Roflumilast promotes memory recovery and attenuates white matter injury in aged rats subjected to chronic cerebral hypoperfusion

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Abstract: Chronic cerebral hypoperfusion (CCH) has been associated with aging-related vascular dementia, including Alzheimer's disease. It can be induced by the four-vessel occlusion/internal carotid artery (4VO/ICA) model in aged rats, resulting in persistent memory deficits, white matter injury, and significant neuronal loss in the hippocampus and cerebral cortex. The phosphodiesterase type 4 inhibitor (PDE4-I) roflumilast has been reported to have pro-cognitive effects in several behavioral paradigms. The present study evaluated the effects of repeated roflumilast treatment in aged rats that were subjected to CCH. After surgery, roflumilast (0.003 and 0.01 mg/kg) was administered intraperitoneally once per day for 29 days. Memory performance was assessed in the aversive radial maze (AvRM) 7, 14, and 21 days after CCH. The effects of roflumilast on hippocampal neurodegeneration and white matter injury were investigated using Nissl and Kluver-Barrera staining, respectively. Western blot and RT-qPCR were used to explore microglial polarization using M1 (Iba-1 and iNOS) and M2 (Arginase-1) markers. Chronic cerebral hypoperfusion caused persistent memory deficits, hippocampal neurodegeneration, and vacuolization and fiber disarrangement in white matter. Repeated roflumilast treatment restored CCH-induced cognitive impairments in aged rats but in the absence of the rescue of hippocampal neurons. Attenuation of white matter injury was detected in the optic tract in aged CCH rats that were treated with roflumilast. In vitro, roflumilast increased Arg-1 gene expression in myelin-laden primary microglia. The present data suggest that roflumilast might be useful for the treatment of cognitive sequelae associated with CCH.

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Maringá, August 21st, 2017.

Dear Editor,

We are sending appended the manuscript entitled "*Roflumilast promotes memory recovery and attenuates white matter injury in aged rats subjected to chronic cerebral hypoperfusion*" authored by Amanda Santiago, Lígia Mendes Soares, Melissa Schepers, Humberto Milani, Tim Vanmierlo, Jos Prickaerts and myself, that we like to submit to *Neuropharmacology*.

We investigated the effects of roflumilast, a selective phophodiesterase type-4 inhibitor, in aged rats with chornic cerebral hypoperfusion (CCH). Roflumilast presents fewer emetic properties than its congenere rolipram, and has been approved by the United States Food and Drug Administration (FDA) for the treatment of peripheral inflammatory disease and severe chronic obstructive pulmonary disease. We showed that repeated roflumilast treatment exerted neuroprotective effects by decreasing neuroinflammation and white matter injury in aged rats, which might contribute to its positive effects on cognition. Our findings indicate that this compound may be useful for the treatment of cognitive sequelae associated with CCH.

I hereby state that the work described has not been submitted elsewhere for publication, in whole or in part, and all the authors listed have contributed to the present work. All authors have approved the manuscript. Financial funds have been provided by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), The Maastricht University and State University of Maringá. Additionally, all animal experiments were executed according to protocols approved by the local Animal Ethical Committee of State University of Maringá, Maringá (CEUA nº 2675220317) and met Brazilian governmental guidelines, thereby minimizing animal suffering and the number of animals used.

We are looking forward to hearing from you soon.

On behalf of all co-authors and with best regards,

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Highlights

- Roflumilast restored cognitive impairments in aged rats with chronic cerebral hypoperfusion (CCH)
- Roflumilast attenuated white matter injury in aged rats with CCH
- Roflumilast changed inflammatory signaling in the central nervous system resident cells
- Roflumilast ncreased *Arg-1* gene expression in myelin-laden primary microglia

Title page

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***Abstract**

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Title

Roflumilast promotes memory recovery and attenuates white matter injury in aged rats subjected to chronic cerebral hypoperfusion

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Abstract

Chronic cerebral hypoperfusion (CCH) has been associated with aging-related vascular dementia, including Alzheimer's disease. It can be induced by the four-vessel occlusion/internal carotid artery (4VO/ICA) model in aged rats, resulting in persistent memory deficits, white matter injury, and significant neuronal loss in the hippocampus and cerebral cortex. The phosphodiesterase type 4 inhibitor (PDE4-I) roflumilast has been reported to have pro-cognitive effects in several behavioral paradigms. The present study evaluated the effects of repeated roflumilast treatment in aged rats that were subjected to CCH. After surgery, roflumilast (0.003 and 0.01 mg/kg) was administered intraperitoneally once per day for 29 days. Memory performance was assessed in the aversive radial maze (AvRM) 7, 14, and 21 days after CCH. The effects of roflumilast on hippocampal neurodegeneration and white matter injury were investigated using Nissl and Kluver-Barrera staining, respectively. Western blot and RT-qPCR were used to explore microglial polarization using M1 (Iba-1 and iNOS) and M2 (Arginase-1) markers. Chronic cerebral hypoperfusion caused persistent memory deficits, hippocampal neurodegeneration, and vacuolization and fiber disarrangement in white matter. Repeated roflumilast treatment restored CCH-induced cognitive impairments in aged rats but in the absence of the rescue of hippocampal neurons. Attenuation of white matter injury was detected in the optic tract in aged CCH rats that were treated with roflumilast. *In vitro,* roflumilast increased *Arg-1* gene expression in myelin-laden primary microglia. The present data suggest that roflumilast might be useful for the treatment of cognitive sequelae associated with CCH.

Keywords: phosphodiesterase inhibitor; roflumilast; chronic cerebal hypoperfusion, memory.

1. Introduction

Phosphodiesterase type 4 (PDE4) enzymes are a subclass of the PDE family of enzymes. PDE4 selectively catalyzes the hydrolysis of cyclic adenosine monophosphate (cAMP), which in turn activates protein kinase A (PKA) and cAMP response element binding (CREB) protein (Conti and Beavo, 2007). Four genes encode different PDE4 enzymes. PDE4A, PDE4B, and PDE4D but not PDE4C are expressed in the central nervous system (CNS; Menitti, 2005). PDE4 is highly expressed in the hippocampus and cerebral cortex (Braun et al., 2007) and has been shown to be involved in signaling cascades that mediate synaptic plasticity and learning and memory processes (Rose et al., 2005; Peng et al., 2014; Heckman et al., 2015).

Experimental evidence indicates that PDE4 inhibition may be a novel approach for treating several brain disorders, such as depression (e.g., O'Donnell & Zhang, 2004), anxiety (e.g., Li et al., 2009), schizophrenia (e.g., Garcia et al., 2016), Alzheimer's disease (Cheng et al., 2010), and multiple sclerosis (Gonzalez-Garcia et al., 2013). PDE4 inhibition has also been shown to play an important role in cognitive function (Bender & Beavo, 2006). For example, the prototypical PDE4 inhibitor (PDE4-I) rolipram improved memory in healthy rats (Rutten et al., 2006) and reversed memory deficits that were induced by scopolamine (Imanishi et al., 1997), MK-801 (Zhang and O'Donnell, 2000; Rutten et al., 2007), and Aβ protein (Gong et al., 2004; Zhuo et al., 2016) in rodents. Rolipram also improved spatial memory in aged rats that presented hypertension (Jabaris et al., 2015a). In models of brain ischemia, rolipram improved memory and emotional deficits, decreased the size of the infarcted area, and attenuated neuronal damage in the hippocampus (Block et al., 1997; Nagakura et al., 2002; Li et al., 2011; Soares et al., 2016). However,

rolipram also produces severe side effects in humans, such as nausea and emesis, which limit its clinical use (Rock et al., 2009).

