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SAVE THE BRAIN!

– STUDIES ON GLP-1-MEDIATED NEUROPROTECTION

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Cover Illustration by Fuad Bahram. Schematic coronal section of a brain with stroke and a GLP-1 peptide.
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Save the Brain!

– Studies on GLP-1-Mediated Neuroprotection
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my Family

ABSTRACT

Background: Patients with type 2 diabetes (T2D) suffer stroke more often and have worse recovery. Moreover, hyperglycemic stroke patients treated with thrombolytic therapy have more intracranial bleedings. Insulin is the gold standard for treatment of hyperglycemia, but has a significant risk of hypoglycemia, especially if rapid normalization of glucose is the goal. Glucagon like peptide-1 based drugs, such as receptor agonists (GLP-1RA) and dipeptidylpeptidase-4 inhibitors (DPP-4i) that raise the endogenous GLP-1 levels are used to treat T2D. Interestingly these drugs have been shown to reduce stroke incidence; and, importantly for this thesis, to exert acute neuroprotective effects in animal models if given before a stroke.
Aim: To determine if GLP-1RA and DPP-4i are neuroprotective in animal models when given after stroke and to identify some of the underlying mechanisms. Furthermore, the aim was to determine the GLP-1 levels in humans after stroke and their relationship to outcome. Finally, to test treating hyperglycemic stroke patients with GLP-1RA early after stroke: in the ambulance.
Study 1: An experimental animal study determining the effect of giving the GLP-1RA exendin-4 (Ex-4) after the onset of stroke. We did this in both young and healthy animals as well as aged and obese/diabetic animals. Experimental stroke was induced by middle cerebral artery occlusion (MCAO). The main finding was that exendin-4 is neuroprotective if given after MCAO, both in young and old animals. The effect was time-sensitive with effect at 1.5 and 3 h after MCAO, but lost at the 4.5 h time point.
Study 2: An experimental animal study where we determined if DPP-4i treatment had neuroprotective properties when started after the onset of stroke. Additionally, we studied if the effect was dependent of the GLP-1 receptor by using a GLP-1R -/- knockout model mouse. The main finding was that DPP-4 inhibitors require chronic pre-treatment to be effective. Furthermore, we showed that the effect is not dependent on the GLP-1 receptor.
Study 3: In rats with diabetes (GK-rats) or without (Wistars) we studied the impact of aging in diabetes on regulatory GABA-ergic interneurons (key cells involved in stroke recovery) and whether treatment with the GLP-1RA exendin-4 could revert the observed changes. Main findings: 1: The number of GABAeric neurons decreased in aged diabetic animals. 2: Treatment with Ex-4 had effect on the subpopulation of GABAeric neurons positive for calbindin.
Study 4: We determined the endogenous GLP-1 levels in patients treated with thrombolytic therapy for ischemic stroke. A group of 59 patients underwent a OGTT at day 2-4 during the hospital stay. A repeat OGTT was performed 3 months later. At the three-month follow-up functional stroke outcome was measured with mRS. 27 healthy controls underwent one OGTT. The main findings were that the GLP-1 level was higher in stroke patients and remained unchanged 3 months later. The GLP-1 level was not associated with functional outcome.
Study 5: A randomized clinical trial examining the feasibility of prehospital treatment with exenatide in hyperglycemic patients (8-15 mmol/L) with suspected stroke. Patients were followed for 24 h. 19 patients were randomized, 8 received exenatide. There was no evidence of a difference in the main outcome of glucose at 4 hours. No adverse events were reported.
Conclusion: Both GLP-1RA and DPP-4i are neuroprotective against stroke. The effect is, however, time sensitive with the need for early initiation after stroke (GLP-1RA) or chronic pre-stroke treatment (DPP-4i). Additionally, the effect of DPP-4i is GLP-1 receptor independent. The findings encourage the use of these drugs for the treatment of diabetes.

LIST OF SCIENTIFIC PAPERS

I. Darsalia V, Hua S, **Larsson M**, Mallard C, Nathanson D, Nystrom T, Sjoholm A, Johansson ME, Patrone C. Exendin-4 reduces ischemic brain injury in normal and aged type 2 diabetic mice and promotes microglial M2 polarization. *PloS one*. 2014;9(8):e103114

II. Darsalia V, **Larsson M**, Lietzau G, Nathanson D, Nystrom T, Klein T, Patrone C. Gliptins-mediated neuroprotection against stroke requires chronic pre-treatment and is glucagon-like peptide-1 receptor independent. *Diabetes, obesity & metabolism*. 2016;18(5):537-41

III. **Larsson M**, Lietzau G, Nathanson D, Ostenson CG, Mallard C, Johansson ME, Nystrom T, Patrone C, Darsalia V. Diabetes negatively affects cortical and striatal GABAergic neurons; an effect that is partially counteracted by Exendin-4. *Biosci Rep*. 2016;36(6).

IV. **Larsson M**, Patrone C, von Euler M, Holst JJ, Nathanson D. GLP-1 secretion in acute ischemic stroke: association with functional outcome and comparison with healthy individuals. *Cardiovasc Diabetol*. 2019;18(1):91.

V. **Larsson M**, Castren M, Lindstrom V, von Euler M, Patrone C, Wahlgren N, Nathanson D. Prehospital exenatide in hyperglycemic stroke - A randomized trial. *Acta Neurol Scand*. 2019;00:1-6.

CONTENTS

LIST OF ABBREVIATIONS

AUC	Area under curve
BBB	Blood brain barrier
BMI	Body mass index
CaBP	Calcium binding protein
CB	Calbindin
Ceder-IR	Cederholm insulin resistance index
CNS	Central nervous system
CPSS	Cincinnati prehospital stroke scale
CR	Calretinin
DM	Diabetes mellitus
DMB	6,7-dichloro-2-methyl-sulfonyl-3-N-tert-butylaminoquinoxaline
DPP-4	Dipeptidyle peptidase-4
ELISA	Enzyme-linked immunosorbent assay
ESDR	End stage renal disease
Ex-4	Exendin-4, same thing as exenatide
FAST	Face arm speech time (-test)
GAD67	Glutamic acid decarboxylase-67
GIP	Gastric inhibitory peptide
GK-rat	Goto-Kakizaki rat
GLM	General linear model
GLP-1	Glucagon like peptide-1
GLP-1R	Glucagon like peptide-1 receptor
GLP-1RA	Glucagon like peptide-1 receptor agonist
HFD	High fat diet
HOMA-IR	Homeostatic model assessment insulin resistance index
HR	Hazard ratio
HRP	Horseradish peroxidase
IHC	Immunohistochemistry
IL	Interleukin
Ip	Intraperitoneal

Iv	Intravenous
IQR	Interquartile range
LPS	Lipopolysaccharide
MACE	Major adverse cardiovascular event
MCAO	Middle cerebral artery occlusion
mRS	Modified Rankin scale
NAFLD	Nonalcoholic fatty liver disease
NIHSS	National Institutes of Health Stroke Scale
NNT	Numbers needed to treat
OGTT	Oral glucose tolerance test
OR	Odds ratio
PACAP	Pituitary adenylate cyclase-activating polypeptide
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PV	Parvalbumin
rtPA	Recombinant tissue plasminogen activator
SDF1α	Stromal cell-derived factor 1α
SICH	Symptomatic intracranial hemorrhage
STZ	Streptozotocin
TNF	Tumor necrosis factor
T-PBS-	TritonX-100 phosphate buffer solution
T1D	Type 1 diabetes
T2D	Type 2 diabetes
VIP	Vasoactive intestinal peptide
WHO	World Health Organization

Molecular weight exenatide: 4186.6 g/mol
1 nmol/kg = 4.186 µg/kg
1 µg/kg = 0.239 mmol/kg

Conversion Insulin:
1 mU = 0.0347 µg
1 µg = 28.8 mU

1 INTRODUCTION

1.1 RELATIONSHIP BETWEEN DIABETES AND STROKE

1.1.1 Prevalence and risk factor

422 million people worldwide had diabetes in 2014, a prevalence of 8,5 % among the adult population (1), out of which 90-95% is type 2 diabetes (T2D) (2). The number of persons living with diabetes is also rapidly increasing, with the sharpest rise in low-income countries and the Eastern Mediterranean Region (1).

Stroke is a vascular disease affecting the brain with sudden loss of neurological function due to neuron cell asphyxia and defined by the World Health Organization (WHO) as: "rapidly developing clinical signs of focal or global disturbance of cerebral function, lasting more than 24 h or until death, with no apparent non-vascular cause" (3). This can be due to either a hemorrhage, or more commonly, approximately 85%, an occlusion in one of arteries supplying the brain tissue causing an ischemic stroke (4, 5). Stroke affects around 21 000 persons every year in Sweden (6) and it is the second most common cause of mortality in the world (7). The estimated cost per year for stroke in Sweden is 12.3 billion SEK, or an average of 600 000 SEK per individual (8).

Diabetes increases the risk for vascular disease complications such as ischemic heart disease and stroke (1, 5). Diabetes is one of the major risk factors for developing ischemic stroke, along with old age, high blood pressure, smoking and atrial fibrillation (5). Patients with T2D have a 2-fold increased risk of having an ischemic stroke, with a favorable time trend over the last decades with the risk coming down from 6-8-fold increase (9-13). Patients with diabetes have higer mortality and recurrence risk (13, 14). Moreover, T2D patients are, in general, affected by more severe neurological impairment and have a worse recovery compared to non-diabetic subjects (15-17). Patients with diabetes not only have less chance of a favorable outcome, but are also at an increased risk of progressive deterioration after a stroke. A recent Swedish cohort study showed that persons with diabetes had a 50 % increased risk of continuing loss of independency in activities of daily living (ADL) between 3 and 12 months after a stroke (18).

The prevalence of diabetes among stroke patients is high. Approximately 20 % have diabetes known prior to the stroke and an additional 16-24 % have previously undiagnosed diabetes (16, 19). The prevalence of abnormal glucose metabolism, not meeting formal criteria for diabetes, is even higher with only 42 % of patients having a normal glucose challenge when tested three months after the stroke (19). In Sweden the diabetes prevalence among stroke patients, according to the national stroke registry (Riksstroke) is 18% for Transitory Ischemic Attack patients and 23 % for stroke patients (6).

This thesis will focus on ischemic stroke and T2D, which is the predominant form of diabetes worldwide. In Sweden approximately 90% of all persons living with diabetes are diagnosed

with T2D according to the Swedish national diabetes registry (20). Herein "diabetes" refers to T2D, unless otherwise specified.

1.1.2 Hyperglycemia, stroke and outcome

As stated above, T2D patients not only suffer strokes more often, they also recover to a lesser extent (15-17, 21). The presence of hyperglycemia at admission for stroke is common (16, 22), and having hyperglycemia on arrival at hospital with an ischemic stroke is an established risk factor for poor outcome, measured e.g. as relief of symptoms, recovery to independent life or even death (14, 23-28). Notably, hyperglycemia appears to be a stronger risk factor for poor outcome in patients not previously diagnosed with diabetes (26, 29).

To date the mechanisms behind the detrimental effects of hyperglycemia on the brain are still unclear. However, several hypotheses are suggested: activation of the hypothalamus-pituitary-adrenal axis (HPA-axis), intracellular acidosis, extracellular glutamate accumulation, worsened brain edema, blood brain barrier (BBB) disruption or hemorrhagic transformation of the infarcted area (21, 30). Some authors have reported a correlation between the level of glucose on admission and the size of infarction (28). Others found no correlation between the perfusion deficit and admission hyperglycemia, while at the same time finding a 2.6-fold risk increase for poor functional outcome 6 months after the stroke (27). In an older study, Toni *et al*. reported a higher risk of death for patients with diabetes but no correlation between admission hyperglycemia and stroke size (31).

Interestingly, electrophysiological disturbances in the contralateral brain hemisphere after stroke has been found in patients with T2D compared with non-diabetic patients (32). This could provide some insight into why patient with T2D recover to a lesser extent.

1.1.3 Diabetes in experimental stroke models

In an experimental study in rats, both diabetic (GK – Goto-Kakizaki a lean T2D model (33)) and normal Wistar rats, Li *et al*. showed that the diabetic rats, despite smaller initial cerebral infarcts, had more edema and hemorrhagic transformation and also performed worse on motor function tests (34). Stroke phenotypes in diabetic animals have been studied in other models of diabetes as well. Additional T2D models include the db/db and ob/ob genetic models involving leptin and resulting in obese, hyperinsulinemic and hyperglycemic animals. In these animal models of T2D, diabetic animals have more extensive strokes (35-37), delayed and changed inflammatory response (38) and shift the inflammatory phenotype towards the pro-inflammatory pathway M1 (39). Furthermore, diabetic animals have more infiltration of neutrophils in the infarcted area and have increased blood brain barrier (BBB) permeability (37). And as for humans, diabetic mice (db/db and db/+) have reduced long-term functional outcome after stroke (39). Increased stroke and BBB breakdown have also been reported by Haley and Lawrence (40) in ob/ob mice. In a rat model with hyperinsulinemic diabetes, Zhang *et al*. found that the stroke volume was unchanged but the rats still had more pronounced deficits in sensorimotor and cognitive function after

experimental stroke (41). In addition, among the rats not subjected to stroke, they found cognitive impairment in the diabetic animals compared with the non-diabetic animals. Diabetes was induced at 13 months of age and this difference was seen after two months of diabetic condition. This lack correlation between stroke size and degree of deficit points towards some kind of underlying disturbance.

Disturbances in brain function after stroke and reduced functional recovery have also been shown in mice with a model of type 1 diabetes (T1D), streptozotocin (STZ) induced diabetes (42). In this study they also found that the animals with diabetes displayed changed activation pattern not only in the primary somatosensory cortex area subjected to experimental stroke, but also in the secondary somatosensory cortex. The study was performed in 3 months old mice that had been diabetic for 1 month before stroke. These findings in a T1D support the generalizability of the negative impact on stroke from diabetes; negative effects seen in other studies comparing for instance GK rats with Wistar rats are not merely strain differences.

1.1.4 Inflammation in the brain after stroke

In the early phase of stroke, circulating inflammatory cells such as lymphocytes and macrophages adhere to the damaged endothelium and migrate into the brain trough the damaged BBB. Several inflammatory cytokines, such as interleukin (IL)-1β, IL-6 and tumor necrosis factor-α (TNF-α) are profoundly elevated in stroke (43-45).

Inflammation is one of the key players in post stroke brain injury (46-49). The ischemic occlusion/reperfusion injury of stroke is a strong pro-inflammatory trigger. Microglia, the macrophage of the central nervous system, is central in the inflammatory response in the brain. This cell type can respond to inflammatory stimulus with different phenotypes, M1 and M2 (50). M1 is a classic pro-inflammatory pathway and M2 is more reparative. Early after stroke the response is mainly of the M2 type but after about a week gradually fades over into pro-inflammatory M1 response (51). Since the M1 response has been shown to be neurotoxic and the M2 response to promote regeneration (52), a prolongation of the M2 response has the potential to be beneficial to an injured brain.

1.1.5 Interneurons

Diabetic individuals are at increased risk from neurological impairment, with stroke being the most common complication in the central nervous system (CNS) (11, 12). But other CNS disorders are also more prevalent in diabetes, such as cognitive impairment and Alzheimer's disease (53-57). Despite an established association between T2D and cognitive impairment, the nature of the impairment at cellular level is far from being well understood. A lot of the attention has been focused on hippocampus due to its importance in memory functions. However, patients with diabetes show many types of sensorimotor problems even without Alzheimer's disease (53). An additional example is that patients with Parkinson's disease and diabetes show a more rapid decline in motor dysfunction when compared with non-diabetic individuals (58). This demonstrates that other areas than hippocampus are likely involved.

1.1.5.1 GABA-ergic neurons

Approximately 5-10 % of the neuron population of the neocortex and the deep brain structure striatum, which is part of the basal ganglia, consists of a set of neurons referred to as interneurons. They are γ-aminobuturic acid (GABA)-signaling and have important modulatory and controlling functions on other neurons (59, 60). Glutamic acid decarboxylase (GAD) is the enzyme responsible for synthesizing GABA and it exists in two isoforms (GAD65 and GAD67). Both are present in the brain, but it is the GAD67 that is mainly expressed in neuronal cell bodies (61). In a rat model study where diabetes was induced by streptozotocin injection (model for T1D), GAD67 was reduced in the brain and was coupled to depressive behavioral change (62). If T2D induces the same changes in GAD67 levels was before our study unknown.

A subset of the GABA-ergic interneurons is characterized by the expression of calcium-binding proteins, calbindin-D 28kD (CB), calretinin (CR) and parvalbumin (PV) (63). In fact, already in 1992 the importance of the calcium binding proteins was suggested in controlling and maintaining healthy CNS (64). They have the capacity to bind and buffer Ca^{2+}, thereby having a protective capacity in Ca^{2+} overload, such as ischemic stroke (64-66). Up-regulation of CB expression in animal models with experimental stroke has been show to be neuroprotective (67). Perhaps through buffering of the excess intracellular Ca^{2+}-levels present after a stroke (68). PV has been shown to play a crucial role in the development of cognitive

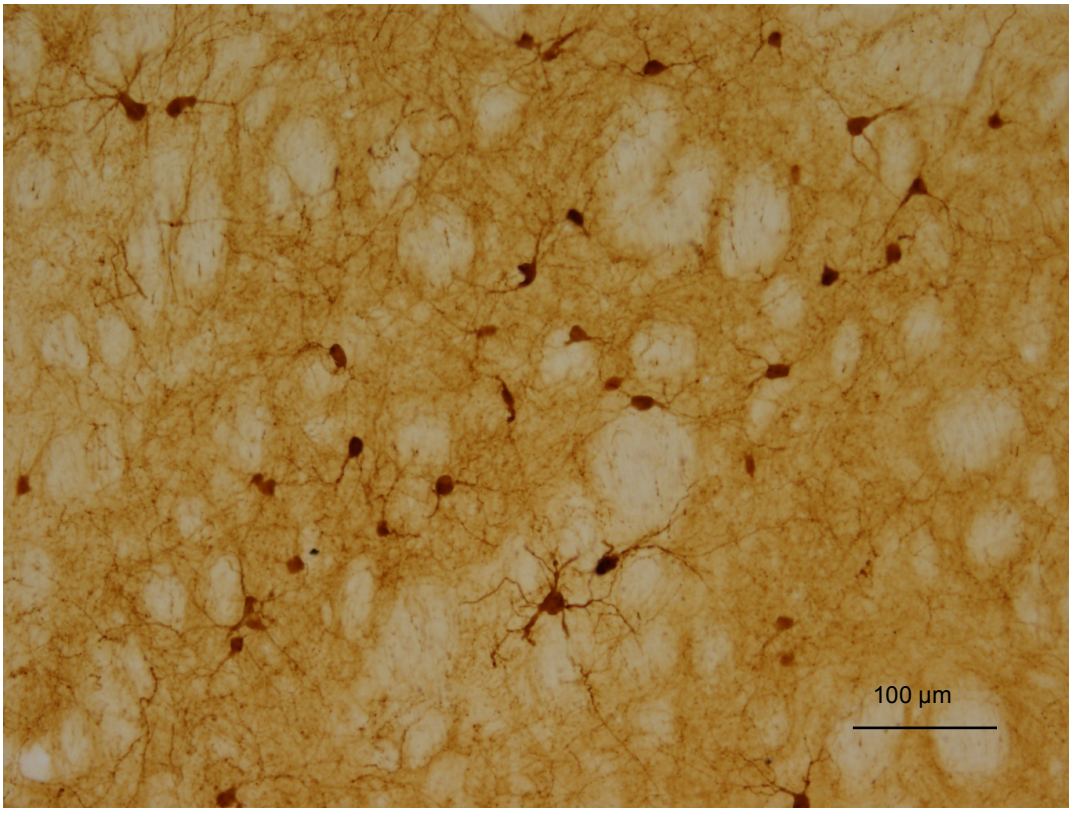

Figure 1. Parvalbumin positive cells in the striatum of 13 months old Wistar rat. Photo: Martin Larsson.

impairment by disturbances in the oscillatory controlling rhythms (see below under energy hypothesis) through which interneurons exert control of the cortico-striatal principal neurons (69). CB has also been demonstrated to play a crucial role in development of Alzheimer's disease (70). Lietzau *et al*. showed in 2016 that T2D negatively impacts the expression of CB in the piriform cortex, a structure involved in olfactory function, and that treatment with the GLP-1R agonist Ex-4 for 6 weeks dramatically increases CB expression (71).

CB positive staining in the striatum also exists in different forms of neurons. The majority not being interneurons, but rather medium size spiny neurons (72) that project out towards *substantia nigra pars reticulata* (73) and participate in the regulation of the dopaminergic motor function control. This could be one of the ways that T2D impacts negatively on Parkinson's disease (58) and could also influence motor control after ischemic injury to the brain. Exenatide treatment has been tested in Parkinson's disease and found to exert beneficial effects on this neurodegenerative disease (74, 75).

