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Research Report

Effects of electroacupuncture on retinal nerve growth factor and brain-derived neurotrophic factor expression in a rat model of retinitis pigmentosa

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ABSTRACT

The aim of this study was to investigate the effect of electroacupuncture (EA) on the progression of retinal degeneration in rats affected by inherited retinitis pigmentosa (IRP) and to correlate this event with the retinal expression of neurotrophins. Thirty-day-old Royal College of Surgeons (RCS) rats were exposed to 25-min-long daily sessions of low-frequency EA for 11 consecutive days. Control-untreated and EA-treated rats were sacrificed 1 h after the last EA session, and their retina removed for biochemical, molecular, and immunohistochemical analyses. Our data revealed that daily sessions of low-frequency EA for 11 days to RCS rats during a critical developmental stage of retinal cell degeneration cause an increase of retinal nerve growth factor (NGF) and NGF high-affinity receptor (TrkA) expression; and increase of outer nuclear layer (ONL) thickness; and enhanced vascularization. These findings suggest the possible beneficial effects of EA treatment in the development of IRP-like retinal degeneration of RCS rats and that the mechanism through which EA might exerts its action on the regulation of NGF and brain-derived neurotrophic factor (BDNF) and/or their receptors in retinal cells.

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Abbreviations:
NGF, nerve growth factor
BDNF, brain-derived neurotrophic factor
VEGF, vascular endothelial growth factor
RCS, Royal College of Surgeons
IRP, inherited retinitis pigmentosa
GC, ganglion cell
INL, inner nuclear layer
IPL, inner plexiform layer
ONL, outer nuclear layer
RPE, retinal pigment epithelium
CNS, central nervous system
EA, electroacupuncture
UT, untreated
PCO, polycystic ovary syndrome
TrkA, tyrosine-kinase A
TrkB, tyrosine-kinase B
SD, Sprague–Dawley

1. Introduction

Nerve growth factor (NGF) is one of the most extensively studied neurotrophic factors produced and released by numerous mammalian cells, such as neuronal, epithelial, endothelial, endocrine, and immune cells (Aloe et al., 2001; Levi-Montalcini, 1987; Sofroniew et al., 2001). NGF is produced by cells of the visual system, and NGF receptors are express by retinal pigment epithelium (RPE), Muller cells, retina ganglion cells (GC), and by photoreceptor (Amendola and Aloe, 2002; Amendola et al., 2003; Carmignoto et al., 1989; Lambiase et al., 1998; Lambiase et al., 2005; Siliprandi et al., 1993). It has been shown that the intraocular administration of NGF protects from retinal GC degeneration after optic nerve transection, ocular ischemia, or experimentally induced ocular damage, thus suggesting a functional role of NGF in the visual system (Carmignoto et al., 1989; Siliprandi et al., 1993). Moreover, ocular NGF administration is able to delay retinal degeneration in animal models of inherited retinitis pigmentosa (IRP), in CH-3 mouse strain (Lambiase and Aloe, 1996), and in Royal College of Surgeon rat (RCS). NGF retrobulbar injection, aimed at stimulating NGF-receptive sites in the retina, is considered an invasive approach because it can produce undesired side effects. Recent studies indicate that it is possible to stimulate endogenous release of NGF both in the peripheral (Stener-Victorin et al., 2000) and in central nervous system (CNS) (Stener-Victorin et al., 2003) by electroacupuncture (EA). EA is a therapeutic method based on the ancient Chinese treatment for illness, pain, or even addiction, which uses fine needles inserted in specific points of the body. Studies published in the last few years indicate that EA can affect neurological and non-neurological disorders. However, the possible biochemical and molecular mechanisms through which EA exerts its effect remain undefined. It has been suggested that low-frequency (1–4 Hz) EA with repetitive muscle contraction results in the activation of physiological processes similar to those resulting from physical exercise (Andersson and Lundeberg, 1995). There is also evidence indicating that EA stimulates the release of different neurotransmitters, both in the CNS and in the peripheral nervous system (PNS) (Bucinskaite et al., 1994; Jansen et al., 1989), and that it can modulates immunological responses and the release of cytokines (Gronlund et al., 2004; Smith et al., 2004). It has also been reported that EA can affect the concentration of NGF in a rat model of polycystic ovaries (PCO) (Stener-Victorin et al., 2000; Stener-Victorin et al., 2003; Yun et al., 2002), in the CNS (Liang et al., 2002), and in the visual system (Chan et al., 2005; Chu and Potter, 2002; Dabov et al., 1985; Smith et al., 2004; Uhrig et al., 2003; Ulett et al., 1997). These observations raised the question of whether EA can influences the presence of neurotrophins and their receptors in the retina of rats affected by IRP. To address this question, we exposed RCS rats to daily sessions of EA during a critical period of retinal degeneration, and the biochemical, structural, and molecular responses of NGF and BDNF in the retinal tissues were examined.

