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Resolvin D1 Halts Remote Neuroinflammation and Improves Functional Recovery after Focal Brain Damage Via ALX/FPR2 Receptor-Regulated MicroRNAs

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Abstract

Remote damage is a secondary phenomenon that usually occurs after a primary brain damage in regions that are distant, yet functionally connected, and that is critical for determining the outcomes of several CNS pathologies, including traumatic brain and spinal cord injuries. The understanding of remote damage-associated mechanisms has been mostly achieved in several models of focal brain injury such as the hemicerebellectomy (HCb) experimental paradigm, which helped to identify the involvement of many key players, such as inflammation, oxidative stress, apoptosis and autophagy. Currently, few interventions have been shown to successfully limit the progression of secondary damage events and there is still an unmet need for new therapeutic options. Given the emergence of the novel concept of resolution of inflammation, mediated by the newly identified ω3-derived specialized pro-resolving lipid mediators, such as resolvins, we reported a reduced ability of HCb-injured animals to produce resolvin D1 (RvD1) and an increased expression of its target receptor ALX/FPR2 in remote brain regions. The in vivo administration of RvD1 promoted functional recovery and neuroprotection by reducing the activation of Iba-1+ microglia and GFAP+ astrocytes as well as by impairing inflammatory-induced neuronal cell death in remote regions. These effects were counteracted by intracerebroventricular neutralization of ALX/FPR2, whose activation by RvD1 also down-regulated miR-146b- and miR-219a-1-dependent inflammatory markers. In conclusion, we propose that innovative therapies based on RvD1-ALX/FPR2 axis could be exploited to curtail remote damage and enable neuroprotective effects after acute focal brain damage.

Keywords Specialized pro-resolving mediators · Inflammation resolution · Neuroinflammation · Remote brain damage · Epigenetics

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Abbreviations

GFAP	Glial fibrillary acidic protein
HCb	Hemicerebellectomy
Rv	Resolvin
RvD1	Resolvin D1
SPM	Specialized pro-resolving mediators

Introduction

A primary brain damage of the central nervous system (CNS), as in traumatic brain and spinal cord injuries, often results in severe functional impairment that is highly dependent on the secondary damage, a cascade of reactive changes occurring in response to the primary insult. Major components of secondary injury are inflammatory responses, including activation of resident glial cells and generation of pro-inflammatory

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mediators [1]. These secondary events amplify the effect of the primary injury and continue in the weeks following the initial insult also in distant areas that are partially affected or unaffected by the primary damage [2, 3] and are critical for the overall clinical profile in many acute CNS pathologies.

This last delayed phenomenon, termed "remote damage" [4], can last for days, weeks, or months and is sustained by many factors, including etiology, alterations of mitochondrial dynamics, autophagy, glial inflammation, and oxidative stress [4–8].

These events are mediated by the up-regulation of genes that play a key role in both the damage and repair of injured neural tissue and whose critical balance between their pro- and anti-survival effects determines injury progression and outcome.

Among the remote damage-associated mechanisms, inflammation is central to death/survival choices [3]. Indeed, inflammation is fundamentally a protective cellular response aimed at removing injurious stimuli and initiating the healing process, but when unresolved, it can become chronic, resulting in organ dysfunction [9, 10] and causing further inflammation and damage also in distally remote regions [6]. Although knowledge of such remote damage-associated mechanisms has considerably improved, there is still an unmet need for new therapeutic options. Hence, the identification of mediators limiting the inflammation and/or involved in the resolution of inflammation is of growing interest and it may provide novel targets for these conditions.

In this context, previously unrecognized metabolites, termed specialized pro-resolving lipid mediators (SPMs), temporally and spatially synthesized from ω-3 essential fatty acids (eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA]), were recently identified as potent mediators that control the magnitude and extent of inflammatory events by activating local resolution programs [11, 12]. Among these, DHA-derived D-series resolvins have received considerable attention in recent years due to their ability to reduce inflammation in different disease models such as kidney injury and cardiovascular and autoimmune disorders [13]. Indeed, recent studies have reported that dietary supplementation of DHA or in vivo administration of DHA-derived metabolites are neuroprotective, attenuate inflammatory responses, and promote functional recovery in several murine models of brain injury [14, 15], including spinal cord injury (as reviewed in [16]).

In this study, we examined the alterations in the "resolution of inflammation" pathway, in particular of two major D-series resolvins, i.e., resolvin D1 (RvD1) and resolvin D2 (RvD2) and the pharmacological effects of resolvins in an in vivo model of focal CNS lesion, focusing on remote changes that are induced by hemicerebellectomy (HCb). HCb is a well-known and highly reproducible model that is used to study remote cell death [8]. In this model, neuronal degeneration is induced by target deprivation and axonal damage of contralateral neurons of the inferior olive (IO) and pontine nuclei (Pn) [8]. Herein, we found that the pro-resolution pathway of RvD1 is altered in HCb-lesioned rats and that the pharmacological treatment of RvD1 promotes functional recovery and protects HCb-lesioned rats against remote neuronal cell death and neuroinflammation.

Materials and Methods

Animals and HCb Lesion

Experiments were performed using 80 adult (70–80 days) male Wistar rats (200–220 g) group-housed in standard cages and maintained under a 12 h light–dark cycle in an air-conditioned facility. The Italian Ministry of Health approved the experimental protocol (authorization no. DM444-2015 PR) in agreement with the guidelines of the European Communities Council Directive 2010/63/EU for the care and use of laboratory animals. All efforts were made to minimize the number of animals used and their suffering. For surgical procedures, the rats were anesthetized by intraperitoneal (i.p.) injections of xylazine (Rompun, 10 mg/kg) and tiletamine and zolepam (Zoletil 100, 25 mg/kg) in a stereotaxic apparatus. The skin of the skull was incised, the occipital bone was drilled and removed, the dura mater incised to expose the cerebellum, and hemicerebellectomy (HCb) was induced by removal of the right cerebellar hemisphere, as described previously [4, 5, 17]. For the control (CTRL) group, surgery was interrupted after the dura lesion was made, and after suturing, the animals were returned to their cages. The different experimental groups are listed in Table 1.

Drugs and Treatments

For the in vivo studies, the following pro-resolving lipid mediator was used: resolvin D1 (7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid, Cayman Chemicals; 0.4 μg /kg, i.p.). Further, the following neutralizing antibody was used: ALX/FPR2 (50 μg/kg, i.c.v. on day 3). All the animals that were treated with RvD1 received one i.p. of RvD1 after HCb lesion and then again every 2 days (at days 3, 5, and 7), whereas a single intracerebroventricular injection (i.c.v.) of anti-ALX/FPR2 at day 3. Treatments in the different experimental groups are listed in Table 1 and antibodies used in Supplementary Table 2.

