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Neuroprotection by Insulin-like Growth Factor-1 in Rats with Ischemic Stroke is Associated with Microglial Changes and a Reduction in Neuroinflammation

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Abstract—We and others have shown that insulin-like growth factor-1 (IGF-1) is neuroprotective when administered systemically shortly following stroke. In the current study, we addressed the hypothesis that microglia mediate neuroprotection by IGF-1 following ischemic stroke. Furthermore, we investigated whether IGF-1 modulates pro- and anti-inflammatory mediators in ischemic brain with a special reference to microglia. Ischemic stroke was induced in normal conscious Wistar rats by infusing the vasoconstrictor, endothelin-1 (Et-1), next to middle cerebral artery (MCA). IGF-1 (300 μg) was injected subcutaneously (SC) at 30 and 120 min following stroke. Microglial inhibitor, minocycline, was injected intraperitoneally (IP) at 1 h before stroke (25 mg/kg) and 11 h after stroke (45 mg/kg). Post-stroke IGF-1 treatment reduced the infarct size and increased the sensorimotor function which coincided with an increase in the number of ameboid microglia in the ischemic cortex. Minocycline treatment abrogated the increase in ameboid microglia by IGF-1, while the effect of IGF-1 in the reduction of infarct size was only partially affected. IGF-1 suppressed mRNA expression of inducible nitric oxide synthase (iNOS) and interleukin (IL)-1β in the ischemic hemisphere, while in purified microglia, only iNOS expression levels were reduced. Our findings show that microglia are a target for IGF-1 and that neuroprotection by IGF-1 coincides with down-regulation of inflammatory mediators which could be instrumental to the beneficial effects.

Key words: inflammation, neuroprotection, microglia, ischemic stroke, IGF-1.

INTRODUCTION

Ischemic stroke is one of the leading causes of death and long-term disability worldwide (Krishnamurthi et al., 2013). Despite substantial advances in experimental studies, recombinant tissue-plasminogen activator (rtPA) remains the only available drug for treatment of ischemic stroke (Albers et al., 2008; Hacke et al., 2008; Lees et al., 2016). However, since rtPA has a narrow therapeutic time window which is four and a half hours after stroke onset, only few stroke patients benefit from this treatment (Albers et al., 2008; Hacke et al., 2008; Lees et al., 2016). Therefore, there is a need to find alternative therapeutic interventions for ischemic stroke that can address the vast majority of ischemic stroke patients. Microglia have attracted a significant amount of attention in preclinical studies as they are the first cells to respond to brain injury and are involved in many neurodegenerative diseases (Hickman et al., 2018). During ischemic stroke, microglia become activated within minutes and undergo morphological changes (Patel et al., 2013; Guruswamy and ElAli, 2017). Depending on the context of ischemic stroke, microglia may either adopt a pro-inflammatory phenotype and secrete mediators that increase neuroinflammation and brain injury, or polarize towards an anti-inflammatory phenotype and release factors that alleviate neuroinflammation and protect neurons (Patel et al., 2013; Guruswamy and ElAli, 2017). The cascade of events that is triggered after the stroke onset, disrupts the blood-brain barrier (BBB) facilitating the infiltration of leukocytes into the ischemic brain (Sandoval and Witt, 2008). Neutrophils are among the early infiltrators into

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Abbreviations: BBB, blood-brain barrier; BDNF, brain-derived neurotrophic factor; EDTA, ethylenediaminetetraacetic acid; Et-1, endothelin-1; FACS, fluorescence-activated cell sorting; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IGF-1, insulin-like growth factor-1; IL, interleukin; iNOS, inducible nitric oxide synthase; MCAO, middle cerebral artery occlusion; NDS, Neurological Deficit Score; NT, neurotrophin; PBS, phosphate buffered saline; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

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the ischemic brain as they increase in numbers within few hours and peak at day 1–3 following stroke onset (Grønberg et al., 2013). The infiltration of neutrophils contributes to further disruption of the BBB, exacerbation of infarct volume and worsening of the behavioral outcome (Grønberg et al., 2013; Herz Josephine et al., 2015; Jickling et al., 2015; Neumann et al., 2015). Macrophages are also involved in the pathophysiology of the ischemic stroke, however, unlike neutrophils, they are hardly detected in the infarcted brain during the early stages of ischemic stroke (Schilling et al., 2003; Grønberg et al., 2013).

Insulin-like growth factor-1 (IGF-1) is a pleiotropic hormone that is involved in many processes in the central nervous system including growth, proliferation, neurogenesis and cell migration (Puglianiello et al., 2000; Mairet-Coello et al., 2009; Nieto-Estévez et al., 2016; Wrigley et al., 2017). Several studies have shown that IGF-1 is neuroprotective when administered after stroke. It has been reported that intranasal (IN) administration of IGF-1 following transient middle cerebral artery occlusion (MCAO) in rats reduced infarct size and improved behavioral outcome which was paralleled by a decrease in apoptotic cells in the ipsilateral hemisphere (Liu et al., 2004). Another study demonstrated that adult rats that were subjected to transient MCAO and treated afterward with intracerebroventricular (ICV) injection of IGF-1 displayed a reduction in infarct size (Bake et al., 2014). In line with these studies, our group previously established that SC or intravenous injection of recombinant human (rh) IGF-1 shortly following endothelin-1 (Et-1)-induced stroke, significantly reduced infarct size in rats (De Geyter et al., 2016, 2013). In addition, central inhibition of IGF-1 receptor function by infusion of the antagonist JB-1 in the lateral ventricle, abrogated the neuroprotection by systemically injected rhIGF-1 in stroke rats (De Geyter et al., 2016). This implies that systemically administered rhIGF-1 exerts central effects in the ischemic hemisphere.

To investigate the underlying mechanisms of the beneficial actions of IGF-1 in the ischemic brain, we addressed the effects of post-stroke IGF-1 treatment on infarct size as assessed by staining with Cresyl Violet and on neuronal damage by using the neuron-specific marker NeuN. Furthermore, we addressed the effects of IGF-1 on the microglial activation and expression of pro- and anti-inflammatory mediators in the ischemic hemisphere and isolated microglia. Our results show that post-stroke IGF-1 preserved neurons, induced microglial changes and reduced neuroinflammation.

EXPERIMENTAL PROCEDURES

Male albino Wistar rats (6–8 weeks old) were obtained from Charles River Laboratories (Germany). After transport, animals remained in the animal facility for acclimatization for at least 7 days under a 12-h light/dark cycle with *ad libitum* supply of standard chow and water. Animals were used for experiments when they weighed 275–300 g. The animals were handled in accordance with the National Guidelines on Animal Experimentation and the study was approved by the Ethical Committee for Animal Experimentation of the Vrije Universiteit Brussel (VUB, project number: 14-278-2).

Surgical operation and induction of stroke

Rats were anesthetized with 3–4% isoflurane, fixed on stereotactic frame and then injected SC with an analgesic (5 mg/kg ketoprofen). After making a midline incision in the skull, a burr hole was drilled and a guide cannula (C317G/SPC, Invivo1, Roanoke, VA, USA) was inserted close to the middle cerebral artery (MCA), using the following coordinates from the Paxinos and Watson atlas (Paxinos and Watson, 2008): anterior–posterior +0.9 mm, medial–lateral +5 mm relative to Bregma and ventral 2.8 mm relative to dura. After surgery, the rats were left to recover for 1 day after which an internal cannula (C317I/SPC, Invivo1, Roanoke, VA, USA) was inserted into the guide cannula followed by infusion in conscious rats of 280 pmol Et-1 (Sigma, St Louis, MO, USA) dissolved in 6 μl iso-osmotic Ringer's solution with a rate of 1 μl/minute. The rats were euthanized 24 h or 72 h after the insult.