Roflumilast is a second-generation selective PDE4-I with fewer emetic properties. It has been approved by the United States Food and Drug Administration (FDA) for the treatment of peripheral inflammatory disease and severe chronic obstructive pulmonary disease (COPD; Rabe, 2011; Tashkin, 2014). Roflumilast was recently shown to ameliorate memory performance in rodents in several behavioral paradigms (Jabaris et al., 2015a; Jabaris et al., 2015b; Vanmierlo et al., 2016). When combined with a subeffective dose of donepezil, a subeffective dose of roflumilast completely restored memory deficits that were induced by the muscarinic receptor antagonist scopolamine in rats (Vanmierlo et al., 2016). To our knowledge, the effects of roflumilast on conditions of cerebral ischemia have not yet been investigated.

Chronic cerebral hypoperfusion (CCH) is an ischemic state whereby cerebral blood flow is gradually and permanently reduced. It has been associated with neurodegenerative conditions, including aging-related vascular dementia and Alzheimer's disease (de la Torre, 2009; Zhao et al., 2014). Chronic cerebral hypoperfusion can be induced by the four-vessel occlusion/internal carotid artery (4VO/ICA) model in aged rats, resulting in persistent memory deficits and consistent neuronal loss in the hippocampus and cerebral cortex (Ferreira et al., 2011; Godinho et al., 2015). It also leads to white matter pathology, characterized by the rarefaction and vacuolization of myelinated fibers, oligodendrocyte death, and microglial activation (Wakita et al., 2002; Farkas et al., 2004). Both hippocampal neurodegeneration and white matter injury are thought to contribute to the cognitive impairments that are frequently observed in conditions of CCH.

The present study investigated the effects of roflumilast on cognitive impairments, neurodegeneration, and white matter injury that were induced by CCH in aged rats. Given the presence of PDE4 in inflammatory cells (microglia and astrocytes) in the brain and the involvement of PDE4 in synaptic plasticity, we also examined the effects of roflumilast on neuroinflammation (i.e., the expression of glial fibrillary acidic protein [GFAP], ionized calcium binding adaptor molecule 1 [Iba1], inducible nitric oxide synthase [iNOS], and arginase-1 [ARG-1]) and markers of neural plasticity (i.e., CREB, phosphorylated CREB [pCREB], brain-derived neurotrophic factor [BDNF], and doublecortin [DCX]) in the hippocampus in aged CCH rats.

2. Materials and Methods

2.1. Animals

Aged male Wistar rats (500-600 g, 20 months old) were acquired from the local *vivarium* of the State University of Maringá (Paraná, Brazil). The animals were housed under controlled temperature (22 ± 1°C) on a 12h/12h light/dark cycle. The animals had free access to tap water and a standard commercial chow diet (Nutrilab-CR1; Nuvital Nutrients, Curitiba, PR, Brazil). All experiments were conducted according to the guideline laid out by the Brazilian College of Animal Experimentation (COBEA) and were approved by the Ethics Committee on animal Experimentation of the State University of Maringá (CEUA nº 2675220317).

2.2. Surgery

The 4VO/ICA model was used to induce CCH (Neto et al., 2005). The 4VO/ICA model consisted in gradually reducing the cerebral blood flow through a permanent

occlusion of vertebral arteries (VA) and both internal carotid arteries (ICA) in 3 stages, with an interstage interval of 4 days. First, the animals were anesthetized with a halothane/oxygen mixture and delivery through a universal vaporizer that was regulated by a bubble flow of 0.5 mL/ min connected to a face mask that was adapted to the rat's nose. The head of the animal was then fixed in a stereotaxic frame for bilateral electrocoagulation of the VA, and a longitudinal incision was made in the dorsal neck at the level of the first cervical vertebra. The tip of a unipolar electrode was inserted into the alar foramen and rotated until the presence of hemorrhage, then immediately stanched by a 3–4 mA electrical current, ensuring complete and irreversible bilateral VA occlusion (VAo; Day 0). Four days later (Day 4), an incision was made in the ventral neck to expose the left ICA, which was carefully dissected and permanently ligated using cotton thread. This procedure was repeated on Day 8 for occlusion of the right ICA. During surgery, core temperature was maintained around 37.5 ºC by a heating blanket. During the first 2 to 3 hours after each surgery stage, the rats were maintained in a warming box (inner temperature, 30 ± 1°C) to avoid eventual brain hypothermia (Seif el Nasr et al., 1992). Animals assigned to the sham-operated groups were subjected to the same surgical procedures as their counterparts but did not receive vessel occlusions.

2.3. Drugs

Roflumilast (a gift from Dr Jos Prickaerts, Maastricht University,The Netherlands) was dissolved in 98% carboximethylcellulose solution (0.5% carboximethylcellulose) and 2% Tween 80 and administered intraperitonally (*i.p*) once a day, for 29 days. The doses of roflumilast (0.003 mg/Kg and 0.01 mg/Kg) were chosen because they were

the most effective doses in recovering the memory deficits induced by the muscarinic acetylcholine receptor antagonist scopolamine in rats (Vanmierlo et al., 2016).

2.4. Experimental Design

Naive rats were trained for 15 days to learn the task in an 8-arms aversive radial maze (AvRM) and then assigned to different treatment groups. Three-stage 4VO/ICA surgery, with an interstage interval of 4 days was performed on days 0, 4, and 8. After full recovery from anesthesia, drug treatments began after the first occlusion stage and continued during 29 days. Retrograde memory performance was assessed in retention memory trials (RMTs), which began 1 week after the last stage of 4VO/ICA surgery and continued for next 3 weeks at a rate of 1 RMT/week (on days 15, 22, and 29). On the day after the last RMT, animals were sacrificed and had their brains removed for histological and biochemical analyses (see Figure 1 for a general timeline).

2.5. Behavioral testing

The 8-arm aversive radial maze

The AvRM was originally concepted by Paganelli et al. (2004), and subsequently modified (Benetoli et al., 2007). It consists in a central polygonal platform radiating 8 arms. At the end of each arm an opening provided access to a dark wooden box. Only one contained the true refuge (a close-ended box, the goal box) that could be shifted from one arm to another between trials. In the remaining arms, the boxes were open-ended, i.e, they had walls like the true box (the goal box), however, lack the bottom. At first, naive animals were habituated to AvRM for one day and then trained for 15 days. The acquisition trial began when the rats were placed individually in the center of the maze, after 30 s the guillotine doors were then opened simultaneously, and the animal

was allowed to explore the entire maze. When the animal entered in a non-rewarded arm (i.e., containing a false goal-box) the guillotine doors of the remaining arms were lowered simultaneously. Upon the rats return to central area, the newly visited arm was closed immediately, and the animal was again confined in the arena for further 10 s. The trial ended when the animal found the goal box or the 4 min period elapsed. If the goal box was not found within 4 min, the rat was placed into the arm containing the correct goal box and gently forced to enter it by the experimenter. The rat remained in the goal box for 1 min, after which it was returned to its home cage.

Each acquisition consisted in 3 trials and at the end of the trials the maze was cleaned and randomly rotated on its central axis. The goal box was randomly moved to any of the other seven arms, although its spatial position in relation to the extra maze cues was kept constant across trials and sessions and was the same for all rats. Behavioral performance was measured by the latency to find the goal box, the number of reference memory errors, and the number of working memory errors. Within a given trial, a reference memory error was counted when the rat visited an arm containing a false goal box for the first time. However, if the rat returned to an arm that had been visited previously during that trial, then a working memory error was recorded.

On the last day of training, four experimental groups were randomly generated: Sham + vehicle (Sham+veh, n= 30), CCH + vehicle (CCH+veh, n = 10), CCH + roflumilast 0.003 mg/Kg (CCH+ROF 0.003, n = 10) and CCH + roflumilast 0.01 mg/Kg (CCH+ROF 0.01, n = 11). Group assignment was balanced based on individual acquisition performance calculated as the mean latency observed across the last 3 days of preoperative training (days −3 to −1). Seven days after the third occlusion stage, cognitive tests of memory retention, i.e., retrograde memory, began. On day 30, the animals were euthanized and had their brains removed for histological analysis.