Detection of Resolvins

CSF from CTRL and HCb animals was kept at − 80 °C. The levels of RvD1 and RvD2 were measured through quantitative competitive Cayman's ELISA kits, based on the competition between free RvD1/RvD2 Tracers for a limited number of

Table 1 Lesion and treatments in the different experimental groups

Groups	Number	Treatment
Ctrl (CTRL)	16	/
Ctrl + resolvin D1 (RvD1)	14	Resolvin D1 0.4 μg/kg, i.p. on days 0, 3, 5, and 7
HCb 1 day	3	/
HCb 3 days	3	/
HCb 5 days	3	/
HCb 7 days	16	/
HCb 7 days + resolvin D1 (HCb + RvD1)	16	Resolvin D1 0.4 μg/kg, i.p. on days 0, 3, 5, and 7
HCb 7 days + resolvin D1 + ALX/FPR2 (HCb + RvD1 + anti-ALX/FPR2)	9	Resolvin D1 0.4 μg/kg, i.p. on days 0, 3, 5, and 7 and ALX/FPR2 0.75 ng/kg, i.c.v. on day 3

CTRL control, *HCb* hemicerebellectomy, *RvD1* resolvin D1, *ALX/FPR2* annexin lipoxin/*N*-formyl peptide receptor 2, *i.p.* intraperitoneal, *i.c.v.* introcerebroventricular

RvD1/RvD2-specific rabbit antiserum binding sites, according to standard procedure (assay sensitivity of 15 pg/ml).

Neurological Evaluation

The Neurologic Severity Score (NSS) was used to evaluate neurological conditions in rats. NSS is a composite of motor, sensory, reflex, and balance tests in which, for each test, one point is awarded for the inability to perform or for the lack of a tested reflex, and zero points are awarded for success. The NSS was evaluated at 24 and 72 h and 5 and 7 days after damage by an investigator who was blind to the experimental groups, as reported [4, 5, 17].

Histology and Immunohistochemistry

Anesthetized animals were perfused transcardially with 4% paraformaldehyde, brains removed immediately, post-fixed in the same paraformaldehyde solution, and transferred to 30% sucrose solution at 4 °C. A series of sections (30-μm-thick) involving pontine nuclei (Pn) were processed for immunohistochemical studies. Following incubation with a solution of primary antibodies, the sections were incubated for 2 h at room temperature with specific secondary antibodies. Sections were examined under a confocal laser scanning microscope (Zeiss LSM700) and analyzed through qualitatively and quantitatively and also by means of Sholl analysis as reported [18] and detailed below.

Qualitative and Quantitative Analyses

Qualitative and quantitative observations were limited to the Pn of the experimental side that was projecting to the lesioned hemicerebellum. Using the Stereo Investigator System (MicroBrightField Europe e.K.), an optical fractionator, stereological design, was applied to obtain unbiased estimates of total Nissl-stained cells. A stack of MAC 5000 controller modules (Ludl Electronic Products Ltd) was configured to interface an Olympus BX 50 microscope with a motorized stage and a HV-C20 Hitachi color digital camera with a Pentium II PC workstation. A three-dimensional optical dissector counting probe (x, y, z dimension of 30 × 30 × 10 μm, respectively) was applied. Five sections for each specimen were analyzed, and Pn was outlined using the × 5 objective, while the × 100 oil immersion objective was used for marking the neuronal cells. The total Pn cell number was estimated according to the formula

$$N = \Sigma Q \times 1/\mathrm{ssf} \times 1/\mathrm{asf} \times 1/\mathrm{tsf}$$

where ΣQ represents the total number of neurons counted in all optically sampled fields of the Pn, ssf is the section sampling fraction, asf is the area sampling fraction, and tsf is the thickness sampling fraction. All quantitative analyses were conducted blinded to the animal's experimental group identity.

Sholl Analysis

Microglia in the Pn were imaged using an optical microscope (DMLB, Leica) equipped with a motorized stage and a camera connected to software (Neurolucida 7.5, MicroBright-Field) that allowed a quantitative 3D analysis of the entire compartment. Only microglia that displayed intact processes unobscured by background labeling or other cells were included in reconstructions. Five cells per animals per group were randomly selected for a total of 60 cells included for analysis. The cell body area, perimeter, number of intersection, number of nodes (branch points), and total length of all processes were measured. To account for changes in the cell's complexity in relation to distance from the cell soma, Sholl analyses were performed for each microglial cell. Concentric circles (radii) were spaced 10 μm apart, originating from the soma. The number of branch points (nodes), processes that intersected the radii, and process length were measured as a function of the distance from the cell soma for each radius [18].

Western Blotting

Total proteins were extracted in RIPA buffer whereas cytosolic proteins in hypotonic buffer. Samples were subjected to SDS–PAGE, transferred to nitrocellulose filters, and immunoreacted with specific primary antibodies and then with the appropriate horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were detected by CDiGit Chemiluminescent Western Blot Scanner (LI-COR Biosciences), as reported [19].

Real-Time qPCR of MicroRNA and Target Genes

TriReagent (Invitrogen, Thermo Scientific, CA, USA) was used to isolate total RNA from rat brain remote regions. MicroRNA analysis was carried out in triplicate with the TaqMan® Individual microRNA assays (Applied Biosystems) according to the manufacturer's instructions. Amplification signal detection was carried out using the Applied Biosystems ViiA 7 Real-Time PCR System. MicroRNAs that displayed threshold cycles (Ct) > 33 were excluded from the analysis. Results were analyzed using the $2^{-\Delta Ct}$ relative quantification method using the small non-coding RNA 4.5S RNA(H) as reference housekeeping microRNA and normalized to β-actin as endogenous control, as reported [20].

Bioinformatics Analysis

MicroRNA.org [21] (http://www.microrna.org/microrna/home.do) database was queried for microRNA target genes, where microRNA-target interactions were predicted with miRanda 3.3a and scores were calculated with mirSVR (< 0. 1) [22].

Statistical Analysis

Statistical analysis was performed through GraphPad Prism 6.0 (GraphPad software for Science, San Diego, CA). All data were expressed as means ± SEM. Differences between groups were compared using Student's *t* test (two groups) or one-way or two-way ANOVA (multiple groups) followed by a Bonferroni post hoc test. The criterion for statistical significance was $P < 0.05$ or less.

