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Abstract

Microglial activation and neuroinflammatory changes are characteristic of the aged brain and contribute to age-related cognitive impairment. Exercise improves cognitive function in aged animals, perhaps because of a modulatory effect on microglial activation. Recent evidence indicates that inflammatory microglia are glycolytic, driven by an increase in 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), an enzyme that is described as the master regulator of glycolysis. Here we investigated whether microglia from aged animals exhibited a glycolytic signature and whether exercise exerted a modulatory effect on this metabolic profile. Young (4 month-old) and aged (18 month-old) mice were trained for 10 days on a treadmill. One day before sacrifice, animals were assessed in the novel object recognition and the object displacement tests. Animals were sacrificed after the last bout of exercise, microglial cells were isolated, cultured for 5 days and assessed for metabolic profile. Performance in both behavioural tests was impaired in sedentary aged animals and exercise attenuated this age-related effect. A significant increase in glycolysis, glycolytic capacity and PFKFB3 was observed in microglia from aged animals and exercise ameliorated these effects, while it also increased the phagocytic capacity of cells. The senescent markers, □-galactosidase and p16INK4A, were increased in microglia from sedentary aged mice, and expression of these markers was significantly decreased by exercise. The data demonstrate that the exercise-related improved cognition is orchestrated by a normalization of the metabolic profile and functionality of microglia.

Keywords	Glycolysis; microglia; neuroinflammation; oxidative metabolism; phagocytosis; IL-1β, exercise.
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Trinity College Dublin
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The University of Dublin

20 November 2019

Dear Editor,

I attach a manuscript entitled “Exercise reverses age-related metabolic changes in microglia reducing their senescence and improving cognition“, which we would like to have considered for publication in BBI. We show in this paper that exercise exerts a significant effect on microglia prepared from aged mice, reducing inflammation, modulating metabolism and decreasing the number of senescent cells. The data link the exercise-associated changes with increased phagocytic capacity of cells. Thus the findings provide previously unreported insight into the effects of exercise on the brain.

Thank you for your consideration,

Yours faithfully

Marina Lynch

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HIGHLIGHTS

The age-related switch to glycolysis in microglia is attenuated by exercise

Glycolytic microglia are functionally impaired

Exercise ameliorates expression of senescent markers in microglia from aged mice

Exercise-induced re-programming of age-related metabolic changes in microglia is accompanied by a reduction in senescent cells

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ABSTRACT

Microglial activation and neuroinflammatory changes are characteristic of the aged brain and contribute to age-related cognitive impairment. Exercise improves cognitive function in aged animals, perhaps because of a modulatory effect on microglial activation. Recent evidence indicates that inflammatory microglia are glycolytic, driven by an increase in 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), an enzyme that is described as the master regulator of glycolysis. Here we investigated whether microglia from aged animals exhibited a glycolytic signature and whether exercise exerted a modulatory effect on this metabolic profile. Young (4 month-old) and aged (18 month-old) mice were trained for 10 days on a treadmill. One day before sacrifice, animals were assessed in the novel object recognition and the object displacement tests. Animals were sacrificed after the last bout of exercise, microglial cells were isolated, cultured for 5 days and assessed for metabolic profile. Performance in both behavioural tests was impaired in sedentary aged animals and exercise attenuated this age-related effect. A significant increase in glycolysis, glycolytic capacity and PFKFB3 was observed in microglia from aged animals and exercise ameliorated these effects, while it also increased the phagocytic capacity of cells. The senescent markers, β-galactosidase and $p16^{INK4A}$, were increased in microglia from sedentary aged mice, and expression of these markers was significantly decreased by exercise. The data demonstrate that the exercise-related improved cognition is orchestrated by a normalization of the metabolic profile and functionality of microglia.

Key words: Glycolysis, microglia, neuroinflammation, oxidative metabolism, phagocytosis, IL-1β, exercise.

1. INTRODUCTION

Microglia play a pivotal role in ensuring the health of the central nervous system and, in particular, neurons. Their neuroprotective role relies on the ability of microglia to sample the microenvironment and react to potentially-damaging stimuli; this depends on the cells' ability to respond to chemotactic signals and become motile, and these changes are often accompanied by an increase in the phagocytic capacity of microglia. Several age-related changes in microglia have been described including increased markers of activation (Griffin et al., 2006; Loane et al., 2009), morphological change (Streit et al., 2004), responsiveness to inflammatory stimuli (Godbout et al., 2005; Henry et al., 2009; Minogue et al., 2007) and the consequent release of inflammatory mediators (Costello et al., 2016; Njie et al., 2012). These changes contribute to the age-related deterioration in neuronal and cognitive function (Norden and Godbout, 2013) and strategies to attenuate microglial activation are associated with improved neuronal function (Loane et al., 2009; Minogue et al., 2007; Norden and Godbout, 2013).

It has been consistently shown that exercise enhances cognitive function in aged animals (van Praag et al., 2005) and this has been linked with increased neurogenesis and production of brain derived neurotrophic factor (BDNF) (Aguiar et al., 2011; Bechara and Kelly, 2013; Jiang et al., 2017; O'Callaghan et al., 2009) and increased hippocampal interleukin (IL)-10 (Gomes da Silva et al., 2013). However exercise also reduces age-related microglial activation particularly in female mice (Kohman et al., 2013) and the accompanying age-related increase in inflammatory cytokines in hippocampus (Barrientos et al., 2011), suggesting that this effect on microglia might contribute to the improved neuronal function.

A number of reports have indicated that exercise can polarize macrophages towards an M2-like phenotype, possibly mediated by peroxisome proliferator-activated receptor (PPAR)γ (Goh et al., 2016) and acute exercise increased expression of the M2-macrophage marker, macrophage galactose-type lectin-1 (MGL1), in obese rats fed on a high fat diet (Oliveira et al., 2013). It has been suggested that exercise induces an M2-like phenotype in microglia (Jiang et al., 2017). This is relevant because it is known that macrophages and microglia that adopt an M2-like phenotype utilize oxidative metabolism as a means of producing ATP (Holland et al., 2018; Rodriguez-Prados et al., 2010), whereas cells that adopt an inflammatory phenotype are metabolically distinct in that they become glycolytic. Specifically lipopolysaccharide (LPS)-stimulated macrophages exhibit increased aerobic glycolysis and impaired oxidative metabolism (Finucane et al., 2019; Galvan-Pena and O'Neill, 2014; Rodriguez-Prados et al., 2010) and the suggestion is that this supports the rapid production of ATP required to mount an immune response. In microglia, stimuli that induce the cells to produce inflammatory cytokines, like amyloid-β (Aβ) or interferon-interferon γ (IFNγ), alone or in combination with LPS, also induce the cells to become glycolytic (McIntosh et al., 2019; Rubio-Araiz et al., 2018). The evidence suggests that this switch to glycolysis is driven by 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB)3, a major regulator of glycolysis (Pilkis et al., 1981; Yalcin et al., 2014), which acts by controlling the conversion of fructose-6-phosphate to fructose-1, 6-bisphosphate (Ros and Schulze, 2013).

Recent evidence from this laboratory has determined that microglia prepared from a transgenic mouse that overexpresses amyloid precursor protein (APP) and presenilin 1 (PS1; APP/PS1 mice) are glycolytic and have compromised phagocytic function (McIntosh et al., 2019). We have suggested that the functional deficit occurs because of cell fatigue since glycolysis is metabolically inefficient, yielding only 2 molecules of ATP per glucose, in contrast with approximately 36 molecules of ATP yielded as a result of oxidative metabolism. Here,

we argued that if exercise can reduce inappropriate microglial activation and neuroinflammation, that it might also impact on microglial metabolism. We report that microglia from aged mice were inflammatory, glycolytic, senescent and functionally-compromised, and that a short exercise programme attenuated these age-related changes.

2. METHODS AND MATERIALS

2.1 Animals

Young (4 months old) and aged (18 months old) C57BL/6 mice were used in these experiments. All experiments were performed under license from the Health Products Regulatory Authority of Ireland in accordance with EU regulations and with local ethical approval (Trinity College Dublin). Animals were housed under controlled conditions (20–22°C, food and water ad lib) and maintained under veterinary supervision.

2.2 Exercise regime

Animals were trained for 10 days on a treadmill for 25 min on day 1 and with training time increasing by 5 min every 2 days. The treadmill was set at a speed of 6.2 m/min for the first 5 min, 8.2 m/min for the next 10 min, 9.2 m/min for the next 10-15 min, 10.2 m/min for the next 5-10 min, and 11.2 m/min for the last 5 min at a 0° incline.

2.3 Behavioural analysis

On the 9th day of the exercise training, the novel object recognition (NOR) and the object displacement (OD) tasks were used to assess non-spatial and spatial memory respectively. For all behavioral assessment, mice were brought from their home cages into the testing room 30 min prior to testing to allow them to acclimatize to the new environment. The apparatus consisted of a black circular open field, 50cm in diameter, placed in a dimly-lit room. Objects

were constructed from standard plastic Lego® blocks, fixed to the floor of the open field 15 cm from the wall and spatial cues were fixed to the walls of the open field. The test arena and the objects were cleaned with 70% alcohol after each trial to ensure the absence of olfactory cues.

For this experiment, the 2-object variant of NOR task was chosen to assay non-spatial memory in sedentary and exercise mice. The task consisted of 2 days of habituation, one session of acquisition (training) and one session of testing. Two days prior to the task, animals were placed into the arena in groups (cage-by-cage) in the absence of objects for a 10 min period. On the second day, mice were habituated individually in the same empty open field for a 5 min period. The acquisition (training) day was 24 h after the second day of habituation, whereupon 2 different objects constructed from standard Lego blocks were placed in the open field. Mice were placed in the arena and allowed to freely explore the 2 objects for 5 min. The criteria for exploration were based on active examination of the object by the animal, with the mouse touching the objects with at least its nose. Measurement of the time spent exploring each object was recorded during the 5 min trial and, on the completion of the task, animals were returned to their home cage. One hour following acquisition, 1 object was replaced by a novel object. Animals were allowed to explore the objects for a 5 min period and the amount of time spent exploring the objects was recorded.

We used the OD task to assess spatial memory in sedentary and exercise mice. The task consisted of 2 days of habituation, one session of acquisition and one session of testing. The habituation phase of the OD task was identical to the habituation for the NOR. One day after the habituation phase, 3 different objects constructed from standard Lego blocks were placed in the open field. Mice were allowed to explore the objects for 5 min. Criteria for exploration were the same used for NOR task. Time spent exploring each object was recorded during the 5 min. One hour following acquisition, 1 of the 3 objects was displaced to a different quadrant

of the open field. Mice were allowed to explore the 3 objects for 5 min. The time spent exploring the objects was recorded following the same criteria used in the acquisition day.

2.4 Tissue preparation

Mice were anaesthetised with sodium pentobarbital (Euthanimal) and transcardially perfused with saline. The brain was dissected free and tissue was fixed in 4% paraformaldehyde for 24 h for immunohistochemical analysis. Following fixation, the tissue was incubated in 30% sucrose for 48 h for cryoprotection and frozen in isopentane. Tissue was mounted in optimal cutting temperature (OCT) solution and 20 µm coronal sections were prepared using a cryostat. Sections were stored at -20°C in freezing solution (30% ethylene glycol, 30% sucrose in PBS) until required for immunohistochemistry. Sections were stained with Iba-1 to identify microglia and co-stained with PFKFB3, Ki67 and 2 markers of senescence, β-galactosidase and $p16^{INK4A}$. For analysis of markers of neuroinflammation and glycolytic enzymes, the hippocampus and cortex were dissected free and prepared for RT-qPCR and western immunoblot. The remaining brain tissue was used to carry out flow cytometry.

