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Research report

Insulin activates microglia and increases COX-2/IL-1 β expression in young but not in aged hippocampus

Clarissa Branco Haas^{a,b,d}, Afonso Kopczynski de Carvalho^a, Alexandre Pastoris Muller^c,
Bart J.L. Eggen^b, Luis Valmor Portela^{a,*}

^a Laboratory of Neurotrauma and Biomarkers, Department of Biochemistry, Programa de Pós-graduação em Bioquímica, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil

^b Department of Biomedical Sciences of Cells & Systems, Section Molecular Neurobiology, University of Groningen, University Medical Center Groningen (UMCG), Groningen, the Netherlands

^c Laboratory of Translational Biomedicine, Pós-graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense (UNESC), Criciúma, SC, Brazil

^d Current address: Department of Anesthesiology, Columbia University Medical Center (CUMC), Columbia University, New York, NY, USA

HIGHLIGHTS

- Insulin persistently activates microglia and increases COX-2 in the hippocampus of young, but not aged rats.
- Insulin resistance is accompanied by a desynchronized hippocampal neuroinflammatory signaling in the aging brain.
- Primary microglia are sensitive to insulin via Akt signaling *in vitro*.
- Microglia in the hippocampus can integrate insulin signaling and neuroinflammatory responses.

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ABSTRACT

Brain insulin resistance and neuroinflammation are known to increase with age. Insulin exerts metabolic roles on neurons and astrocytes, but its effects on microglia is unclear. In this study we investigated whether insulin affected microglia in the hippocampus of young and aged rats. We injected intracerebroventricular (i.c.v.) insulin (20 mU) or vehicle for five days and evaluated microglial inflammatory markers in the hippocampus of young (3 months) Wistar rats. Increased microglial activation (Iba-1⁺CD68⁺ cells) and COX-2/IL-1 β levels in the hippocampus were found. Since the aged brain is an experimental model for brain insulin resistance and chronic neuroinflammation we submitted aged rats (22 months) to i.c.v. insulin/vehicle administration and found no significant increase in Iba-1⁺CD68⁺ microglia or COX-2/IL-1 β levels. To further investigate whether insulin triggered transient or persistent proinflammatory responses, young rats were evaluated eight-days after the last insulin injection. Microglia were persistently activated, and COX-2 levels remained elevated in the hippocampus, which paralleled increased spatial memory performance in the Morris Water Maze behavioral task. To determine if microglia were directly responsive to insulin, primary microglia were challenged with insulin and increased Akt Ser473 phosphorylation, a protein activated by the insulin receptor, was detected. These data suggest that microglia in the hippocampus integrate insulin signaling and neuroinflammatory responses and that this signal is disrupted during chronic inflammation. In our concept, the disruption between microglia activation by insulin signaling is a new pathological mechanism behind insulin resistance in the aging brain.

1. Introduction

Microglia are a specialized and dynamic population of central nervous system (CNS) parenchyma-resident macrophages that are able to detect tissue damage and (micro) environmental variations (Hanisch

and Kettenmann, 2007). Although, microglia are the primary innate immune cells in the brain, their CNS-tailored functions go beyond their well-characterized roles in neuroinflammation (Salter and Beggs, 2014). Microglia also have important functions in non-immune brain physiology and are reported to contribute to learning and memory

* Corresponding author at: Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul – UFRGS, Rua Ramiro Barcelos, 2600 anexo, 90035-003, Brazil.

E-mail address: roskaportela@gmail.com (L.V. Portela).

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Fig. 1. Insulin triggers microglia activation in the hippocampus. (A) Confocal microscopy representative images of microglia (Iba-1-green), activated microglia (CD68-red) and nuclei (DAPI-blue) markers in both young and aged vehicle/insulin treated animals in hippocampal sub regions Cornu Ammonis 1 (CA1), Cornu Ammonis 3 (CA3), and Dentate Gyrus (DG); Scale bar = 25 μ m. (B) Microglial activation (Iba-1⁺CD68⁺ cells/Iba-1⁺ cells) in CA1 (n = 4 animals per group). (C) Microglial activation (Iba-1⁺CD68⁺ cells/Iba-1⁺ cells) in CA3 (n = 4 animals per group). (D) Microglial activation (Iba-1⁺CD68⁺ cells/Iba-1⁺ cells) in DG (n = 4 animals per group). Two-way ANOVA followed by Tukey multiple comparisons poshoc test. Results are expressed as mean $\pm$ SEM. *p $\leq$ 0.05; **p $\leq$ 0.01; ns: p > 0.05.