IGF-1 and minocycline treatment

A dose of 300 μg of rhIGF-1 (Ipsen NV, Merelbeke, Belgium) or its vehicle was prepared in 0.4 ml saline and injected SC at 30 and 120 min after stroke induction. Minocycline (minocycline·HCl, Sigma, ST-Louis, USA) was dissolved in milliQ water. A first dose of 25 mg/kg minocycline or its vehicle was injected IP 1 h before stroke induction and a second dose of 45 mg/kg was given 11 h after the insult.

Therefore, the following four groups had been assigned to the rats:

(1) vehicle minocycline (M) + vehicle IGF-1 (I)
(2) vehicle (M) + IGF-1
(3) minocycline + vehicle (I)
(4) minocycline + IGF-1

Rats were euthanized 24 h after stroke induction for analysis of short-term effects of IGF-1 or 72 h to follow up the long-term effect of IGF-1.

Neurological Deficit Score (NDS)

The sensorimotor function of the rats was assessed at 24 h or 72 h after stroke induction using the NDS as described by Garcia et al. (1995). The NDS consists of six parameters: response to vibrissae touch, outstretching of forepaws, symmetrical movement of forelimbs, climbing, spontaneous movement and body proprioception. The NDS ranges from a minimum score of 3 to a maximum score of 18. A high score is positively correlated with sensorimotor performance.

Measurement of infarct volume

After stroke induction, rats were euthanized by IP injection of an overdose of sodium pentobarbital (Ceva

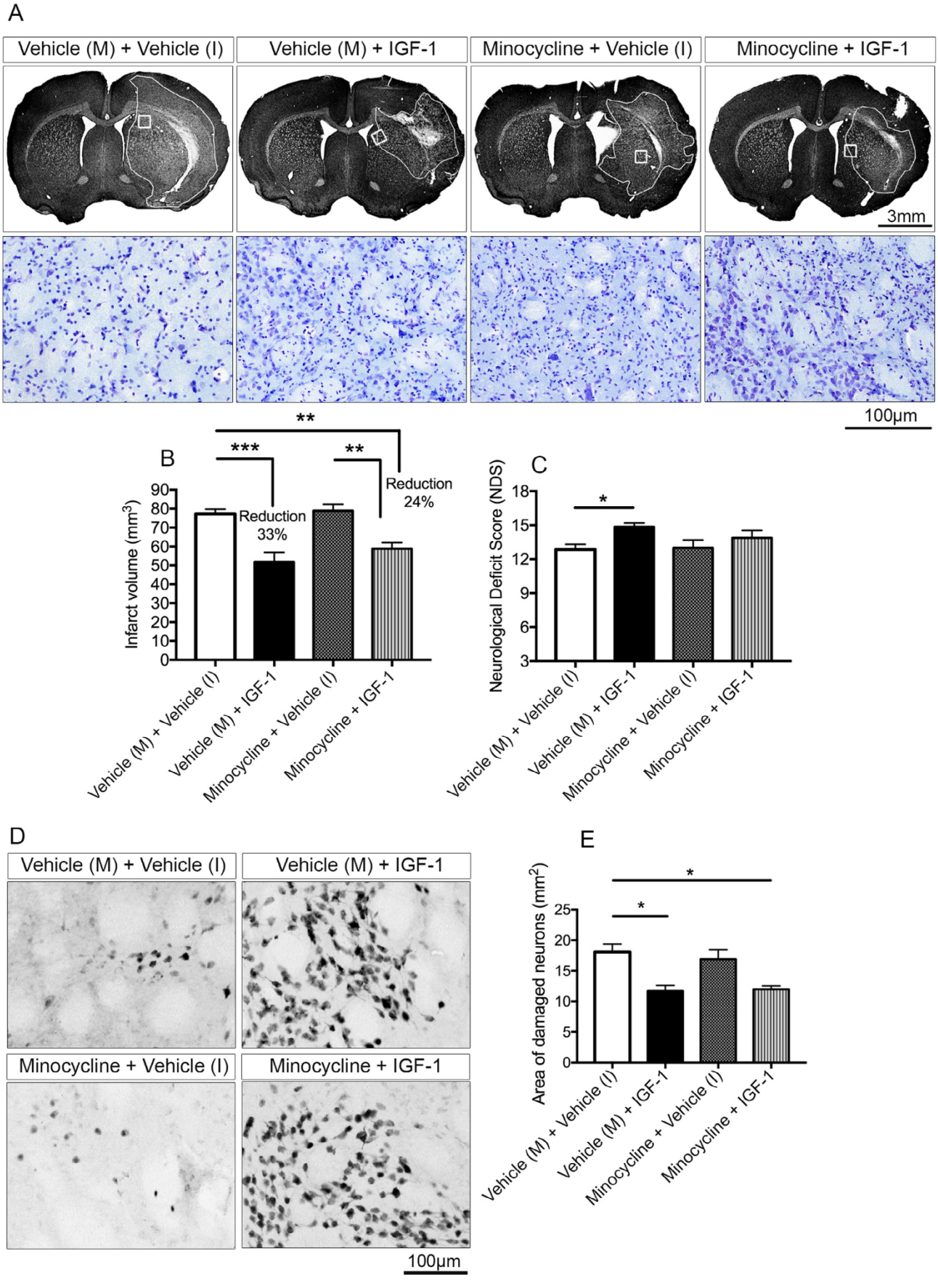
Santé Animale, Brussels, Belgium) after which rats were transcardially perfused with saline and 4% phosphate-buffered paraformaldehyde solution (pH 7.2) for 5 min each. Next, brains were quickly collected and post-fixed in 4% paraformaldehyde, after which they were sectioned into 50 μm slices using a vibratome (Leica VTS1000, Bensheim, Germany) and kept at 4 °C in phosphate buffered saline (PBS) containing 0.01% sodium azide. Brain sections were taken every 200 μm and mounted onto gelatin-coated slides. The brain slices were stained with 0.5% Cresyl Violet acetate (C5042, Sigma) and the infarcted area was measured on digitized images using Image J software (NIH, v 1.43). The infarct volume was estimated according to the following formula: $v = d \times \Sigma a$, where (v) is the infarct volume (mm^3), (d) is the distance (200 μm) between the upper (rostral) surfaces of two consecutive analyzed brain slices and (a) is the surface areas of infarct size (Avendaño et al., 1995). The correction of edema was calculated using the following formula: infarct volume × (volume of contralateral hemisphere/volume of ipsilateral hemisphere).

Immunohistochemistry

Iba-1 (ionized calcium binding adaptor molecule-1) staining was used to determine microglial morphology. Two or three 50 μm thick brain sections were selected for each sample between 0.90 mm anterior and 0.26 mm posterior to Bregma. Brain sections were incubated with 3% H_2O_2 in water for 30 min followed by 0.1% Triton X-100 in water for 15 min and 20% pre-immunized normal goat serum in PBS for 30 min at room temperature. The brain sections were washed with PBS containing 0.1% Tween-20 between the incubation steps. Next, brain sections were incubated overnight with rabbit anti-iba-1 (1:1000 dilution in 20% normal goat serum in PBS; Wako, Japan) at 4 °C followed by incubation with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:100 dilution in 20% normal goat serum in PBS; NA934V, GE Healthcare, UK) for 4 h at room temperature. To detect the presence of neurons, brain sections were stained with NeuN. The staining procedure was done as previously described for Iba-1 staining. Brain sections were incubated overnight at 4 °C with anti-NeuN (1:1000 dilution in 20% normal goat serum in PBS; MAB377 Millipore, Temecula, USA). Next day, brain sections were incubated with the secondary antibody horseradish peroxidase-conjugated sheep anti-mouse IgG (1:200 dilution in 20% normal goat serum in PBS; NA931V, GE Healthcare, UK) for 4 h at room temperature. Antibody binding was visualized using the diaminobenzidine substrate chromogen kit (Dako Cytomation, Glostrup, Denmark). Stained sections were mounted onto 3-aminopropyltriethoxylane (APES)-coated slides and left to dry at room temperature overnight after which the slides were cover-slipped using DPX mounting medium and scanned using an Aperio CS2 scanner (Leica, Belgium). Quantification of the cells was performed using Imagescope software (version 12.1.0.5029, Leica, Belgium). The regions for quantification were selected as previously described by De Geyter et al. (2013). Briefly, 1 mm^2 was selected in three regions in the striatum and two regions in the cortex of the ipsilateral hemisphere. Activation of microglia was manually counted using free hand annotation tool and expressed as mean number of cells/mm^2. Since the rats that were euthanized 72 h after stroke induction displayed aggregates of microglia/macrophages in the ipsilateral hemisphere, activation of microglia/macrophages was evaluated by measuring Iba-1 staining intensity using the Aperio Positive Pixel Count v9 algorithm in the Imagescope software. To measure the Iba-1 intensity in the striatum, we selected the whole striatum. However, since the infarcted cortex sustained minor damages in some brain sections, we measured Iba-1 intensity in the cortex by selecting 1 mm × 2 mm in the same region as previously described (De Geyter et al., 2013). Finally, to correct inter-assay variability, the staining intensity of ipsilateral hemisphere was subtracted from the intensity of contralateral hemisphere. We quantified the effects of IGF-1 on the neuronal loss by measuring the surface area of the regions displaying low levels of NeuN-staining in the infarcted hemi-

