HHS Public Access

Author manuscript

Stroke. Author manuscript; available in PMC 2021 March 01.

Published in final edited form as:

Stroke. 2020 March ; 51(3): 958–966. doi:10.1161/STROKEAHA.119.027315.

Brain cleanup as a potential target for post-stroke recovery: the role of RXR in phagocytes

Shun-Ming Ting, MS, **Xiurong Zhao, MD**, **Guanghua Sun, MD**, **Lidiya Obertas, BS**, **Mercedes Ricote, PhD**[#], **Jaroslaw Aronowski, MD, PhD**[*]

Department of Neurology, University of Texas HSC, McGovern Medical School, Houston, Texas, USA

Abstract

Background and Purpose: Phagocytic cells, such as microglia and blood-derived macrophages, are a key biological modality responsible for phagocytosis-mediated clearance of damaged, dead, or displaced cells that are compromised during senescence or pathological processes, including after stroke. This process of clearance is essential to eliminate the source of inflammation and to allow for optimal brain repair and functional recovery. Transcription factor, retinoic-X-receptor (RXR) is strongly implicated in phagocytic functions regulation, and as such could represent a novel target for brain recovery after stroke.

Methods: Primary cultured microglia and bone marrow macrophages were used for phagocytic study. Mice with deleted RXRα in myeloid phagocytes (Mac-RXRα$^{-/-}$) were subjected to transient middle cerebral artery occlusion-(MCAo) to mimic ischemic stroke, and then treated with RXR agonist bexarotene. RNA-sequencing and long-term recovery was evaluated.

Results: Using cultured microglia, we demonstrated that the RXRα promotes the phagocytic functions of microglia toward apoptotic neurons. Using mice with deleted RXRα in myeloid phagocytes(Mac-RXRα$^{-/-}$), we have shown that despite behaving similarly to the control at early time points (up to 3d, damage established histologically and behaviorally), these Mac-RXRα$^{-/-}$ mice demonstrated worsened late functional recovery and developed brain atrophy that was larger in size than that seen in control mice. The RXRα deficiency was associated with reduced expression of genes known to be under control of the prominent transcriptional RXR partner, PPARγ, as well as genes encoding for scavenger receptors and genes that signify microglia/macrophages polarization to a “reparative” phenotype. Finally, we demonstrated that the RXR agonist, bexarotene, administered as late as 1d after MCAo, improved neurological recovery and reduced the atrophy volume as assessed 28d after stroke. Bexarotene did not improve outcome in Mac-RXRα$^{-/-}$ mice.

[*]**CORRESPONDENCE:** Jaroslaw Aronowski, Department Neurology, University of Texas, McGovern Medical School, 6431 Fannin Street, Houston Texas, 77030. Tel:713-500-7059, j.aronowski@uth.tmc.edu.

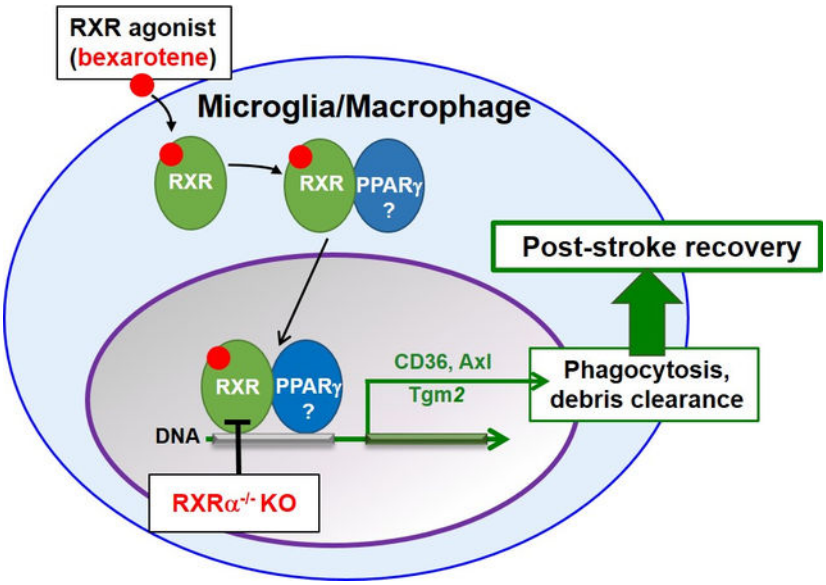
[#]Area of Myocardial Pathophysiology, Centro Nacional de Investigaciones Cardiovasculares Carlos III, Madrid, Spain

Disclosures

None.

Conclusions: Altogether, these data suggest that phagocytic cells control post-stroke recovery and that RXR in these cells represent an attractive target with exceptionally long therapeutic window.

Graphical Abstract



Keywords

Cerebral ischemia; Retinoid X Receptor; Recovery; Phagocytic Microglia

Subject Code:

Cerebrovascular Disease/Stroke; Ischemic Stroke; Basic Science Research

INTRODUCTION

Ischemic stroke is the leading cause of long-term neurological disability[1]. Multifactorial cell death pathways triggered by cerebral ischemia lead to brain cell death and destruction of a large mass of brain tissue[2]. This infarcted tissue, enclosed by the otherwise functional brain, acts as a reservoir for various cytotoxic and pro-inflammatory molecules that harm the adjacent healthy tissue, leading to augmented acute damage and secondary injury. In addition to adverse biochemical effects, the infarcted tissue forms a biological and physical barrier hampering neural reorganization, repair, and ultimately neurological recovery. Thus, in order to enable effective recovery, cellular debris and dead cells need to be cleared from the stroke-injured brain through a process involving phagocytosis. Microglia and infiltrating blood-derived macrophages (microglia/macrophages, MΦ) are the main phagocytes involved in this process, thus their proper function is fundamental to post-stroke recovery.