2. Results

2.1. *General observations*

Daily exposure of RCS rats to low-frequency EA at 2 Hz for 11 consecutive days does not affect recovery time from anesthesia and causes no changes in other behavioral manifestations, such as feeding behavior, social behavior with littermates, and body weight.

2.2. *Effect of EA on NGF and NGF receptors*

Fig. 1 shows the expression of NGF protein (Fig. 1A) and its mRNA (Fig. 1B) in the retina of RCS rats exposed to daily EA session for 11 consecutive days. The expression of NGF and NGF mRNA in the retina of EA-treated RCS rats is higher compared to that of age-matched RCS-untreated (UT) rats.

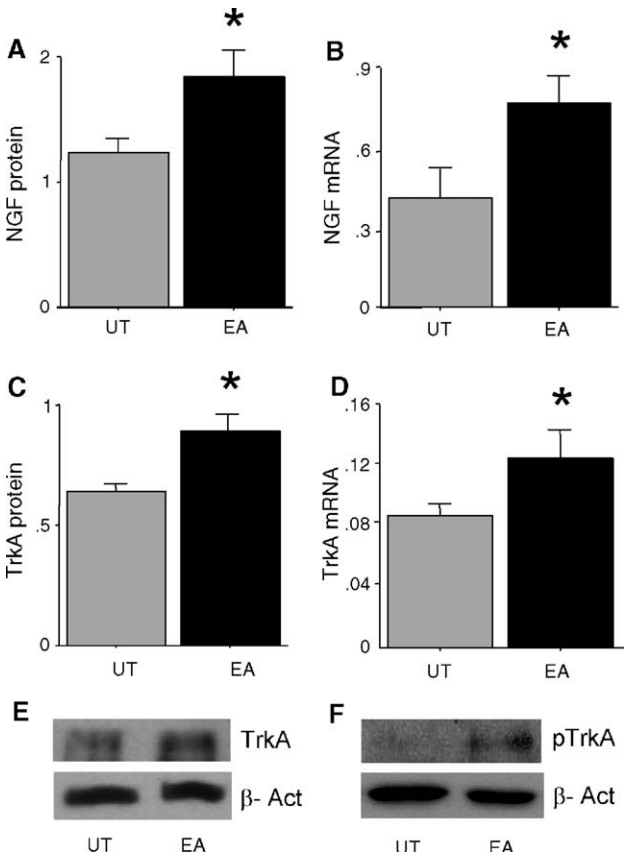

Fig. 1 – Presence of Nerve Growth Factor (NGF) protein (A), mRNA-NGF (B), TrkA protein (C and E), and its mRNA (D) in the retina of Royal College of Surgeons (RCS) rats exposed (EA) and non-exposed (UT) to electroacupuncture (EA) for 11 consecutive days. Note the enhanced expression of NGF and TrkA and their respective mRNAs in the retina of rats exposed to EA compared to controls. Note also that non-phosphorylated TrkA (E) and phosphorylated TrkA (F) are upregulated.

This difference is statistically significant ($P < 0.05$). No changes in the concentration of NGF protein or mRNA-NGF were found in the retina of Sprague–Dawley (SD) rat strain exposed to EA (data not shown).

The retina of EA-treated and -untreated (UT) rats were also used to determine the expression of high-affinity NGF receptors. As shown in Figs. 1C–E, both TrkA protein (Figs. 1C and E) and its mRNA (Fig. 1D) are more expressed in the retina of RCS rats exposed to EA compared to those of untreated rats ($P < 0.05$). EA also enhances the expression of the phosphorylated TrkA (pTrkA) (Fig. 1F), the activated form of TrkA, suggesting an active interrelationship between NGF/TrkA in the retina of EA-treated rats.

To explore the possible functional significance of these changes, we immunostained sections of control and EA-treated rats with TrkA antibodies. As illustrated in Fig. 2A, TrkA immunoreactivity is more present at 30 days of life, a time that coincides with weaning, and it decreases and almost disappears at 41 days of age. As reported in Figs. 2B–D, EA treatment for 11 days increase TrkA-positive cells in INL and GC.

2.3. *Effect of EA on retinal vascularization*

Because vascularization plays a pivotal role in tissue protection, the distribution of blood vessels in the retina of UT- and EA-treated RCS rats was also investigated. As shown in Fig. 3, EA induces a mild blood vessel hypertrophy (Fig. 3B) compared to UT rats (Fig. 3A). The number of blood vessels, evaluated as area occupied by vessels in each microscopic field, is higher in EA-treated rats when compared to the untreated group. The computer-based image analysis (Fig. 3C) revealed that this increase is statistically significant ($P < 0.05$). Moreover, the VEGF protein in the retina of EA-treated RCS rats is more expressed (D) than in UT RCS rats. EA treatment does not affect blood vessel distribution and VEGF expression in the normal retina of SD rats (not shown).