Results

The Pro-Resolution Pathway of RvD1 Is Altered in HCb-Lesioned Rats Since several evidences reported extensive neuroinflammatory and neurodegenerative changes upon HCb lesion at remote regions (Fig. 1a), we first wondered whether injured animals exhibited possible alterations in the "resolution of inflammation" pathway, by assessing the levels of two major D-series resolvins, i.e., resolvin D1 (RvD1) and resolvin D2 (RvD2), and the expression of their target receptors. Analyzing the CSF of lesioned (HCb) and control (CTRL) animals after 7 days of injury, we observed a reduction in RvD1 and RvD2 levels, particularly significant for RvD1 (Fig. 1b). By contrast, no variation in the levels of both pro-resolving lipid mediators upon HCb injury was observed in the periphery, namely in plasma (Fig. S1A). Thus, we next investigated the ability of HCb animals to respond to RvD1 and to activate pro-resolution programs in remote brain regions (i.e., pontine nuclei, Pn) by measuring the expression of its target receptor ALX/FRP2 also after 7 days of injury. Western blotting analysis revealed a significant up-regulation of ALX/FPR2 during HCb (Fig. 1c), which was also corroborated by immunohistochemical and confocal analysis, showing a markedly stronger staining of this receptor in HCb animals compared to CTRL brains. Interestingly, double immunofluorescence of ALX/FPR2 and GFAP, a marker of astrocytes, demonstrated that ALX/FPR2 was strongly induced exclusively in reactive astrocytes (Fig. 1d) whereas double-labeling of ALX/FPR2 with Iba1, a microglial marker or with NeuN, a neuronal marker, showed that neither microglial (Fig. S2) nor neuronal (Fig. S2B) cells did express ALX/FPR2 in Pn.

RvD1 Promotes Functional Recovery and Protects HCb-Lesioned Rats against Remote Neuronal Cell Death and Neuroinflammation Given the alteration of RvD1 pathway during HCb, in terms of its production and expression of its key molecular targets, we next tested the pharmacological actions of RvD1 in HCb animals (Table 1). Upon HCb, rats were evaluated for neurological conditions as measured by Neurological Severity Score (NSS) 24 h after lesion and then every 2 days in presence or absence of i.p. injection of RvD1 (0.4 μg/kg at days 0, 3, 5, and 7) (Fig. 2a). We found that systemic treatment with RvD1 (HCb + RvD1) promoted functional recovery as early as 3 days after lesion compared to untreated animals (HCb) (Fig. 2b). This beneficial effect on neurological symptoms was maintained also at days 5 and 7, whereby HCb animals receiving RvD1 treatment significantly showed constantly reduced NSS scores. Furthermore, against a marked reduction in neuronal cells, paralleled by an increase in cytochrome-c (Cyt-c) release during HCb, animals receiving RvD1 displayed a neuroprotective profile, inasmuch as RvD1 protected Pn neurons from HCb-induced degeneration by increasing neuronal survival (Fig. 2c) and by reducing Cyt-c expression and release (Fig. 2c, d). These neuroprotective effects were also coupled with a marked reduction of HCb-induced reactive astrocytes and microglia. Accordingly, we observed a significant decrease in the number of GFAP-positive astrocytes and of Iba1-positive microglial cells in RvD1-treated animals as shown both by immunohistochemical (Fig. 3a) and western blotting (Fig. 3b) analyses. Intriguingly,

