

# Glucagon-like peptide-1 receptor activation reduces ischaemic brain damage following stroke in Type 2 diabetic rats

Vladimer DARSALIA\*, Shiva MANSOURI\*, Henrik ORTSÄTER\*, Anna OLVERLING\*, Nino NOZADZE\*, Camilla KAPPE\*, Kerstin IVERFELDT†, Linda M. TRACY†, Nina GRANKVIST\*, Åke SJÖHOLM\* and Cesare PATRONE\*

\*Diabetes Research Unit, Department of Clinical Science and Education, Södersjukhuset, Karolinska Institutet, Stockholm, Sweden, and †Department of Neurochemistry, Stockholm University, Stockholm, Sweden

## A B S T R A C T

Diabetes is a strong risk factor for premature and severe stroke. The GLP-1R (glucagon-like peptide-1 receptor) agonist Ex-4 (exendin-4) is a drug for the treatment of T2D (Type 2 diabetes) that may also have neuroprotective effects. The aim of the present study was to determine the efficacy of Ex-4 against stroke in diabetes by using a diabetic animal model, a drug administration paradigm and a dose that mimics a diabetic patient on Ex-4 therapy. Furthermore, we investigated inflammation and neurogenesis as potential cellular mechanisms underlying the Ex-4 efficacy. A total of seven 9-month-old Type 2 diabetic Goto–Kakizaki rats were treated peripherally for 4 weeks with Ex-4 at 0.1, 1 or 5 µg/kg of body weight before inducing stroke by transient middle cerebral artery occlusion and for 2–4 weeks thereafter. The severity of ischaemic damage was measured by evaluation of stroke volume and by stereological counting of neurons in the striatum and cortex. We also quantitatively evaluated stroke-induced inflammation, stem cell proliferation and neurogenesis. We show a profound anti-stroke efficacy of the clinical dose of Ex-4 in diabetic rats, an arrested microglia infiltration and an increase of stroke-induced neural stem cell proliferation and neuroblast formation, while stroke-induced neurogenesis was not affected by Ex-4. The results show a pronounced anti-stroke, neuroprotective and anti-inflammatory effect of peripheral and chronic Ex-4 treatment in middle-aged diabetic animals in a preclinical setting that has the potential to mimic the clinical treatment. Our results should provide strong impetus to further investigate GLP-1R agonists for their neuroprotective action in diabetes, and for their possible use as anti-stroke medication in non-diabetic conditions.

## INTRODUCTION

Stroke is the primary cause of disability in adults and the third most common cause of death. Stroke is often associated with comorbid health conditions that increase the risk and severity of stroke [1,2]. T2D (Type 2 diabetes)

is one such comorbid disease where premature stroke represents one of the most common and serious long-term complications [3–6]. The risk of stroke is increased 2- to 6-fold in patients with T2D [6] with greater risk for stroke recurrence and higher mortality rate [6,7]. Finally, a pre-diabetic state with impaired glucose tolerance

**Key words:** exendin-4 (Ex-4), Goto–Kakizaki (GK) rat, middle cerebral artery occlusion (MCAO), neurogenesis, neuroprotection. **Abbreviations:** BrdU, bromodeoxyuridine; bw, body weight; CNS, central nervous system; DAPI, 4',6-diamidino-2-phenylindole; DCX, doublecortin; Ex-4, exendin-4; GK, Goto–Kakizaki; GLP-1R, glucagon-like peptide-1 receptor; IHC, immunohistochemistry; MCA, middle cerebral artery; MCAO, MCA occlusion; SVZ, subventricular zone; T2D, Type 2 diabetes. **Correspondence:** Professor Åke Sjöholm (email ake.sjoholm@ki.se) or Dr Cesare Patrone (email cesare.patrone@ki.se).

is often detected in stroke patients following hospital admission, and such patients generally exhibit a poor prognosis [8,9].

GLP-1R (glucagon-like peptide-1 receptor) agonists are novel treatments against T2D [10], which may also have direct neuroprotective effects besides their gluco-regulatory action. These molecules interact with the G-protein-coupled GLP-1R and enhance glucose-dependent insulin secretion [11]. Exenatide [synthetic Ex-4 (exendin-4)] is a stable GLP-1 analogue isolated from the saliva of the 'Gila monster' lizard [12]. It is resistant to degradation and is approved in both Europe and the U.S.A. for the clinical treatment of T2D [11].

GLP-1R is expressed throughout the brain [13,14] and GLP-1 and Ex-4 can cross the blood-brain barrier [15]. GLP-1R agonists have been reported to be neuroprotective [16,17], neurogenic [18,19], enhance synaptic plasticity [20] and improve different forms of learning [16,19]. Furthermore, anti-inflammatory CNS (central nervous system) effects of GLP-1 have been reported [21–23]. Intracerebroventricular administration of Ex-4 15 min before stroke reduced ischaemic damage in mice [24]. Intraperitoneal injection of Ex-4 2 h before ischaemia and 1 h after reperfusion protected hippocampal CA1 neurons in a global ischaemia model in gerbils [25]. Finally, acute intravenous administration of Ex-4 following ischaemia reduced the infarct volume. However, the neuroprotective effect was lost if Ex-4 was administered 3 h after stroke [26]. These studies were performed in young healthy non-diabetic mice.

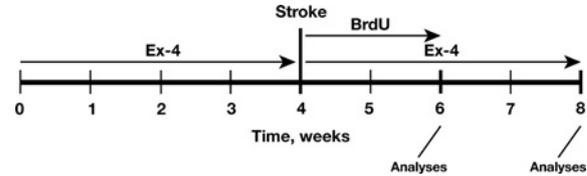
Although the effects of GLP-1R activation in the brain are promising, more research is needed to understand the mechanisms behind its action and whether GLP-1R activation is effective in the diabetic brain.

In the present study, we tested the anti-stroke efficacy of Ex-4 in diabetes by using middle-aged diabetic GK (Goto-Kakizaki) rats. The GK rat is a non-obese Wistar substrain, which spontaneously develops T2D early in life [27] and where Ex-4 treatment reduces hyperglycaemia [28].