Table 1. List of primers

Gene	Forward primer (5′–3′)	Reverse primer (5–3′)	Reference
YWHAZ	CCCTCAACTTCTCTGTGTTCTAC	TGCTGTCTTTGTACGACTCTTC	
SDHA	TACTGTTGCAGCACAGGGAG	CAGTCAGAGCCTTTCACGGT	
BDNF	GACACATTACCTTCCAGCATCT	GGATGGTCATCACTCTTCTCAC	
NT-3	CGTCCCTGGAAATAGTCATACG	GCCACGGAGATAAGCAAGAA	
NT-4	GCGTCAGTACTTCTTCGAGAC	GACTGTTTAGCCTTGCATTCTG	
IL-10	AGTGGAGCAGGTGAAGAATG	GAGTGTCACGTAGGCTTCTATG	
IL-6	CTTCACAAGTCGGAGGCTTAAT	GCATCATCGCTGTTCATACAATC	
TGF-β	CCGCAACAACGCAATCTATG	CTTCCCGAATGTCTGACGTATT	
NGF	CGTGCTGTTTAGCACCCA	GTGAGTCCTGTTGAAGGAGATT	
Ym 1	TCTCCATCCGACACTGGAATA	TGCTGGAAATCCCACAATGA	
CD206	CAGCAACTTGACCAACAATGAG	GTAGCCATCGACATCCATGTAA	
TNF α	CCACACCGTCAGCCGATT	TCCTTAGGGCAAGGGCTCTT	Doorn et al. (2015)
Iba-1	GCCTCATCGTCATCTCCCCA	AGGAAGTGCTTGTTGATCCCA	Doorn et al. (2015)
GFAP	CAGACTTTCTCCAACCTCCAG	CTCCTGCTTCGAGTCCTTAATG	Doorn et al. (2015)
IL-1β	AAAGAAGAAGATGGAAAAGCGGTT	GGGAACTGTGCAGACTCAAACTC	Doorn et al. (2015)
iNOS	AACTTGAGTGAGGAGCAGGTTGA	CGCACCGAAGATATCCTCATGA	Doorn et al. (2015)


A
Vehicle (M) + Vehicle (I)
Vehicle (M) + IGF-1
Minocycline + Vehicle (I)
Minocycline + IGF-1
3mm
100μm
B
Infarct volume (mm3)
Reduction 33%
Reduction 24%
**
***
**
C
Neurological Deficit Score (NDS)
*
D
Vehicle (M) + Vehicle (I)
Vehicle (M) + IGF-1
Minocycline + Vehicle (I)
Minocycline + IGF-1
100μm
E
Area of damaged neurons (mm2)
*
*

sphere using Image J software. Surface areas were corrected for edema according the following formula: surface area of the regions showing low levels of NeuN-staining × (surface area of contralateral hemisphere/surface area of ipsilateral hemisphere).

Purification of microglia by fluorescence-activated cell sorting (FACS)

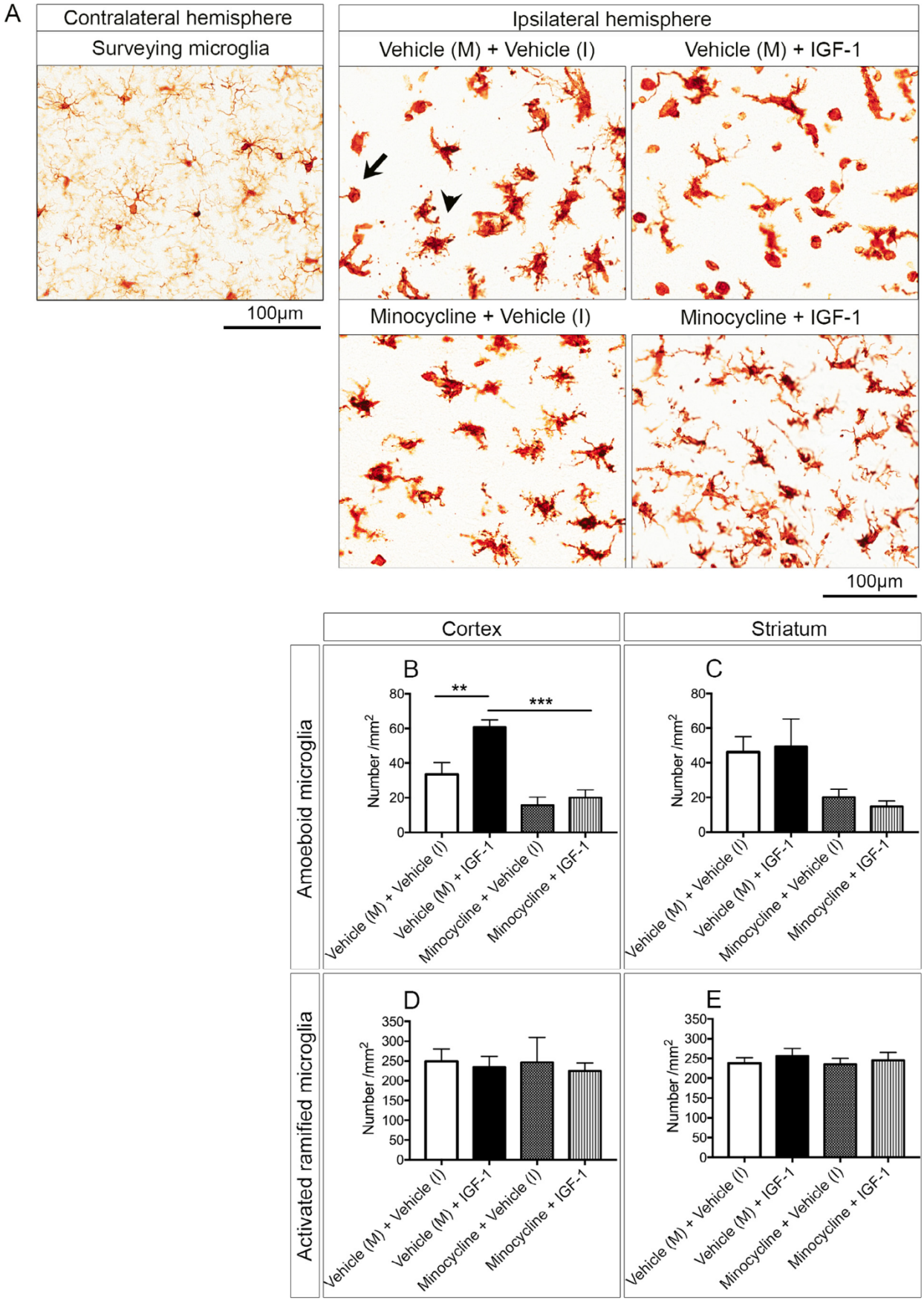
Rats were sacrificed by IP injection with an overdose of sodium pentobarbital 24 h after stroke induction and transcardially perfused with saline for 10 min. Next, brains were collected and kept on ice in medium A with Phenol Red (HBSS with Phenol Red containing 0.6% glucose and 15 mM HEPES). Since the yield of purified microglia from one hemisphere was low, we pooled the ipsilateral hemispheres from two rats receiving the same treatment and considered them as one biological replicate. Hemispheres were mechanically homogenized using a glass potter and homogenates were filtered over a 70 μm cell strainer and then centrifuged (220*g*, 10 min, acceleration 9, brake 9 at 4 °C). The pellets were resuspended in 1 ml of 22% percoll (Sigma-Aldrich BVBA, Overijse, Belgium) diluted in NaCl (1.5 M) and myelin gradient buffer (solution contains 0.78 g/l $NaH_2PO_4{\cdot}H_2O$ that was adjusted to pH 7.4 by adding 3.56 g/l $Na_2HPO_4{\cdot}2H_2O$ and then, the following substances were added: 8 g/l NaCl, 0.4 g/l KCl and 2 g/l glucose) and separated into 3 tubes (50 ml). The tubes were filled with 25 ml of percoll (22%) and overlaid carefully with 3 ml cold PBS. After centrifugation (950*g*, 20 min, acceleration 4, brake 0, at 4 °C), the supernatant was aspirated carefully and the pellets were resuspended in washing buffer (PBS containing 2 mM EDTA) and then, microglia were enriched using anti-CD11b/c-conjugated microbeads (Miltenyi Biotec, Leiden, The Netherlands) according to the manufacturer's protocol. Briefly, cells were incubated with CD11b/c microbeads (20% in washing buffer) for 15 min at 4 °C and subsequently resuspended in 1 ml of washing buffer and centrifuged (300*g*, 10 min, acceleration 9 and brake 9, at 4 °C). Then, pellets were resuspended in 500 μl washing buffer and passed through an MS column (Miltenyi Biotec, Leiden, The Netherlands) that was placed on a magnetic field. Thereafter, the MS column was removed from the magnetic field and the retained $CD11b/c^+$ cells were eluted with 1 ml washing buffer. The collected $CD11b/c^+$ cells were washed and taken in medium A without Phenol Red (HBSS without Phenol Red, containing 15 mM HEPES, 0.6% glucose and 1 mM EDTA) and incubated for 30 min at 4 °C in the dark with the following antibodies: CD11b –V450 (1:50; BD Biosciences, Erembodegem, Belgium), CD45 –PE/Cy7 (1:50; Biolegend, San Diego, USA), CD3 – Brilliant Violet 605 (1:300; BD Biosciences, Erembodegem, Belgium), His48 –PE (1:200; eBioscience, San Diego, USA), ACSA-1 –APC (1:40; Miltenyi Biotec, Leiden, The Netherlands) and CD163 –FITC (1:50; Acris, Herford, Germany). After a washing step, cells were resuspended in medium A without Phenol Red and kept on ice until microglia sorting. The cell viability dye, 7-Aminoactinomycin D (7-AAD, BD Biosciences, Erembodegem, Belgium), was added to the cells shortly before starting FACS analysis to discriminate between dead and live cells. Cell doublets and dead cells ($7\text{-}AAD^+$) were excluded from the analysis and cells with the following phenotype: $CD11b^+/CD45^{int}/His48^-/CD163^-/CD3^-/ACSA1^-$ were sorted on a FACSAria III (BD Biosciences, Erembodegem, Belgium). The purity of isolated microglia was at least 95% as assessed by FACSAria III and FlowJo software (v10.4.2, Ashland, OR, USA). The main contaminating fractions were astrocytes ($ACSA1^+$) macrophages ($CD163^+$), neutrophils ($His48^+$) and lymphocytes ($CD3^+$).