For MΦ to engage in phagocytosis, they are regulated by various external (e.g. cytokines, DAMPS) and internal (e.g. PPARγ; Nrf2, and scavenger receptors) factors provided by the

local environment (e.g. tissue injury) or pharmacologic agents affecting these pathways[3–8]. Pro-phagocytic MΦ are associated with anti-inflammatory and trophic phenotype, which is often referred to as the "reparative" phenotype that plays a beneficial role in tissue repair[9–13]. Recent studies demonstrated that soon after ischemic injury, the "reparative" MΦ phenotype is transiently enhanced, in part through release of IL-4 by the ischemia-primed neurons[4]. However, over time the abundance of the "reparative" MΦ declines, while the prevalence of pro-inflammatory, potentially harmful MΦ phenotype sharply increases[4, 14, 15]. The balance between the numbers of "reparative" vs. "harmful" MΦ could play an instrumental role in phagocytosis-mediated cleanup and post-stroke recovery.

Retinoid-X-receptor (RXR) is a ligand-dependent, transcription factor in the nuclear receptor superfamily[16]. It regulates metabolism and immune responses, including inflammation resolution[8, 17]. The RXRα isoform is uniquely abundant in macrophages, where it plays many essential functions, including tuning the expression of many genes associated with phagocytosis, including engulfment of myelin debris in a model of EAE[7, 8, 17]. PPARγ is a transcriptional partner of RXR. They form the heterodimer RXR:PPARγ[18–20] that regulates target gene expression by binding to conserved DNA sequences, termed *peroxisome-proliferator response elements*. We and others have reported that the activation of PPARγ could polarize MΦ toward the "reparative" phenotype, with enhanced phagocytic function toward dead/damaged tissues and various cellular debris. This would lead to more efficient clearance of damage-associated cellular debris after ischemic stroke and intracerebral hemorrhage[3, 4, 21]. Although PPARγ-selective ligands are sufficient for PPAR:RXR dimerization and binding to PPRE (to activate transcription), RXR activation by its selective agonist may lead to homodimerization and/or formation of heterodimers with other nuclear receptors[8]. Thus, RXR agonists could potently activate not only PPARγ[22–25] but also other transcriptional processes, e.g. through LXR[26], which could potentially improve MΦ functions in stroke-affected brain.

Here we explored the role of RXRα in MΦ as a factor involved in regulating MΦ function in promoting brain tissue clearance and neurological functional recovery after ischemic stroke.

MATERIAL AND METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request. All animal studies followed the guidelines outlined in *Guide for the Care and Use of Laboratory Animals* from the National Institutes of Health and were approved by the Animal Welfare Committee of the UT-Health. All studies were performed using a randomization (coin toss) approach and all the analyses were performed by investigators blinded to the treatment assignments (animals were coded for the group allocation). Animals were fed a standard rodent diet, and housed in standard cages on a 12-hour inverted light–dark cycle. Experiments included male and female animals at ages of 4–6-months. Behavioral analyses were conducted from the hours of 10:00AM-to-4:00PM.

See supplement material for detailed description of all the methods.

Mac-RXRα$^{-/-}$ mice.

We used conditionally-disrupted RXRα mice in myeloid cells. The experimental mice were progeny of LyzM-Cre^{+}/RXRα$^{-/-}$ mice crossed with RXRα^{LoxP}. The genotypes of mice were age- and gender- matched littermates, either RXRα^{LoxP} wild-type control (LyzM-Cre^{-}/RXRα^{LoxP}) or Mac-RXRα$^{-/-}$ knockout (LyzM-Cre^{+}/RXRα^{LoxP})[27].

Molecular signaling and genotyping.

The sequence of PCR primers used to genotype LyzM-Cre mice are included in the supplement and follow earlier report[27].

Administration of RXR agonist.

For *in vitro* experiments, microglia were pre-incubated for 24h with 0.5μM bexarotene (BEX, Sigma), and then exposed to dead neurons (DN) to assess phagocytosis. DMSO (0.1%) was used for vehicle control. For *in vivo* studies, BEX in 3%DMSO was administered intraperitoneally at 5mg/kg, first at 24h after surgery and then once a day for a total of 7d. DMSO (3%) was used for vehicle control.

Ischemia model in mice.

Transient (60min) focal ischemia was induced by unilateral MCA/CCAo[4]. We experienced no mortality.

Brain atrophy volume measurement.

The post-ischemic atrophy infarction volume was measured using indirect method as previously described[4].

Neurological deficits (NDS) measurement.

Behavioral tests in mice were conducted in a quiet and low-lit room by an experimenter blinded with respect to the treatment groups. A battery of behavioral tests, including foot fault, postural flexing, and corner turn, and arrive at a combination score, as reported[4]. All animals survived to the terminal endpoint.

RNA isolation and quantitative reverse transcription-polymerase chain reaction (RT-qPCR).

The RNA extraction and SYBR Green-based RT-qPCR were performed as we described[3, 4, 28]. The sequences of primers are listed in Table-1-(Supplement). The expression fold-change was calculated using the delta-delta Ct method.

Primary brain glial culture and microglia isolation.

The cortical cultures were prepared as we described[4]. After a total of 14–21d in culture, the microglia were harvested by shaking the co-cultures and plated at a density of 1–4×10^{5}cells/ml.

Bone marrow-derived macrophages (BMM) isolation.

Bone marrow was harvested from 2–4mo old mice and processed as describe[29].

Phagocytosis assay for dead neurons (DN).

We assessed phagocytosis of dead neurons as earlier reported[30]. Briefly, DN were generated using gamma irradiation. DN were added to the microglia or BMM cultures at 50:1/DNs:Microglia/BMM for 1.5h. Phagocytosed DN were visualized using neuronal-specific class III β-tubulin (Tuj1) antibody and the amount of the $Tuj1^+$ neurons per phagocyte was analyzed on still microscope images with ZEN blue edition software (Zeiss).

RNA-sequencing and analysis for microglia.