## MATERIALS AND METHODS

### Ex-4 bioactivity assay

To ascertain the bioactivity of Ex-4 (kindly provided by Amylin), insulin secretion was measured from islets of Langerhans isolated from non-diabetic male Wistar rats. Batches of 15 islets per group were maintained for 30 min at 37°C in a buffer consisting of 3 or 20 mM glucose, 125 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl<sub>2</sub>, 1.3 mM CaCl<sub>2</sub> and 25 mM Hepes, titrated to pH 7.4 with NaOH and supplemented with 1 mg/ml BSA (fraction V; Roche Diagnostics) with/without 10 nM Ex-4. After incubation, aliquots of buffer were frozen pending analyses of released insulin by ELISA (Mercodia).



**Figure 1** Experimental design and drug administration paradigm

### Animals and experimental groups

A total of 42 7–9-month-old male diabetic GK rats were used. All experiments were conducted according to the 'Guide for the Care and Use of Laboratory Animals' published by U.S. National Institutes of Health (NIH publication no. 85-23, revised 1985) and approved by the regional ethics committee for animal experimentation.

Before the start of the Ex-4/PBS treatments, baseline fasting blood glucose concentrations were measured and the animals were assigned to the different treatment groups so that mean blood glucose values were equalized. Treatment groups thus created were tested for normality using the D'Agostino and Pearson omnibus normality test. All rats received intraperitoneal injections of Ex-4 [0.1, 1 or 5 µg/kg of bw (body weight) ( $n = 10$ )] or PBS ( $n = 12$ ) for 4 weeks twice daily before being subjected to stroke. Ex-4 and PBS injections were continued for another 2 or 4 weeks following the stroke. To assess neurogenesis, all rats received daily intraperitoneal injections of the thymidine analogue BrdU (bromodeoxyuridine; 50 mg/kg of bw) for 2 weeks following stroke. Rats were killed for immunohistochemical analyses at 2 or 4 weeks after stroke. The experimental design is illustrated in Figure 1.

### MCAO [MCA (middle cerebral artery) occlusion]

MCAO is a stroke animal model that closely resembles the most common form of human stroke [29]. Rats were anaesthetized by spontaneous inhalation of 1.5% isoflurane through a snout-mask. Body temperature was maintained at 37–38°C using a heating pad. Stroke was induced by the intraluminal filament technique [29]. Briefly, common and external carotid arteries were ligated, and the internal carotid artery was temporarily closed. A monofilament was advanced through the internal carotid artery to the origin of the MCA, the wound was closed and the animal was allowed to wake up and was placed in its cage. After 90 min of occlusion, the animals were anaesthetized again and the filament was withdrawn. The surgeon performing the operation was blinded to the treatment groups.

## Immunocytochemistry

Animals were deeply anaesthetized and perfused transcardially with 4% (w/v) paraformaldehyde. The brains were extracted and submersed in 20% sucrose in phosphate buffer overnight. Then 40- $\mu$ m-thick coronal sections were cut using sliding microtome and stained as free-floating sections. The following primary antibodies were used: mouse anti-Ki67 (1:200 dilution; Novocastra), a marker of cell proliferation; goat anti-DCX (doublecortin) (1:400 dilution; Santa Cruz Biotechnology), a marker for migrating neuroblasts; mouse anti-NeuN (1:100 dilution; Millipore), a neuronal marker; rabbit anti-Iba1 (1:1000 dilution, Wako Chemicals), a marker for microglia; mouse anti-ED1 (1:200 dilution; Serotec), a marker of activated microglia; rat anti-BrdU (1:200 dilution; Santa Cruz Biotechnology), to assess neurogenesis in combination with NeuN; and rabbit anti-GLP-1R (1:50 dilution; Abcam). In the sections stained for GLP-1R, DAPI (4',6-diamidino-2-phenylindole) was used to visualize all of the cells. Sections were incubated with primary antibodies overnight at 4°C in phosphate buffer containing 3% appropriate serum and 0.25% Triton X-100. Primary antibodies were detected by use of appropriate fluorescent Cy3 (indocarbocyanine)-, Cy2 (carbocyanine)- (both from Jackson ImmunoResearch) or biotin-conjugated (Vector) secondary antibodies (1:200 dilution). Sections were incubated with secondary antibodies for 2 h at room temperature (approx. 21°C) in phosphate buffer containing 3% of the appropriate serum and 0.25% Triton X-100. For chromogenic visualization, biotinylated secondary antibodies, avidin-biotin complex (ABC kit; Vector) and diaminobenzidine were used.

## Tissue damage evaluation and cell quantification

Tissue damage evaluation and cell counting were performed by an investigator blinded to the experimental groups. For tissue damage evaluation, the NeuN-labelled tissue sections were displayed live on the computer monitor and the area of contralateral hemisphere and the area of the intact ipsilateral tissue were measured in every section containing stroke damage using NewCast (Visiopharm) software. To compensate for the stroke-induced morphological tissue changes, the infarct volume was calculated by subtracting the volume of remaining tissue in the ipsilateral hemisphere from the volume of the contralateral hemisphere.

Immunoreactive cells were counted using either a computerized non-biased setup for stereology, driven by NewCast software, or by using an Olympus BX40 epifluorescence/light microscope. The number of neurons (NeuN) and microglia (Iba1 and ED1) cells was quantified using the optical fractionator method [31,32]. Briefly, brain sections were displayed live on the

computer monitor and the striatum and cortex delineated at low magnification. Quantifications were performed using a  $\times 100$  oil-immersion lens with numeric aperture of 1.30 or a  $\times 40$  dry lens with numeric aperture of 0.75. Ten evenly spaced sections in parallel-cut series through the entire striatum were included. Random sampling was carried out using the counting frame, which systematically was moved at predefined intervals so that  $\sim 200$  immunoreactive cells were counted. The total number of cells was estimated according to the optical fractionator formula [31,32].