(Parkhurst et al., 2013). Other important microglia functions are trophic neuronal support, removal of cellular debris, synaptic pruning, injury repair and homeostatic support for living neurons, which is crucial for the neural circuit remodeling and synaptic plasticity (Galatro et al., 2017; Janssen et al., 2017; Salter and Beggs, 2014).

Microglia sense disturbances in brain homeostasis and turn out to be “activated”, a phenotype characterized by morphological changes, e.g. process retraction and thickening, and upregulation of a broad spectrum of cellular membrane surface antigens, such as CD68 (O’Neil et al., 2018). Microglial activation is accompanied by expression of several intracellular molecules including the COX enzyme family followed by intracellular and extracellular expression of cytokines (Akundi et al., 2005). Neuroprotective functions of COX-2 are already described, which include contributions to learning and memory processing (Bazan, 2006; Choi et al., 2009; Teather et al., 2002). Although substantial amount of evidences had identified both IL-1 β and IL-6 as important

cytokines with potential neuroprotective functions (Borsini et al., 2015; Frakes et al., 2014; Gruol, 2015; Seibert and Masferrer, 1994; Tak and Firestein, 2001; Takemiya et al., 2017) indicating that proinflammatory cytokines responses are not only detrimental to the brain (Biber et al., 2014), IL-1 β and IL-6 neurophysiological and neuroprotective signals still need further scrutiny.

During aging, microglia adopt a more activated and sensitized state phenotype referred to a “primed”, resulting in an exaggerated inflammatory response to an inflammatory stimulus (Perry and Holmes, 2014; Raj et al., 2014). Aging is associated with persistent changes in the brain microenvironment, and chronic neuroinflammatory stimulation has been proposed as a key factor associated with microglial dysfunction and a mechanism for inducing the age-related functional deficits (Holtman et al., 2015; Karch and Goate, 2015; Norden et al., 2015; Spittau, 2017). However, although aged microglia may adopt an undesirable phenotype, the mechanistic effectors of this reprogramming

are not well comprehended.

Insulin has diverse functions in the CNS, including neurotrophic signaling (Bomfim et al., 2012; Rhea et al., 2019). Brain insulin administration improved memory in young but not in aged rats, which was related with a disrupted connection between both insulin and BDNF pathways in the aged hippocampus (Haas et al., 2016). Accordingly, experimental rodent models and studies with aged patients have consistently demonstrated a strong mechanistic association between deficient insulin signaling, cognitive disruption and Alzheimer's disease (Ghasemi et al., 2013; Gutches, 2014; Lopez-Otin et al., 2013; Steculorum et al., 2014; Talbot et al., 2012). The role of insulin in neurons has been extensively investigated, but only recently its role in glial cells has received the necessary attention. For instance, insulin signaling in hypothalamic astrocytes controls the availability of glucose to neurons, which is crucial for the regulation of whole body energy homeostasis (Garcia-Caceres et al., 2016). Recently, it was demonstrated that insulin receptor (IR) and upstream proteins (IRS-1 and IRS-2) are expressed in human primary astrocytes and microglia supporting the notion that insulin may directly bind to and influence microglia activity (Spielman et al., 2015), but, it is still unclear the downstream microglial insulin signaling (Akt-GSK3- β), how insulin affects microglia activation *in vivo*, and, similarly as occurs in aged neurons (Rodriguez-Rodriguez et al., 2017), if primed microglia are resistant to insulin.

In order to address the effect of insulin on microglia *in vivo*, we performed insulin infusions directly into the brain of young and aged rats and analyzed their activation, and COX-2, IL-6 and IL-1 β expression in the hippocampus. To detect a direct insulin-mediated response by microglia, we performed *in vitro* experiments using primary microglia cultures.