Cultured microglia from Mac-$RXR\alpha^{-/-}$ and $RXR\alpha^{LoxP}$ mice were incubated with 0.5μM BEX or 0.1%DMSO for 24h. We used 2×10^6 cells for RNA extraction. RNA sequencing, including library construction and data analysis, was performed by Novogene Inc. The clean reads were mapped to the reference genome using STAR software, and the mapping results were visualized with Integrative Genomics Viewer. Differential expression analysis was performed using DESeq2 R package; Enrichment analysis, including gene ontology (GO) enrichment and Kyoto encyclopedia of genes and genomes (KEGG) pathway, was done using clusterProfiler software.

Statistical analysis.

All data are expressed as mean±SEM. All statistical analyses were performed using the GraphPad Prism 7 and InStat. Repeated-measures two-way analysis of variance (ANOVA) followed by Tukey post-hoc test was used to evaluate differences among groups at different time points in behavioral tests. Two-way ANOVA followed by Tukey post-hoc test was used to analyze data with two grouping variables. Remaining data were analyzed using one-way ANOVA followed by Tukey post-hoc test. Non-paired t-test was used when two groups are compared.

RESULTS

Gene profile of RXRα-deficient microglia suggest altered phagocytic and reparative functions

We have reported that PPARγ promotes phagocytic activities and “reparative” capacities of microglia[3, 4], suggesting that RXRα, a transcriptional partner of PPARγ, may (co)regulate microglia’ contribution to brain repair after stroke. To probe this notion, we now performed gene profile analysis of microglia from conditional RXRα knockout mice (Mac-$RXR\alpha^{-/-}$), with RXRα deletion selectively targeting myeloid phagocytes (Fig-I&II-Supplement), in presence or absence of RXR agonist, bexarotene (BEX). $RXR\alpha^{LoxP}$ mice were used as control.

First, the genome mapping results of RXRα confirmed the successful deletion of RXRα exon 4 in microglia from Mac-$RXR\alpha^{-/-}$ mice (Fig-I-C-Supplement). Next, the analysis of differential gene expression showed limited differences in gene expression between microglia from Mac-$RXR\alpha^{-/-}$ and $RXR\alpha^{LoxP}$ mice, with only a few differentially expressed genes (DEGs; Fig-1A). However, after exposure to the BEX, $RXR\alpha^{LoxP}$ microglia showed a total of 386 DEGs (Fig-1B), and this induction of gene expression by BEX was reduced (20-DEGs) in Mac-$RXR\alpha^{-/-}$ microglia (Fig-1C). Gene ontology (GO) enrichment analysis of

RXRα^{LoxP} microglia showed that BEX-enhanced signaling pathways included lipid transport and metabolism (Apoe, Abca1, Srebf2), tissue repair, and importantly, phagocytosis (CD36, Axl, Mertk, Tgm2; Fig-1D). The extent of activation of these RXR-mediated signaling pathways by BEX was diminished in Mac-RXRα$^{-/-}$ microglia. In addition, activation of RXR with BEX suppressed migration, proliferation, and leukocyte activation pathways (Ctsc, Cx3cr1, CD86) in RXRα^{LoxP} microglia (Fig-1D). These results provide useful insights into the role of RXR activation in microglial phagocytic and reparative processes. Furthermore, the results of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed that activation of RXR with BEX enhanced PPAR:RXR signaling pathways (Fig-III-Supplement), confirming the important role of PPAR in RXR signaling in microglia.

Loss of RXRα impairs phagocytic capacity of microglia/macrophages (MΦ)

The optimal brain remodeling and post-stroke recovery necessitates effective removal of pro-inflammatory infarcted tissue from the affected brain, process that requires MΦ and could be regulated by RXR. Thus, our next step was to determine if RXR improves the phagocytic capacity of microglia and macrophages. We harvested the microglia and bone marrow-derived macrophages from Mac-RXRα$^{-/-}$ mice and RXRα^{LoxP} and exposed them to mouse apoptotic neurons (target of phagocytosis) to establish an index of phagocytosis, based on the amount of internalized neurons per phagocyte (Fig-2). We found that, compared to control cells, the phagocytic capacity of Mac-RXRα$^{-/-}$ microglia (Fig-3A,B) and macrophages (Fig-3C,D) were significantly compromised, confirming that RXRα deficiency is detrimental for phagocytosis. In agreement with the promoting role of RXR, in a parallel experiment we showed that activation of RXR with BEX augmented phagocytic efficacy of microglia and macrophages in an RXRα-dependent fashion (Fig-3).

These results suggest that RXRα in both microglia and macrophages is important for efficient phagocytosis.

In ischemia-affected brain, selective deletion of RXRα in MΦ is associated with reduced expression of genes that control reparative functions of phagocytes

To probe the role of RXR in MΦ after ischemic stroke, we subjected Mac-RXRα$^{-/-}$ mice and RXRα^{LoxP} (littermate control) mice to a transient MCA/CCA occlusion. By measuring the infarct volume at 3d after stroke, we established that Mac-RXRα$^{-/-}$ and the RXRα^{LoxP} mice have indistinguishable infarctions (8.98±3.19mm^3 vs. 8.10±2.81mm^3 for Mac-RXRα$^{-/-}$ and RXRα^{LoxP}, respectively; p=0.78, n=6/group), suggesting that RXRα in MΦ does not modulate susceptibility to ischemia during the acute stages of injury.

However, MΦ are well-known for their reparative functions, properties that are controlled by RXR and could be important during repair after stroke. Granulocytes express negligible levels of RXRα[31] (also see Fig-II-Supplement), and as such were not considered as factor contributing to the outcome in this study.