For evaluation of SVZ (subventricular zone) cell proliferation, SVZ cells immunoreactive for Ki67 were counted. DCX is a marker for newly produced neuroblasts from neurogenic brain areas and DCX-positive cells have been shown to migrate into the ischaemic striatum [33]. Therefore neuroblast production was quantified by counting cells positive for DCX in stroke-damaged striatum. NeuN is a ubiquitous nuclear marker for mature neurons. Thus, neurogenesis was assessed by counting NeuN/BrdU-positive cells in stroke-damaged striatum. These quantifications were performed in three evenly spaced (400  $\mu$ M) brain sections on the side ipsilateral to stroke, starting at 0.7 mm anterior to Bregma.

## Statistics

Statistical analyses were performed using one-way or two-way ANOVA, followed by Bonferroni's or Dunnett's post-hoc test. Differences between groups were considered statistically significant when  $P < 0.05$ . Results are presented as means  $\pm$  S.E.M.

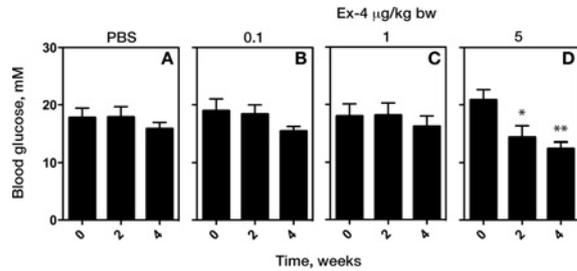
## RESULTS

### Ex-4 reduces hyperglycaemia in GK rats

The bioactivity of Ex-4 was shown as its efficacy to augment several fold the glucose-induced insulin secretion in islets of Langerhans isolated from adult non-diabetic rats (results not shown). Treatment with Ex-4 slightly reduced glycaemia in GK rats, although this effect did not attain statistical significance in the groups treated with 0.1 and 1  $\mu$ g/kg of bw. Only the group treated with Ex-4 at 5  $\mu$ g/kg of bw showed a statistically significant reduction in glycaemia after 2 and 4 weeks of treatment (Figures 2A–2D). Animals were fasted for 3 h before blood glucose measurements were performed.

### GLP-1R is expressed by neurons in the brain of diabetic rats

To assess GLP-1R expression in the brain, we immunostained brain sections for GLP-1R. The results showed a widely dispersed expression of GLP-1R throughout the brain which co-localized with NeuN-positive-neurons (Figure 3).



**Figure 2** Ex-4 effects on glycaemia in GK rats

(A–D) Fasted blood glucose levels in GK rats treated with PBS ( $n = 12$ ), or EX-4 at 0.1 ( $n = 10$ ), 1 ( $n = 10$ ) or 5  $\mu\text{g}/\text{kg}$  of bw ( $n = 10$ ) for 4 weeks before inducing a stroke. Bars represent means  $\pm$  S.E.M. \* $P < 0.05$  and \*\* $P < 0.01$  compared with week 0 using one-way ANOVA, followed by Bonferroni's post-hoc analysis.

### Ex-4 exhibits dose-dependent anti-stroke efficacy

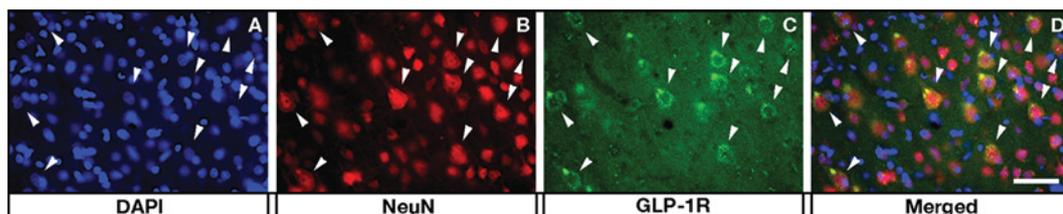
In order to determine the potential anti-stroke efficacy mediated by Ex-4, the measurement of infarct volume was assessed at both 2 and 4 weeks after stroke. Since no difference was recorded between 2 and 4 weeks after stroke between the treatment groups, results from 2- and 4-week time points were pooled. The measurement of the infarct volume revealed that treatment with Ex-4 at 5  $\mu\text{g}/\text{kg}$  of bw resulted in a significant reduction of tissue damage after stroke (Figures 4A, 4C, and 4D). Although not statistically significant, the treatment with Ex-4 at 0.1 and 1  $\mu\text{g}/\text{kg}$  of bw showed a noticeable trend towards a reduction of ischaemic tissue damage (Figure 4D).

In order to assess the neuroprotective effect of Ex-4 with greater accuracy than infarct volume measurement, NeuN-positive neurons were quantified in both stroke-damaged striatum and cortex using the optical fractionator method (see the Materials and methods section). As for the volume measurements, no difference was recorded in the number of surviving neurons between 2 and 4 weeks after stroke between the treatment groups. Thus results from 2- and 4-week time

points were pooled. Results in Figure 4(E) show that, following a stroke induced by MCAO, the least amount of neurons remained in the stroke-damaged striatum and cortex of the PBS-treated group. Furthermore, in the two groups of animals treated with Ex-4 at 0.1 and 1  $\mu\text{g}/\text{kg}$  of bw, the activation of GLP-1R resulted in a statistically significant effect above control with an increased number of surviving neurons in the stroke-damaged striatum and cortex (Figure 4E). Treatment with Ex-4 at 5  $\mu\text{g}/\text{kg}$  of bw further increased the number of surviving neurons compared with both PBS and the two lower doses of Ex-4 (Figure 4E). When neuronal survival was evaluated separately in the striatum and cortex, the neuronal quantifications showed that a statistically significant neuroprotective effect in striatum was only achieved with Ex-4 at 5  $\mu\text{g}/\text{kg}$  of bw, although a dose-dependent trend was also noticeable with the two lower Ex-4 doses (Figure 4F). This neuroprotective effect was much stronger in the cortex, where Ex-4 treatment at 0.1 and 1  $\mu\text{g}/\text{kg}$  of bw also resulted in statistically significant neuroprotection (Figure 4G).