2. Results

2.1. Insulin triggers microglia activation in the young but not in the aged hippocampus

To evaluate the insulin effects on microglia *in vivo*, we performed Iba-1/CD68 co-staining (Fig. 1a) to access microglia activation in the hippocampus. We found that after five days of i.c.v. insulin treatment, microglia of young rats were more activated in CA1 ($p = 0.022$) (Fig. 1b) and CA3 ($p = 0.009$) (Fig. 1c) hippocampal subregions when compared to controls. No significant alterations were detected in the DG ($p = 0.165$) (Fig. 1d). During aging, CNS develops resistance to insulin signaling and increased neuroinflammation (Haas et al., 2016; Raj et al., 2017). Next, we performed the same treatment in aged rats with insulin to assess whether aged (primed) microglia might be resistant to insulin challenges. Interestingly, insulin did not further increase the number of already present activated microglia. (Fig. 1b-d). As expected, and in conjunction with previous observations (Ojo et al., 2015; Solano Fonseca et al., 2016), naïve aged rats showed significantly increased microglial activation in the hippocampus in comparison to young rats – CA1 ($p = 0.001$) (Fig. 1b) and DG ($p = 0.002$) (Fig. 1d).

2.2. Insulin increases COX-2 and IL-1 β expression in the hippocampus of young rats

Trying to elucidate the neuroinflammatory molecules that would link to microglia activation in young rats, we determined COX-2, IL-1 β and IL-6 expression levels in the hippocampus. Insulin significantly increased COX-2 levels in young rats ($p = 0.003$), an almost 3-fold increase compared to the young vehicle group was observed. In aged animals, we did not detect an increase in COX-2 levels in response to insulin (Fig. 2a, b). In young animals, this profile was accompanied by increased levels of IL-1 β ($p = 0.038$) (Fig. 2c) but not IL-6 (Fig. 2d). In contrast, the level of IL-6 level was decreased in aged animals after insulin treatment ($p = 0.031$) (Fig. 2d).

2.3. Microglia remain activated after 8 days of insulin treatment in young rats

Next, we determined the persistence of the neuroinflammatory response to insulin in young Wistar rats. Young rats were subjected to the same protocol of 5-days insulin (i.c.v., 20 mU) and analyzed microglia activation 8 days after the last administration (Fig. 3a). We found that microglia were persistently activated in the CA3 ($p = 0.015$) (Fig. 3c), but not in the CA1 region ($p > 0.05$) (Fig. 3b). To determine the neurological impact of insulin and if this treatment was neuroprotective during this 8 day-period, we performed a Morris water maze test, as previously described by us and others (Adzovic et al., 2015; Haas et al., 2016). We confirmed that relative to vehicle, insulin treatment increased spatial memory performance in young rats ($p = 0.020$) (Sup. Fig. 2).

2.4. COX-2 but not IL-1 β expression persists after 8 days of insulin treatment

To investigate if the prolonged CA3 microglial activation (Fig. 3) is accompanied with a persistent increased expression of COX-2 and IL-1 β , we measured these inflammatory markers 8 days after the last insulin treatment. COX-2 levels remained increased in the hippocampus as previously observed (Fig. 1) when compared to vehicle ($p = 0.0409$) (Fig. 4a, b). The increased IL-1 β levels observed after 5 days of insulin treatment (Fig. 2c) returned to baseline levels at day 8 post-administration (Fig. 4c), and IL-6 levels were not significantly altered (Fig. 4d).

2.5. Primary microglia cells are responsive to insulin

To determine if microglia are responsive to insulin, we used primary microglia cell culture and analyzed the phosphorylation and activation of proteins AKT and GSK3- β , which are downstream signals of the insulin receptor. Cells exposed to insulin (1000 ug/mL) for 30 min displayed increased AKT phosphorylation ($p = 0.003$) (Fig. 5a), but GSK3- β phosphorylation was not found altered (Fig. 5b). Incubation with the PI3K inhibitor wortmannin prevented insulin-induced AKT phosphorylation (Sup. Fig. 1), confirming that, insulin receptor downstream signals occur via PI3K and AKT in microglia.

3. Discussion

Our findings imply that the inflammatory responses to insulin are different between young and aged microglia. This notion is supported by the increased number of CD68-positive microglia in the CA1 and CA3 hippocampal subfields of young but not in aged rats in response to insulin. Complementary, the analysis of the inflammatory markers associated with microglia highlighted a concomitant higher expression of COX-2 and IL-1 β in the hippocampus of young rats, and these elevated COX-2 levels were sustained until at least 8 days after insulin administration. Aged rats did not respond to insulin challenges with microglial activation and increased hippocampal levels of IL-1 β /COX-2 as was observed in young animals, suggesting a complementary mechanism associated with brain insulin resistance during aging process. Also, we confirmed in primary microglia cultures, that insulin activates the phosphorylation of AKT (Fig. 5 and Sup. Fig. 1), which signals downstream of the insulin receptor (Fernandez and Torres-Aleman, 2012). These cellular cultures were generated from neonatal mouse pups, suggesting that microglia in young mice and rats can respond to insulin and that this responsiveness is lost or perturbed with aging.