Thus, to gain more insight into this process, we performed the gene expression profiling in ischemia-affected hemisphere at 3d after MCA/CCAo in Mac-RXRα$^{-/-}$ and RXRα^{LoxP} mice. Since the important role of RXRα in MΦ includes transcriptional control of lipid

metabolism and phagocytic pathways, we probed for genes reflecting these functions, including lipoprotein lipase (LPL), CD36, and CD206 (Fig-4). When compared to RXRα^{LoxP}, Mac-RXRα$^{-/-}$ mice showed reduced expression of genes encoding for 1) growth factors that modulate reparative processes and tissue remodeling, e.g. bFGF and VEGF; and 2) scavenger receptors that modulate phagocytosis, e.g. CD36, CD163, CD206, CD204, and ABCA1, which are also regarded as biomarkers for a "reparative" phenotype of MΦ (Fig-4). One limitation of this experiment is that it does not selectively analyze MΦ, but the whole brain. However, similarities in gene expression profile between the cultured microglia and whole brain (e.g. genes signifying phagocytosis), suggest strong contribution of MΦ toward whole brain profile. Overall, this data suggests the important role of RXRα in MΦ in modulating phagocytosis and reparative processes in the stroke-affected brain.

RXRα in MΦ assist in recovery after focal cerebral ischemia

As demonstrated above, RXRα deficiency in MΦ does not affect acute ischemic damage. However, the gene profile analyses and analysis of phagocytic activities, suggest that RXRα in MΦ could promote the reparative phenotype of MΦ important in long-term recovery after stroke.

Thus, to test this hypothesis, we subjected Mac-RXRα$^{-/-}$ and RXRα^{LoxP} mice to MCA/CCAo and then monitored the animals' neurological deficit over 28d, the time necessary to achieve infarcted tissue clearance. We found that the neurological deficit at 28d was significantly worse in Mac-RXRα$^{-/-}$, as compared to RXRα^{LoxP} mice (Fig-5A,B), signifying a beneficial role of MΦ RXRα during the recovery process. Responses were similar for male and females (Fig-IV-Supplement). After completing the behavioral assessment at d28, we also measured brain atrophy (missing tissue) volume, and established that Mac-RXRα$^{-/-}$ had larger atrophy than the RXRα^{LoxP} mice (Fig-5C; gray vs. black), especially among male mice (Fig-V-Supplement), suggesting that RXRα in MΦ is needed to limit maturation/progression of brain atrophy. To emphasize the relevance of brain atrophy volume, at the end of the experiment (d28), we revealed a positive correlation between the atrophy volume and neurological deficit (which was similar for both genotypes) (Fig-5D). This provided new and important information: histological damage at 4 weeks accurately predicts functional outcome. Also, these results suggest that approaches aimed at activating RXRα may represent a target for improving stroke recovery.

Bexarotene improves post-stroke recovery after focal cerebral ischemia with a one-day therapeutic window

Our next experiment investigated RXRα as a therapeutic target for post-stroke recovery. Since our *in vivo* experiment with Mac-RXRα$^{-/-}$ suggests that RXRα plays a role during the post-acute recovery process, we tested the therapeutic relevance of RXR activation to post-stroke recovery. We used clinically translational BEX to activate RXR. We subjected Mac-RXRα$^{-/-}$ and the control mice to MCA/CCAo and then administered BEX at 24h after stroke and then daily for seven days. We found that control animals receiving BEX showed the most robust neurological recovery, with a significant improvement seen at d14 after the stroke that persisted until d28, the end of the experiment (Fig-5A; red- vs. black-lines). The beneficial effects of BEX on recovery were not detected on d3 and d7, suggesting again that

BEX acts through mediating processes involved in secondary injury or repair. Notably, we did not see beneficial effects of BEX on post-stroke recovery in Mac-RXRα$^{-/-}$ mice (Fig-5A–B; blue- vs. gray-lines), suggesting that at least in part, the role of BEX in recovery is through modulation of RXRα activity in MΦ.

After concluding behavioral assessments (d28), we measured the brain atrophy to determine the tissue loss at the site of ischemic injury. In agreement with the neurological assessment, BEX-treated control mice demonstrated reduced brain atrophy volumes (Fig-5C; red vs. black). The beneficial effect of BEX on brain tissue preservation was lost in Mac-RXRα$^{-/-}$mice.

We did not detect major differences in any measured responses between male and female mice (Fig-IV&V-Supplement).

DISCUSSION

Our hypothesis was that MΦ-mediated brain cleanup (e.g. phagocytosis-mediated removal of dead tissue) may play essential role in post-stroke recovery. To test this hypothesis, we took advantage of a well-established role of RXRα as key regulator of phagocytic activities of myeloid MΦ and the use of Lysozyme M-Cre to delete RXRα in these cells. The neutrophils express RXRα at the negligible level[31] (and Fig-II-Supplement), and as such were not considered as contributor to the process. Using *in vitro* phagocytosis assay, we determined that microglia and macrophages respond similarly to RXR modulation, thus we did not differentiate between these cells, regarding their contribution to post-stroke recover. Using reversible ischemia model, a model that in the era of thrombolysis and thrombectomy has major translational value[32], we established that Mac-RXRα$^{-/-}$ mice have worse post-stroke recovery, as compared to control mice, despite similar level of the initial damage (up to 3d post-ictus), based on the infarct volume and neurological deficit assessment. In agreement with a beneficial role for RXR during the post-stroke recovery phase, repetitive activation of RXR with the clinically-relevant RXR-activating agent BEX, initiated as late as 24h after the stroke onset, improved the recovery rate, as assessed with validated sensory-motor tests[4]. Our *in vitro* functional data with MΦ in culture indicate that one of the important roles RXRα plays in MΦ is to enhance phagocytosis[8, 17], a process that is responsible for brain cleanup and inflammation resolution through removal of damage-associated cellular debris. Also, our RNA-sequencing data suggest the importance of RXRα in phagocytic and reparative phenotype activation. Although the phenotypic difference between RXRα-deficient and proficient microglia is limited in the absence of stimulus, activating RXR with BEX significantly increases expression of genes involved in lipid transport and metabolism, tissue remodeling, and phagocytosis in RXRα-proficient (and not deficient) MΦ. All of these pathways are essential for cleanup and repair processes in brain tissue after stroke. Furthermore, the PPARγ signaling pathway is upregulated in RXRα-proficient but not RXRα-deficient microglia after exposure to BEX, suggesting an eminent role for PPARγ in RXRα transcriptional regulation. We and others have previously demonstrated that MΦ-mediated cleanup is, in part, under the control of PPARγ, a transcriptional partner of RXR, which in MΦ regulates the expression of several scavenger receptors, molecules that are critical for debris clearance during phagocytic engulfment[17, 33].