2.6. Histology: Cresyl violet and Fast Blue staining

Half of the rats were deeply anesthetized with 50 mg/kg sodium thiopental (Thiopentax; Cristália, SP, Brazil) and transcardially perfused with 0.9 % saline followed by Bouin's fixative (22 ml/min for 7–10 min) using a peristaltic pump. Following decapitation, the head was immersed in crushed ice (1–2 ◦C) for 2 h. The brain was carefully removed, the cerebellum was discarded, and the hemispheres were sectioned coronally at the level of the optic chiasm into two blocks that were post fixed in Bouin's solution for 3 to 5 days. The blocks were dehydrated, cleaned, and embedded paraffin according to standard procedures. A rotating microtome (LEICA RM2445, Wetzlar, Germany) was used to cut 7 µm coronal slices at a stereotaxic hippocampus level between -3.6 to -4.3 mm posterior to bregma.

For cresyl violet staining (Nissl), the brain slices were hydrated by immersion (5 min) in serial, decreasing alcohol concentrations (100%, 95%, 70%) in distilled water and then incubated in 0.3% cresyl violet solution for 5–7 min. Excessive stain was removed by briefly immersing the slides in a 95% alcohol solution, followed by dehydration in absolute alcohol. The tissue was cleared in xylene for 5 min and cover slipped with Permount®. Images from the hippocampus were obtained with the aid of a binocular microscope (400x magnification, Olympus BX41, Tokyo, Japan) coupled to a color, high-performance device camera (QColor, Ontario, Canada). Cells presenting a well-delimited, spherical form with a distinct nucleus and nucleolus were considered viable. Those that had shrunken cell bodies or surrounding empty spaces were considered destined to die and excluded from counting. The number of preserved cells was quantified in the CA1, CA2, CA3 and CA4 hippocampal subfields. For each animal, the measurements were taken in both cerebral hemispheres, summed and

normalized to the mean of the Sham+veh group, which was considered 100%. Normalized values were used to represent the percentage of normal-looking pyramidal neurons in each group.

In an adjacent set of slides, luxol fast blue die (Kluver Barrera´s staining) was used for semi quantitative assessment of white matter. After standand deparafinization and hydratation, the slides were immersed in luxol fast blue 0.1% overnight. Then the slides were rinsed in ethanol 70% and water to remove the excess of luxol fast blue. Lithium carbonate 0.05% was used to differentiate white matter. The slides were dehydratated with 95% and 100% alcohol followed by xylene during 5 min. The slides were mounted in gelatin coated slides using Permount and coverslips. After recorded, the images were evaluated by measuring the integrated optical density (IOD) using ImageJ software (National Institutes of Health, Bethesda, MD, USA). To evaluate the IOD the images was converted to gray scale, the background subtracted and the integrated optical density (IOD) obtained. The IOD measurements were determined in prefixed areas located in *corpus callosum* (0.27 mm^2) and *optic tract* (0.16 mm^2). Results were presented as the mean ± S.E.M. of three sections/animal.

2.7. Western blot analysis

Another half of the animals were decapitated, their brains carefully removed and the whole hippocampus isolated by microdissection and snap-frozen in liquid nitrogen. The tissue samples were lysed with lysis buffer (50 mM Tris, 600 mM NaCl, 1 mM EDTA) containing protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged for 20 min at 4ºC at 12000 rpm. The supernatant was assayed for total protein concentrations using the Bio-Rad Lowry Protein Assay (Bio-Rad Laboratories Inc., Hercules, USA).

Hippocampal homogenates (30 µg protein each) in sample buffer were separated on a 15% (Iba 1 and BDNF), 12% (GFAP, ARG-1, pCREB, CREB and DCX) or 8% (iNOS) SDS-PAGE gel, using a total of eight different blots to measure all different proteins. After protein transfer into a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, USA), membranes were blocked (2% BSA in TBS buffer) and incubated with the primary antibody at 4 °C overnight at the following dilutions: rabbit anti-GFAP (1:2000; Abcam, Cambridge, MA, USA), rabbit anti-IBA1 (1:1000; Wako Chemicals, Richmond, VA, USA), rabbit anti–iNOS (1:1000; Sigma-Aldricht, Saint Louis, MO, USA), mouse anti-ARG – 1 (1:800; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti–pCREB (1:100; Cell Signaling, Danvers, MA,USA), mouse anti-CREB (1:500; Cell Signaling, Danvers, MA,USA), rabbit anti-BDNF (1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-DCX (1:500; Abcam, Cambridge, MA, USA) and mouse anti-GAPDH (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After a washing step with TBS, membranes were incubated for 2 h with horse radish peroxidase-conjugated donkey anti-rabbit IgG (1:2500 Abcam, Cambridge, MA, USA) or donkey anti-mouse IgG (1:2000; Abcam, Cambridge, MA, USA) and were finally developed using ECLplus® (Invitrogen, Carlsbad, CA, USA). In order to assess the protein control (GAPDH) all blots were stripped with harsh stripping buffer (20% SDS 10%, 12.5% Tris HCl 0.5M and 0.8% ß-mercaptoethanol in H_2O). Bands were visualized using ChemiDoc Imaging Systems (Bio-Rad, Hercules, CA, USA). Intensities of specific bands were quantified using ImageJ (NIH, Bethesda, MD, USA) and normalized to GAPDH protein levels. Data were presented as % of the Sham+veh group (control).

2.8. Primary microglia isolation

Primary mixed glial cultures were isolated and cultured as described previously (O'Meara et al., 2011). In brief, mixed glial cultures were prepared from postnatal d0 mouse cerebral cortices of C57Bl6/J mice (Envigo, Venlo Netherlands B.V) and cultured in high glucose DMEM medium supplemented with 10% FCS and 100U pen/100µg strep/ml. Mixed glial cultures were used to generate microglia-enriched glial cultures by separating the microglia from the astrocyte monolayer by orbital shaking followed by purification by differential adhesion to plastic after 14 days in culture. Purified microglia were seeded on poly-L-lysine (5 µg/ml; Sigma-Aldrich) coated 24-well plate at a density of 250.000 cells/well. Primary microglia were treated with 10µM roflumilast or vehicle (1:1000 DMSO) in high glucose DMEM medium supplemented with 10% FCS, 100U pen/100µg strep/ml and 15% L929 conditioned medium for 24h in presence or absence of myelin (100µg/ml; kindly provide by Dr. J.F.J. Bogie, Hasselt University, Hasselt, Belgium). Subsequently, microglia were treated with lipopolysaccharide (LPS, 100ng/ml; Sigma-Aldrich, Bornem, Belgium) for 6h preceding lysis for qPCR or for 18h preceding nitrite quantification.

2.9. Nitrite quantification

Supernatants of roflumilast and or LPS-stimulated primary mouse microglia were collected to quantify nitric oxide (NO) release. Using a Griess reagent system nitrite production in the supernatant was measured as stable surrogate marker of NO production according the manufacturers' instructions (Promega, Leuven, Belgium).

2.10. RNA isolation and RT-qPCR

Primary murine microglia were lysed in Qiazol (Qiagen, Venlo, The Netherlands) preceding RNA isolation, and total RNA was prepared using the RNeasy mini kit

(Qiagen), according to manufacturer's instructions. RNA concentration and quality was determined with a NanoDrop spectrophotometer (Isogen Life Science, IJsselstein, The Netherlands). RNA was reverse transcribed to cDNA using the qScriptTM cDNA synthesis kit (Quanta Biosciences, Gaithersburg, USA). Quantitative PCR was subsequently conducted on a StepOnePlus detection system (Applied biosystems, Gaasbeek, Belgium). Relative quantification of gene expression was performed using the comparative Ct method. Data were normalized to the most stable reference genes. Primers were chosen according to literature or designed using Primer-Express (http://www.ncbi.nlm.nih.gov/tools/primer-blast), and details of primers used are shown in Table 1.

