Involvement of Nitric Oxide Through Endocannabinoids Release in Microglia Activation During the Course of CNS Regeneration in the Medicinal Leech

KARIM ARAFAH, DOMINIQUE CROIX, JACOPO VIZIOLI, ANNIE DESMONS, ISABELLE FOURNIER, AND MICHEL SALZET*
Laboratoire de Spectrométrie de Masse Biologique Fondamentale et Appliquée (FABMS), EA 4550, Université Lille 1, Cité Scientifique, Villeneuve d'Ascq F-59655, France

ABSTRACT
The medicinal leech is notable for its capacity to regenerate its central nervous system (CNS) following mechanical trauma. Using an electrochemical nitric oxide (NO)-selective electrode to measure NO levels, we found that the time course of NO release in the injured leech CNS is partially under the control of endocannabinoids, namely, N-arachidonyl ethanolamide (AEA) and 2-arachidonyl glycerol (2-AG). Relative quantification of these endocannabinoids was performed by stable isotope dilution (2AGd8 and AAEd8) coupled to mass spectrometry in course of regeneration process or adenosine triphosphate (ATP) treatment. Data show that 2-AG levels rose to a maximum about 30 min after injury or ATP treatment, and returned to baseline levels 4 h after injury. In same conditions, AEA levels also rapidly (within 5 min) dropped after injury or ATP treatment to the nerve cord, but did not fully return to baseline levels within 4 h of injury. In correlation with these data, chemoattraction activities of endocannabinoids on isolated leech microglial cells have been shown *in vitro* and *in vivo* reflecting that control over NO production is accompanied by the controlled chemoattraction of microglia directed from the periphery to the lesion site for neuronal repair purposes. Taken together, our results show that in the leech, after injury concurrent with ATP production, purinergic receptor activation, NO production, microglia recruitment, and accumulation to lesion site, a fine imbalance occurs in the endocannabinoid system. These events can bring explanations about the ability of the leech CNS to regenerate after a trauma and the key role of endocannabinoids in this phenomenon.   © 2013 Wiley Periodicals, Inc.

INTRODUCTION

In pathological conditions such as those resulting from trauma, ischemia, hypoxia, or neurodegenerative diseases, the vertebrate central nervous system (CNS) increases the pro-inflammatory response and its production of nitric oxide (NO). This overproduction of NO first involves the directly affected neurons and then the immune-related cells in the brain, mainly the resident microglial cells. In the latter case, NO overproduction becomes a hallmark of their activated state. As a consequence, the intracellular calcium concentration and the formation of reactive oxygen species both increase, as does the activation of neuronal apoptosis. In parallel, the endocannabinoid pathways have been shown to be strongly activated (Eljaschewitsch et al., 2006; Panikashvili et al., 2001). Indeed, the depolarization of the plasma membrane increases the intracellular calcium concentration, and this rapidly activates lipid precursors of the most strongly evoked endocannabinoids: anandamide (N-arachidonyl ethanolamide (AEA)) and 2-arachidonyl glycerol (2-AG).

AEA is an agonist at the seven-pass transmembrane cannabinoid receptor type 1 (CB1r) (Devane et al., 1992), which is widely expressed in the vertebrate CNS by neurons, astrocytes, glial, and microglial cells. AEA-mediated activation of CB1r has been correlated with neuroprotection via the MKP-1 pathway (Eljaschewitsch et al., 2006). However, the biological effects of AEA on the vertebrate brain are complex, because it can also produce deleterious effects when it binds to rat neurons via the microglial transient receptor potential vanilloid 1 (TRPV1) receptor (Maccarrone et al., 2000). The related endocannabinoid 2-AG (Sugiura et al., 1995), which is released by the vertebrate CNS following injury (Walter et al., 2003), binds both CB1r and the related cannabinoid receptor type 2 (CB2r) with high affinity (Sugiura and Waku, 2000). CB2r is specifically expressed in the vertebrate brain on the surface of immune system cells (Felder and Glass, 1998), such as microglial cells (Carlisle et al., 2002; Carrier et al., 2004). The CB2r-mediated chemoattraction of dendritic cells by 2-AG *in vivo* has been documented (Maestroni, 2004), and an antioxidant effect of this lipid has been reported to be neuroprotective (McCarron et al., 2003).

It is currently hypothesized that, in vertebrates, AEA and 2-AG mediate neuroprotection by combining a chemoattractant effect on microglial cells (Franklin and Stella, 2003) with control of the immune response (Eljaschewitsch et al., 2006). In microglial cells, CB1 receptors are always weakly expressed, whereas the expression of CB2 receptors is modulated according to the cell's state of immune activation. Thus, reports indicate that CB2r is not expressed on the surface of resting mammalian microglial cells, but is highly expressed when these cells are responsive and primed. Cabral et al. (2008) have reported a "window" of functional relevance during which activation of the CB2r modulates microglial cells activities.

However, in mammal's spinal cord injury model, several groups have shown those spinal microglia are activated by endocannabinoids not through CB-receptors but by TRPV1 (Chen et al., 2009; Kim et al., 2006; Schilling and Eder, 2009). TRPV1 mediates the release of neurotransmitters, such as glutamate and Calcitonin gene related peptide in the dorsal mouse horn, which can subsequently activate glia. TRPV1 plays a role in the activation of spinal glia in mice with nociceptive, inflammatory, and neuropathic pain (Chen et al., 2009; Shirakawa et al., 2010), through NADPH oxidase-mediated reactive oxygen species generation in microglia (Schilling and Eder, 2009, 2010).

Similarly leech known by its capacity to regenerate neurites and synapses after a nerve cord injury and to restore functional central synaptic circuitry following injury involves the accumulation of microglial cells at the lesion site in the nerve cord (Baylor and Nicholls, 1971; Jansen and Nicholls, 1972; Morgese et al., 1983; Salzet and Macagno, 2009; von Bernhardi and Muller, 1995). Recent data suggest that this microglia accumulation in the leech CNS is essential for the sprouting of injured axons to occur (Ngu et al., 2007). In addition, real-time amperometric measurements have shown that experimental lesions trigger the quick release of low amounts of NO during the first ~20 min (Kumar et al., 2001; Shafer et al., 1998). This NO production at the lesion site is required for the initial step of CNS regeneration process because treatment with the NO inhibitor N-omega-nitro-L-arginine methyl ester (L-NAME) inhibits the first peak of NO release and brain regeneration as well (Chen et al., 2000). Nevertheless, a high concentration of NO in the leech brain produced by a NO donor like spermine inhibits the regeneration process (Chen et al., 2000; Kumar et al., 2001). Moreover, by targeting NO synthase and cGMP in microglial cells, Duan et al. (2003) showed that these activated cells accumulate at the lesion site in a time-dependent manner, suggesting that a higher amount of NO is produced hours after the lesion. The cannabinoid agonists, anandamide and CP 55940, stimulate the release of NO from leech CNS in a concentration-dependent manner, whereas the antagonist, SR 141716A, did not. This process can be antagonized by preincubating the ganglia for 2 min with the NO synthase inhibitor, N-omega-nitro-L-arginine methyl ester (L-NAME), as well

as exposing the ganglia to the cannabinoid antagonist SR 141716A, demonstrating the specificity of the process. The level of NO release following exposure of the cells to 10^{-6} M anandamide is ~14 nM (Stefano et al., 1997b). Thus, endocannabinoid receptor binding sites (Stefano et al., 1997b) and fatty acid amide hydrolase (Stefano et al., 1998) have also been detected in leech nervous system pointing the putative presence of an endocannabinoid system in the medicinal leech involved in the inhibition of presynaptic release of dopamine though NO production (Stefano et al., 1997a). To assay for possible physiological activity of these cannabinoids in regeneration, a series of *in vitro* studies with several cannabinoid and vanilloid receptor agonists and antagonists have been tested (Meriaux et al., 2011). Neurites sprouted from the cut nerve in less than a week for the control preparations and in less than 3 days for preparations exposed to either of the agonists, capsaicin, and arvanil, whereas no outgrowth was observed with exposure to either of the antagonists tested, AEA, and capsazepin (Meriaux et al., 2011). These data further suggest that endocannabinoids also play key roles in CNS regeneration, mediated through the activation of leech vallinoid receptors (TRPVs), as revealed *Hirudo* genome and expressed sequence tag screening where at least two putative TRPVs were identified (Meriaux et al., 2011).