Examples include CD36, Axl, and Mertk, proteins that we and others have implicated in the post-intracerebral hemorrhage cleanup process[3, 34]. Thus, one possible scenario is that RXR-mediated pro-phagocytic effects are achieved through activation of transcriptional activities of the RXR:PPARγ complex through direct RXR activation. Indeed, this cooperative interaction appears to be the likely scenario. By using primary rat microglia (data not included), we documented that activation of RXR with BEX effectively augmented PPARγ agonist (rosiglitazone)-induced phagocytic activity. In a more direct experiment, we showed that PPARγ antagonist could reverse BEX-augmented microglia-mediated phagocytosis (data not included). Finally, our targeted analyses of the gene expression profile in the ischemia-affected brain tissue from Mac-RXRα$^{-/-}$ mice, suggest that the loss of RXRα in MΦ coincided with the reduced expression of prototypic PPARγ-regulated genes such as lipoprotein lipase, CD36, and catalase, as well as PPARγ itself. Collectively, this data indicate that the inhibition (knockout) or stimulation (BEX) of RXR in microglia is mirrored by similar responses of PPARγ, suggesting that under our experimental conditions, the activity of RXR in MΦ is effectively coupled to PPARγ.

Various cellular mechanisms are engaged in the regulation of phagocytic/endocytic functions conducted by MΦ. One such essential mechanism, especially in relation to damage associated with the septic form of inflammation such as that caused by ischemia-induced damage, is through expression of various scavenger receptors. These membrane-associated proteins are intrinsic to various forms of MΦ and play key roles in the post-injury tissue cleanup and repair[35, 36]. We established here that the RXRα deficiency in MΦ is associated with the reduced expression of several scavenger receptors in the brains of mice subjected to MCA/CCAo. These changes, as measured 3d after the stroke, included reduced expression of CD36 (the defining member of class B scavenger receptors with a well-established role in efferocytosis), CD163 (scavenger receptor for hemoglobin:haptoglobin complexes and other cell degradation products[37, 38]), CD206 (mannose receptor that is involved in engulfment of several mannose-bearing serum glycoproteins[39]), and CD204 (SR-A/class A scavenger receptor involved in various phagocytic/endocytic functions[40]). It has to be emphasized that these gene expression changes were unlikely related to the extent of initial ischemic damage, as Mac-RXRα$^{-/-}$ and the control mice demonstrated indistinguishable infarct volume and behavioral deficit at the time point when gene analysis was performed. It has to be independently noted that the above scavenger receptors are also often recognized as biomarkers for the “reparative” phenotype of MΦ. This is often referred to as the M2 phenotype, MΦ with stronger phagocytic capabilities, anti-oxidative activities, and able to more effectively generate anti-inflammatory cytokines and trophic factors[13].

A reason for why the atrophy volume at four weeks after stroke was larger in Mac-RXRα$^{-/-}$ mice as compared to the control mice cannot be clearly deduced from the experimental data collected in this study. We know that the initial stroke-induced injury was similar in the control and Mac-RXRα$^{-/-}$ mice. Thus, it is justified to assume that the differences in lesion size could be due to pathogenic processes that took place at some later stages of ischemic lesion maturation. Also, since these changes occurred as a result of modification primarily limited to MΦ, we assume that at least one of the MΦ functions that is under control of RXR is causally related to differences in the ischemic outcome in these animals. As we discussed earlier, one key function of MΦ is to conduct phagocytosis-mediated cleanup to achieve a

better and faster elimination of dying cells and cellular debris, the main source of cytotoxicity and deleterious inflammation. In agreement with the existing data[7, 8], our *in vitro* experiments suggest that RXR indeed plays an important role in optimizing the phagocytic function of MΦ, and as such RXR activation (e.g. with BEX) after stroke could enhance the cleanup and reduce the injury to the brain tissue caused by toxic byproducts of ischemic injury. Finally, since the behavioral deficit across the genotypes showed a positive correlation with the brain atrophy volume, we believe that it is unlikely that the sole neuronal plasticity (independent of the total lesion size) within the peri-lesional brain tissue could account for the behavioral improvement. This once again suggests that the cleanup process could account for a key mechanism associated with functional outcome.

In conclusion, we propose that RXR could represent an attractive and clinically-relevant target for improving post-stroke recovery through modification of MΦ.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Sources of Funding

This work was supported by grant from National Institute of Health - NINDS - 1R01NS084292 to JA. Additional funding from Spanish Ministerio de Ciencia, Innovación y Universidades (MCNU) (SAF2017-90604-REDT-NurCaMein, RTI2018-095928-B-I00) to MR permitted to study RXR deletion in the phagocytic cells. The Centro Nacional de Investigaciones Cardiovasculares is supported by the Instituto de Salud Carlos III (ISCIII), the MCNU and the Pro-CNIC Foundation, and is a Severo Ochoa Center of Excellence (SEV-2015-0505).