2.5 Preparation of microglia from young and aged mice

Tissue was homogenised using the gentle-MACS Dissociator (Miltenyi Biotec, UK) in combination with the AdultBrain Dissociation Kit (Miltenyi Biotec UK) according to the manufacturer's instructions. The dissociated tissue was filtered and washed with Dulbecco's phosphate-buffered saline (D-PBS; PBS containing calcium (100mg/l), magnesium (100mg/l), glucose (1000mg/l), and pyruvate (36mg/l)). Samples were centrifuged (3000 x g, 10 min) to remove cell debris and the resultant supernatant was centrifuged (300 x g, 10 min) to remove red blood cells. The pellet containing the microglia was resuspended in D-PBS,

microglia were incubated with CD11b microbeads (Miltenyi Biotec. UK) and magnetically-separated using the QuadroMACS separator (Miltenyi Biotec, UK) according to the manufacturer's instructions. Samples were resuspended in PBS containing 0.5% fetal bovine serum and centrifuged (300g, 10 min). The final microglial pellet was resuspended in PBS (75 µl) and cells were seeded in 48-well plates ($2.5x10^4$ cells/well; final volume 200 µl) and cultured in Dulbecco's modified Eagle's medium (cDMEM) supplemented with macrophage colony stimulating factor (M-CSF; 100 ng/ml; R&D Systems, UK) and granulocyte macrophage colony stimulating factor (GM-CSF; 100 ng/ml; R&D Systems, UK). Microglia were cultured for 5 days and media was changed every second day. On day 5, the metabolic profile of cells was assessed using SeaHorse Extracellular Flux (XF96) Analyser (SeaHorse Bioscience, US), or cells were assessed for phagocytic function.

2.6 Preparation of microglia from neonatal mice

Microglia were prepared as previously described (Costello et al., 2015). Briefly, isolated mixed glia from cortical tissue of C57BL/6 neonatal mice were cultured in T25 cm^2 flasks in cDMEM supplemented with M-CSF (100ng/ml; R&D Systems, UK) and GM-CSF (100 ng/ml; R&D Systems, UK) for 10-12 days, after which time non-adherent microglia were seeded in 24-well plates ($1x10^5$ cells/well) and cultured for a further 2 days. Medium was replaced with fresh cDMEM ± IL-1β (100 ng/ml; R&D System, UK) for 24 h. Following treatment, cells were harvested for analysis of metabolism using Seahorse technology or for protein expression by western immunoblotting or immunocytochemistry.

2.7 Metabolic analysis

To undertake real-time metabolic analysis of cells, microglia were seeded ($6x10^4$ cells/well; 180 µl) on SeaHorse 96-well cell culture plates. The sensor cartridge was hydrated by adding

SeaHorse XF Calibrant solution (200µl; SeaHorse BioScience, US) to each well of the utility plate and left overnight in a CO_2-free incubator at 37°C. Prior to the assay, cells were washed twice with the appropriate assay medium (90 µl) according to the manufacturer's instructions, assay medium was added to give a final volume of 180 µl/well and the plate was incubated in a CO_2-free incubator (37°C, 1 h). For the mitochondrial stress test, oligomycin (20 µM; AbCam, UK), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (20 µM; FCCP; Sigma-Aldrich, UK) and antimycin A (40 µM; Sigma-Aldrich, UK) were loaded into the appropriate ports for sequential delivery. For the glycolytic flux test, glucose (10 mM), oligomycin (20 µM) and 2-deoxy-D-glucose (2-DG; 500 mM; all Sigma-Aldrich, UK) were prepared in glycolytic flux assay media and similarly loaded into the appropriate ports. Following calibration, oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured every 8 min for 96 min during which time the appropriate compounds were injected sequentially at 24 min intervals. ECAR and OCR were automatically calculated using the SeaHorse XF96 software and 4-6 replicates were assessed for each separate sample.

2.8 PCR

mRNA expression of IL-1β, CD11b and CD68 was assessed in samples of hippocampal homogenate from young and aged mice by RT-PCR. RNA was isolated from microglia using the Nucleospin® RNAII KIT (Macherey-Nagel, Duren, Germany) and cDNA was prepared using High-Capacity cDNA RT kit according to the manufacturer's instructions (Applied Biosystems, UK). Real-time PCR was performed with predesigned Taqman gene expression assays (IL-1β (Mm00434228_m1), CD11b (Mm00434455_m1) and CD68 (Mm03047343_m1); Applied Biosystems, UK) using an Applied Biosystems 7500 Fast Real-Time PCR machine (Applied Biosystems, Germany). Samples were assayed as previously described (Costello et al., 2016) with β-actin (Mm00407939_s1) as the endogenous control to

normalize gene expression data. Gene expression was calculated relative to the endogenous control samples and to the control sample.

2.9 Western immunoblotting

Samples of hippocampal homogenate were prepared for western immunoblotting using the supernatant obtained in the second step of the Nucleospin® RNAII KIT (Macherey-Nagel, Duren, Germany). The supernatant was incubated for 30 min in acetone at -20^{o}C and centrifuged (10 min, 12000 rpm). Pellet was resuspended in 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS buffer). Samples of microglial homogenate was prepared in lysis buffer (composition in mM: Tris-HCl 10, NaCl 50, $Na_4P_2O_7.H_2O$ 10, NaF 50, containing 1% each of Igepal, phosphatase inhibitor cocktail I and II, and protease inhibitor; Sigma, UK.) All the samples were equalized for protein, added to 5x SDS sample buffer (composition: Tris-HCl 100mM, pH 6.8, 4% SDS, 2% bromophenol blue, 20% glycerol; Sigma, UK), boiled (95°C, 5 min) and applied to 10-15% SDS gels. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane, non-specific binding was blocked (5% Marvel in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBS-T)) and membranes were incubated overnight at 4°C with the antibodies raised against PFKFB3 (1:750), hexokinase I (1:1000), lactate dehydrogenase (LDH; 1:1000) (AbCam, US), IL-1β (1:750; Prepotech, UK) or Iba-1 (1:500; Wako , Japan); all IgG, raised in rabbit, diluted in 5% non-fat dried milk/TBS-T). Membranes were washed and incubated (room temperature, 2 h) with a secondary horseradish peroxidase (HRP)-linked anti-rabbit antibody (1:2000 in 5% milk in TBS-T). Immunoreactive bands were detected using WesternBright enhanced chemiluminescent (ECL) substrate (Advansta, US). Images were captured using the Fujifilm LAS-4000 imager and densitometry analysis was carried out using ImageJ (http://rsb.info.nih.gov/).

2.10 Flow cytometry

Brain tissue was lysed in cRPMI using a tissue lyser (Qiagen, US). Brain homogenate was resuspended in isotonic Percoll (40%; 5 ml), layered over 5 ml of 70% isotonic Percoll (70%; 5 ml) and centrifuged (1300xg; 20 min). Mononuclear cells were removed from the interface of the Percoll gradients and passed through a 70 μm filter. Cells were washed and incubated with a live/dead stain (LIVE/DEAD Fixable Aqua Dead Cell Stain Kit; Life Technologies, UK; 1:600) and Fcγ block (BD Biosciences, US; 1:50). Cells were washed and surface stained with antibodies specific for CD45 (clone 30-F11, BioLegend, US; 1:200), CD11b (clone M1/70, eBioscience, US; 1:200), Ly6G (clone 1A8, BioLegend, US; 1:600) and Ly6C (clone AL-21, BD Biosciences, US; 1:200). Cells were analysed using an LSR Fortessa flow cytometer (BD Biosciences, US) and the data were analysed with FloJo software. Analysis of the stained populations was performed by gating on single, live cells.

2.11 IL-1β ELISA

Supernatant from microglial cells were assessed for IL-1β secretion as previously described (Dempsey et al., 2016). Briefly, 96-well plates (Nunc-Immuno plate with Maxisorp surface, Denmark) were coated with goat anti-mouse IL-1β capture antibody (R&D Systems, US), incubated overnight at room temperature and blocked with 1% BSA in PBS (300 ml/well) for 1 h at room temperature. Duplicate samples and standards were added (100 μl/well) and incubated for 2 h at room temperature. Plates were washed (0.05% Tween 20/PBS) and incubated with biotinylated goat anti-mouse IL-1β detection antibody (1% BSA in PBS; 100 μl/well) for 2 h at room temperature. Plates were washed and incubated with HRP conjugated streptavidin (100 μl/well; 1% BSA in PBS) for 20 min, and washed again. Substrate solution (100μl/well; 1:1 H_2O_2:tetramethylbenzidine; Sigma-Aldrich, UK) was applied and incubated

in darkness for 20 min at room temperature. The reaction was stopped using 1 M H_2SO_4 (50 µl/well) and absorbance was read at 450 nm with a BioTek Synergy HT microplate reader.

2.12 Analysis of CCL5

CCL5 concentration was assessed in samples of homogenized tissue prepared from mice in the 4 treatment groups using a mouse/rat CCL5/RANTES Quantikine ® ELISA kit (R&D systems, US). Samples (50µl) were added to a pre-coated 96-well plate and incubated (2 h, room temperature). Samples were washed with the provided buffer, antibody (100 µl, mouse RANTES conjugated antibody) was added to each well, and samples were incubated (2 h, room temperature) and washed. Substrate solution was added to each well and incubation proceeded (30 min, room temperature) after which time the provided STOP solution was added. Optical density was assessed immediately using a BioTek Synergy HT microplate reader set to 450nm with a wavelength 540nm correction.

2.13 Analysis of phagocytosis and chemotaxis and Aβ engulfment

Aβ engulfment was assessed in microglia isolated from young and aged mice treated for 2 h with $A\beta_{1\text{-}40}$ (5 μM). Microglia were fixed in 4% PFA and washed in PBT (PBS+1% Triton X-100) prior to staining. Cells were blocked (1 h, PBT+3% BSA), before incubation overnight at 4°C with rabbit anti-Iba1 (1:1000; Wako, Japan) and mouse anti-6e10 (1:500; Biolegend, US). Coverslips were washed and incubated (2 h, room temperature) with Alexa Fluor 546 donkey anti-rabbit IgG (1:1000) and Alexa Fluor 488 donkey anti-mouse IgG (1:1000), and mounted in ProLong Gold with the nuclear marker DAPI (Thermo Scientific, US). Images (8 fields/animal; 40X magnification) were acquired with a Zeiss AX10 Imager A1 microscope. Analysis of images was undertaken with ImageJ software; the number of $A\beta^+$ microglia was assessed and the % of Iba^+ cells that engulfed Aβ was calculated.

In some experiments, uptake of latex beads (fluorescent yellow-green; 1.0 μm particle size; Sigma-Aldrich, Ireland) was assessed in microglia isolated from young and aged mice. Microglial cells ($6x10^4$ cells/well) were plated onto coverslips coated with poly-D-lysine (5 μg/ml; Merck Millipore Ltd, UK). After 5 days in vitro, latex beads (0.025% in fresh cDMEM media) were added to the wells, incubation continued for 4 h, cells were washed, fixed in 4% paraformaldehyde (PFA) and washed in PBT (PBS+1% Triton X-100) prior to staining. Cells were blocked (1 h in PBT containing bovine serum albumin (BSA; 3%)), incubated overnight (4°C) with primary antibody (rabbit anti-Iba-1; Wako, Japan 1:1000), washed and incubated (2 h; room temperature) with the secondary antibody (Alexa Fluor 546 donkey anti-rabbit IgG (1:1000)). Cells were mounted, fixed and assessed as indicated above. The number of latex $beads^+$ $Iba\text{-}1^+$ microglia and the number of beads per cell (<5 beads/cell; 5-10 beads/cell or >10 beads/cell) were calculated.