In this article, we demonstrate that the lesioned leech nerve cord can control its concentration of NO through endocannabinoids releasing. For this, we use for the first time a selective amperometric system for dynamic measurements of NO over a period of 20 h following injury. We highlight then a relationship between the 2-AG-mediated secretion of NO and the chemotaxis of microglial cells accumulated at the lesion site during the initial steps of inflammation. We determine the involvement of the 2-AG in microglial cell recruitment through the *in vitro* and *in vivo* chemotaxis assays. In addition to this chemoattractant effect on microglia, 2-AG controls the kinetics of NO production in the injured leech nerve cord. This report furnishes novel insights regarding the involvement of the endocannabinoids and the microglial response.

MATERIALS AND METHODS
Leech Dissections

Adult medicinal leeches (*Hirudo* sp.) were purchased from Ricarimpex (Eysines, France). Upon arrival, the animals were kept in artificial pond water without any further feeding. In preparation for regeneration assays, the complete CNS, including all ganglia and noninjured interganglionic connectives, was dissected from animals anesthetized in 10% ethanol (20 min at room temperature) according to established procedures (Nicholls and Baylor, 1968). Tissues were bathed at room temperature in sterile Ringer's solution (115 mM NaCl, 1.8 mM CaCl$_2$, 4 mM KCl, 10 mM Tris maleate pH 7.4) for 7 h before performing experiments.

Real-time NO measurements

For all NO determinations, freshly dissected leech connectives flanked by their segmental ganglia were pinned into a 35-mm dish containing Sylgard polymer in the bottom. The connectives were then injured using a pair of fine forceps.

Chemicals

The 2-AG and the solvents [acetonitrile (ACN) and ethanol] were purchased from Sigma-Aldrich (St. Louis, MO) and AEA was obtained from Calbiochem (Merck Chemicals, Nottingham, UK) and prepared in ethanol. The selective cannabinoid receptor 1 antagonist AM-251 was purchased from Cayman Chemicals (Ann Arbor, USA). The selective cannabinoid receptor 2 antagonists AM-630 was purchased from Tocris (Tocris Bioscience, Ellisville, USA). Both antagonists were diluted in ACN solution.

Technical description

An electrochemical NO-selective electrode encased in a protective stainless steel sleeve covered with a gas-permeable NO-selective Nafion membrane (World Precision International, Apollo 4000) (Xian et al., 2000) was used to oxidize the NO at the working electrode into an amperometric signal monitored by a computer. With this approach, neither the nitrite and nitrate ions nor interfering species such as dopamine, ascorbate, and ʟ-arginine can react with the electrochemical sleeve.

Biological preparation and NO records

Connectives were first pinned and bathed in a 35-mm dish. After crushing the connective with fine forceps, the Nafion extremity of the sleeve was put a 2 mm above the injured site of the leech connective. Surrounding the lesion site, the NO release from the crushed site went through the NO-selective membrane and was immediately oxidized by the electrochemical cell thereby yielding real-time NO measurements with very high sensitivity (limit of detection <1 nM) and accuracy. The quantity of NO (moles) released from the biological sample was calculated after calibration of the electrochemical cell and following manufacturer's recommendations. Real-time measurements of NO release from the injured connectives were performed with an amplifier Apollo 4000 and recorded with the Apollo software (World Precision Instruments, Stevenage, UK). All graphs described the quantity of NO (intensity scale in the y-axis) related to the time course.

Endocannabinoid treatments

AEA was dissolved in ethanol and maintained as a stock 20 mM solution. 2-AG, AM-251, and AM-630 were dissolved in ringer containing ACN. AEA (10 μM) and 2-AG (30 μM) were carefully deposited onto the crushed site of connectives and NO release was simultaneously monitored and recorded. Due to their hydrophobic properties, the antagonists of the mammalian cannabinoid receptors 1 and 2, AM-251 and AM-630, respectively (30 μM), were directly injected into the lesion site of the connective using a syringe equipped with a 1 μm diameter tip glass needle before NO recordings.

Chemotaxis Assays and Staining Experiments

Microglial cell isolation

To assess the *in vitro* effect of synthetic cannabinoids on leech microglial cell chemotaxis according to the procedure of Croq et al. (2010), the entire CNSs from six leeches were dissected as described above and each ganglion was carefully decapsulated using a fine pair of forceps. Neurons, microglial cells, and other nerve cells were mechanically resuspended by gentle scraping in Ringer solution. After filtration of the cell suspension into a 7 μM diameter nylon mesh, microglial cells were harvested and thus separated from the others according to their size. A centrifugation step (1000g for 10 min) at room temperature gave a cell pellet, which was gently suspended in 300 μL of complete medium (Leibovitz L-15 basal medium (Gibco, Invitrogen) complemented with foetal bovine serum 10% (m:v), 0.6% glucose (m:v), 2 mM ʟ-Glutamin, 100 μg/mL gentamycin, 1000 UI/mL penicillin/streptomycin, 10 mM Hepes, all reagents from Invitrogen (France)).

Microglia chemotaxis assays *in vitro*

The effects of synthetic AEA and 2-AG on microglial cell recruitment were determined using the *in vitro* double-P assay (Kohidai, 1995) for chemotaxis purposes with minor modifications (Croq et al., 2010; Schikorski et al., 2009; Tahtouh et al., 2009, 2012). Chemotaxis experiments were carried out in triplicate and their results are expressed as the mean of the microglial cell number $\pm$ S.D. A one-way repeated analysis of variance (ANOVA; P value $= 0.01$) was performed for all the results and the significance of differences was assessed with the Holm-Sidak test $\alpha = 0.01$ (SigmaStat 3.11).

Microglial chemotaxis assays *ex vivo*

Leech CNS cell nuclei were counterstained incubating the cultured nerve cords for 20 min at room temperature

with propidium iodide 5 μg/mL (Invitrogen, France) diluted in Ringer's solution. Particular attention was focused on the microglial cells present at the lesion site of the injured connectives. Microglia accumulation at the lesion site consecutive to treatments with cannabinoid receptor agonists (AEA, 2-AG) or antagonists (AM-251, AM-630) was then directly assessed by confocal microscopy (Zeiss LSM 510, USA). A treatment with a 1:1000 solution of ACN was done as a negative control. In each experiment, successive stacks of 4.96 μm were imaged in the area surrounding the lesion site and the microglia was counted and reported to a volume of 4.10^5 μm^3. Experiments were carried out in triplicate (counting were done in two different connectives from three different leeches per condition). The results were expressed as mean ± S.D. and significant differences were performed with a student t-test (α = 0.5; $P \leq 95\%$) (SigmaStat 3.11).

Immunocytochemistry

An immunocytochemical assay for CB2 receptor-like investigation in the leech was performed on isolated microglial cells and leech neurons (Croq et al., 2010; Schikorski et al., 2009; Tahtouh et al., 2009, 2012). An antibody targeting the extracellular loop of the CB2 rat receptor was chosen for the experiments.

Microglial cell staining at the lesion site

Leech cell nuclei were stained in the connectives for 20 min at room temperature with a 1:5000 Hoechst 33258 dye solution (Invitrogen) diluted in Ringer's solution. Particular attention was focused on the microglial cells present at the lesion site of the injured connectives. *In vivo* microglia accumulation at the lesion site consecutive to treatments with cannabinoid receptor agonists (AEA, 2AG) or antagonists (AM251, AM630) was then directly assessed using a FITC filter on a Leica microscope.