Interneurons have, furthermore, been described to have important impact on higher cerebral functions such as behavior, learning, memory and decisions making which has been reviewed by Pennartz *et al*. (76). Interneurons are a part of the striatal activity involving these higher cerebral functions, such as learning habits, acquiring motor skills and as modulator of motor function (77). Good function of these cerebral functions is crucial to achieve good recovery after brain injury, such as a stroke.

1.1.5.2 Energy hypothesis of interneuron vulnerability

Complex neuronal functions depend on precise spatial and temporal coordination of the neurons in cortical networks (78). This coordination is provided by network oscillations that enable synchronization of the neural function (79, 80). The cortical network oscillations depend on the inhibitory fast-spiking PV positive interneurons to produce gamma oscillations (30-100 Hz) (81-83). These fast spiking PV positive interneurons have, compared to other interneurons and pyramidal cells, been shown to have higher numbers of mitochondria and more cytochrome c (a crucial protein in the respiratory chain of the mitochondria), which both are signs of high energy expenditure (84). The gamma oscillations of the PV interneurons provide a fundament for information processing in the brain, and their high energy consumption makes them vulnerable to effects of metabolic stress (85).

The vulnerability of the PV+ interneuron induced gamma oscillations in models of stroke has been demonstrated. Hippocampal ischemia by experimental stroke disrupts the gamma oscillations (86). In an experiment with transient global cerebral ischemia (5 min) GABA-ergic network activity was show to decrease in the somatosensory cortex in spite of recovered excitability of the neurons (87).

1.1.5.3 Interneurons Diabetes and Stroke

Disruptions of the gamma oscillations, network activity or Ca^{2+}-buffering capacities of the interneurons provide one potential mechanism for T2D induced vulnerability to stressors

such as stroke and could be part of the explanation for early cognitive decline. Studies addressing and determining the effect diabetes has on interneurons are warranted to understand how diabetes affects stroke.

1.2 GLP-1

Glucagon like peptide-1 (GLP-1) is a peptide hormone that is part of the gut hormones potentiating the insulin response to a meal: incretins. The incretin effect refers to the finding by Nauck *et al*. that glucose given orally results in a higher insulin response compared to glucose given intravenously at an equal rate (88-90). The incretin effect constitutes approximately 50 % of the total insulin response to an oral intake (90). GLP-1 is a 30 amino acid long peptide synthesized and secreted from endocrine epithelial L-cells in the intestines in response to a meal (91-93).

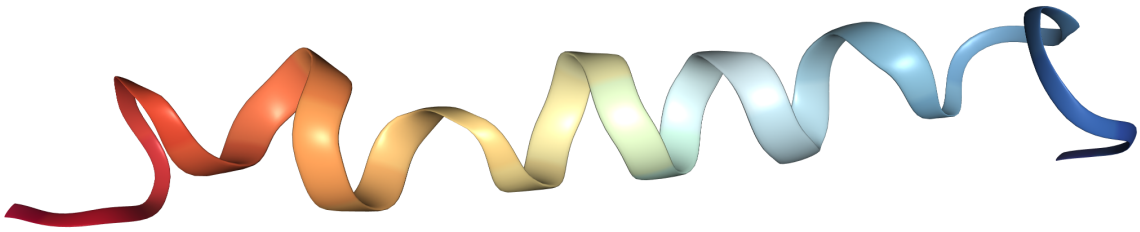

Figure 2. 3D-structure of GLP-1-(7-36)-amide. Image from the Research Collaboratory for Structural Bioinformatics Protein Data Base (rcsb.org) of PDB ID 1D0R.
DOI: 10.2210/pdb1D0R/pdb.

1.2.1 The anti-hyperglycemic action of GLP-1

GLP-1 stimulates insulin release by binding to the GLP-1 receptor (GLP-1R) on pancreatic β-cells. The GLP-1R is a transmembrane-spanning G-protein coupled receptor. This stimulates formation of cyclic AMP (cAMP) and downstream protein kinase A (PKA) that in turn leads to rising intracellular calcium levels and exocytosis of insulin (94, 95). For this to occur, concomitant hyperglycemia must also be present (96-98). Thus, the glucose lowering effect of GLP-1 disappears when euglycemia is reached. The binding of the GLP-1 on the pancreatic GLP-1R also results in increased gene transcription and insulin production, β-cell proliferation and reduced apoptosis (94, 99). The islet δ-cells in the pancreas also express GLP-1R and its activation results in increased somatostatin release (99, 100). Additionally, GLP-1 rapidly suppresses glucagon release from the islet α-cells which is of great benefit as glucagon release is often paradoxically increased in T2D (101). The suppression of glucagon release is likely through indirect mechanism of inhibitory signals from the β-cells and through somatostatin release from the δ-cells (93). Furthermore, GLP-1 receptor agonists (GLP-1RA)

have been shown to effectively suppress glucagon secretion in C-peptide negative T1D (102). This, in turn, indicates that β-cells are not necessary for the effect on glucagon suppression.

The combined action of insulin release and glucagon suppression results in an antihyperglycemic effect (103).

The GLP-1 release in T2D in response to an oral load of glucose in relation to i.v. glucose is less pronounced when compared to normal subjects (104). The loss of the incretin effect has been speculated to be an important part of the pathophysiology behind the development of T2D (105).

1.3 GLP-1R AGONISTS AND DPP-4 INHIBITORS

1.3.1.1 GLP-1R agonists

Degradation of GLP-1 by DPP4

Endogenous GLP-1 secreted by the L-cells is rapidly degraded (5 minutes) by the enzyme dipeptidyl-peptidase 4 (DPP-4) by means of proteolysis (106). The intact form of GLP-1 amide (7-36) is degraded into a largely inactive form (9-36). DPP-4 exists both circulating in the plasma and on the endothelium (107). GLP-1 is only degraded by the DPP-4 but several other substrates for DPP-4 exist, e.g. gastric inhibitory peptide (GIP) and stromal cell-derived factor 1α (SDF1α) (107, 108). The short half-life of native GLP-1 makes it unsuitable for use as an anti-diabetic drug.

Degradation resistant GLP-1 analogues

In the saliva from the Gila monster lizard (*Heloderma suspectum*) a peptide with 53 % amino acid homology with human GLP-1 has been found (109). This peptide, named exendin-4 (Ex-4), is a potent agonist to the GLP-1R (109). The altered amino-acid sequence makes Ex-4 resistant to degradation by DPP-4 and the half-life of Ex-4 is markedly prolonged in comparison with endogenous GLP-1, shown both in rat (110) and human (111). Synthetic Ex-4 is named exenatide and it is the first GLP-1R agonist available as a drug for anti-diabetic treatment. After a subcutaneous (s.c.) injection, the maximum concentration of around 60 pmol/L of exenatide, is reached after approximately 2 h and its half-life is approximately 3.5 h (111). The maximum concentration is about three times higher than the fasting concentration of total GLP-1 or 30-60 times higher than the fasting active GLP-1 amide (7-36). Exenatide was first introduced in the U.S. where it was approved in 2005.

GLP-1R antagonist

Ex-4 also exists in a truncated form (9-39). This works as a potent GLP-1R antagonist and co-administration of Ex-4 and the truncated Ex-4 (9-39) cancels out the Ex-4 effect (109). This is of interest in research studies.

GLP-1 receptor agonists for clinical use

Since the introduction of exenatide, several other GLP-1RA have reached the market with different mechanisms for enabling prolonged actions. Liraglutide and semaglutide are GLP-1RA with a much greater amino-acid sequence homology with endogenous human GLP-1 (97 % for liraglutide and 94 % for semaglutide). They have a free fatty acid attached to the peptide allowing binding to albumin, which thereby serves as a circulation depot and prolongs the half life to 12-14 hours and 1 week, respectively (92, 112). This project uses the first GLP-1RA to be introduced on the market, exenatide, for GLP-1R activation. This and other GLP-1RA on the market are reviewed in the section 1.4.2 Clinical trials of GLP-1RA and DPP-4i.

1.3.1.2 DPP-4 Inhibitors

Another approach to enhance the GLP-1R activation is inhibition of the DPP-4 degradation of GLP-1. Several drugs working through this mechanism are now available for clinical use. Inhibition of DPP-4 results in a 2-3-fold increase in GLP-1 levels in plasma after meal (113). In a study by Ahren *et al.* (113), a 4-week treatment with the DPP-4 inhibitor (DPP-4i) vildagliptin resulted in lower post-prandial glucose levels without a concomitant change in insulin levels pointing to an insulinotropic effect with enhanced release in relation to the glucose level. Vildagliptin also reduced the glucagon levels. These effects are all in line with GLP-1 action. In this thesis, the DPP-4i linagliptin was used. It has been associated with neuroprotection (114, 115), which is further described below under the section 1.7 DPP-4 inhibition, Neuroprotection and Ischemic Stroke.

1.3.2 GLP-1 effects beyond the anti-hyperglycemic action

Receptors for GLP-1 are widespread in the body and exist not only in the primarily glucose controlling organs such as pancreas and liver. Apart from the pancreatic β-cells and δ-cells, high levels have been found on endothelial cells, myocardial cells, the gastrointestinal tract and the kidney (94). GLP-1 receptors have also been found in the brain, both on neurons (116) and on microglia (117). GLP-1 is in fact also produced in the caudal brainstem (118). These non-glucose functions are reviewed by Drucker (94). The cerebral effects and neuroprotective properties of GLP-1 are expanded on below in a separate paragraph, 1.6 GLP-1, Neuroprotection and Ischemic Stroke.

GLP-1R are present on both endothelium and vascular and myocardial myocytes (119). In a mouse model Ban *et al*. showed that administration of GLP-1 increased glucose uptake, cAMP release, left ventricular pressure and coronary artery blood flow in isolated hearts (119). Ban *et al*. also show that GLP-1 has effects not mediated directly through the GLP-1R. Both animal (120, 121) and clinical (122-124) studies have shown that GLP-1R activation results in protection from cardiac ischemia, but the exact mechanisms are still unclear. For example in mouse knockout models (GLP-1R -/-, globally or selective in cardiomyocytes (CM -/-)). Complete loss of GLP-1R (GLP-1R -/-) had no impact on the outcome of experimental myocardial infarcts, but administration of Ex-4 no longer provided positive

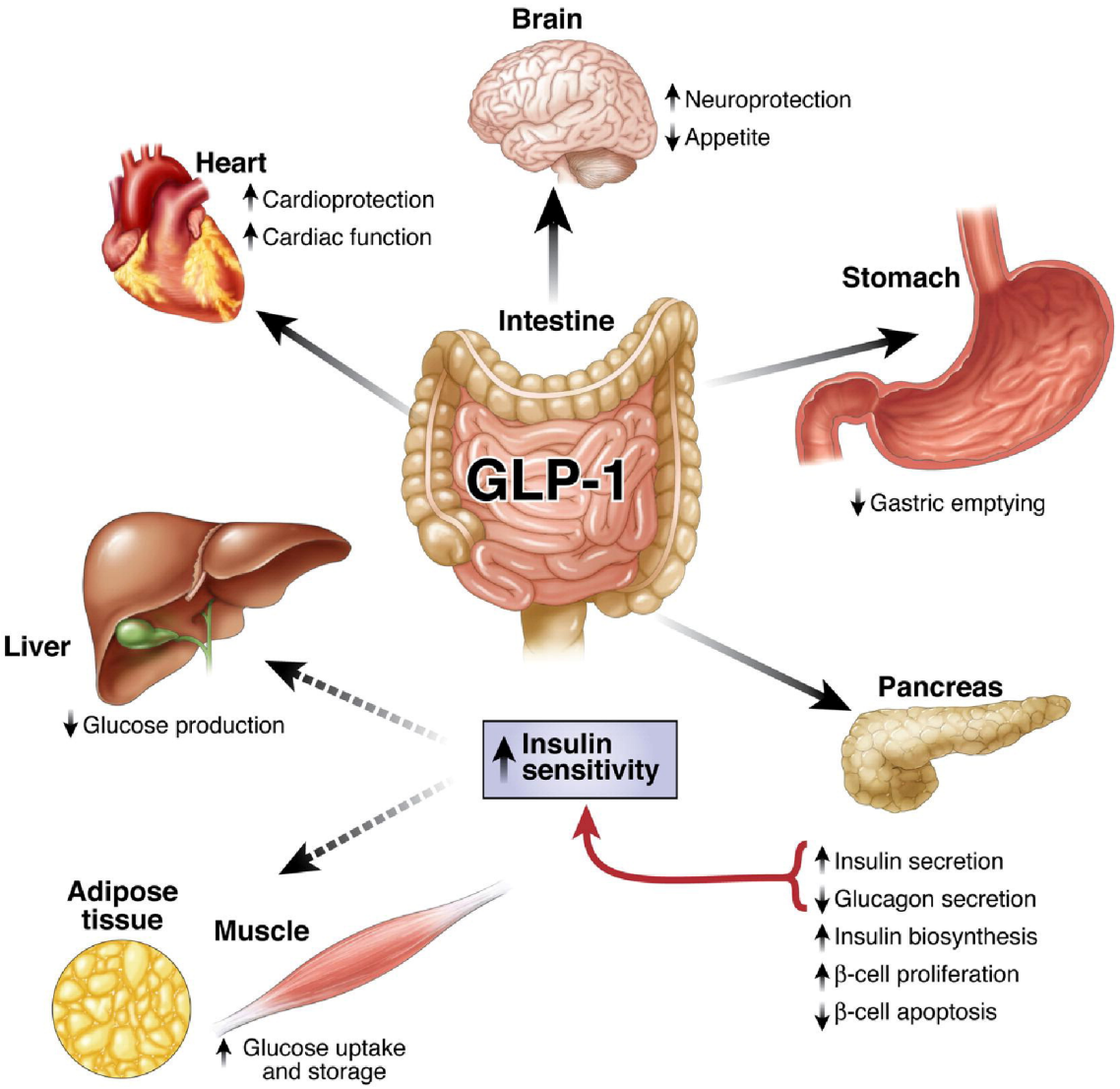

Figure 3. GLP-1 actions in peripheral tissues. ©Elsevier, reproduced with permission from DOI: 10.1053/j.gastro.2007.03.054.

effect on cardiac outcome as seen in clinical studies (122-124). Furthermore, and surprisingly, use of a different GLP-1R agonist, liraglutide, in animals that lacked the GLP-1R in cardiomyocytes (GLP-1R CM -/-) still resulted in cardioprotection. This indicates that the GLP-1R in the CM is not essential for cardiac protection (121). The field has been reviewed by Ussher (125). In diabetic patients with congestive heart failure the GLP-1R agonist exenatide has been tried for acute treatment of congestive heart failure. The patients' cardiac index (cardiac output divided with body surface) increased as a result of a positive chronotropic effect (126). Exenatide has also been shown to increase the myocardial salvage index in acute coronary syndromes (122).

It has been increasingly established, and received more attention, that inflammation has an important role in the development of cardiovascular disease (127). Inflammation is in fact one key element in understanding post stroke brain damage (46-49). GLP-1 based treatments

have been shown to reduce inflammation in vascular disease as well as neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease (118, 128-130).

Increased satiety and reduced spontaneous caloric intake by infusion of GLP-1 has been shown in humans, first described by Flint in 1998 (131). GLP-1 slow down gastric emptying (132), which could explain the increased satiety. Additionally it could be a partial explanation of the effects on glucose, with delayed uptake from the intestines as a result of slowed gastric emptying. It could also be one of the mechanisms underlying the nausea reported by approximately 25 % of patients on GLP-1RA as treatment for diabetes (133-135). The prevalence of nausea and vomiting appears to be dose-dependent (135). The reduced gastric emptying is believed to be via effect on vagus nerve (136) and has been shown to be mediated by cholinergic mechanisms (137). There is, however, also evidence of a direct cerebral mediation of the nausea effect (138).

1.4 CLINICAL HUMAN STUDIES

Several clinical studies throughout the years have examined prevention of cardiovascular disease in diabetes. The landmark studies in this category of trials are summarized in Table 1. The main outcome is listed, together with stroke specific outcome. Many of these trials showed cardiovascular benefit from the various intensified treatment strategies. They failed, however, to show stroke specific benefit with the exception STENO-2 (multifactorial intervention).

For the GLP-1RA, on the other hand, there is evidence that such treatment can prevent stroke, both in epidemiologic research and clinical trials.

Study (Ref)	Year	Study Size	Intervention	Follow up (years)	Primary outcome	Primary outcome HR (95 % CI)	Stroke outcome	Stroke specific outcome HR/RR (95 % CI)
UKPDS (139)	1998	3 867	Intensive treatment	10	Comb. of macro and micro	0.88 (0.79-0.99) (microvasc reduction, macro neutral)	Stroke	1.11 (0.81-1.51)
ADVANCE (140)	2008	11 140	Intensive glucose control	5.0	Comb. of macro and micro	0.90 (0.82-0.98)	Non-fatal stroke	1.02 (0.85-1.24)
ACCORD (141)	2008	10 251	Intensive treatment	3.5	MACE	0.90 (0.78-1.04)	Nonfatal stroke	1.06 (0.75-1.50)
STENO-2 (142)	2008	160	Multifactorial intervention	13.1	Death	0.54 (0.32-0.89)	Stroke	RR 0.2 (CI not reported)
VADT (143)	2009	1 791	Intensive treatment	5.6	MACE	0.88 (0.74-1.05)	Stroke	0.78 (0.48-1.28)
ORIGIN (144)	2012	12 537	Basal insulin	6.2	MACE	1.02 (0.94-1.11)	Stroke	1.03 (0.89-1.21)

Table 1. Landmark studies of cardiovascular prevention in type 2 diabetes with stroke specific outcome.

1.4.1 Epidemiologic GLP-1RA and DPP-4i studies

Epidemiologic studies support stroke-preventing effects of GLP-1RA treatment in patients with T2D. The first epidemiologic study was published in 2011 (145) and has been supported by a larger retrospective observational study (146). These two studies show large risk reduction of cardiovascular events with approximately 20 % and 50 % respectively (145, 146). In the first study, by Best *et al.*, only a combined outcome measure of cardiovascular events was reported as outcome. But in the second, more specific outcome data was reported for heart failure, myocardial infarction and stroke individually. In the second study, by Paul *et al.*, exenatide treated patients were compared with T2D patients not on exenatide. Data was reported for patients in three groups: exenatide without insulin, exenatide with insulin and insulin without exenatide. Stroke was reported separately with comparisons between patients treated with exenatide *vs*. patients treated with exenatide + insulin (HR 0.50; 95 % CI 0.28-0.84) and exenatide + insulin *vs*. insulin (HR 0.38; 95 % CI 0.27-0.54) (146). Oral anti diabetic agents were used in both groups. The results were unchanged even if patients with previous cardiovascular disease were excluded. In this type of retrospective cohort study there is a risk of confounding by indication, i.e. that there is a reason that determined if a patient received exenatide or not which in turn is the real reason behind the observed differences. One such confounder could be that younger patients were more likely to get the new treatment (exenatide). In fact exenatide treated patients were younger. They tried to adjust for this and several other potential confounders, and also used the statistical method called propensity score matching to further try to adjust for this. The risk of confounding must, however, be kept in mind when interpreting these studies.

In an epidemiologic study on the use of DPP-4i in T2D patients with end stage renal disease (ESRD) on dialysis treatment Chan *et al*. found a very large risk reduction for all cause mortality (HR 0.43; 95 % CI 0.39-0.47) and also marked reduction in stroke incidence (HR 0.77; 95 % CI 0.61-0.97) (147). Propensity score was used for matching and mean follow up time was 1.8 years, which is in line with the follow up time in the large clinical studies SAVOR, TECOS and EXAMINE. This study was, in contrast with the clinical RCTs, performed in patients with ESRD and the rate of events was high. The most common DPP-4 inhibitor in the study was sitagliptin.

1.4.2 Clinical trials of GLP-1RA and DPP-4i

Several clinical studies of both GLP-1R agonists and DPP-4 inhibitors have recently been published and we now have quite a substantial body of evidence showing cardiovascular safety from these drugs and in some cases also good protection against cardiovascular disease (CVD) including stroke. Two trials have, apart from overall cardiovascular benefit, also demonstrated stroke specific benefit, SUSTAIN-6 (148) and REWIND (149).

For the DPP-4 class several large clinical randomized cardiovascular safety trials have been finalized in the last couple of years. These include the DPP-4 inhibitors saxagliptin (SAVOR) (150), sitagliptin (TECOS) (151), alogliptin (EXAMINE) (152), linagliptin (CARMELINA)

(153) and CAROLINA (154). These large studies were primarily constructed as cardiovascular safety studies with placebo comparator. They all, in short, show cardiovascular safety, but there is no positive protective effect in terms of cardiovascular outcome. Worth mentioning is also that in the SAVOR study there was an increase in hospitalization for heart failure that did not occur in the other trials. No reduction in stroke incidence was seen in these studies. At the same time it must be kept in mind that it is not the same thing to influence the occurrence of a cardiovascular event such as a stroke and to modulate and potentially mitigate the negative outcome of a stroke (i.e. to be neuroprotective in the context of a stroke).