2.4. *Effect of EA on BDNF and BDNF receptors*

To further explore the effect of EA on the synthesis and release of neurotrophins in the retina of RCS rats, we also measured

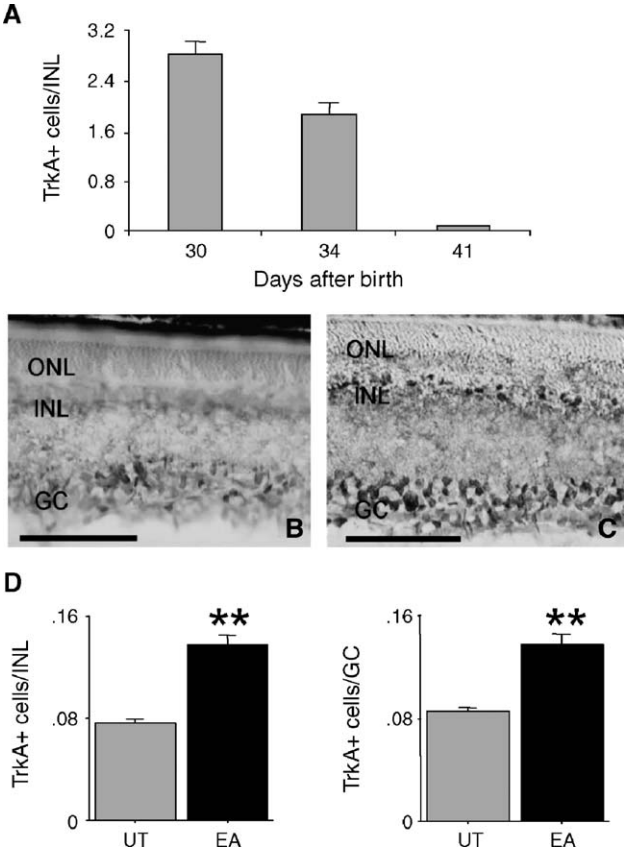

Fig. 2 – Expression of TrkA immunoreactivity (A) in cells of the Inner Nuclear Layer (INL) of Royal College of Surgeons (RCS) untreated (UT) retina at 30, 34, and 41 days of postnatal age. The study revealed that TrkA immunoreactivity in these cells is more expressed in early postnatal life and it is nearly absent at 7 weeks of age. Qualitative and quantitative analyses of TrkA immunoreactivity in the INL and the ganglion cells (GC) of UT and electroacupuncture (EA)-treated rats are shown in panels B, C, and D, respectively. Note the significant increase of TrkA positivity cells of EA-treated rats ($P < 0.01$). Scale bars = 100 μm.

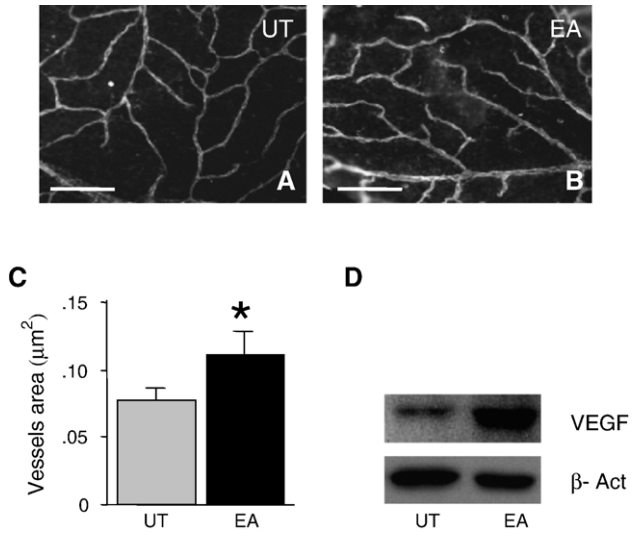

Fig. 3 – Distribution of blood vessels in the retina of untreated (UT) and electroacupuncture (EA)-treated rats showing that EA induces a mild blood vessels hypertrophy (B) compared to controls (A). The number of blood vessels (C), evaluated as field area occupied by vessels, is higher in the retina of EA-treated rats when compared to UT rats; this difference is statistical significance, $P < 0.05$; scale bars = 100 μm. Vascular endothelial growth factor (VEGF) protein expression (D) in the retina of EA-treated Royal College of Surgeons (RCS) rats is higher compared to those of UT rats.

the concentration of BDNF and the expression of its receptor, TrkB. As reported in Fig. 4, EA causes a significant increase in BDNF (Fig. 4A), TrkB proteins (Figs. 4C and E), and TrkB-mRNA (Fig. 4D) ($P < 0.05$), whereas BDNF-mRNA remains unchanged (Fig. 4B).