Endocannabinoid quantification

Extraction. Endocannabinoid level measurement was performed in course of regeneration by MALDI-TOF mass spectrometry. Twenty-four isolated nerve cords at six different time course of regeneration from 0 to 240 min were collected in physiological saline. Each set of four nerve cord sections (three ganglia and three connectives) was used to titrate the anandamide (AEA) and 2-AG. Internally deuterated anandamide, AEA 4 μg/mL (Cayman Chemical, 390050) and 2-AG 4 μg/mL (Cayman Chemical, 362160) were added to each sample before lipid extraction. The Folch method (chloroform/methanol (3:1; v/v)) was performed for *Hirudo* CNS total lipid extraction (Meriaux et al., 2011). The chloroformic phase containing endogenous and deuterated endocannabi-

noids (AEA and 2-AG) was harvested and submitted to a relative quantification using MALDI-TOF Mass spectrometry (Ultraflex II, Bruker Daltonics, Bremen, Germany).

Mass spectrometry

A 6 mg sample of 2.5 DHB (149357) matrix was mixed with 3 mg of lithium chloride (L-9650; Sigma-Aldrich, St. Louis, MO) salt in 100% acetone lithiated matrix (chromasolV 34850; Sigma-Aldrich, St. Louis, MO) and lipid extracts (1:1, v:v) were mixed and dropped onto a 384 stainless steel sample plate (Bruker Daltonics) for mass spectrometric analysis. Lithium salt combined with DHB in acetone is known to be effective for determination of non polar long-chain lipids, hydrocarbons, and polymers by MALDI (Stubiger et al., 2008). Under these conditions, 3 mg of lithium chloride salt with 6 mg of 2,5-DHB was dissolved in 100% acetone. Lithiated matrix and lipid extracts (1:1, v/v) were mixed and dropped onto a 384 stainless steel sample plate (Bruker Daltonics Bremen, Germany). Mass spectrometric analyses were performed in positive reflector mode with an UltraFlex II MALDI-TOF/TOF instrument (Bruker Daltonics, Bremen, Germany) equipped with a Smartbeam laser having a repetition rate up to 200 Hz and controlled by FlexControl 3.0 (Build184) software (Bruker Daltonics, Bremen, Germany). The spectra were treated with FlexAnalysis 3.0 (Build 96) software. Linearity of the mass spectrometry measurements was determined with endocannabinoid standards by measuring the ratio of their accumulated (8000 laser smartbeam shots) area spectra over the corresponding internal deuterated standard one. The measurements obtained for accumulated spectra showed linear responses within the referenced range. Relative quantification of 2-AG and AEA was performed by stable isotope dilution (2-AGd8 and AEAd8). The presence of 2-AG and AEA was determined by observing that their respective ions of m/z 385.29 and 354.29 were isolated and fragmented according the positive LIFT mode of the MALDI-TOF mass spectrometer and compared with their corresponding standards (Meriaux et al., 2011). A one-way ANOVA was used for statistical analysis of the measurements of 2-AG, for which the data passed both the normality and equal variance tests, whereas a Mann-Whitney U test was used for the AEA data. The Holm-Sidak method for multiple comparisons versus control group was used to determine significance at the 0.01 level, indicated by asterisks.

RESULTS
Real-Time Measurements of Nitric Oxide Released by Injured Leech Nerve Cords

In leech, there is no macrophage. The blood cells appear to share morphological characteristics with hya-

line cells, also called plasmatocytes, which represent the most prevalent type of differentiated circulating cells in invertebrates. Because of lack of conservation between mammalian macrophage markers, such as CD14 and CD61, and leech orthologs, well-described macrophage markers could not be detected in these cells (Boidin-Wichlacz et al., 2012). In leeches, as in mammals, microglial cells, considered as the resident phagocytic cells of the CNS, have been demonstrated to respond rapidly for neural protection or healing after CNS injury (Chen et al., 2000; Croq et al., 2010; Duan et al., 2003, 2009; Masuda-Nakagawa et al., 1990; McGlade-McCulloh et al., 1989; Morgese et al., 1983; Salzet and Macagno, 2009; Schikorski et al., 2008, 2009; Tahtouh et al., 2009, 2012). Thus, to explore the relationship between NO amount and microglial cell recruitment to the lesion site, we measured the release of NO in crushed connectives treated with either agonists or antagonists to the putative CB2-like receptor, thought to be implicated in leech microglia activation in vertebrates. Freshly dissected nerve cords were either crushed or crushed and stimulated with different drugs or control solutions injected in connectives (Figs. 1 and 2). Using an amperometric apparatus, we detected and monitored NO release from injured nerve connectives (Fig. 1). As a basal determination of the NO released from uncrushed and untreated connectives, the amperometric apparatus for NO measurements began its recording 2 h before the experiments (T_{-2h}) (Fig. 1). The level of NO release was monitored for the following 20 h (Fig. 1A). The average starting time of NO pulses relative to the basal level corresponded to the amount of 29.7 nM (± 6.33) (Fig. 1A). This value constitutes the baseline of NO release from uncrushed connectives. In control conditions (crushed connectives), we observed a range of NO concentration varying from 23.37 to 36.03 nM during the 4 h period following injury, this amount gradually increased in the next 4 h (Fig. 1A). In order to quantify the effect of 2-AG and AM-630 on NO production, we compared the time course of its release after injury (Fig. 1C,D). To overcome the fluctuation linked to individual variation of NO production within the first 4 h period postinjury, we assigned the concentration of 40 nM as the reference threshold for the NO release time course. This value is significantly over the indicated variability range of controls and constitutes a good reference to evaluate the effect of drug treatments on NO production in leech nerve cords. In this context, the control condition reached the reference threshold 4 h 30′ following the injury of the nerve cords (Fig. 1A). Several concentrations of 2-AG, AEA, and AM-630 have been tested in order to get sufficient NO release (data not shown). However, based on previous data on intact leech nerve cord, nanomolar to micromolar concentrations have been tested (Stefano et al., 1997ab, 1998, 2002). Best results were obtained with micromolar concentration. Tests with injured nerve cord have been also performed using Biological MicroElectro-Mechanical System compatible with microfluidic circulation and electromagnetic propagation. Production of NO was observed and measured in the far-THz spectral domain (Abbas et al., 2009). In this case, NOS activity immediately reached a peak of intensity and then decreased over time, never returning to the pre-injury level as we detect in amperometric method.

Experimental treatments revealed a different time course of NO release in injured or injured and treated nerve cords (Figs. 1 and 2). The injection of 2-AG (30 μM) at the lesion site induced a faster and stronger production of NO in the first 4 h after lesion, reaching the reference threshold 1 h 25′ after injury (Fig. 1C). The injection of the CB2r antagonist AM-630 (30 μM) blocked the activation of NO production that remained at the initial, basal level of production until 8 h postinjury (Fig. 1D). As a negative control, the vehicle solution of the 2-AG and the AM-630, ACN, was injected at the lesion site. In that case, the NO release that resulted was similar to that observed in control experiments, demonstrating that ACN does not interfere with NO production in this experimental system.

In mammals, Carlisle and colleagues defined a short period where microglia might express functional CB2r involved in microglia activation. By analogy, we determined in injured nerve cords, the effects of instant or 1 h-delayed injection of agonist and antagonist of the CB2-like receptor on the production of NO by activated microglia (Fig. 2). In control experiments (crushed connectives), the amount of NO reached the reference threshold 267.17 min (± 47.06) after lesion. The injection of the 2-AG (30 μM), simultaneously to the lesion reduced this period to 133 min (± 20.15), indicating an inhibitory effect on NO release. The 1 h-delayed treatment with the same concentration of 2-AG provoked a faster increase in NO production attending the 40 nM reference level 90 min (± 14.88) after lesion. By contrast, blocking the CB2-like receptor in the crushed connectives with the AM-630 (30 μM) caused a significant delay of the NO pulses to 389.1 (± 13.15). No significant differences were observed with the 1 h-delayed AM-630 treatment. Finally, to exclude any possible effect of the ACN vehicle used to dissolve the 2-AG and the AM630 solutions, we injected a 1:1000 solution of ACN into the crushed connectives. No significant difference was found with the control condition, thereby suggesting that ACN did not interfere with the timing of NO release after crushing the nerve cord.

Taken together, these results suggest a strong and fine regulation in the kinetics of NO secretion by the 2-AG in injured connectives of leech CNS.