Chemotaxis was assessed in microglia isolated from young and aged mice. Cells were plated (115,000 cells/cm^2) onto the transwell insert on a Boyden chamber (pore size 8μm; Corning Inc, UK). After 5 days, media ± ATP (300 μM; Sigma-Aldrich, Ireland) was added to the bottom

on the Boyden chamber. Cells were incubated for 6 h at 37ºC. Non-migrating cells from the upper side of the cell culture insert were removed and migrating cells that remained on the membrane were fixed (4% PFA, 30 min), washed 3 times and mounted in ProLong Gold with the nuclear marker DAPI (Thermo Scientific, US). Images (8 fields; 20X magnification) were acquired with a Zeiss AX10 Imager A1 microscope. Analysis of images was undertaken with ImageJ software, the number of migrated microglia was assessed and chemotaxis percentage was calculated using spontaneous migration as control.

2.14 Immunohistochemistry

PFKFB3, Ki67, β-galactosidase and $p16^{INK4A}$ were assessed by immunostaining. Tissue was incubated with citrate (20 min, 85ºC) and thereafter with pepsin (20 min, room temperature) to facilitate antigen retrieval. Samples were washed, tissue was permeabilised (10 min; PBS with 0.1% Triton-X100), blocked (1 h; 5% BSA with 0.1% Triton-X100) and incubated (overnight, 4°C in primary antibodies goat anti-Iba-1 (LSBio Inc., US; 1:1000), rabbit anti-PFKFB3 (Abcam, UK; 1;250), mouse anti-ki67 (Leica Biosystems, 1;250), chicken anti-β-galactosidase (Abcam, UK; 1;750) or mouse anti-$p16^{INK4A}$ (Abcam, UK; 1;200). Tissue was incubated (2 h, room temperature) with Alexa Fluor® 546 donkey anti-rabbit IgG (1:1000), Alexa Fluor® 633 donkey anti-goat IgG (1:1000), Alexa Fluor® 594 donkey anti-chicken IgG (1:1000) or Alexa Fluor® 488 donkey anti-mouse IgG (1:1000) and mounted in ProLong®Gold with the nuclear marker DAPI (ThermoScientific, US).

β-Galactosidase was also assessed in microglia that were/were not treated with IL-1β using a β-Galactosidase detection kit (Abcam, UK) according to the manufacturer's instructions. Briefly, cells were fixed (15 min in fixative solution), washed, incubated with β-galactosidase staining solution (overnight, 37ºC) and viewed under a microscope. Images (8 fields; 40X magnification) were acquired with a Zeiss AX10 Imager A1 microscope and analysis of images

was undertaken with ImageJ software. The number of β-galactosidase^{+} microglia was assessed and expressed as a % of the total number of cells.

2.15 Statistical analysis

Data are reported as the mean ± SEM and the number of experiments is indicated in each case. Statistical analysis was carried out using Student's t-test for independent means or ANOVA as appropriate. The significance level was set at $p < 0.05$.

3. RESULTS

3.1 Age-related switch in glycolysis in microglia is associated with impaired function

Recent evidence suggests that when microglia are exposed to inflammatory stimuli, like IFNγ or LPS+Aβ, they switch their metabolism towards glycolysis (Holland et al., 2018; Rubio-Araiz et al., 2018) and, similarly, microglia from APP/PS1 mice that have an inflammatory phenotype are glycolytic (Holland et al., 2018; McIntosh et al., 2019). In all studies to date, the switch to glycolysis is correlated with increased PFKFB3. To assess whether a similar change in microglia from aged mice, we assessed PFKFB3 and demonstrate here that there was also an age-related increase in PFKFB3 (*$p < 0.05$; student's t-test for independent means; Figure 1A,B), suggesting a switch to glycolysis. We show that this is associated with compromised microglial function; thus phagocytosis of Aβ (Figure 1C,D) and chemotaxis (Figure 1E) were both significantly reduced in microglia from aged, compared with young, mice (*$p < 0.05$; student's t-test for independent means). In addition, microglia from aged mice released significantly more IL-1β compared with microglia from young mice (*$p < 0.05$; student's t-test for independent means; Figure 1F) adding to the evidence that the phenotype of microglia from aged mice is glycolytic and inflammatory (McIntosh et al., 2019).

3.2 Exercise modulates age-related changes in microglia and improves cognition

It is well established that exercise attenuates several age-related changes in mice including inflammatory changes (Barrientos et al., 2011; Kohman et al., 2013) and that this may contribute to the exercise-related improved cognition. Here, we assessed behaviour in novel object recognition and novel object displacement tasks in young and aged mice that were assigned to sedentary or exercise groups. The data confirmed the earlier findings, in aged rats, of an exercise-related improved performance in both tasks (Birch and Kelly, 2019). Specifically, the mean discrimination ratio data show that there was an age-related deficit in both (*p <0.05, ***p <0.001 Bonferroni post hoc test; young sedentary vs aged sedentary; Figure 2A,B) and that these changes were ameliorated by exercise ($^{++}p< 0.01$; $^{+++}p < 0.001$; aged sedentary vs aged exercise).

Age-related deficits in neuronal function have been linked with many changes and these include neuroinflammatory changes (Costello et al., 2016) and here we chose to assess hippocampal IL-1β mRNA and protein as indicators of neuroinflammation. The data demonstrate that there was an age-related increase in IL-1β mRNA in hippocampus that was attenuated by exercise ($^{\dagger}p < 0.05$; main effect of age, 2 way ANOVA; $^{\S}p < 0.05$; main effect of exercise; Figure 2C,D). A significant age x exercise interaction in IL-1β protein was observed ($p < 0.01$; 2 way ANOVA). Post hoc analysis revealed that IL-1β was significantly increased in tissue from aged compared with young mice (*p < 0.05, young sedentary vs aged sedentary; Bonferoni post hoc test) and that exercise prevented this age-related change ($^{+++}p < 0.001$; aged sedentary vs aged exercise).

These findings suggest that microglia in the aged brain adopt an inflammatory phenotype and the age-related increases in mRNA expression of CD11b and CD68 are consistent with this ($^{\dagger\dagger}p < 0.01$; main effect of age, 2 way ANOVA; Figure 2F,G). Exercise did not attenuate these age-

related changes although evaluation of Iba-1 by western immunoblotting indicated a significant main effect of exercise ($^{\S}p < 0.05$; 2 way ANOVA; Figure 2H).

3.3 Microglia from aged mice are glycolytic and exercise restores metabolic homeostasis

Having established that exercise attenuated the age-related increase in IL-1β mRNA and protein, we argued that if inflammation drives glycolysis as previously suggested (Finucane et al., 2019; Galvan-Pena and O'Neill, 2014; Rodriguez-Prados et al., 2010), then exercise should impact on microglial metabolism. Therefore we examined the metabolic profile of microglia prepared from the 4 cohorts of mice in this study. There was an age-related increase in ECAR (Figure 3A) and analysis of mean glycolysis and mean glycolytic capacity revealed a significant age x exercise interaction. Post hoc analysis indicated that both were significantly increased in microglia from aged sedentary, compared with young sedentary, mice ($^{**}p<0.01$; Bonferroni post hoc test; Figure 3B,C) and that these age-related increases were attenuated in aged animals that were assigned to the exercise group ($^{+}p <0.05$). OCR was decreased in microglia from aged, compared with young, mice (Figure 3D) and, although there was no significant change age- or exercise-related change in basal respiration (Figure 3E), there was a significant age-related decrease in maximal respiration ($^{\dagger}p < 0.05$; main effect of age, 2 way ANOVA; Figure 3F).

PFKFB3 is a master regulator of glycolysis (Pilkis et al., 1981; Yalcin et al., 2014) and we have determined that its expression correlates with a switch to glycolysis in microglia from neonatal (Holland et al., 2018; Rubio-Araiz et al., 2018) and adult (McIntosh et al., 2019) mice. Here, immunohistochemical analysis of PFKFB3 revealed a marked increase in the number of PFKFB3^{+} Iba-1^{+} cells in sections from aged sedentary, compared with young sedentary, mice (Figure 4A) and analysis of the mean values indicated a statistically significant increase ($^{***}p <0.001$; Bonferroni post hoc test; 2 way ANOVA; Figure 4B). Consistent with this, PFKFB3 was

increased in hippocampal tissue prepared from aged, compared with young, mice as indicated in the sample immunoblot (Figure 4C) and in the analysis of the mean data (*p <0.05; Bonferroni post hoc test; Figure 4D), and this change was attenuated in aged mice that were assigned to the exercise group ($^{+}$p <0.05; Bonferroni post hoc test). We also examined hippocampal expression of hexokinase II and LDH, which catalyze the first and last steps in glycolysis and are rate-limiting. With respect to hexokinase, the data indicate that there were significant main effects of age (††p < 0.01) and exercise (§§p < 0.01; Figure 4E). There was no age-related effect on LDH but a significant decrease with exercise was observed (§p < 0.05; main effect of exercise; Figure 4F).

3.4 IL-1β triggers glycolysis in microglia

The small molecule inhibitor of the inflammasome, MCC950, attenuates inflammation-induced glycolysis in macrophages (Finucane et al., 2019) and microglia (Rubio-Araiz et al., 2018) indicating a key role for IL-1β. Here we considered that IL-1β, which is increased as a result of inflammasome activation in microglia from aged, compared with young, mice, might be a factor in driving glycolysis. To assess this, we prepared microglia from neonatal mice, incubated them in the presence and absence of IL-1β and assessed ECAR. IL-1β increased ECAR (Figure 5A) and significantly increased both glycolysis and glycolytic capacity (*p < 0.05; student's t-test for independent means; n = 6 and 9 in control and IL-1β-treated microglia; Figure 5B,C). Consistent with a key role for PFKFB3 in driving glycolysis, the data also show that IL-1β increases PFKFB3 in microglia (*p < 0.05; student's t-test for independent means; Figure 5D).

Previous studies revealed that macrophages and also lymphocytes infiltrate the brain with age and this is associated with neuroinflammation and a deficit in synaptic function (Barrett et al., 2015; Costello et al., 2016). The evidence suggests that infiltrating immune cells

markedly increase inflammation, negatively impact on cognitive function and are a source of inflammatory cytokines including IL-1β (Browne et al., 2013; Costello et al., 2016; McManus et al., 2014; McQuillan et al., 2010). Since exercise had a modest effect on microglial activation, the primary source of IL-1β in the brain, we assessed whether it might impact on infiltration of peripheral immune cells. First we assessed CCL5, which plays a role in recruitment of macrophages and T lymphocytes (Strazielle et al., 2016; Zhou et al., 2018) and demonstrate that it was significantly increased in tissue from aged, compared with young, mice (***$p < 0.001$; Bonferroni post hoc test; Figure 6A) and that exercise attenuated this age-related increase ($^{+}p < 0.05$; Bonferroni post hoc test). Analysis of data from flow cytometry revealed age-related increases in the infiltration of macrophages, monocytes, lymphocytes and neutrophils in the brain of aged, compared with young, animals (*$p < 0.05$; ***$p < 0.001$; Bonferroni post hoc test; Figure 6B-E) and exercise ameliorated these changes ($^{+}p < 0.05$; $^{++}p < 0.01$; $^{+++}p < 0.001$; Bonferroni post hoc test).