2.5. *Effect of EA on retinal structural configuration*

Fig. 5 compares the histological configuration of the ONL, INL, and GC in the retinal of wild type, UT RCS and EA-treated RCS age-matched rats, showing no changes in the GC layer, but a clear difference in the ONL and INL cell layers. Quantitative analysis of coded retinal sections indicated that the ONL and INL thickness increases significantly ($P < 0.01$ and $P < 0.05$, respectively) after exposure to EA.

3. Discussion

The present study examined the effect of daily exposure to EA for 11 consecutive days occurring in the retina of RCS rats affected by IRP. It was found that EA treatment starting at 30 days of postnatal life, which represents the beginning of a critical period of retinal cell degeneration in this rat strain (Strauss et al., 1998; Amendola and Aloe, 2002), induces (1) an increase of NGF and BDNF proteins; (2) a delay of retinal cell degeneration; (3) changes of the structural organization of cells localized in the INL and GC layers; and (4) up-regulation of VEGF, a factor that plays a functional role in blood vessels growth and differentiation. It was also found that EA enhances the expression of mRNA-TrkA in the retina and of TrkA immunoreactivity in GC and in the cells of the INL, suggesting that EA promotes not only the synthesis and the release of NGF in the retina but, most probably, also its functional activity. The fact that in UT rats the number of TrkA-positive cells in the retina decreases significantly and that exposure to EA either blocks or delays this trend is consistent with this hypothesis. The observation that EA exposure has no effect on NGF presence and on blood vessels distribution in the retina of SD rat strain suggests that EA can exerts its effect on degenerating, but not on normal retina.

The mechanisms through which EA promote these effects in the retina of RCS rats are at present not clear. Because previous studies indicate that the administration of NGF in mice (Lambiase and Aloe, 1996) and rats (Amendola and Aloe, 2002; Amendola et al., 2003; Lenzi et al., 2005) with IRP delays the progressive degeneration of retinal cells, it is possible that a mechanism through which EA mediate this action is by enhancing endogenous NGF synthesis and release.

This hypothesis is supported by recent studies showing that EA can exert its effect by promoting the synthesis and release of neurotrophic factors in the brain (Liang et al., 2002; Yun et al., 2002) and in the peripheral nervous system (Manni

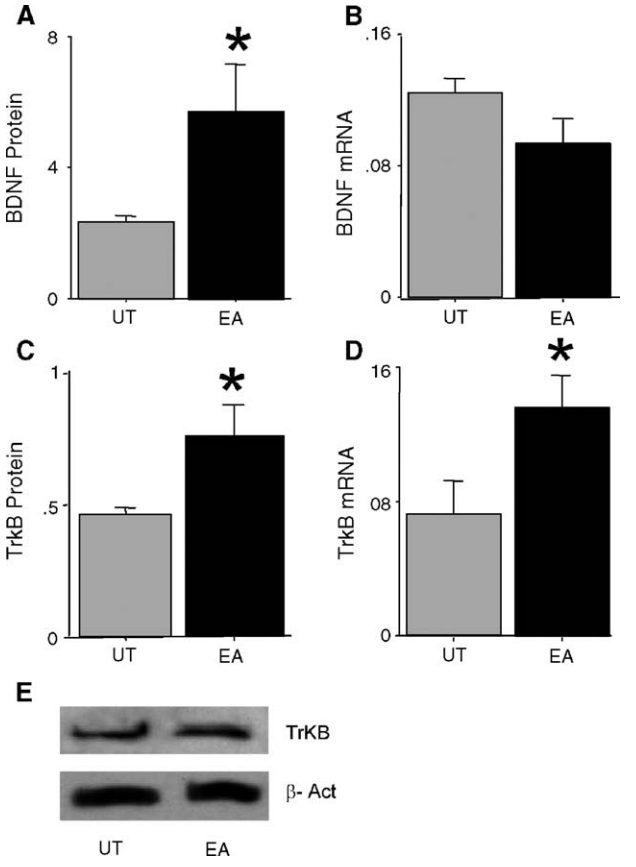

Fig. 4 – Presence of BDNF protein (A), mRNA-BDNF (B), TrkB protein (C and E), and its mRNA (D) in the retina of RCS rats exposed and of non-exposed (UT) to EA for 11 consecutive days. Note the enhanced presence of BDNF protein, but not of its mRNA, whereas TrkB protein and its mRNA are both more express after exposure to EA.