AEA and 2-AG-Induced Microglial Chemotaxis

In vitro

Modulation of chemotaxis by AEA and 2-AG was assayed *in vitro* on isolated microglial cells harvested from freshly dissected leech CNSs. This approach (see
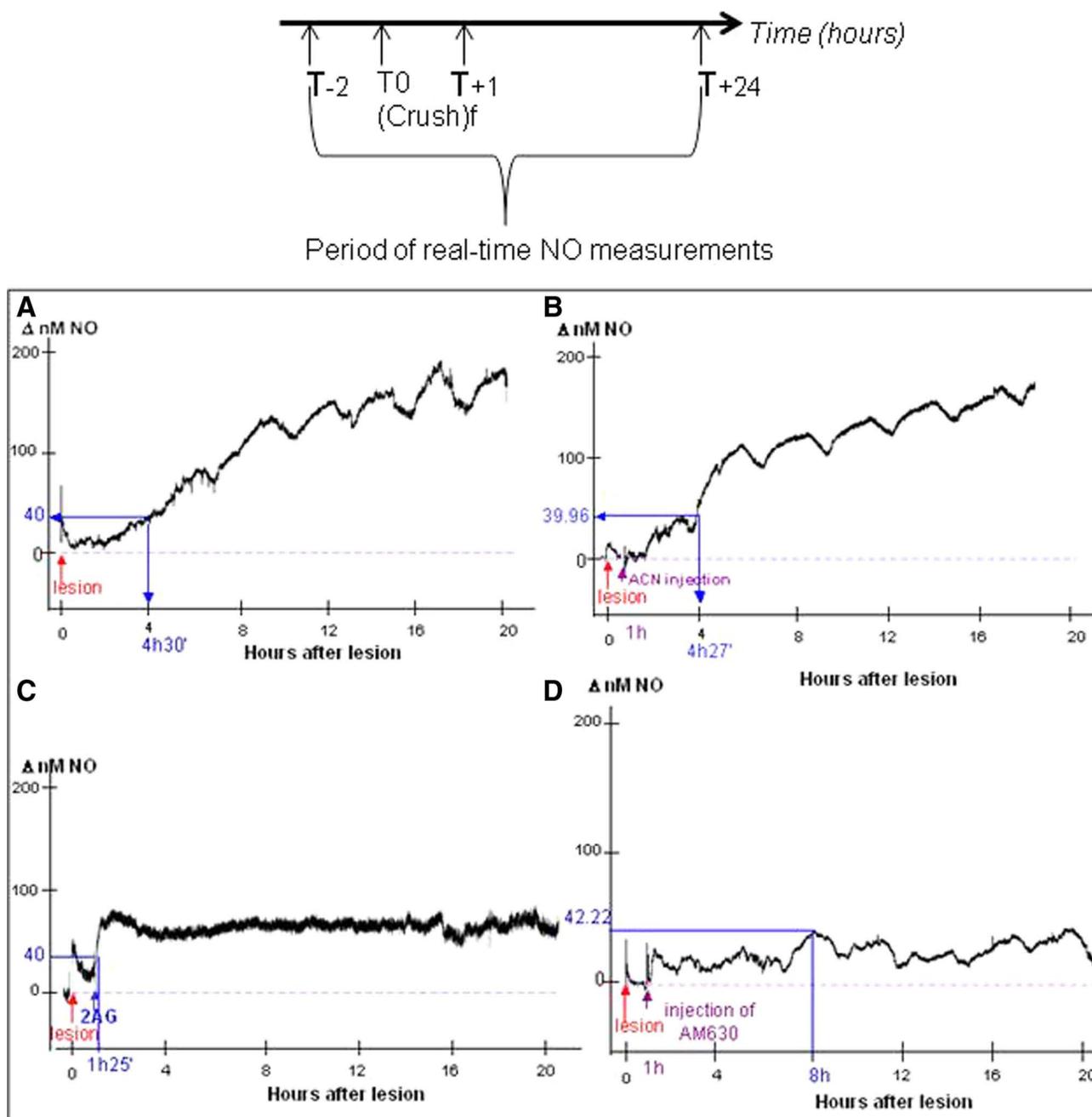
Fig. 1. Real-time NO recording illustrations showing the NO profile from crushed connective (control condition (**A**)) and crushed connective treated with either acetonitrile (**B**), 30 μM 2-AG (**C**) or 30 μM AM-630 (**D**). The figures depict the variation in NO concentration (in nM) over the course of the experiment (20 h). These results show that the kinetics of NO production in the control condition and the ACN-treated condition are similar, suggesting that stimulation of the crushed connectives with ACN only weakly influences the kinetics and concentration of NO. In contrast, 2-AG and AM-630 strongly modulate the NO profile in its kinetics as well as its concentration. While 2-AG speeds up the process allowing the connective to produce NO waves, AM-630 downregulates this process in comparison with the control and the connective treated with ACN (*n* = 6). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

"Materials and Methods") was used previously to determine the effects of two other chemoattractants, one related to EMAPII (Schikorski et al., 2009) and the other to IL-16 (Croq et al., 2010) or C1q (Tahtouh et al., 2009). In this context, microglial cells were harvested from the CNSs of freshly dissected leeches. The previous observations of the modulation of cannabi-

noid-related chemotaxis of microglial cells were investigated directly in isolated microglia. As shown in Fig. 3, at concentrations of 10 μM both AEA and 2-AG attracted about 10× as many microglial cells (45–50%) as the negative control (~5%; L-15 culture medium without the reagents). Interestingly, this difference becomes much larger at higher concentrations (30 μM)
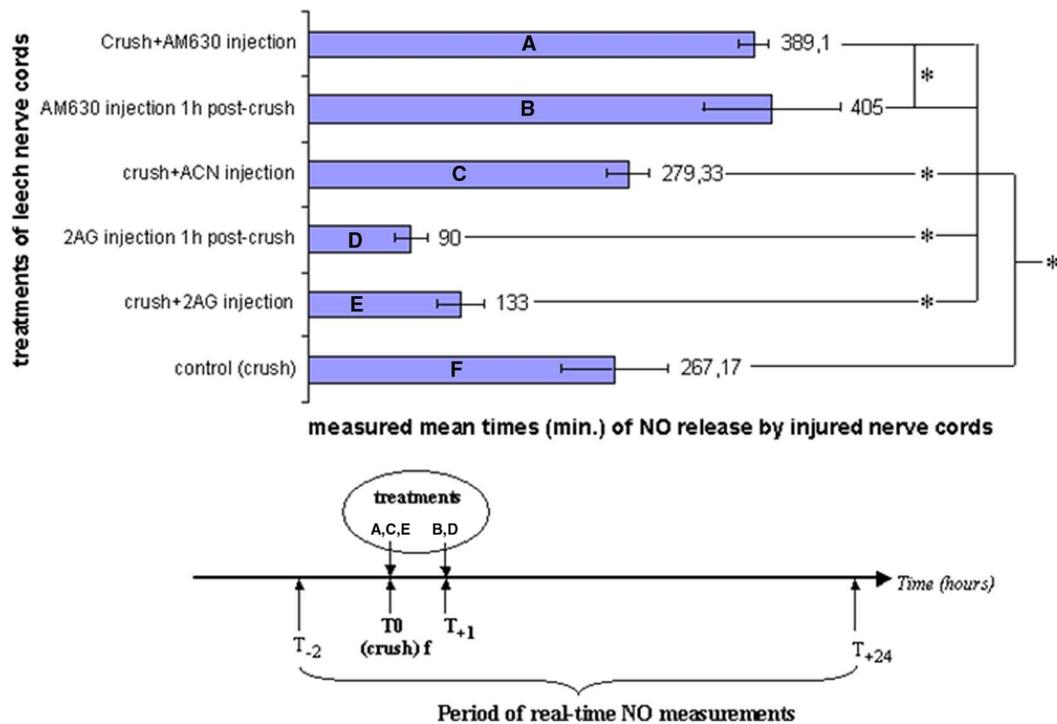
Fig. 2. The CB receptor agonist 2-AG and the selective antagonist AM-630 modulate NO release in the injured leech CNS independently from the initial time-point of stimulation (simultaneously with crush or 1 h post-crush). Significant differences have been established in the timing of NO release at the lesion site between control (**F**) and treated connectives (**A**, **B**, **D**, and **E**). Stimulation of the nerve cord with 30 μM 2-AG induced significant NO production at the injury site (D and E) in comparison with the control condition (F). Treatment with AM-630 (30 μM) (A and B), which is known to have a high affinity for the CB2 receptor, significantly delayed NO release in the injured nerve cord compared with the control. Moreover, no significant NO release related to the ACN treatment was evident in comparison with the control. All statistics were performed with SigmaStat 3.11 using a one-way repeated-measures ANOVA test $P < 0.01$ with the *post hoc* Holm-Sidak test $\alpha = 0.05$. Significant differences are denoted by asterisks. All the treatment conditions presented significant differences when compared with the control condition (A–E vs. F). The AM-630 conditions did not share significant differences with each other (A vs. B) but were different from all other conditions. Finally, the (**C**–**E**) conditions were significantly different from each other ($n = 6$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