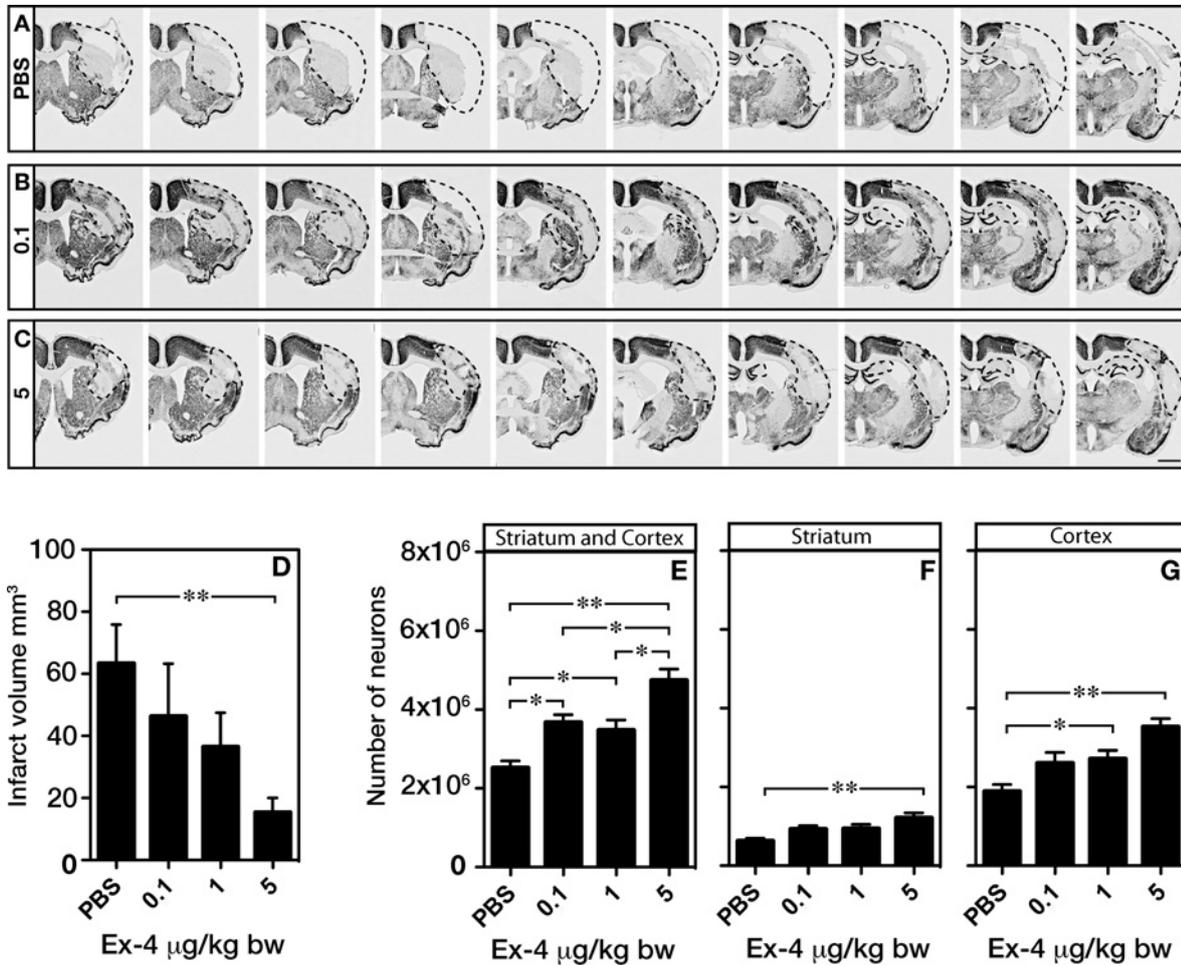
### Ex-4 treatment significantly reduces microglia infiltration after stroke, but has a marginal effect on microglia activation

To evaluate the magnitude of microglia infiltration, reflecting inflammatory levels, Iba1-immunoreactive cells were counted using the optical fractionator method (see the Materials and methods section). At 2 weeks after stroke, the number of Iba1-positive microglial cells within the stroke-damaged hemisphere (cortex plus striatum; Figure 5A) and in cortex and striatum separately (Figures 5C and 5E) was similar in the PBS-treated and Ex-4-treated groups. However, at 4 weeks after stroke, the number of Iba1-positive cells in stroke-damaged hemisphere was significantly different between the groups treated with Ex-4 and PBS (Figure 5B). When we evaluated microglia infiltration at 4 weeks in the ipsilateral striatum and cortex separately, it became apparent that the Ex-4 treatment had no effect on microglia infiltration in the striatum (Figure 5D), while a



**Figure 3** GLP-1R expression in the cortex of the GK rat brain

(A) DAPI staining (general cell marker); (B) neurons positive for NeuN (neuro-specific marker); (C) GLP-1R-positive cells; (D) merged channels for NeuN and GLP-1R. Arrowheads indicate cells positive for DAPI, NeuN and GLP-1R respectively. Images were taken using a  $\times 40$  dry lens with 0.75 numeric aperture and ColorView II FW digital camera driven by the Cell<sup>h</sup> imaging software. Brightness/contrast was adjusted and final images resized using Adobe Photoshop software. (D) Scale bar, 50  $\mu\text{m}$ .



**Figure 4 Neuroprotective effect of Ex-4**

(A–C) Photomicrographs of representative ischaemic damage after treatment with PBS, or Ex-4 at 0.1 or 5  $\mu\text{g}/\text{kg}$  of bw. Dotted lines outline the ischaemic damage. (C) Scale bar, 2 mm. (D) Volume of the infarct. (E–G) Stereological quantification of NeuN-positive cells in stroke-damaged striatum and cortex. Groups, PBS ( $n = 12$ ), or Ex-4 at 0.1 ( $n = 10$ ), 1 ( $n = 10$ ) or 5 ( $n = 10$ )  $\mu\text{g}/\text{kg}$  of bw. Values are means  $\pm$  S.E.M. \* $P < 0.05$  and \*\* $P < 0.01$  between groups using one-way ANOVA, followed by Bonferroni's post-hoc test.