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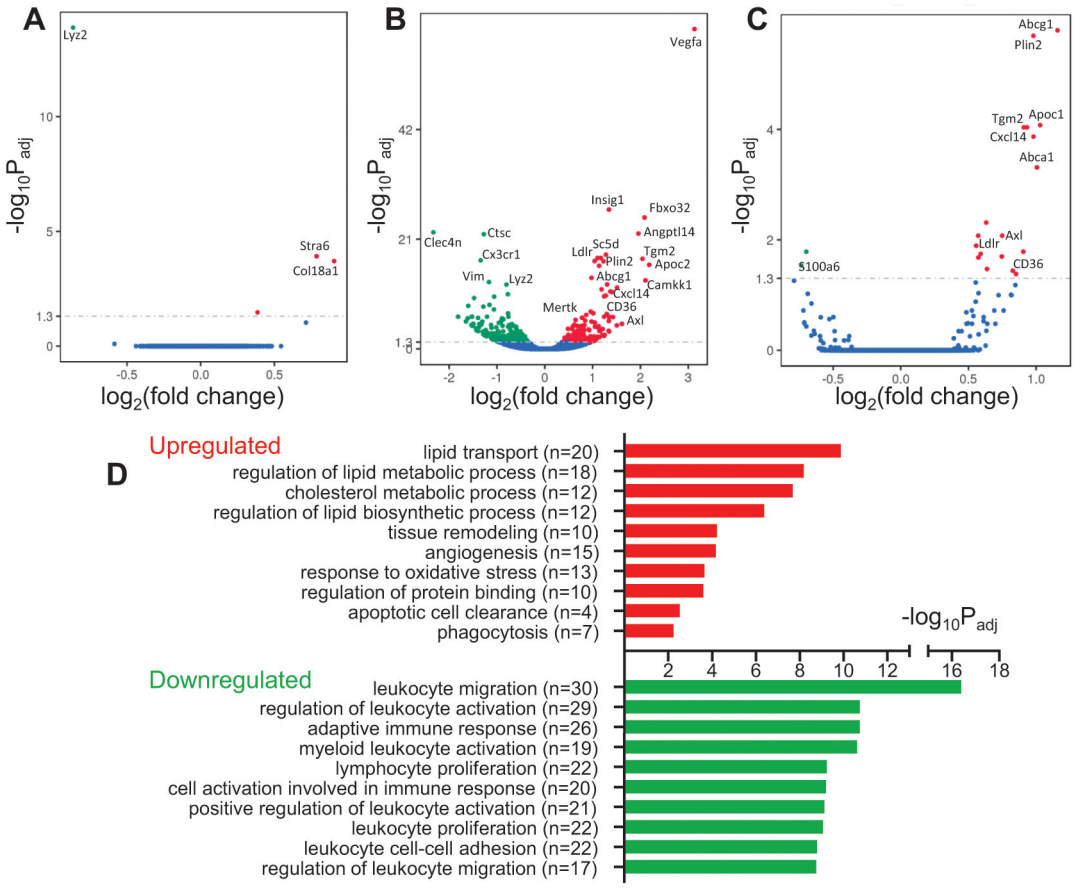

Figure 1. Diminished reparative phenotype of microglia from Mac-RXRα−/−mice; RNA-seq analysis

(A) Mac-RXRα$^{-/-}$ vehicle compared to RXRα^{LoxP} vehicle with four differentially expressed genes (DEGs), **(B)** RXRα^{LoxP} BEX compared to RXRα^{LoxP} vehicle with 386 DEGs, and **(C)** Mac-RXRα$^{-/-}$ BEX compared to Mac-RXRα$^{-/-}$ vehicle with 20 DEGs. **(D)** Gene ontology enrichment analysis showed pathways that were significantly upregulated and downregulated by RXR agonist BEX in RXRα^{LoxP} microglia. Primary cultured microglia from Mac-RXRα$^{-/-}$ and RXRα^{LoxP} mice were incubated with 0.5μM BEX or vehicle control (0.1%DMSO) for 24h before RNA isolation. Each RNA sample was extracted from 2×10^{6}cells. Three samples were analyzed in each RXRα^{LoxP} group and two samples in each Mac-RXRα$^{-/-}$ group.

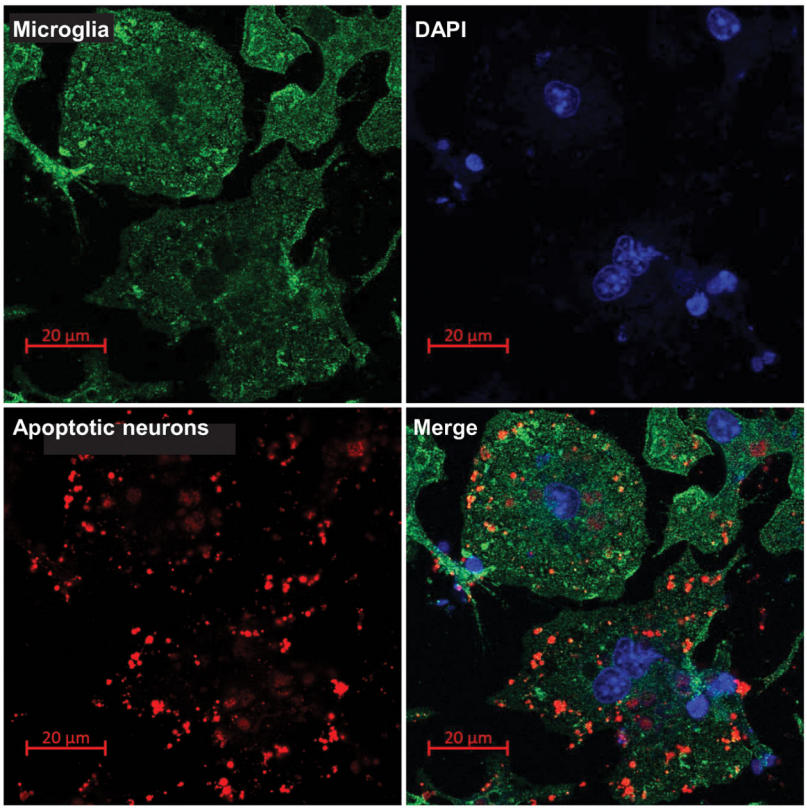

Figure 2. Microglia phagocytose apoptotic neurons

Representative micrograph illustrating primary cultured microglia ($CD68^+$ cells/green) phagocytosing apoptotic neurons (neuronal tubulin 1/$Tuj1^+$/red), employed for establishing the phagocytosis index.

