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INTRAVITREOUS INTERLEUKIN-2 TREATMENT AND INFLAMMATION MODULATES GLIAL CELLS ACTIVATION AND UNCROSSED RETINOTECTAL DEVELOPMENT

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Abstract—Interleukin-2 (IL-2) plays regulatory functions both in immune and nervous system. However, in the visual system, little is known about the cellular types which respond to IL-2 and its effects. Herein, we investigated the influence of IL-2 in the development of central visual pathways. Lister Hooded rats were submitted to multiple (at postnatal days [PND]7/10/13) or single (at PND10) intravitreous injections of phosphate-buffered saline (PBS) (vehicle), zymosan, or IL-2. IL-2 receptor α subunit was detected in the whole postnatal retina. Chronic treatment with either PBS or IL-2 increases retinal glial fibrillary acidic protein (GFAP) expression, induces intravitreous inflammation revealed by the presence of macrophages, and results in a slight rearrangement of retinotectal axons. Acute zymosan treatment disrupts retinotectal axons distribution, confirming the influence of inflammation on retinotectal pathway reordering. Furthermore, acute IL-2 treatment increases GFAP expression in the retina without inflammation and produces a robust sprouting of the intact uncrossed retinotectal pathway. No difference was observed in glial cells activity in superior colliculus. Taken together, these data suggest that inflammation and interleukin-2 modulate retinal ganglion cells development and the distribution of their axons within central targets. **© 2011 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: IL-2, critical period, development, superior colliculus, plasticity, glial cells.

Interleukin-2 (IL-2) is a pro-inflammatory cytokine that plays multiple regulatory functions not only related to the immune but also to the nervous system. This cytokine exerts its effects through binding to membrane heterotrimeric receptors composed by α, β, and γ subunits (Kono et al., 1993). Both IL-2 and IL-2R are widely distributed in the CNS (Araújo et al., 1989) and at the cellular level, they are potentially synthesized by neurons and glial cells (Petitto and Huang, 2001; Araujo and Cotman, 1995; Gabryel et al., 2004). Several studies have pointed out the role of IL-2 in different aspects of brain development such as neuronal survival, proliferation, differentiation, and, of particular interest, neurite outgrowth and synaptic plasticity (Sholl-Franco et al., 2001a,b; Saneto et al., 1986; Otero and Merrill, 1997; Shen et al., 2010; Tancredi et al., 1990). Consistently with its role in plasticity, IL-2 treatment of hippocampal slices before and after tetanic stimulation disrupts both the induction and maintenance of long-term potentiation (LTP) (Tancredi et al., 1990). Hippocampal neurons exhibit increased number and length of neurites after IL-2 administration (Shen et al., 2010). Alternatively, IL-2 has also been associated with neurodegeneration and other neuropathological conditions such as optic neuritis, diabetic retinopathy, and endogenous uveitis, which results in ocular complications of 76% of pediatric patients (Zandian et al., 2009; Ho et al., 2011; Wang et al., 2009; McAllister et al., 1995; Johnsen-Soriano et al., 2010; Barton et al., 1993, 1995; Touchard et al., 2010; Sijssens et al., 2007).

Inflammation has been related to neuroplasticity (Rosi, 2011; Leon et al., 2000). It has been reported that intravitreous inflammation induced by zymosan or lens injury triggers axonal outgrowth after optic nerve crush (Lorber et al., 2008; Yin et al., 2003). Moreover, neuroinflammation has been associated with deficits in synaptic plasticity in different neurological diseases (Centonze et al., 2009; Lyons et al., 2007). Indeed, it recently demonstrated a reduction in hippocampal LTP and long-term depression in a chronic brain inflammation model (Min et al., 2009a,b). Additionally, glial fibrillary acidic protein (GFAP) is upregulated in response to neuroinflammation (Walsh et al., 2011; Deren et al., 2010), indicating that astrocytes activation could be related to the adjustment of synaptic efficacy, through neuroactive factors release.