Microglia are a highly ramified cell type in close contact with synapses, supporting an ideal microenvironment for synaptic network connectivity (Wu et al., 2015). Here, the activation of microglia, particularly in the CA1 and CA3 subregions of young rats, suggests that insulin influences the expression profile of inflammatory mediators and spatial memory processing, which is a function highly dependent on the

Fig. 2. Insulin increases COX-2 and IL-1 β in young rats' hippocampus. (A) Representative images of western blot detection of COX-2 expression in young and aged animals' hippocampus. (B) Quantification of western blot detection of COX-2 expression ($n = 5-6$ animals per group). (C) ELISA analysis of interleukin 1 β (IL-1 β) in young and aged ($n = 7-10$ animals per group) Wistar rats' hippocampus. (D) ELISA analysis of interleukin 6 (IL-6) in young and aged rats ($n = 5-6$ animals per group). Results are expressed as average $\pm$ SEM. * $p \leq 0.05$; ** $p \leq 0.01$; ns: $p > 0.05$.

hippocampus. We found that the pattern of protein expression in microglia of young rats after insulin treatment was accompanied by increased performance in the acquisition and test phases of the Morris Water Maze (MWM) task (Sup. Fig. 2) corroborating an association between microglia phenotypes and memory function (Haas et al., 2016). Although the positive effects of both COX-2 and IL-1 β on memory were already described by others (Bazan, 2006; Donzis and Tronson, 2014; Goshen et al., 2007; Takemiya et al., 2017; Teather et al., 2002), our results primarily connect this signature with insulin mediating higher brain functions, likely through microglia. Also, it was previously demonstrated that COX gene expression in the hippocampus of 24 months-old rats was not altered (Aid and Bosetti, 2007) corroborating with our finding that COX-2 protein level are not changed in 22 months-old. However, it should be taken into consideration that neither the referred study nor our study provided evidences to discard possible abnormalities in COX enzymatic activity due to aging.

The resulting response of microglia to insulin challenges seems to be impaired during aging, which is coincident with the decline in the peripheral and central insulin signaling previously described (Steculorum et al., 2014), moreover, animal models of diabetes showed CSF decreased insulin levels (Nameni et al., 2017a,b). Also, despite controversies, it was already demonstrated that the brain produces a small amount of insulin, albeit is still unknown the exact function and whether this synthesis might contribute to extracellular levels within a brain affected by aging (Aid and Bosetti, 2007). Adult rats manifest impaired glucose control during the early periods of the aging process likely associated with deficits in the peripheral insulin signaling (Arnold et al., 2018). We found a resistant profile in the glucose tolerance test (GTT) in 180 days old rats compared to 60 days old expressed by a higher peak of blood glucose levels after the intraperitoneal (i.p.) administration of a sucrose load (Sup. Fig. 3A) and a significantly higher area under the curve (AUC) (Sup. Fig. 3B). The levels of insulin in the cerebrospinal fluid (CSF) of fed animals were not changed in aged rats (22 months old), compared to young ones (3 months old) (Sup. Fig. 4A), however the insulin level on hippocampus were decreased on aged animals compared to young (Sup. Fig. 4B) pointing a possible impairment on insulin delivery or activation on hippocampus of the aged animals.