The pre-sort FACS analysis data were used to determine the percentages of neutrophils and macrophages using FlowJo software.

RNA isolation and qPCR

Brain homogenate from two pooled ipsilateral hemispheres from rats that received a similar treatment, were prepared and the total RNA was extracted using the Qiagen RNeasy Lipid Tissue Kit (Qiagen). Total RNA from purified microglia was extracted using the Qiagen RNeasy micro kit (Qiagen). RNA was reversed transcribed using the GoScript™ Reverse Transcriptase kit (Promega, The Netherlands). The Primers in Table 1 were either obtained from Doorn et al. (2015), or designed by (PrimerQuest tool, Integrated DNA Technologies, Iowa, USA). The qPCR was executed in 96-well plates (Applied Biosystems, CA, USA) using an ABI 7900HT Real-Time PCR system (Applied Biosystems, CA, USA). cDNA was quantified using the SYBR green Master mix (Life Technologies Europe BV, Merelbeke, Belgium). The housekeeping genes succinate dehydrogenase complex subunit A (SDHA) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide (YWHAZ) were selected as reference genes as their expressions were stable in different treatment groups. The Ct values of each sample were normalized relative to the mean of two reference genes and the gene expression was quantified using ΔCt (Kwapiszewska

Fig. 1. Comparison of infarct size and sensorimotor function between different treatment groups. (**A**) Representative micrographs of Cresyl Violet-stained brain sections. The first row shows the infarct volume in different treatment groups. The outlined regions in the first row are shown at higher magnification in the second row. (**B**), (**C**) show the effect of minocycline and IGF-1 on the infarct size (**B**) and the NDS (**C**) that were assessed 24 h after the insult. (**D**) Representative micrographs of NeuN-staining in different treatment groups. (**E**) Shows the effect of different treatments on the neuronal loss in the ipsilateral hemisphere. The number of animals in each group was as follows: Vehicle (M) + vehicle (I) ($n = 7$ for infarct volume and NDS and $n = 5$ for NeuN-staining), minocycline + vehicle (I) ($n = 7$ for infarct volume and NDS and $n = 4$ for NeuN-staining) and minocycline + IGF ($n = 8$ for infarct volume and NDS and $n = 3$ for NeuN-staining). Vehicle (M) + IGF-1 ($n = 6$ for infarct volume, $n = 7$ for NDS and $n = 3$ for NeuN-staining). *Significance $P < 0.05$, **Significance $P < 0.01$, ***Significance $P < 0.001$.


A
Contralateral hemisphere
Surveying microglia
100µm
Ipsilateral hemisphere
Vehicle (M) + Vehicle (I)
Vehicle (M) + IGF-1
Minocycline + Vehicle (I)
Minocycline + IGF-1
100µm
Cortex
Striatum
Amoeboid microglia
Activated ramified microglia
B
C
D
E
Number /mm2
**
***
Vehicle (M) + Vehicle (I)
Vehicle (M) + IGF-1
Minocycline + Vehicle (I)
Minocycline + IGF-1

et al., 2012) according to the following formula: $\Delta Ct = \text{mean } Ct_{\text{reference genes}} - Ct_{\text{gene of interest}}$.

Statistics

All samples were coded and the analysis was done in a blinded manner. Statistical analysis was done using GraphPad Prism 7 software (version 7.0a for Mac OS, GraphPad Software, La Jolla, CA, USA) and the data are presented as mean ± standard error of the mean. Results were considered statistically significant when $P < 0.05$. Statistical differences in infarct volume, NeuN-staining and microglia activation were assessed using a one-way analysis of variance (ANOVA) with the Bonferroni's multiple-comparison *post hoc* test in case of multiple comparisons or the Student's *t*-test for comparisons between two groups. The differences in NDS were analyzed using the Kruskal–Wallis with Dunn's multiple correction and a Mann–Whitney test for multiple comparisons and differences between two groups, respectively. Statistical analysis of qPCR data was done by unpaired student's *t-test* using the ΔCt values of different treatment groups. The differences in neutrophil infiltration between different groups were analyzed by the Mann–Whitney test.

RESULTS

The role of microglial activation in neuroprotection by IGF-1

To address the role of microglia and activation of these cells in neuroprotection by IGF-1, we treated rats with IGF-1 in the presence and the absence of minocycline which inhibits microglial activation.