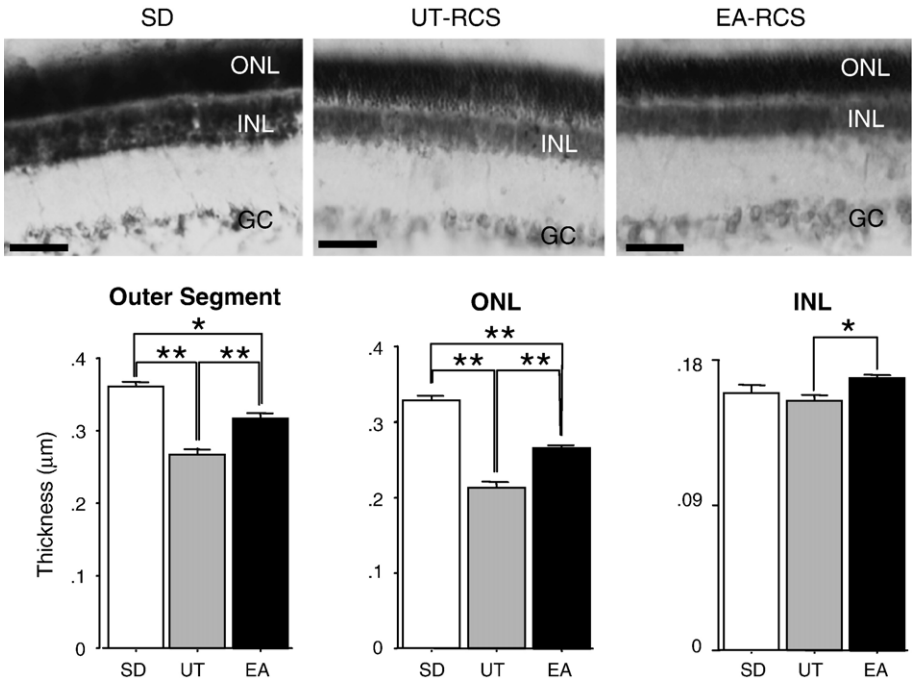

Fig. 5 – Histological preparation of the retina of healthy SD rats, RCS UT and EA-treated rats showing the structural configuration of the ONL, INL, and GC layer. Quantitative computerized analyses of serial comparable sections stained with toluidine blue indicate that the thickness of these structures increases following exposure to EA for 11 consecutive days. These differences are statistically significant. SD = Sprague–Dawley retina; UT = untreated RCS retina; EA = EA-treated RCS retina; *$P < 0.01$, **$P < 0.001$; scale bars = 50 μm.

et al., 2005b; Manni et al., 2005c; Stener-Victorin et al., 2000; Stener-Victorin et al., 2003; Stener-Victorin and Lindholm, 2004), and by stimulating the release of other endogenous biological mediators, such as peptides (Andersson and Lundeberg, 1995; Cho et al., 2004; Smith et al., 2004; Uhrig et al., 2003) and neurotransmitters (Yoon et al., 2004), or inducing beneficial effect in patients with keratoconjunctivitis sicca (Gronlund et al., 2004).

The present study also demonstrates that exposure of EA to RCS rats enhances the basal level of BDNF and the expression of its receptor TrkB in the retina. This observation indicates that EA can also stimulate the synthesis and release of other neurotrophins. Whether this latter is a direct effect of EA or an effect mediated by NGF up-regulation is not clear. Findings showing that retrobulbar administration of NGF increases the BDNF protein and its mRNA in the retina (Lenzi et al., 2005), and that transplanted Schwann cell lines transfected with BDNF construct sustain retinal structure and function in RCS rats (Lawrence et al., 2004) suggest that the increase of BDNF after EA treatment might be mediated by NGF up-regulation.

Because NGF has been shown to enhance blood vessels growth (Samii et al., 1999) and VEGF synthesis and release (Calzà et al., 2001; Emanueli et al., 2002; Manni et al., 2005a; Raychaudhuri et al., 2001), it might be also possible that the enhanced expression of retinal VEGF might also be linked to the local up-regulation of NGF. However, to assess the validity of this hypothesis, further studies with animal models affected by vascular deficits need to be done.

In summary, our findings indicate that EA treatment in rats with IRP, starting at the beginning of retinal degeneration, delays retinal cell loss and that one mechanism through which this process can take place might be the local up-regulation of NGF and BDNF. Whether prolonged EA treatment can further enhance the synthesis of NGF and BDNF in the retina of RCS rats and promote a longer lasting protection from retinal cell remains to be established.

4. Experimental procedures

4.1. *Animals*

For this study, we used Royal College of Surgeons (RCS) rats, a rat strain that developed inherited retinal degeneration. The genetic defect in RCS rats causes the inability of the retinal pigment epithelium (RPE) to phagocytose shed photoreceptor outer segments (Strauss et al., 1998). As previously reported, the retinal ONL cell degeneration begins early in postnatal life (20–30 days of postnatal life) and then this degeneration steadily proceeds reaching at about 70 days of life almost the complete loss of ONL layer (Strauss et al., 1998; Amendola and Aloe, 2002). For our study, we used 30-day-old RCS rats and a group of Sprague–Dawley (SD) rats strain of the same age to further explore the effect of EA on non-degenerating retina. Animals were maintained on a 12-h light/dark cycle and were provided with food and water ad libitum. For the housing care and experimental procedures, we followed the guidelines indicated by our intramural animal ethical committee, the Ethical Commission on animal experimentation of the CNR in conformity with National and International laws (EEC council

directive 86/609, OJ l 358, 1, December 12, 1987). Moreover, all efforts were made to minimize animal suffering and to reduce the number of animals used.