Insulin serves as a hub for several physiological roles underlying cell

survival (Bruning et al., 2000) and hypothalamic glucose support from astrocytes to neurons (Garcia-Caceres et al., 2016). Besides, insulin has been previously proposed to integrate peripheral and central immune signals (De Felice and Ferreira, 2014), and not coincidentally, insulin resistance has been connected with increased neuroinflammation and aged-related abnormalities (Lopez-Otin et al., 2013; Steculorum et al., 2014), our study shows that at basal conditions, the microglia in aged animals are more activated than in young ones. such finding is in agreement with others (Shoham et al., 2019) and with the sustained inflammation hypothesis in the course of aging (Wyss-Coray and Mucke, 2002). Also, when insulin was injected in aged rats there was neither hippocampal microglia activation nor increased IL-1 β production, but a decrease in IL-6 hippocampal levels. Although we showed that primary microglia are responsive to insulin and leads to increase IL-1 β production, we cannot rule out that in our *in vivo* experiments this cytokine originates from other cells in the CNS. However, this limitation does not negate the possibility that microglia might be enrolled in neuroprotection even at activated state and expressing pro-inflammatory molecules, albeit persistent inflammation represents a substrate for unfavorable neurological outcomes (Heneka et al., 2014; Saijo and Glass, 2011). Importantly, long-term i.c.v. infusions of insulin in young rats prevented neuroinflammatory and memory impairment outcomes induced by lipopolysaccharide (LPS) (Adzovic et al., 2015) reinforcing its direct importance as a neuroinflammatory mediator. Although the relationship between hippocampal neuroinflammation, microglia phenotypes and cognitive decline during aging is largely discussed in the current literature in both experimental and clinical studies (Tangestani Fard and Stough, 2019), here the absence of a functional endpoint is a limitation for future clinical interpretations of the present results.

Also, as previously conceptualized by others, neuroinflammation is a result of integrated reactions that take place in astrocytes and microglia but further involves a concomitant exchange of signals with neurons (Tjalkens et al., 2017). Remarkably, i.c.v. insulin infusion diffuses through the brain tissue and may activate IR signals in neuronal and astrocytic cells thereby impacting microglia physiology, or alternatively intrinsic microglial mechanisms may be affected by the insulin administration and propagate effects to the neighbor cells, or both (Butterfield et al., 2014). Although this particular question is still

Fig. 3. Microglia activation triggered by insulin is persistent in CA3 hippocampal subregion after 8 days of insulin treatment. (A) Confocal microscopy representative images of microglia – Iba-1 (green), activated microglia – CD68 (red) and nuclear – DAPI (blue) markers in both young and aged vehicle/insulin treated animals in the sub regions Cornu Ammonis 1 (CA1), Cornu Ammonis 3 (CA3), and Dentate Gyrus (DG); Scale bar = 25 μm. (B) Microglial activation (Iba-1⁺CD68⁺ cells/Iba-1⁺ cells) in CA1 (n = 4 animals per group). (C) Microglia activation (Iba-1⁺CD68⁺ cells/Iba-1⁺ cells) in CA3 (n = 4 animals per group). (D) Microglia activation (Iba-1⁺CD68⁺ cells/Iba-1⁺ cells) in DG (n = 4 animals per group). Results are expressed as average ± SEM. *p ≤ 0.05; **p ≤ 0.01; ns: p > 0.05.

unsolved, our present findings bring subsidies to further investigate the interplay between insulin and the triad astrocyte-neuron-microglia mediating neuroinflammatory responses. Another question rises from the immunological bidirectional communication between periphery and brain. It is known that peripheral inflammatory cytokines, such as IL-6, IL-1β, TNF-α, cross the blood brain barrier and cause increased expression of transcriptional factor NFκ-β within the brain cells accompanied by locally increments in the pro and anti-inflammatory effectors (Fishel et al., 2005). Conceptually exacerbated production of pro-inflammatory mediators oppose central and peripheral insulin signaling and may be a risk factor of neurodegeneration and cognitive problems (Akiyama et al., 2000). However, these studies often use strategies to exacerbate the global inflammatory status with LPS injections and diet-induce obesity and DM type 2. We already showed that “normal aged rats” did not respond to insulin infusions (same strategy used in the present study) with increased spatial memory performance, which predicts a lack of association between the reported microglial phenotypes and inflammatory markers with spatial memory outcomes. This assumption is in line with the effects of insulin in young rats.

In conclusion, insulin signaling caused a persistent microglia activation in the hippocampus of young rats, together with increased expression of inflammatory molecules COX-2 and IL-1β, and microglia were sensitive to insulin via the AKT signaling pathway. In aged brains, an apparent lack of responsiveness to insulin was detected, indicative of aging-associated insulin resistance of the CNS. In our concept, the rupture between the neuroinflammatory insulin signaling in the hippocampus and memory processing is a new pathological mechanism behind insulin resistance and cognitive decline in the aging brain.

4. Materials and methods

4.1. In vivo experiments

In vivo experiments were performed using 3 and 22-month-old male Wistar rats under approval of the UFRGS Local Ethic Committee for Animal Experimentation (CEUA); project number: #26376. Wistar rats were housed in groups of maximum five animals, in a room with controlled temperature, under a 12 h light/dark cycle and with free access to food and water.