3.5 Microglia from aged animals exhibit signs of senescence that are ameliorated by exercise

We asked whether the shift to glycolysis, which is an inefficient means of generating ATP, might affect microglial viability. To assess this, we used immunohistochemical analysis to evaluate the expression of 2 markers of cell senescence, β-galactosidase and $p16^{INK4a}$, in Iba-1^{+} cells. β-galactosidase is upregulated and accumulates in lysosomes of senescent cells because it identifies lysosomes which expand in these cells, whereas $p16^{INK4a}$ is a cell cycle inhibitor that also accumulates in senescent cells. It plays a role in inhibiting cyclin dependent kinase (CDK)-induced phosphorylation of retinoblastoma protein (pRb); hypophosphorylated pRb is a potent cell cycle inhibitor. The data show that the number of β-galactosidase^{+} Iba-1^{+} cells and $p16^{INK4a+}$ Iba-1^{+} cells was significantly increased in aged, compared with young, mice (**$p<0.01$; ***$p < 0.001$; Bonferroni post hoc test; Figure 7A-D)

and that these age-related increases were significantly attenuated in aged animals that were assigned to the exercise group ($^{++}p < 0.01$; Bonferroni post hoc test). In an effort to explain this decrease in senescent cell numbers, we asked whether there might be an increase in microglial cell turnover and show that exercise significantly increased $Ki67^+$ $Iba\text{-}1^+$ cells in the hippocampus of aged mice ($^*p < 0.05$; student's t-test; Figure 7E,F).

3.6 IL-1β increases β-galactosidase and reduces phagocytosis in microglia

To assess whether IL-1β might contribute to cell senescence, we prepared microglia from neonatal mice, incubated them in the presence or absence of IL-1β and assessed β-galactosidase immunoreactivity. The data show that IL-1β significantly increased β-galactosidase immunoreactivity in these cells ($^{***}p < 0.001$; student's t-test; Figure 8A,B). It might be predicted that cells which exhibit signs of senescence will also exhibit a deterioration in function and here we show that IL-1β reduced microglial uptake of latex beads (Figure 8C); specifically, IL-1β significantly decreased the number of phagocytic cells (Figure 8D) and also the phagocytic capacity of cells as indicated by the decrease in the number of latex beads within cells ($^*p < 0.05$; $^{**}p < 0.01$; student's t-test; Figure 8E).

3.7 Age-related senescence in microglia impacts on function

Since exercise ameliorated the age-related microglial glycolysis, and reduced the number of senescent microglia, we considered that it may also have a beneficial effect on phagocytic function and therefore assessed the phagocytosis in microglia from aged and young mice that were assigned to the sedentary and exercise groups. We found that there was a significant exercise-related decrease in the number of cells that phagocytosed less than 5 beads ($^{\S}p < 0.05$; main effect of exercise; 2 way ANOVA; Figure 9A,B) and an exercise-related increase in

the number of cells that phagocytosed more than 10 beads ($^{\S}p < 0.05$; main effect of exercise; Figure 9C), regardless of age.

4. DISCUSSION

The key findings presented here are that exercise revitalises microglia, attenuating the age-related increase in expression of the senescent markers, β galactosidase and $p16^{INK4A}$, and it decreases the age-related shift towards an inflammatory and glycolytic phenotype, reducing the dependence of microglia on metabolically-inefficient glycolysis. The data indicate that there is an age-related increase in IL-1β, which increases glycolysis, and this may derive from the infiltration of immune cells or from senescent cells, both of which are reduced in microglia of aged mice assigned to the exercise subgroup. These changes are associated with improved cognitive function.

The increase in PFKFB3 immunoreactivity demonstrated in microglia from aged mice suggests that these cells may be glycolytic because this enzyme is a known driver of glycolysis (Pilkis et al., 1981; Yalcin et al., 2014), because of the previous studies indicating that inflammation-induced glycolysis is consistently associated with increased PFKFB3 (Holland et al., 2018; McIntosh et al., 2019; Rubio-Araiz et al., 2018) and because inhibiting PFKFB3 prevents the LPS+Aβ-induced glycolysis (Finucane et al., 2019). The evidence presented links this change with reduced function as indicated by compromised phagocytosis and chemotaxis. It is not clear why microglial function is impaired but one possibility is that an increase in dependence on glycolysis will reduce the cells' metabolic efficiency since glycolysis produces only 2 molecules of ATP per glucose molecule compared with >32 from oxidative metabolism. It has been reported many times, especially in macrophages, that cells which are inflammatory are also glycolytic (Galvan-Pena and O'Neill, 2014) and, consistently, the present data show that microglia from aged mice release more IL-1β.

The evidence indicates that exercise attenuates the age-related increase in IL-1β in the hippocampus and, given that inflammation and cognitive function are intimately related, it was predictable that, as previously shown, exercise also restored performance in the novel object recognition and object displacement tasks. Exercise-related improvement in cognitive and neuronal function in aged animals is a consistent finding (Birch and Kelly, 2019; Svensson et al., 2015), although the mechanism involved remains a subject of debate. On the one hand, some experimenters have linked the improvement with decreased IL-1β (Gibbons et al., 2014; Speisman et al., 2013); others have reported no change in inflammatory cytokines, but an increase in hippocampal IL-10, following exercise (Gomes da Silva et al., 2013) leading to the proposal that the beneficial effects of exercise on cognition in aged animals may derive from its anti-inflammatory actions. However exercise-induced neurogenesis, accompanied by increased expression of BDNF has also been proposed as the key contributor to the improved cognitive function in aged animals (Gibbons et al., 2014; Kelly, 2018; Speisman et al., 2013).

Activated microglia are a likely cell source of IL-1β and the age-related increases in mRNA expression of CD11b and CD68, described here, are indicative of microglial activation, supporting similar findings from a previous study (Gibbons et al., 2014). However exercise did not alter expression of either marker, perhaps because of the exercise regime used here. It has been shown that a longer period of exercise, 8-10 weeks wheel running in 22 month-old female mice, decreased Iba-1^{+} CD86^{+} MHCII^{+} microglia in the hippocampus (Kohman et al., 2013).

Recent evidence has indicated that microglia that exhibit an inflammatory phenotype switch their metabolism towards glycolysis (Holland et al., 2018; McIntosh et al., 2019; Rubio-Araiz et al., 2018) and here, we demonstrate that ECAR, as well as glycolysis and glycolytic capacity were increased in isolated microglia from aged, compared with young, mice and that exercise ameliorated these age-related changes. Interestingly, there was an age-related decrease in

oxidative metabolism in microglia but while exercise corrected one aspect of the shifting metabolism in microglia, glycolysis, it did not impact on OCR.

We have consistently reported that PFKFB3 is increased in microglia that adopt a glycolytic phenotype (Holland et al., 2018; McIntosh et al., 2019; Rubio-Araiz et al., 2018), suggesting that this enzyme plays a key role in the metabolic switch in microglia as it does in other cells, notably macrophages (Finucane et al., 2019; Galvan-Pena and O'Neill, 2014; Holland et al., 2018) and cancer cells (Ros and Schulze, 2013). Here we have consolidated this observation showing that the age-related increase in glycolysis in microglia from aged mice is accompanied by an increase in the proportion of $PFKFB3^{+}$ $Iba\text{-}1^{+}$ cells and protein expression of PFKFB3 in microglia. The strong link between glycolysis and PFKFB3 was further supported by the observation that exercise attenuated the age-related change in these measures. Two other enzymes that play significant roles in cell metabolism are hexokinase, which catalyses the first step in the glycolytic pathway and LDH which catalyses the production of lactate from pyruvate, shunting pyruvate away from the TCA and the electron transport chain. In addition to the effect of exercise on PFKFB3, the present data indicate that protein expression of both enzymes was decreased in microglia from mice that were assigned to the exercise group. Overall, the findings indicate that exercise profoundly affects microglial phenotype and, to our knowledge, this is the first evidence that exercise is capable of inducing such an effect.

The underlying cause of the age-related shift to glycolysis in microglia remains to be determined but one possibility is the increase in IL-1β. This idea is supported by the finding that IL-β increases glycolysis in microglia and this may be mediated by an increase in PFKFB3. The parallel changes in glycolysis and PFKFB3 add to the correlation between these factors that we have previously reported in microglia and to the evidence that PFKFB3 is a key enzyme in driving glycolysis.

Microglia are the primary cell course of IL-1β in the brain but in age and in neuropathology peripheral immune cells infiltrate the brain and provide an additional source of IL-1β (Browne et al., 2013; Costello et al., 2016; McManus et al., 2014; McQuillan et al., 2010). Thus we (Costello et al., 2016) and others (Moraga et al., 2015; Ritzel et al., 2019; Unger et al., 2018) have reported that there is an age-related increase in infiltrating immune cells and, at least in our hands, macrophages from aged animals are sensitized to inflammatory stimuli (Barrett et al., 2015) and release IL-1β in response to soluble extract obtained from brain tissue of aged mice (Costello et al., 2016). The present data concur with these findings providing evidence of increased monocytes, macrophages, lymphocytes and neutrophils in the brains of aged, compared with young mice. Interestingly there was little evidence of infiltrating cells in aged mice that were assigned to the exercise group and this correlates with the decrease in IL-1β and is consistent therefore consistent with the idea that infiltrating cells may contribute to the age-related increase in IL-1β.

Production of IL-1β relies on inflammasome activation and the endogenous activator(s) of the inflammasome in the aged brain remain to be identified. Possibilities include DAMPs that may be released from dying cells or signalling to the brain as a consequence of age-related increased circulating inflammatory molecules (Bruunsgaard and Pedersen, 2003). An alternative source of IL-1β is senescent cells that are characterised by their secretory phenotype, the so-called senescence-associated secretory phenotype; the secretome includes cytokines like IL-6 but also IL-1 (Borodkina et al., 2018; Meyer et al., 2017). Senescent cells also produce other factors including reactive oxygen species and cathepsin B (Coppe et al., 2010) that are known inflammasome activators, as well as matrix metalloproteinases that can release IL-1β in an inflammasome-independent manner at least in eosinophils (Esnault et al., 2019). We assessed 2 markers of microglial senescence, β-galactosidase and $p16^{INK4a}$; β-

galactosidase, specifically the senescence-associated isoform, increases because lysosomes expand in senescent cells. $p16^{INK4a}$ levels accumulate with age and, because it binds to cyclin D dependent kinases, CDK4 and CDK6 displacing cyclin D, it cause growth arrest and ultimately cell senescence (Baker and Petersen, 2018). The data indicate that both markers were increased in Iba-1^+ cells in sections prepared from aged mice and this is consistent with data from a previous study that reported an age-related increase in $p16^{INK4a}$ (Ritzel et al., 2019). Importantly, the age-related increases in both senescence markers were attenuated in sections from aged exercised mice. Thus, there is a positive correlation between senescent microglia and IL-1β suggesting that a causal relationship between the two might exist. We show that exercise also upregulated microglial turnover in hippocampus of aged mice as indicated by the increase in $Ki67^+$ $Iba1^+$ cells and this may, in part, explain the reduction in senescent cells in aged mice that were assigned to the exercise group.