4.2. *Treatment*

From a western perspective, EA is a non-pharmacological method known to trigger a number of reactions at spinal level and, centrally, in the brain (Andersson and Lundeberg, 1995; Sato et al., 2002). Low-frequency EA (1–4 Hz), with intensity high enough to cause muscle twitches, probably excites low and high threshold mechanoreceptors (Noguchi et al., 1999), and particularly a group of receptors found in muscles, indicated as ergoreceptors (Kniffki et al., 1981; Kaufman et al., 1983) that are physiologically activated during muscle contractions. Based on these and other studies, it has been suggested that EA with repetitive muscle contraction results in the activation of physiological processes similar to those resulting from physical exercise (Andersson and Lundeberg, 1995). Our rats were exposed to 25-min daily session of EA stimulation for 11 consecutive days starting at day 30 after birth. During each treatment, rats were anaesthetized superficially with an intraperitoneal (i.p.) injection of a mixture of ketamine (50 mg/kg; Parke Davis, Warner Lambert Nordic AB, Sweden) and Rompun (20 mg/kg; Bayer, Bayer AG, Germany). The points chosen for stimulation were bilateral in the mm. biceps femoris and biceps brachii. These points were chosen to evoke the maximal supraspinal effects of the treatment (Stener-Victorin et al., 2000) and are not directly related to traditional Chinese acupoints. Indeed, such kind of stimulation has been proven effective in the activation of supraspinal pathways, centrally mediated regulation of sensory and autonomic systems and in the regulation of central nervous system neurotransmitters and neuromodulators (Stener-Victorin and Lindholm, 2004). The needles (Hegu; Hegu AB, Sweden) were inserted to depths of 0.5–0.8 cm and then attached bilaterally, via clip electrodes, to an electrical stimulator (CEFAR ACUS II, Cefar, Sweden). Each pair of electrode needles was inserted at the same point bilaterally. The points were electrically stimulated with a low burst frequency of 2 Hz; each pulse was a square electric wave and had duration of 180 μs, length of 0.1 s, and internal burst frequency of 80 Hz. The intensity (1.5–2 mA) was monitored, checking for local muscle contractions to reflect the activation of muscle–nerve afferents (A-delta fibers and possibly C fibers). The location and type of stimulation were the same in all rats. RCS-untreated (n = 10) and RCS EA-treated (n = 10) rats, as well as SD-untreated (n = 5) and SD EA-treated rats (n = 5), were sacrificed 1 h after the last session, with an overdose of anesthetic. Eye bulbs were enucleated and frozen in a –80 °C freezer, or postfixed with 4% paraformaldehyde dissolved in 0.1 M phosphate buffer, pH 7.4, for 24 h and then overnight in the same buffer containing 20% sucrose and 0.01% sodium azide.

4.3. *NGF and BDNF protein determination*

Quantification of NGF and BDNF was performed using NGF Emax Immunoassay System (Promega Italia, Italy) and BDNF Emax Immunoassay System (Promega Italia, Italy) following the manufacturer's instruction. NGF and BDNF concentrations in the retina were expressed as pg/g of tissue and all assays were performed in duplicate (Amendola et al., 2003; Tirassa et al., 2000; Weskamp and Otten, 1987).

4.4. *RT-PCR ELISA*

The expression of NGF-, BDNF-, TrkA-, and VEGF-mRNA was evaluated, on the retina of animals treated for 11 daily EA sessions, using the reverse transcriptase (RT)-PCR enzyme-linked immunosorbent assay (ELISA) protocol, exactly as previously described by Tirassa et al. (2000). Total mRNA was extracted from control (n = 5) and treated (n = 5) different retina tissues using RNAgent Total RNA Isolation System (Promega Italia, Italy). Complementary DNA (cDNA) was synthesized from 2 μg of total RNA using 200 U of M-MLV reverse transcriptase enzyme (Promega Italia, Italy) in 20 μl of total volume reaction containing 500 ng Oligo(dT)12–18 primer (Promega Italia, Italy), 30 U of RNAsin ribonuclease inhibitor (Promega Italia, Italy), and 0.5 mM of dNTPs (Promega Italia, Italy) in 5× Reaction Buffer (250 mM Tris–HCl, pH 8.3, 375 mM KCl, 15 mM MgCl2, 50 mM DTT). The mixture was incubated at 42 °C for 1 h and the reaction was terminated with a further incubation at 95 °C for 5 min.