Fig. 4. Hippocampal COX-2 persists increased after 8 days of insulin treatment. (A) Representative images of COX-2 immunodetection in the hippocampus. (B) COX-2 expression level (n = 6–7 animals per group) acquired from the western blot analysis. (C) Interleukin 1 β (IL-1 β) and (D) interleukin-6 levels were measured in the hippocampal homogenates of young and aged Wistar rats through ELISA assays (n = 7–8 animals per group). Results are expressed as average $\pm$ SEM. *p = 0.05; **p = 0.01; ns: p > 0.05.

Fig. 5. Insulin triggers AKT phosphorylation in primary microglia. Qualitative analysis of insulin pathway proteins by western blot. (A) p-Akt-Ser473 and Akt immuno-detection in primary microglia stimulated with insulin (1000 ng/mL) or vehicle (PBS) for 30 min (n = 6–7 replicates per group) (B) p-GSK3 β Ser9 and GSK3 β immuno-detection in primary microglia stimulated with insulin (1000 ng/mL) or vehicle (PBS) for 30 min (n = 6–7 replicates per group).

4.1.1. Glucose tolerance test – GTT

Wistar rats (3 and 6 months) were used for glucose tolerance test (GTT) evaluation. Glucose homeostasis was determined as previously described (Muller et al., 2008) using the glucose tolerance test (GGT), which was performed prior to animal sacrifice. A concentrated glucose solution (2 mg/g i.p.) was injected into mice fasted for 12 h, and blood was collected from a small puncture on the tail at 0, 30, 60, and 120 min after injection. Blood glucose level was measured with glucometer (AccuChek Active, Roche Diagnostics, USA), and the area under the curve was used to compare the glucose tolerance among groups.

4.1.2. Cerebrospinal fluid sampling

Before the surgery for cannula implantation the anesthetized rats were placed in the stereotaxic equipment (Insight, São Paulo, Brazil) and 100–120 μ L of cerebrospinal fluid (CSF) was collected from the cisterna magna with an insulin syringe similar as previously described by Osés et al. (2019). Samples contaminated with blood cells were discarded.

4.1.3. Surgery and intracerebroventricular treatments

First animals were submitted to surgery for cannula implantation. Animals were anesthetized using intraperitoneal (i.p.) ketamine (100 mg/kg) and xylazine (10 mg/kg) and placed in stereotaxic equipment (Insight, São Paulo, Brazil). A cannula (27-gauge 9-mm) was positioned according to the following coordinates: 0.9 mm posterior from the bregma, 1.5 mm right of the midline and 2.6 mm ventral to the superior cranial surface. Animals were left to recover for 2 days, and on the third day post-surgery, when the rats already displayed normal food and water consumption as well as spontaneous locomotion the intracerebroventricular (i.c.v.) treatment was started. Young and aged rats received i.c.v. insulin 20 mU (Humulin R[®]; Lilly) or vehicle (NaCl 0.9%) once a day for five consecutive days injected through the cannula implanted in the fourth ventricle. After i.c.v. administrations, rats were randomly allocated in two different groups: i) Twenty-four hours after the last insulin administration, a group of both young and aged rats were euthanized by decapitation and the brain samples were collected for further analysis; and ii) After 8 days of the last insulin administration a second group comprising only young rats were euthanized and

brain samples were also processed for further analysis.