Although the emphasis on the study of senescent cells has been focussed mainly in cancer research, Streit and colleagues reported the presence of dystrophic, senescent microglia in the aged human brain (Streit et al., 2004) that are found adjacent to degenerating neurons (Streit et al., 2009). Indeed this group has proposed that microglial senescence, rather than microglial activation, is the significant factor in driving age-related deterioration of neuronal function because senescent cells lose their neuroprotective function. The present findings support this proposal since the age-related increases in both senescence markers were attenuated in sections from aged exercised mice and this paralleled the improved cognitive function. Indeed the possibility that senescent cells may promote neurodegenerative changes has been proposed (Baker and Petersen, 2018).

Analysis in cultured microglia indicated that IL-1β increased one marker of senescence, β-galactosidase and, in parallel with this, it decreased phagocytosis. Thus not surprisingly, the homeostatic function of microglia is altered by increasing senescence but while inflammatory

molecules have been shown to decrease phagocytic function in some circumstances (Sarlus and Heneka, 2017), this is not always the case (Ferreira et al., 2011).

Here we asked whether phagocytic function might be reduced *in vivo* where there was evidence of microglial senescence and the data showed that this was the case suggesting a causal link between the two. We have proposed that cell function may be compromised if cells shift their metabolism to the more metabolically-inefficient glycolysis and we have reported that microglia from APP/PS1 mice which have a glycolytic phenotype, have reduced ability to phagocytose Aβ (McIntosh et al., 2019). The present data add another factor, microglial senescence, that negatively impacts on function and demonstrate that when the age-related shift to glycolysis in microglia is attenuated by exercise, the number of senescent microglia decreases and the phagocytic capacity of cells is increased.

The recognition that exercise profoundly affects brain health has driven the burgeoning interest in determining the mechanism by which this is achieved. A significant emphasis has been placed on exploring the well-described neurogenesis and several studies have established a role for neurotrophic factors, primarily BDNF. The present data point to alternative mechanism by which exercise improves cognitive function in aged animals and this pivots around attenuating the age-related microglial senescence and associated age-related shift to glycolysis. The proposal is that these changes limit the neuroprotective functions of microglia while this is restored by exercise.

5. CONCLUSIONS

The recognition that exercise profoundly affects brain health has driven the burgeoning interest in determining the mechanism by which this is achieved. A significant emphasis has been placed on exploring the well-described neurogenesis and several studies have established a role for neurotrophic factors, primarily BDNF. The present data point to

alternative mechanism by which exercise improves cognitive function in aged animals and this pivots around attenuating the age-related microglial senescence and associated age-related shift to glycolysis. The proposal is that these changes limit the neuroprotective functions of microglia while this is restored by exercise.

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FIGURE LEGENDS

Figure 1. Age exerts a negative impact on microglial function

Microglia were prepared from aged and young mice as described in Methods.

A,B. PFKFB3 immunoreactivity was significantly increased in microglial prepared from aged, compared with young, mice (*p < 0.05;). C-E. Phagocytosis of Aβ (E,D) and chemotaxis (E) were significantly reduced in microglia from aged, compared with young, mice (*p < 0.05). F. Microglia from aged mice released significantly more IL-1β compared with microglia from young mice (*p < 0.05). Data are presented as means ± SEM and assessed by the student's t-test for independent means (n=5).

Figure 2. Exercise attenuates the age-related inflammation and cognitive dysfunction.

A,B. Mice were trained in the NOR and NOD tests and tested after 9 days of exercise training and the discrimination ratio (DR) was calculated. Significant age and exercise effects were observed (NOR, age: $F(1,26)=30.25$, $p<0.0001$, exercise: $F(1,26)= 59.56$, $p<0.0001$; NOD, age: $F(1,27)=22.61$, $p<0.0001$, exercise: $F(1,27)=24.45$, $p<0.0001$; 2 way ANOVA) and post hoc analysis identified a significant age-related decrease in NOR and NOD (*p < 0.05; ***p < 0.001; Bonferoni post hoc test) and a significant beneficial effect of exercise ($^{++}p< 0.01$; $^{+++}p < 0.001$). C-H. Hippocampal tissue was prepared for analysis of mRNA expression and western immunoblotting as described in the methods. Significant age effects were observed in mRNA expression of IL-1β (C; $F(1,13)=8.15$, †p < 0.05; 2 way ANOVA), CD11b (F; $F(1,42)=19.75$, ††p < 0.01; 2 way ANOVA) and CD68 (G; $F(1,24)=28.39$, ††p < 0.01; 2 way ANOVA) and a significant exercise effect was observed in the case of IL-1β mRNA ($F(1,13)=14.99$, $^{§}p < 0.05$; 2 way ANOVA). Post hoc analysis of processed (17kDa) IL-1β revealed a significant age-related increase (*p<0.05, Bonferoni post hoc test) that was significantly attenuated in sample from exercised mice ($^{+++}p<0.005$, Bonferoni post hoc test). Analysis of Iba-1 by western

immunoblotting revealed a significant exercise effect (H; $F(1,27)=10.38$, $^{\S}p < 0.05$; 2 way ANOVA). Data are presented as means ± SEM (n=8-13).

Figure 3. Age triggers a switch to glycolysis in microglia that is ameliorated by exercise

Microglia were prepared from the brains of young and aged, sedentary and exercised, mice and assessed for ECAR or OCR using the SeaHorse Extracellular Flux (XF96) Analyser. For ECAR, measures were taken at baseline and after addition of glucose (80mM), oligomycin (9μM) and 2-deoxyglucose (500mM) as indicated by the arrows. To assess OCR, measures were taken at baseline and after addition of oligomycin (20μM), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (20μM; FCCP) and antimycin A (40μM) as indicated by the arrows.

A-C. The bioenergetic profile (A) indicates that ECAR was greatest in microglia from aged exercise mice and posthoc analysis of mean glycolysis (B) and glycolytic capacity (C) indicated significant age-related increases ($^{**}p < 0.01$; 2 way ANOVA with post hoc Bonferoni test) that were significantly ameliorated in the aged exercise group ($^{+}p <0.05$).

D-F. The OCR bioenergetics profile (G) and maximal respiration (I) indicate that oxidative metabolism was decreased in microglial cells from aged animas ($F(1,22)=7.40$, $^{\dagger}p<0.05$; 2 way ANOVA). Basal respiration was unchanged (H). Data (A-C and G-I) are presented as means ± SEM (n =6-8).

Figure 4. Exercise inhibits the age-related changes in metabolic enzymes

Co-localization of PFKFB3 and Iba-1 was performed by immunohistochemistry. The sample immunograph indicates that there was a marked increase in co-localization of PFKFB3 and Iba-1 (A), and analysis of the mean data show that there was a significant age-related increase in the percentage of Iba-1^{+} cells that stained positively for PFKFB3 (B; $^{***}p < 0.001$; ANOVA

with post hoc Bonferoni test). Exercise significantly attenuated the age-related change ($^{+}p < 0.05$; n = 4-7).

C-F. Samples of hippocampal homogenate were used to assess the protein expression of hexokinase II, PFKFB3 and LDH by western immunoblot as indicated in the sample immunoblot (C). Analysis of the mean data indicate that there was a significant age-related increase in PFKFB3 from age, compared with young, mice (D; $^{*}p < 0.05$; 2 way ANOVA with post hoc Bonferoni test) that was significantly ameliorated in the aged exercise group ($^{+}p < 0.05$). Significant main effects of age ($F(1,14)=5.68$, $^{\dagger\dagger}p < 0.01$; 2 way ANOVA) and exercise ($F(1,14)=4.16$, $^{\S\S}p < 0.01$; 2 way ANOVA) were observed in hexokinase (D) and a significant effect of exercise was observed in LDH (F; $F(1,14)=8.27$, $^{\S}p < 0.05$; 2 way ANOVA). Data are presented as means ± SEM (n=8-13).

Figure 5. IL-1β triggers glycolysis in microglia

Microglia were prepared from neonatal mice, incubated ± IL-1β and assessed for metabolic profile, senescence and function as described above.

A-C. IL-1β increased ECAR (A) and analysis of the mean data indicated a significant IL-1β-induced increase in glycolysis (B) and glycolytic capacity (C; $^{*}p < 0.05$). D. Analysis by western immunoblotting revealed that IL-1β increased PFKFB3 as indicated by the sample immunoblot and the mean densitometric data (D; $^{*}p < 0.05$).

Figure 6. Exercise decreases the infiltration of immune cells into the brain

A single cell suspension was prepared from brain tissue of mice in each of the treatment groups as described in the methods and these samples were used to carry out flow cytometry and CCL5 concentration was assessed in the homogenate.

Post hoc analysis of the mean data indicated that there was a significant age-related increase

in CCL5 (A; ***p < 0.001; ANOVA with post hoc Bonferoni test) that was attenuated in aged exercise mice ($^{+}$p <0.05). Similarly age-related increases in the infiltration of macrophages, monocytes, lymphocytes and neutrophils were observed in the brain of aged, compared with young, animals (*p < 0.05; ***p < 0.001; Bonferroni post hoc test) and exercise significantly attenuated the age-related change ($^{+}$p <0.05; $^{++}$p <0.01). Data are presented as means ± SEM (n =7-8).

Figure 7. Exercise decreases expression of markers of cell senescence

A-D. Expression of the senescence markers, β-Gal and $p16^{INK4A}$ in Iba-1^{+} cells was evaluated by immunohistochemistry in brain slices of young and aged, sedentary and exercised, mice. The sample immunographs indicate that β-Gal^{+} Iba-1^{+} cells (A) and $p16^{INK4A+}$ Iba-1^{+} cells (C) were increased in sections from aged sedentary mice. Post hoc analysis of the data identified a significant age-related increase in β-Gal^{+} Iba-1^{+} and $p16^{INK4A+}$ Iba-1^{+} cells (B,D; **p < 0.01; ***p < 0.001; Bonferoni post hoc test) and a significant beneficial effect of exercise ($^{++}$p< 0.01). E,F. Exercise enhanced microglial turnover as indicated by the increase in Ki67^{+} Iba1^{+} cells (*p < 0.05; student's t-test for independent means). Data are presented as means ± SEM (n=5-6).

Figure 8. IL-1β increases β-galactosidase and reduces phagocytosis in microglia

Microglia were prepared from neonatal mice, incubated ± IL-1β and assessed for β-galactosidase immunoreactivity and phagocytic function.

A,B. IL-1β increased β-galactosidase immunoreactivity and significantly increased the number of β-galactosidase^{+} cells (B; ***p < 0.001). C,D. IL-1β also decreased phagocytosis of latex beads by microglia (C), and specifically the proportion of phagocytic microglia (D; *p < 0.05) and the phagocytic capacity of the cells as indicated by the number of beads/cell (E; **p <

0.01). Data are presented as means ± SEM and assessed by the student's t-test for independent means (n=5).

Figure 9. Impact of exercise on phagocytic capacity of microglia

Microglia, prepared young and aged, sedentary and exercised, mice, were assessed for phagocytic capacity by evaluating engulfment of latex beads. Representative images are shown (A). The percentage of phagocytic cells with less than 5 beads per cells (B) and phagocytic cells with more than 10 beads per cell (C) were analysed. Exercise decreased the number of cells with <5 beads ($F(1,16)=5.19$, $^{\$}p < 0.05$; 2 way ANOVA) and increased the number of cells with >10 beads and a main effect of exercise was observed in both measures, ($F(1,16)= 6.66$, $^{\$}p < 0.05$; 2 way ANOVA). Data are presented as means ± SEM (n=5 with duplicates).