of the drugs because of opposite dose-dependences: 2-AG recruited more cells (~70% vs. 55%) at 30 μM than it does at 10 μM, whereas AEA recruited fewer (~29% vs. 48%). Although these two lipid-derived compounds have similar structures derived from arachidonic acid, the chemotaxis results suggest that AEA and 2-AG modulate microglial attraction differently depending on the dose and the regulatory context (Fig. 3).

In vivo

To better understand the link between endocannabinoids and NO-mediated microglia recruitment in leech, we treated injured nerve cords with NO-Synthase inhibitor (L-NAME) and with AEA and 2-AG as well as two cannabinoid receptor antagonists (AM-251 and AM-630) (Fig. 3). Basal microglial cell accumulation at the lesion site (Fig. 4A) was impaired using the specific NO synthase inhibitor L-NAME (Fig. 4B). AEA and 2-AG were added simultaneously to the crush and microglial cells were assessed for chemotaxis during lesion of the leech CNS (Fig. 4C,D). The AEA (10 μM) blocked the microglial cell migration to the lesion site (Fig. 4C). An opposite effect was obtained using 2-AG that, at the same concentration, highly increased the accumulation of these cells at the axotomyzed nerve cord (Fig. 4D) in comparison with the control condition (Fig. 4A). In the same experimental conditions, the treatment with the CB1-like receptor antagonist AM-251 (30 μM) did not inhibit the microglial recruitment. In contrast, the addition the of CB2-like receptor antagonist AM-630 blocked the chemotaxis process (data not shown). The counting of stained microglia at the lesion site of each experimental condition are reported in Fig. 5A. The treatments of nerve cords with AEA, AM-251, and AM-630 significantly reduced the accumulated microglia at the lesion site in comparison with control conditions and 2-AG treated nerve cords, confirming the role on microglial activation of 2-AG.

Ex vivo

Ex vivo experiments were also perform to confirm the *in vivo* data. Freshly isolated leech microglial cells were incubated either with a preincubated solution containing the primary antibody and its specific blocking peptide (1:500) or without primary antibody (Fig. 5B). In
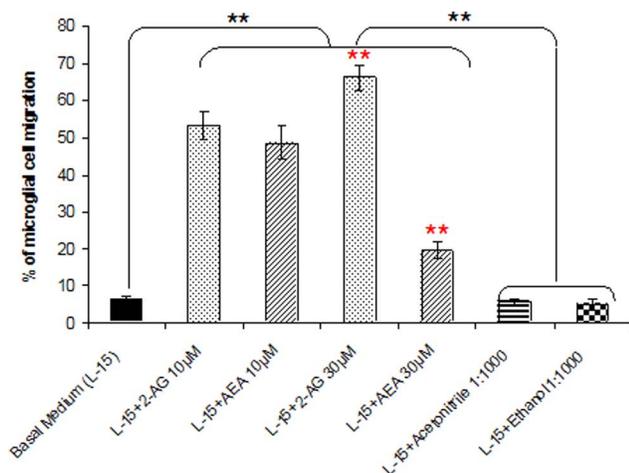
Fig. 3. Chemotactic effects of cannabinoids on isolated microglia from total leech nerve cord. The results are expressed as the percent of microglial cells that migrate compared with the L-15 control condition. In each set of experiments, cell counts were performed in triplicate at the beginning of the chemotaxis test and at its end. The asterisks denote that the indicated samples were significantly different from each other (one-way repeated ANOVA, P value = 0.01, Holm-Sidak test α = 0.01; SigmaStat 3.11). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

each set of experiments, images of 5× and 20× magnification were recorded with both FITC (Fig. 5B-1–B-3) and Hoechst (Fig. 5B-4–B-6) filters of a Leica instrument. In the control condition without the CB2r primary antibody (Fig. 5B-3,B-6), no microglial staining was observed (Fig. 5B-3) even though microglial cells were indeed discernible by the nuclear Hoechst 33258 dye (Fig. 5B-6). In contrast with the control condition, the CB2r primary antibody successfully recognized the isolated microglial cells (Fig. 5B-1) in accordance with the Hoechst 33258 staining results (Fig. 5B-4). A second negative control using the primary antibody saturated with its specific blocking peptide before performing the immunostaining experiment revealed only nonspecific binding in the slide (Fig. 5B-2), whereas Hoechst 33258 allowed staining of microglial cells on the same slice (Fig. 5B-4).

AEA and 2-AG Titrations

Following injury

To better understand whether endocannabinoid levels in the leech change after injury and in course of microglia movement and migration, relative quantification of these endocannabinoids was performed by stable isotope dilution (2-AGd8 and AEAd8) coupled to mass spectrometry (Fig. 6A,B). As shown in Fig. 6C, 2-AG levels rose from 1.174 ($\pm$0.123) nmol/mg of CNS tissue to a maximum of 4.392 ($\pm$0.088) nmol/mg of CNS tissue about 30 min after injury, and returned to baseline levels 4 h after injury. In contrast, AEA levels rapidly (within 5 min) dropped from 17.710 ($\pm$0.481) nmol/mg of CNS tissue to

0.088 ($\pm$0.009) nmol/mg of CNS tissue after injury, but did not fully return to baseline levels within 4 h of injury (Fig. 6D).

Following treatment with 100 μM ATP

Recent data in leeches showed that injury releases adenosine triphosphate (ATP) into the extracellular space, activating the microglia through gap junction channels called innexons located in the cell membrane (Bao et al., 2007; Dykes et al., 2004; Firme et al., 2012; Kandarian et al., 2012). Thus, in the leech, innexin membrane channels releasing ATP from glia are required for migration and accumulation of microglia after nerve injury (Samuels et al., 2010). As our results suggest a strong and fine regulation in the kinetics of NO secretion by the 2-AG in injured connectives of leech CNS. Because, moreover, ATP is known to increase production of 2-AG in cultured murine microglia (Witting et al., 2004), ATP and a crush, which releases ATP, might also increase production of cannabinoids in the leech. Thus, it is of interest to measure endocannabinoid level of ATP treatment. As shown in Fig. 7A, 2-AG levels rose about 14-fold from baseline levels 30 min after treatment with 100 μM ATP, and returned to baseline levels 60 min after treatment. Similarly, 2-AG levels rose to a maximum about 30 min after injury, and returned to baseline levels 4 h after injury (Fig. 6C). In contrast, AEA levels dropped soon after treatment with ATP, beginning to recover by 10 min after treatment. AEA levels also rapidly (within 5 min) dropped after injury to the nerve cord, but did not fully return to baseline levels within 4 h of injury (Fig. 7B).

These results showed that ATP increased production of 2-AG. Duan et al. (2009) have previously demonstrated that application of 100 μM ATP caused maximal movement of microglia in leech nerve cords via purinergic receptors. Taken together, ATP released directly or indirectly by injury activates microglia to move before stimulating (30 min) 2-AG production associated to NO modulation for microglia cells accumulation to the lesion site.