4.1.4. Immunohistochemistry and confocal microscopy

Immunohistochemistry was conducted as previously described (Haas et al., 2016). After euthanasia, brains were collected to perform immunohistochemistry. After the brain fixation in 4% paraformaldehyde, in 0.1 M phosphate buffer, at pH 7.4 for 24 h, they were transferred to PBS and cut into coronal sections (50 μm) with a Vibratome (Leica, Buffalo, USA). Of every eight slices, one was collected and permeabilized for 15 min. (Triton X-100 in PBS 0.1 M) and blocked (BSA 2%, Triton X-100, TBS) for 1 h. Primary antibodies Iba-1 (1:1000; #019-19741; Fujifilm WAKO, Richmond, USA) and CD68 (1:500; #MCA341R; AbD Serotec, Hercules, USA) were incubated overnight, followed by the secondary antibodies Alexa Fluor 594/488 (1:1000; #A32731; #A32744; Invitrogen, Waltham, MA) for 2 h at room temperature. Nuclei marker DAPI (0.0001%; #28718-90-3; Santa Cruz Biotech., Dallas, USA) was used for 25 min at 4 °C. Pictures were captured by the entire thickness Z stack for analysis. After images acquisition using confocal microscope (Olympus FV1000, Shinjuku, Japan) they were analyzed using Image J® software, (NIH, Bethesda, USA). The number of microglia cells expressing both Iba-1 and CD68 were quantified from a total of six slices per animal covering the 3 hippocampal subregions CA1, CA3, and DG. Three regions of interests (ROIs) (100 μm^2) per hippocampal subregion were placed always at the same position for all images. The average of cell counts by the three ROIs per subregion per slice was multiplied by 10, and the results were expressed in cells/ mm^2 . For expressing the results in Figs. 1 and 3, The percentage of CD68-positive microglia over total microglial cells was calculated as previously described (Peng et al., 2014) taking in account the total number of microglial cells that varies depending on the individual animal. For that we calculated the percentage of cells displaying Iba-1/CD68 colocalization (activated microglia)/ mm^2 by the total number of cells expressing Iba-1 (total microglial number)/ mm^2 per animal. We analyzed such parameters for each hippocampal subregion (CA1, CA2 and DG). Total number of cells used for percentage of CD68-positive microglia calculation can be found in the Supplementary Tables 1 and 2 regarding results on Fig. 1 and Supplementary Tables 3 and 4 regarding results on Fig. 3.

4.1.5. Western blot

For *in vivo* experiments hippocampal tissue were homogenized in lysis buffer (EDTA 2 mM, protease and phosphatase inhibitor cocktails I and II (Sigma, St. Louis, USA) 0.001%, Tris HCl 50 mM, pH 7.4, glycerol 10% and Triton X-100 1%) and centrifuged (15,000 g/10 min). For *in vitro* experiments, microglia were washed twice in PBS and a non-denaturing cell lysis buffer (CST, Danvers, MA) was used accordantly the manufacturer's instruction. Resultant protein suspensions were measured using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA) and further diluted in sample buffer (SDS 10%, glycerol 40% and bromophenol blue 0.25%; Sigma, St. Louis, USA) to a final concentration of 1 $\mu\text{g}/\mu\text{L}$. 20–40 μg protein were separated by weight using electrophoresis SDS-PAGE gel and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA) in transfer buffer (25 mM Tris, 150 mM glycine, 20% v/v methanol). Blocking procedures were performed according to the primary antibodies' datasheets using nonfat dry milk (Bio-Rad, Hercules, CA) or BSA (Sigma, St. Louis, USA). After blocking, membranes were washed 2 times with Tween-TBS and once with TBS and the primary antibodies p-Akt^{Ser473} (1:1000; #4060; CST, Danvers, MA), Akt (1:2000; #2920; CST), p-GSK3- β ^{Ser9} (1:1000; #9323, CST), GSK3- β (1:1000; #9315, CST), COX-2 (1:1000; #160112, Cayman, Ann Arbor, USA), β -Actin (1:5000–1:10000; #Ab6276, Abcam, Cambridge, MA), were incubated overnight at 4 °C. After primary antibody incubation, membranes were washed 3 times, and the HRP-conjugated anti-rabbit or anti-mouse IgG secondary antibodies (1:5000; #NA9310; #NA9340, GE, Little Chalfont, UK) were incubated 2 h at room temperature. Proteins of interest were

immunodetected by chemiluminescence using both Image Quant LAS 4000 (GE, Little Chalfont, UK) or ChemiDocMP® (Bio-Rad, Hercules, CA). Protein expression was quantified using both ImageLab 5.2.1® (Bio-Rad, Hercules, CA) and Image J® (Rockville, USA). The immunoccontent of each target protein was divided by the constitutive protein present in each sample and after that normalized by the relative expression of the target in the control group (%).

4.1.6. ELISA assays

For hippocampus homogenates (prepared using the same protocol described in the Western Blot section) IL-1 β and IL-6 (#RAB0278 #RAB0311; Sigma, St. Louis, USA) kits were used. The protocols were performed according to the instructions of the manufacturers. Samples and standards were assessed in duplicates and the coefficient of variation was less than 5% for both assays. For CSF insulin levels an ELISA Insulin kit (Millipore Ref# EZRMI-13 K) was used and the technical procedures were performed accordingly to the manufacturer instructions. For measurements using ELISA in hippocampus homogenates the results were corrected by the concentration of total protein which was measured using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA).