Post-stroke IGF-1 treatment resulted in a 33% reduction in infarct volume as assessed by Cresyl Violet staining (Fig. 1A, B). Minocycline injection did not influence the infarct size (Fig. 1A, B). Moreover, co-administration of minocycline did not significantly affect the beneficial effect of IGF-1 on the infarct volume as shown in Fig. 1A, B.

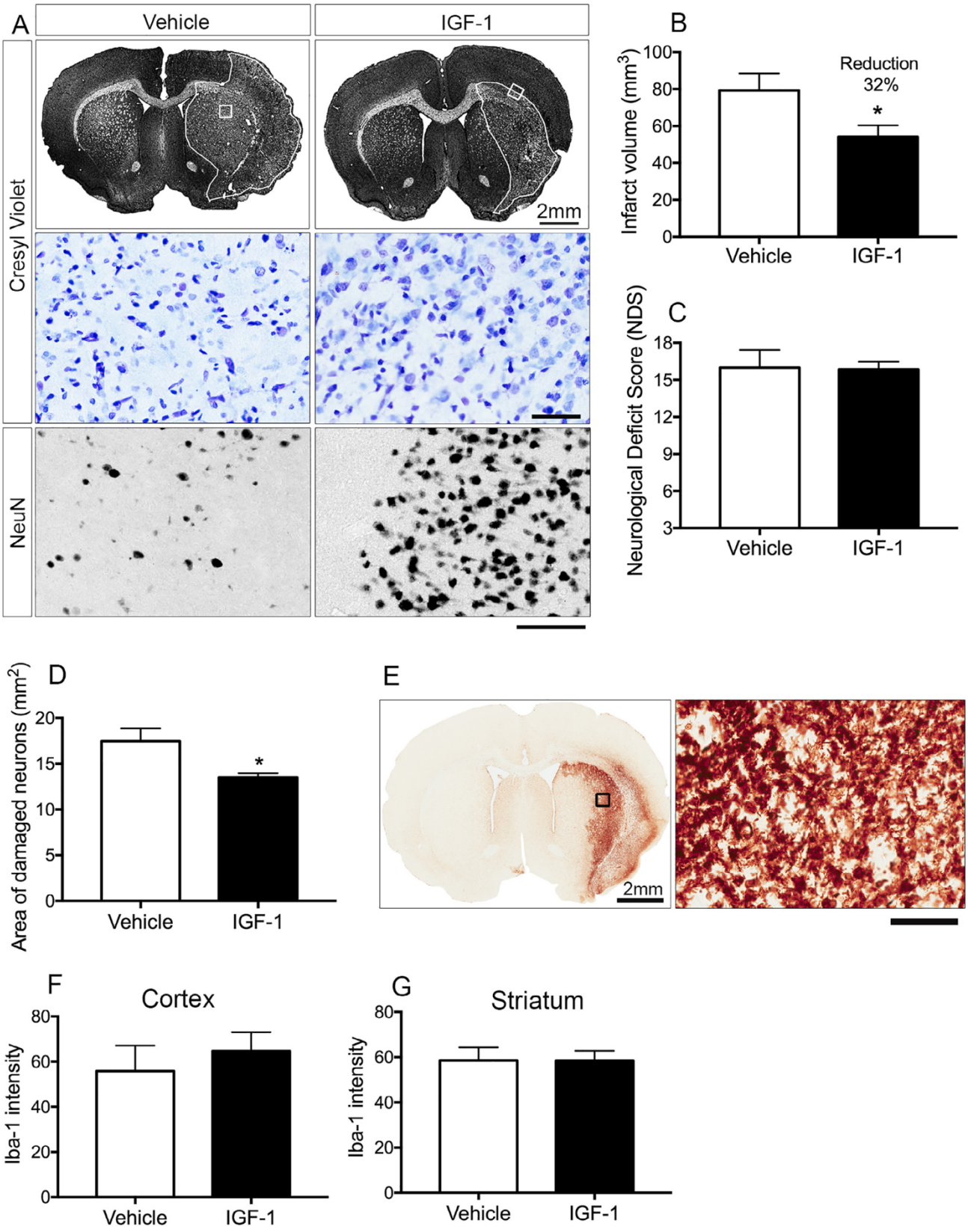
To determine whether IGF-1 and minocycline improve the sensorimotor behavior, the NDS test was assessed 24 h after the insult. Fig. 1C shows that IGF-1 treatment significantly improved sensorimotor function, whereas minocycline treatment was without effect. Notably, the improvement in sensorimotor function by IGF-1 was abolished or reduced by co-treatment with minocycline (Fig. 1C).

To assess whether IGF-1 treatment protects neurons in the infarcted hemisphere, we performed NeuN-staining on brain sections and quantified the surface of the regions in the ipsilateral hemisphere displaying low levels of NeuN-staining. It appeared that the infarcted region displayed low levels of $NeuN^+$ cells, while the non-infarcted region showed relatively large numbers of $NeuN^+$ cells (data not shown). Furthermore, control- and minocycline-treated rats with relatively large infarct sizes displayed extensive neuronal damage in the infarcted hemisphere (Fig. 1A, B, D), while the decrease in the infarct volume in animals treated with IGF-1 and IGF-1 in combination with minocycline coincided with a decrease in neuronal damage (Fig. 1A, B, D). Neurological damage as assessed by measuring the infarcted surface on NeuN-stained sections was significantly reduced by IGF-1. However, the decrease in neuronal damage by IGF-1 in the presence of minocycline did not reach significance (Fig. 1E).

Effect of IGF-1 and minocycline on the activation of microglia

Microglia and macrophages are often indistinguishable from each other as they both express similar markers including Iba-1 and share similar morphological and functional features (Patel et al., 2013). However, since microglia are activated within minutes following stroke, while macrophage infiltration into the ischemic hemisphere takes more than one day (Schilling et al., 2005, 2003), we used the Iba-1 expression as a marker for microglia activation and for morphological analysis to distinguish different stages of microglia activation after stroke. We characterized the activated microglia based on morphological features discriminating two phenotypes i.e., activated ramified microglia and ameboid microglia. Unlike surveying microglia that are found in the contralateral hemisphere and are characterized by thin and long ramifications (Fig. 2A), activated ramified microglia have dense cell bodies with thick and relatively short ramifications (Fig. 2A, arrowhead). On the other hand, ameboid microglia are round-shaped and have very small or no ramifications (Fig. 2A, arrow). Ameboid microglia might secrete toxic substances including reactive oxygen species (ROS) that exacerbate inflammation and brain injury (Socodato et al., 2015) or phagocytose damaged brain tissue and protect viable neurons following ischemic stroke (Kawabori et al., 2015). Both activated ramified and ameboid microglia are predominantly found in the ipsilateral hemisphere of ischemic brain.

Fig. 2. Effect of minocycline and IGF-1 treatment on the activation of microglia 24 h after stroke onset. (**A**) Representative images of the surveying microglia in the contralateral hemisphere and activation of microglia in different treatment groups in the ipsilateral hemisphere that show the morphology of ameboid microglia (arrow) and activated ramified microglia (arrowhead) in the infarcted cortex. (**B**) and (**C**) display the differences in the density of ameboid microglia (number/mm^2) between different treatment groups in the cortex and striatum, respectively. (**D**) and (**E**) show the effect of IGF-1 and minocycline on the clusters of activated ramified microglia (number/mm^2) in the cortex and striatum, respectively. The number of animals per group for ameboid and activated ramified microglia in striatum was as follows: Vehicle (M) + vehicle (I) ($n = 7$), vehicle (M) + IGF-1 ($n = 6$), minocycline + vehicle (I) ($n = 6$), minocycline + IGF-1 ($n = 5$). The number of animals per group for Ameboid and activated ramified microglia in cortex: Vehicle (M) + IGF-1 ($n = 6$), minocycline + vehicle (I) ($n = 4$), minocycline + IGF-1 ($n = 5$), whereas activated ramified and ameboid microglia in cortex of vehicle (M) + vehicle (I) treated group were ($n = 7$) and ($n = 6$), respectively. **Significance $P < 0.01$ and ***Significance $P < 0.001$.