The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was co-amplified with TrkA, NGF, BDNF, and VEGF and used to normalize sample to sample variability. PCR amplification was carried out using a 50-μl reaction mixture containing: cDNA, 5 μl in 10× buffer, 200 mM dNTPs, 1.5 mM MgCl2, 5 U of Taq DNA Polymerase (Promega Italia, Italy), 30 pmol of TrkA rat primer (5′-Bio-CCATCGTGA-AGAGTGGCCTC-3′; 5′-CCAGCTCTGTGAGG-ATCCAG-3′), 12.5 pmol of NGF human primer (5′-Bio-TCCACCCACCCAGTC-TTCCA-3′; 5′-GCCTTCCTGCTGAGCACACA-3′), 7.5 pmol BDNF rat primer (5′-Bio-AGCTGAGCGT-GTGTGACAGT-3′; 5′-TCCA-TAGTAAGGGCCCGAAC-3′), or 1.5 pmol GAPDH primer (5′-Bio-CACCACCATGGAGAAGGCCG-3′; 5′-GATGGATGACCTTGGCC-AGG-3′), and cDNA, 4 μl in 10× buffer, 200 mM dNTPs, 1.5 mM MgCl2, 2.5 U of Taq DNA Polymerase (Promega Italia, Italy), and 12.5 pmol of VEGF primer (5′-Bio-CTCACCAAAGCCAGCACA-CATA-3′; 5′-ACCGCCTTGGCTTGTCACAT-3′). A sample containing all the reaction reagent except cDNA was used as PCR-negative control in any amplification. Mixture containing TrkA primers and GAPDH primers were incubated at 95 °C for 5 min, for 35 cycles of amplification (1 min at 95 °C, 1 min at 52 °C and 2 min at 72 °C), and then at 72 °C for 5 min, using a GeneAmp PCR System 9700 (Applied Biosystem, CA, USA). Mixture containing NGF or BDNF and GAPDH primers for 35 cycles of amplification (1 min at 95 °C, 1 min at 55 °C, and 2 min at 72 °C) and then at 72 °C for 5 min in a GeneAmp PCR System 9700 (Applied Biosystem, CA, USA). Mixture containing VEGF and GAPDH primers were incubated for 30 cycles of amplification (1 min at 95 °C, 1 min at 58.5 °C, and 2 min at 72 °C) and then at 72 °C for 5 min in a GeneAmp PCR System 9700 (Applied Biosystem, CA, USA). PCR products were electrophorized in 2% agarose gel together with a DNA ladder of 100 base pairs (GIBCO) and visualized by ethidium bromide.

To allow the detection of PCR product by ELISA, we used biotinylated sense primers. To assess the presence of amplified probes, we coated microplates (Maxisorp Nunc) with

50 μg/ml of avidin (Sigma Chemical, Italy) in a coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and incubated for 2 h at 37 °C. After incubation, free sites were saturated with 2% blocking solution (Boheringer Mannheim, Germany) in a coating buffer at 4 °C overnight. Biotinylated PCR products were diluted in PBS countering 3% Bovine Serum Albumin (PBSB) and were distributed in duplicate (100 μl/well) in avidin-coated microplates and incubated for 1 h at room temperature. After incubation, microplates were washed three times with PBS containing 0.02% Tween-20 (washing buffer). DNA were denatured using 0.25 M NaOH at room temperature for 8 min, then were added to the microplates 100 μl/well of 4pmol/ml of digoxygenin-labeled probes of TrKA (5′-Dig-TGCACA-GTTTTCCAGGAGAGGGACTCC-3′), BDNF (5′-Dig-TAACCCATGGGATTACACTTGGT-CACGTAG-3′), NGF (5′-Dig-TCGATGCCCCGGCACCCACTCTCAACAGGA-3′), VEGF (5′-Dig-GTTCTATCTTTCTTTGGTCTGCATTCACAT-3′), or GAPDH (5′-Dig-ACAATCTTGA-GTAGATTGTCATATTTC-TCG-3′) in DIG Easy Hybridisation buffer (Boheringer Mannheim, Germany) and incubated at 42 °C for 2 h. After washing, anti-digoxigenin POD-coupled antibody (Boheringer Mannheim, Germany) was added (1:1000 in PBSB) and incubated for 1 h at 37 °C. Reaction was developed by TMB (3,3′-5,5′-tetramethylbenzidine; 0.6 mg in citrate buffer, pH 5.0) and blocked after 30 min with 2 M of HCl. The amount of amplified products was measured as optical density at 450/690 nm (OD, 450/690) using a Dynatech ELISA Reader 5000. GAPDH level (OD 450/690) was used to normalize the relative differences in samples size, integrity of the individual RNA, and variation in reverse transcription efficiency.