Gene	Forward	Reverse
Cyca	TATCTGCACTGCCAAGACTGAGT	CTTCTTGCTGGTCTTGCCATTCC
Tbp	TGGGATTGTACCACAGCTCCA	CTCATGATGACTGCAGCAAACC
Tnfa	CCAGACCCTCACACTCAG	CACTTGGTGGTTTGCTACGAC
Arg-1	GTGAAGAACCCACGGTCTGT	GCCAGAGATGCTTCCAACTG
iNos	GGCAGCCTGTGAGACCTTTG	GCATTGGAAGTGAAGCGTTTC

2.11. Statistical analysis

SAS 9.3 software was used for the analysis, which followed as described previously (Godinho et al., 2015; Zaghi et al., 2016). Briefly, the behavioral and histological data were examined for assumptions of normality and homocedasticity. Since behavioral data did not follow normal distribution (D'Agostino and Pearson omnibus test) and homoscedasticity (Levene's test), we firstly determined the covariance model to which the row data fit better. To this end, the following information criteria were used: (i) Akaike's information criterion (AIC), (ii) Akaike's information criterion corrected (AICC), (iii) Bayesian information criterion (BIC), and (iv) the Likelihood ratio test. The autoregressive (AR) covariance structure was then used. A

two-way analysis of variance (ANOVA) for repeated-measures was performed to quantify memory performance across the various days of testing, with *Group* as the between-subjects factor and *Trial* (testing day) as the within-subjects factor. When memory performance was expressed by the parameters 'total latency' and 'total number of errors', the one-way ANOVA was used for between-group comparisons. If a main effect of Group was found for the behavioral data, Tukey-Kramer's multiple range test was used to distinguish between groups. Paired Student's t-test was applied for within-group comparisons of memory performance measured prior to and after ischemia. Once the histological data also did not follow a normal distribution and homoscedasticity, the generalized linear model with a Poisson distribution was used for the count data (i.e., the number of Nissl-stained cells) and the generalized linear model with a gamma distribution was used for continuous data (i.e., the IOD for Kluver Barrera-stained cells and Western blot data). To evaluate survival data a proportion-like *t*-test was used. Primary mouse microglia data were analyzed using two-way ANOVA (roflumilast x LPS) and Tukey's multiple comparison *post hoc* test. Values of $p < 0.05$ were considered statistically significant. Extreme values were excluded by means of Dixon´s principles of exclusion of extreme values (Dixon, 1959).

3. Results

3.1. Survival rate

Overall, 69 animals entered the experiment. No death was observed in the Sham+veh group (0/30, 100% survival rate). In comparison to Sham+veh group, however, a significant effect of CCH was detected, as seen by 6 deaths in 16 animals (6/16, 62.5% survival rate, $p < 0.01$). Roflumilast 0.01 mg/kg (0/11, 100% survival rate)

but not roflumilast 0.003 mg/kg (2/12, 83.3% survival rate) reduced the mortality in aged rats subjected to CCH (p = 0.1).

3.2. Roflumilast prevents memory impairment in CCH aged rats

Fig 2. shows the effect of roflumilast (ROF) on retrograde memory performance in aged rats that were subjected to CCH and evaluated in the AvRM test. An increase in latency, number of reference memory errors, and number of working memory errors indicates that the rats forgot the spatial location of the goal box that was learned during preoperative training. The longitudinal analysis of data (Fig. 2b,2c,2d) revealed a highly significant main effect of *Group* for all the three parameters ($F_{3,171}$=7.72 to 17.39, p < 0.001). Although a global effect of Trial was not detected by ANOVA for any of the three parameters ($F_{2,171}$=0.63 to 3.13, $p > 0.05$), the number of reference and working memory errors increased consistently across time in the CCH+veh group, suggesting a progressive deterioration of memory. The *post hoc* analysis revealed that both latency and number of errors were significantly elevated in the CCH+veh group (p < 0.0001), indicating a state of retrograde amnesia in that group. CCH-induced loss of retrograde memory is also evident by the parameters total latency and total number of errors that were summed over the RMTs (Fig 2e, 2f, 2g; $F_{3,60}$=10.69 to 17.07, p < 0.0001 to 0.001, CCH+veh in comparison to Sham+veh group). This memory deterioration following CCH was further confirmed by a within-group analysis that compared the total latency and total number of errors that were measured prior to and after CCH. This analysis revealed that all three parameters consistently increased from preoperative training to postoperative RMTs in the CCH+veh groups (t = -6.05 to -4.22, p < 0.0001 to 0.01). This did not occur, however, in Sham+veh rats (t = -1.53 to -0.45, p > 0.05). Collectively, these results indicate that CCH caused the rats to forget the task that they

learned prior to CCH (i.e., retrograde amnesia), an effect from which they did not spontaneously recover during the experiment. This memory deficit caused by CCH and reflected by all three parameters and analyses, was significantly alleviated by roflumilast, independently of the doses used (Fig 2b, 2c, 2d; $p < 0.0001$ to 0.05, and Fig 2e, 2f, 2g; $p < 0.0001$ to 0.01, CCH+ROF (0.003 mg/Kg and 0.01 mg/Kg) in comparison to CCH+veh; Fig. 2e, 2f, 2g; $p = 0.10$ to 0.70, CCH+ROF (0.003 mg/Kg and 0.01 mg/Kg) preoperative *vs.* CCH+ROF (0.003 mg/Kg and 0.01 mg/Kg) postoperative).

3.3. Roflumilast fails to restrain hippocampal neurodegeneration while protects white matter from ischemic injury in CCH aged rats

The results of the histological analysis are illustrated in Fig. 3. CCH caused significant reduction in the number of intact appearing neurons in the hippocampus, evidenced by neurons that had shrunken cell bodies or surrounding empty spaces which were considered to have died in the Nissl staining (Fig. 3b; χ^2 =184.5, $p < 0.0001$; CCH+veh *vs*. Sham+veh, $p < 0.0001$). This neurodegenerative effect of CCH was not counteracted by roflumilast, independently of the doses used [$p > 0.05$, CCH+ROF (0.003 mg/Kg and 0.01 mg/Kg) *vs*. CCH+veh].

Regarding the results of Kluver-Barrera staning, no significant difference between groups considering rarefaction or fiber disarrangement of white matter in the *corpus callosum* (Fig. 3c; χ^2 =3.03, $p = 0.38$). Differences were found, however, in the *optic tract* (Fig. 3d; χ^2 =20.01, $p = 0.0002$). Overall, CCH+veh group exhibited more vacuolization and fiber disarrangement, reflected by a significant decrease in the IOD when compared with Sham+veh group ($p < 0.0001$). Otherwise, repeated roflumilast treatment (0.003 mg/Kg and 0.01 mg/Kg) prevented the white matter injury in the *optic*

tract when compared to CCH+veh group (CCH+ROF 0.003, p = 0.002; CCH+ROF 0.01, $p < 0.0001$).

3.4. Roflumilast changes inflammatory response in the hippocampus of CCH aged rats

Western blot analysis revealed differences in the protein levels of GFAP (χ^2 = 17.13, p = 0.0007), Iba-1 (χ^2 = 10.65, p = 0.01), ARG-1 (χ^2 = 9.06, p = 0.02) but not iNOS (χ^2 = 5.54, p = 0.13) in the hippocampus of aged rats (Fig. 4). Treatment with roflumilast 0.01 mg/kg induced an increase in the levels of GFAP (p = 0.0004), Iba-1 (p = 0.008) and ARG-1 (p=0.01) in aged CCH rats as compared to CCH+veh group.

3.5. Roflumilast suppresses inflammation in primary microglia and induces expression of Arg-1 in presence of myelin

In vitro roflumilast reduced LPS-induced NO production ($F_{1,12}$ = 11.41; $p < 0.01$) and downregulated gene expression of *iNos* ($F_{1,11}$=12.45; $p < 0.05$) and *Tnfa* ($F_{1,12}$ = 5.11; $p < 0.05$) in primary microglia (Fig. 5a, 5b, 5c). Otherwise, roflumilast increased *Arg-1* gene expression in myelin-laden primary microglia ($F_{1,10}$ = 57.69; $p < 0.001$) , in particular under inflammatory conditions ($p < 0.001$; Fig. 5d).