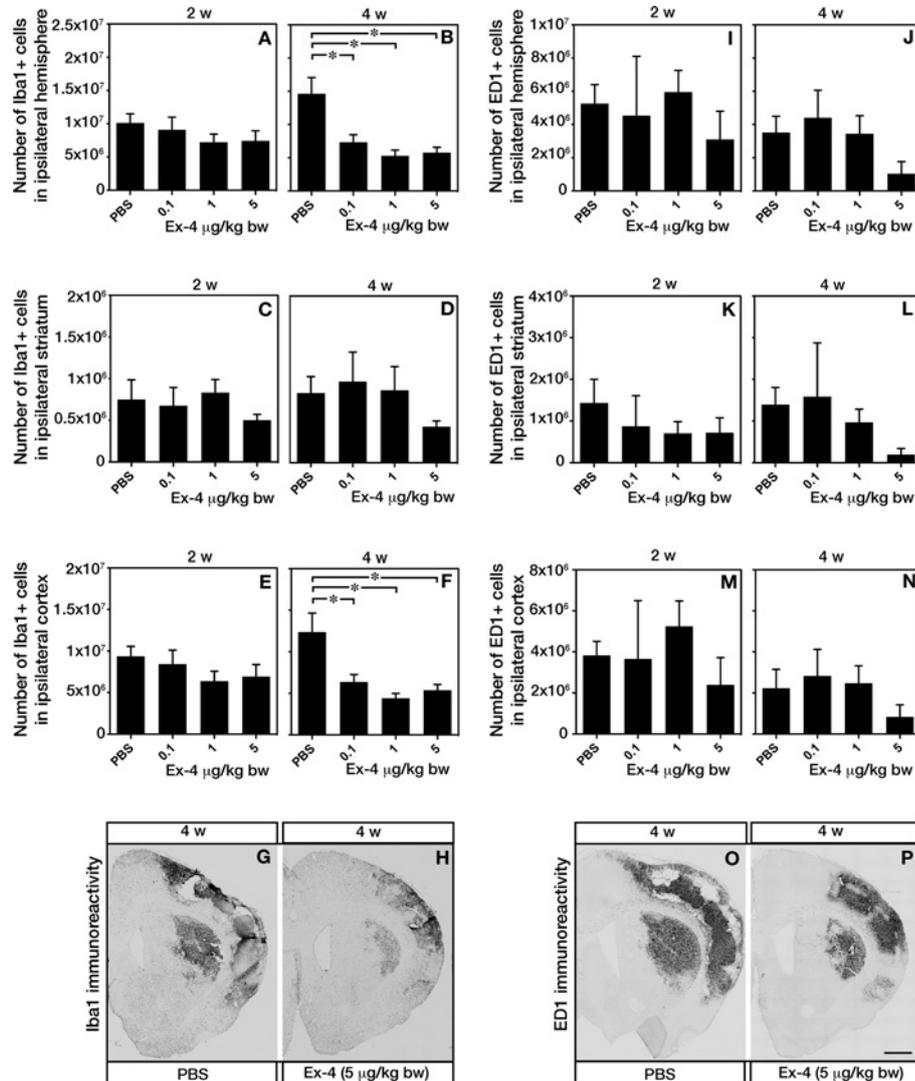
statistically significant effect was recorded in the cortex between each of the Ex-4 treated groups and PBS (Figure 5F).

To assess microglia activation, ED1-positive cells were quantified in a similar manner by using the optical fractionator method. As for Iba1, at 2 weeks after stroke, the number of ED1-positive microglial cells was similar in all experimental groups, both in striatum and cortex separately or pooled together (Figures 5I, 5K and 5M). At 4 weeks after stroke, the number of ED1-positive cells also did not decrease significantly (Figures 5J, 5L and 5N). However, a strong trend towards a reduction of the number of ED1-positive microglial cells in the group treated with Ex-4 at 5  $\mu\text{g}/\text{kg}$  of bw was observed in striatum and cortex both pooled together or when analysed separately (Figures 5J, 5L and 5N).

### Ex-4 treatment increases stem cell proliferation and neuroblast formation without affecting stroke-induced neurogenesis

The SVZ of the lateral ventricle and the hippocampal dentate gyrus are the two brain areas in which adult neurogenesis occurs [34]. To study the effect of Ex-4 treatment on stroke-induced neurogenesis, all animals were given daily injections of the thymidine analogue BrdU for 2 weeks following MCAO. Brains were analysed 2 or 4 weeks after stroke (Figure 1).

Ki67-expressing cells were counted to evaluate stem/progenitor cell proliferation in the SVZ of the brain. The results show that, at 2 weeks after stroke, Ex-4-treated animals showed 2-fold greater number of proliferating cells in the SVZ as compared with the