Linagliptin was compared with an active comparator (glimepiride) in the longest existing clinical trial of a DPP-4 inhibitor, the CAROLINA trial (154). It randomized 6 033 patients with T2D and met the primary endpoint of non-inferiority of linagliptin compared to glimepiride, but not superiority. It was, however, safe from a cardiovascular perspective.

For GLP-1R agonists, six major clinical trials have been published: lixisenatide (ELIXA) (155), liraglutide (LEADER) (156), semaglutide (SUSTAIN-6) (148), albiglutide (HARMONY) (157), a once-weekly preparation of exenatide (EXSCEL) (158) and dulaglutide (REWIND) (149). None of the study protocols in these trials allowed for assessment of functional outcome in the patients that suffered ischemic stroke. Only data on time to first event, and the nature of the event, was recorded.

The results, in summary, show a preventive effect of GLP-1RA against incident stroke. Below some of the trials are elaborated on. The GLP-1RA and DPP-4 trials are summarized in Table 2, with a Forest plot visually plotting the main outcome and the stroke specific outcome of the GLP-1RA trials in Figure 4. Overall estimates in the Forest plots were calculated by combining the studies weighted with inverse-variance.

1.4.2.1 LEADER

The LEADER-study (156) was originally planned as a cardiovascular safety trial of liraglutide and the primary endpoint was non-inferiority in comparison with placebo on a composite endpoint of death from cardiovascular causes, nonfatal myocardial infarction or nonfatal stroke. A total of 9 340 patients at high risk for cardiovascular events (established cardiovascular disease or chronic heart failure) were randomized and the average follow up time was 3.8 years. HR for the primary outcome was 0.87 95 % CI 0.78-0.97. P-value for non-inferiority was <0.001 and p=0.01 for superiority.

The LEADER study is the first clinical GLP-1RA trial to show a reduction of cardiovascular events in T2D. The risk reduction for nonfatal stroke was 11 %, but not significant (HR: 0.89, 95 % CI: 0.72-1.11).

1.4.2.2 SUSTAIN-6

SUSTAIN-6 (148) was also originally designed as a non-inferiority study comparing semaglutide with placebo. Semaglutide is a GLP-1R agonist that closely resembles liraglutide (97 % homology) but has small amino acid modifications and a different fatty acid attached to it making the clinical half-life of semaglutide markedly prolonged to approximately 1 week (112). This allows for a once-weekly administration.

SUSTAIN-6 included high-risk individuals with established cardiovascular disease, chronic heart failure or chronic kidney disease. The main outcome was a three point MACE of cardiovascular death, nonfatal myocardial infarction or nonfatal stroke. There was a 26% reduction in the primary endpoint (HR 0.74, 95% CI of 0.58-0.95). P for non-inferiority was <0.001, p for superiority was 0.02.

The SUSTAIN-6 showed, similar to the LEADER-study, solid evidence for cardiovascular risk reduction. More interesting from a stroke perspective is that SUSTAIN-6 showed a pronounced stroke preventive effect. The risk reduction was 39 % for nonfatal stroke (HR 0.61, 95 % CI 0.38-0.99). This marked stroke-preventive effect of semaglutide may be indicative of a specific neuroprotective action but this remains to be elucidated. There was no data on functional outcome of the stroke.

1.4.2.3 EXSCEL

EXSCEL is a randomized controlled trial of once-weekly exenatide compared with placebo. 14 752 patients were randomized with a median follow up time of 3.2 years (158). Primary analysis was non-inferiority for major cardiovascular events, which the study achieved (p<0.001). Secondary analysis was for superiority, which they did not show. HR 0.91, (95 % CI 0.83-1.00), p=0.06. HR for fatal or non-fatal stroke was 0.85 with a 95 % CI of 0.70-1.03.

1.4.2.4 HARMONY

HARMONY is a randomized controlled trial of albiglutide given once-weekly compared to placebo (157). 9 463 patients were included and followed for a median of 1.5 years. The trial demonstrated non-inferiority with a p<0.0001 and also superiority on the primary composite endpoint of cardiovascular death or non-fatal cardiovascular events such as myocardial infarction and stroke: HR 0.78 (95 % CI 0.68-0.90). The stroke specific outcome of fatal or non-fatal stroke was not significant with a HR of 0.86 (95 % CI 0.66-1.14), p=0.30. Of note is that this overall superiority outcome was achieved after the short study period of 1.5 years. The study period was actually prolonged after the primary objective was met in order to allow for potential adverse events to surface.

1.4.2.5 REWIND

REWIND is a randomized controlled trial of once-weekly dulaglutide compared with placebo in patients with T2D above the age of 50 years (149). This study differs somewhat from the other GLP-1RA trials in that it included both patients with established cardiovascular disease

(31.5 %) and a high proportion of patients with risk factors without previous CVD (68.5 %). It was a superiority trial with the primary outcome defined as first occurrence of a MACE. HR for primary outcome was 0.88, (95 % CI 0.79-0.99), p=0.026. All components of the combined outcome measure (MACE) showed consistent results and the stroke specific outcome of non-fatal stroke showed a HR 0.76 (95 % CI 0.61-0.95), p=0.017. No data was presented on functional outcome stroke.

	Study	Drug	Author/ Year	Study Size	Avg. follow up (years)	Primary outcome	Primary outcome HR (95 % CI)	Stroke specific outcome HR (95 % CI)
GLP-1	REWIND (149)	Dulaglutide	Gerstein, 2019	9 901	5.4	MACE	HR 0.88 (0.79-0.99)	0.76 (0.61-0.95)
	HARMONY (157)	Albiglutide	Hernandez, 2018	9 463	1.5	MACE	HR 0.78 (0.68-0.90)	HR 0.86 (0.66-1.14)
	EXSCEL (158)	Exenatide	Holman, 2017	14 752	3.2	MACE	HR 0.91 (0.83-1.00)	HR 0.85 (0.70-1.03)
	LEADER (156)	Liraglutide	Marso, 2016	9 340	3.8	MACE	HR 0.87 (0.78-0.97)	HR 0.86 (0.71-1.06)
	SUSTAIN-6 (148)	Semaglutide	Marso, 2016	3 297	2.1	MACE	HR 0.74 (0.58-0.96)	HR 0.61 (0.38-0.99)
	ELIXA (155)	Lixisenatide	Pfeffer, 2015	6 068	2.1	MACE	HR 1.02 (0.89-1.17)	HR 1.12 (0.79-1.58)
DPP-4	CAROLINA (154)	Linagliptin	Rosenstock, 2019	6 042	6.3	MACE	HR 0.98 (0.84-1.14)	HR 0.86 (0.66-1.12)
	CARMELINA (153)	Linagliptin	Rosenstock, 2019	6 979	2.2	MACE	HR 1.02 (0.89-1.17)	HR 0.91 (0.67-1.23)
	SAVOR (150)	Saxagliptin	Scirica, 2013	16 492	2.1	MACE	HR 1.00 (0.89-1.12)	HR 1.11 (0.88-1.39)
	TECOS (151)	Sitagliptin	Green, 2015	14 671	3.0	MACE (4-point)	HR 0.98 (0.89-1.08)	HR 0.97 (0.79-1.19)
	EXAMINE (152)	Alogliptin	White, 2013	5 380	3.3	MACE	HR 0.96 (≤1.16*)	HR 0.91 (0.55-1.50)

Table 2. Clinical trials investigating cardiovascular effects of GLP-1 RA and DPP-4i treatment on patients with T2D. *Primary outcome used one-sided CI, p<0.001 for noninferiority, p=0.32 for superiority. MACE: major adverse cardiovascular event, HR: Hazard ratio, CI: confidence interval.

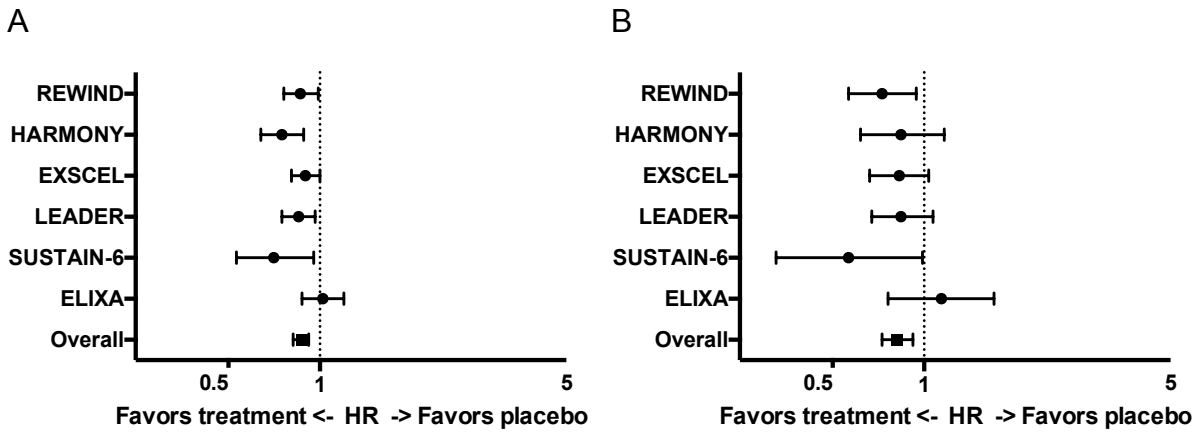

Figure 4. Forest plots of main outcome (A) and stroke specific outcome (B) in GLP-1RA cardiovascular outcome trials. Bars indicate 95 % CI.

1.5 TREATMENTS IN STROKE AND DIABETES

To *prevent* the occurrence of cardiovascular events and death is not the same thing as *improving outcome* after an event. Diabetes affects the outcome after stroke and influence the effect treatments, such as rtPA, have.

1.5.1 Treatment with rtPA in the light of hyperglycemia and outcome

During the last decades intravenous treatment with recombinant tissue plasminogen activator (rtPA) has been established as standard treatment for patients with ischemic stroke as a means to dissolve the formed thrombus causing the tissue asphyxia. The treatment must be started within 4.5 h of stroke onset with a number needed to treat (NNT) of 4.5 if given at 1.5 h post onset and a NNT of 14 when given at 4.5 h post onset in order to achieve an excellent functional outcome defined as modified Rankin Scale (mRS) 0-1 (159). The use of rtPA is, however, associated with a significant risk of symptomatic intracranial hemorrhage (SICH); approximately 1-10 %, dependent on a number of risk factors including time from onset to treatment and also on the definition used (159-163). The risk is increased when hyperglycemia is present, regardless of diabetes or not (163-165).

There is, furthermore, evidence that hyperglycemia in the acute phase is detrimental, regardless of rtPA-therapy but particularly so if reperfusion with rtPA is achieved (166, 167). Using diffusion-weighted imaging Ribo *et al*. found that the ischemic lesion growth was 2.7 times faster in the presence of hyperglycemia during occlusion (166). Hyperglycemia has even been indicated to counteract the beneficial effects of reperfusion (167). In a study by Poppe *et al*. on stroke patients treated with rtPA, admission hyperglycemia was associated with an increase in the relative risk of death within 90 days (RR 1.5 [95 % CI 1.2-1.9]), SICH (RR 1.69 [95 % CI 0.95-3.0]) and decreased chance of favorable outcome (RR 0.7 [95 % CI 0.5-0.9]) (165). The risks for death, SICH and unfavorable outcome were incremental with increasing admission glucose, even within the non-diabetic group.

A study examining more than 16 000 individuals treated with rtPA for ischemic stroke included in the global Safe Implementation of Treatments in Stroke International Stroke Thrombolysis Register (SITS-ISTR) [www.sitsinternational.org] showed a strong association with death, SICH and unfavorable outcome with increasing hyperglycemia (164). The association was of dose-response type between risk and the level of hyperglycemia. The association was stronger in patients without previously diagnosed diabetes. In a study by Mazya from the SITS-ISTR, a clinical scoring system was developed to predict the risk of SICH; the odds ratio (OR) for SICH in hyperglycemia (defined as >10 mmol/L) was 2.1 (95 % CI 1.7-2.6, $p<0.001$) (163). Notably, in this clinical scoring model, the only potentially modifiable risk factors in preparation for rtPA treatment were hyperglycemia and hypertension. The other risk factors were: aspirin and/or clopidogrel therapy, high National Institutes of Health Stroke Scale (NIHSS) score, old age, high weight, onset to treatment > 3h and history of hypertension.

1.5.2 Antidiabetic treatment in acute stroke

The evidence points to a detrimental effect of hyperglycemia in the acute phase of ischemic stroke and that the beneficial effect of rtPA is diminished. Thus, achieving normoglycemia in the acute phase, preferably before recanalization therapy, has potential for a positive effect for the patients with ischemic stroke. Randomized clinical trials aiming for euglycemia with intensive insulin treatment in hyperglycemic stroke patient have so far been unable to show benefit for intensive insulin treatment in the acute phase (168). Additionally, the studies have often been started at a late time point (see Table 3).

In the GIST-UK study by Gray *et al.,* where insulin-potassium-glucose infusion was used in patients with stroke, no benefit was shown despite achieving lower plasma glucose (169). The treatment was, however, on average started 13 h after stroke onset and there was sustained hypoglycemia (>30 minutes below 4.0 mmol/L) in 15 % of the patients (169). The SHINE-trial published 2019 is the most recent of these insulin trials (170). Treatment was started somewhat earlier, at 8.6 hours, but did not either show any benefit from insulin treatment. In fact the Cochrane collaboration has in a systematic review of this field of research concluded that maintaining glucose in a narrow normal range does not provide any benefit in terms of neurological outcome or survival (171).

A potential explanation for the lack of effect in these intensive insulin trials may be that they have investigated glucose lowering in stroke at a too late time point, (8.6 and 13.3 in the hours after stroke onset in the large trials) (169, 170); at which a majority of the neurons in the *penumbra* are probably highly ischemic and/or already dead.

Furthermore, in the intensive insulin treatment studies, a high frequency of hypoglycemia was reported in the insulin intervention groups. Hypoglycemia may be especially detrimental to neurons under ischemic stress. In Table 3 trials investigating intensive glucose treatment in stroke patients with time to treatment initiation and occurrence of hypoglycemia are listed. Most do not report functional outcome, except Johnson 2019 and Gray 2007, which are both neutral.

Intervention on multiple targets at the same time has also been proposed as a way of achieving transferability of neuroprotective strategies from animal models to human stroke patients (172). Such as starting a therapy early, with a glucose normalization treatment that does not carry with it risk for hypoglycemia and at the same has neuroprotective properties even in euglycemic conditions. This type of strategy could be of paramount importance for neuroprotection to be efficacious.

Reference	Patients	*N*	Intervention	Time to treatment onset (h)	Hypoglycemia cut off (mmol/L)	Hypoglycemia Proportion
Bruno, 2004 (173)	Ischemic stroke <12 h	24	Iv and sc insulin	Not reported	<3.0	46 %
Walters, 2006 (174)	Ischemic stroke <24 h	13	Iv insulin	9.1	<3.5*	23*
Gray, 2007 (169)	All stroke <24 h	460	Iv glucose-potassium-insulin	13.3	<4.0 for >30 min.	15.7 %
Bruno, 2008 (175)	Ischemic stroke <12 h	31	Iv insulin	Not reported	3.3	35 %
Kreisel, 2009 (176)	Ischemic stroke <24 h	20	Iv insulin	17.1	3.3	23 events
Kruyt, 2010 (177)	Ischemic stroke <24 h, tube & iv insulin, iv insulin only	10 13	Tube feeding & iv. insulin or iv insulin	Not reported	3.5 3.5	20 30.7 %
Johnston, 2009 (178)	Ischemic stroke <24 h, Intensive control & moderate control	24 25	Iv insulin	12.3 10.6	<3.1	30 % 4 %
McCormick, 2010 (179)	Ischemic stroke <24 h	25	Iv insulin	20.8	4.0	76 %
Staszewski, 2011 (180)	Ischemic stroke <12 h	26	Iv insulin	6	<3.3	8 %
Rosso, 2012 (181)	Ischemic stroke <6 h	85	Iv insulin	Not reported	<3.0 <3.6	5.7 % 19.5 %
Johnston, 2019 (170)	Ischemic stroke <12 h, Glucose >6.1 if T2D, >8.8 if no DM. T1D excluded.	581	Iv insulin, computer aided.	Median 8.6h.	Severe: <2.2	2.6 % Aborted treatment in 144 patients: 39 % due to hypoglycemia

Table 3. Trials of intensive glucose control with insulin in stroke. *N* denotes number of patients in intensive treatment group. *Not presented in article original article, found in review by Kruyt from 2010, (168).

1.6 GLP-1, NEUROPROTECTION AND ISCHEMIC STROKE

In an ischemic stroke, the affected area can be divided in a core and a *penumbra*. The core is the area of the brain where the cells die very quickly. The *penumbra* is the surrounding area of the brain where some circulation is still present which keeps the neurons alive for up to 6 to 8 hours and make them potentially salvageable if an intervention is put in place, otherwise it will progress to infarction (182). So far pre-clinically promising therapies targeting the neurons of the *penumbra* have failed when attempted in clinical trials (183). This could be due to many different factors such as difference in underlying biology as well as timing of administration in relation to stroke, dose or route of administration. Experimental animal studies are, furthermore, often performed in animal models without important comorbidities such as diabetes or performed in young animals and thereby differing substantially from the clinical situation with old and often comorbid patients.

1.6.1.1 GLP-1 can pass the Blood Brain Barrier

Endogenous GLP-1 has been shown to pass the BBB by means of diffusion in mouse (184). The GLP-1R analogue exendin-4 has been shown to rapidly pass the BBB, but the rate might be limited in high concentrations (185). Additionally there is also evidence that the GLP-1R analogues liraglutide and lixisenatide can pass the BBB (186). Passage of the BBB has thus

been demonstrated in rodents, but not shown in man. GLP-1 receptors are expressed in the brain (116, 187).

1.6.2 GLP-1 treatment in experimental stroke models

GLP-1 based therapies have been studied and shown to exert neuroprotective effect in stroke models. Importantly, most studies on neuroprotection in the central nervous system (CNS) with GLP-1RA and DPP4i have been performed under non-diabetic conditions. One of the first studies to demonstrate neuroprotective effects of Ex-4 was a study by Li *et al*. from 2009 where they saw a reduction in stroke volume and improvement in locomotor activity after an experimental model of stroke (MCAO – middle cerebral artery occlusion) in Sprague-Dawley rats (188). An interesting finding in this study was that the neuroprotective effect was lost in GLP-1R -/- knockout mice indicating that the effect is receptor-dependent. However, a major limitation of this study in terms of clinical relevance was the timing of administrating of Ex-4: before induction of the stroke. Moreover, the route of administration – direct intracerebral injection into a lateral ventricle is not applicable in a clinical setting. Nevertheless, this study demonstrated the neuroprotective potential of GLP-1R agonists.

Ex-4 given peripherally has also been shown to be neuroprotective in the experimental stroke model MCAO. In a study from our group, by Darsalia *et al.*, pretreatment with intra peritoneal (i.p.) Ex-4 for four weeks pre stroke was effective in reducing the stroke volume and increased the number of surviving neurons (189). The study also showed effect on decreased inflammatory microglia infiltration and increased stem cell proliferation in diabetic rats (GK). The effect was dose dependent and discernible at a clinical dosing of 0.1 µg/kg. This study showed that clinical dosing and peripheral administration could give neuroprotection in stroke, but the treatment was started before the stroke making it a positive study for those on the drug already but of limited value for someone not on the drug and suffering a stroke.

Additionally, pre-stroke treatment with clinical dosing of Ex-4 (0.1 µg/kg) has been shown to have neuroprotective capabilities independent of effect on glucose in the aforementioned study by Darsalia *et al.* (189).

Briyal *et al*. evaluated the effect of Ex-4 as pretreatment before (7 days) MCAO on stroke volume and also on markers of oxidative stress and reported a reduction in stroke volume and reduced oxidative stress (190). The effect of Ex-4 has also been shown in gerbils in a hippocampal cerebral ischemia model (191).

Demonstration of neuroprotective efficacy from GLP-1RA treatment has also been shown when given post stroke. Teramoto *et al.* showed that intravenous administration of Ex-4 given immediately after, or 1 h, after reperfusion resulted in reduced stroke volume and improved functional outcome (192). In this study the effect was lost if Ex-4 was given 4 h post stroke when measured as stroke volume.

The GLP-1R analogue liraglutide has also been tested by Briyal *et al. (193)* and been shown to be neuroprotective and to reduce apoptosis and oxidative stress when used as a 2-week pre-treatment before MCAO. The effect was seen both in diabetic and non-diabetic animals. As model of diabetes they used STZ (a model of T1D). This implies insulin independent, and perhaps, glucose independent effects of liraglutide. Liraglutide has been shown to be neuroprotective with reduced infarct volume and up-regulation of vascular endothelial growth factor (VEGF) in the cortex, but not in the striatum (194) in non-diabetic rats (Wistar). The once-weekly preparation semaglutide (with 97% sequence homology with liraglutide), has also in a recent publication from 2019 been shown to be neuroprotective with reduced infarct size and better neurological recovery (195).