3.6. Roflumilast does not affect expression of neuronal plasticity markers in the hippocampus of CCH aged rats

As shown in Fig. 6, no significant differences was detected in the protein levels of pCREB/CREB (χ^2 = 1.48, p = 0.68), BDNF (χ^2 = 1.10, p = 0.77) or DCX (χ^2 = 0.64, p = 0.88) in the hippocampus of CCH aged rats (Fig 6). Neither CCH nor CCH+roflumilast

(0.003 mg/Kg and 0.01 mg/Kg) were able to alter the hippocampal expression of these plasticity markers in CCH aged rats.

4. Discussion

Aging and CCH are important risk factors and contributors to vascular dementia, including Alzheimer's disease (de La Torre, 2009). The present study found that the selective PDE4-I roflumilast increased survival rates and improved cognitive performance in aged rats that were subjected to CCH. Roflumilast also attenuated CCH-induced white matter injury in the optic tract in aged rats and changed inflammatory signaling in the CNS resident cells. To our knowledge, this is the first study that evaluated the effects of roflumilast under conditions of CCH and white matter pathology. The present findings support the use of roflumilast as a therapeutic strategy for the treatment or prevention of cognitive disorders associated with CCH.

Persistent memory deficits are consistently observed following the 4VO/ICA model of CCH in aged rats (Pereira et al., 2012; Romanini et al., 2013; Ferreira et al., 2014; Godinho et al., 2015). Accordingly, we found that aged CCH rats performed worse in the AvRM than control sham animals. These findings demonstrate that long-term memory that was acquired before 4VO/ICA did not spontaneously recover, indicating a condition of persistent retrograde amnesia. Moreover, the cognitive deficits that were induced by CCH in aged rats may be interpreted as a correlate of functional cognitive impairments that are observed in humans with cerebrovascular deficiency (Luckhaus et al., 2008).

Repeated treatment with roflumilast (0.003 and 0.01 mg/kg) attenuated CCH-induced cognitive impairments in aged rats in the hippocampus-dependent task

evaluated in the AvRM. Our results are consistent with previous studies that reported that the prototypical PDE4-I rolipram improved learning and memory function under several ischemic conditions (Imanishi et al., 1997; Nagakura et al., 2002; Li et al., 2011; Soares et al., 2016). Previous studies suggested that the pro-cognitive effects of rolipram are attributable to the activation of CREB through the PKA/CREB signaling pathway. Jabaris et al. (2015a) showed that the PDE4-I rolipram and its congener roflumilast ameliorated hypertension-induced learning deficits via cAMP/CREB signaling in the hippocampus. Indeed, the regulation of cAMP levels in the hippocampus by PDE4 is critically involved in mechanisms of memory formation, such as long-term potentiation (Ricciarelli and Fedele, 2015). Therefore, roflumilast might improve cognitive performance in aged CCH rats by elevating cAMP and its downstream effectors. However, in the present study, neither CCH nor roflumilast treatment affected the levels of pCREB, BDNF and DCX in the hippocampus of aged CCH rats. This finding is supported by previous studies showing significant decrease in the levels of plasticity markers including DCX an BDNF in aged brains (Shetty et al., 2005; Calabrese et al., 2013). In conditions of global brain ischemia, Choi et al. (2012) have shown lower extent of cell proliferation, neuroblast differentiation and neuronal maturation in aged gerbil in comparison to ischemic adult animals. Whether similar changes occur in the hippocampus of CCH aged animals remains to be better evaluated.

Roflumilast exerts antiinflammatory and immunomodulatory effects (Hatzelmann and Schudt, 2001), which might also have contributed to the functional recovery that was observed in aged CCH rats. Curiously, we detected an increase in the levels of GFAP, Iba-1 and Arg-1 in the hippocampus of aged CCH rats. There are indications that brain inflammation is driven by different microglial phenotypes,

i.e., M1 and M2, and/or by the balance between pro- and anti-inflammatory cytokines produced by activated microglia. While M1 microglia as a pro-inflammatory subtype, has been associated with tissue damage, M2 microglia as an anti-inflammatory subtype, has been shown to facilitate repair and regeneration. Moreover, microglia phenotype may shift form one phenotype to another dependent on microenvironment, time and brain area assessed after an insult (Harry et al., 2013). With aging, in particular, microglia may display diminished ability to shift from a pro-inflammatory to an anti-inflammatory state to regulate injury and repair (Koellhoffer et al., 2017). The present results did not show a clear polarization toward M1 (Iba-1) and M2 (Arg-1) phenotypes in response to CCH or repeated roflumilast in aged CCH rats. However, roflumilast increased the expression of the antiinflammatory protein *Arg-1* in myelin-laden microglia under inflammatory conditions. Roflumilast also decreased the expression of Tnfα and iNos and reduced NO secretion. Therefore, roflumilast appears to attenuate inflammation in microglia by suppressing LPS-induced NFκB signaling, which is consistent with previous findings in macrophages (Park et al., 2016).

Poor cognitive performance is often observed after CCH in aged rats and has been associated with hippocampal neurodegeneration (Romanini et al., 2013; Godinho et al., 2015). Our results indicated that aged CCH rats exhibited cognitive impairments that were associated with hippocampal cell loss. Repeated treatment with roflumilast, however, did not impact CCH-induced hippocampal cell loss in aged rats. These findings were somewhat unexpected because the other PDE4-I, rolipram, conferred neural protection against neuronal damage in the hippocampus that was induced by focal and global cerebral ischemia (Block et al., 1997; MacDonald et al., 2007; Li et al., 2011; Soares et al., 2016). Interestingly, we

previously reported that the PDE3-I cilostazol, which increases cAMP and cGMP levels, and the PDE5-I sildenafil, which increases cGMP levels, prevented hippocampal and cortical neurodegeneration in aged CCH rats (Ferreira et al., 2013; Godinho et al., 2015). Consequently, one speculation is that neuroprotection following treatment with a PDE3-I or PDE5-I in aged CCH rats may be attributable to an increase in the delivery of energy substrates to the brain as a consequence of cerebrovascular dilatation that is induced by elevated levels of cGMP. On the other hand, the cognitive effects of roflumilast in the absence of histological neuroprotection might indicate that the rescue of neurons in the hippocampus is not necessarily required for memory recovery after CCH in aged rats.

White matter injury is often observed under conditions of aging, hypertension, and cerebrovascular disease and has been associated with cognitive decline and vascular dementia in the elderly population (Boone et al., 1992; Bolandzadeh et al., 2012; Biffi et al., 2016). Chronic cerebral hypoperfusion leads to the rarefaction and/or vacuolization of myelinated fibers, axonal damage, and the activation of inflammatory cells (i.e., microglia and astrocytes) in the white matter in rats (Ihara et al., 2001; Wakita et al., 2002; Cho et al., 2006; Lee et al., 2006; Watanabe et al., 2006). In the present study, aged CCH rats exhibited white matter injury, reflected by a decrease in the IOD measurements in the optic tract (Kluver-Barrera staining). However, no significant effect of CCH was detected in the *corpus callosum* in aged ischemic rats. Indeed, the optic nerve and optic tract are the most vulnerable structures that are affected by CCH (Wakita et al., 2002) because they receive blood supplies from carotid circulation via the ophthalmic artery, anterior cerebral artery, and distant vertebral circulation (Takamatsu et al., 1984). Such vascular pathways were affected in the present study using the 4-VO/ICA model of CCH.

Although the behavioral consequences of white matter pathology in aged CCH rats are still unclear, a link has been suggested between white matter injury and cognitive impairments in the conventional radial maze in CCH mice (Shibata et al., 2007). Moreover, the NOTCH3 gene is linked to white matter injury in humans, and ischemic NOTCH3 knockout mice performed worse than wildtype controls in the novel object recognition task (Blasi et al., 2014). White matter tracts connect broadly distributed neuronal networks that coordinate several aspects of cognitive function, and white matter injury, consequent inflammatory processes, and hippocampal neurodegeneration may have contributed to memory deficits in CCH rats in the present study.