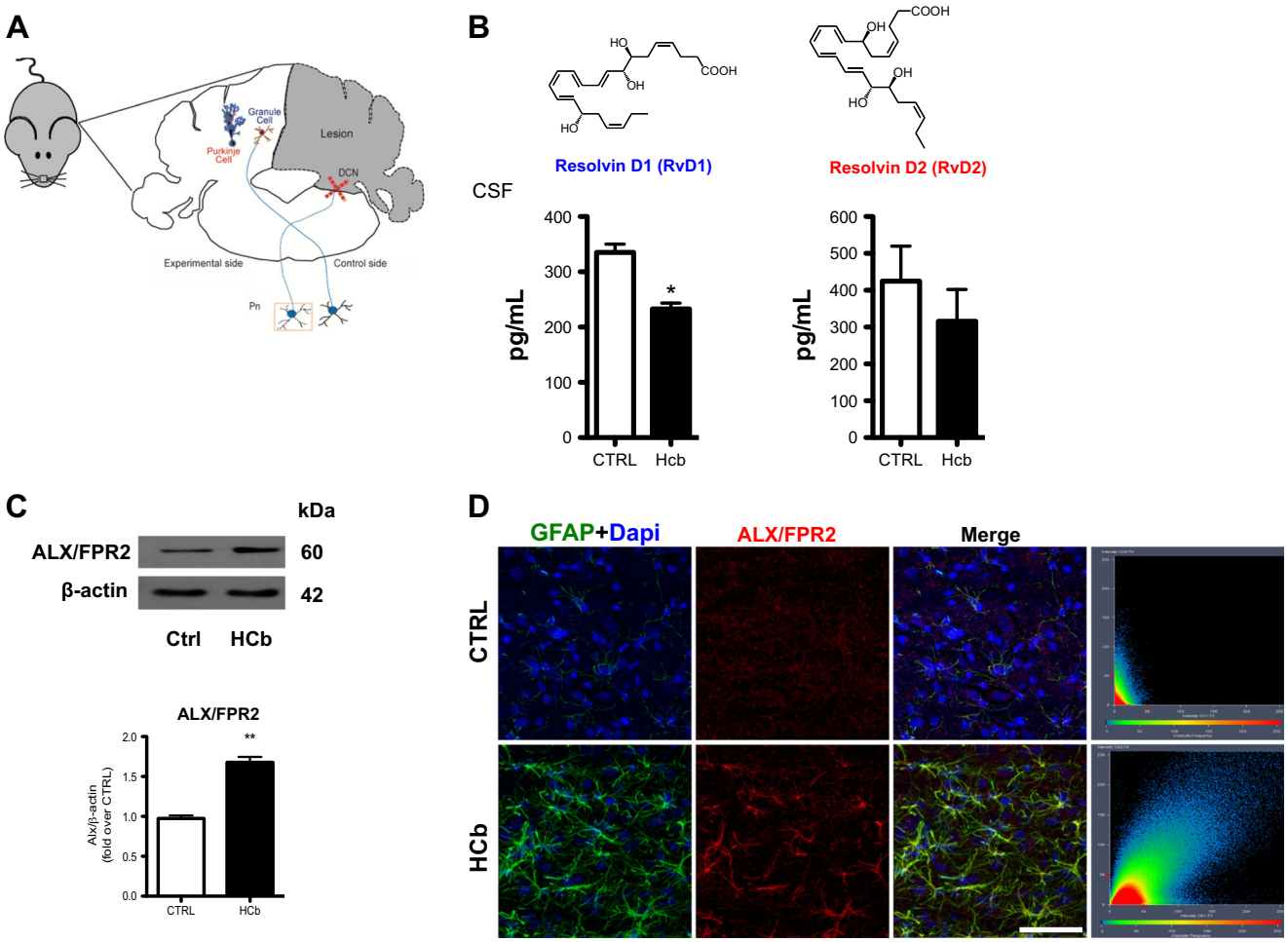


Fig. 1 The pro-resolving pathway of RvD1 is altered in HCb. **a** Schematic of the hemicerebellectomy (HCb) model. Due to the crossed input–output organization of the cerebellar connections, unilateral lesion of a cerebellar hemisphere induces axonal lesions and subsequent degeneration of the contralateral (experimental side) pontine nuclei (Pn), with sparing of Pn on the ipsilateral side (control side). **b** Quantification of RvD1 and RvD2 levels in cerebrospinal fluid (CSF) of CTRL and HCb animals by ELISA. $^{*}P < 0.01$ by Student's *t* test. **c** Western blotting bands and densitometry of ALX/FPR2 receptors in Pn. $^{**}P < 0.01$ by Student's *t* test. **d** Immunofluorescence staining of ALX/FPR2 receptor. Double-labeled and merged confocal images of GFAP (green) plus DAPI counterstaining (blue) and ALX/FPR2 receptor (red) in Pn of CTRL and HCb animals. The last panel represents the graphic representation (scatter plot) of the correlation coefficient of Pearson (PCC) for quantifying the co-localization between the ALX/FPR2 and GFAP in CTRL and HCb animals (PCC = 0.56 in HCb vs PCC = 0.00 in CTRL). Higher PCC values correspond to a strong co-localization (Scale bar = 50 μm). Results are mean ± SEM or representative of $N = 6$ rats

when attentively observing the morphology of Iba-1+ microglial cells, we noticed that RvD1 treatment was associated with morphological changes. Thus, we performed Sholl analysis and found that at 7 days after HCb microglial cells of axotomized Pn displayed a higher degree of complexity and ramification compared to CTRL, presenting a larger cell body (Fig. 3c, gray arrowhead) and thicker and more branched processes (Fig. 3c, white arrowhead), as well as longer ramifications, increased number of intersections and nodes, larger ramification perimeter, and area (Fig. S4A-E). Conversely, treatment with RvD1 showed a de-ramified morphology compared to HCb, showing significantly thinner and less ramified processes (Fig. 3c). Similarly, microglia process length, intersections, and nodes, as well as total perimeter and area, were significantly reduced in RvD1-treated animals (Fig. S4A-E).

RvD1 Regulates the Expression of Pro-Resolving MicroRNAs and of their Target Genes in HCb-Lesioned Rats Since selected microRNA (miR-21, miR-142, miR-146b, miR-208, miR-219, and miR-203) profiles have been shown to be temporally regulated by RvD1 and ALX/FPR2 in acute self-limited inflammation in mice [23], we asked whether they could also be involved in the neuroprotective and anti-inflammatory effects exerted by RvD1 in the HCb model. Thus, we investigated the expression of microRNA in the remote regions of HCb and HCb + RvD1-treated animals, finding that all the candidates examined were detectable, except miR-208. Among the different candidates, only miR-146b and miR-219a-1-3p were significantly up-regulated in HCb + RvD1-treated animals compared to HCb animals (Fig. 4b). Next, we