Intranasal administration of Ex-4 has also been shown to be neuroprotective (non-diabetic mice, MCAO) (196). In this study, the animals were pre-treated 7 days before MCAO. The authors claim the effect to be through anti-apoptotic pathways.

Another way of stimulation the GLP-1R has been published regarding neuroprotection. Zhang *et al*. (197) have recently reported that a quinoxaline; 6,7-dichloro-2-methyl-sulfonyl-3-N-tert-butylaminoquinoxaline (DMB) that acts as an allosteric modulator of the GLP-1R and works as an agonist on the receptor also confers neuroprotection. In this study DMB was given orally 30 minutes before MCAO and compared with Ex-4 that was injected 30 min before MCAO. DMB showed neuroprotection, which also was demonstrated with Ex-4 (40 μg/kg). The same group has furthermore published a study demonstrating anti-stroke effects using a long-acting polymeric GLP-1, pro-GLP-1, to activate the GLP-1R (198).

In summary there are preclinical studies on treatment of acute stroke with GLP-1R agonists. Either by pretreatment *before* stroke (188-191, 193, 196-198), or as acute treatment *after* the onset (192, 194, 195, 199).

1.7 DPP-4 INHIBITION, NEUROPROTECTION AND ISCHEMIC STROKE

As with GLP-1R agonists, DPP-4i has been studied as a neuroprotective drug. There is evidence that this strategy has neuroprotective potential but not at the same level as for GLP-1R agonists. A proof of concept study was when intracerebral administration of a DPP-4 inhibitor in 2012 was shown to reduce the cortical infarct volume in experimental stroke with endothelin induced MCAO (eMCAO) (200). The DPP-4i linagliptin has been shown to increase the number of surviving neurons after MCAO in the cortex of mice in a study where linagliptin was given during 4 weeks before and 3 weeks after MCAO (115). In this study the neuroprotective effect by linagliptin was seen both in normal and diabetic mice, pointing towards glucose independent mechanisms. A different DPP-4 inhibitor, alogliptin, has also been shown to be neuroprotective if given 3 weeks before induction of experimental stroke (201). The authors measured brain-derived neurotrophic factor (BDNF), which was increased in the treated group and they speculate if this is part of the mechanism behind the

neuroprotective effect, potentially via up-regulation of the number of GABA-ergic synapses where BDNF has been found to play a role (202). BDNF has also been shown to enhance the GABA release (203). GABA and its role in signaling is discussed further below in the interneurons section.

Another potential mechanism through which DPP-4 inhibitors might have neuroprotective properties is stimulation of neural stem cell proliferation. After a stroke, neurogenesis has been shown to take place in the striatum, but some of the new neurons are probably preformed neuroblasts that could remigrate from the olfactory bulb to the striatum and replace dead neurons post stroke (204, 205). In *Regulatory Peptides* Darsalia *et al*. show that the DPP-4i linagliptin enhances neural stem cell proliferation after experimental stroke (206). This effect was, however, only seen in diabetic animals and the effect was not seen in vitro indicating indirect effects (pathways).

Treatment with the DPP-4i linagliptin has been shown to, irrespective of glycemic control, improve cerebrovascular dysfunction and remodeling in GK-rats (207). Additionally, high-dose-treatment with linagliptin (10 mg/kg/day) in db/db mice was able to improve performance in cognitive tests (water maze and passive avoidance) after cerebral ischemia (208). This occurred without a concomitant reduction in blood sugar. The animals were highly hyperglycemic throughout the study (glucose well in excess of 20 mmol/L), pointing to other mechanisms than regulation of glycemia as the main effect mediator.

SDF-1α is, in addition to GLP-1, a substrate for DPP-4 and has been shown to be important in the efficacy of DPP-4i (209-213).

The efficacy of DPP-4i in affecting the inflammatory polarization of macrophages also been studied. Zhuge *et al.* have tested the DPP-4i linagliptin and sitagliptin in young mice given high fat diet (HFD) to become obese and simulate the T2D metabolic syndrome patient (214). They showed that the DPP-4i shift the M1/M2 polarization towards the reparative M2. Linagliptin was more effective than sitagliptin. The effect was studied in fat and liver tissue which is not directly transferable to stroke, but it is interesting since the effect has been shown in GLP-1R treated diabetic mice (199).

1.8 SUMMARY OF INTRODUCTION

- Diabetes is a strong risk factor for neurological complications, with stroke as the most common CNS complication.
- Recovery after an ischemic stroke is impaired in T2D patients.
- The risk of complications to acute stroke treatment with rtPA is higher in hyperglycemic patients.
- Trials of glucose normalization with insulin have failed to show patient benefit.
- GLP-1RA treatment is proven to reduce ischemic stroke incidence.
- GLP-1-based therapies are safe and have the potential for pleiotropic, non glucose-mediated, effects that protect against ischemic stroke.
- Impaired interneuron function could be one of the explanations behind why diabetes causes CNS-complications and why persons with diabetes have worse outcome after stroke.
- GLP-1RA has potential to amend some of the impairments in interneuron functions.

2 AIMS OF THESIS AND INDIVIDUAL STUDIES

2.1 OVERALL AIM

To establish the role of GLP-1 in ischemic stroke and to determine whether GLP-1 based treatments can be used to counteract metabolic changes in patients with stroke and mitigate the detrimental effects of ischemic stroke.

2.1.1 Study 1 Exendin-4 in mice

To determine the neuroprotective efficacy of GLP-1RA exendin-4 given after stroke and to study its effect on the inflammatory response.

2.1.2 Study 2 Linagliptin in mice

To determine the neuroprotective efficacy of the DPP-4 inhibitor linagliptin given after stroke and to establish if the effect was dependent on the GLP-1 receptor.

2.1.3 Study 3 GABA-ergic neurons in rats

To establish if, and how, aging and T2D affect cortical and striatal GABA-ergic neurons. The aim was also to determine if potential changes could be counteracted by treatment with the GLP-1RA Ex-4.

2.1.4 Study 4 OGTT among rtPA treated Stroke patients

To determine if endogenous GLP-1 secretion is altered in the acute phase of stroke and if the GLP-1 levels in acute stroke are correlated to functional outcome of the stroke.

2.1.5 Study 5 Exenatide in ambulance

To evaluate the feasibility of starting exenatide treatment of hyperglycemia in the ambulance in patients with suspected stroke.

3 MATERIAL AND METHODS

3.1 STUDY DESIGN

The thesis is based on a combination of experimental animal studies and human clinical studies. The different studies had the following designs.

3.1.1 Study 1

An experimental animal study. A total of 128 male C57Bl mice were used in the experiments. It was divided into four sub-studies to test:

1. The potential neuroprotective efficacy of 5 µg/kg Ex-4 started 1.5 and 3 hours post MCAO on 2 months old mice. (n=31)
2. The potential neuroprotective efficacy of 50 µg/kg Ex-4 started 1.5, 3 and 4.5 hours post MCAO on 2 months old mice. (n=47)
3. The potential neuroprotective efficacy of 50 µg/kg Ex-4 started 1.5 and 3 hours post MCAO on mice that were obese and diabetic by exposure to HFD for 12 months. (n=27)
4. The potential effect of 50 µg/kg Ex-4 started 1.5 hours post stroke on the inflammatory response after MCAO on 2 months old mice. (n=23)

Treatment was continued in all sub-studies with 0.2 µg/kg Ex-4 daily for 7 days until sacrifice except for the inflammation sub-study that was ended at 3 days (to be concurrent with M2 microglia response after stroke).

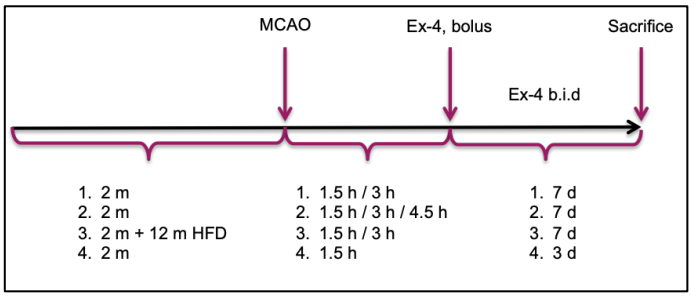

Figure 5. Experimental design Study 1. MCAO: middle cerebral artery occlusion, Ex-4: exendin-4, HFD: High fat diet, m: months, d: days, b.i.d: twice daily

The animals intended for immunohistochemistry were deeply anesthetized and perfused transcardially with saline followed by paraformaldehyde (4%, ice cold). Animals intended for tissue collection were perfused with saline only.

3.1.2 Study 2

An experimental animal study. A total of 117 male mice were used in the experiments. 35 glp-$1^{-/-}$ and 82 wild type (C57Bl). The glp-$1r^{-/-}$ mice have a mutation in the gene for the GLP-1 receptor making it inactive. They develop normally, have lower levels of circulation insulin and have higher glucose levels during an OGTT (215).

The study was divided in four sub-studies. The objective was to test:

1. **Neuroprotective efficacy of linagliptin given acutely in comparison with chronic treatment before stroke.** 50 mg/kg of linagliptin (n=12) or vehicle (n=13) was

injected intravenously and MCAO surgery started immediately after (30 minutes of occlusion was used). For the chronic setting p.o. treatment of linagliptin 10 mg/kg (n= 9) or vehicle (n=10) was used.

2. **Neuroprotective efficacy of linagliptin in the absence of the GLP-1 receptor.** Knockout mice glp-1r$^{-/-}$ were used. Chronic treatment with linagliptin (n=9) or vehicle (n=7) was used according to the same protocol as in sub-study 1. MCAO was performed with 30 minutes of occlusion.
3. **Neuroprotective efficacy of Ex-4 in the absence of the GLP-1 receptor.** Knockout mice glp-1r$^{-/-}$ were used. Ex-4 0.1 µg/kg intraperitoneally (i.p.) (n=12) or vehicle (n=7) was given for four weeks before MCAO and three weeks after. For comparison eleven wild type mice were given Ex-4 (n=5) or vehicle (n=6) according to the same protocol. MCAO was performed with 30 minutes of occlusion.
4. **The effect of linagliptin on cerebral GLP-1 levels and DPP-4 activity**. 10 weeks old mice were chronically treated (n=19) for four weeks of linagliptin or vehicle before sacrifice or given acute treatment (n=8) with linagliptin iv. or vehicle and sacrificed after 4 hours.

The animals intended for immunohistochemistry were deeply anesthetized and perfused transcardially with saline followed by paraformaldehyde (4 %, ice cold). Animals intended for tissue collection were perfused with saline only.

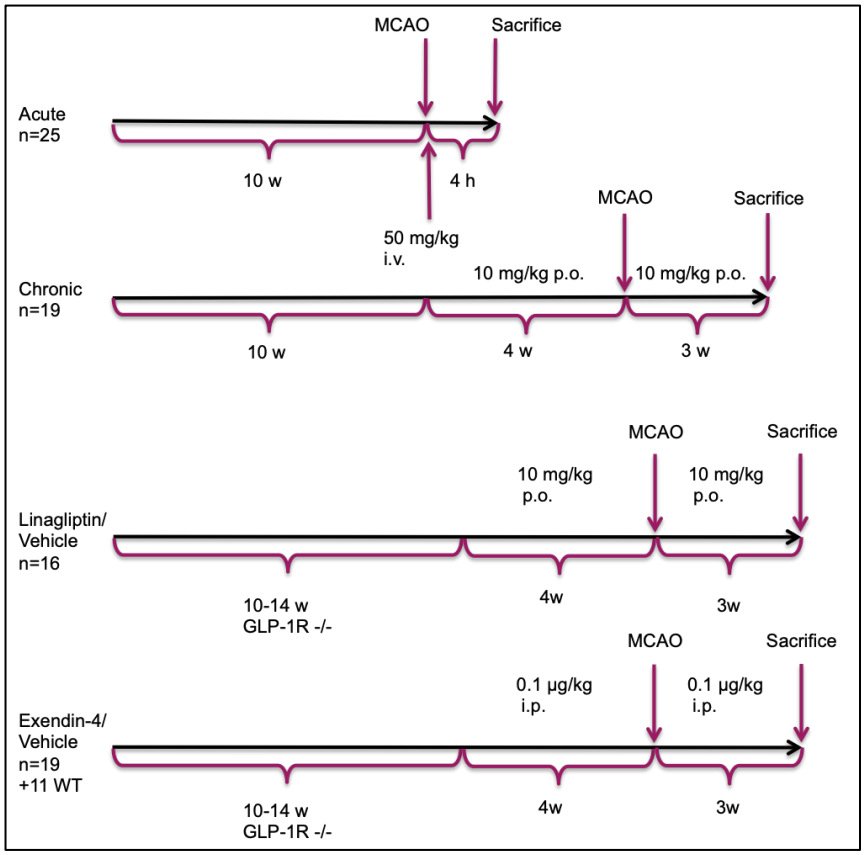

Figure 6. Experimental design Study 2. MCAO: middle cerebral artery occlusion, w: weeks i.p.:intraperitoneal, WT: wild type mice, GLP-1r -/-:GLP-1 receptor knockout mice.

3.1.3 Study 3

An experimental animal study. A total of 43 rats were used (31 GK-rats and 12 Wistar rats). The experiment was divided into two sub-studies to test:

1. The effect of T2D and aging on GABA-ergic neurons. 13 months old GK and Wistar (non diabetic) rats were compared to 3 months old GK and Wistar rats.
2. The potential of Ex-4 to reverse the pathological changes on GABA-ergic neurons. 9 months old GK rats were treated with clinical dosing of Ex-4 for 6 weeks.

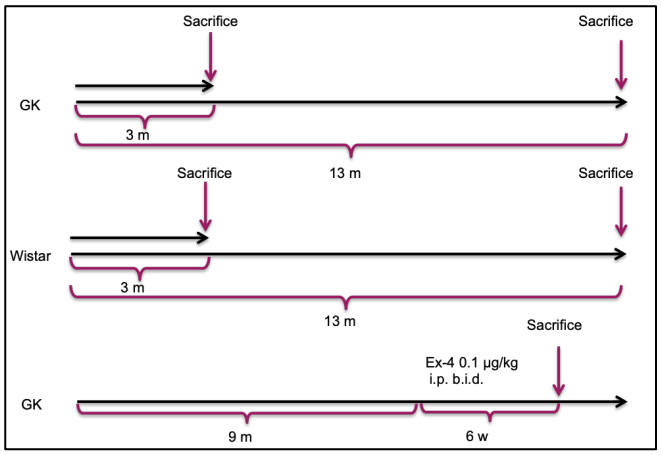

Figure 7. Experimental design Study 3. GK: Goto-Kakizaki rats, Ex-4: exendin-4, ip.: intraperitoneal, b.i.d.: twice daily, m: months, w: weeks.

The animals were deeply anesthetized; blood was collected through cardiac puncture and then perfused transcardially with saline followed by paraformaldehyde (4 %, ice cold).

3.1.4 Study 4

A clinical study on patients that had undergone rtPA treatment for ischemic stroke and had no known diabetes. The participants underwent a 75 g OGTT at day 2-4 after admission. They were then invited back for a follow-up OGTT 3 months later. Functional outcome was scored at the second visit.

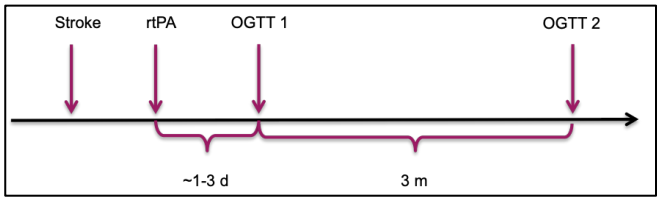

Figure 8. Outline of study events in Study 4. rtPA: recombinant tissue plasmin activator, OGTT: oral glucose tolerance test, d: days, m: months.

The study was open for inclusion between January 2014 and January 2017. A total of 379 patients were treated with rtPA at the hospital during this time period. A research nurse consecutively screened patients for eligibility. The recruited population is a subset of the total rtPA treated population at Södersjukhuset that had no pharyngeal palsy, as swallowing was necessary for the OGTT, and needed to be in a condition that allowed informed consent. This makes the subpopulation slightly younger and with somewhat lower NIHSS-scores compared to the total rtPA treated population. Patients who at discharge from hospital or at the three-month follow up were diagnosed with a condition other than stroke (stroke mimic) were excluded from the study. A total of 59 patients were included.

As a comparator, a group of healthy individuals were recruited by advertisement. They underwent a single OGTT and anthropometric data was collected.

3.1.5 Study 5

A randomized clinical trial performed in the pre-hospital arena in patients with acute suspected stroke. Four ambulance stations with Södersjukhuset as primary target hospital participated (Farsta, Nacka, Värmdö and Södermalm). Ambulance nurses were individually trained and delegated to include patients and to receive the informed consent of the included patients. A total of 109 ambulance nurses were trained in the study.

The study was registered prior to first randomization on the EU clinical trials site: www.clinicaltrialsregister.eu registered as "PROLOGUES - Prehospital lowering of glucose in Stroke" (Identifier 2011-002780-16).

The patients were screened, included and randomized in the ambulance and were followed for 24 hours. If randomized to exenatide, treatment was initiated in the ambulance. During the first 4 hours glucose was checked every hour, and thereafter every 4 hours.

The study was open for inclusion between May 2013 and May 2018. Inclusion and exclusion criteria were chosen so they were possible to determine in a stressful prehospital environment. Inclusion criteria were: ≥ 1 point on the FAST-test (Face Arm Speech Time), symptoms of stroke with a duration less than 6 hours, capillary plasma glucose 8-15 mmol/L, age ≥ 18 and signed informed consent. Exclusion criteria were: T1D, antidiabetic treatment other than metformin, pregnancy, cirrhotic liver disease, regular hemo- or peritoneal dialysis, GCS < 4 or GCS verbal < 5, signs of pharyngeal palsy or previously known dementia.

Due to slow recruitment two changes to the protocol were made regarding the inclusion/exclusion criteria. The interval for glucose was widened from 10-15 mmol/L in the beginning to 8-15 mmol/L and a change was made to also allow for metformin treated patients to be included.

3.2 ANIMAL MODELS OF DIABETES (STUDY 1 AND 3)

In this thesis two animal models of diabetes have been used.

3.2.1 High fat diet (Study 1)

The use of high fat diet (HFD), with 60 % of energy from fat, is a way of inducing a T2D-like phenotype in mice. It also induces concomitant conditions seen in human T2D such as nonalcoholic fatty liver disease (NAFLD) and chronic inflammation (216). It takes months to induce diabetes in this way and the result is mild hyperglycemia. This is, however, a strength when modeling human T2D that usually also takes time to develop. When studying a disease

such as stroke that most often occurs in aged humans, a model of aged animal is better suited to produce transferable results.

HFD was started at the age of 2 months and was continued for 12 months to induce diabetes. Fasting glucose in the HFD group was 11±3 mmol/L.

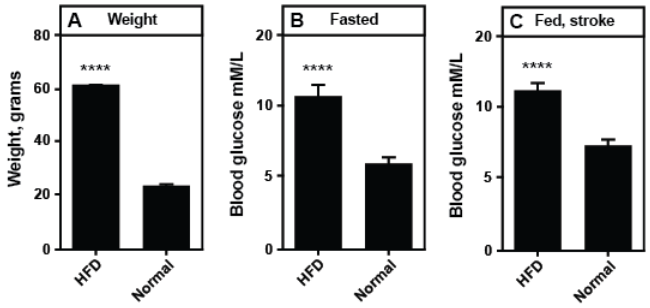

Figure 9. Effect of HFD on mice in Study 1. Body weight (A), fasting plasma glucose (B) or plasma glucose in the fed state at time of MCAO (C). HFD: 14 months old mice treated with high fat diet for 12 m, normal: 2 months old healthy mice. Bars indicate SEM, ** denotes p<0.0001. Adapted and reproduced under creative commons attribution license (CC BY 4.0) from DOI: 10.1371/journal.pone.0103114**

3.2.2 Goto-Kakizaki rats (study 3)

The Goto-Kakizaki-rat (GK-rat) is a non-obese model of T2D. It was developed by selective inbreeding of Wistar rats, and is consequently a derivative of the Wistar rat strain (33). They spontaneously develop hyperglycemia. With increasing age, they progress to insulinopenia as well. The GK-rats develop β-cell dysfunction similar to human T2D patients (217). They also develop common T2D complications seen in T2D patients (218).