Roflumilast attenuated cognitive impairments that were induced by CCH and protected white matter from ischemic injury, possibly by suppressing local inflammation. To our knowledge, this is the first demonstration of the protective effects of roflumilast against white matter injury that is induced by CCH. These results are supported by early studies that showed that the selective PDE4-I rolipram prevented oligodendrocyte death and leukocyte infiltration that were induced by contusive spinal cord injury in adult rats. Rolipram also improved both descending and ascending long-tract axonal conductivity, leading to functional recovery (Beaumont et al., 2009; Costa et al., 2013).

In summary, the present study found that roflumilast increased survival rates, promoted memory recovery, and attenuated white matter injury in aged rats that were subjected to 4VO/ICA. These findings suggest that roflumilast may be useful as a pharmacological strategy for improving memory deficits that are induced by CCH.

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Conflict of interest

The authors declare no conflict of interest, except for Jos Prickaerts who has a proprietary interest in the PDE4 inhibitor roflumilast.

Legends

Figure 1. Experimental design. Naïve aged rats were trained during 15 days in the 8-arms aversive radial maze (AvRM) until achieving asymptotic learning performance. Then, they were subjected to chronic cerebral hypoperfusion by the four-vessel occlusion/internal carotid artery (4VO/ICA) in 3 stages, with an interstage interval of 4 days or sham-operation. Vehicle or roflumilast (0.003 mg/kg and 0.1mg/kg) were *i.p.* administered daily during 29 days. The treatment started one hour after the first stage of sham or CCH surgeries. On days 15^{th}, 22^{nd} and 29^{st} after surgery, rats were subjected to the retention memory trials (RMT) behavioral testing. After the last RMT, rats were sacrificed and their brains were processed for histology and western blot analysis. VA-o, vertebral artery occlusion; ICA-o, internal carotid artery occlusion.

Figure 2. Roflumilast (ROF) prevents memory impairment in aged CCH rats. (a) Schematic representation of the 8-arm aversive radial maze. Upper panel: temporal distribution of memory performance expressed by the parameters (b) latency, (c) number of reference memory errors, and (d) number of working memory errors measured on each retention memory trials (RMT). Lower panel: (e) Total latency and (f and g) total number of errors summed over RMTs. Preoperative training performance (T) is expressed as the mean (upper panel) or sum (lower panel) of the last 3 days of training. Values are means ± SEM. *p < 0.05, ***p < 0.001 vs. Sham+veh; $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01, $^{\#\#\#}p$< 0.001 vs. CCH+veh for between-group comparisons during RMT. $^{\$\$}p$ < 0.01, $^{\$\$\$}p$ < 0.001, within-group comparisons between pre- and post-CCH performance. Sham+veh (n = 30), CCH+veh (n = 10), CCH+ROF 0.003 (n = 10), CCH+ROF 0.01 (n = 11).

Figure 3. Roflumilast (ROF) fails to restrain hippocampal neurodegeneration while protects white matter from ischemic injury in aged CCH rats. (a) Representative diagram illustrating a coronal brain section containing the hippocampus, *corpus callosum* and optic tract (Franklin and Paxinos, 2008). (b) Number of intact neurons in the hippocampus. (c and d) IOD of Kluver-Barrera staining in the *corpus callosum* and optic tract. (e-h) Representative photomicrographs of Nissl staining in the CA1 hippocampal subfield. Representative photomicrographs of Kluver-Barrera staning in the *corpus callosum* (i - l) and optic tract (m – p). Data are presented as % of the Sham+veh group. Values are mean ± SEM. *p < 0.05; **p < 0.01, ***p < 0.001 vs. Sham+veh group; $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01, $^{\#\#\#}p$ < 0.001 vs. CCH+veh group. Sham+veh (n = 6), CCH+veh (n = 5), CCH+ROF 0.003 (n = 5), CCH+ROF 0.01 (n = 5).

Figure 4. Roflumilast (ROF) changes inflammatory response in the hippocampus of CCH aged rats. Levels of (a) glial fibrillary acidic protein (GFAP), (b) ionized calcium binding adaptor molecule 1 (Iba1), (c) inducible nitric oxide synthase (iNOS) and (d) arginase-1 (ARG-1) in the hippocampus as measured by western blot and corrected for GAPDH. (e) Representative blots of GFAP, Iba1, iNOS and ARG-1 protein levels. Data are presented as % of the Sham+veh group. Values are mean ± SEM. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. CCH+veh group. Sham+veh (n = 6), CCH+veh (n = 5), CCH+ROF 0.003 (n = 5), CCH+ROF 0.01 (n = 6).

Figure 5. Roflumilast (ROF) suppresses inflammation in primary microglia and induces expression of *Arg1* in presence of myelin debris.

Primary mouse microglia were cultured and stimulated with or without LPS (100ng/ml) to determine the influence of ROF (10 µM) or vehicle (DMSO) on relative (a) *iNos* and (b) *Tnfa* mRNA expression levels. (c) The influence of ROF (10 µM) or vehicle (DMSO) were also determined in presence or absence of LPS (100 ng/ml) on nitrite production. (d) In presence of myelin debris (100 µg/ml), the influence of ROF (10 µM) or vehicle (DMSO) on relative mRNA expression of *Arg-1* was determined with or without LPS (100 ng/ml) stimulus. The dashed line represents the vehicle treated condition without LPS or myelin. qPCR data were accomplished using comparative *Ct* method. Data were normalized to the most stable reference genes, determined by Genorm (*Tbp* and *Cyca*), Values are mean ± SEM and data were analyzed in a 2-way ANOVA using Tukey's multiple comparison tests. Significance is indicated by $*p < 0.05$ (n=3-4 per group) .

Figure 6. Roflumilast (ROF) does not affect hippocampal expression of plasticity markers in aged CCH rats. Levels of hippocampal (a) cAMP regulated element-binding protein (CREB), phosphorylated CREB (pCREB), (b) brain-derived neurotrophic factor (BDNF) and (c) doublecortin (DCX) as measured by western blot and corrected for GAPDH. (d) Representative blots of pCREB, CREB, BDNF and DCX protein levels. Data are presented as % of the Sham+veh group. Values are mean ± SEM. Sham+veh (n = 6), CCH+veh (n = 5), CCH+ROF 0.003 (n = 5), CCH+ROF 0.01 (n = 6).

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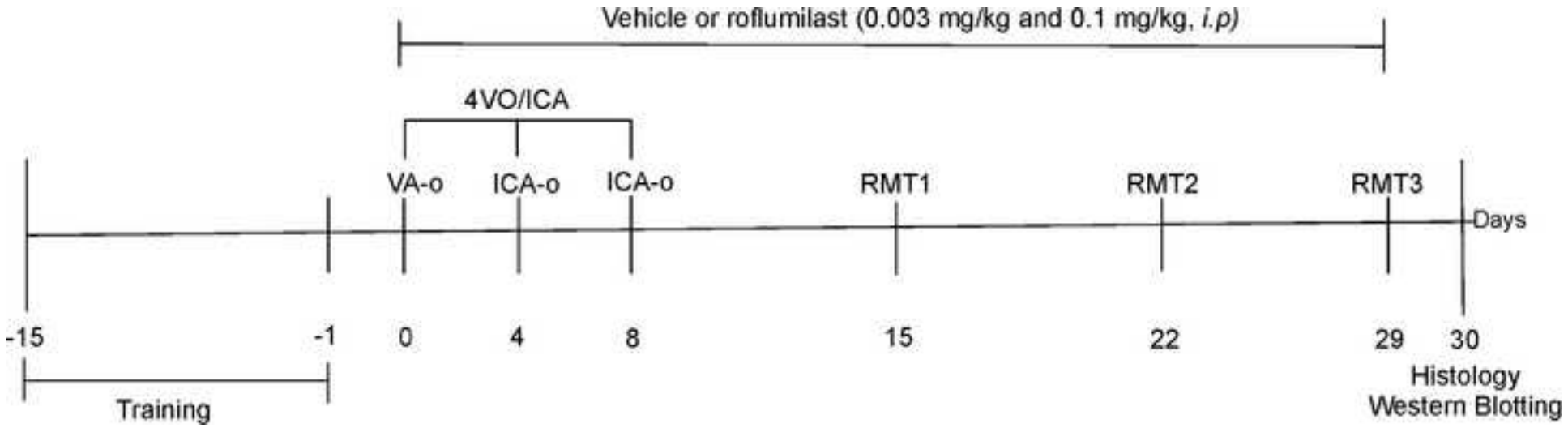