**Figure 5** Magnitude of brain inflammation following Ex-4 treatment at 2 and 4 weeks after stroke

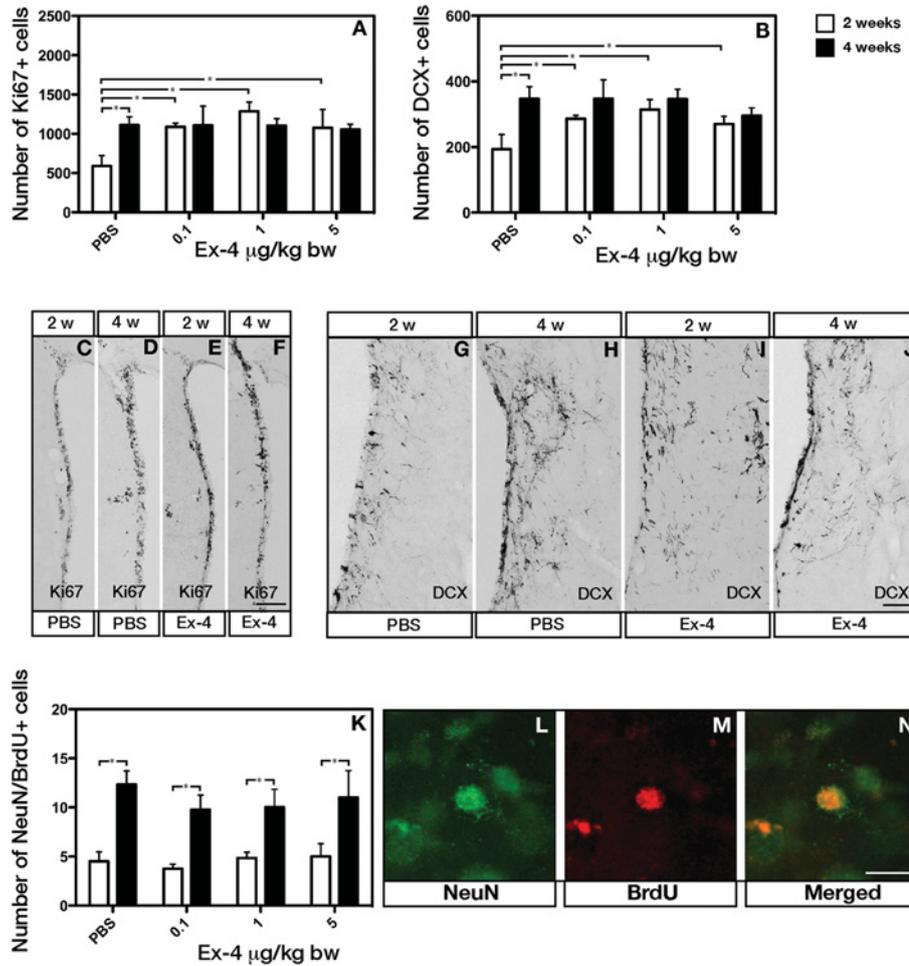
(A, B) Stereological quantification of Iba1-positive microglial cells in the stroke-damaged hemisphere at 2 (A) and 4 (B) weeks after MCAO. (C, D) Stereological quantification of Iba1-positive microglial cells in the stroke-damaged striatum at 2 (C) and 4 (D) weeks after stroke. (E, F) Stereological quantification of Iba1-positive microglial cells in the stroke-damaged cortex at 2 (E) and 4 (F) weeks after MCAO. (G, H) Representative images of Iba1 immunoreactivity in the groups receiving PBS (G) or Ex-4 at 5  $\mu\text{g}/\text{kg}$  of bw (H) at 4 weeks after stroke. (I, J) Stereological quantification of ED1-positive microglial cells in the stroke-damaged hemisphere at 2 (I) and 4 (J) weeks after stroke. (K, L) Stereological quantification of ED1-positive microglial cells in the stroke-damaged striatum at 2 (K) and 4 (L) weeks after MCAO. (M, N) Stereological quantification of ED1-positive microglial cells in the stroke-damaged cortex at 2 (M) and 4 (N) weeks after stroke. (O, P) Representative images of ED1 staining in the group treated with PBS (O) and Ex-4 at 5  $\mu\text{g}/\text{kg}$  of bw (P) at 4 weeks after stroke. (P) Scale bar, 2 mm. Groups at 2 weeks, PBS ( $n = 6$ ) or Ex-4 at 0.1 ( $n = 5$ ), 1 ( $n = 5$ ) and 5 ( $n = 5$ )  $\mu\text{g}/\text{kg}$  of bw. Groups at 4 weeks, PBS ( $n = 6$ ) or Ex-4 at 0.1 ( $n = 5$ ), 1 ( $n = 5$ ) and 5 ( $n = 5$ )  $\mu\text{g}/\text{kg}$  of bw. Values are means  $\pm$  S.E.M. \* $P < 0.05$  between groups using one-way ANOVA, followed by Dunnett's post-hoc test.

PBS group (Figures 6A, 6C and 6E), with no statistical differences between the different Ex-4 doses (Figure 6A). However, 4 weeks after stroke, no difference in the number of Ki67-positive cells between PBS- and Ex-4-treated groups was recorded (Figures 6A, 6D and 6F).

Stroke-induced neuroblast production was evaluated by quantifying the number of DCX-positive cells in the striatum. The DCX-positive neuroblast quantification revealed that, at 2 weeks after stroke, Ex-4 treatment

led to a 1.5-fold increase in neuroblast production compared with the PBS-treated group (Figures 6B, 6G and 6I). However, at 4 weeks after stroke, no difference in neuroblast production between PBS- and Ex-4-treated animals was recorded (Figures 6B, 6H and 6J).

Double labelling for the neuronal marker NeuN and the proliferation marker BrdU was used to identify mature neurons that were generated after stroke in striatum. At 2 weeks after stroke, the number of



**Figure 6** Stroke-induced neural stem/progenitor cell proliferation and neurogenesis in Ex-4-treated GK rat SVZ and striatum

(A) Number of Ki67-positive cells in SVZ ipsilateral to stroke at 2 and 4 weeks after stroke. (B) Number of DCX<sup>+</sup> neuroblasts at 2 and 4 weeks after stroke in the stroke-damaged striatum. White and black bars represent animals killed at 2 and 4 weeks respectively. (C, D) Representative images of Ki67 immunoreactivity in the ipsilateral SVZ of PBS-treated rats at 2 and 4 weeks after stroke respectively. (E, F) Representative images of Ki67 immunoreactivity in the ipsilateral SVZ of rats treated with Ex-4 at 5 μg/kg of bw at 2 and 4 weeks after stroke respectively. (F) Scale bar, 200 μm. (G, H) Representative images of DCX immunoreactivity in the ipsilateral striatum of PBS-treated rats at 2 and 4 weeks after stroke respectively. (I, J) Representative images of DCX immunoreactivity in the ipsilateral striatum of rats treated with Ex-4 at 5 μg/kg of bw at 2 and 4 weeks after stroke respectively. (J) Scale bar, 100 μm. (K) Number of NeuN/BrdU<sup>+</sup> neurons in stroke-damaged striatum at 2 (white bars) and 4 (black bars) weeks after stroke. (L–N) Photomicrographs of a NeuN/BrdU<sup>+</sup> neuron in stroke-damaged striatum. (L) NeuN, (M) BrdU and (N) merged channels showing double staining. (N) Scale bar, 20 μm. Groups at 2 weeks, PBS (*n* = 6) or Ex-4 at 0.1 (*n* = 5), 1 (*n* = 5) and 5 (*n* = 5) μg/kg of bw. Groups at 4 weeks, PBS (*n* = 6) or Ex-4 at 0.1 (*n* = 5), 1 (*n* = 5) and 5 (*n* = 5) μg/kg of bw. Values are means ± S.E.M. \**P* < 0.05 between groups using two-way ANOVA, followed by Bonferroni's post-hoc test. w, weeks.