The young GK rats (3 months) had slight fasting hyperglycemia, approximately 9 mmol/L compared to 6 mmol/L for the Wistars. In the 13 months old GK rats, the fasting hyperglycemia was approximately 18 mmol/L. The plasma insulin levels were lower in the

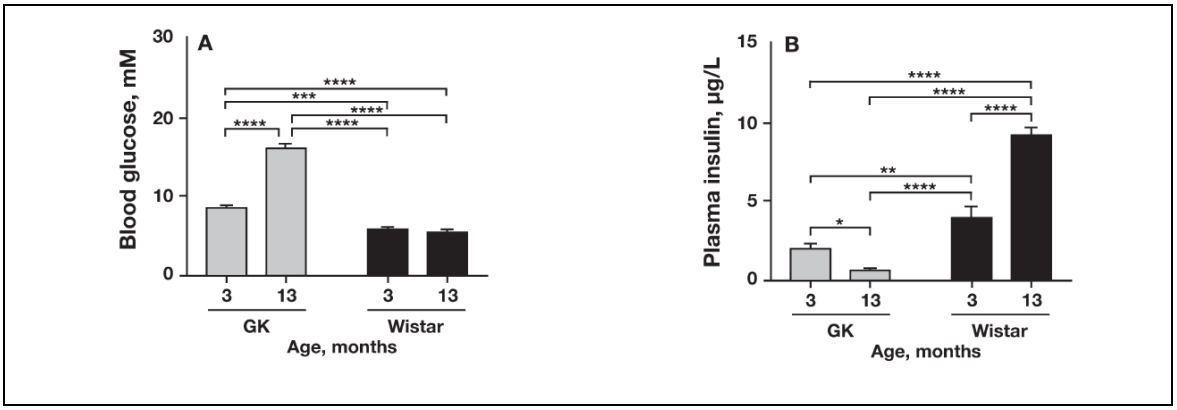

Figure 10. Glycemia and development of insulinopenia in the GK rats compared to Wistar rats. 3 and 13 months old. Fasted blood glucose (A) and fed plasma insulin (B). Bars indicate SEM. *, **, * and **** denote p<0.05, 0.01, 0.001 and 0.0001 respectively. ©IOS Press, adapted and reprinted from DOI: 10.3233/JAD-131958**

GK rats compared to the Wistars. At 3 months the insulin level was approximately 2 µg/L in the GK-rats compared with 4 µg/L for the Wistars.

In the sub-study with Ex-4 treatment, the fasting glucose levels in GK rats were approximately 10 vs. 6 mmol/L for the control group and Ex-4 treated group respectively.

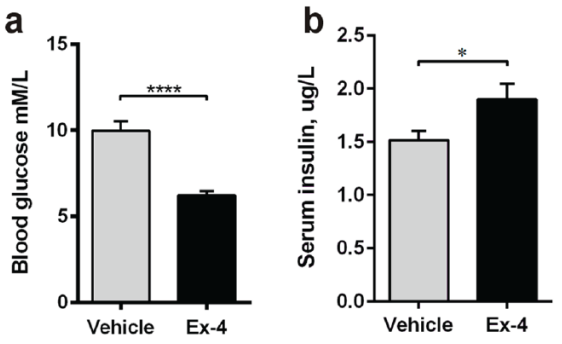

Figure 11. Effect of exendin-4 (Ex-4) on fasting blood glucose (A) and fed serum insulin (B) in the 9 months old GK rats. Bars indicate SEM. * and ** denote p=<0.05 and 0.0001 respectively. Adapted and reproduced under creative commons attribution license (CC BY 3.0) from DOI: 10.18632/oncotarget.6823.**

3.3 TRANSIENT MIDDLE CEREBRAL ARTERY OCCLUSION (STUDY 1 AND 2)

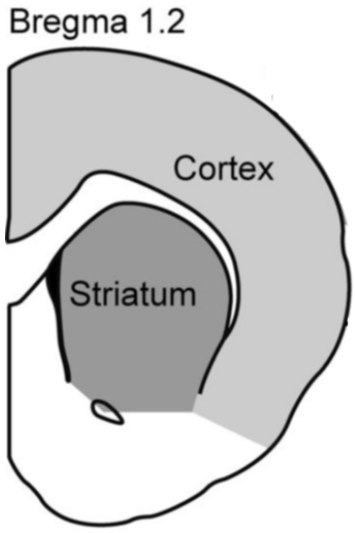

Figure 12. Hemisphere of rodent brain with structures Striatum and Cortex indicated. Reproduced and adapted under the Creative Commons Attribution License 4.0 (CC BY 4.0) from DOI: 10.1042/BSR20160437

Middle cerebral artery occlusion (MCAO) is a technique to induce a focal ischemic stroke involving the striatum and cortex through occlusion and reperfusion that results in an ischemic infarction.

In brief, this is how the procedure is done: After induction of anesthesia the carotid arteries on the left side are exposed. The external carotid artery is ligated and through a small incision in the external carotid artery a monofilament suture coated with silicone is inserted into the internal carotid artery. It is advanced to the point of resistance were it blocks the origin of the middle cerebral artery. After the filament is positioned the wound is closed and anesthesia stopped allowing the animal to wake. After 30 minutes of occlusion time, anesthesia is restarted and the filament removed again. Throughout the procedure and ischemic period, body temperature is maintained between 36-38 °C by a heating lamp. After the procedure the animals are transferred to a heated box where they are kept for two hours until they regain full consciousness. The surgeon performing the procedure is blinded to treatment allocation.

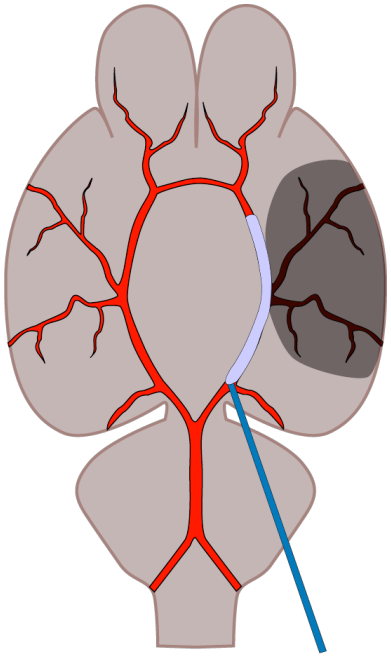

Figure 13. Schematic picture of MCAO. A rodent brain seen from below. A monofilament (blue) is introduced through the internal carotid artery on the left side and blocks the middle cerebral artery, thereby inducing an ischemic stroke.

3.4 IMMUNOHISTOCHEMISTRY (STUDY 1-3)

3.4.1 Tissue preparation

The animals intended for immunohistochemistry were deeply anesthetized and perfused transcardially with saline followed by paraformaldehyde (4 %, ice cold). The brains were extracted and post-fixed over night in 4 % paraformaldehyde at 4 °C. They were then transferred to a solution of sucrose (20 %) in phosphate buffer until they sank. The brains were then cryo-sectioned in 40 µm thick slices with a sliding microtome.

For immunohistochemistry (IHC) the slices were then stained as free-floating sections. A general description of procedure follows below. Different markers were detected by use of various primary antibodies, see below.

1. The sections were rinsed three times in phosphate buffer solution (PBS).
2. The sections were quenched with H_2O_2 (3 %) in methanol (10 %) to block endogenous peroxidase activity.
3. The sections were rinsed PBS three times.
4. Blocking with serum from the animal species of the secondary antibody in T-PBS (PBS with the detergent Triton X-100). This is to reduce background staining and to increase membrane permeability.
5. Incubation with primary antibody in normal serum, typically over night.
6. Washing three times in T-PBS.
7. Incubation with Biotinylated secondary antibody against the species of the primary antibody for 2 hours (1:200, Vector Laboratories).

8. Sections washed three times in T-PBS.
9. Incubation for 1 h with Avidin-Biotin-Complex-Solution (ABC-Solution). This step produces large complexes of avidin-biotin bound to the secondary antibodies. The enzyme horseradish peroxidase is bound to the complexes.
10. Sections washed 3 times in PBS.
11. Sections incubated with DAB/H_2O_2 solution for approximately 2-5 minutes until color develops. DAB (3,3'-diaminobenzidine) is a substrate for HRP and produces a brownish color.
12. Sections washed 3 times.
13. Mounting of sections on glass
14. Sections were dehydrated by ethanol and Xylene.
15. Covering with cover glass and xylene based coverslip medium.

3.4.2 Histology markers

3.4.2.1 NeuN (Study 1 and 2)

Neuronal Nuclei (NeuN) is a neuron specific marker. Antibodies against NeuN stain most neuron types and is present in a wide range of species including rodents and humans (219). It is the product of the gene Fox-3 and function as a splicing regulator (220). For IHC the primary antibody anti-NeuN from Millipore, MA, USA, was used in a concentration of 1:100.

3.4.2.2 Cresyl violet (Study 3)

A basic dye that can be used to stain the Nissle substance in neurons – granules of the endoplasmatic reticulum. 0.1 % Cresyl Violet acetate (Sigma-Aldrich) was used. It is a general neuron stain that also stains myelin cells. For neuron quantification only cells with neuron morphology were counted.

3.4.2.3 GAD67 (Study 3)

Glutamic acid decarboxylase-67 (GAD67) is an enzyme responsible for producing the inhibitory neurotransmitter GABA (61). It was detected by mouse anti-GAD67 (1:500, Merck Millipore), overnight incubation at 4 °C.

3.4.2.4 Calcium binding proteins (Study 3)

A subgroup of the regulatory interneurons are characterized by the presence of calcium binding proteins (CaBP), Calbindin-28kD, Parvalbumin and Calretinin(63). They were detected by following primary antibodies: rabbit anti-Calbindin-28kD (1:1500, Abcam), rabbit anti-Parvalbumin (1:1500, Abcam) and rabbit anti-Calretinin (1:1500, Vector Laboratories).

3.5 QUANTITATIVE ANALYSIS OF HISTOLOGIC SAMPLES (STUDY 1-3)

Histologic evaluation was performed in a computer aided manner using the software NewCast (Visiopharm, Hoersholm, Denmark) connected to an Olympus BX51 epifluorescent/light microscope. The number of cells, cell volume and stroke volume was evaluated in a blinded manner.

The cryo-sectioning of the brains was made with serial cutting. This means that each cut brain slice was binned serially, resulting in for example 10 sets of slices (10 bins) with an offset of the cut thickness, 40 μm, between each set. Slice 1, 11, 21 and so forth would go in the first bin. Slice 2, 12, 22 and so forth would go in the second bin. This number of series that was cut creates a Section Fraction, (SF).

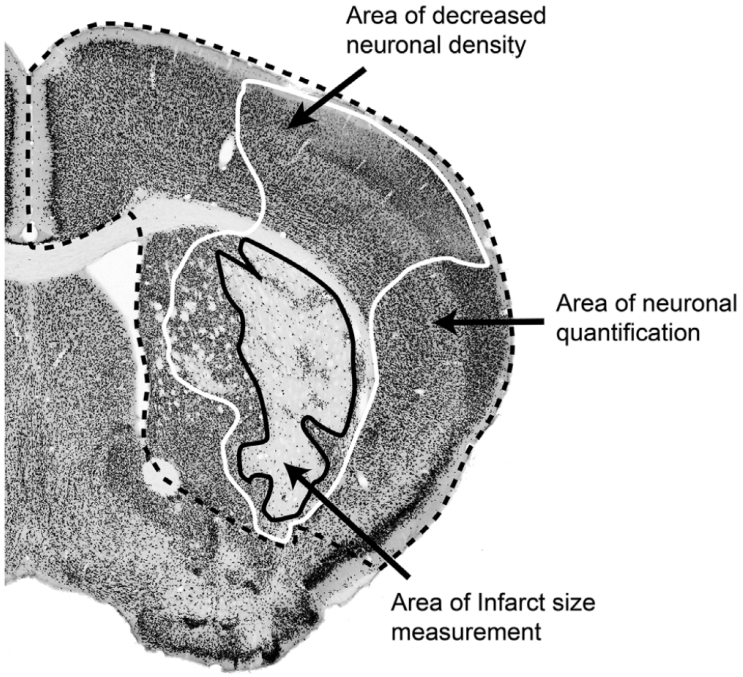

Figure 14. Representative photograph taken with light microscope. NeuN immunostained brain section of stroke damaged mouse brain (MCAO). Black line delineate area of volume measurement (stroke size) Withe line delineate area of reduced cell density post MCAO. Dashed line indicates area of quantification. Reproduced under creative commons attribution license (CC BY 4.0) from DOI: 10.1371/journal.pone.0103114.

3.5.1 Stroke volume measurement

By use of the software, the area of undamaged brain tissue in the ipsilateral hemisphere was measured in the brain sections containing stroke. In the same tissue sections, the areas of the contralateral hemispheres were measured. Stroke area was calculated by subtracting the measured area in the ipsilateral hemisphere from the contralateral area. Each section was cut 40 μm thick and by knowledge of the binning, of sections during cutting [Section Fraction (SF)] volume can be calculated from the area measurements.

3.5.2 Cell number

The optical fractionator method was used for cell number counting (221, 222). The area of interest for quantification is outlined in a computer software on all sections of interest. Counting then takes place within a counting frame that is moved at regular intervals (steps) over the entire delineated area with a random starting point. The counting frame is a virtual computer-added frame that is superimposed on the image by the software; cells within it are counted and a set of counting rules decide which cells touching any of the frame borders are counted. The step length is chosen so that on average 100-200 cells are counted. The total delineated area divided by the number of steps gives a step area. Step area divided by the area of the counting frame gives area fraction, (AF). The total number of cells (N) within the area of quantification is then calculated by the fractionator formula.

Formula for estimation: $N = n \times SF \times AF$

N = estimated total number of cells.

n = counted number of cells

SF = Section Fraction – number of series that was cut

AF = Area fraction – step area/frame area

For a cell density measure, total number of cells was estimated within sampled volume and then normalized on standardized unit, e.g. per mm^3. Total volume of the structure of interest was measured in the same way stroke volume was measured.

The counting was set up so that the same area was counted within the respective study; position relative to Bregma or midline determined by appearance of brain structures such as the striatum.

3.5.3 Cell volume quantification

The nucleator technique was used (223). The area of interest for volume measurement of cell volume was delineated. A counting frame was moved in a stepwise manner over the area and measurements were made on the cells within the counting frame. The measurement was then performed on cells that fell within the frame by clicking at the approximate center of each cell and measuring the distance to the cell boundary along random (computer generated) axes projecting out from the center. The software then estimates the volume of each cell using the nucleator principle. Between 100-200 cells were measured for each individual animal.

3.6 INFLAMMATION STUDIES (STUDY 1 AND 3)

3.6.1 Study 1

One of the sub-studies aimed to determine the effect of Ex-4 on post stroke inflammation. During the sacrifice the animals were transcardially perfused with saline (0.9 %, ice cold), brains divided along the midline (to separate stroke from non-stroke side) and then snap-frozen in dry ice. The frozen brain samples were then homogenized with cold RNAse free PBS. Gene expression of pro-inflammatory cytokines was then analyzed using real-time PCR.

The effect of Ex-4 on cytokine levels in microglia cell cultures was also studied. Whole brains of young mice (day 2-3 after birth) were homogenized and microglia cells cultured and enriched. They were then stimulated with lipopolysaccharide (LPS) (10 ng/mL, List Biological Laboratories Inc., Cambell, CA) with or without Ex-4 (10 nmol/L). Cytokine levels were measured with Bio-Plex Pro Assay (Bio-Rad Laboratories, Inc., CA).

3.6.2 Study 3

In the sub-study with 6 weeks of treatment with Ex-4 or vehicle in GK-rats, serum cytokines (IL-1β, MCP-1, IL-6, IL-10 and TNF-α) was measured with the Bio-Plex Multiple Cytokine Assay (Bio-Rad Laboratories, Inc., CA).

3.7 GLP-1 RECEPTOR KNOCKOUT MODEL

An experimental model with a null-mutation in the gene for the GLP-1 receptor (GLP-1 -/-) was used in Study 2 to determine if the neuroprotective effect from DPP-4 inhibitors is mediated through the GLP-1 receptor. These mice develop normally and have normal feeding behavior, but develop higher glucose levels during a glucose challenge and also have somewhat higher fasting glucose (215).

3.8 DPP-4 ACTIVITY (STUDY 2)

To measure the effect of linagliptin treatment on DPP-4 activity in the acute vs. chronic linagliptin treatment, study plasma was collected from the mice at sacrifice. The activity was measured by fluorescence assay at Boehringer Ingelheim Pharma GmbH & Co (224). The activity levels were then normalized to the level in vehicle treated mice.

The DPP-4 activity in the brain itself was established by extraction and freezing of the brains immediately after sacrifice. The frozen brains were homogenized and DPP-4 activity established in the same way as described above.

3.9 OGTT (STUDY 4)

On hospital day 2-4 a 75 g fasting oral glucose tolerance test was performed. Venous samples were drawn at 0, 60, 90 and 120 minutes. Plasma was separated and frozen at -70 °C for batch analysis.

3.9.1 Glucose analysis

Glucose during the OGTT was measured as plasma glucose by the HemoCue 201+ system (HemoCue AB, Ängelholm, Sweden).

3.10 INSULIN AND INSULIN RESISTANCE (STUDY 4)

Plasma insulin was measured as batch analysis from frozen samples at the Karolinska University Laboratory by electrochemiluminescence.

3.10.1 Cederholm Insulin Resistance Index

To estimate the insulin resistance, we used the Cederholm Insulin Resistance Index (Ceder-IR) (225). This index is a stronger predictor of cardiovascular disease than other indices, e.g. HOMA-IR (225, 226).

The Ceder-IR is defined as 100 divided by Cederholm sensitivity index. Insulin resistance and sensitivity index is calculated by:

$$Ceder\text{-}IR = \frac{100}{Cederholm\ sensitivity\ index}$$

$$Cederholm\ sensitivity\ index = \frac{\left(\frac{glucose\ uptake\ rate}{mean\ blood\ glucose}\right)}{\log mean\ insulin}$$

Glucose uptake rate was defined as (in a 75 g OGTT): [75,000 (mg)/120 (min)] + [[0-min glucose (mmol/L) – 120-min glucose (mmol/L)] x 0.19 x body weight (kg) x 180/120 (min)]. 0.19 x body weight calculates glucose space and 180 is the conversion factor of glucose between mmol/L to mg/L. **Mean blood glucose** was defined as glucose at 0 minutes + 120 minutes divided by 2. **Log mean insulin** was defined as log (Insulin at 0 minutes + 120 minutes divided by 2). Described in detail in the work by Zethelius and Cederholm (225).

3.11 GLP-1 ANALYSIS

GLP-1 in the linagliptin study (Study 2) was measured as active GLP-1 with a commercially available ELISA kit (K150JWC-1, Mesoscale, Gaithersburg, USA). This kit detects both the GLP-1 (7-36)-amide and the 7-37 form.

In Study 4 GLP-1 was measured as total GLP-1 by batch analysis of the frozen samples by radioimmunoassay using the antiserum 89390. It detects both the active form of GLP-1 (7-36 amide) and the primary metabolite (9-36 amide). It detects but less than 0.1 % of the GLP-1 7-7 form, glucagon, gastric inhibitory peptide (GIP) and vasoactive intestinal peptide (VIP) (227). Detection threshold was 1 pmol/L and the coefficient of variation was < 6 %.

3.12 CLINICAL INVESTIGATIONS

3.12.1 FAST/CPSS

In study 5, a likely diagnosis of suspected stroke was made using the clinical investigation tool Face-, Arm-, Speech-test (FAST). A stroke is suspected by the presence of any of the acronym components: asymmetry or impaired movement of one side of the face, weakness in one arm or speech impairment. FAST is a development of the Cincinnati Prehospital Stroke Scale (CPSS) but instead of using a pre-specified term to determine speech abnormality (The patient repeats "The sky is blue in Cincinnati" or the Swedish equivalent "*Det är vackert väder idag*"), speech abnormality is adjudicated in the normal interaction with the patient. The CPSS has good reproducibility and validity (228).

The sensitivity and specificity for detection of stroke is 66 % and 88 % respectively with one element of the scale being abnormal (228).

In the study, we used one point on the FAST scale in combination with clinical suspicion of stroke in the opinion of the ambulance nurse as a requirement for inclusion.

3.12.2 NIHSS

The National Institutes of Health Stroke Scale (NIHSS) is clinical examination scale that can be used to rate the severity of a stroke (229). The scale is comprised of motor function tests, sensory evaluations, level of consciousness, neglect and language ability. Higher scores indicate a larger stroke with more severe functional deficits. The score has been used in numerous stroke trials to assess stroke severity, including the pivotal rtPA trials. NIHSS has been shown to strongly predict the outcome of a stroke with the likelihood of excellent outcome at three months dropping by 17 percent for each additional point on the NIHSS (230).

In Study 4, NIHSS was used to compensate for initial stroke severity when examining the relationship between GLP-1 and three-month outcome. In Study 5, NIHSS was included as baseline characteristic.