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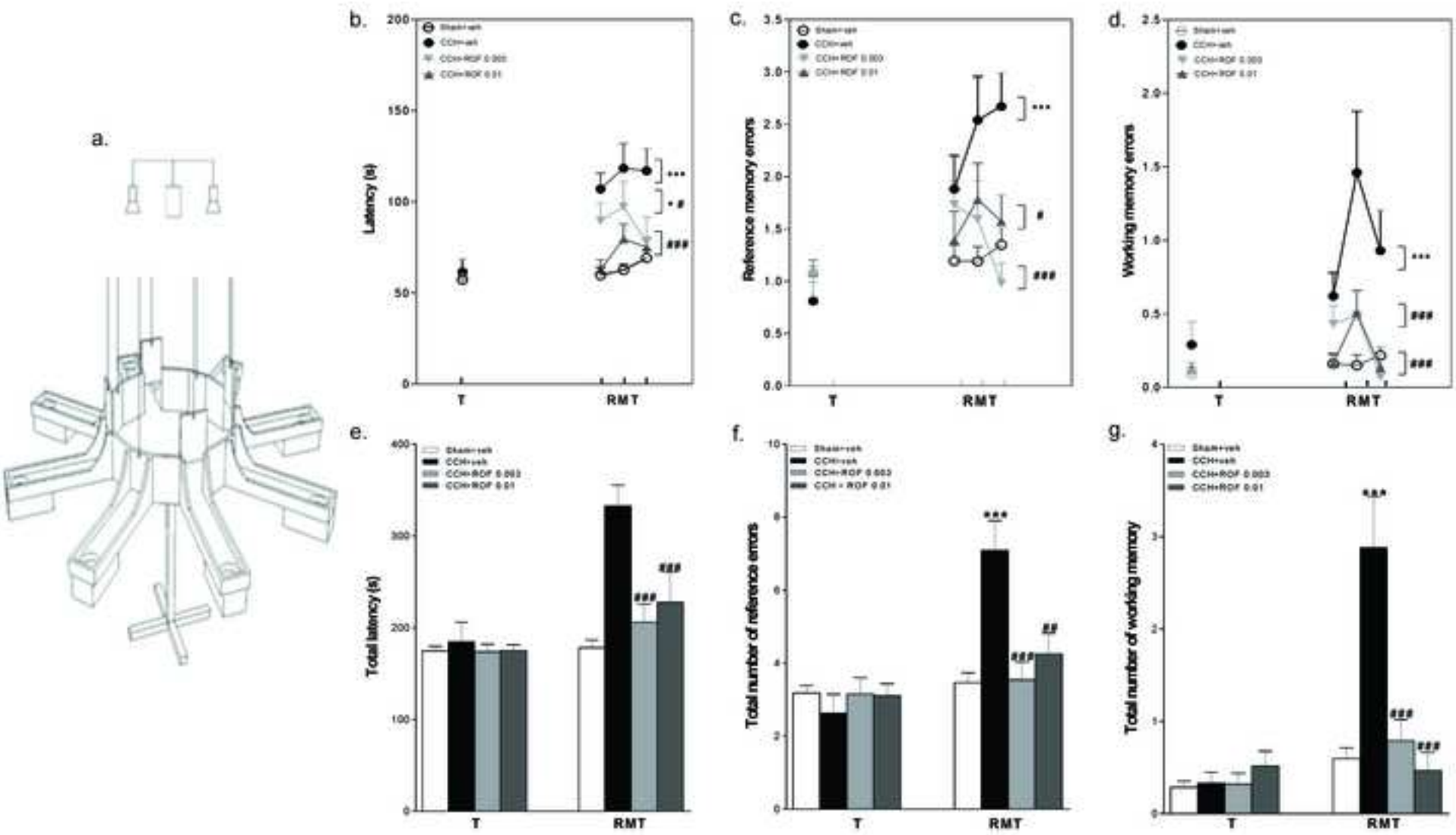

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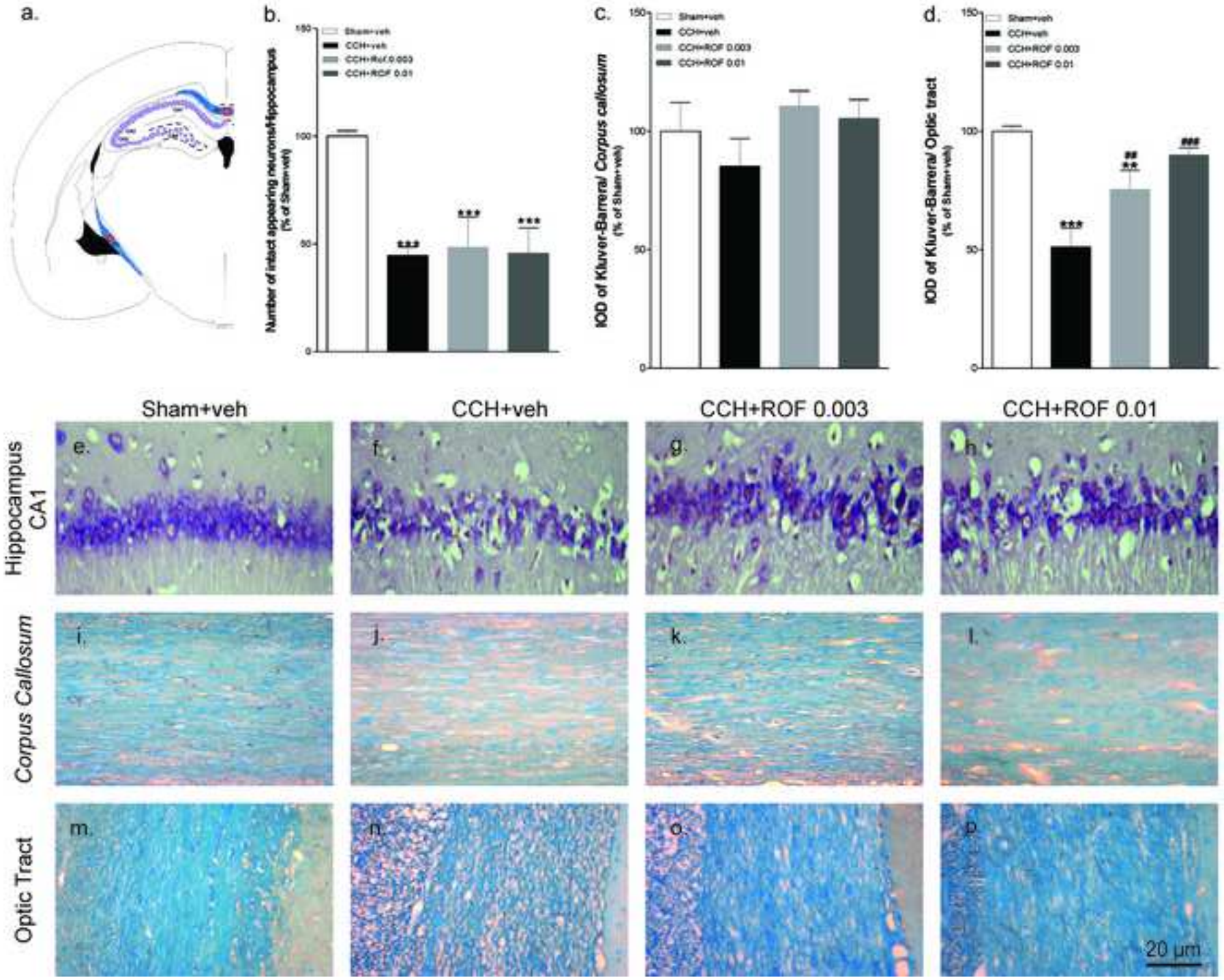