NeuN/BrdU-positive neurons was similar in both PBS- and Ex-4-treated groups (Figure 6K). At 4 weeks after stroke, the number of NeuN/BrdU-positive neurons increased 2-fold in all groups without any differences between the PBS- and the Ex-4-treated groups (Figure 6K).

Similar quantifications were performed in the subgranular zone and granule cell layer of the dentate gyrus of the hippocampus. In this case, Ex-4 treatment had no detectable effect on either cell proliferation or neurogenesis (results not shown).

## DISCUSSION

In the present study, we show the anti-stroke efficacy of Ex-4 in a preclinical setting that mimics the clinical situation. Although successful in animal models, anti-stroke treatment strategies based on early intervention with neuroprotective drugs have not yet proven their efficacy in the clinic [35,36]. On the other hand, therapeutic strategies during the later and chronic phase of stroke have been largely unsuccessful both in preclinical experiments and clinical trials [36–38]. This

failure is likely due to two main factors. First, principal differences between the research animal models and typical stroke patients. The majority of stroke patients are elderly [39], where comorbidities such as diabetes and hypertension are common [2,35]. By contrast, in most of the preclinical experiments the efficacy of drugs is tested on animals that are both young and healthy. It has been calculated that to date, for six drugs reviewed systematically, only 10% of publications which studied the efficacy of these drugs had used animals with hypertension or diabetes, and none of the experiments reported efficacy in aged animals [35]. Secondly, preclinical drug administration paradigms are often not relevant for the clinical situation. For example, in many cases the preclinical efficacious doses of these drugs vastly (several orders of magnitude) differed from doses used in clinical practice [40]. Therefore, the successful implementation of preclinical achievements is largely dependent on how closely the experimental paradigms match the likely clinical scenario.

In the present study, we have evaluated the anti-stroke efficacy of the GLP-1R analogue Ex-4. The neuroprotective effect of Ex-4 administered immediately or within an hour after ischaemia in non-diabetic adult mice has been previously demonstrated [25,26]. However, the neuroprotective effect was not seen when Ex-4 was administered after a 3 h delay [26]. These results are clearly interesting and demonstrate GLP-1R-mediated acute neuroprotection. However, their applicability to the clinical setting is remote due to the relatively short effective intervention time-window (up to 1 h) and the Ex-4 doses employed that were up to 100 times higher (10  $\mu\text{g}/\text{kg}$  of bw) when compared with the clinical dose [26].

To closely simulate the way Ex-4 is used in clinical practice, we have used middle-aged Type 2 diabetic GK rats, among which one group was treated with Ex-4 at 0.1  $\mu\text{g}/\text{kg}$  of bw, which corresponds to the clinical dose that is used in Type 2 diabetic patients. Since Ex-4 is a marketed drug for the treatment of T2D, our rationale was to mimic the clinical setting of Type 2 diabetic patients on Ex-4 treatment, where the peripheral and chronic administration of Ex-4 will likely occur both before and after stroke. Therefore, we have treated GK rats in a similar manner and administered clinically relevant doses of Ex-4 for several weeks both before and after stroke. We demonstrate here, for the first time, anti-stroke efficacy of Ex-4 in a preclinical setting that mimics the clinical situation.

When we evaluated the tissue damage by measuring the infarct volume, only the highest dose of Ex-4 (5  $\mu\text{g}/\text{kg}$  of bw) showed a statistically significant effect in comparison with the control group, whereas the two lower Ex-4 doses (0.1 and 1  $\mu\text{g}/\text{kg}$  of bw) produced a noticeable but not statistically significant trend towards reduction of the infarct volume. Closer examination revealed 'thinning' of

neuronal density and 'patchy' cell loss in these animals as opposed to the more severe cell loss in the control group. The quantification of surviving NeuN-positive neurons using the optical fractionator method revealed that when counts from striatum and cortex were combined, the two lower doses of Ex-4 significantly increased the neuronal survival (indicated by increased neuronal counts). Since infarct volume measurement does not account for neuronal loss in tissue surrounding the infarct, we suggest that this type of assessment is an accurate and precise addition for evaluating stroke damage and neuronal survival. Unlike the measurements of the infarct volume, which dramatically changes depending on the time after stroke and may lead to misinterpretation of the results [42,43], the stereological quantification of surviving neurons the using optical fractionator method provides accurate information and helps in avoiding such pitfalls.

The Ex-4-mediated efficacy was dose-dependent, with 5  $\mu\text{g}/\text{kg}$  of bw being the most effective dose in the study. Ex-4 has been reported to cross the blood-brain barrier in normal mice [15] and GLP-1R is expressed on NeuN-positive neurons.

Although the anti-stroke effect mediated by Ex-4 was found to be dose-dependent, it was not linear between the three administered doses. One possibility to explain this non-linearity is that Ex-4 at the two lower doses may act via the known GLP-1R, while the neuroprotective effect mediated by the highest dose of Ex-4 is also occurring via a GLP-1R-independent pathway recently described [44]. Linearity may also not be expected considering that the incretin effect by Ex-4 occurs on top of already existing GLP-1 levels in the blood. Finally, although Ex-4 can pass the blood-brain barrier [15], it is hard to predict what amount of Ex-4 can reach both striatum and cortex.

The neuroprotective effect of Ex-4 was stronger in the cortex than in the striatum. To investigate whether this effect is due to differences in GLP-1R expression in cortex and striatum, quantitative studies will have to be performed at earlier time points than the ones used in our study thus better targeting the neuroprotective window (hours to few days after MCAO). While the possibility that the cortical and striatal neurons react differently to Ex-4 treatment cannot be ruled out, this difference is most likely due to the characteristics of the stroke model that we have employed. In this model, ischaemic damage originates in the striatum and then spreads across the overlaying cortex depending on the duration of the MCAO. Thus, as cortex contains more ischaemic penumbral area, the neuroprotective intervention can be more effective.