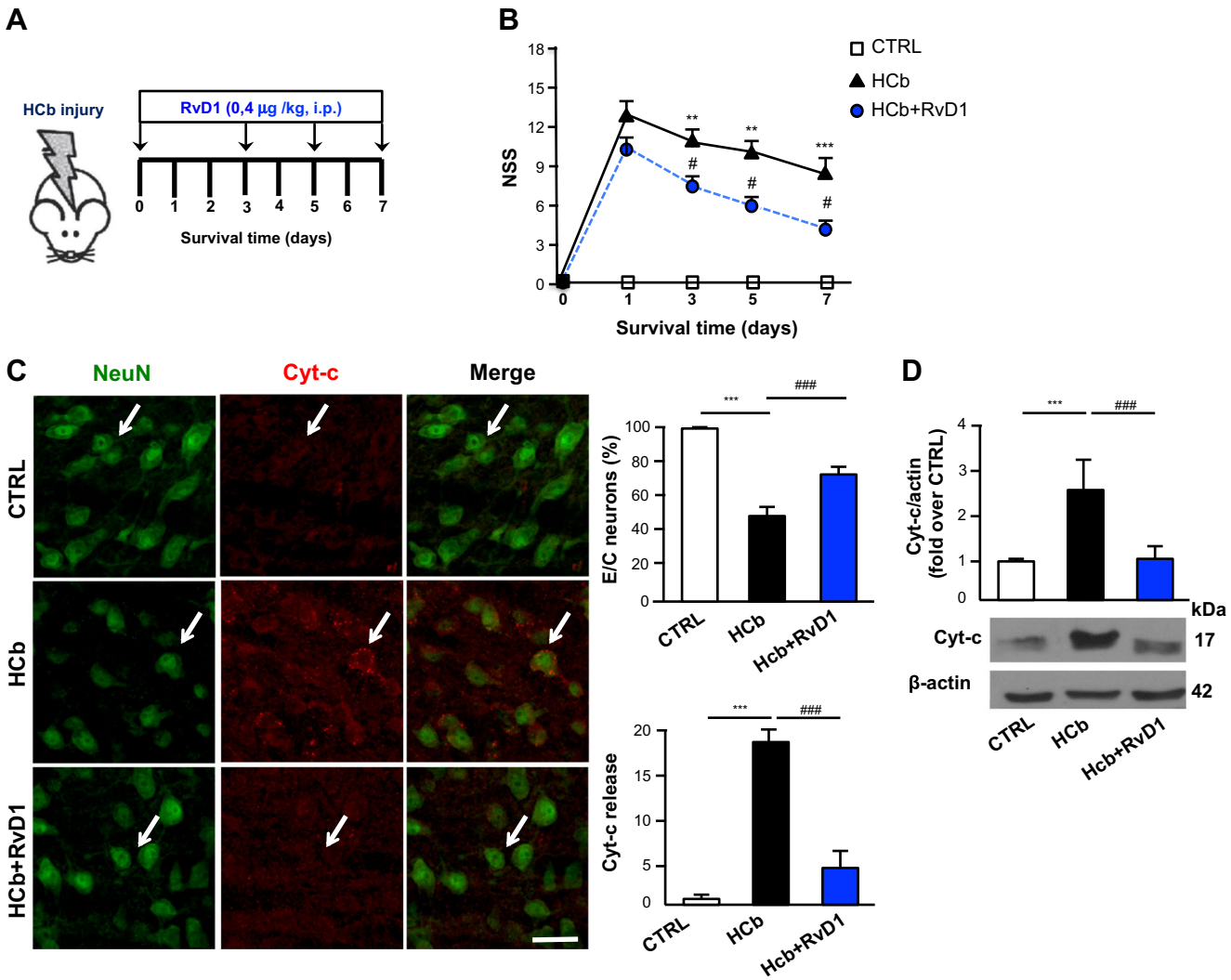


Fig. 2 RvD1 promotes functional recovery and protects from neuronal cell death. HCb rats were left untreated or treated with RvD1 at days 0, 3, 5, and 7 (0.4 µg /kg, i.p.). **a** Treatment protocol employed in the study. **b** Time course of neurological recovery (NSS). $^{**}P < 0.01$ and $^{***}P < 0.001$ (CTRL vs HCb) and $\#P < 0.05$ (HCb vs HCb + RvD1) by two-way ANOVA followed by Bonferroni's post hoc test. **c** Immunofluorescence staining and merged confocal images of NeuN-positive neurons (green) and cytochrome-c (cyt-c, red) in Pn of CTRL, HCb, and HCb + RvD1 animals (scale bar = 25 µm). $^{***}P < 0.001$ vs CTRL and $^{\#\#\#}P < 0.001$ vs HCb. **d** Western blotting bands and densitometry of cyt-c. $^{***}P < 0.001$ vs CTRL, $^{\#\#\#}P < 0.001$ vs HCb by one-way ANOVA followed by Bonferroni's post hoc test. Results are mean ± SEM for $N = 6$ rats

sought to investigate some of the gene targets that could be regulated by miR-146b and miR-219a-1-3p. To do so, the microRNA.org database was queried to search for putative mRNA targets regulated by the microRNAs of interest (Fig. 4a). Among these, we focused on those genes with functions in immune systems, namely, TLR4 under the regulation of both miR-219-1-3p and miR-146b as well as CD200 under the regulation of miR-219-1-3p. In addition, we also investigated NF-kB and IL6R, known to be targeted by miR-146b according to the literature [23, 24]. As shown in Fig. 4c, RvD1 treatment was associated with a significant reduction in IL6R and TLR4. These findings suggest that RvD1-induced protective effects in HCb might be associated with modulation of specific microRNA-regulated inflammatory genes (Supplementary Table 3).

RvD1 Neuroprotective Effects Are Mediated by ALX/FPR2

Since ALX/FPR2 was significantly up-regulated in Pn during HCb (Fig. 1c–e), we hypothesized that it could play a critical role in mediating RvD1-induced protective effects. Thus, we investigated the effects of inhibiting ALX/FPR2 downstream pathways. In order to choose the appropriate time-point at which pharmacologically antagonizing RvD1 receptor, we first assessed its time-course expression during HCb lesion. The western blotting analysis of ALX/FPR2 revealed a bell-shaped expression, with a significant up-regulation at 3 and 5 days after HCb lesion and a down-

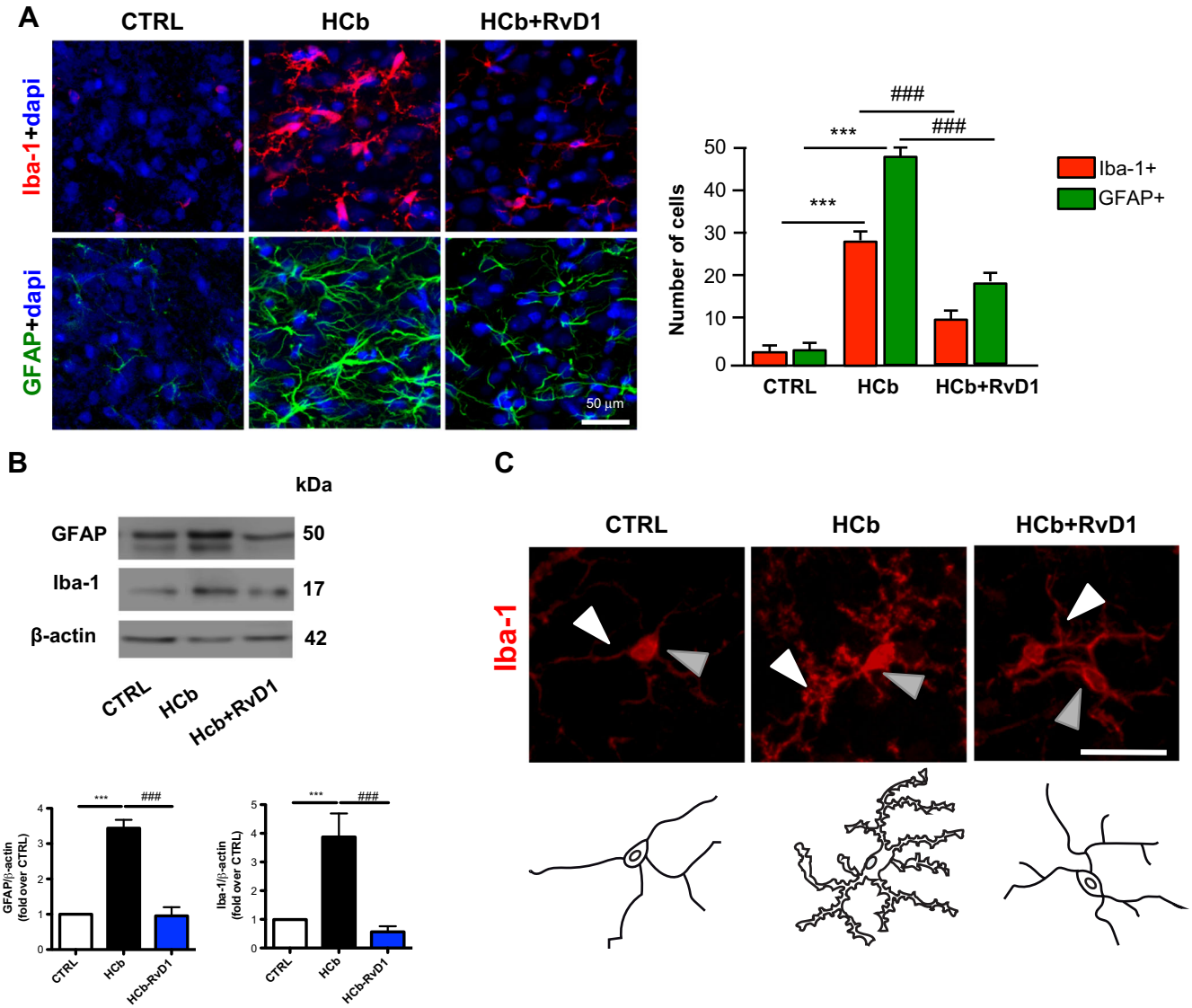


Fig. 3 RvD1 reduces microglia and astrocyte responses. **a** Immunofluorescence staining of microglia and astrocytes upon RvD1 treatment. Double-labeled and merged confocal images of Iba-1 (red) plus DAPI counterstaining (blue) (upper panel) and GFAP (green) plus DAPI counterstaining (blue) (lower panel) in Pn of CTRL, HCb, and HCb + RvD1 animals (scale bar = 50 µm). $^{***}P < 0.001$ vs CTRL; $^{\#\#\#}P < 0.001$ vs HCb. **b** Western blotting bands and densitometry of Iba-1 and GFAP. $^{***}P < 0.001$ vs CTRL. $^{\#\#\#}P < 0.001$ vs HCb by one-way ANOVA followed by Bonferroni's post hoc test. **c** Immunofluorescence staining of microglia upon treatment with RvD1. Single labeled images of Iba-1 in Pn of CTRL, HCb, and HCb + RvD1 groups (scale bar = 25 µm) and representative images of 3D-reconstructed microglia phenotypes. Results are mean ± SEM or representative of $N = 6$ rats