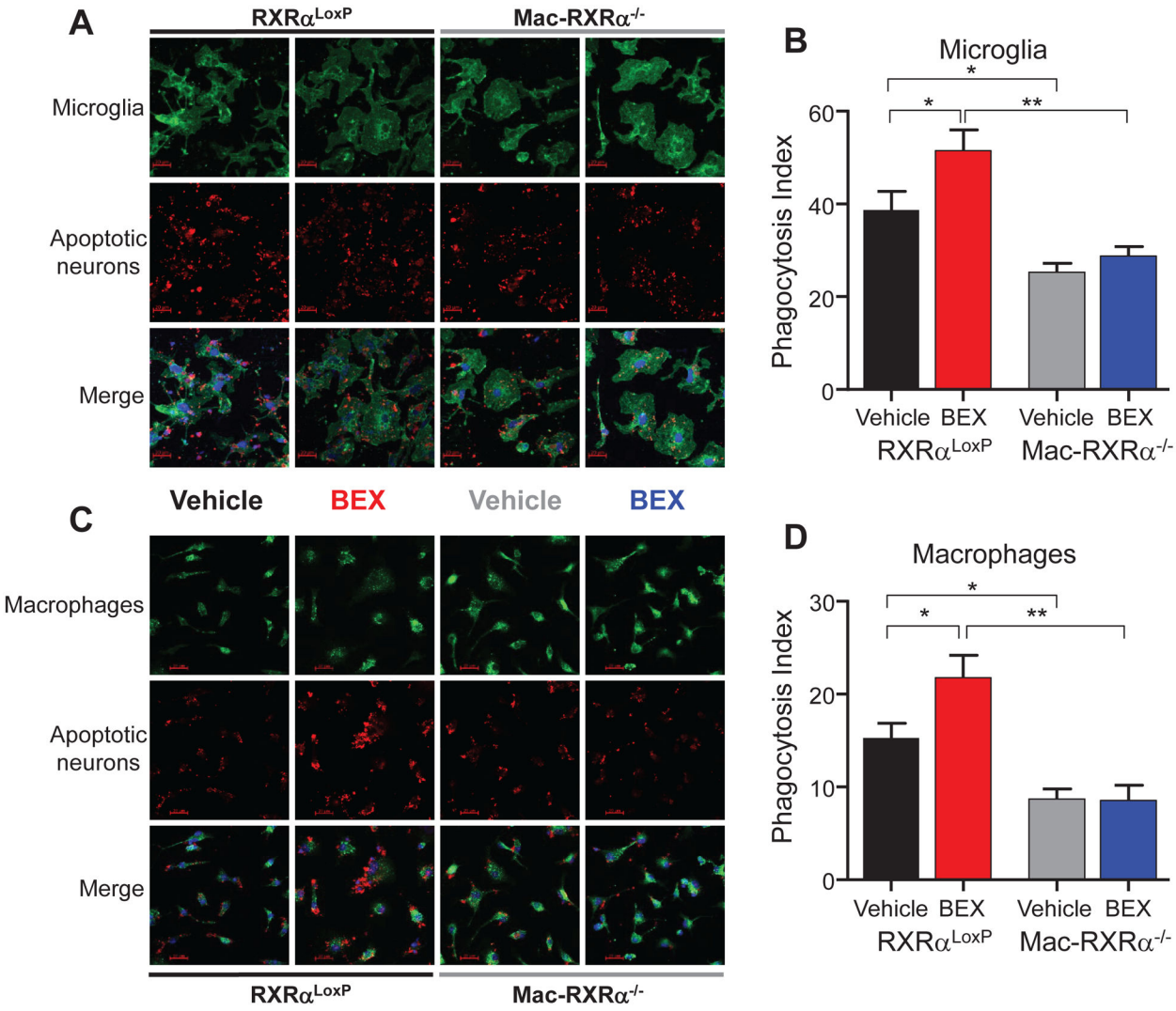

Figure 3. Loss of RXRα in microglia or macrophages impairs phagocytosis

The primary microglia (**A,B**) in culture or bone marrow-derived macrophages (BMM; **C,D**) were harvested from RXRα^{LoxP} and Mac-RXRα$^{-/-}$ pup brains and treated with 0.1%DMSO (vehicle) or bexarotene (BEX, 0.5μM). 24h later, cells were exposed to mouse apoptotic neurons. Phagocytic index was determined at 1.5h by measuring the amount of engulfed apoptotic neurons per individual phagocyte, based on Tuj1^{+} pixel count in each analyzed cell. The boundary of phagocyte was establish using CD68 immuno-labeling. Nuclei were stained with DAPI (blue). **(B,D)** Bar graphs demonstrating the phagocytic index. The data are expressed as mean±SEM (n=50 microglial cells/condition). *p<0.05. **p<0.01. Two-way ANOVA followed by pairwise comparison.