4.5. *Western blot analysis*

Retina tissues from 7 different rats' eyes were homogenized in a sample buffer (20 mM Tris–acetate, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, pH 7.5) and centrifuged at 10000 × *g* for 25 min at 4 °C. Supernatants were then used for Western blotting and ELISA. Thirty micrograms of total protein of each sample was dissolved with a loading buffer (0.1 M Tris–HCl buffer, pH 6.8, containing 0.2 M DTT, 4% SDS, 20% glycerol, and 0.1% bromophenol blue), separated by 12.5% SDS–PAGE, and electrophoretically transferred to a polyvinylidene fluoride membrane (PVDF) overnight. Membranes were then incubated overnight at 4 °C with polyclonal rabbit anti-TrkA 1:1000 (Santa Cruz Biotechnology, CA, USA), monoclonal mouse anti-TrkB 1:1000 (BD Transduction Laboratories, CA, USA), monoclonal mouse anti-VEGF 1:500 (Santa Cruz Biotechnology, CA, USA), and monoclonal mouse anti-pTrkA 1:1000 (Santa Cruz Biotechnology, CA, USA), the activated form of NGF receptor TrkA (Yang et al., 1997), and 45 min at room temperature with monoclonal mouse anti-β-actin 1:10000 (Sigma, MO, USA). Blots were developed with an enhanced chemiluminescent (ECL) assay (Amersham Bioscience UK limited, England) as chromophore. The optical density of β-actin bands was used as an internal control of difference in sample loading.

4.6. *Histological analysis*

Eye bulbs were serially sectioned with cryostat at 20-μm thickness. Eye sections of untreated and EA-treated RCS rats were stained with toluidine blue and the structural organization of the retina analyzed under a Nikon Eclypse E600 microscope equipped with a Nikon DMX 1200 digital camera connected to a personal computer with a computerized image analysis system. GC, Inner Nuclear layer (INL), Inner Plexiform layer (IPL), Outer Segment, ONL, photoreceptor layer, and the total retina thickness were measured in 12 sections, in three point each session (right edge, left edge, and central), coming from 4 different eye bulbs per experimental group. Thickness was established using the Nikon-Lucia image processing and analysis program.

4.7. *Immunohistochemistry for TrkA*

Sections from four different eye bulbs per experimental group were air dried to improve section adherence and washed in 0.1 M phosphate buffer and 0.1% triton (PBST). They were incubated for 10 mins at room temperature with 3% hydrogen peroxide and 10% methanol in 0.1 M phosphate buffer (PBS), then for 1 h with 10% normal goat serum (NGS) in PBST and after an additional wash, incubated overnight at 4 °C with 2% NGS and polyclonal rabbit anti-TrkA 1:70 (Santa Cruz Biotechnology, CA, USA) in PBST. After having been washed, the slides were then incubated for 2 h at room temperature with polyclonal biotynilated goat anti-rabbit 1:300 and immunoperoxidase staining was performed using an ABC Vectastain Elite Kit (Vector Laboratories, CA, USA). The labeled sections were analyzed under a Nikon Eclypse E600 microscope equipped with a 20× objective and a Nikon DMX 1200 digital camera connected to a PC computer, with the aid of a computerized image analysis system. The length of GC and INL was measured by using the Nikon-Lucia image processing and analysis program, and measurements were standardized between the experimental groups using the same calibration system. Positive cells were determined in 20× magnification images, through the ratio between cells measured and length of GC or INL.

4.8. *Retina blood vessels distribution*

Retina were isolated and mounted flat on glass slides, air dried, and incubated overnight with biotinylated Griffonia *simplicifolia lectin* I (Vector Laboratories, CA, USA), 10 μg/ml in 0.1 M phosphate buffer, and 0.1% triton. Griffonia *simplicifolia lectin* I, a member of the lectin family, is used to visualize blood vessels because it has the capacity to bind glycoconjugates proteins on erythrocytes, with a particular specificity for *N*-acetylgalactosamine and α-galactose residues (Lenzi et al., 2005). Retina were then exposed to rhodamine-conjugated avidin (Vector Laboratories, CA, USA). Blood vessels present in 14 non-overlapping fields of different retina (n = 4) were counted and compared. Because the image analyzer determines the optical density of thresholding operation, measurements were standardized between groups using the following criteria: (1) all measurements were conducted after the same calibration of the image analysis system; (2) thresholding was carried out to the same value for each image; and (3) binary analysis was calibrated to a range of 4–250 arbitrary units.

4.9. *Statistical analysis*

One-way analysis of variance (ANOVA) was used to analyze biochemical data, the distribution of vessels on retina and morphological analyses. Post hoc comparisons within logical sets of means were performed using the Tukey–Kramer test.

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