4.2. In vitro experiments

Male and female C57Bl/6 mice pups were used to perform experiments *in vitro*. C57Bl/6 pups were provided by the Central Animal Facility, CUMC, University of Groningen. All experiments were performed according to the experimental animal guidelines of the University of Groningen, The Netherlands and approved by the local IACUC.

4.2.1. Microglia cell culture and treatments

Primary neonatal microglia culture was performed according (Schaafsma et al., 2015) with modifications. C57Bl/6 mice of postnatal day P0-P2 (female and male) were sacrificed by decapitation and the forebrains were removed from the skull and maintained in Dissection Medium (DM) – Hanks Bovine Salt Serum (HBSS; Lonza), 45% D-(+)-Glucose (Sigma, St. Louis, USA), 1 M HEPES (Lonza), 100 U/mL Penicillin/100 mg/mL Streptomycin (Sigma, St. Louis, USA). Meninges, brain stem and cerebellum were separated, and the forebrains were minced and washed with DM. Next, a chemical dissociation step was performed, the minced tissue was incubated in 1 $\times$ DNase (Sigma, St. Louis, USA) – 2.5% trypsin (Lonza, Maastricht, The Netherlands) – DM, during 25 min, 37 °C, mixing gently every 5 min. The chemical dissociation was stopped by addition of heat inactivated (HI) 20% Fetal Bovine Serum (FBS – Gibco, Waltham, USA) containing 1 $\times$ DNase and 0.1 mg/mL trypsin inhibitor (Sigma, St. Louis, USA) in DM. Tissue was then followed by a quickly wash using 10% HI FBS with 1 $\times$ DNase in DM following by a mechanical dissociation using glass pipette until a cell suspension was obtained. The cell suspension was washed in microglia medium – MM (4.5 mg/mL glucose – 0.584 mg/mL L-glutamine – Dulbecco's Modified Eagle Medium (DMEM – Gibco), 10% HI FBS (Gibco), 1 mM sodium pyruvate (Lonza), 100 U/mL Penicillin-100 mg/mL Streptomycin) and centrifuged (10 min.; 960 rpm; 12 °C). The cell pellet was re-suspended in MM and plated in T75 culture flasks (1.5 brains/flask) that where cultured at 37 °C in a humidified atmosphere at 5% CO₂. In the day after, the medium was refreshed and then the medium changes were done every 3–4 days. After 7–10 days, 33% L929 (ATCC, Manassas, USA) fibroblast-conditioned medium containing M-CSF was added to the microglia medium in order to stimulate microglia proliferation. After 11–15 days of culture, when cells reached 100% confluence, microglia were obtained by mitotic shake-off using an orbital shaker (1 h; 150 rpm; 37 °C). After seeding, microglia were cultured in 50:50 MM supplemented with post shake-offs medium. The microglia primary cell culture purity was accessed by Western Blot for Iba-1 microglial marker (1:1000; #019-19741 Fujifilm-WAKO; Neuss,

Germany) compared to neuronal β -III-Tubulin (1:5000; #ab7751; Abcam, Cambridge, UK), astrocytes GFAP (1:1000; #Z033420-2; Agilent; Dako, Santa Clara, CA) and oligodendrocytes MBP (1:1000; #AB980; Sigma-Millipore) markers (Sup. Fig. 1). Insulin, wortmannin and DMSO (Sigma) were used to treat the cells and respective concentrations and incubation times can be found in the figure legends. Cells were always submitted to drug treatments at a maximum of 2 days after the shake-off.

4.3. Statistical analysis

Analysis between 4 groups with two variances (treatment and age) were performed by two-way Analysis of Variance (ANOVA) followed by multiple comparisons Tukey *posthoc* test. For 2 groups analysis unpaired Student's *t*-test was used. GraphPad Prism® Software was used for statistical analysis and graphs preparation. Data were presented always as average $\pm$ S.E.M. *P* values $\leq$ 0.05 were considered statistically significant. Exact *p* values can be found in the respective result descriptions.

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Conflict of interest

The authors state that no personal, scientific or financial conflict of interest exists.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.brainres.2020.146884>.

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