Ex-4 stimulates insulin secretion [45] and a number of previous studies have reported decreased ischaemic brain injury when hyperglycaemic levels of diabetic animals were normalized using insulin [46,47]. In our study, however, the treatment with Ex-4 at 0.1 and 1  $\mu\text{g}/\text{kg}$  of

bw significantly reduced stroke injury without showing a significant impact on hyperglycaemia (Figures 2B and 2C). Thus we suggest that, in our case, the reduction in neuronal damage mediated by Ex-4 at 0.1–1  $\mu\text{g}/\text{kg}$  of bw is largely independent of glycaemic effects. Only the treatment with Ex-4 at 5  $\mu\text{g}/\text{kg}$  of bw resulted in a significant reduction in glycaemia (Figure 2D), although these animals still remained hyperglycaemic. This dose of Ex-4 was also the most effective in reducing the ischaemic brain injury, and therefore a partial contribution of the Ex-4-mediated glucose lowering to the reduction of the ischaemic damage cannot be ruled out. To entirely exclude the possibility that Ex-4 induces neuroprotection by lowering glucose, future studies will be done using blood glucose-lowering drugs, titrated to achieve the same glycaemic effects as Ex-4, in head-to-head comparisons. Interestingly, and in support of our findings, Teramoto et al. [26] recently reported that the anti-stroke efficacy by GLP-1R activation in non-diabetic mice occurred with unchanged insulin levels, suggesting that its CNS effects are not due to changes in glycaemia.

At 2 weeks after stroke, all groups showed similar numbers of Iba1-positive microglial cells in the injured brain hemisphere. However, at 4 weeks following stroke, significantly fewer Iba1-positive microglial cells were detected in the cerebral cortex of Ex-4-treated groups. The inflammation process in the brain after stroke may last for several weeks and is believed to have both beneficial and detrimental effects that largely depend on the time after the stroke onset [48,49]. As evident from Figures 5(A), 5(B), 5(E) and 5(F), the difference in Iba1-positive numbers at 4 weeks is mainly attributed to the increase of microglia infiltration from 2 weeks and onwards in the PBS group, whereas it remained largely unchanged in Ex-4-treated groups. We interpret our results as the Ex-4 treatment after 4 weeks had halted the microglia infiltration, but had not reduced its magnitude compared with the 2 weeks time point. Furthermore, since the change in microglia infiltration after Ex-4 treatment did not correlate either with the size of the ischaemic lesion or the stroke-induced stem cell proliferation and neurogenesis, it is unclear whether the modulation of microglia infiltration by Ex-4 has any significant impact on the ischaemic damage and stroke-induced neurogenesis. Although it is possible that the decreased microglia infiltration could influence neurogenesis at later time-points, new experiments using these later-time points will be needed to answer this question.

Obesity and diabetes impair adult neurogenesis and animal studies have shown decreased neurogenesis in diabetic animal models [50–54]. Stroke induces neurogenesis in both rodents and humans [33,55–57]. However, it is known that the great majority of the newborn neuroblasts induced by stroke do not reach the mature state due to poor survival [58]. Since our previous

work has shown that Ex-4 enhances neurogenesis in non-diabetic rodents [18,19], in this work we studied whether Ex-4 had the potential to enhance stroke-induced neurogenesis and/or to promote survival of the newly generated neurons in the diabetic brain. Our results show that Ex-4 treatment significantly increased stem/progenitor cell proliferation in the SVZ and neuroblast production in the striatum. However, this effect was only noticeable at 2 weeks after stroke, whereas no differences between the groups were observed after 4 weeks of Ex-4 treatment. The kinetics of stroke-induced neural stem/progenitor cell proliferation in GK rats have not been yet characterized. However, it is conceivable that at this later time point, any proliferative effect of Ex-4 treatment may be masked by the proliferative effect induced by stroke.

Although at 2 weeks after stroke, Ex-4 treatment increased stem cell and neuroblast proliferation, this treatment did not result in any increase in the generation of mature neurons. Both at 2 and 4 weeks after stroke all groups showed similar numbers of NeuN/BrdU-positive neurons, although the number of NeuN/BrdU-positive neurons at 4 weeks was 2-fold higher compared with 2 weeks in both PBS and Ex-4 treatment groups. This is most likely due to neuroblast differentiation [33]. Thus, although a greater number of proliferating cells and neuroblasts at 2 weeks after stroke in the Ex-4 treated groups did not result in an increase in neuronal differentiation, our results provide evidence that Ex-4 indeed stimulates the formation of new brain cells following stroke. Whether these Ex-4-induced proliferating cells can contribute to the reduction of the ischaemic damage remains to be investigated. However, future therapeutic strategies based on the combination of Ex-4 with neuro-surviving factors, might have the potential to increase the survival and neuronal differentiation of these newly generated cells.

In conclusion, we report here a pronounced anti-stroke, neuroprotective and anti-inflammatory effect of peripheral and chronic Ex-4 treatment in middle-aged diabetic animals in a preclinical setting that has the potential to mimic the clinical treatment conditions. Our results are strong motivating factors to further investigate GLP-1R agonists for their neuroprotective action in diabetes, as well as for their possible use as anti-stroke therapies in non-diabetic conditions.

## AUTHOR CONTRIBUTION

Vladimir Darsalia performed and designed the stroke experiments, performed part of the immunohistochemical studies, contributed to the discussion, wrote the paper, performed stereology analysis, and acquired and processed the images and Figures. Shiva Mansouri performed the immunohistochemical and screening of ischaemic

damage, and contributed to the discussion. Henrik Ortsäter provided expertise in diabetes, contributed to the discussion and performed the bioactivity studies of Ex-4. Anna Olverling performed the immunohistochemical neurogenesis experiments. Camilla Kappe performed GLP-1R immunohistochemical experiments. Kerstin Iverfeldt and Linda M. Tracy designed and performed part of the inflammation studies. Nino Nozadze performed immunohistochemical inflammation studies. Nina Grankvist performed bioactivity studies of Ex-4. Åke Sjöholm provided expertise in diabetes, the GK rats, conceived the research plan, contributed to the discussion and edited the paper prior to submission. Cesare Patrone contributed to the discussion, conceived, designed and co-ordinated the research plan, the stroke experiments and wrote/edited the paper prior to submission.

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