DISCUSSION

As in mammalian, the roles of the AEA/2-AG and NO pathways in activated microglia remain controversial. In this study, we first demonstrated the immediate production of NO when a lesion in the leech nerve cord occurs. Then, we showed that this rate of production decreased over time (Fig. 8). Indeed, a delay between 3 h 30 min and 4 h after the lesion was systematically observed. During this delay, the damaged nerve cord enhanced NO production at the lesion site and after that, the experiments described in this report put forward a regulation of the NO rate at the lesion site over several hours.
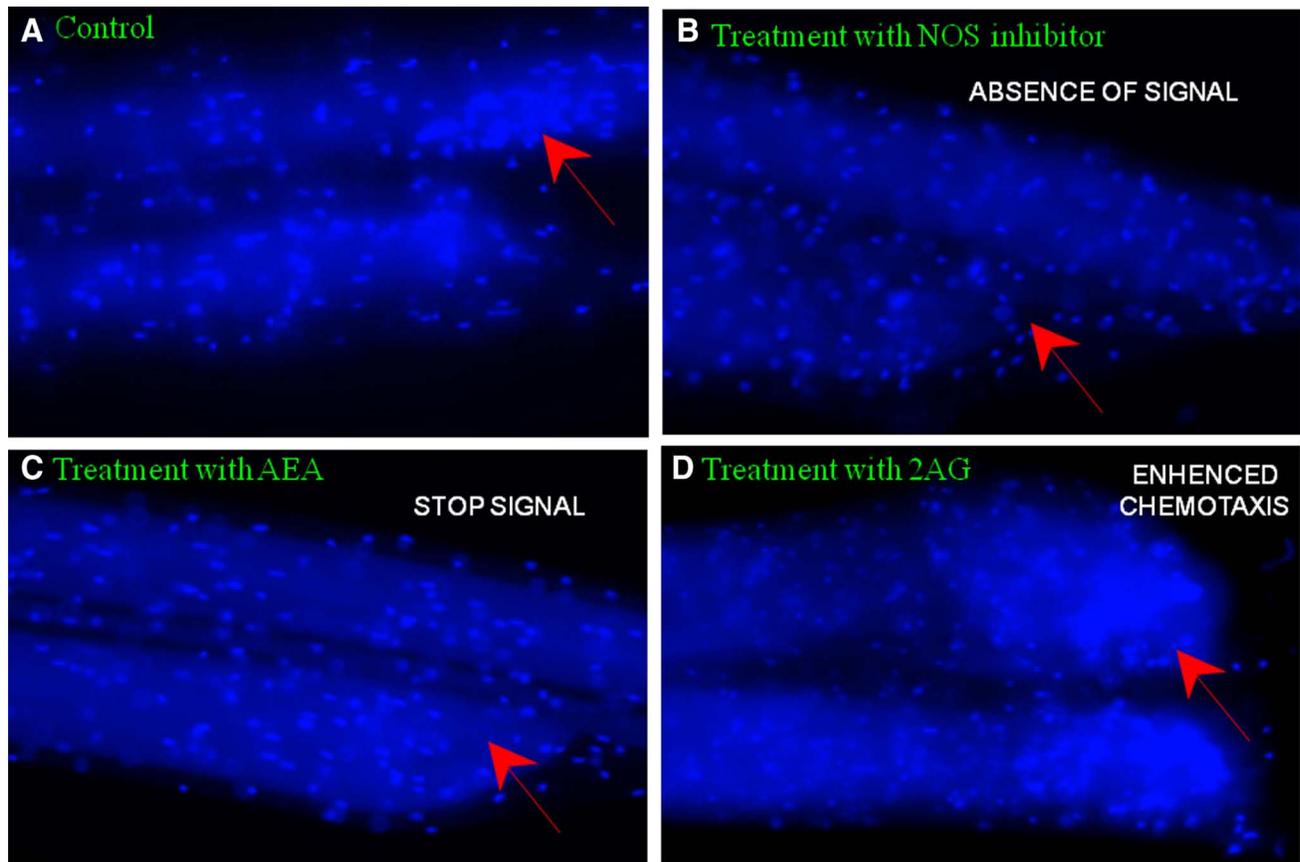
Fig. 4.   *Ex vivo* chemotactic effects on microglia by drug treatments. Confocal microscopy images showing connectives microglial cells accumulated (red arrow) to the crush site 6 h after lesion. Control microglia accumulation is shown in (**A**). Images (**C**) and (**D**) show the effect of CB1 and CB2 receptor ligand AEA and 2-AG, respectively, on microglia chemotaxis. In image (**B**), microglial cells are displayed recruited after treatment with NO synthase inhibitor L-NAME. Drugs were added simultaneously to axotomy of nerve cords. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The present report brings insights regarding the complementary chemotactic effects of the AEA and the 2-AG over the microglia in injured leech nerve cord. Treatment of leech connectives with the specific CB2 receptor antagonist AM-630 diminished the recruitment of microglial cells in both *ex vivo* and *in vivo* experiments. The co-stimulation with the 2-AG and the AM-630 at the connective crush site failed to reverse the accumulation of microglia. This latter result may suggest that 2-AG promote chemotaxis and direct recruitment of microglial cells to the site of injury through the CB2-like receptor in the leech. In parallel, the treatment of injured connectives with this physiological concentration of 2-AG released upon CNS injury in the leech provoked a specific response of NO production at the lesion site just after the stimulation. This observation is consistent with the binding and thus activation of the CB2-like receptor subsequently inducing the release of NO (Stefano et al., 1997b). Interestingly, the simultaneous crush and treatment of connectives with the same concentration 1 h after the crush stimulated NO production more rapidly. This observation suggests that microglial cells are more responsive to 2-AG 1 h after the lesion than immediately after the