3.12.3 mRS

Modified Rankin Scale (mRS) is a stroke outcome scale. It ranges from 0 (no symptoms) to 5 (severe disability, in need of constant nursing care) (231). Sometimes a grade 6 (dead), is added. In Study 4 favourable outcome after the stroke was defined as mRS 0-1 (232).

mRS	Definition
0	No symptoms.
1	No significant disability. Able to carry out all usual activities, despite some symptoms.
2	Slight disability. Able to look after own affairs without assistance, but unable to carry out all previous activities.
3	Moderate disability. Requires some help, but able to walk unassisted.
4	Moderately severe disability. Unable to attend to own bodily needs without assistance, and unable to walk unassisted.
5	Severe disability. Requires constant nursing care and attention, bedridden, incontinent.

Table 4. Description of the modified rankin scale (mRS). Sometimes a 6 is added: dead.

3.13 STATISTICAL METHODS (ALL PAPERS)

In Study 1, Student's unpaired t-test was used for assessing differences in inflammatory markers in microglia cultures. One-way analysis of variance (ANOVA) with Tukey's multiple comparison correction was used to compare the stroke volume estimates and NeuN counts. Inflammatory markers in the *in-vivo* (sub-study 4) part of Study 1 were compared with Kruskal-Wallis one-way analysis of variance followed by Dunn's multiple comparisons test.

In the animal stroke study with linagliptin (Study 2), Student's unpaired test was used.

In the interneuron Study (Study 3), two-way ANOVA with Tukey's multiple comparisons correction was used to analyze differences and allow for interaction between age and diabetes. Cytokine levels were analyzed by Student's unpaired t-test.

In the clinical OGTT study (study 4), a Univariate General Linear Model (GLM) was used to compare fasting levels of GLP-1, insulin and glucose between patients and controls. For comparison of the fasting GLP-1 and insulin measures between the acute phase and the three-month follow-up the non-parametric test Wilcoxon Signed Ranks Test was employed. Glucose and insulin were analyzed by two-way ANOVA. Huynh-Feldt correction was used to adjust for sphericity violation. Logistic regression was used to determine the association between GLP-1 and stroke outcome. In this analysis, mRS was grouped as favorable if mRS was 0-1 or unfavorable if mRS ≥ 2, all other variables of non-binary nature was treated as continuous.

In the expanded analyses of GLP-1 and glucose (using data from 0, 30, 90 and 120 minutes) between the stroke patients and the healthy controls during the OGTT, a Univariate General Linear Model was used in the same way as the analysis of only fasting levels, but with repeated measurements. This was not presented in the paper in Cardiovascular Diabetology. When comparing GLP-1 levels between the acute phase and the three-month follow up two-way ANOVA was used instead of the Wilcoxon Signed Ranks Test.

In the study examining exenatide in the ambulance (Study 5), analysis of p-glucose at 4 h and analyses of the AUC of capillary glucose during the first 4 h and 24 hours were made with the non-parametric Mann-Whitney U-test as primary analysis. A complementary analysis was made with linear regression to allow for adjustments (age, sex and BMI).

In Study 1-3 GraphPad Prism v. 5 & 6 (GraphPad Software, CA, USA) were used for the statistical analyses. In Study 4-5 SPSS v23.0.0.3 (IBM Corp, Armonk, NY, USA).

3.13.1 Missing data

Overall, the level of missing data was very low. There were no missing data points from the animal studies. In Study 4, one GLP-1 measurement was missing from one person in the control group. Since ANOVA requires complete cases for analysis, that individual was excluded in those analyses. In Study 5, data on venous glucose measurements at 4 hours was missing for three individuals and for some of the capillary glucose measurements. This was handled with different forms of sensitivity analyses and with replacement of capillary values for the missing venous as outlined in the manuscript.

4 ETHICAL CONSIDERATIONS

Study 1-3 were performed in accordance with the "Guide for Care and Use of Laboratory Animals" published by the National Institutes of Health in U.S. These studies were approved by the animal research ethical board at the Agricultural department and the local ethics board for animal research ("Stockholms södra djurförsöksetiska nämnd"). When designing the studies we used the principle of 3 R: Replace, Reduce and Refine. It was not possible to replace the animals with other methods as we studied complex interactions in the brain after stroke, which is not possible to study in, for example, a cell culture. From previous studies, both our own and performed by others, we had knowledge of the group size needed to detect meaningful differences. We could thereby keep the number of animals in each experimental group low and did not need to overuse the number of animals. The animals could furthermore be used in multiple experiments. The brains were extracted and serially cut (binned) ensuring up to 10 and equivalent sets of sections that could be stained with different markers. The research group had furthermore several scientific questions regarding different parts of the brain that could be examined in the same sections, reducing the need for sacrificing more animals. The type of information and knowledge gained form the animal studies is not possible to gather in any other way, neither in cell cultures nor in humans. I therefore consider the performed research ethical.

The clinical studies (Study 4-5) were performed in accordance with the Declaration of Helsinki and were approved by the regional ethical board at Karolinska Institutet (Study 4) or the Swedish Central Ethics board (Study 5). Informed consent was obtained from all participants.

In Study 4, the patients were all without known diabetes, but had suffered a stroke. Diabetes is a well-known risk factor for stroke and OGTT is a standard procedure to establish if someone has diabetes or impaired glucose tolerance. The procedure they went through was therefore something that would (at least should) have been undertaken regardless of if they participated in the study or not. The extra blood samples taken during the OGTT were taken through a peripheral cannula and not through repeated punctures, reducing suffering and pain. The volume of blood, about 30 mL, is not considered harmful. The risk to which the healthy controls were exposed was also very limited and they had the benefit of having their metabolic status (glucose tolerance) measured as a form of check up.

Study 5 was more complex from an ethical viewpoint. Stressed patients with potential acute brain injury were asked to participate in a clinical trial. The problem of including patients in the pre-hospital arena is further elaborated on in the discussion part. The drug they were exposed to, exenatide, was however already an approved drug for the treatment of diabetes with known properties and side effects. We also made some adaptions of the protocol from the beginning to minimize potential harm to the study subjects. Our hypothesis was that hyperglycemia is harmful in the acute phase. We therefore did not want to include patients with very pronounced hyperglycemia in a study with a drug not previously tested in this setting. We consequently set a threshold of 15 mmol/L in glucose as a maximum for

inclusion. It is not possible to perform this type of study in alternative models such as animals or cell cultures. Additionally, our hypothesis was that GLP-1RA treatment is beneficial with glucose lowering capacity without concomitant risk of hypoglycemia. This was of potential benefit to the patients and the risk they were exposed to was limited so we found the research to be reasonable and could be motivated from an ethical point of view.

5 RESULTS

5.1 STUDY 1 EXENDIN-4 IN MICE

In this study we aimed to determine the neuroprotective efficacy of GLP-1RA Ex-4 given after stroke and to study its effect on the inflammatory response.

In short, our results showed that Ex-4 given after stroke was neuroprotective, both in young animals and old diabetic animals. Additionally, Ex-4 shifted the inflammatory response to a more reparative response, M2.

5.1.1 Exendin-4 reduces effect of stroke in dose- and time-dependent manner.

Administration of Ex-4 immediately after MCAO-induced stroke resulted in an increase in the number of NeuN-positive neurons in the striatum of 2-month-old mice. In sub-study 1 (5 µg/kg *bolus*) the increased neuronal number was detected if *bolus* was given 1.5 hours after MCAO ($p<0.05$), but lost if given after 3 hours. In sub-study 2 (50 µg/kg *bolus*) there was an increase in NeuN-positive neurons both at treatment initiation at 1.5 ($p<0.001$) and 3 hours ($p<0.05$). No effect was seen if treatment was started 4.5 hours after stroke. No effect was seen in the cortex and there was no difference in stroke volume. Figure 15.

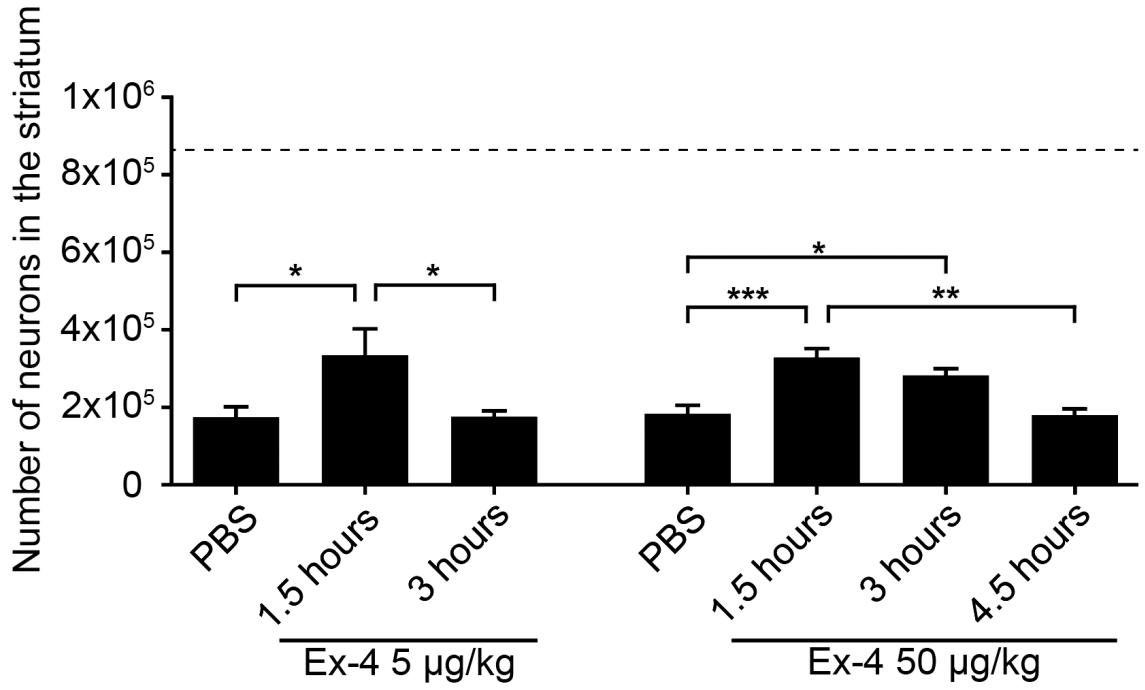

Figure 15. Neuroprotective effect of Exendin-4 in the striatum after stroke in normal adult mice. Time point indicates how long after MCAO Ex-4 treatment was initiated. Dashed line show number of neurons on contralateral side. Ex-4: exendin-4, PBS: Phosphate buffer solution (placebo). Bars indicate SEM, *, **, and * represent $p<0.05$, $p<0.01$ and $p<0.001$ respectively. Adapted and reproduced under creative commons attribution license (CC BY 4.0) from DOI: 10.1371/journal.pone.0103114**

5.1.2 Exendin-4 is neuroprotective in aged diabetic and obese mice

In 14 months old and diabetic mice, treatment with Ex-4 was neuroprotective in MCAO induced stroke, both when initiated 1.5 and 3 hours post MCAO ($p<0.05$ and $p<0.05$). In contrast to the young animals, the effect was seen in the cortex but not in the striatum.

5.1.3 Exendin-4 treatment affect microglia phenotype

Ex-4 treatment initiated 1.5 hours post MCAO did not affect the inflammatory response pattern of inflammatory markers such as, MCP-1, IL-1β and TNF-α as measured by real-time PCR. However, the microglia phenotype response pattern was shifted towards a more reparative, M2 response, with up-regulation of gene expression of CD206, ARG1 and YM1/2 in the Ex-4 treated animals.

5.2 STUDY 2 LINAGLIPTIN IN MICE

Here the goal was to determine the neuroprotective efficacy of the DPP-4i linagliptin given after stroke and to establish if the effect was dependent on the GLP-1 receptor.

In short, our results show that the DPP-4i linagliptin was neuroprotective if it was given chronically before and continued after experimental stroke. The study also showed that the effect was independent of the GLP-1 receptor.

In wild type mice there was no difference in the stroke volume or the number of NeuN-positive neurons when linagliptin was given acute iv. at MCAO. By contrast chronic treatment started four weeks before MCAO increased the number of surviving neurons by approximately 30 % compared to vehicle, $p=0.003$ (Figure 16). The difference in stroke volume with approximately 40 % smaller stroke volume in linagliptin treated animals was not significant, $p=0.06$.

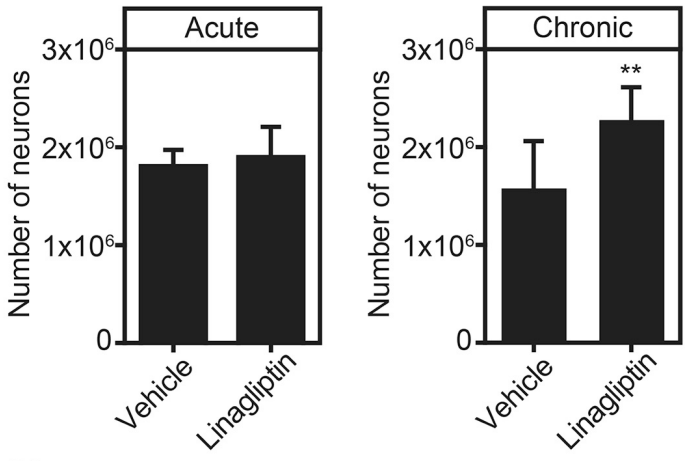

Figure 16. Neuroprotective effect of linagliptin in stroke. Number of NeuN-positive neurons in the striatum and cortex. Linagliptin was given either acutely after MCAO or chronically for 4 weeks before MCAO. Bars indicate standard deviation. ** represent p<0.01. © 2016 John Wiley and Sons, reproduced and adapted with permission from DOI: 10.1111/dom.12641.

DPP-4 activity was significantly reduced in both the acute treatment and chronic treatment groups, with GLP-1 levels increased approximately 10 times, $p<0.0001$.

In the GLP-1 receptor knockout mice, linagliptin treated animals had an approximately 55 % reduction in stroke volume compared to vehicle treated, $p=0.008$. The GLP-1RA Ex-4 had no effect in the GLP-1 receptor knockout animals. Figure 17.

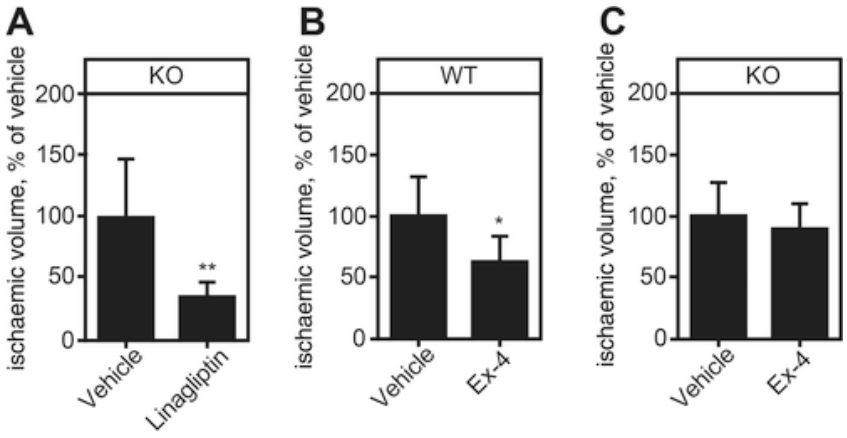

Figure 17. Linagliptin induced neuroprotection is GLP-1R independent. GLP-1R -/- (KO) and wild type (WT) mice treated chronically for 4 weeks with vehicle/linagliptin p.o. or vehicle/Ex-4 before MCAO. Treatment continued 3 weeks after MCAO. A: Ischemic volume quantifications in KO mice after chronic linagliptin treatment. B, C: Ischemic volume quantification in WT and KO mice after chronic Ex-4 treatment. Bars indicate standard deviation. * denote $p<0.05$, ** denote $p<0.01$. © 2019 John Wiley and Sons, reproduced with permission from DOI: 10.1111/dom.12641.

5.3 STUDY 3 GABA-ERGIC NEURONS IN RATS

The aim was to establish if, and how, aging and T2D affect cortical and striatal GABA-ergic neurons. The aim was also to determine if potential changes could be counteracted by treatment with the GLP-1RA Ex-4.

In short, our results showed that diabetes negatively affects GABA-ergic neurons in aging rats and that the effect is partially counteracted by Ex-4.

The overall density of neurons in the striatum and cortex was not different in the Wistar or GK-strains, neither at 3 nor 13 months, as tested with Cresyl Violet staining.

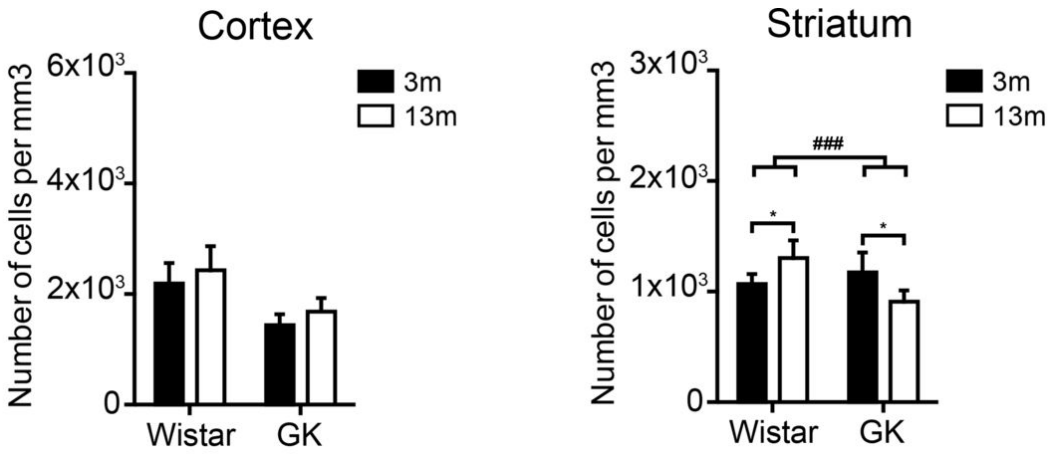

Figure 18. The effect of aging in type 2 diabetes in Wistar and GK-rats. GAD67 positive cells in the cortex and striatum. Bars indicate standard deviation. Two-way ANOVA was used to test group difference and the interaction between strain and age. 3m: 3 months old, 13m: 13 months old. * represent p<0.05, ### represent p<0.001 for interaction. Adapted and reproduced under creative commons attribution license (CC BY 4.0) from DOI: 10.1042/BSR20160437.

The study investigated the interaction effect of aging and diabetes on GABA-ergic interneurons. There was an increase in striatal GAD67-positive neurons in the old non-diabetic Wistar rats ($p<0.05$) vs. a reduction in the old diabetic GK-rats ($p<0.05$), $p<0.001$ for interaction. This interaction was not present in the cortex where neither strain had any difference in the neuronal density in the old vs. young animals. See Figure 18.

Three specific subtypes of interneurons were also stained for, Calbindin (CB), Calretinin (CR) and Parvalbumin (PV). There was a large reduction in the number of CB-positive cells in the 13-month-old compared to the 3-month-old GK rats, with around 45 % reduction in the cortex ($p<0.0001$) and 80 % reduction in the striatum ($p<0.001$). There was a similar trend in the striatum for CR, but it did not reach statistical significance after correction for multiple testing.

5.3.1 Exendin-4 partially counteracts T2D changes in aging

In sub-study 2, Ex-4 treatment for 6 weeks before sacrifice gave a 90 % increase in the number of CB-positive cells in the striatum ($p<0.01$). No difference was detected in the cortex. For GAD67, CR and PV no difference was found.

5.4 STUDY 4 OGTT IN rtPA TREATED STROKE PATIENTS

In this clinical study, the aim was to determine if endogenous GLP-1 secretion is altered in the acute phase of stroke and if the GLP-1 levels in acute stroke are correlated to functional outcome of the stroke.

In summary, the results show a higher GLP-1 level in patients that recently suffered an ischemic stroke compared to healthy controls. There was no association between the GLP-1 level in the acute phase of stroke and functional outcome.

A total of 379 patients were treated with rtPA for ischemic stroke at Södersjukhuset, Stockholm, Sweden between January 2014 and January 2017. Patients were consecutively screened by a research nurse for eligibility. 59 patients were included in the study and underwent the first OGTT. 51 underwent the second OGTT. 7 were lost to follow-up or declined a second OGTT and 1 patient died during follow-up. A control group of healthy volunteers was included for comparison (n=27). Characteristics of the stroke and control groups are shown in Table 5. As all patients treated with rtPA at Södersjukhuset are included in a quality of care registry. Characteristics of the entire rtPA treated cohort are included for comparison.

The included patients compared to the total rtPA treated cohort were younger (70.6 vs. 74.5 years), comprised of more males (66 vs. 51 %) and had lower NIHSS scores (4 vs. 6). Compared to the stroke patients, the group of healthy controls that underwent OGTT was younger (44.6 vs. 70.6 years), lower BMI (22.7 vs. 26.2) and had fewer men (40.7 vs. 66.1).