Retinotectal pathways have been used as a model to understand the mechanisms underlying the development of central connections and also structural plasticity that follows injury of the visual system (Trindade et al., 2011; Mendonça et al., 2010; Penedo et al., 2009; Gomes et al., 2009; Oliveira-Silva et al., 2007; Campello-Costa et al., 2000, 2006; Serfaty et al., 2005; Mclaughlin et al., 2003). The development of retinotectal topography takes place within a critical period that comprises the first two/three postnatal weeks (Serfaty et al., 2005; Serfaty and Linden, 1994; Simon and O'Leary, 1992). The adult pattern of connections emerges from a diffuse innervation, observed

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Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein; HRP, horseradish peroxidase; IL-2, interleukin-2; LTD, long-term depression; LTP, long-term potentiation; PBS, phosphate-buffered saline; PND, postnatal day; SC, superior colliculus; TMB, 3,3,5,5′-tetramethylbenzidine.

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in newborns, and is characterized by small clusters of uncrossed retinotectal projection at the ventral border of the superior colliculus (SC) overlapped by contralateral terminals (Serfaty and Linden, 1994).

The aim of this work was to investigate the influence of chronic or acute intravitreous IL-2 treatments upon visual system development. We analyzed the expression of IL-2 receptor in retinal tissue and the effect of IL-2 on glial activation both in the retina and superior colliculus. We also examined the distribution of uncrossed retinotectal axons in response to both treatments. We showed that inflammation and IL-2 treatment influence the sprouting of distant retinotectal terminals.

EXPERIMENTAL PROCEDURES

All animal use procedures were in strict accordance with The National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Local Animal Care Committee (CEUA-UFF) under protocol 130/09. Experiments were designed to minimize the number of animals used, and care was taken to avoid pain and suffering.

Materials

Horseradish peroxidase (HRP) type VI, 3,3,5,5′-tetramethylbenzidine (TMB), and 4′,6-diamidino-2-phenylindole (DAPI) were obtained from Sigma Chemical Co. (St. Louis, USA). Recombinant human IL-2 (specific activity, 2.0×10^8 U/mg) was from Peprotech (USA). Anti-IL2Rα and anti-α-actin primary antibodies, as well as the anti-rabbit secondary antibody were obtained from Santa Cruz Biotechnology (USA). Anti-GFAP primary antibody and anti-mouse secondary antibody were obtained from Cell Signaling (USA). Anti-ED1 CD11b (mouse) primary antibody was obtained from Chemicon (USA). Secondary fluorescent antibodies were obtained from Cell Signaling (USA). Biotinylated anti-mouse IgG, rat pre-adsorbed and ABC Elite complex were obtained from Vector laboratories (USA). ECL plus chemiluminescent kit and hyperfilm PVDF (polyvinylidene difluoride membranes) were obtained from Amersham Biosciences (Piscataway, NJ, USA). All other reagents were of analytical grade.

Experimental groups and intravitreous injection

Lister Hooded rats were divided in two groups. One group received chronic intravitreous treatment with IL-2 (50 U/0.5 μl) in the right eye at postnatal days (PND) 7/10/13. The other group received a single intravitreous injection of zymosan (60.5 μg/0.5 μl) or IL-2 (156 U/0.5 μl) in the right eye at PND10. Control-matched groups received phosphate-buffered saline (PBS) injections as vehicle. Briefly, animals were anesthetized with ketamine (100 mg/kg) and xilazine (5 mg/kg) and submitted to PBS, IL-2, or zymosan injection into the vitreous chamber through a small hole made at the dorsal limbus. Animals presenting lens lesions were discarded. Tissues obtained from both groups were processed for western blot, immunohistochemistry, and histochemistry.