regulation at day 7 (Fig. S3). Thus, we decided to neutralize ALX/FPR2 receptor at day 3, being the first time-point at which its protein level was significantly evident in axotomized Pn. Intracerebroventricular administration of neutralizing ALX/FPR2 antibody at day 3 in presence of systemic treatment with RvD1 significantly prevented the functional recovery observed in HCb animals treated with RvD1, when compared to HCb (Fig. 5a). Furthermore, it completely abolished the effects of RvD1 in reducing the density of Iba1 and GFAP immunoreactive cells (Fig. 5b). Interestingly, the RvD1-induced changes in the morphology of activated microglia during HCb were also counteracted upon neutralization of ALX/FPR2, with microglial cells returning back to a more ramified and hypertrophic morphology (Figs. 5c and S4A-E). Additionally, to test whether ALX/FPR2 was involved in mediating the RvD1-induced neuroprotective effects via modulation of miR-146b and miR-219a-1-3p, we also evaluated whether its neutralization could modulate the microRNA-regulated circuits previously shown to be affected by RvD1. In particular, blocking ALX/FPR2 not only significantly down-regulated both microRNAs (Fig. 5d) but also up-regulated IL6R and TLR4 (Fig. 5e, f), suggesting that RvD1 exerts its neuroprotective effects via ALX/FPR2 receptor and its

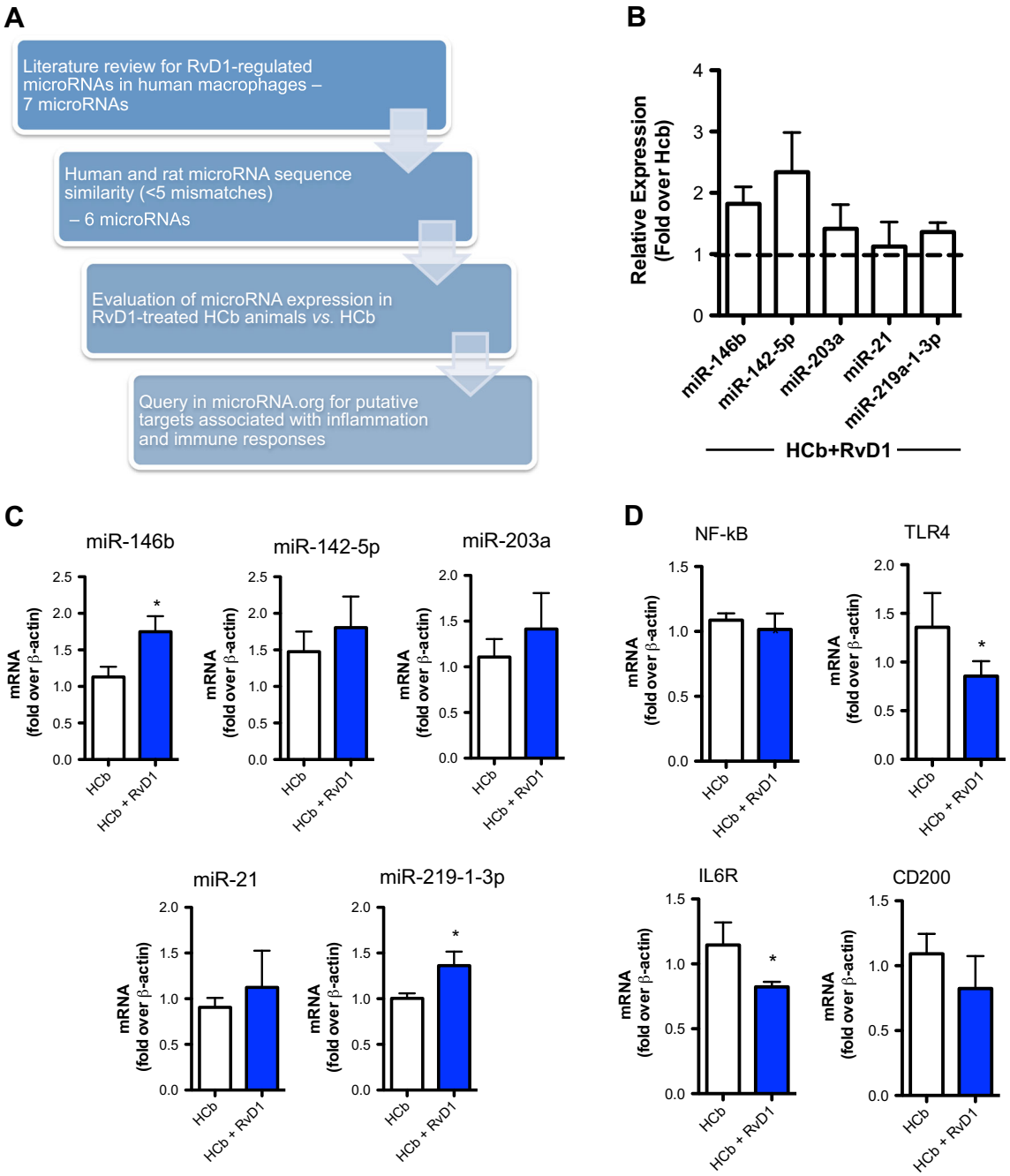


Fig. 4 RvD1 down-regulates the expression of pro-inflammatory markers regulating specific microRNAs. **a**, **b** qRT-PCR of selected microRNAs. *$P < 0.05$ vs HCb by one-way ANOVA followed by Bonferroni's post hoc test. **c**, **d** qRT-PCR of the chosen target genes (via bioinformatics tools) of the selected microRNAs (broken lines represent control values). *$P < 0.05$ vs HCb by Student's t test. Results are mean ± SEM for $N = 6–8$ rats

regulated microRNAs that mainly impact on the neuroinflammation induced by HCb injury (Fig. 5e).