A
Vehicle
IGF-1
Cresyl Violet
NeuN
2mm
B
Infarct volume (mm3)
Reduction
32%
*
Vehicle
IGF-1
C
Neurological Deficit Score (NDS)
Vehicle
IGF-1
D
Area of damaged neurons (mm2)
*
Vehicle
IGF-1
E
2mm
F
Cortex
Iba-1 intensity
Vehicle
IGF-1
G
Striatum
Iba-1 intensity
Vehicle
IGF-1

We evaluated the effect of IGF-1 treatment on the density of different types of activated microglia in three regions in striatum and two regions in the cortex as explained by De Geyter et al. (2013). IGF-1 treatment enhanced the number of ameboid microglia in the infarcted cortex, but not in the striatum (Fig. 2A–C). The treatment with the microglial inhibitor, minocycline, resulted in attenuation of the increase in number of ameboid microglia by IGF-1 in the cortex (Fig. 2A, B). Furthermore, neither minocycline nor IGF-1 affected the number of activated ramified microglia in the cortex and striatum (Fig. 2A, D, E).

Long-term effects of IGF-1 neuroprotection

Since most cellular and biochemical events in the ischemic cascade are initiated within 24 h after the insult, assessment of infarct size and neuronal damage after 24 h is useful to unravel the working mechanism of the beneficial effects of IGF-1. To investigate whether the beneficial effects of IGF-1 are sustainable, we performed a separate experiment to test the efficacy of IGF-1 treatment at 72 h after the stroke. Our results show that the beneficial effect of IGF-1 is still present after three days. IGF-1 reduced the infarct size and neuronal damage in the infarcted hemisphere (Fig. 3A, B, D). However, the NDS score was not improved by IGF-1 (Fig. 3C). Interestingly, Iba-1 staining showed that the ipsilateral hemisphere contained an increased level of aggregates of microglia/macrophages (Fig. 3E) compared to this level at 24 h (Fig. 2A). Hence, we assessed the level of microglia/macrophages activation using the Iba-1 staining intensity as a measure. Our results show that IGF-1 did not affect the staining intensity in the striatum or the cortex (Fig. 3F, G).

Regulation of inflammatory mediators in the ischemic hemisphere by IGF-1

To determine the underlying mechanisms of IGF-1 neuroprotection, we investigated whether IGF-1 targets neuroinflammation during the acute phase of ischemic stroke. Therefore, we measured the transcripts of pro- and anti-inflammatory mediators in the ischemic hemisphere 24 h following stroke. We found that IGF-1 treatment significantly down-regulated gene expression of the pro-inflammatory mediators, inducible nitric oxide synthase (iNOS) and interleukin (IL)-1β (Fig. 4) in the ischemic hemisphere. Furthermore, the Iba-1 transcripts were substantially decreased by IGF-1 treatment, while the reduction in glial fibrillary acidic protein (GFAP) expression did not reach significance (Fig. 4). The expression of the pro- and anti-inflammatory mediators, IL-6, transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), IL-10, neurotrophin (NT)-3 and nerve growth factor (NGF) were also reduced by IGF-1 treatment, but these differences did not reach statistical significance (Fig. 4). Likewise, a small non-significant decrease in mRNA expression of chitinase 3-like 3 (Chi3l3/Ym1) and mannose receptor c-type 1 (Mrc1/CD206) by IGF-1 was observed (Fig. 4). The transcripts of neurotrophic factor, brain-derived neurotrophic factor (BDNF) was slightly up-regulated by IGF-1, although the effect was not significant (Fig. 4). On the other hand, mRNA expression of NT-4 was not affected by IGF-1 (Fig. 4).

IGF-1 modulates the inflammatory mediator iNOS in microglia that are present in the ischemic hemisphere

After having established that IGF-1 markedly suppressed Iba-1 transcripts in the ischemic brain, we asked whether IGF-1 targets microglia and regulates their inflammatory transcripts in the ischemic hemisphere. Therefore, we pooled ipsilateral hemispheres from two rats receiving the same treatment and considered them as one sample. Next, we isolated the microglia by FACS to measure mRNA expression of inflammatory mediators. Fig. 5 reveals that IGF-1 reduced iNOS transcripts in microglia following stroke. In addition, IGF-1 slightly increased mRNA expression of TGF-β and decreased the level of Iba-1 (Fig. 5), but these effects were not significant. In contrast, IGF-1 did not modulate the mRNA expression of IL-1β and TNF-α (Fig. 5).

IGF-1 does not change the relative abundance of neutrophils in the ischemic hemisphere

To investigate whether IGF-1 blocks the infiltration of neutrophils into the infarcted brain, we measured the proportions of neutrophils in the ischemic hemisphere using FACS analysis. Briefly, after CD11b/c cell enrichment by magnetic cell sorting, we performed a pre-sort FACS analysis using the following gating strategy: After exclusion of doublets and dead cells, CD11b was plotted against CD45. The $CD11b^{+}/CD45^{hi}$ cell populations were gated (Fig. 6A) and further analyzed based on the expression of His48 and CD163 (Fig. 6B). Neutrophils were determined as $CD11b^{+}/CD45^{hi}/His48^{+}$ and the FACS analysis demonstrated

Fig. 3. Beneficial effects of IGF-1 at 72 h after stroke onset. (**A**) Representative micrographs of Cresyl Violet-stained sections from vehicle- and IGF-1- treated rats. The outlined squares in the first row are shown at higher magnification in the second row. The third row shows representative micrographs of NeuN-staining in IGF-1- and vehicle- treated rats. Quantification of infarct volume and NDS are displayed on graphs (**B**), (**C**), respectively. (**D**) Shows the effect of IGF-1 on the neuronal loss in the ipsilateral hemisphere. Aggregates of microglia/macrophages in the ipsilateral hemisphere are shown in (**E**). The outlined region in the left micrograph, is represented at higher magnification in the right micrograph. The effect of IGF-1 on the Iba-1 staining intensity in the infarcted cortex and striatum are shown in (**F**) and (**G**), respectively. Scale bars of the second and fourth rows in (**A**) = 50 μm and 100 μm, respectively. Scale bar of the right micrograph in (**E**) = 100 μm. The number of animals per group was as follows: Vehicle ($n = 4$) and IGF-1 ($n = 7$ for NDS and infarct volume, $n = 5$ for Iba-1 intensity in the cortex and NeuN-staining and $n = 6$ for Iba-1 intensity in striatum). *Significance $P < 0.05$.