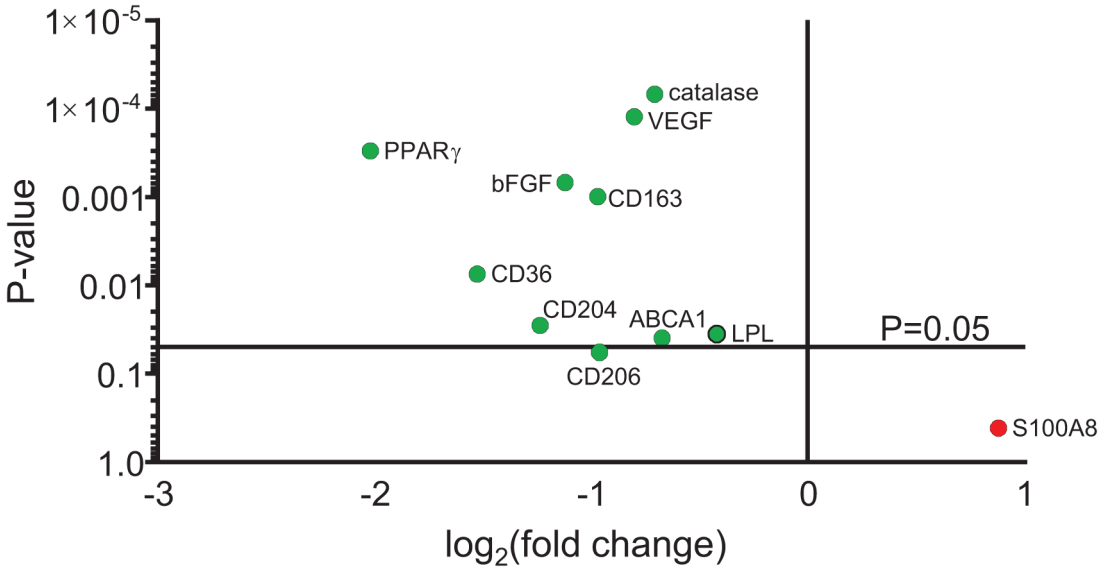

Figure 4. In ischemia-affected brain, RXRα deletion in MΦ leads to reduced expression of genes that signify reparative functions of microglia

Gene expression profile (by RT-qPCR) for the indicated genes in ischemia-affected ipsilateral cortices of RXRα^{LoxP} and Mac-RXRα$^{-/-}$ mice, 3d after MCA/CCAo. Gene expression fold-change was calculated using delta-delta Ct method with RXRα^{LoxP} mice as base line and GAPDH as reference gene. N=6 mice/group.

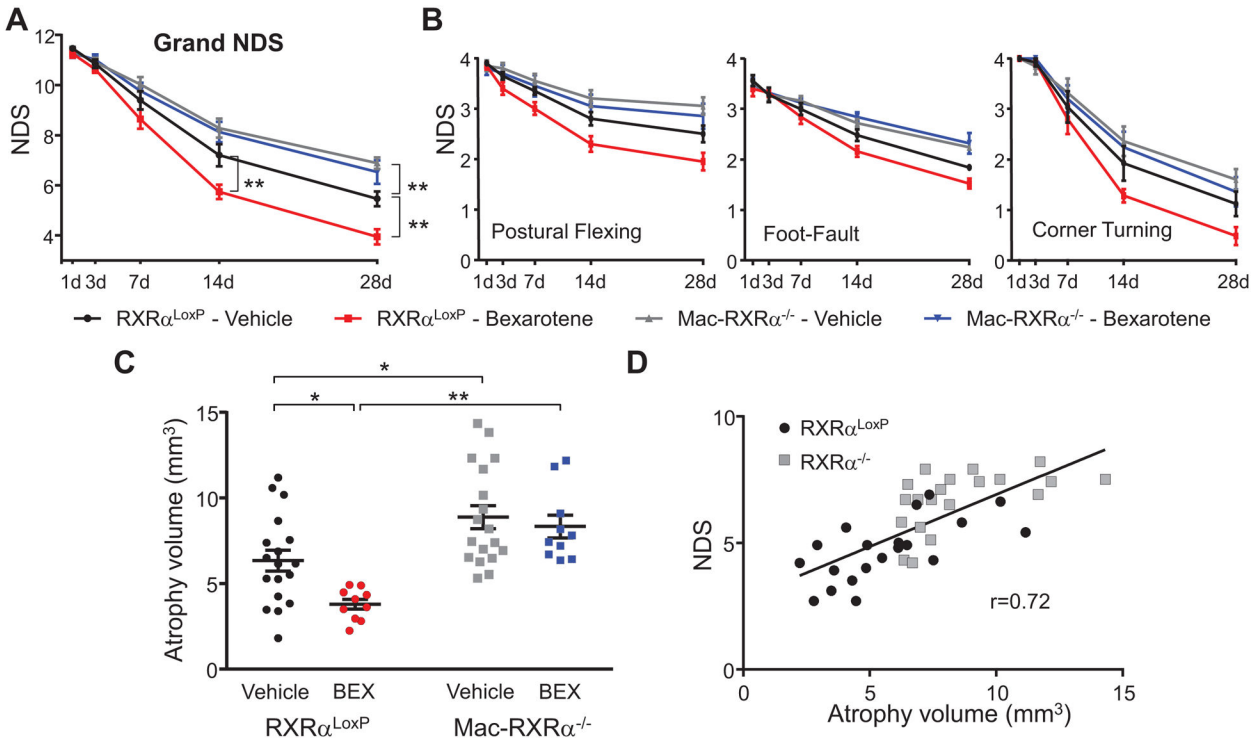

Figure 5. Neurological deficit outcomes and brain atrophy volumes 28d after MCA/CCAo

The Mac-RXRα$^{-/-}$ and RXRα^{LoxP} mice were subjected to 60min of MCA/CCAo. BEX, 5mg/kg, or vehicle was injected intraperitoneally, starting at 24h after MCA/CCAo and then daily for 7d. The composite neurological deficit scores (Grand NDS) **(A),** and the individual neurological deficit score (NDS) including postural flexing, foot fault, and corner turning **(B)** on d1 through d28 are demonstrated. Data are expressed as mean±SEM (n=10/group). **p< 0.01. Repeated measure two-way ANOVA followed by pairwise comparison. **(C)** The brain atrophy volume at the site of ischemic injury at d28 was measured. Data are expressed as mean±SEM (n=18 in each vehicle group and n=10 in each BEX group). *p<0.05, **p<0.01. Two-way ANOVA followed by pairwise comparison. **(D)** Correlations between brain atrophy volumes and neurological deficit scores at d28 were calculated by Pearson's coefficient r-(r=0.725, p<0.001).