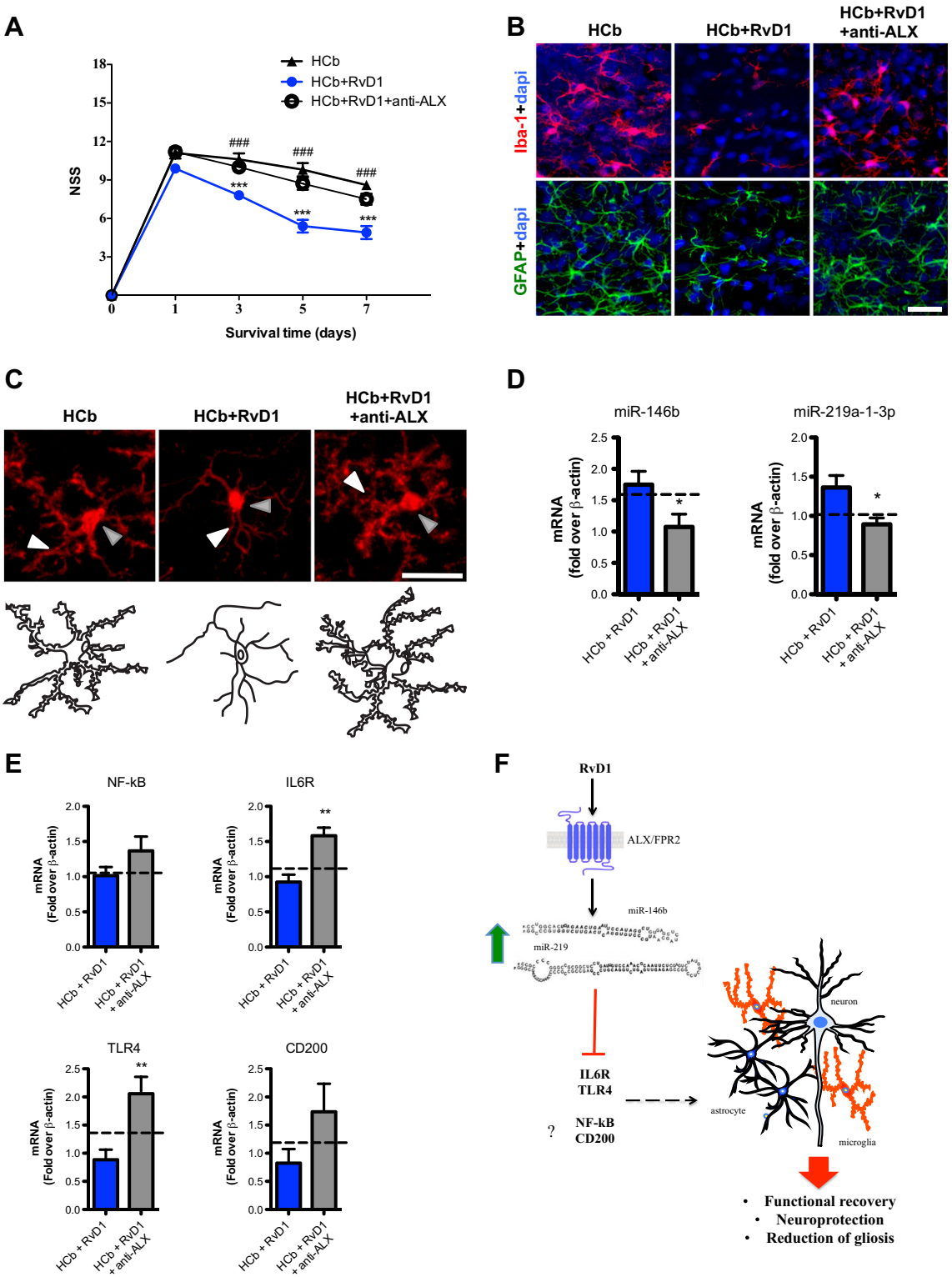
Discussion

In the present work, we reported for the first time that RvD1, a specialized pro-resolving lipid mediator (SPM) derived from DHA, promotes functional recovery and halts glial activation and neuronal death in an in vivo model of remote damage. Over the past 20 years, there has been an increased interest in the health benefits and therapeutic potential of omega-3 polyunsaturated fatty acids (ω-3 PUFAs) in neurodegeneration and neuroinflammation due to their anti-oxidant, anti-inflammatory, anti-apoptotic, and neuroprotective properties [14, 15], making them safe and very strong candidates for rapid

translation to the clinic [25, 26]. Interestingly, it seems that only DHA but not EPA exerts neuroprotective and anti-inflammatory effects in murine models of SCI [27], suggesting that DHA-derived bioactive metabolites could be responsible for such actions. In this study, we used the HCb model, a reliable and valid paradigm in which neuronal degeneration can be investigated remotely from the primary site of injury, where effects of the primary damage can confound the study of neuropathological changes such as neuronal apoptotic cell death and neuroinflammation [5, 8]. Herein, we asked if after brain damage, an alteration of the pro-resolution program mediated by specific SPMs might occur in remote regions and its possible role on remote damage and on functional recovery.

Our data reporting reduced CSF levels of RvD1, and to a lesser extent of RvD2, coupled with an increased expression of ALX/FPR2 receptor, demonstrating for the first time that injured animals undergo a profound reorganization of the "pro-resolving system" in terms of endogenous lipid mediators and their molecular targets. Of note, the up-regulation of ALX/FPR2 in the Pn was exclusively on astrocytes while neuronal and microglial cells seemed to lack this receptor as shown in the immunohistochemistry data. This observation extends previous findings on the increased levels of glial ALX/FPR2 in the injured brain of Alzheimer's disease patients [28], where it seems that this glial receptor is involved in amyloid-β internalization [29]. Furthermore, in the study of Wang and colleagues, the presence of ALX/FPR2 was also observed in pyramidal neurons in different regions of the hippocampus, with a particular role in promoting migration and differentiation of neural stem cells in both rats and mice [30, 31]. Our lack of detection of this receptor on axotomized neuronal cells and on microglia suggests that the heterogeneity in the pathophysiology, the type of neuronal population affected, and the species considered may account for the observed differences. Furthermore, its marked expression on astrocytes also suggests that these cells could indirectly mediate the observed RvD1-induced neuroprotective effects. Indeed, astrocytes are the most abundant cell types in the whole CNS and regulate almost every physiological aspect of the brain, from nutritional and neuro-signaling support, synaptic plasticity, and constitution of the blood brain barrier [32] to also immune regulation through astrocyte-microglia cross talk [33].