Figure 1

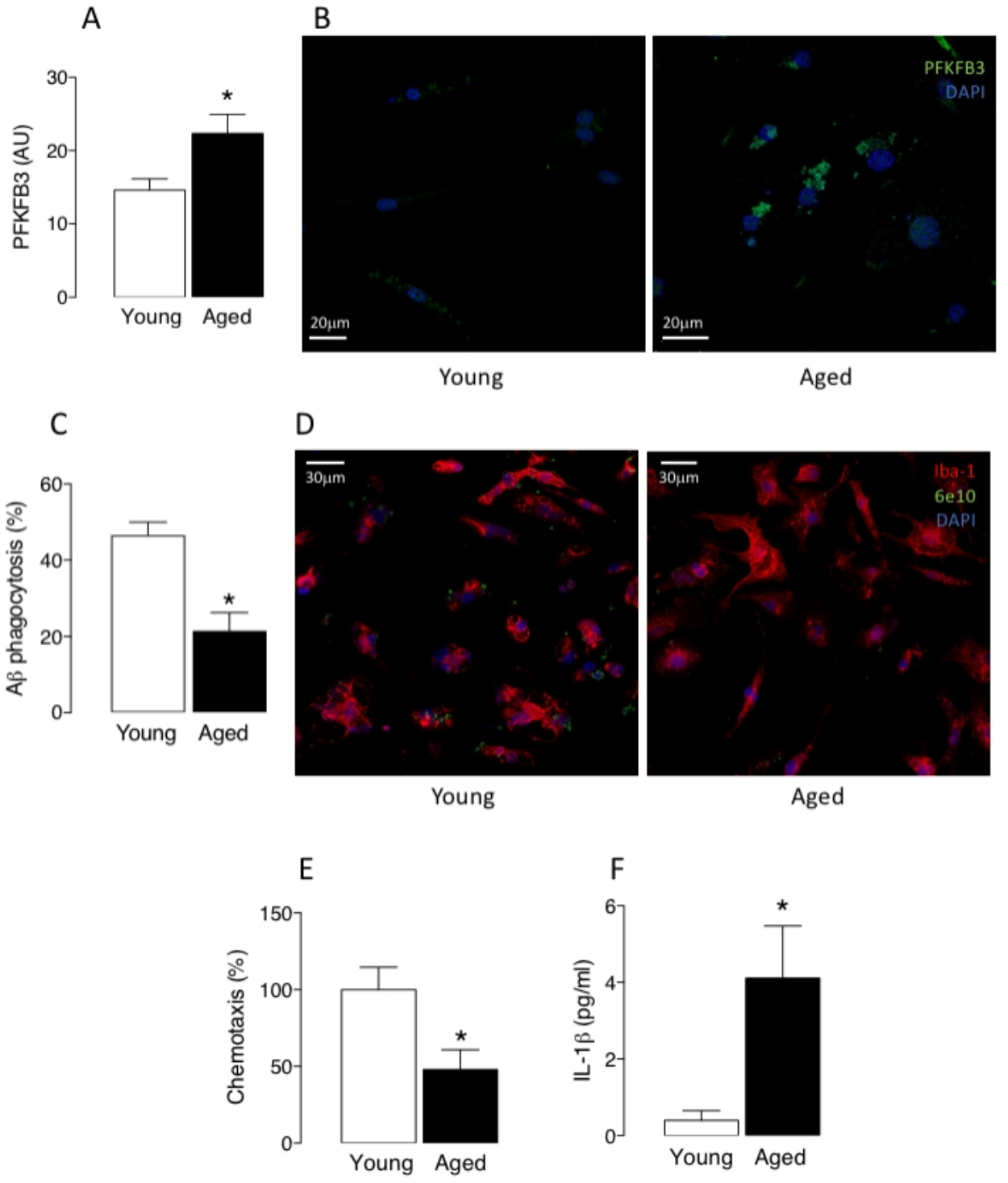

A
B
PFKFB3 (AU)
30
20
10
0
*
Young
Aged
20μm
20μm
PFKFB3
DAPI
Young
Aged
C
D
Aβ phagocytosis (%)
60
40
20
0
*
Young
Aged
30μm
30μm
Iba-1
6e10
DAPI
Young
Aged
E
F
Chemotaxis (%)
150
100
50
0
*
Young
Aged
IL-1β (pg/ml)
6
4
2
0
*
Young
Aged

Figure 2

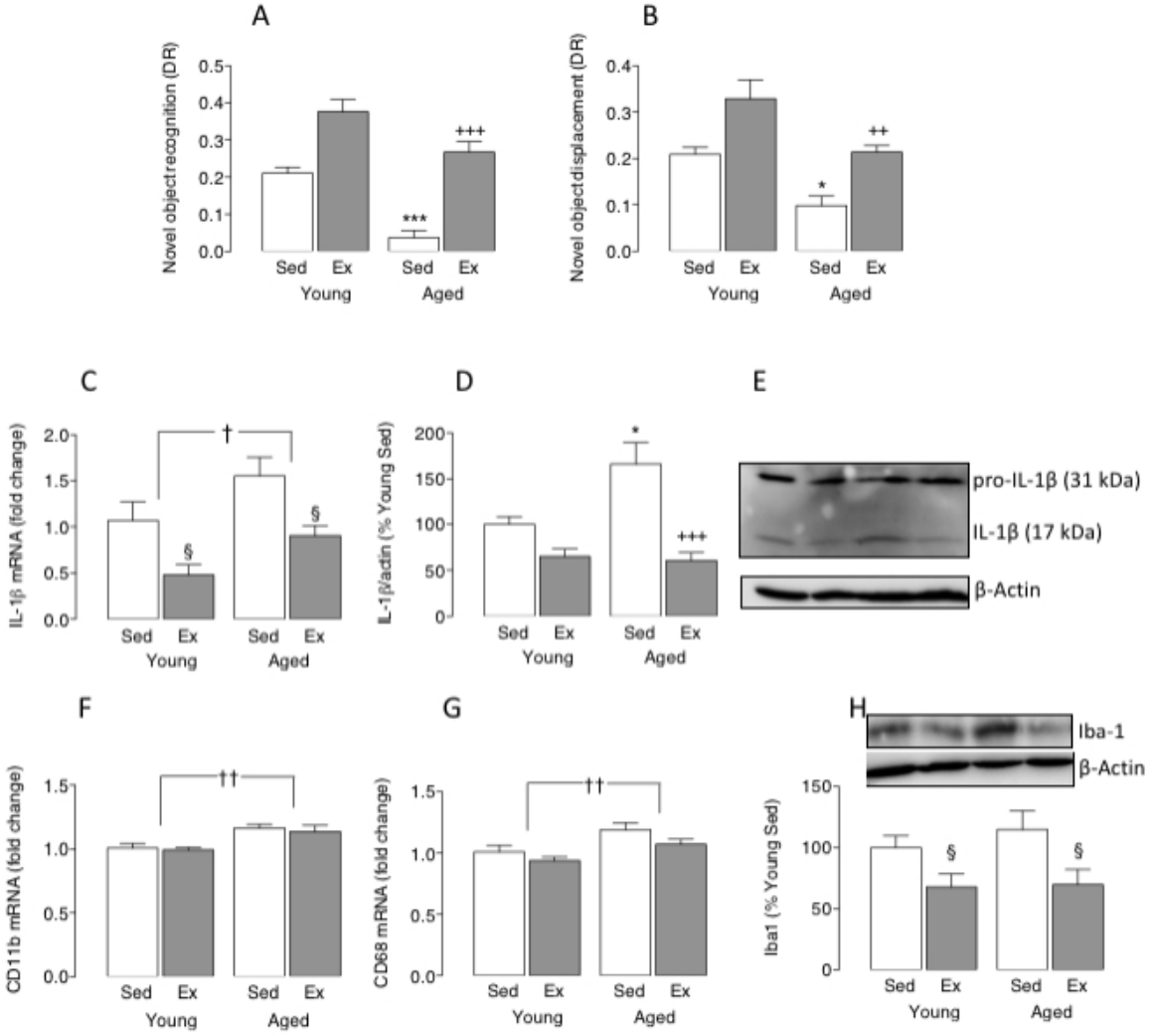

A
Novel object recognition (DR)
0.5
0.4
0.3
0.2
0.1
0.0
***
+++
Sed Ex Sed Ex
Young Aged
B
Novel object displacement (DR)
0.4
0.3
0.2
0.1
0.0
*
++
Sed Ex Sed Ex
Young Aged
C
IL-1β mRNA (fold change)
2.0
1.5
1.0
0.5
0.0
†
§
§
Sed Ex Sed Ex
Young Aged
D
IL-1β/actin (% Young Sed)
200
150
100
50
0
*
+++
Sed Ex Sed Ex
Young Aged
E
pro-IL-1β (31 kDa)
IL-1β (17 kDa)
β-Actin
F
CD11b mRNA (fold change)
1.5
1.0
0.5
0.0
††
Sed Ex Sed Ex
Young Aged
G
CD68 mRNA (fold change)
1.5
1.0
0.5
0.0
††
Sed Ex Sed Ex
Young Aged
H
Iba-1
β-Actin
Iba1 (% Young Sed)
150
100
50
0
§
§
Sed Ex Sed Ex
Young Aged

Figure 3

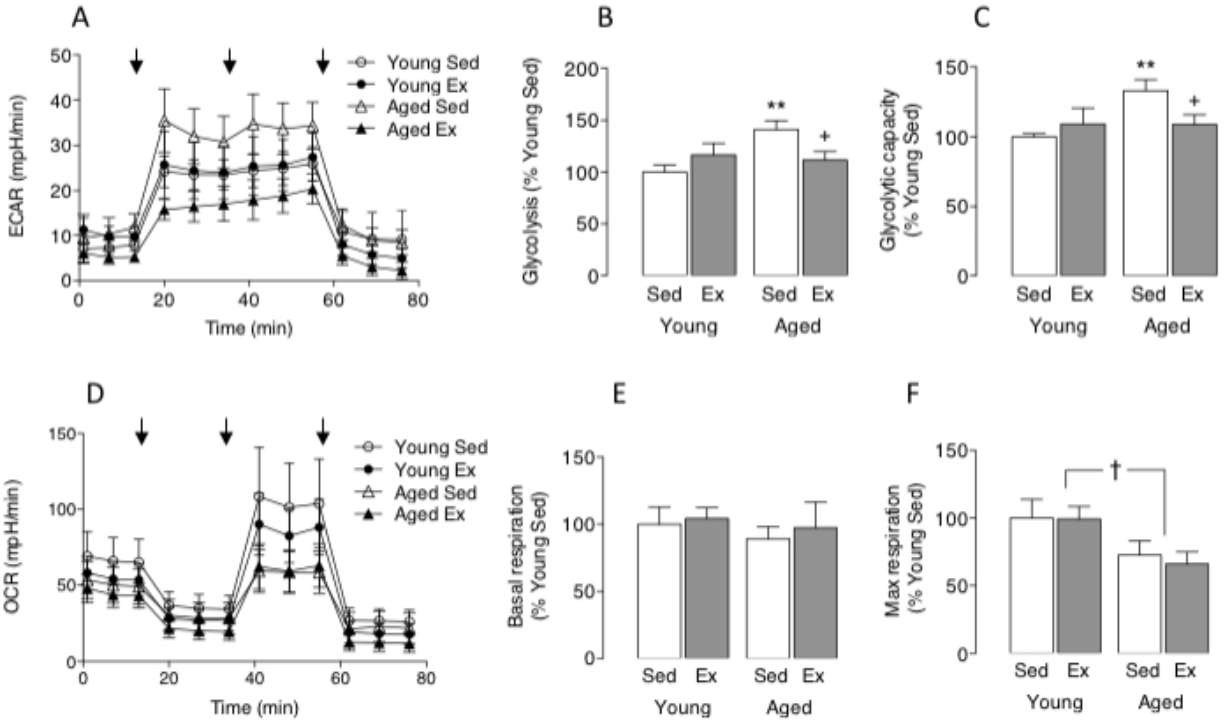

A
ECAR (mpH/min)
Time (min)
Young Sed
Young Ex
Aged Sed
Aged Ex
B
Glycolysis (% Young Sed)
Sed Ex Sed Ex
Young Aged
C
Glycolytic capacity (% Young Sed)
D
OCR (mpH/min)
E
Basal respiration (% Young Sed)
F
Max respiration (% Young Sed)