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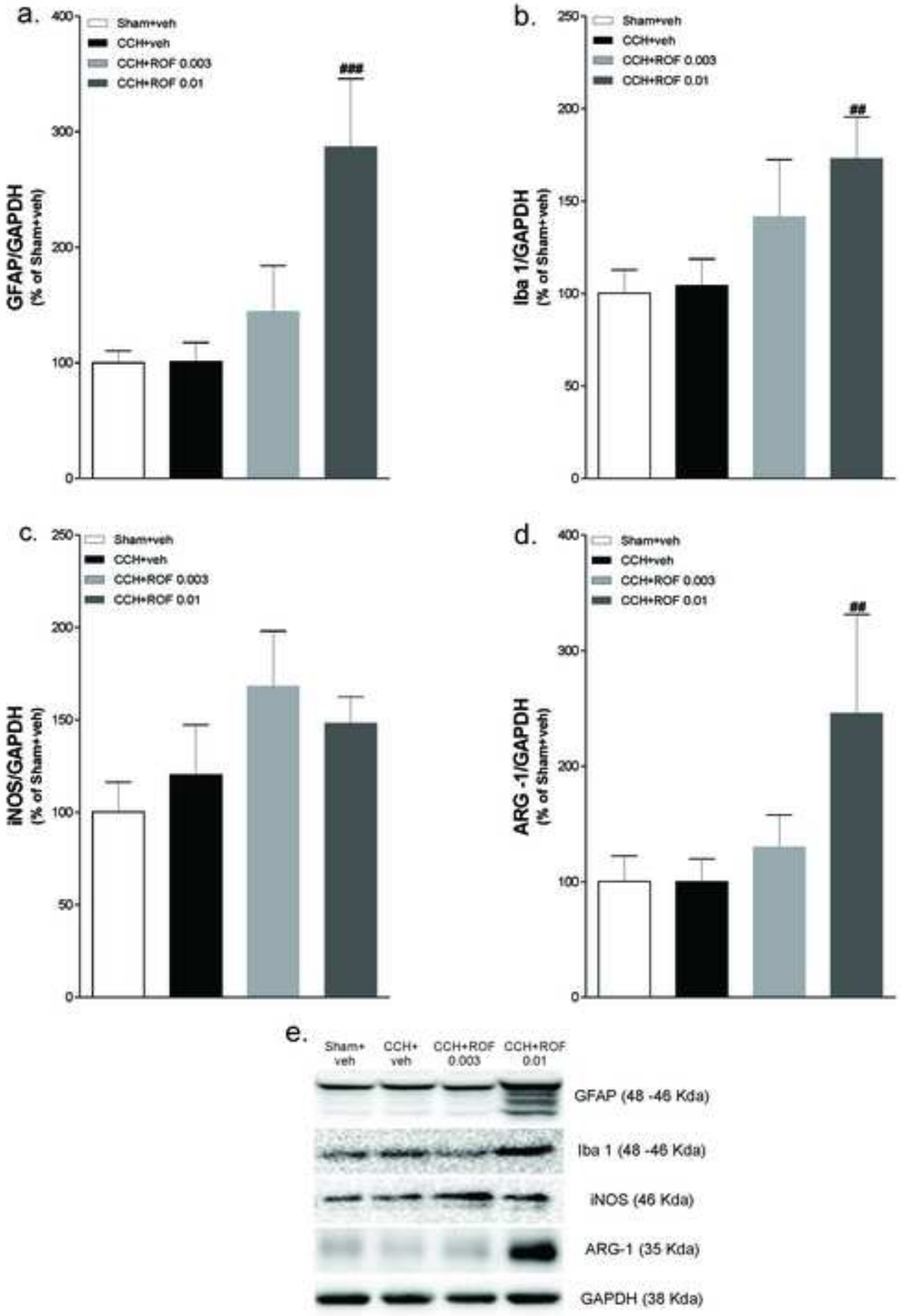

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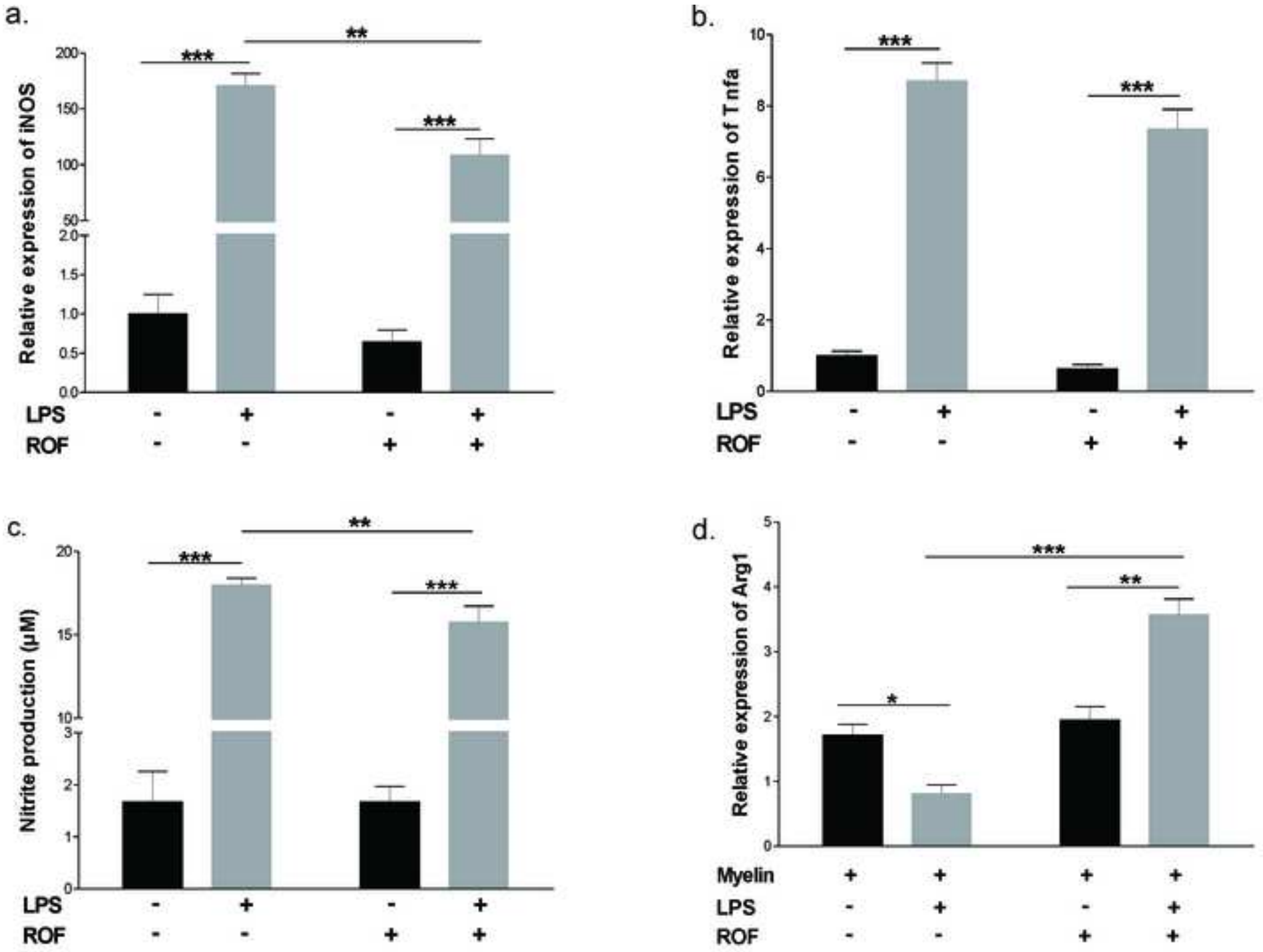

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