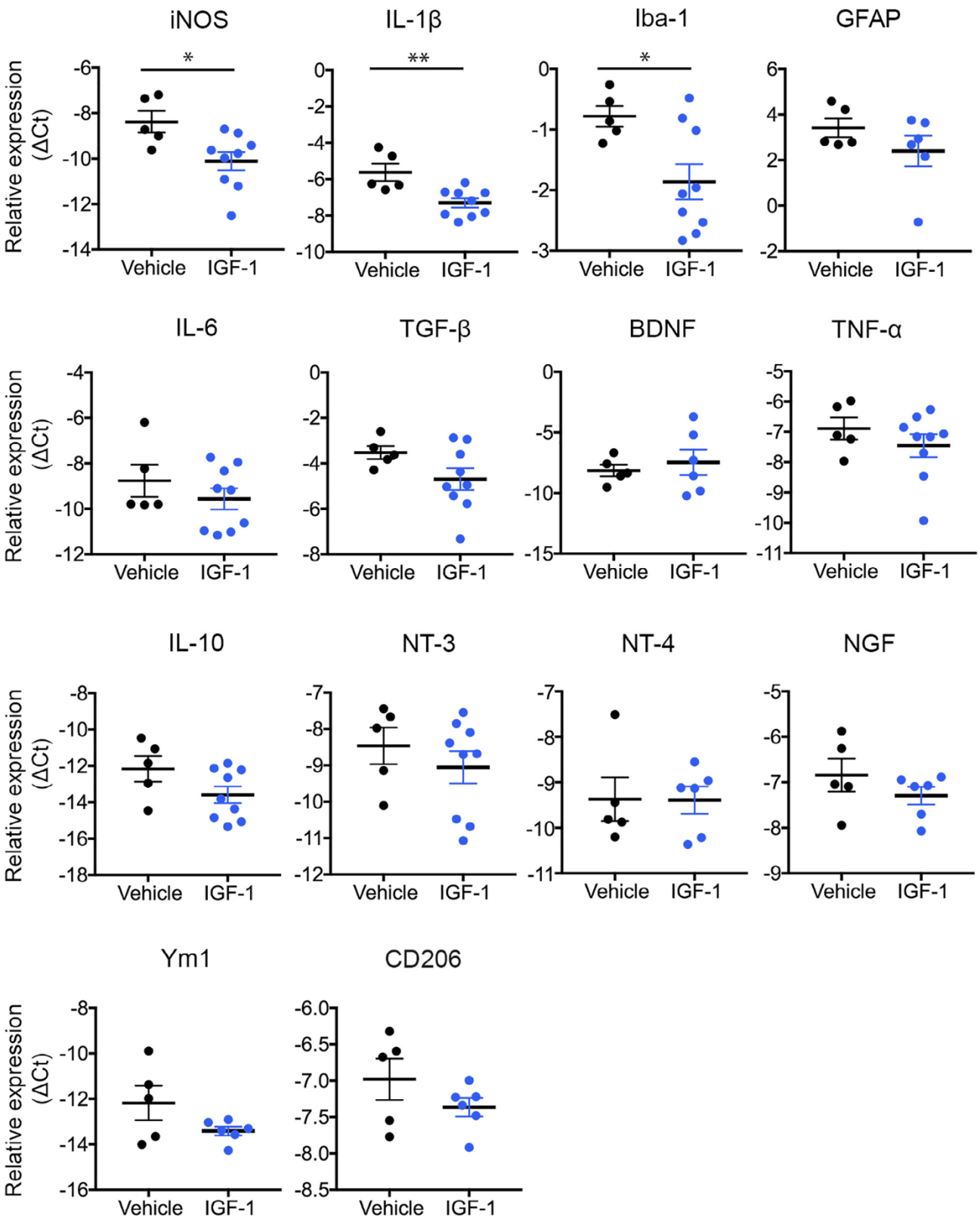


Fig. 4. Effect of IGF-1 on the mRNA expression of pro- and anti-inflammatory mediators and markers 24 h following stroke. Two ipsilateral hemispheres from two rats that received same treatment, were pooled and considered as one biological replicate. Next, total RNA was extracted and transcribed into cDNA which was quantified by qPCR. The number of samples per group was as follows: Vehicle- (n = 5) and IGF-1-treated groups (n = 6 for BDNF, GFAP, NT-4, NGF, Ym-1, CD206 and n = 9 for all other genes). *Significance $P < 0.05$ and **Significance $P < 0.01$.

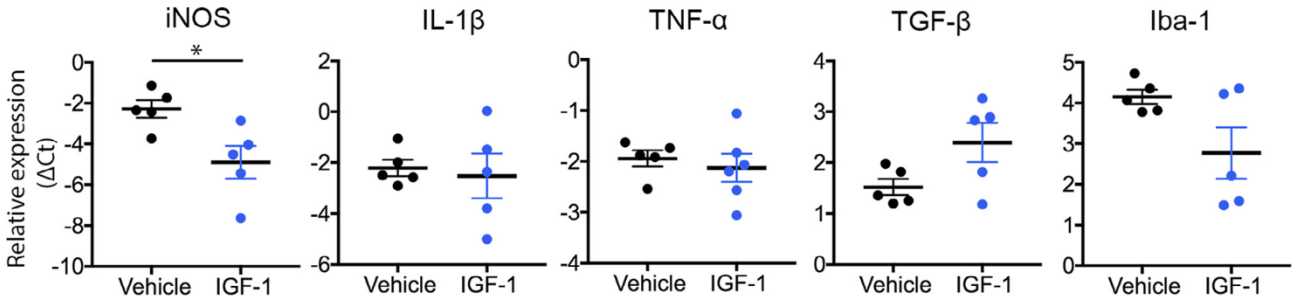


Fig. 5. Modulation of pro-inflammatory mediator transcripts by post-stroke IGF-1 treatment in microglia. Microglia were purified from ischemic hemispheres 24 h after the insult. Two ipsilateral hemispheres from rats that received the same treatment were combined and considered as one sample after which microglia were purified. The total RNA was isolated from purified microglia and subsequently transcribed to cDNA which was subjected to qPCR analysis. The number of samples per group was as follows: Vehicle- (n = 5) and IGF-1-treated rats (n = 6 for TNF-α and n = 5 for other genes). *Significance $P < 0.05$.

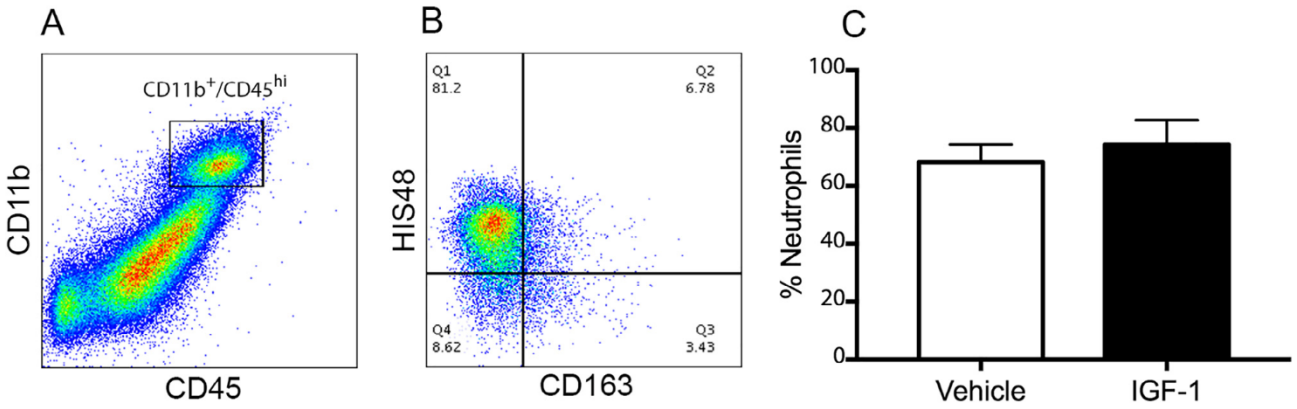


Fig. 6. Effect of IGF-1 on the neutrophil proportions in the ipsilateral hemisphere. (**A**), (**B**) show the gating strategy of infiltrated neutrophils into the infarcted region. (**C**) Represents the proportions of neutrophils in the ipsilateral hemisphere of vehicle- (n = 4) and IGF-1-treated rats (n = 3).

that IGF-1 treatment did not affect the proportions of neutrophils in the ischemic hemisphere (Fig. 6C).

DISCUSSION

The results in the present paper corroborate our previous findings (De Geyter et al., 2013) that IGF-1 decreases infarct volume and improves sensorimotor function when injected SC 24 h following induction of ischemic stroke (Fig. 1A–C).

Our results are in agreement with observations that high serum levels of IGF-1 correlate with a good neurological outcome in stroke patients (Åberg et al., 2011; Tang et al., 2014; Saber et al., 2017). We and others have shown before that systemic IGF-1 administration is indeed neuroprotective in preclinical models for ischemic stroke (Liu et al., 2004; Bake et al., 2014; De Geyter et al., 2016). In regard to these observations the results of De Magalhaes Filho et al. (2017) were remarkable. They showed that selective depletion of IGF-1 receptors in forebrain neurons was beneficial in a murine model for ischemic stroke. However, the depletion also led to increased serum levels of IGF-1 which could be operative in neuroprotection through receptors on microglia or other glia in the brain.