Figure 4

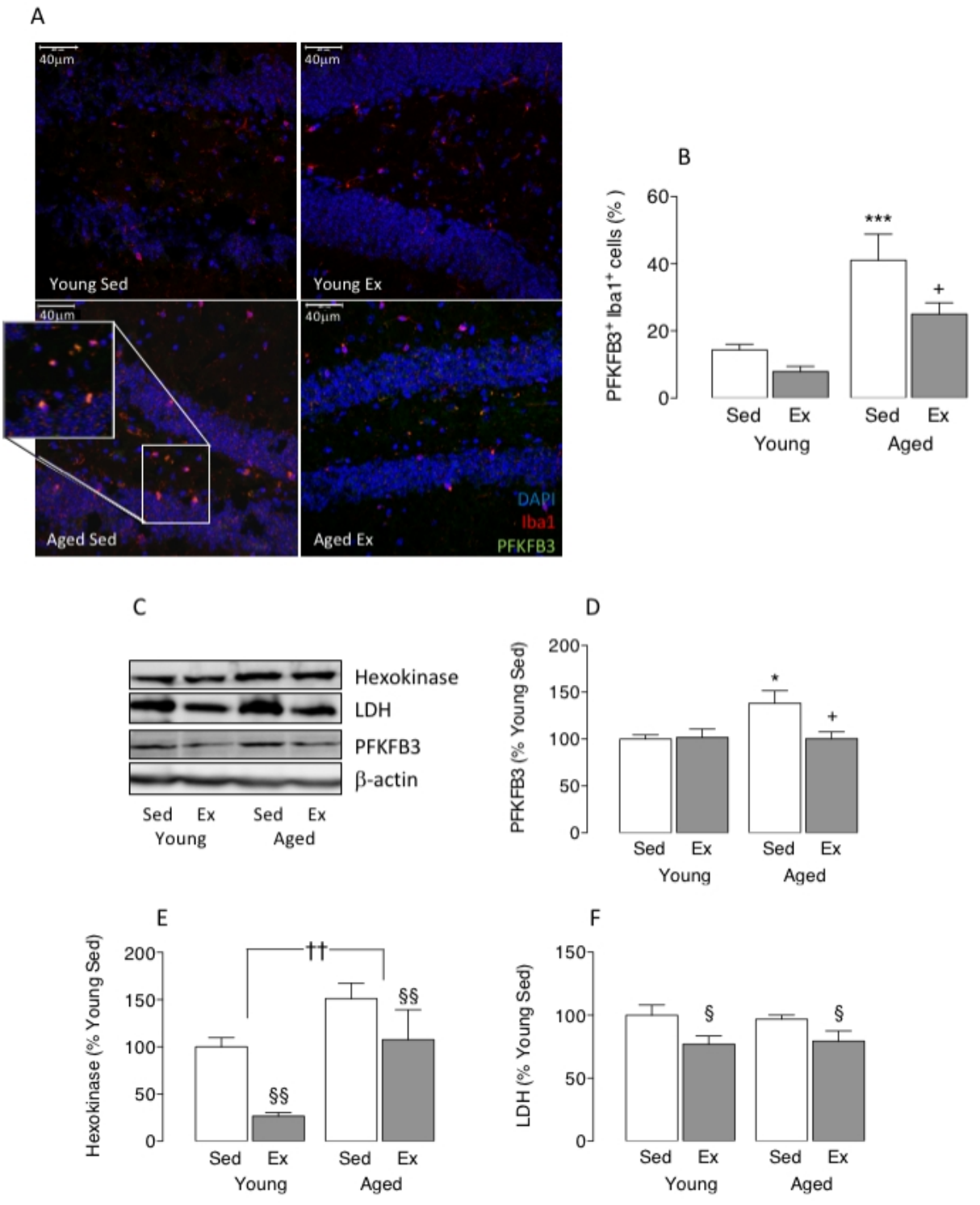

A
40µm
Young Sed
Young Ex
Aged Sed
Aged Ex
DAPI
Iba1
PFKFB3
B
PFKFB3+ Iba1+ cells (%)
60
40
20
0
***
+
Sed
Ex
Young
Aged
C
Hexokinase
LDH
PFKFB3
β-actin
D
PFKFB3 (% Young Sed)
200
150
100
50
*
E
Hexokinase (% Young Sed)
††
§§
F
LDH (% Young Sed)
§

Figure 5

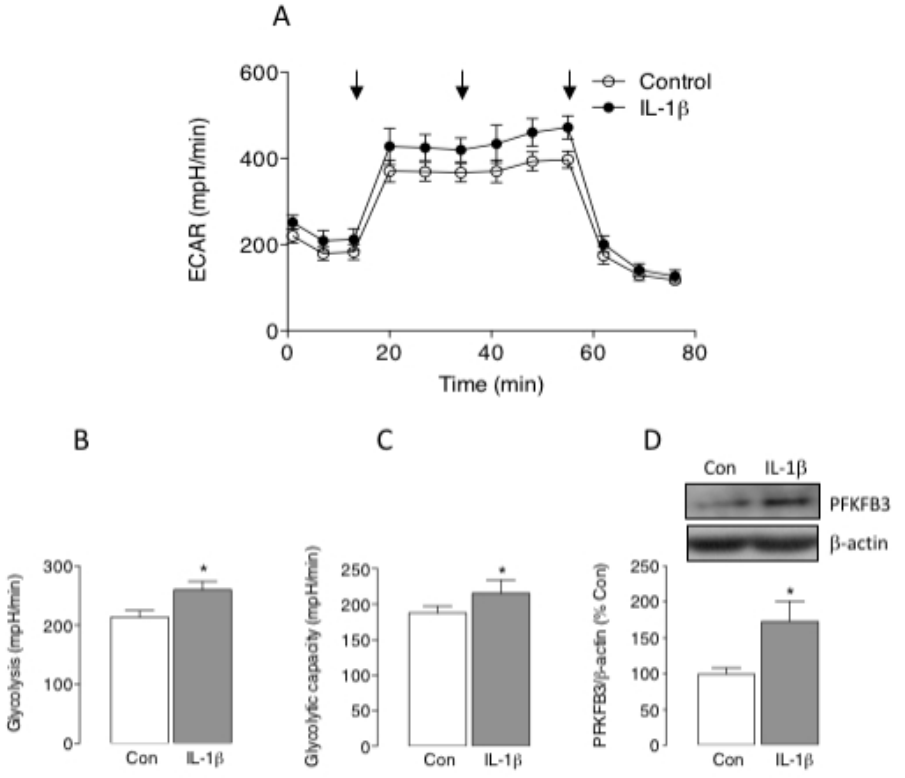

A
600
400
200
0
ECAR (mpH/min)
0
20
40
60
80
Time (min)
Control
IL-1β
B
Glycolysis (mpH/min)
300
200
100
0
Con
IL-1β
*
C
Glycolytic capacity (mpH/min)
250
200
150
100
50
0
Con
IL-1β
*
D
Con
IL-1β
PFKFB3
β-actin
PFKFB3/β-actin (% Con)
250
200
150
100
50
0
Con
IL-1β
*

Figure 6

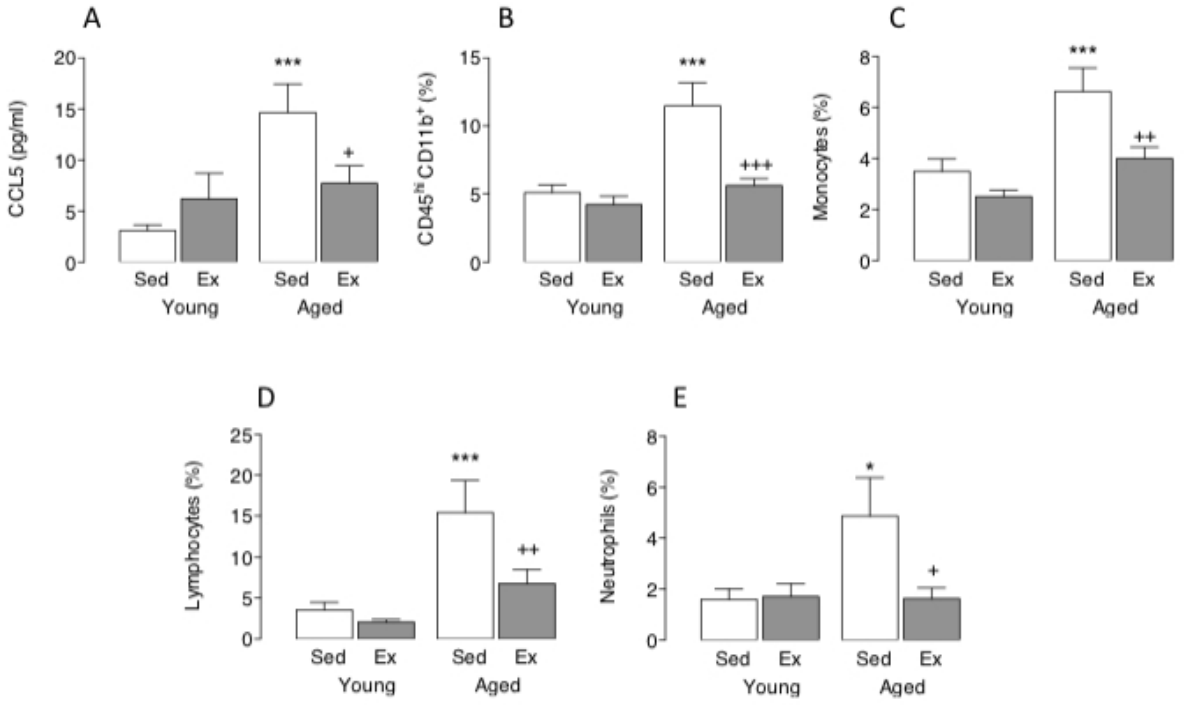

A
CCL5 (pg/ml)
20
15
10
5
0
***
+
Sed
Ex
Sed
Ex
Young
Aged
B
CD45hi CD11b+ (%)
15
10
5
0
***
+++
Sed
Ex
Sed
Ex
Young
Aged
C
Monocytes (%)
8
6
4
2
0
***
++
Sed
Ex
Sed
Ex
Young
Aged
D
Lymphocytes (%)
25
20
15
10
5
0
***
++
Sed
Ex
Sed
Ex
Young
Aged
E
Neutrophils (%)
8
6
4
2
0
*
+
Sed
Ex
Sed
Ex
Young
Aged

