS and/or IFN- γ (Mizuno et al., 1994; Williams et al., 1996; Lee et al., 2002; Seo et al., 2004). Arimoto et al. (2007) reported that infusion of IL-10 after LPS injection in SN reduced LPS-induced loss of dopaminergic neurons at the time that downregulated microglial activation. So IL-10 could exert a neuroprotective role through the inhibition of microglia activation (Lodge et al., 1996; Hu et al., 1999; Ledebor et al., 2000; Molina-Holgado et al., 2001b; Sawada et al., 1999; Kremlev and Palmer, 2005). According to these results, one potential explanation about difference in inflammatory factors activation in the two areas studied here could be the long-term induction of anti-inflammatory cytokines in striatum.

In summary, we show evidence supporting that cellular density influences the pro- or anti-inflammatory response to LPS, which could have physiological relevance in brain regions with high microglial cell density such as SN. Consequently further studies remains to establish which specific factors contribute to disturb the pro-/anti-inflammatory balance in areas where inflammatory stimuli induce neurodegeneration. Actually a precise regulation of the amplitude of the cytokine signalling pathways is essential to keep inflammatory response controlled and well-balanced so that it exerts protection against tissue insults without induce damage.

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Figure legends

Figure 1. Cell density differentially influenced the induction of mRNAs of proinflammatory and antiinflammatory factors after N13 microglia stimulation with LPS.

mRNA expression of TNF- α , iNOS and IL-10 after LPS stimulation in N13 microglia was measured by RT-real time PCR. Assays were performed at three different cell densities: 0.25, 0.5 and 1×10^6 cells/well. (A) TNF- α mRNA was significantly up-regulated after LPS treatment and the induction was directly related to cell density, with higher expression in cultures with higher N13 density. (B) LPS stimulation also up-regulated the mRNA expression of iNOS in N13 microglia and the induction was also directly related to cell density. (C) Up-regulation of mRNA expression of the anti-inflammatory cytokine IL-10 after LPS treatment was inversely related to cell density.

Data are expressed as mean $\pm$ S.D of three independent experiments. * $p < 0.05$, significant differences compared to control culture. **a** $p < 0.05$, significant differences between the three densities. **b** $p < 0.05$, significant differences between low and medium cell density cultures (0.25 and 0.5×10^6 cells/plates) and cultures with high density (1.0×10^6 cells/well).

Figure 2. The kinetics of expression or release of proteins also revealed the influence of cell density.

(A) Upper panel is showing quantification of TNF- α as well as a representative immunoblot experiment in which TNF- α protein (membran-bound, 26 kDa) is detected in N13 cells. TNF- α expression was stimulated by LPS, showing a positive correlation with microglia density mainly at 6 hours poststimulation. Lower panel shows immunoblot of supernatant samples. Released TNF- α (17 kDa) was only evident at the highest density (1.0×10^6 cells/well) and at the later time studied (24h). (B) Expression of iNOS protein shows also a positive correlation with microglia density at any time studied. Although 6 hours poststimulation the increase was not observed at low density culture, it was already evident in cultures with medium and high

densities of cells. The lower panel is showing a representative immunoblot in which iNOS is detected. (C) Release of the antiinflammatory IL-10 was assayed in supernatants by ELISA. After LPS stimulation, release of IL-10 was upregulated at the three cell densities studied reaching maximum values at 24 hours after LPS-stimulation. Conversely to proinflammatory proteins, release of IL-10 was inversely related to cell density. Data are expressed as the mean of cytokine concentration (pg) per numbers of cells in the well, from three different experiments.

In A and B data are expressed as percentage of difference respect to control values. One control well was assayed for each cell density at any time point studied. The data are the mean $\pm$ S.D of three independent experiments. * $p<0.05$, significant differences between the three densities. **a** $p<0.05$, significant difference between low and medium cell density cultures (0.25 and 0.5×10^6 cells/plates) and cultures with high density (1.0×10^6 cells/well).

Figure 3 . Temporal profile of TNF- α , iNOS and IL-10 after intracerebral injection of LPS.

RT-real time PCR analysis of mRNAs of TNF- α , iNOS and IL-10 in SN and striatum of LPS injected rats. Assays were done at different times after LPS injection and for each time point mRNAs of at least three different animals were pooled and measures were done in triplicate. (A) TNF- α mRNA was up-regulated after LPS injection as early as 3 hours postinjection, and reached control values at 14 days postinjection. Although increases of mRNA were higher in SN, statistical differences were not found between SN and striatum. (B) Shown is the temporal profile of iNOS mRNA expression. The highest expression of this molecule was observed at 6-14 hours postinjection and reached control values by 3 days postinjection in SN and striatum. Significant difference of iNOS mRNA increase were found when compared SN and striatum. (C) Representative Western blots of iNOS expression. LPS injection clearly induced the expression of iNOS protein at 14-24 hours postinjection in both areas but the amount of protein observed in SN at 14 hours was largely bigger than in CD. iNOS protein was barely detected at 72 hours

postinjection in both areas. (D) LPS induced expression of IL-10 mRNA in both areas but the increase was more intense and sustained in striatum than in SN. SN: substantia nigra; CD: striatum.

Data are expressed in A, B and D, as mean $\pm$ S.D. For each time point at least three different animals were pooled and mRNA measures were done by triplicate. Saline injection did not affect either TNF- α mRNAs, iNOS mRNA or IL-10 mRNA expression at any time. * $p<0.05$, significant differences compared to saline animals. **a** $p<0.05$, significant differences when compared SN and striatum.

Figure 4- Temporal changes in GAD65 mRNA after LPS-injection

mRNA coding for GAD65 protein was analyzed by Real-time PCR using as template cDNA from rat striatum or SN injected with LPS (4 μ g or 2 μ g respectively). Saline injection did not affect mRNAs expression at any times. (A) Expression of mRNA GAD65 in SN decreased after LPS injection from 14 hours post-injection and still lasted at 7 days. (B) Conversely to SN, LPS injection did not modify expression of GAD65 mRNA in striatum, except for a slight increase at 7 days post-injection. (C) At seven days, co-administration of LPS with S-methylisothiourrea (3 nmol), a specific iNOS inhibitor, or with SB203580 (2.5 nmol), a p38 MAP kinase inhibitor, both reverted the LPS induced decrease of GAD65 mRNA in SN.

Data are expressed as mean $\pm$ S.D (A and B). For each time point at least three different animals were pooled and mRNA measures were done by triplicate. In C, data are expressed as the ratio in relation to saline injected animals. *: Bonferroni $P<0.05$, with respect to saline injected.