Characteristic	OGTT in Stroke (*n*=59)	OGTT controls (*n*=27)	Total rtPA cohort (*n*=379)
Age, years	70.6 (61.5-76.2)	44.6 (27.9-68.3)	74.5 (65.3-83.3)
Male Sex (%)	66.1	40.7	51.4
NIHSS before rtPA	4 (3-6)	-	6 (3-12.5)
mRS at discharge	1 (1-3)	-	2 (1-4)
BMI, kg/m^2	26.2 (23.4-29.3)	22.7 (21.2-26.0)	24.9 (22.9-28.4)
eGFR, ml min^{-1} $[1.73m]^{-2}$	70 (59-76)	-	64 (51-76)
Admission glucose mmol/L	6.8 (6.0-7.5)	-	6.7 (5.9-8.0)
Hypertension	54 %	-	56 %
Systolic BP, mmHg	154 (140-176)	-	157 (140-173)
Diastolic BP, mmHg	89 (80-98.5)	-	83.5 (75-93)
Atrial fibrillation, *n* (%)	8 (14 %)	-	20
Current smokers, *n* (%)	6 (10 %)	-	12
Prev. diabetes, *n* (%)	0 (0 %)	0 (0 %)	15
Pathologic OGTT, *n* (%)	34 (58 %)	3 (11 %)	-
Normal glucose tolerance	25 (42 %)	24 (89 %)	-
Fasting hyperglycemia	11 (19 %)	1 (4 %)	-
Impaired glucose tolerance	31 (53 %)	3 (11 %)	-
Diabetes	3 (5 %)	0 (0 %)	-

Table 5. Baseline characteristics of the stroke patients, healthy controls and total rtPA treated cohort. Values presented as median (IQR) or (%). IQR; interquartile range, NIHSS: National Institutes of Health Stroke Scale, mRS: modified Rankin scale, BMI: body mass index, eGFR: estimated glomerular filtration rate, BP: blood pressure. Adapted under creative commons attribution license (CC BY 4.0) from DOI: 10.1186/s12933-019-0896-z.

5.4.1 Patients treated with rtPA for ischemic stroke have higher GLP-1 levels

During the OGTT performed on day 2-4 in the acute phase of stroke in patients that were treated with rtPA for ischemic stroke samples were taken at 0, 60, 90 and 120 minutes. The same time-points were sampled in the healthy controls. The stroke patients had higher levels of fasting GLP-1 compared to the healthy controls (25.1 vs. 18.0 pmol/L, p=0.004). Figure 19A. In a univariate GLM model taking all time-points into account the estimated marginal means for stroke patients and controls showed a consistent overall difference at 31.1 vs. 26.0 pmol/L (difference 5.1 pmol/L, 95% CI 1.1 - 9.2 pmol/L; p=0.013), Figure 20.

There was no evidence of difference in the levels of fasting glucose between the stroke patients and the control group (5.3 vs. 5.6 mmol/L, p=0.33) (Figure 19C) or the levels of fasting insulin (13.2 vs. 9.8 mU/L, p=0.20) (Figure 19A). The stroke patients did, however, develop higher glucose at the end of the OGTT (7.8 vs. 6.1 mmol/L, p<0.001. Figure 19C.

There was no difference in the fasting GLP-1 levels between the acute phase of stroke and the three-month follow up (25.8 vs. 25.6 pmol/L, p=0.80). Neither was there any difference between the acute phase and the three-month follow up in a repeated measures two-way ANOVA taking all time-points during the OGTT into account with the estimated marginal means 31.7 vs. 33.6 pmol/L (difference 1.85 pmol/L 95 % CI, -1.6 – 5.3 pmol/L, p= 0.29), Figure 20B.

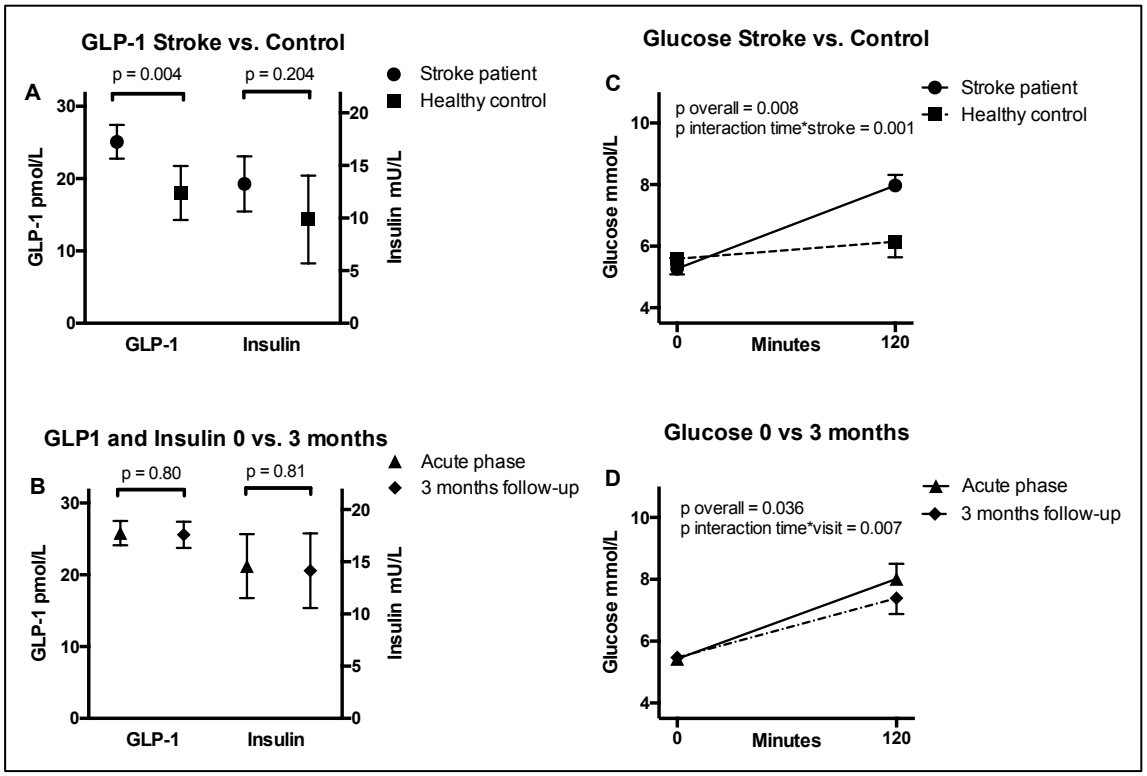

Figure 19. Fasting plasma GLP-1 and insulin (A and B) and plasma glucose (C and D) from a 120 min OGTT. Data is presented as mean values, bars indicate 95 % CI. In panel A and C the graphs show means adjusted for sex, age and BMI. Reproduced under creative commons attribution license (CC BY 4.0) from DOI: 10.1186/s12933-019-0896-z

5.4.2 No association between GLP-1 in the acute phase after stroke and outcome

There was no association between the GLP-1 levels measured in the acute phase after stroke and the three-month functional outcome defined as mRS ≥2. This remained unchanged, no matter if fasting GLP-1 was used as predictor, or if other measures e.g. AUC of GLP-1 during the OGTT, was used. The unadjusted results were the same as if adjustment was made for NIHSS, sex, age and BMI. Unadjusted OR for unfavorable outcome for fasting GLP-1 as predictor was 0.99 (95% CI 0.89-1.10, p=0.85), Table 6.

	Unadjusted OR (95 % CI)	p-value	Adjusted* OR (95 % CI)	p-value
GLP-1 at 0 minutes	1.03 (0.95-1.12)	0.50	0.99 (0.89-1.10)	0.85
GLP-1 AUC at stroke	1.01 (0.99-1.03)	0.29	0.99 (0.95-1.02)	0.50
GLP-1 rise AUC	1.00 (0.99-1.03)	0.44	0.99 (0.96-1.03)	0.60
GLP-1 max rise	1.00 (0.98-1.03)	0.78	0.98 (0.94-1.02)	0.32

Table 6. Odds ratios (OR) for GLP-1 as predictor for unfavorable outcome *Adjusted for age, sex, initial National Institutes of Health Stroke Scale (NIHSS), Cederholm insulin resistance index (Ceder-IR) and atrial fibrillation.

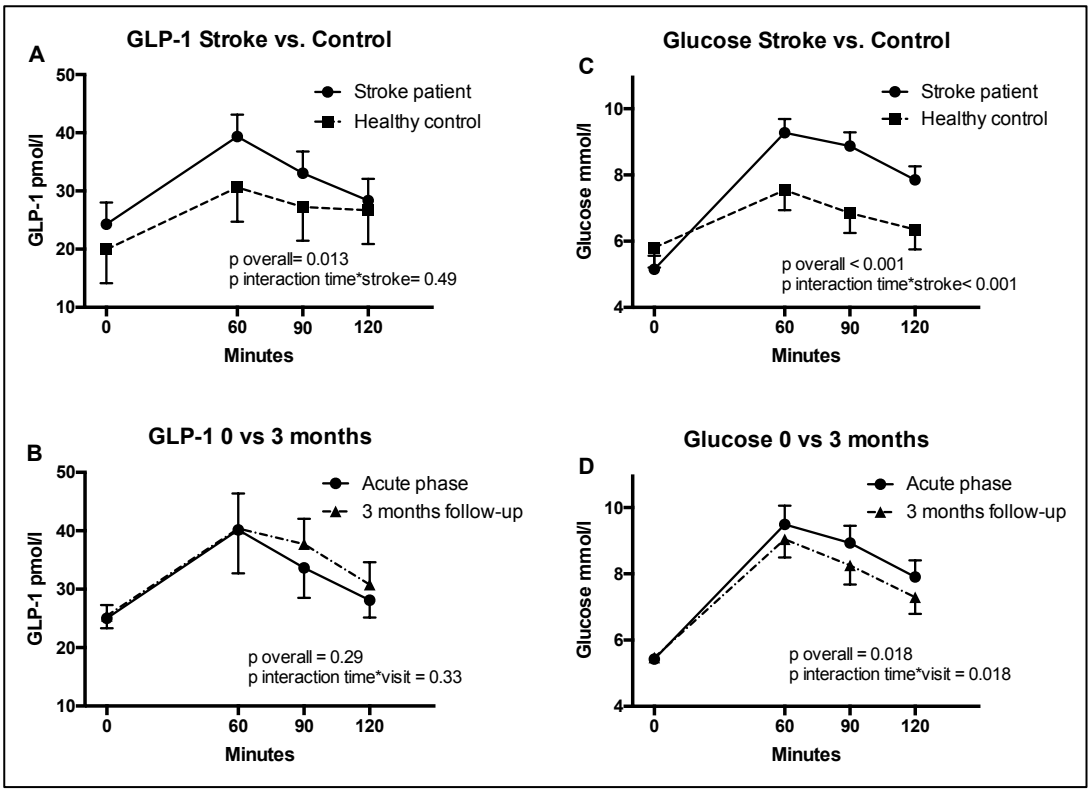

Figure 20. Plasma GLP-1 (A and B) and plasma glucose (C and D) during a 120 min OGTT. Data is presented as mean values, bars indicate 95 % CI. In panel A and C the lines show hypothetical curves during a 120 min OGTT for a male aged 62 years with BMI 25 (population means).

5.5 STUDY 5 EXENATIDE IN AMBULANCE

A randomized pilot trial with the aim to evaluate overall feasibility of treating hyperglycemia starting in the ambulance among patients with suspected stroke.

In short, the study found no evidence of a glucose lowering effect but the treatment administration was feasible without adverse events.

In total, 19 patients were included between May 2013 and May 2018. The study was stopped early due to slow recruitment. 8 were randomized to exenatide. Baseline characteristics are found in Table 7. The treated group was slightly younger (71 vs. 80 years) and had higher NIHSS-score on admission (median of 4.5 vs. 1).

Characteristic	Control (IQR)	Exenatide (IQR)
Age, years	80 (63-89)	71 (54-82)
Male sex, *n* (%)	6 (55 %)	4 (50 %)
BMI, kg/m^2	26.4 (23.2 – 31.7)	27.2 (21.6 – 29.5)
cP-Glucose, mmol/L	9.6 (9.0-10.8)	9.6 (8.4-10.6)
vP-Glucose, mmol/L	9.4 (7.9-11.2)	8.8 (6.9-10.9)
Systolic blood pressure, mmHg	165 (130-190)	150 (124-175)
Diastolic blood pressure, mmHg	89 (80-100)	80 (76-98)
Heart rate	88 (72-90)	80 (68-89)
NIHSS	1 (0-4)	4.5 (1-8)
Discharge diagnosis		
Ischemic Stroke, *n*	9	4
Hemorrhagic Stroke, *n*	0	2
Non-Stroke, *n*	2	2
Treated with rtPA, *n*	1	0

Table 7. Baseline characteristics of the included patients in the prehospital exenatide study. Data is presented as median with interquartile range unless otherwise specified. IQR: interquartile range, BMI: body mass index, cP-Glucose: capillary plasma glucose, vP-Glucose: venous plasma glucose, NIHSS: National Institutes of Health Stroke Scale, rtPA: recombinant tissue Plasminogen Activator. © 2019 John Wiley and Sons, reproduced and adapted with permission from DOI: 10.1111/ane.13166.

5.5.1 Slow recruitment analysis

The study was stopped early due to slow inclusion after having included 19 of the target 42 patients. Slow inclusion was identified early in the course of the trial and several attempts to address this were made. The inclusion and exclusion criteria were changed after applications for amendments to the protocol. At the onset of the trial, for instance, the inclusion criterion for glucose was 10-15 mmol/L. This was first expanded to 9-15 mmol/L and finally to 8-15 mmol/L. Originally, all persons with previously known diabetes were excluded, but later changed to previously known diabetes treated with anything other than lifestyle/diet or metformin.

A recruitment failure analysis was made in 2015 at the largest participating ambulance station, responsible for approximately 25 % of ambulance transports to the study hospital and 50 % of the study ambulances. The first four months of 2015 were analyzed, at which time the inclusion range for glucose was 9-15 mmol/L. Among the non-included patients, 143 dispatches for suspected stroke were made. After ambulance nurse evaluation 69 patients remained, and out of these, 13 had the required glucose of 9-15 mmol/L. 11 would have been excluded, mainly for problems pertaining to obtaining informed consent. 2 patients were true recruitment failures. The initial 13 eligible patients would have been increased to 19 with a glucose inclusion range of 8-15 mmol/L. As a result, the inclusion range was expanded after an approved protocol amendment.

5.5.1.1 93 Ambulance Nurses

The study was required to arrange a personal delegation to each participating ambulance nurse to inform the patients and obtain informed consent. This meant that each time a trained nurse switched jobs a potential study recruiter was lost. Repeated study trainings were arranged in an effort to keep the pool of nurses informed and able to include patients. In total 93 nurses were trained and individually delegated. Regular informal contacts with the participating stations also took place, aside from the trainings, to maintain motivation. ML, as study physician, was also available around the clock throughout the study period for telephone consultations regarding the study.

5.5.2 Glucose outcome

There was no evidence of difference in glucose level at 4 hours, the primary glucose endpoint. Exenatide treated vs. control group: 7.6 vs. 7.0, p=0.56. A linear regression model with adjustment for age, sex and BMI did not alter the result: 7.1 vs. 7.4, p=0.76. Complementary analyses to measure glucose exposure as AUC was also performed, but no evidence of a difference was found. See Figure 21, p-values denote statistical tests of AUC 0-4 hours and 0-24 hours.

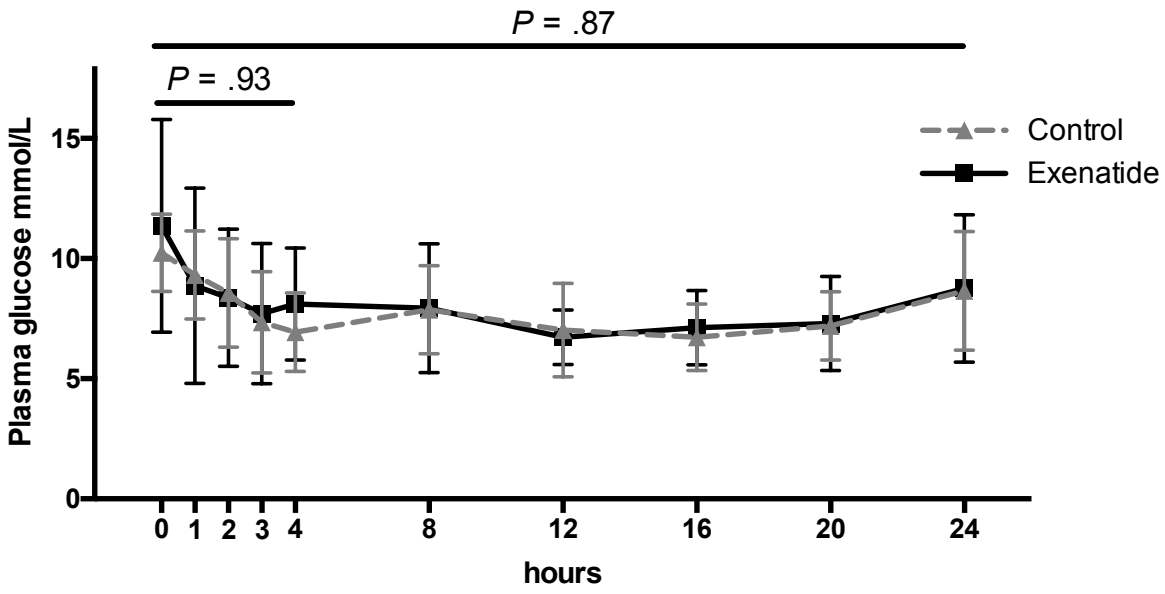

Figure 21. Capillary plasma glucose. P-values in figure represent Mann-Whitney U-test of AUC 0-4 h and 0-24 h. Bars indicate standard deviation. © 2019 John Wiley and Sons, reproduced permission from DOI: 10.1111/ane.13166.

5.5.3 Adverse events

Adverse events were recorded throughout the study period. No major adverse events and no hypoglycemic episodes were reported. One patient experienced nausea and vomiting, but had done so prior to inclusion. This patient suffered from a basilar thrombosis which, rather than the treatment, was considered to be the most likely explanation.

6 DISCUSSION

Stroke can be a debilitating disease. Persons with diabetes are at greater risk of suffering an ischemic stroke and experience a worse functional outcome. This thesis has focused on how to mitigate the effects of ischemic stroke by means of GLP-1-mediated neuroprotection. Importantly, the experimental work was performed by using a clinically relevant set-up. This facilitates the potential transfer of the findings, and thereby increases the value of scientific endeavor through translational research.

For this purpose, we have studied GLP-1RA treatment given after the onset of stroke, both in an experimental animal study (Study 1) and parallel to this, with a similar setup, we randomized suspected stroke patients in a clinical trial evaluating prehospital treatment with the GLP-1RA exenatide (Study 5). We have also studied if neuroprotection could be achieved by means of increasing the endogenous GLP-1 levels through, at the onset of stroke, inactivation of the GLP-1-degrading enzyme DPP-4 by the administration of linagliptin (Study 2). Furthermore, we tried to find explanations as to why persons with diabetes show impaired recovery after stroke by studying a subset of key regulatory neurons in stroke recovery: interneurons (Study 3). Additionally, in Study 3, we also evaluated whether GLP-1RA treatment could have a positive impact on these cells. Finally, we studied the endogenous GLP-1 levels in patients exposed to acute ischemic stroke in comparison to healthy individuals and the potential association between endogenous GLP-1 levels and functional outcome (Study 4).

6.1 GLP-1 RECEPTOR ACTIVATION IS NEUROPROTECTIVE POST STROKE AND TIME DEPENDENT

In Study 1, we showed that it is possible to achieve neuroprotection in experimental stroke through activation of the GLP-1 receptor by giving Ex-4 after the onset of stroke. The effect persisted if the treatment initiation was delayed up to 3 hours after the onset of stroke. However, the effect was lost at 4.5 hours, highlighting the need for a timely intervention. This puts the time-window for effective intervention in the same time-frame as rtPA (233). Furthermore, the effect was more pronounced at the earlier time point. To be efficacious, the treatment should therefore be started as soon as possible, perhaps already in the ambulance en-route to hospital. The treatment effect was not dependent on hyperglycemia/diabetes. This is in agreement with surrounding literature, with the majority of neuroprotection trials having shown GLP-1RA efficacy in non-diabetic animals, e.g. (188, 190-192, 194-198). This opens up for the potential use of these drugs in non-diabetic stroke patients as well.

Study 1 also demonstrated a dose-dependent relationship of the neuroprotective effect strengthening the evidence of a true (causal) effect. The initial *bolus* doses we used in this post-stroke study, 5 μg/kg and 50 μg/kg of Ex-4, were higher than usual clinical dosing of 0.1 μg/kg twice daily. Our study used, however, the standard clinical dose in the prolonged phase (for one week after stroke). Interestingly, the protective effect of exenatide 5 μg/kg and

50 µg/kg were similar at the 1.5-hour time-point in our study, but at the 3 hour intervention only the high dose was neuroprotective. Noteworthy is also that there is evidence for a protective effect of the clinical dose as well, albeit in pre-stroke treatment (189).