injury where microglia might be in a "resting" state. Blockade of the CB2-like receptor by AM-630 revealed an inhibition of NO secretion and the time course of NO production was significantly delayed in comparison with the control condition. In this way, we demonstrated that at least part of the NO controlled production by the injured leech nerve cord was related to the 2-AG-activated CB2-like receptor, as already observed in experiments using Guinea pig mast cells (Vannacci et al., 2004). The mechanisms by which the resting microglia becomes responsive are not fully understood. However, we hypothesize that the NO immediately released by the damaged neurons (the first peak of secreted NO detected in the injured leech CNS with the amperometric apparatus) might stimulate the resting microglial cells present at the lesion site and allow them to be responsive for CB2-like receptor expression. These results suggest that, during the responsive step, leech microglia might express functional CB2 receptors at the cell surface, and that, when these receptors are activated by the 2-AG, NO might be produced and participate in cell recruitment as a chemical gradient from the lesion site towards the periphery. In contrast, the AEA released at the lesion site decreased in concentra-
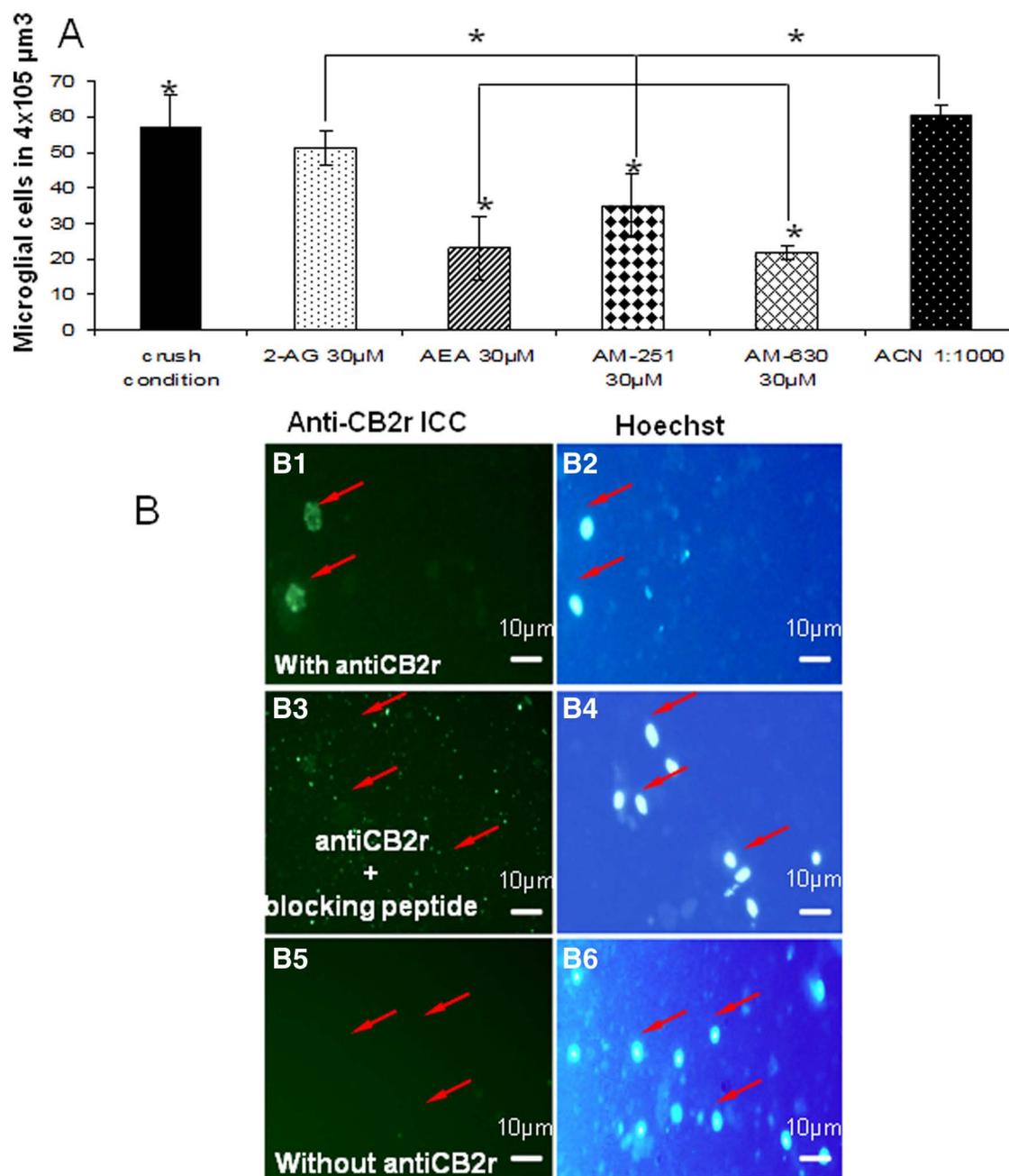
Fig. 5. **A**: Quantification of microglial cells recruited at the lesion site upon drug treatment (microglia nuclei were stained with propidium iodide (5 µg/mL). **B**: Immunochemistry investigation of CB2-like receptor in microglia isolated from *Hirudo* CNS. A rabbit anti-rat CB2r polyclonal antibody, coupled to secondary FITC anti-rabbit Ab, positively stained microglial cells (**B1**). Negative controls were performed following the same protocol either adding a preincubation step mixing the antibody with its selective blocking peptide (**B2**) or using the secondary antibody alone (**B3**). To establish the position of the microglial cells, some of them indicated by arrows, samples were counterstained with the nuclear dye Hoechst 33258 (**B4–B6**). Scale bars = 10 µm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

tion after the lesion, and it can activate NO release by targeting the AEA binding receptor leech TRVP1-like receptor (Meriaux et al., 2011) and inhibiting the accumulation of microglia at the lesion site. This pathway has been described as leading to a microglia stop signal (Salzet, 2000). The dual activity of these two endocannabinoids working in apparent opposition might be a

way to control microglial cell recruitment to the lesion site. However, both molecules control the long-term release of NO, as shown in our time course measurements of NO after stimulation of injured connectives with cannabinoids. This can be explained by the immunosuppressive response of microglial cells described both in vertebrate (Ehrhart et al., 2005) and inverte-
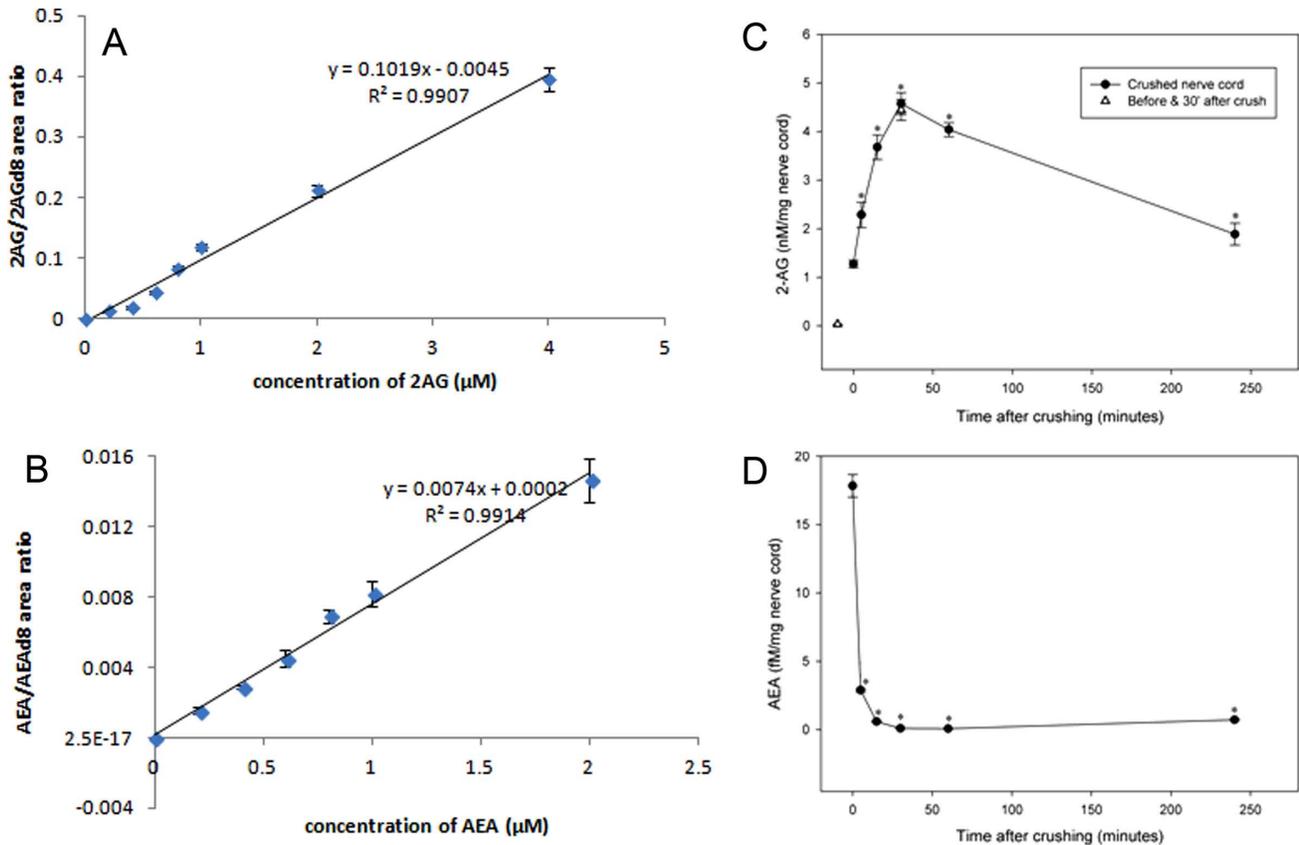
Fig. 6. MALDI TOF measurement of cannabinoids in injured nerve cords. **A** and **B**: Calibration curves for determination of endocannabinoids with plots and parameters of linear regression (see "Materials and Methods"). Curve for determination of 2-AG (A) and AEA (B) in crushed nerve cords. **C** and **D**: Cannabinoids titration: Levels of 2-AG (C) and AEA (D) after the nerve cord was crushed (closed circles, $n = 6$). In a separate set of six animals, the levels before crushing and 30 min after crushing were also measured (open triangles). Asterisks indicate levels significantly different from the first time point, nominally at 0 min, at $P < 0.01$, as described in "Materials and Methods." [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