Although it has been shown that activation of microglia leads to secretion of detrimental mediators that can affect stroke outcome, activated microglia can also protect neurons by secretion of supportive neurotrophic factors and scavenging of cell debris (Patel et al., 2013; Guruswamy and ElAli, 2017). The latter observation is in line with a study showing that depletion of microglia, augments infarct volume and impairs behavioral outcome after stroke induction which was also paralleled by an increase in the levels of pro-inflammatory mediators (Jin et al., 2017). In the current paper, we show that systemic IGF-1 treatment has differential effect, in which it increases the density of ameboid microglia in the cortex, but not in striatum of the ischemic hemisphere (Fig. 2A–C). This effect could be due to the fact that the core of the infarct, which includes necrotic tissue is situated in the striatum, whereas the penumbra which contains metabolically inactive, but potentially salvageable tissue, is predominantly located in the cortex. To further explore the role of microglial activation in neuroprotection by IGF-1, we treated the rats with minocycline, an inhibitor of microglial activation. Interestingly, minocycline treatment abolished the augmentation in the number of ameboid microglia by IGF-1 in the cortex (Fig. 2A, B). The reduction of infarct size by IGF-1 in the presence of minocycline, suggests that the increase in number of ameboid microglia is not or hardly instrumental to the decrease in infarct size by IGF-1. On the other hand, the decline in augmentation of the NDS by IGF-1 in minocycline-treated rats (Fig. 1C), indicates that the increment of ameboid microglia density could be functional with respect

to the neurological outcome. Improvement of the neurological outcome does not only depend on the number of rescued neurons, but also on their functionality and it could be that ameboid microglia exert beneficial effects on neuronal function. Consistent with this finding, a deficiency in Triggering Receptor Expressed on Myeloid Cells 2 (TREM2), which is responsible for polarizing activated microglia towards the ameboid type, leads to impaired neurological recovery following ischemic stroke (Kawabori et al., 2015).

Another explanation for the absence of neurological recovery could be that minocycline exerts deleterious effects on stroke outcome when injected at high doses (Matsukawa et al., 2009).

Our finding that minocycline had no effect on infarct volume and sensorimotor function, contradicts with previous studies that showed minocycline has beneficial effects on stroke outcome. Hayakawa et al. have shown that IP administration of minocycline following stroke for 14 days improved survival rate and neurologic impairment that was accompanied by significant decrease in microglia activation in mice (Hayakawa et al., 2008). Furthermore, administration of minocycline 4 days following ischemic stroke that continued till four weeks, induced neurogenesis, improved motor function and decreased microglia activation in rats (Liu et al., 2007). Since we investigated the effect of minocycline after 24 h of stroke induction, while other studies tested its effect for several days or weeks after stroke onset, it could be possible that administration of minocycline for 1 day is not sufficient to elicit neuroprotection and improves sensorimotor function.

It is clinically relevant to test the long-term effect of drugs. Therefore, we tested the efficacy of IGF-1 treatment on infarct volume and sensorimotor function at 72 h following stroke. We established that post-stroke IGF-1 treatment decreased infarct volume (Fig. 3A, B). It should be noted that the reduction in infarct size at 72 h after the insult does not go along anymore with augmentation of sensorimotor function, although IGF-1 significantly decreased the area of neuronal damage in the ipsilateral hemisphere (Fig. 3A, D). Notably, the sensorimotor function increased in both treated and untreated animals and it could be that functional recovery after 24 h depends on other processes than neuroprotection occurring within the first 24 h. Alternatively, a longer treatment period with IGF-1 could also further increase the sensorimotor function at 72 h. Indeed Schäbitz et al. demonstrated long-term effects in a rat model with ischemic stroke by daily treatment with IGF-1 (Schäbitz et al., 2001).

Our results confirm the findings in the literature (Liu et al., 2004) that systemic administration of IGF-1 has long-term neuroprotective effects. Furthermore, it has been shown that microglia/macrophages increase in numbers and peak at 3–7 days (Grønberg et al., 2013) which is in line with our findings that show aggregates of microglia/macrophages in the ipsilateral hemisphere (Fig. 3E). Since IGF-1 did not affect the Iba-1 intensity in the striatum and cortex (Fig. 3F, G), neuroprotective effect of IGF-1 at 72 h of stroke onset seems to be independent of microglial activation.

To further explore the role of microglia and modulation of neuroinflammation by IGF-1 on neuroprotection, we assessed the expression of inflammatory mediators in the ischemic hemisphere and in isolated microglia. We found that IGF-1 treatment resulted in a decreased expression of IL-1β and iNOS mRNA in the ischemic hemisphere (Fig. 4), suggesting that IGF-1 induces neuroprotection by modulation of neuroinflammation. We previously delivered definitive evidence that systemically injected IGF-1 exerts its effects centrally through IGF-1 receptors in the brain (De Geyter et al., 2016). In the current paper, we aimed to obtain more evidence for the role of microglia as a target for IGF-1 in the brain. Therefore, we purified microglia from the ischemic hemisphere of vehicle- and IGF-1- treated rats and subsequently analyzed the mRNA content. It appeared that IGF-1 treatment decreased the expression of iNOS in these microglia, while the expression of IL-1β was not changed (Fig. 5). Since our results indicate that IGF-1 decreases the expression of inflammatory mediators in the ischemic hemisphere and that this effect is, at least in part, mediated by microglia, it could be that IGF-1 induces neuroprotection by polarizing microglia towards anti-inflammatory phenotype. However, since the anti-inflammatory mediators (TGF-β, IL-10, NT-3, NT-4 and NGF) and anti-inflammatory markers (Ym1 and CD206) were not significantly regulated by IGF-1, this issue needs to be further addressed maybe by using a higher number of rats for the gene expression studies. A role for microglia in neuroprotection through suppression of neuroinflammation has been suggested in several preclinical stroke studies. The histone deacetylase inhibitor sodium butyrate, induced neuroprotection in mice subjected to MCAO by suppressing the gene expression of TNF-α and iNOS, and promoting the expression of IL-10 in microglia (Patnala et al., 2017). Another study showed that a reduction in infarct size by pre-stroke injection of salvianic acid coincided with an impairment of microglial activation and down-regulation of IL-6 and IL-1β (Zhuang et al., 2017).

Since IGF-1 did not significantly reduce GFAP mRNA expression in the ischemic brain (Fig. 4), we suggest that IGF-1 targets astrocytes and down-regulates mRNA expression of IL-1β. The possible interaction of IGF-1 with astrocytes could be mediated directly or indirectly via microglia. The latter possibility is in accordance with the recent observation that depletion of microglia in mice, exacerbates the secretion of pro-inflammatory mediators including IL-1β by astrocytes (Jin et al., 2017).

A diminution of inflammatory mediators could also lead to a decreased infiltration of circulating neutrophils leading to neuroprotection. It has been shown that an interleukin-1-receptor antagonist (IL-1Ra) diminished the infiltration of neutrophils, which in turn decreased infarct volume and improved BBB disruption (Pradillo et al., 2012). Furthermore, neutralization of IL17A, a cytokine produced by γδ T cells, by IL-17A-blocking antibody reduced neutrophil invasion and brain injury following

stroke (Gelderblom et al., 2012). Although IGF-1 significantly suppressed neuroinflammation following stroke, IGF-1 did not modulate the proportion of neutrophils (Fig. 6C) in the ischemic hemisphere. Moreover, we found that the proportion of infiltrated macrophages in the ipsilateral hemisphere following stroke was negligible (data not shown), which is consistent with other studies that showed macrophages do not infiltrate the ischemic hemisphere after 1 day of stroke onset (Schilling et al., 2005, 2003).

Neuroprotection by IGF-1 goes along with a reduction in inflammatory mediators in the ischemic hemisphere, which could be instrumental to the reduction of the infarct volume and neurological deficits. The observation that IGF-1 treatment also decreases iNOS production in the microglia from the ischemic hemisphere, suggests that microglia are a direct or indirect target for IGF-1 in neuroprotection.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

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