Previous studies in hyperglycemic stroke patients have all used insulin, and failed to show benefit from glucose reduction. They were, however, often started 8-12 hours or later after stroke onset, see Table 3. This delay before insulin intervention could be an explanation to why insulin trials have failed. The shorter time interval, between stroke onset and treatment, could be why our GLP-1RA treatment was neuroprotective where insulin failed. Furthermore, an additional disadvantage for insulin is its ability to cause hypoglycemia, and that it often does (see Table 3), with potential negative effects for a brain already under stress. GLP-1RA treatment, on the other hand, does not induce hypoglycemia by itself as the glucose lowering effect goes away once euglycemia is reached (96-98). Moreover, since the neuroprotective effect of GLP-1RA is not dependent of hyperglycemia or diabetes, regulation of glycemia at an earlier time point alone is unlikely the sole explanation of its neuroprotective efficacy.

Many previous studies have used young and healthy animals in the model (190, 192-194). This reduces their transferability, since stroke mainly occurs in aged and comorbid patients. Our study evaluated the effect not only in young animals but also in aged and obese/diabetic animals, induced by HFD. The neuroprotective efficacy was similar in the aged animals as well; with neuroprotection up to 3 hours post MCAO. We found, however, a difference in where the protective effect occurred. In the young animals the protective effect was shown in the striatum, but in the aged and comorbid animals the effect was seen in the cortex. This difference is hard to explain with certainty, but could be a consequence of different sensitivity to stroke in different areas of the brain with larger strokes resulting in larger *penumbra* zones. Most likely it is in the *penumbra* that the treatment effect occurs. The ischemic damage was more pronounced in the striatum of the young animals compared to the striatum in the aged and obese animals. For the cortex, the situation was opposite. Changes in the vascularization of the brain have been shown to occur in diabetes (234, 235) and could cause the different response to MCAO in old/diabetic and young/healthy animals. Although speculative, this could be an explanation.

6.2 THE STRUGGLE WITH INCLUDING STROKE PATIENTS IN A PREHOSPITAL CLINICAL TRIAL

The concept of early intervention being the key to successful neuroprotection was behind the clinical randomized trial of exenatide given in the ambulance to hyperglycemic patients with suspected stroke (Study 5). This was a study mainly focusing on the overall feasibility of early intervention with GLP-1RA in hyperglycemic patients with symptoms of acute stroke.

The study was powered to detect a 2 mmol/L difference in p-glucose at 4 hours with 42 patients included, but was stopped after 5 years with 19 included patients. The study found no

evidence of that exenatide given in the ambulance results in more rapid normalization in plasma glucose, but too few patients were included for a certain conclusion. A main finding of the study was in fact the difficulties one faces in including patients in the prehospital environment. Any study in humans must be based on informed consent, as described in the declaration of Helsinki (236). This ethical concern induces challenges in trials on patients with acute stroke. As patients are in a situation of great stress and since stroke is a disease with a high prevalence of cognitive impairment (237) and aphasia (238) obtaining an informed consent based on complex information is difficult. A further hamper is the stressful working conditions for the caregivers in the prehospital setting. Our recruitment failure analysis is indicative of the aforementioned obstacles. The main reasons for exclusion of otherwise suitable study subjects were related to difficulty obtaining informed consent, such as dementia, confusion or reduced level of consciousness.

Another difficulty was the constant turnover of nurses. The ambulance nurse collected the informed consent from the participating patients on individual delegation. Every time a nurse left their employment, a new one had to be trained and motivated. We strived to keep constant contact with the ambulance stations to ensure that nurses were up to date and able to include patients. In total 93 nurses were trained and individually delegated throughout the trial. A complication for maintaining this contact is the decentralized structure of the ambulance services. The ambulance service is, as a natural consequence of the treat and retrieve mission, constantly on the roll. This makes assembling them in one place and time to inform them more difficult. Furthermore, introducing new devices (injection syringe for exenatide), drugs (exenatide) and study glucose meters that the ambulance nurse had little previous knowledge about was another barrier. To support the participating nurses the study team offered a study physician (M.L.) constantly on call around the clock throughout the study for consultations.

There were, however, no adverse events or other complications in connection with the administration of the study drug in the ambulances and in this regard, the prehospital treatment with exenatide could be considered feasible.

6.3 LINAGLIPTIN REQUIRES PRE-TREATMENT BUT NOT GLP-1 RECEPTORS

Previous studies have shown that DPP-4i treatment is neuroprotective if given chronically *before* and after stroke in animal experimental models. We therefore aimed to determine the neuroprotective efficacy of the DPP-4i linagliptin initiated at stroke, as we had done with the GLP-1RA Ex-4. We found no evidence of a neuroprotective effect when linagliptin was given acutely at stroke. Effect of chronic treatment before and after stroke was, however, shown in Study 2, which is in line with previous DPP-4i studies (115, 201). The efficacy was shown in non-diabetic animals, indicating that it was glycemia-independent. Again this is in line with previous studies. A very interesting discovery of our study was that the

neuroprotective effect is not dependent on the presence of a GLP-1 receptor. In fact, in a knockout model deficient of the GLP-1 receptor, the DPP-4i neuroprotective effect was still present with chronic linagliptin treatment. On the other hand, treatment with a direct GLP-1RA (Ex-4) was without effect. This is the first evidence showing that the neuroprotective effect of linagliptin is mediated through alternate mechanisms, apart from elevation of endogenous GLP-1, at least in part.

DPP-4 is not a substrate specific degradation enzyme, with several other peptides apart from GLP-1 being degraded by it, including: SDF1α, GIP and PACAP (107, 108). These peptides have been shown to be neuroprotective (209, 239, 240). Inhibition of DPP-4 leads to modest elevations of all its substrates. A combination of several peptides could be responsible for the neuroprotective efficacy of DPP-4i, an effect not dependent on diabetes. An additional study, by our group, has been able to show that the neuroprotective efficacy by DPP-4i is mediated through SDF1α (209). Indeed both experimental and clinical studies have shown that SDF1α is an important mediator of the effect from inhibition of DPP-4 (210-213).

6.4 POTENTIAL CELLULAR MECHANISMS INVOLVED IN THE NEUROPROTECTIVE EFFECT

The evidence for GLP-1RA induced neuroprotection has now been confirmed in various studies (188-199). However, the cellular mechanisms by which these effects are mediated are still not clear. Potential explanations could be found in the regulation of inflammation and regulatory neurons, both shown to be affected by GLP-1RA treatment (Study 1 and Study 3). Inflammation is causing damage after stroke (46-48). Exenatide has been shown, in *pre stroke* treatment, to reduce the number of inflammatory microglia cells (189, 191, 192). Our *post stroke* treatment study did not result in reduction of pro-inflammatory cytokines (as measured by gene expression) but did, interestingly, show a shift in the inflammatory phenotype of microglia towards the so-called M2 response. This M2 phenotype has been linked to CNS repair (52). This shift in inflammatory response type could be one of the mechanisms behind the beneficial role of GLP-1RA.

In aging animals with diabetes, we found that the number of regulatory GAD67-positive neurons was reduced (Study 3). This occurred in the striatum, which is the same anatomical structure that also demonstrated a dramatic reduction in the number of CB-positive neurons. This decrease in CB-positive neurons has also been confirmed by others, both in rats and mice (241). Striatal CB-positive neurons are linked to the regulation of dopaminergic neurons (242) and the striatum is involved in motor control and skills making it important in recovery from stroke but it is also affected in motor diseases such as Parkinson's disease and Huntington's disease (243). Studies have indeed shown a positive effect from GLP-1RA treatment in Parkinson's disease and Huntington's disease apart from the animal studies on stroke (75, 188, 244, 245). Our study shows that treating middle-aged diabetic GK rats with Ex-4 can increase the number of CB-positive neurons, thereby establishing a potential

mechanism for the beneficial role of GLP-1RA in these situations. It remains to be shown whether the changes in CB-positive neurons reflect changes in CB protein expression or in cellular number. In this regard, we did not observe any apoptosis at the time of sacrifice although cell death at earlier stages of the aging process could have occurred.

6.5 ELEVATED GLP-1 AMONG STROKE PATIENTS

The relationship between endogenous GLP-1 levels and diabetes/obesity and cardiovascular disease has been unclear. Some studies have found diminished GLP-1 responses to OGTT in diabetes with a negative association with BMI (246) while others have shown higher levels in diabetic patients and a positive association with BMI (247, 248).

Several treatment studies have now shown a reduction in stroke incidence with GLP-1 treatment (148, 149, 249), but no trial has investigated if GLP-1RA treatment influences the outcome of stroke (250). It has, as discussed above been shown in animals, but not in humans. To study the relationship between endogenous GLP-1 and stroke outcome we performed OGTT studies on patients that had recently had an ischemic stroke treated with rtPA (OGTT performed 1-3 days after the stroke). We found that the GLP-1 levels were higher among the stroke patients compared to healthy controls. This was true both when looking at fasting levels and the response during the OGTT. In the study, we unfortunately did not take samples at 30 minutes after glucose loading. That would probably have reflected the peak response optimally. Both fasting levels and the post-challenge levels were, nonetheless, consistently elevated among the stroke patients.

Interestingly, we found no evidence of a difference in GLP-1 response to OGTT or in the fasting GLP-1 levels between the acute phase of stroke and a follow-up three months later. The fact that the GLP-1 levels in the acute phase and at the three-month follow-up remained unchanged argues against the observed difference being a consequence of the acute stroke. More likely it is a chronic and underlying difference; probably reflecting the levels before the stroke, although this is, of course, speculative. It could mean that it signals of a phenotype with increased stroke risk, indicative of an underlying pathophysiologic process. This is an area where previous studies have been conflicting. In a Swedish study on patients about to undergo valvular heart surgery, the levels were also found to be elevated, both the level of active and total GLP-1 (247). Furthermore, a Japanese study also found elevated GLP-1 levels to be associated with risk factors for cardiovascular disease, independently from diabetes (248). While at the same time the opposite has been reported, with lower GLP-1 levels in patients with established coronary disease (251). This difference is somewhat puzzling; it could be a consequence of progressive disease with first elevation and as a metabolic syndrome progress development of GLP-1 secretion dysfunction, but will have to be investigated further in the future.

The circulating endogenous levels did not show any association to the functional outcome of the stroke. One potential explanation for this lack of association could be that the endogenous GLP-1 elevation is much lower compared to a pharmacologic treatment dose. A recent article (2019) in *Cell Reports* has also demonstrated that endogenous GLP-1 and GLP-1RA exert their effect through different pathways (252).

The published article originating from Study 4 only included the fasting values of GLP-1, as we did not have data on the GLP-1 levels 30 minutes after the glucose challenge. GLP-1 secretion usually peaks at 30 minutes (253) and in order not to be potentially misleading only fasting levels were included. However, an additional analysis of the entire OGTT with all four time-points gave similar results with the same difference between stroke patients and healthy controls. This consistency in results makes it, in my opinion, unlikely that the results would have differed if data at 30 minutes were available.

7 CONCLUSION

GLP-1RAs are neuroprotective and can be used to attenuate the impact of ischemic stroke. What is very important to emphasize from a clinical perspective is that neuroprotection can be achieved not only if the treatment is given before the onset of stroke (clinically relevant for T2D patients already on GLP-1-based treatment when suffering from stroke), but also if it is started after a stroke has already occurred (clinically relevant for virtually any stroke patient). Efficacy of post stroke initiated treatment is, however, time-dependent and treatment should be initiated as soon as possible. DPP-4 inhibition also exerts neuroprotective effects, but needs chronic pre-stroke treatment for efficacy. This discrepancy could be explained by the fact that neuroprotection induced by DPP-4 inhibition is not dependent on GLP-1. Furthermore, the efficacy of GLP-1RA and DPP-4i is not dependent on hyperglycemia. The endogenous level of GLP-1 is elevated in patients that recently suffered a stroke, and it most likely represents a chronic elevation; the significance of this will have to be elucidated in the future.

The available data from large randomized trials have shown that GLP-1RAs reduce the incidence of cardiovascular events, such as stroke. Our experimental data further encourages the increased use of both GLP-1RA and DPP-4i in T2D with high stroke risk. Perhaps it should be the first add-on after metformin, to not only prevent stroke, but to also improve recovery in the event of a stroke.

8 FUTURE DIRECTIONS

That GLP-1RA can be used to prevent cardiovascular events is known (reduced incidence), but there is a gap of knowledge in whether such treatment can impact the outcome of the event itself. Therefore studies with GLP-1RA focusing on the outcome after a stroke would be very interesting to see. Perhaps legacy studies after the large randomized DPP-4i trials could be performed.

A limitation to the transferability of results from many experimental animal studies to patient care is the difference in doses used. This includes ours, where a high initial dose was used before the follow-up with clinical dosing. Testing of higher dose treatments in acute settings with neuroprotective aims in humans would be interesting. Higher doses would have a better chance to rapidly result in effective levels in the brain tissue and this may be important in time sensitive treatments such as post stroke neuroprotection.

Studies on neuroprotection with GLP-1RA and DPP-4i have shown that it works under diabetic conditions, but also in subjects without diabetes. Therefore, further studies on the mechanisms behind the neuroprotective effect are warranted. We propose two possible mechanisms in this regard: the regulation of interneurons and the inflammatory phenotype shifting. SDF-1α is another example. These should be further studied but also other paths of inquiry should be pursued.

The fact that GLP-1RA is effective both with and without the presence of concomitant diabetes makes a somewhat different prehospital study in suspected stroke a tempting target. A study including patients with all glucose values, not only hyperglycemic, has the potential to be easier to perform, and could possibly establish GLP-1 as a baseline treatment in stroke.

9 LIMITATIONS OF THE STUDIES

First, limitations that are more general to study 1-3 will be discussed followed by more study-specific limitations.

9.1 EXPERIMENTAL ANIMAL STUDIES (STUDY 1-3)

A major limitation of the studies is the fact that they are carried out in animals, reducing the transferability of the results. They are also all evaluated on the basis of mainly immunohistochemistry and to some extent inflammation markers. They lack functional data such as grip strength or rotarod evaluations of motor function. A weakness with immunohistochemical (IHC) staining is that reduction in number of staining-positive cells has an unclear significance. It is possible that the cells truly have gone into necrosis or apoptosis, but they could also be temporarily stunned with affected antigen presentation that with time would have reverted. When using IHC the specificity of the antibodies is important as well, and the dependence on this is a limitation.

9.2 STUDY 1

The study used high initial dosing of exenatide (5 and 50 μg/kg), albeit with the follow up dose being the same as clinical dose of 0.1 μg/kg twice daily. This high initial dose reduces the transferability. Another limitation is that the treatment was given for 1 week only. It is possible that a longer follow-up treatment would extend the time-window of effect after stroke. Moreover, how this time window transfers to humans is not clear. The time point of evaluation, one week after stroke, is also quite short in relation to recuperation after stroke in humans.

9.3 STUDY 2

The study shows that the treatment effect is not dependent of a GLP-1 receptor. The study does not, however, show through what mechanism the effect is mediated. This is a limitation in understanding how it works and how it could be transferred to a clinical setting. This study was also only performed in young animals where neuroplasticity possibly is better than in aged brains. A further limitation of the study, which to some extent is discussed previously, is that an enzyme inhibitor is not specific. DPP-4 inhibition elevates the level of GLP-1, but other enzyme substrates as well will be increased. This makes inference on the mechanism that causes the observed effect harder.

9.4 STUDY 3

A limitation of this study is that the exact functional role of each of the cell types tested for in the brain is not clearly known and established. This makes interpretation of the significance of the findings harder but does not in itself challenge the existence of differences between the normal Wistar and the diabetic GK strain. Another limitation is that the observed differences could be caused by animal strain differences rather than by an effect of diabetes. The GK rat

is, however, a derivative of the Wistar rat. It is also important to keep in mind that in relation to stroke, this was a pilot study performed in rats without stroke aimed to determine if T2D impacts interneurons and if there is potential for GLP-1RA treatment. To determine how interneurons/CB-positive neurons would respond to stroke and GLP-1RA would need a separate study.

9.5 STUDY 4

The GLP-1 levels were only measured after the stroke event, and estimating the levels before stroke is speculative. A limitation of this study is also the fact that GLP-1 was not measured at 30 minutes post challenge, missing the probable maximum. This was a mistake in study design. Inclusion of patients with diabetes, in addition to those without, would have increased the external validity of the study. The appropriateness of glucose loading diabetic patients in the acute phase of stroke could, on the other hand, be questioned. Furthermore, there was a difference in the age and BMI between the stroke patients and the control group. It was adjusted for, but one cannot rule out the risk of residual confounding.

9.6 STUDY 5

The main limitation is the low number of included patients. A larger sample would have been needed to reliably look at the glucose reduction outcome. Detecting side-effects and adverse events is also hard in randomized trials. The low number of treated patients means that we would only have a fair chance to detect adverse events expected in around 35 % according to the rule of three (254). The study was not designed as a neurological outcome study, but that would of course be of higher interest than glucose related outcomes. The short follow-up is another limitation; longer follow-up with repeated dosing would have been interesting. The study included patients in the ambulance, which in some cases turned out to have other diagnoses than stroke, making the cohort a less clearly defined. From a safety and feasibility perspective of prehospital interventions this might be less of a problem though since it reflects the real-life situation of prehospital care; not everything turns out to be what it appeared to be in the beginning.

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11 POPULÄRVETENSKAPLIG SAMMANFATTNING

Personer med typ 2 diabetes har större risk att få en stroke och när de drabbas återhämtar de sig sämre. Om man har högt blodsocker när man får propplösande behandling mot stroke, så kallad trombolys, är risken större att drabbas av en blödning som komplikation. Standardbehandling mot högt blodsocker är insulin. Men insulinbehandling innebär samtidigt en risk att blodsockret sjunker för lågt, speciellt om snabbt behandlingsresultat är målet.

GLP-1 är ett hormon som utsöndras från tarmen när vi äter och hjälper kroppen att reglera blodsockret efter en måltid. GLP-1-baserade läkemedel är en läkemedelsgrupp som används mot typ 2 diabetes och som genom sin verkningsmekanism inte riskerar att ge för lågt blodsocker. GLP-1-baserade behandlingar har i studier visat sig kunna minska risken för stroke och annan hjärtkärlsjukdom. I tidigare djurförsök har man kunna se att det också minskar effekten av en stroke.

Målet med detta forskningsprojekt har varit att närmare undersöka de hjärnskyddande effekterna av GLP-1-baserade läkemedel genom djurförsök och studier på människor.

Studie 1: I denna djurexperimentella studie kunde vi se att behandling med GLP-1 läkemedlet exenatid givet *efter* stroke var hjärnskyddande både i unga samt i feta/gamla djur. Vi såg effekt när behandlingen startades upp till 3 h efter stroke, men den försvann vid 4.5 h.

Studie 2: I denna djurexperimentella studie använde vi ett läkemedel, linagliptin, som höjer de kroppsegna nivåerna av bl.a. GLP-1, men även påverkar flera andra ämnen. Vi kunde här se att linagliptin behöver ges *före* stroke för att vara skyddande. Vi kunde emellertid även se att linagliptin inte behöver GLP-1-receptorer för att vara hjärnskyddande.

Studie 3: Vi undersökte om åldrandet i hjärnan hos djur med diabetes påverkar en speciell typ av nervceller, så kallade interneuron, annorlunda än hos djur utan diabetes. Dessa neuron har till uppgift att reglera övriga nervcellers aktivitet. Vi kunde se att djur med diabetes hade lägre nivåer av dessa neuron, men också att påverkan delvis kunde motverkas genom behandling med GLP-1 läkemedlet exenatid.

Studie 4: Här undersökte vi på vilken nivå de kroppsegna nivåerna av GLP-1 ligger hos patienter som nyligen haft en stroke. Vi fann att de var förhöjda, både kort efter en stroke och var oförändrade 3 månader senare. Den kroppsegna nivån av GLP-1 i akutskedet hade ingen koppling till hur utfallet efter stroke blev.

Studie 5: En ambulansstudie där patienter med högt blodsocker och misstänkt stroke lottades till att få standardbehandling, eller att i ambulansen få GLP-1-läkemedlet exenatid. Vi kunde inte se att detta påverkade blodsockret, men studien blev för liten för att kunna bedöma detta. Den var också ett test för att se om behandlingen medförde några problem, vilket vi inte kunde se.

Sammanfattningsvis: GLP-1 baserad behandling är hjärnskyddande, men tidskritisk. Behandling behöver startas inom enstaka timmar för exenatid och måste ha pågått före stroke för linagliptin. Fynden uppmuntrar till ökad användning av dessa läkemedel vid diabetes.

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