brate models (Salzet, 2000). This cross-talk pathway between the endocannabinoid and NO systems may be due to the regulation of the CB2-like receptor carried by immune cells of the leech CNS. As a consequence of these observations, all the dynamic measurements of NO produced at the lesion site might be attributed to the movement of the microglia in which CB2-like receptor activation might directly be linked to the activated state of cells after injury. Moreover, the 2-AG has been shown to enhance and drive the chemotaxis of microglial cells in a dose-dependent manner in the mouse microglial cell line BV-2 (Walter et al., 2003). This result is supported by a recent report suggesting that 2-AG could act through the autocrine/paracrine system to chemoattract microglial cells after brain insult (Cabral et al., 2008). Previous pharmacological studies done on rat and mouse macrophage-like cells including microglia showed the CB2 receptor as playing a crucial role in the early inflammatory process, thereby implicating microglia in this process as well (Cabral et al., 2008). The CB2 receptor is expressed very early in the different activation steps of microglia, thus describing a "window" of functional relevance for the expression of the CB2r in the plasma membrane of brain immune cells including microglial cells. During this initial activation period, characterized by morphology variation from resting to responsive cells, microglia acquires CB2r in correlation with chemotaxis and phagocytosis. The next steps of activation, named "primed" and "responsive," respectively, were distinguished from the initial step of CB2 receptor expression. It has been hypothesized that in the mouse microglial cell line BV-2, when the diffusible lipid 2-AG increases, the microglia become responsive, change cell morphology and start to accumulate at the lesion site in relation with the activation of the CB2-like receptor expressed in microglia lamellipodia (Walter et al., 2003). A better understanding of the implications of the endocannabinoid system is currently a very attractive field of investigation because reports have implicated dysregulation of this system in many brain disorders (Maresz et al., 2007; Sinor et al., 2000).

In *Hirudo*, long-term concentration of NO remains in the nanomolar range after injury (largely below 200 nM in the control condition) and can decrease until 50% of the concentration in the control response when
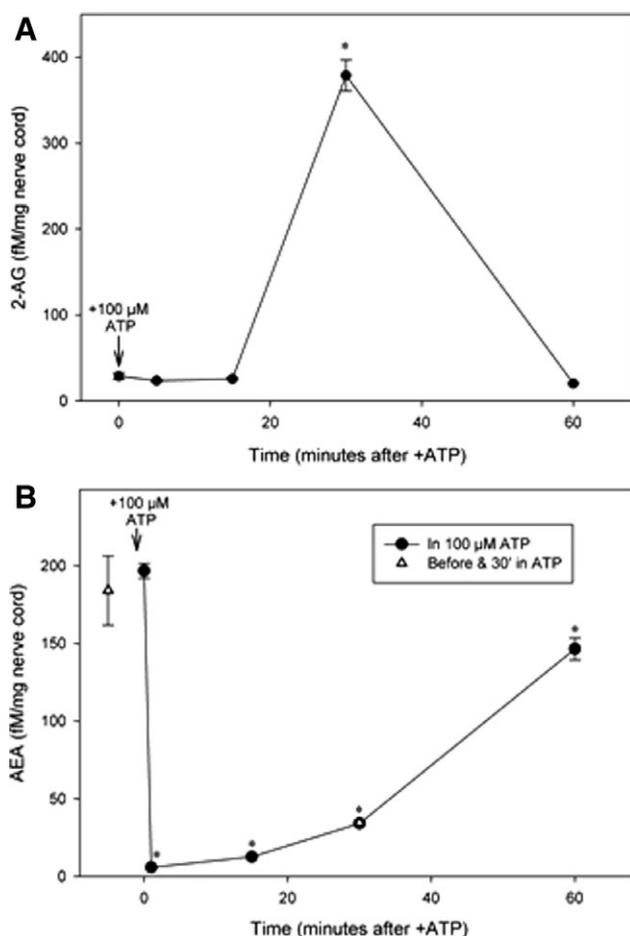
Fig. 7. MALDI TOF measurement of cannabinoids in cords treated with ATP. **A**: Levels of 2-AG after the nerve cord was bathed in 100 μM ATP (closed circles, $n = 5$). **B**: Levels of AEA after the nerve cord was bathed in 100 μM ATP (closed circles, $n = 5$). In a separate set of five animals, the levels before adding ATP and 30 min afterward were also measured (open triangles). Asterisks indicate levels significantly different from the first time point, nominally at 0 min, at $P < 0.01$, as described in "Materials and Methods."
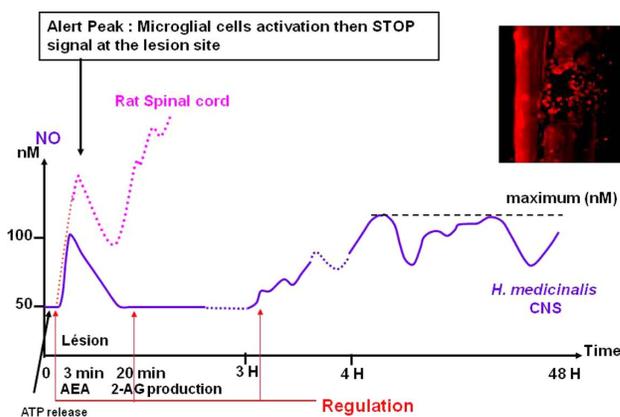

Fig. 8. Scheme of time course of NO release modulation in leech and mammals after nerve cord injury. ATP and endocannabinoids release and microglial cells activation are also represented. An accumulation of microglial cells is depicted at the lesion site of an axotomized leech nerve cord by confocal imaging using propidium iodide. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

an agonist and antagonist of the CB2-like receptor are added concurrently with the lesion. In comparison with the leech, the neuroprotective effects of these endogenous cannabinoids on Vertebrate have been reported in several experimental models including *in vitro* studies in rat (Mechoulam, 2002; Nagayama et al., 1999) and *in vivo* studies led in rat and mouse models (Pryce et al., 2003; Veldhuis et al., 2003). However, controversial effects have been widely ascribed to the well-studied endocannabinoid AEA. Previous studies described them with inhibition of the neuroinflammation process and neuroprotection via activation of the MKP-1-related CB1 receptor pathway in microglial cells during inflammation of the vertebrate CNS (Eljaschewitsch et al., 2006). In contrast, a high concentration of AEA after an injury in the vertebrate CNS can also activate another type of receptor named transient vanilloid receptor 1 (TRPV1), by which AEA can enhance proinflammatory with nociceptive and neurotoxic effects in the rat CNS (Kim et al., 2007). Some new data in leech

nociception studies performed by Burell group seems to indicate that endocannabinoids can modulate nociceptive synaptic transmission through a TRVP-like receptor activation (Li and Burrell, 2009, 2010). In recent screening of *Hirudo* genome, three TRVPs have been identified (Meriaux et al., 2011). We also established that leech nerve cord segments placed in culture, subjected to different concentrations of AEA, 2-AG, capsaicin, arvanil, and capsazepin, and checked for neurite outgrowth from a cut connective nerve at various intervals after lesion shown neurites sprouting in less than a week for the control preparations and in less than 3 days for preparations exposed to either 2-AG, capsaicin, and arvanil, whereas no outgrowth was observed with exposure to AEA or capsazepin (Meriaux et al., 2011). For the latter molecules, a repair process has apparently occurred, because a brown coloration can be observed that indicates a wound healing process is taking place instead of neuronal regeneration. The data argue in favor of the involvement in the long-term regeneration process of leech TRPV1-like receptor (Meriaux et al., 2011) and a balance between TRPV and G-protein-coupled receptors (GPCRs) expressing binding sites to endocannabinoid agonists and antagonists.

CONCLUSIONS

Taking into account all the data, we suggest that in the leech, after injury, endocannabinoids are released at different levels compared with those in the mammalian CNS. Moreover, because these compounds are both derived from arachidonic acid and have similar structures, an interesting possibility is that AEA and 2-AG modulate the attraction of microglia by each binding to different receptor that mediates a different response. Taken together, our results show that in the leech, con-

current with NO production and microglia recruitment, a fine imbalance occurs in the endocannabinoid system (Fig. 8). These events may promote neuronal survival by regulating immune functions such as NO production in the microglia. This data can bring explanations about the ability of the leech CNS to regenerate after a trauma and also further insights to explain the limited regeneration in mammalian CNS.

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