Immunohistochemistry

At PND14, all animals were deeply anesthetized with an overdose of anesthetic and sacrificed by transcardially perfusion with saline (0.9% NaCl) followed by 4% paraformaldehyde in 0.1-M phosphate buffer, pH 7.2, for 20 min. Eyes and brains were removed and cryoprotected overnight in a series of sucrose solutions (15, 20, and 30%). Eyeballs were oriented in aluminum chambers filled with OCT (optimum cutting temperature) before freezing. Ten-μm-thick transverse sections of retinal and brain tissues were cut in a cryostat and mounted on gelatin-coated slides. A group of retinal sections were stained with Cresyl Violet (Nissl staining). Alternatively, sections were maintained at −20 °C until processing for immunohistochemistry. The orientation procedure ensured that sectioning through the tissues was orthogonal to the surface of the retina. Brain sections were taken through the coronal plane. For GFAP, IL-2 receptor α subunit (IL-2Rα), and ED1 CD11b immunodetection, tissues were pre-treated with Triton X-100, then incubated with a blocking solution (1% bovine serum albumin, BSA, in PBS, 0.1 M, pH 7.4) for 30 min. Subsequently, slides were incubated overnight at 4 °C with mouse anti-GFAP polyclonal antibodies (1:100), mouse anti-ED1 CD11b (1:500), or mouse polyclonal anti-IL-2Rα (1:200) diluted in blocking solution. Anti-GFAP-treated and anti-ED1 CD11b-treated retinal slices were incubated with biotinylated anti-mouse IgG, rat pre-adsorbed (1:200) for 90 min at room temperature, followed by ABC Elite complex (1:50) for further 90 min. Sections were then incubated (5–10 min) with 0.1-M Tris–HCl, pH 7.4, containing 0.04% of 3.3′-diaminobenzidine (DAB) and 0.02% H_2O_2. Afterward, sections were washed with PBS, dehydrated in alcohol, cleared in xylol, and coversliped with Entellan (Merck). Anti-IL-2Rα-treated retinal slices and anti-GFAP-treated brain slices were reacted with Alexa 555 (1:200; red) fluorescent secondary antibody and Alexa Fluor 488 (1:500, green), respectively, diluted in PBS-BSA 1%, for 2 h, at room temperature, coversliped using fluorescence medium (4% n-propylgalate in phosphate buffer/80% glycerol), and conserved at 4 °C, protected from light. Control sections were processed in the absence of primary antibody. Sections were analyzed in a Zeiss Axioskope microscope under bright field and fluorescence optics.

Western blot

The analysis of GFAP and α subunit of IL-2 receptor expression were carried out in total protein extracts prepared from retinal tissue and superior colliculus of PND14 animals. For analysis of retinal IL-2Rα, total protein content was extracted from retinal tissue ranging from PND0 to PND60. Animals were deeply anesthetized with an overdose of anesthetic and decapitated. Retinae and superior colliculi were dissected and homogenized in ice-cold lysis buffer (20-mM Tris–HCl, pH 7.4, 10-mM $MgCl_2$, 0.6-mM $CaCl_2$, 0.5-mM EDTA, 0.05% Triton X-100, 1-mM PMSF, DTT) with protease inhibitors. Protein concentration was measured using the method of Bradford (Bradford, 1976). Samples (40-μg total protein/lane) were resolved in SDS/PAGE (10% with a 4% concentrating gel) and electro-transferred to PVDF membrane. Transfer was verified by staining the membrane with Ponceau Red, which indicated equal loading among the lanes. Membrane was incubated in blocking buffer (5% defatted milk in Tris-buffered saline, pH 7.6, containing 0.1% Tween 20—TBS-T) for 2 h at room temperature. Incubation with anti-GFAP antibody (1:2500) or anti-IL-2Rα (1:500) was carried out overnight, at 4 °C. After washing with TBS-T, membranes were incubated with anti-mouse secondary antibody (1:5000) in TBS-T during 1 h, at room temperature. Following washing with TBS-T and TBS, membranes were incubated with ECL plus chemiluminescent kit during 5 min. To assure equal loading of the samples, after capturing the bands on hyperfilm, membranes were re-probed and tested for alpha-actin immunoreactivity. Briefly, after the first exposure, membranes were washed with TBS and incubated with 0.2-M glycine, pH 2.2, for 30 min at orbital rotation. Membranes were then blocked as described earlier and incubated with rabbit anti-alpha-actin antibody (1:2000) overnight. Then, membranes were washed and incubated with anti-rabbit secondary antibody (1:3000) and developed as described previously. The densitometry analysis was performed using Gelplot analysis macro in ScionImage Software, Version 4.03 (Scion Corporation, MD, USA).

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