The alteration of the pro-resolving system observed in our model led us to assume that in vivo administration of RvD1 could resolve some neuropathological hallmarks occurring in remote brain areas after injury and on functional recovery. Indeed, not only systemic RvD1 treatment of injured animals attenuated neuronal cell death and promoted functional recovery, with animals showing a significantly higher neuronal survival and neurological recovery, but also strongly reduced gliosis, with astrocytes and microglial cells being less abundant compared to injured brains. This is in line with recent evidence that several SPMs, including RvD1, are indeed neuroprotective and improve neuronal survival in vitro [34]. Moreover, we also observed that the treatment with RvD1 somehow significantly affected microglia morphology, reducing the HCb-induced transition into more ramified and thick processes. Although still controversial, microglia are dynamic cells whose morphological changes, which occur at different developmental stages, seem to be associated with their functional activities [35]. In particular, ramified microglia act as surveying cells by actively sensing the surrounding environment via dynamic processes, whereas hyper-ramified microglia are characterized by increasing branching processes and the secretion of pro-inflammatory mediators. Bushy morphology represents intermediate microglial activation and amoeboid or "phagocytic" microglia are highly motile with few processes and participate in phagocytosis or in inflammatory functions [35, 36]. Although the exact relationship between morphological changes and production of either pro- or anti-inflammatory mediators is poorly understood, our results suggest that RvD1 reduces the overall ramification of microglia, presumably making them more prone to migrate and get ready to activate pro-resolving programs. Accordingly, RvD1 and RvE1 (an EPA-derived SPM) have been reported to inhibit microgliosis and pro-inflammatory cytokine release in primary microglial cultures [37, 38], with the former involving a mechanism that required regulation of miRNA expression [38] and promotion of IL-4-induced and alternatively activated microglia [39]. On the contrary, another DHA-derived SPM termed neuroprotectin D1 (NPD1) was shown to induce a ramified microglial phenotype in the choroid layer of the eye [40], yet NPD1 reduced microglia recruitment and the ramified microglia were non-injuring and resting, not only corroborating the evidence that DHA-derived SPMs are indeed neuroprotective but also suggesting that the mechanism of neuroprotection can entail different microglial morphologies. It is important to add that since Iba-1 can also stain macrophages migrated from the periphery, our data also suggest that this lipid can also impact on recruitment and activation of infiltrated monocytes/macrophages, which are also involved in brain neuroinflammation [41]. However, these effects of RvD1 on microglia are not direct but could rather be mediated by soluble factors released by astrocytes. RvD1 also shortens the interval of spontaneous recovery of neurological functions, and this is in line with the whole literature on SPMs, whose main role is probably that of quantitatively shortening resolution indices that take into account the temporal activation of resolution programs. Moreover, although establishing a link between sparing of neuronal death in a selected brain area and improvements in functional recovery is always risky, we cannot exclude that systemic RvD1 treatment might influence outcomes by acting directly on neurons or on brain centers and intracellular signaling pathways that differ from those that we have investigated.



To date, mechanisms through which resolution of inflammation is achieved are of considerable interest. Along this line, microRNAs act as posttranscriptional or translational repressors of numerous genes [42], including several genes involved in the overall regulation of immunity and inflammation [43, 44]. Our data identified a novel resolution circuit where RvD1 activates its receptor ALX/FPR2 to specifically regulate miR-219a-1-3p and miR-146b and their downstream targets TLR4 and IL6R, and to a lesser extent NF-kB and CD200. These results are consistent with other

◀ **Fig. 5** ALX/FPR2 receptor mediates RvD1-induced neuroprotective effects. HCb rats were left untreated or treated with RvD1 in presence or absence of anti-ALX antibody (750 μg/kg, i.c.v. on day 3). **a** Time course of neurological recovery (NSS). $^{***}P < 0.001$ vs HCb and $^{\#\#\#}P < 0.001$ vs HCb + RvD1 by one-way ANOVA followed by Bonferroni's post hoc test. **b** Double- labeled and merged confocal images of Iba-1 (red) plus DAPI counterstaining (blue) (upper panel) and GFAP (green) plus DAPI counterstaining (blue) (lower panel) in pontine nuclei of HCb, HCb + RvD1, HCb + RvD1 + anti-ALX groups (scale bar = 50 μm). **c** Single-labeled images of Iba-1 in pontine nuclei of HCb, HCb + RvD1, HCb + RvD1 + anti-ALX groups (scale bar = 25 μm) and representative images of 3D-reconstructed microglia phenotypes. **d**, **e** qRT-PCR of selected microRNAs and target genes (broken lines represent HCb values). $^{*}P < 0.05$ vs HCb. $^{**}P < 0.05$ vs HCb by Student's *t* test. Results are mean ± SEM for $N = 6$ rats. **f** Schematic representation of the RvD1-dependent effects on HCb-induced remote damage via selective ALX/FPR2-regulated microRNAs

studies in mice and humans, where RvD1 down-regulated the microRNA-regulated expression of NF-kB and its downstream pro-inflammatory cytokines. These microRNAs, together with others such as miR-21 and miR-208a, and in turn their target genes, seem to belong to a complex network of regulatory molecules that promote resolution of acute inflammation [23]. The failure of RvD1 to significantly modulate miR-21 and miR-208a expression in our model could reflect differences in either species or brain areas. As for the RvD1/ALX-regulated microRNA targets, both miR-146b and miR-219a-1-3p target the TLR4, which is strongly expressed upon acute and chronic inflammation and whose role is pivotal in promoting the activation of NF-kB pathway. The observed RvD1-induced down-regulation of miR-219a-1-3p and miR-146b-mediated TLR4 not only is in agreement with previous reports showing that this SPM down-regulates this pathogen-recognition receptor [45] but also suggests that its pro-resolving activity is operated by reducing a receptor that is also involved in binding endogenous molecules produced as a result of tissue injury [46]. The anti-inflammatory role of RvD1 in HCb brain injury via its ALX/FPR2-regulated microRNAs is also explicated by a significant down-regulation of IL6 receptor, possibly attenuating the inflammatory activity of IL6, which has been recently reported to amplify the TLR4 /NF-kB pathway [47]. Overall, it is plausible that miR-219a-1-3p and miR-146b are both implicated in the observed neuroprotective and anti-inflammatory effects of RvD1 by decreasing key pro-inflammatory markers.

In conclusion, our study provides novel evidence for an alteration of the pro-resolution pathway in remote regions after focal brain damage, the reinstatement of which by systemic administration of RvD1 promotes functional recovery and reduces neuroinflammation via microRNA-dependent activation of resolution programs. Failure of activating endogenous anti-inflammatory and neuroprotective signaling at sites of secondary injury may be relevant not only for better understanding the pathogenesis and the molecular mechanism of brain injuries but also for the development of selective therapies against these conditions.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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