Figure 7

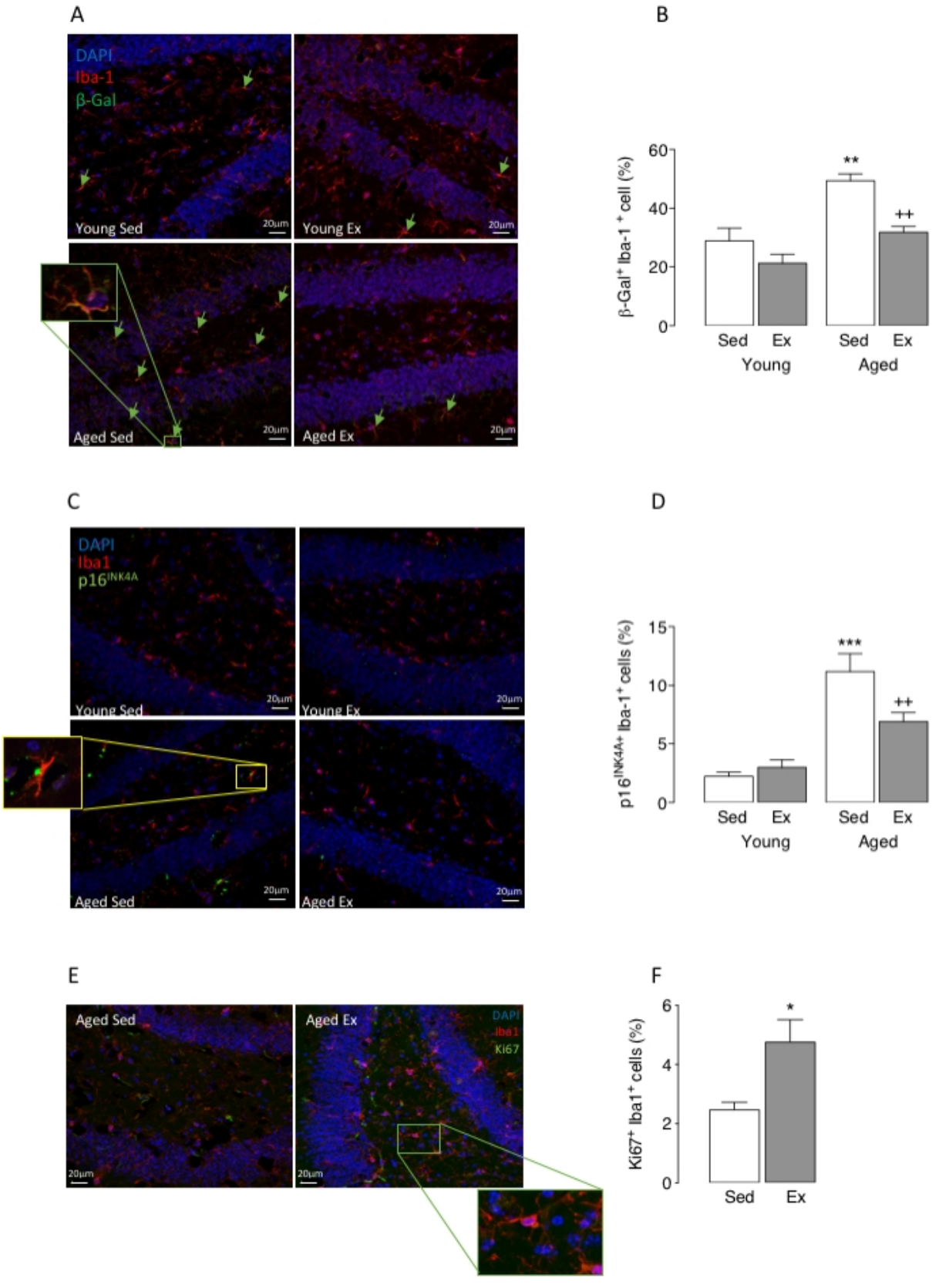

A
DAPI
Iba-1
β-Gal
Young Sed
Young Ex
Aged Sed
Aged Ex
20μm
B
β-Gal+ Iba-1+ cell (%)
60
40
20
0
**
++
Sed
Ex
Young
Aged
C
DAPI
Iba1
p16INK4A
Young Sed
Young Ex
Aged Sed
Aged Ex
20μm
D
p16INK4A+ Iba-1+ cells (%)
15
10
5
0
***
++
Sed
Ex
Young
Aged
E
Aged Sed
Aged Ex
DAPI
Iba1
Ki67
20μm
F
Ki67+ Iba1+ cells (%)
6
4
2
0
*
Sed
Ex

Figure 8

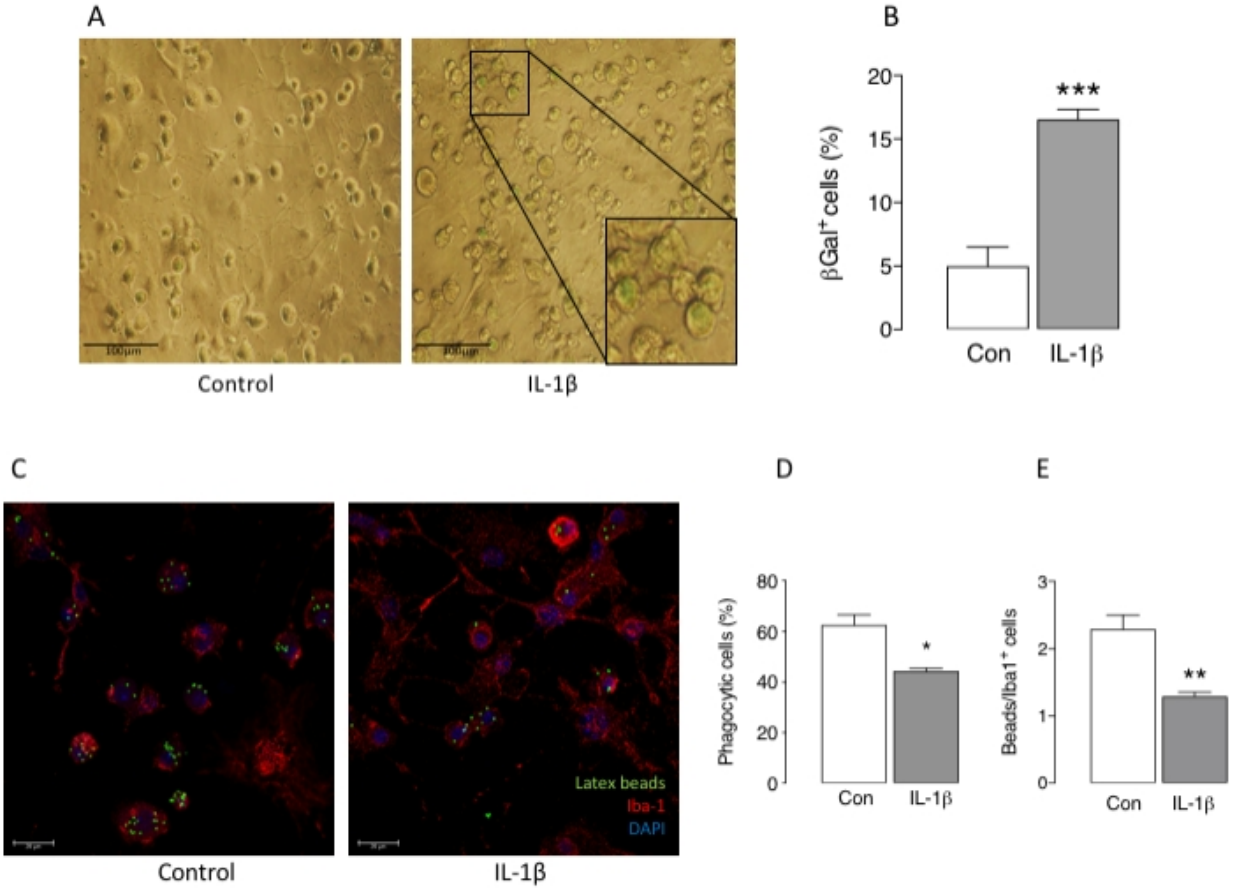
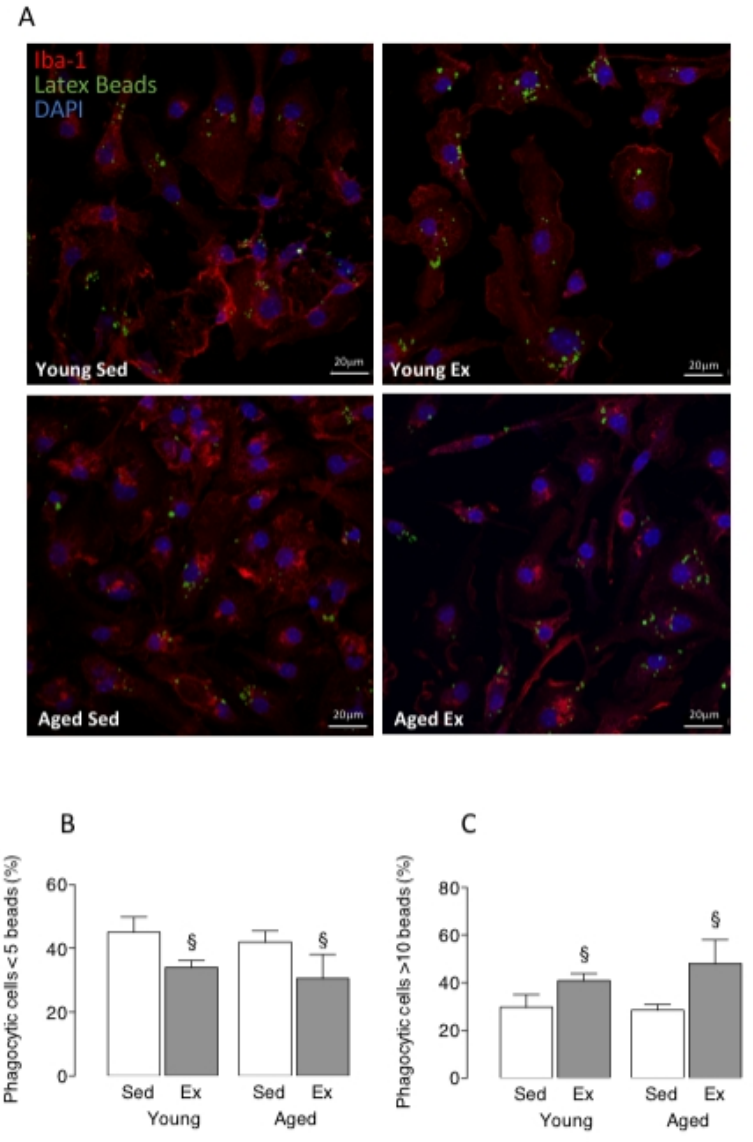

A
Control
IL-1β
B
βGal+cells (%)
20
15
10
5
0
***
Con
IL-1β
C
Latex beads
Iba-1
DAPI
Control
IL-1β
D
Phagocytic cells (%)
80
60
40
20
0
*
Con
IL-1β
E
Beads/Iba1+ cells
3
2
1
0
**
Con
IL-1β

Figure 9


A
Iba-1
Latex Beads
DAPI
Young Sed
Young Ex
Aged Sed
Aged Ex
20µm
B
Phagocytic cells <5 beads (%)
60
40
20
0
§
Sed
Ex
Young
Aged
C
Phagocytic cells >10 beads (%)
80
60
40
20
0